Traffic of Kv4 K^+ channels mediated by KChIP1 is via a novel post-ER vesicular pathway

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The traffic of Kv4 K^+ channels is regulated by the potassium channel interacting proteins (KChIPs). Kv4.2 expressed alone was not retained within the ER, but reached the Golgi complex. Coexpression of KChIP1 resulted in traffic of the channel to the plasma membrane, and traffic was abolished when mutations were introduced into the EF-hands with channel captured on vesicular structures that colocalized with KChIP1(2–4)-EYFP. The EF-hand mutant had no effect on general exocytic traffic. Traffic of Kv4.2 was coat protein complex I (COPI)–dependent, but KChIP1-containing vesicles were not COPII-coated, and expression of a GTP-loaded Sar1 mutant to block COPII function more effectively inhibited traffic of vesicular stomatitis virus glycoprotein (VSVG) than did KChIP1/Kv4.2 through the secretory pathway. Therefore, KChIP1seems to be targeted to post-ER transport vesicles, different from COPII-coated vesicles and those involved in traffic of VSVG. When expressed in hippocampal neurons, KChIP1 co-distributed with dendritic Golgi outposts; therefore, the KChIP1 pathway could play an important role in local vesicular traffic in neurons.

Introduction

The Kv4 channels, are rapidly inactivating A-type voltage gated K^+ channels that are important for controlling neuronal firing frequency, preventing the back propagation of action potentials, and regulating the repolarization phase of action potentials (Pak et al., 1991; Serodio et al., 1994; Dilks et al., 1999). The potassium channel interacting proteins (KChIPs) interact directly with the NH_2-terminal domains of the pore-forming Kv4 α-subunits, and act as integral components of the channels (An et al., 2000; Bahring et al., 2001; Scannevin et al., 2004; Zhou et al., 2004); KChIPs and Kv4 channels colocalize in neurons (Rhodes et al., 2004). Studies in heterologous expression systems showed that the association with KChIPs increases the surface expression and Kv4 current density, and overall, restores more native-like properties to the Kv4 current (An et al., 2000; Bahring et al., 2001; Hatano et al., 2002; Holmqvist et al., 2002; Morohashi et al., 2002; Shibata et al., 2003; Takimoto et al., 2002). In central nervous system neurons, Kv4 channels are concentrated somato-dendritically where they are crucial regulators of postsynaptic excitability and are modulators of synaptic plasticity (Sheng et al., 1992; Maletic-Savatic et al., 1995); reduced expression of Kv4.2 is associated with hyperexcitable neuronal firing in conditions, such as epilepsy (Bernard et al., 2004). Therefore, understanding the regulation of Kv4 channel expression and traffic by KChIPs is of major interest (Birnbaum et al., 2004).

KChIPs belong to the family of EF-hand neuronal calcium sensor (NCS) proteins that are expressed predominantly in neuronal cells, but also are found in other cell types, such as cardiac myocytes and smooth muscle cells (An et al., 2000; Burgoyne and Weiss, 2001; Amberg et al., 2002). NCS proteins undergo conformational changes upon binding Ca^{2+}, and transduce Ca^{2+} signals by modulating target protein activity (Burgoyne and Weiss, 2001; Burgoyne et al., 2004). Four KChIP subtypes are known, one of which (KChIP1) is NH_2-terminally myristoylated like the other NCS proteins. Different NCS proteins use their myristoyl tail in differing ways. Recoverin, hippocalcin, neurocalcin δ, and the visinin-like proteins have a Ca^{2+}-myristoyl switch mechanism that provides Ca^{2+}-dependent membrane targeting (Zozulya and Stryer, 1992; Tanaka et al., 1995; Ivings et al., 2002; O'Callaghan et al., 2003b; Spilker and Braunewell, 2003), whereas NCS-1 and KChIP1 have their myristoyl tails exposed constantly and are membrane associated, independently of Ca^{2+} (O'Callaghan et al., 2002, 2003a; O'Callaghan and Burgoyne, 2004). Myristoylation and correct targeting of KChIP1, which is determined by residues in the minimal myristoylation sequence, are required for the KChIP1 stimulation of the traffic of Kv4.2 channels to the plasma membrane.

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Abbreviations used in this paper: ARF, ADP-ribosylation factor; COP, coat protein complex; ERGIC, ER-Golgi intermediate compartment; KChIP, potassium channel interacting protein; NCS, neuronal calcium sensor; VSVG, vesicular stomatitis virus glycoprotein.

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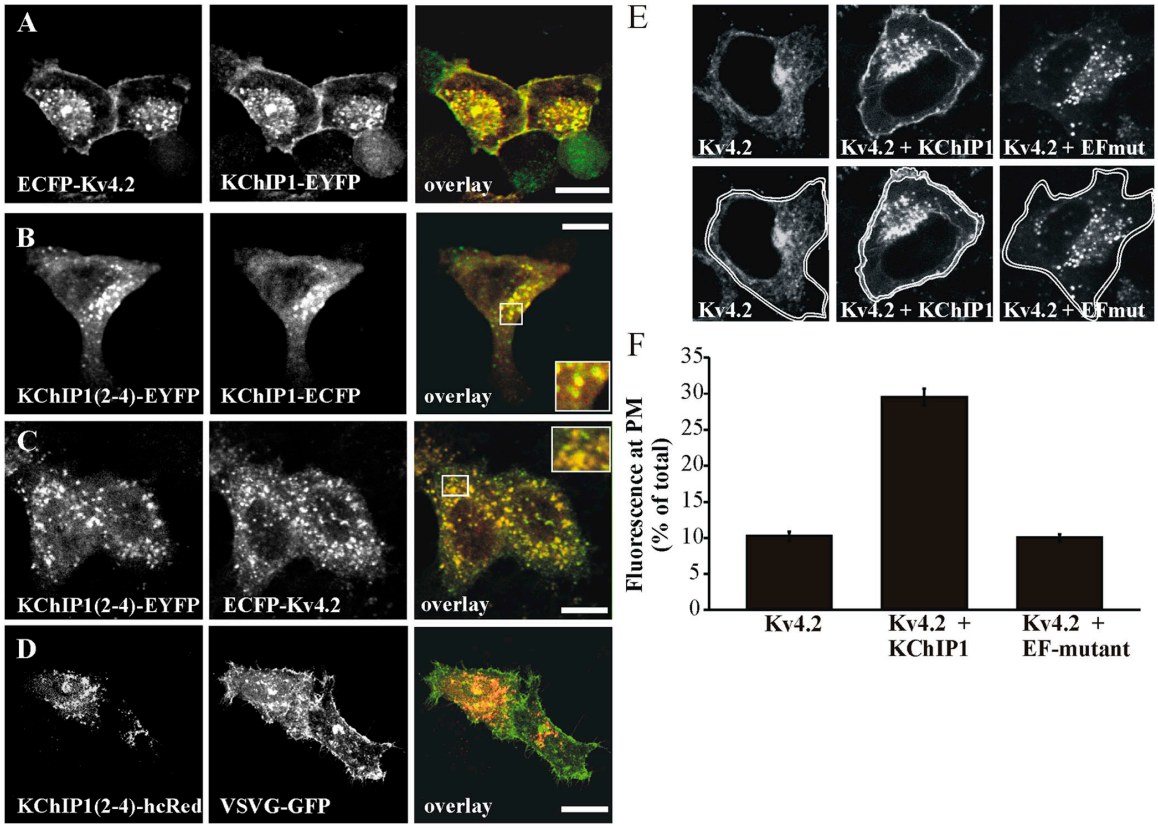

Figure 1. **Effects of the KChIP1 EF-hand mutant on membrane traffic.** (A) In HeLa cells coexpressing KChIP1-EYFP, the Kv4.2 channel traffics to the plasma membrane. The KChIP1 EF-hand mutant (KChIP1(2–4)-EYFP) is targeted to the same vesicles as is KChIP1-ECFP (B), but in contrast to KChIP1-EYFP, it disrupts traffic of ECFP-Kv4.2 to the plasma membrane and traps the channel in vesicles (C). The EF-hand mutant does not disrupt traffic of VSVG-GFP to the plasma membrane over a 5-h period at permissive temperature (D). The color overlays show coexpressed proteins in red and green with colocalization appearing in yellow. Bars, 10 μm. Quantification of the effect of KChIP1-EYFP and KChIP1(2–4)-EYFP on ECFP-Kv4.2 traffic to the plasma membrane. (E) HeLa cells were transfected to express ECFP-Kv4.2 alone or in combination with KChIP1-EYFP or the EF-hand mutant KChIP1(2–4)-EYFP. ECFP-Kv4.2 fluorescence was imaged and quantified by drawing regions of interest around the outside and the inside of the plasma membrane (bottom panels) to allow determination of the percentage of total fluorescence at the plasma membrane. (F) Mean data derived from 25–29 cells expressing ECFP-Kv4.2 alone or coexpressing KChIP1-EYFP or KChIP1(2–4)-EYFP.

A minimal NH_2-terminal myristoylation sequence of KChIP1 targets to the same vesicle structures as does KChIP1 itself. Following transfection, these structures enlarge and become ER-Golgi intermediate compartment 53 (ERGIC-53)–positive, which is consistent with blockade of KChIP1-containing intermediates in their traffic from the ER to Golgi (O'Callaghan et al., 2003a).

The issues that we addressed here are when does KChIP1 interact with Kv4.2 channels to promote their surface expression, and where in the secretory pathway is Ca^{2+} binding to KChIP1 required for membrane traffic? The channel by itself can reach as far as the Golgi apparatus, but interaction of the two proteins during ER-to-Golgi traffic mediates the traffic to the plasma membrane in a coat protein complex 1 (COPI)–dependent manner. KChIP1 expressed alone is targeted to a novel type of post-ER transport vesicle that differs from COPII-coated vesicles. We suggest that KChIP1 is targeted to vesicles that represent a distinct non–COPII-coated post-ER vesicle population, and support the idea of multiple pathways from the ER to the Golgi. KChIP1's role in the traffic of Kv4.2 channels could provide a mechanism for local Ca^{2+}-dependent traffic of channels in neuronal dendrites.

Results

Localization of expressed Kv4.2 and KChIP1 and the effect of abolishing the Ca^{2+}-binding ability of KChIP1 on traffic of Kv4.2

When expressed in HeLa cells alone, KChIP-1 localizes to punctate vesicular structures (Fig. S1; available at http://www.jcb.org/cgi/content/full/jcb.200506005/DC1). Expressed Kv4.2 is concentrated within an intracellular perinuclear compartment (An et al., 2000; Bahring et al., 2001; Hatano et al., 2002) (O'Callaghan et al., 2003a). In transfected HeLa cells, this compartment corresponds to the Golgi complex (Fig. S1 and Fig. S2), which suggests that Kv4.2 is not retained in the ER and can traffic to the Golgi complex.

Coexpression of Kv4.2 with KChIP1 results in a marked change in the intracellular localization of both proteins, with redistribution to the plasma membrane (Fig. 1 A). The ability of KChIP1 to traffic Kv4.2 beyond the Golgi complex must mean that the two proteins interact at some stage during ER-to-Golgi or intra-Golgi traffic. It was shown that the functional effects of KChIP1 on the Kv4.2 channel—including the increase

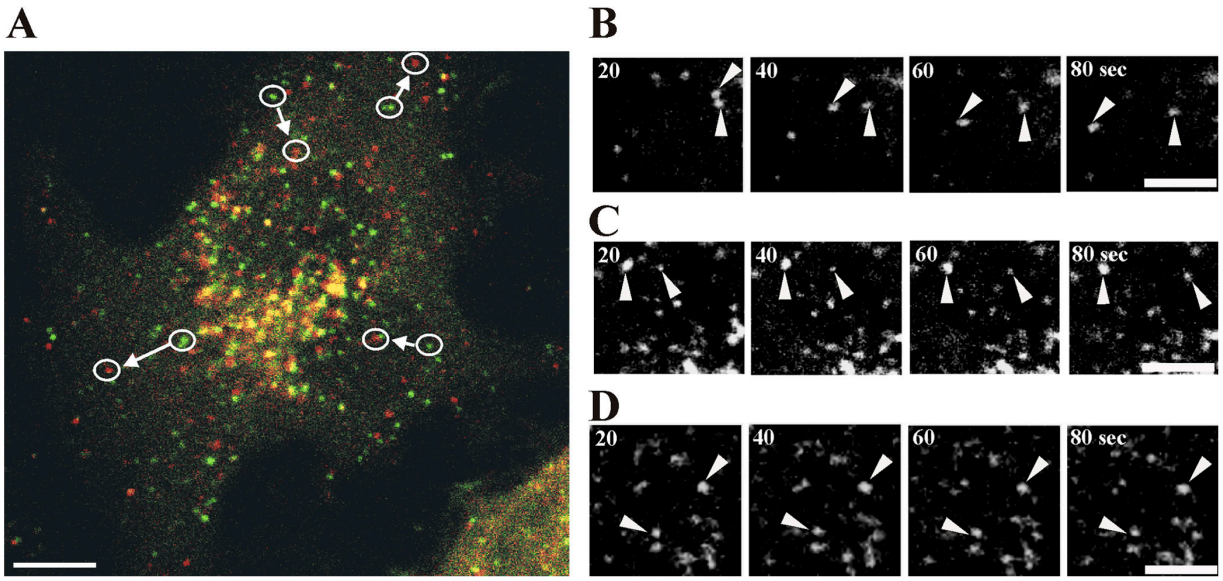

Figure 2. **The mobility of KChIP1-EYFP–labeled vesicles.** HeLa cells were transfected to express KChIP1-EYFP and the dynamics of the KChIP1 vesicles were imaged by time-lapse confocal laser scanning microscopy at 20°C. (A) Movement of the vesicles is shown in an overlaid image of a cell at the beginning and 180 s later with the initial image in green and the later image in red. Stationary vesicles appear in yellow. Examples of vesicles showing directed stop-go motion are circled, with their direction of travel indicated by the arrows. (B) Image series show the movement of a KChIP1-EYFP–labeled vesicle in a control cell, with a neighboring one remaining stationary at this time interval (marked by arrow heads). (C) The mobility is not affected by the EF-hand mutation of KChIP1. (D) Incubation in 10 μM nocodazole for 1 h resulted in complete loss of the mobility of the KChIP1-EYFP–labeled structures. Bars, 10 μm (A) or 5 μm (B–D).

in Kv4.2 current density, which was measured electrophysiologically—are Ca^{2+} dependent, whereas the interaction between the two proteins is not (An et al., 2000). It is not known why the Ca^{2+}-binding ability of KChIP1 is essential and where in channel traffic the Ca^{2+}-dependent step occurs. We used the same triple EF mutant of KChIP1 (KChIP1(2–4)-EYFP) as described in An et al. (2000), which could still bind to Kv4. The localization of KChIP1 itself was not affected by the EF-hand mutation, because KChIP1-ECFP and KChIP1(2–4)-EYFP overlapped in punctate structures in coexpressing cells (Fig. 1 B), and hence, the targeting of KChIP1 was not dependent on Ca^{2+}. In contrast to wild-type KChIP1, the coexpression of the EF-hand mutant with ECFP-Kv4.2 did not allow traffic of the channel to the plasma membrane in any of the cells that were examined (Fig. 1 C). Surprisingly, the channel did not simply remain in the Golgi region as in cells that express Kv4.2 alone, but instead had a punctate distribution like KChIP1(2–4)-EYFP. In all cells that were examined, >50% of the punctate spots were positive for KChIP1(2–4)-EYFP and ECFP-Kv4.2 (see color overlay). The effect of KChIP1-EYFP and the EF-hand mutant on traffic of ECFP-Kv4.2 to the cell surface was quantified by determining the level of fluorescence at the cell periphery and in the cell as a whole (Fig. 1 E). Coexpression with KChIP1-EYFP resulted in a threefold increase in fluorescence at the cell surface. Coexpression with KChIP1(2–4)-EYFP did not increase the percentage of ECFP-Kv4.2 that reached the cell surface (Fig. 1 F). These data are consistent with previous electrophysiologic analyses (An et al., 2000). The EF-hand mutant did not affect membrane traffic in general, because vesicular stomatitis virus glycoprotein (VSVG)—expressed as ts045 VSVG-GFP (a well characterized protein for following constitutive exocytic vesicular traffic [Presley et al., 1997])—did not colocalize with KChIP1(2–4) and was able to traffic to the plasma membrane (Fig. 1 D).

By immunostaining coexpressing HeLa cells for markers of the secretory pathway, we examined to which intracellular compartment ECFP-Kv4.2 had been redistributed by KChIP1(2–4)-EYFP. The channel no longer overlapped with the cis-Golgi marker, β-COP, which suggested that its traffic to the Golgi apparatus had been disrupted (Fig. S3 D; available at http://www.jcb.org/cgi/content/full/jcb.200506005/DC1). The punctate structures with which Kv4.2 was associated also showed no overlap with calnexin or Sec13 (marker of ER exit sites), but showed partial overlap with ERGIC-53 (Fig. S3, A–C). This was similar to what was observed previously for KChIP1-EYFP vesicles, which also had partial overlap with ERGIC-53 (O'Callaghan et al., 2003a). The effects of KChIP1(2–4)-EYFP on the distribution of ECFP-Kv4.2 were not due to disruption of the intracellular organelles themselves, because the ER, ERGIC, and Golgi apparatus all appeared in their usual pattern as seen in untransfected cells (Fig. S3, A–D). In summary, abolishing the Ca^{2+}-binding ability of KChIP1 prevented Kv4.2 from reaching the Golgi complex, which suggested that the site of interaction of KChIP1 and Kv4.2 is at a pre-Golgi level, and that it is at this stage that Ca^{2+} binding to KChIP1 is required.

KChIP1 is associated with nonconventional post-ER transport vesicles

KChIP1-EYFP expressed alone is targeted via its myristoyl tail to punctate structures, which do not colocalize completely with any known intracellular markers. However, they do overlap

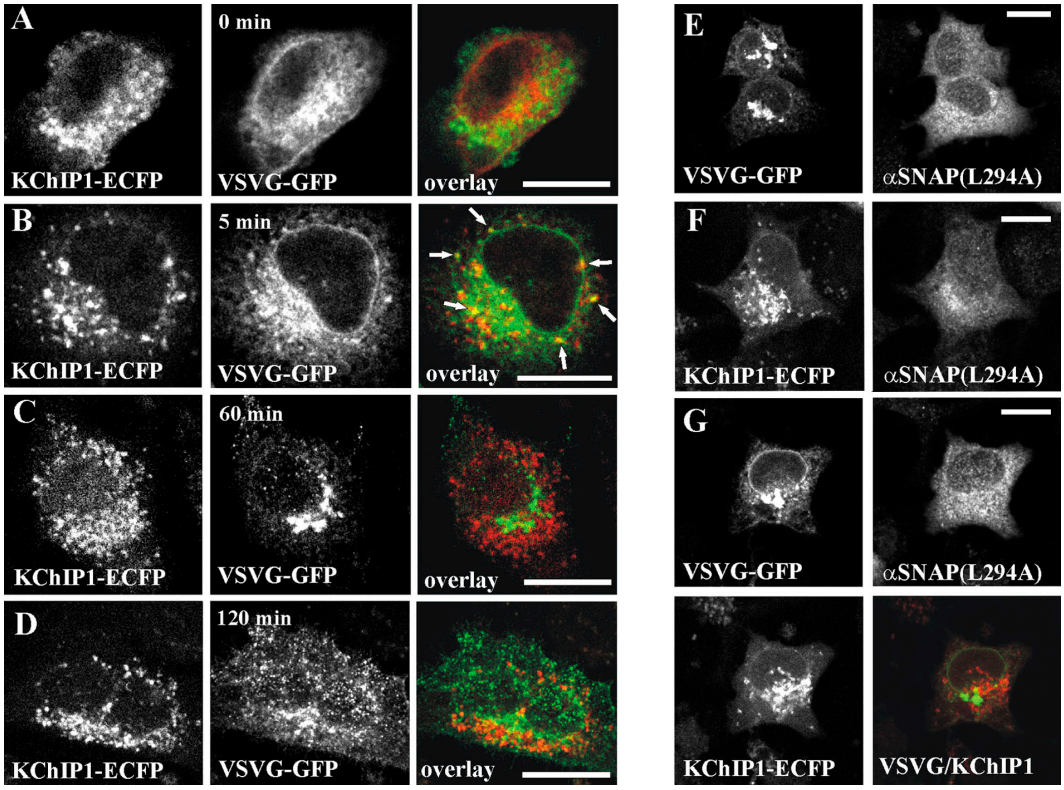

Figure 3. **Ts045 VSVG-GFP and KChIP1-ECFP in the secretory pathway and effect of inhibiting traffic.** HeLa cells were cotransfected with KChIP1-ECFP and ts045 VSVG-GFP and were incubated at 40°C overnight, which causes retention of ts045 VSVG-GFP in the ER. The temperature was reduced to 32°C and the cells were fixed after various time intervals as indicated in the images. At 40°C, ts045 VSVG-GFP is retained in the ER (A); upon the shift to permissive temperature for 5 min, it exits the ER and appears in punctate structures, which are likely to be post-ER vesicles (B). It then traffics to the Golgi apparatus over the next 60 min (C) and further to the plasma membrane in secretory vesicles over a 120-min period (D). The localization of KChIP1-ECFP and ts045 VSVG-GFP is shown individually and in color overlay (KChIP1-ECFP in red and ts045 VSVG-GFP in green) with colocalization seen in yellow (the arrows point at vesicles that overlap and appear in yellow). (E and F) HeLa cells were cotransfected with plasmids encoding pcDNA3-αSNAP(L294A) and VSVG-GFP (E) or KChIP1-ECFP (F) or were triple-transfected with all three constructs (G). αSNAP(L294A), which was visualized by immunostaining the cells with anti-αSNAP antibody and using Texas red–streptavidin, inhibits vesicular traffic throughout the secretory pathway. Ts045 VSVG-GFP was allowed to traffic at 32°C for 5 h before fixation of the cells. The localizations of the proteins are shown individually and in color overlay with colocalization seen in yellow. Bars, 10 μm.

partially with ERGIC-53–positive structures. The minimal myristoylation motif, KChIP1(1–11)-EYFP, leads to progressive enlargement of the ERGIC because of blockade of traffic that is mediated by COPI, and then overlaps completely with ERGIC-53, β-COP, and ECFP-Kv4.2 (O'Callaghan et al., 2003a). Live cell imaging revealed that a proportion of the KChIP1-EYFP–labeled structures were mobile (Fig. 2, A and B). Vesicles within the perinuclear region essentially were stationary, but vesicles that are more peripheral showed random movement over short distances (<2 μm) or showed directed stop-go movement over distances ≤20 μm at rates of ≤0.15 μm/s. The directed movement occurred into and away from the perinuclear region (Fig. 2 A). The EF-hand mutant did not affect mobility of the vesicles (Fig. 2 C). Both types of movement were microtubule dependent, because incubation with nocodazole did not affect the morphology of the vesicles, but did cause them to become immobile (Fig. 2 D). These findings are consistent with the KChIP structures being transport vesicles.

We compared KChIP1 with ts045 VSVG-GFP, as a marker of the constitutive secretory pathway (Presley et al., 1997). At a restrictive temperature of 40°C, ts045 VSVG-GFP has a folding defect that retains it in the ER (Griffiths et al., 1985; Beckers et al., 1987; Bergmann, 1989). Transfer to a permissive temperature of 32°C allows the protein to exit from the ER and traffic to the Golgi apparatus and then on to the plasma membrane. We examined the localization of ts045 VSVG-GFP and KChIP1-ECFP in cotransfected HeLa cells to determine whether ts045 VSVG-GFP colocalizes with KChIP1-ECFP during its traffic through the secretory pathway. Cotransfected HeLa cells were incubated overnight at 40°C. The next day, they were transferred to 32°C, and captured by fixation after various time intervals. Before the temperature reduction to 32°C (0 min), ts045 VSVG-GFP appeared in a reticular pattern as it was retained in the ER with no overlap visible with KChIP1-ECFP (Fig. 3 A). Within 5 min of the shift to 32°C, vesicular structures began to appear—potentially vesicles budding off the ER—and a small proportion colocalized with KChIP1-ECFP–labeled vesicles (Fig. 3 B). Upon a temperature shift to 32°C for 15 min, ts045 VSVG-GFP redistributed to a perinuclear Golgi-like region and no longer overlapped with KChIP1-ECFP. The same situation was observed at 30 min (not depicted) and 60 min (Fig. 3 C). After 120 min at 32°C, ts045 VSVG-GFP was found in numerous punctate structures and on the plasma membrane. The punctate structures, likely to be vesicles that were trafficking

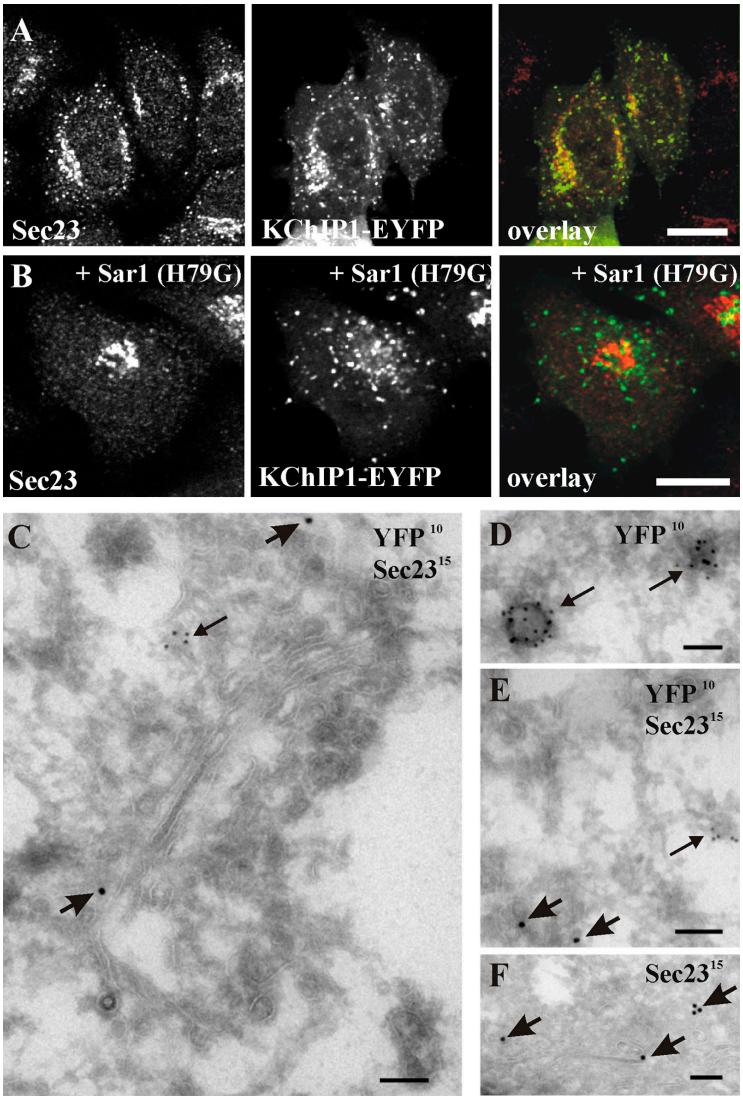

Figure 4. **KChIP1-labeled vesicles are not COPII coated.** (A and B) HeLa cells were transfected to express KChIP1-EYFP or KChIP1-EYFP together with the constitutively active Sar1(H79G) mutant. The cells were immunostained with anti-Sec23, a COPII coat component, with visualization with Texas red–streptavidin. The color overlays show KChIP1-EYFP in green and Sec23 in red (colocalization appear in yellow). No colocalization between Sec23 and KChIP-EYFP can be observed in cells expressing Sar1(H79G). Bars, 10 μm. (C–F) Immunolabeling of frozen sections of transfected HeLa cells with anti-GFP to detect KChIP1-EYFP using 10 nm gold and anti-Sec23 using 15 nm colloidal gold. All micrographs are from cotransfected cells, but in some images, only labeling with one antibody is detectable. Small arrows indicate 10 nm gold and large arrows indicate 15 nm gold. Bars, 100 nm.

from the Golgi apparatus to the plasma membrane, showed no colocalization with KChIP1-ECFP–labeled vesicles (Fig. 3 D). By 180 min at 32°C, ts045 VSVG-GFP had reached the plasma membrane, with a component still in the Golgi apparatus; again, no colocalization was seen with KChIP1-ECFP (unpublished data). These findings demonstrate that KChIP1-ECFP–labeled vesicles are part of a post-ER, pre-Golgi traffic system and are distinct from the bulk of those that are involved in traffic of VSVG from the ER to the Golgi complex. The colocalization of KChIP1-ECFP with a small subset of vesicles that contains ts045 VSVG-GFP, within 5 min after a shift to permissive temperature, suggests that KChIP1 vesicles may be close to a subset of ER exit sites that is used by VSVG or that there may be a small overlap between the two pathways.

Further support for KChIP1 targeting to immediate post-ER vesicles that are distinct from those in VSVG traffic was provided by the finding that coexpression of the dominant negative αSNAP(L294A) mutant (Barnard et al., 1997) did not result in colocalization of KChIP1-ECFP and ts045 VSVG-GFP (Fig. 3 G). This experiment should result in the accumulation of nonfusogenic vesicles after their budding. The αSNAP (L294A) mutant has a decreased ability to stimulate NSF ATPase activity, and thereby blocks SNARE function inhibiting vesicular traffic throughout the secretory pathway by inhibition of membrane fusion (Barnard et al., 1997; Christoforidis et al., 1999; Lanoix et al, 1999; Mironov et al., 2003). Even after 5 h at the permissive temperature (32°C), ts045 VSVG-GFP did not reach the plasma membrane and was localized tightly in the perinuclear region (Fig. 3 E). Vesicles that were labeled with KChIP1-ECFP in cells that were doubly transfected with αSNAP-(L294A) (Fig. 3 F) were distributed less diffusely than in normal cells, but did not colocalize with VSVG-GFP in triply transfected cells (Fig. 3 G). This indicates that the two proteins reside on different vesicle populations.

KChIP1 is associated with non-COPII-coated post-ER transport vesicles

We tested other intracellular markers for KChIP1-labeled vesicles, including P23 and P24, members of the P24 protein family. These proteins may be cargo receptors in ER-derived COPII transport vesicles, and therefore, were candidates to test for KChIP1-containing vesicles (Blum et al., 1999). EGFP-P23

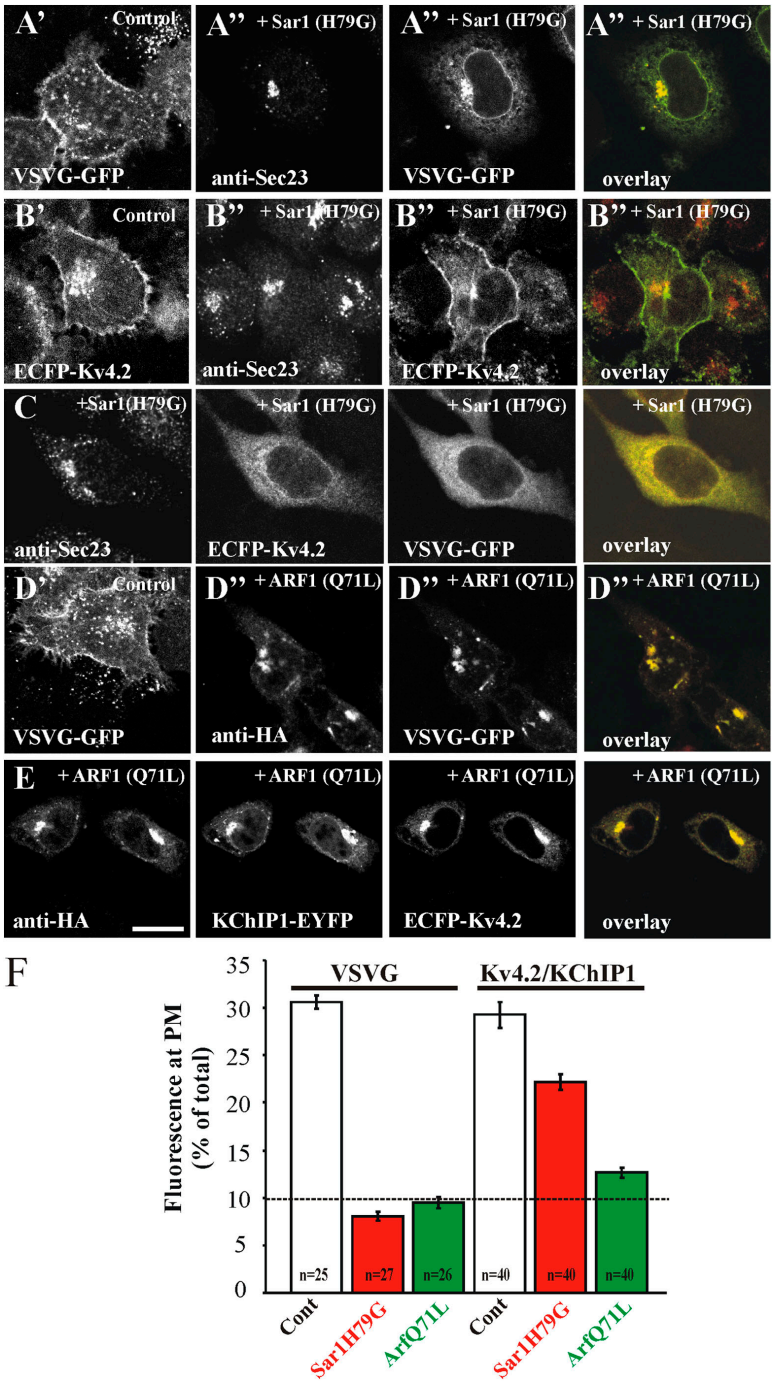

Figure 5. **Effect of disrupting COPII or COPI on the traffic of VSVG and Kv4.2 to the cell surface.** (A) HeLa cells were transfected to express VSVG-GFP in the absence (A′) or presence (A″) of Sar1(H79G). After transfection, cells were maintained at 37°C for 24 h. Sar1(H79G)-transfected cells were stained with anti-Sec23. In cells cotransfected with Sar1(H79G), its effectiveness was checked by examining distribution of Sec23, which became concentrated in a perinuclear region. Traffic of VSVG to the plasma membrane was inhibited by the Sar1 mutant. (B) HeLa cells were cotransfected with KChIP1-EYFP and ECFP-Kv4.2 in the absence (B′) or presence (B″) of Sar1(H79G), and its effectiveness was checked by examining distribution of Sec23, which became concentrated in a perinuclear region. Traffic of Kv4.2 to the plasma membrane was not prevented by Sar1(H79G). (C) HeLa cells were transfected to express VSVG-GFP and ECFP-Kv4.2 in the presence of Sar1(H79G). The traffic of both proteins out of the ER was inhibited and they can be seen to be colocalized in the overlay. (D) HeLa cells were transfected to express VSVG-GFP in the absence (D′) or presence (D″) of ARF1(Q71L). ARF1(Q71L)-transfected cells were stained with anti-HA to detect the ARF1(Q71L). Traffic of VSVG to the plasma membrane was inhibited by the ARF1 mutant. (E) HeLa cells were cotransfected with KChIP1-EYFP, ECFP-Kv4.2 and ARF1(Q71L). Traffic of Kv4.2 to the cell surface was blocked by ARF1(Q71L). Bars, 10 μm. The color overlays show VSVG-EGFP or ECFP-Kv4.2 in green and anti-Sec23 in red (A and B), VSVG-GFP in green and ECFP-Kv4.2 in red (C) or with anti-HA staining in red (D and E) with colocalization seen in yellow. (F) VSVG-EGFP or ECFP-Kv4.2 fluorescence was imaged in control cells or cells expressing Sar1(H79G) or ARF1(Q71L), and quantified by drawing regions of interest around the outside and the inside of the plasma membrane to allow determination of the percentage of total fluorescence at the plasma membrane. Data derived from the indicated number of cells are expressed as mean ± SEM.

and EGFP-P24 localized to punctate structures that were concentrated in the perinuclear region. However, they did not show overlap with KChIP1-hcRed in cotransfected HeLa cells (unpublished data). We also did not see a convincing overlap of KChIP1-EYFP and the COPII coat protein, Sec23 (Fig. 4 A). One problem with labeling COPII-coated transport vesicles is that the COPII coat can be lost rapidly after budding from the ER (Aridor et al., 1995; Stephens et al., 2000). Uncoating of the COPII-coated vesicles is initiated by GTP hydrolysis by Sar1, and can be inhibited by expression of the constitutively active Sar1(H79G) mutant (Aridor et al., 1995; Rowe et al., 1996). In the presence of the Sar1(H79G) mutant, Sec23-labeled structures accumulated in a perinuclear region (Fig. 4 B), as would be expected if COPII vesicles reached the ERGIC but could not uncoat and fuse with this compartment. In contrast, the KChIP1-EYFP–labeled vesicles did not have an altered distribution (Fig. 4 B). No colocalization was observed, at the light microscope level, between Sec23 and KChIP1 in cells that coexpressed Sar1(H79G) (Fig. 4 B). This suggested that the KChIP1-EYFP–labeled vesicles were not COPII-coated vesicles; however, to confirm this, immunoelectron microscopy was performed. Structures containing KChIP1-EYFP were detected using anti-GFP and were found to be vesicles that were similar in size (50–100 nm) to vesicles that were labeled for

Sec23. Again, minimal overlap was observed, and in most cases, structures were labeled for YFP or Sec23 alone. Only 8 of 175 vesicles (4.8% colocalization) were labeled for YFP and Sec23 in control cells, and 7 of 151 vesicles (4.9%) in cells expression Sar1(H79G).

Certain cargos, such as procollagen, can traffic from the ER to the Golgi in non–COPII-coated structures. Nevertheless, these all require COPII for their budding from the ER (Mironov et al., 2003; Stephens and Pepperkok, 2004). To test whether Kv4.2/KChIP1 require COPII for traffic through the secretory pathway, the effect of Sar1(H79G) on traffic was examined. Its expression blocked traffic of all previously studied cargos to the cell surface (Kuge et al., 1994; Aridor et al., 1995, 2001; Stephens and Pepperkok, 2004), in some cases, by preventing export from the ER. We used the same experimental conditions to follow VSVG and Kv4.2/KChIP1, which resulted in a similar extent of traffic of each to the plasma membrane (Fig. 5 A', B', D', and F). To assess the effect of Sar1(H79G), the cells were stained with anti-Sec23 to verify the effectiveness of the mutant in redistributing Sec23 to the perinuclear region in the transfected cells. Only cells in which this could be established were examined for traffic of VSVG or Kv4.2/KChIP1. Expression of Sar1(H79G) effectively inhibited traffic of VSVG-EGFP to the plasma membrane (Fig. 5 A). In all cells that we examined, VSVG-EGFP remained in the ER and in perinuclear structures where it overlapped with Sec23. From the quantitative analysis of peripheral VSVG-EGFP, it was clear that inhibition of traffic to the plasma membrane essentially was complete, because the residual level of peripheral fluorescence (~10% shown by the broken line in Fig. 5 E) is the same as that found using this assay with proteins that are unable to traffic out of the ER (Fig. S5; available at http://www.jcb.org/cgi/content/full/jcb.200506005/DC1) or beyond the Golgi (Fig. 1). In contrast, expression of Sar1(H79G) did not prevent Kv4.2 from reaching the plasma membrane when it was expressed in the presence of KChIP1, despite redistribution of Sec23 (Fig. 5 B). Plasma membrane expression was visible in 24/40 (60%) of the cells. Perinuclear ECFP-Kv4.2 fluorescence was observed often in Sar1(H79G)-expressing cells, as it was in control cells, but this did not overlap substantially with Sec23. Quantification supported the conclusion that significant traffic of Kv4.2/KChIP1 to the plasma membrane still occurred in the presence of Sar1(H79G) (Fig. 5 F). Interestingly, when ECFP-Kv4.2 was expressed without KChIP1, its exit from the ER was inhibited by Sar1(H79G). This also was seen in cells that expressed VSVG-GFP; in all cases examined in which traffic of VSVG-GFP was blocked, ECFP Kv4.2 was retained in reticular ER-like structures and overlapped completely with VSVG-GFP (Fig. 5 C), unlike the normal Golgi localization of the channel (Figs. S1 and S2).

For comparison with Sar1(H79G), parallel experiments were performed using a GTP-loaded ADP-ribosylation factor 1 (ARF1) mutant (Zhang et al., 1994), which would have similar effects on coat disassembly, but on COPI vesicles. Expression of ARF1(Q71L) effectively blocked traffic of VSVG (Fig. 5 D) and Kv4.2/KChIP1 (Fig. 5 E) to the cell surface (Fig. 5 F) with their retention in the modified ARF1(Q71L)-positive Golgi complexes. These experiments show that traffic of Kv4.2/KChIP1, when expressed together, is fully dependent on COPI, but does not have a strict requirement for COPII.

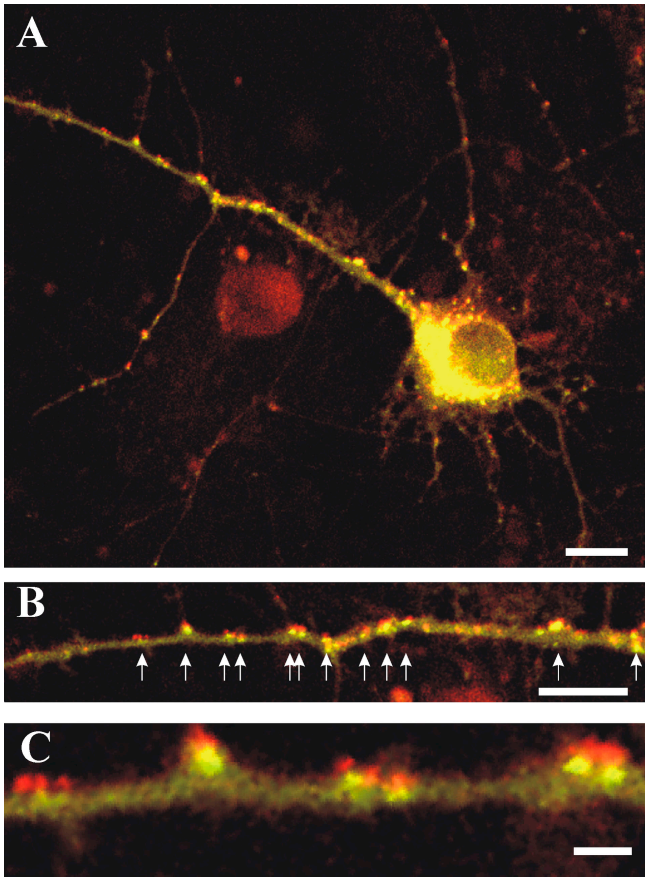

Figure 6. **Co-distribution of KChIP1-EYFP with Golgi structures in rat hippocampal neurons.** Primary neuronal cultures were transfected to express KChIP1-EYFP (green) and Golgi-ECFP (red). The confocal images show the presence of both proteins in the cell body and at specific sites along the dendrites. B and C show higher magnifications of a dendrite; the arrows in B indicate the Golgi outposts. Bars, 10 μm (A and B) or 2 μm (C).

KChIP1 co-distributes with dendritic Golgi outposts in hippocampal neurons

We used HeLa cells in this study because these do not express KChIP proteins or Kv4 channels endogenously, and allow analysis of their individual targeting and trafficking. To test whether the same situation occurred in neurons we examined primary cultures of rat hippocampal neurons. Endogenous KChIP1 was found in punctate structures in neurons (Shibata et al., 2003), and KChIP1-EYFP was targeted to intracellular punctate structures in the cell body (Fig. S4, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200506005/DC1) and was distributed along the dendrites (Fig. S4 C) in proximal and distal areas (>120 μm from the cell body). In contrast, the closely related NCS1-EYFP was found on the plasma membrane (Fig. S4, D and E), as seen in HeLa cells (O'Callaghan et al., 2002, 2003a). In dendrites, it did not show evidence of a punctate localization (Fig. S4 F). Hippocampal neurons were shown to possess satellite secretory pathways with ER exit

sites and associated Golgi outposts distributed in dendritic processes a long distance from the cell body (Pierce et al., 2001; Horton and Ehlers, 2003; Aridor et al., 2004). We examined the relationship of KChIP1-containing structures to these outposts in hippocampal neurons that were cotransfected with KChIP1-EYFP and Golgi-targeted ECFP. Both proteins were expressed in the perikarya and were present in the dendrites (Fig. 6). As expected, KChIP1-EYFP did not overlap exactly with the Golgi marker along the dendrites, but showed a marked co-distribution so that essentially every Golgi outpost had an associated KChIP1-EYFP–labeled structure.

Discussion

Many characterized channels and receptors are retained within the ER, and require association of escort proteins or oligomerization to mask their ER-retention signal and allow them to traffic beyond the ER (Ma and Jan, 2002). In contrast, we found that Kv4.2 can traffic to the Golgi apparatus by itself. In fact, no functional ER retention signal could be found in the Kv4.2 N-terminal domain with which KChIP1 interacts, and which determines the intracellular retention of the Kv4.2 subunits when they are expressed alone (Bahring et al., 2001; Shibata et al., 2003). In addition, mutations that prevent tetramerization of Kv4.2 recently were reported to result in a different intracellular localization from the wild-type channel, because this form is retained in the ER (Kunjilwar et al., 2004) but can traffic to the cell surface in cells that express high levels of KChIP3. We observed a more ER-like distribution of this mutant form of Kv4.2 in HeLa cells that did not colocalize with KChIP1, and that KChIP1 was unable to stimulate traffic of this ER-retained form of Kv4.2 to the cell surface (Fig. S5).

The KChIP1 EF-hand mutant (KChIP1(2–4)-EYFP), which is unable to bind Ca^{2+}, failed to promote traffic of the channel to the plasma membrane, and inhibited Kv4.2 from reaching the Golgi apparatus. This suggested that the two proteins interact in pre-Golgi structures and that traffic of these to the Golgi requires Ca^{2+} bound to KChIP1. Instead, KChIP1(2–4)-EYFP seems to capture Kv4.2 on the post-ER vesicles to which it is targeted, which is consistent with the demonstrated ability of the EF-hand mutant to bind Kv4.2 (An et al., 2000). The organelles of the secretory pathway did not show any morphologic abnormalities, which confirmed that the punctate distribution of Kv4.2 that was caused by KChIP1(2–4)-EYFP was not due to fragmentation of the Golgi, but was a result of blockade of ER-to-Golgi traffic of the channel. In addition, exocytotic traffic, in general, was not disrupted by coexpression of the mutant because it did not prevent the VSVG protein from reaching the plasma membrane. The requirement for a Ca^{2+}-sensing protein as an escort protein for the traffic of Kv4 channels to the cell surface could be consistent with this being a distinct process from the simple masking of a retention signal, and instead, could allow specific Ca^{2+}-dependent control of the traffic of the Kv4 channels to the cell surface. However, it is possible that the Ca^{2+} binding is simply a structural requirement, rather than being due to sensing of changes in Ca^{2+} concentration if KChIP1 binds Ca^{2+} constitutively at resting Ca^{2+} levels. Bacterially expressed KChIP1 in vitro undergoes a measurable conformational change on Ca^{2+} binding with an affinity of ~60–100 nM (unpublished data). Analysis of the resting Ca^{2+} concentration in neuronal dendrites found this to be in the range of 40–65 nM (Sabatini et al., 2002). Kv4 channels are constitutively present on dendritic plasma membrane, but activity-dependent targeting of Kv4.2 channels from the cell body to synaptic sites has been demonstrated in cerebellar granule neurons (Shibasaki et al., 2004), which indicated how regulation of Kv4 channel localization could provide a dynamic link between synaptic activity and dendritic excitability. The distribution of expressed KChIP1 in hippocampal neurons indicated an association with Golgi outposts in the dendrites (Horton and Ehlers, 2003). It remains to be determined whether KChIP1 can stimulate local traffic of Kv4 channels (e.g., through the satellite secretory pathway in dendrites) (Pierce et al., 2001; Horton and Ehlers, 2003; Aridor et al., 2004).

The specific targeting of KChIP1 via its myristoyl tail is functionally important for promoting Kv4.2 channel traffic to the plasma membrane (O'Callaghan et al., 2003a). Other KChIPs are membrane targeted by distinct mechanisms, including palmitoylation (Takimoto et al., 2002). There is not likely to be competition in mediating channel traffic between KChIP proteins with differing targeting mechanisms, because they seem to be expressed in distinct classes of neurons (Rhodes et al., 2004). In our attempt to characterize KChIP1 vesicles, we show that they are different from vesicles that contain P23/P24 proteins, which cycle constitutively between peripheral elements of the ERGIC and the cis-Golgi network (Blum et al., 1999). The ts045 VSVG-GFP did not colocalize with KChIP1 vesicles at any stage after exit from the ER during its traffic through the secretory pathway. A partial colocalization with VSVG-GFP after 5 min at 32°C was observed that would be consistent with KChIP1 being associated with vesicles that exit from nearby, but distinct, regions of the ER, or alternatively, a partial overlap of the two pathways. A significant distinction between VSVG-GFP and KChIP1 vesicles is supported by the data from the use of the α-SNAP mutant. To test whether we observed little overlap of KChIP1 with VSVG because the vesicles are too transient to be detected, but the proteins otherwise traffic on the same pathway, we blocked vesicular traffic throughout the secretory pathway by overexpression of the dominant negative αSNAP (L294A) mutant (Barnard et al., 1997), which was shown not to inhibit VSVG exit and budding of vesicles from the ER, but did prevent their subsequent fusion (Mironov et al., 2003). We examined whether KChIP1 and VSVG accumulate in the same compartment, but no colocalization was found; this showed that most of the KChIP1 resides on vesicles that traffic along a ER-to-Golgi pathway that is distinct from the one that is involved in VSVG traffic.

Although transport from the ER to the ERGIC is mediated mainly by COPII-coated vesicles, the vesicles that KChIP1 associated with were not COPII coated. This comes from the data on cells that express the Sar1(H79G) mutant (Kuge et al., 1994; Aridor et al., 1995). This constitutively active

Sar1 mutant blocks traffic to the Golgi complex (Kuge et al., 1994), and results in accumulation of COPII vesicles (Oka and Nakano, 1994) which then have a perinuclear distribution (Stephens and Pepperkok, 2004). The distribution of KChIP1 vesicles was not affected by the expression of Sar1(H79G) nor did it colocalize with COPII coat components. It was reported that some ER-to-Golgi carriers, such as those that transport procollagen, bud off the ER but do not label with COPII; this indicated that COPII is not the only mediator of cargo export from the ER (Stephens and Pepperkok, 2001, 2002). As for KChIP1-containing vesicles, these carriers were described to move from the ER to the Golgi in a microtubule-dependent manner (Mironov et al., 2003). Despite the lack of COPII on the structures that are involved in procollagen traffic, these structures require COPII for their budding from the ER, and Sar1(H79G) inhibited traffic to the cell surface of all cargos that have been examined (Mironov et al., 2003; Stephens and Pepperkok, 2004). We found substantial traffic of Kv4.2/KChIP1 to the plasma membrane in cells that express Sar1(H79G) under the same conditions that totally blocked traffic of VSVG. A partial inhibition of Kv4.2/KChIP1 traffic by Sar1(H79G) was seen; however, it is unclear whether this was due to a direct requirement for COPII for budding of Kv4.2-containing vesicles from the ER or to a more general effect in the cell of the inhibition of other traffic from the ER. Therefore, we cannot be certain that traffic of Kv4.2/KChIP1 is fully COPII independent. However, the effectiveness of Sar1(H79G) was emphasized by the fact that when expressed without KChIP1, the Kv4.2 channel remained in the ER and colocalized with VSVG-GFP in all cells that were examined. This suggests that interaction with KChIP1 switches the exit of Kv4.2 from the ER from COPII to non-COPII vesicles. In yeast, COPI vesicles also can bud from the ER (Bednarek et al., 1995), and COPI was involved at an early step in ER exit in mammalian cells (Stephens and Pepperkok, 2002). Traffic of Kv4.2 in the presence of KChIP1 was blocked nearly completely at the Golgi complex by the GTP-bound ARF mutant, ARF(Q71L), which indicated that its traffic is COPI dependent. The GDP-bound ARF(T31N) mutant was shown to inhibit ER export (Dascher and Balch, 1994). When Kv4.2 is expressed without KChIP1, it colocalizes with β-COP (O'Callaghan et al., 2003a), and ARF(T31N) blocks its transport to the Golgi (unpublished data). When KChIP1 was expressed with Kv4.2, ARF(T31N) did inhibit exit of Kv4.2 from the ER in some transfected cells (unpublished data), but as reported previously (Dascher and Balch, 1994), the effect of this mutant on ER export was variable between cells. Therefore, it is possible that exit of Kv4.2 from the ER in the presence of KChIP1 involves COPI, rather than COPII, vesicles.

Overall, the results of this study support the conclusion that KChIP1 vesicles represent a distinct non-COPII, post-ER vesicle type that is separate from those that are involved in other commonly studied forms of constitutive traffic. The existence of Ca^{2+}-dependent vesicular traffic of Kv4.2 channels provides evidence for a potential mechanism for the regulation of localized vesicular transport of integral membrane proteins, either from the neuronal cell body to dendrites or from the satellite secretory pathway in dendrites to the dendritic plasma membrane (Pierce et al., 2001; Horton and Ehlers, 2003; Aridor et al., 2004). This could have an important role in modifying functional cell surface channels, and thus, contribute to synaptic plasticity.

Materials and methods

Plasmids

The pcDNA3-Kv4.2 construct was made by insertion of the human Kv4.2 sequence (NM_012281) amplified from the AB1701_A07 plasmid (Origene Technologies, Inc.) by PCR, into the pcDNA3.1(+) vector (Invitrogen). The primers contained endonuclease sites (underlined) to facilitate this cloning. The sense primer was 5′-CTTCAGAATTCCATGGCGGCGGGGGGTGGCA-3′ (EcoRI) and the antisense primer was 5′-ATTCCTCTAGACTTACAAAGCAGAAACTC-3′ (XbaI). The KChIP1-pHcRed construct was made by cutting out the KChIP1 sequence from the KChIP1-EYFP construct (O'Callaghan et al., 2003a) and inserting it into the pHcRed-Tandem-N1 vector (EVROGEN), using the endonucleases, SacI and EcoRI. The pcDNA3-Sar1(H79G) construct was made by the insertion of the Sar1(H79G) mutant amplified from the Sar1(H79G)pET-3c vector (gift from W.E. Balch, Scripps Research Institute, La Jolla, California), by PCR, into the pcDNA3.1(-) vector (Invitrogen). The primers contained endonuclease sites (underlined) to facilitate this cloning. The sense primer was 5′-GATGCTAGCATGTCCTTCATATTTGACTGG-3′ (NheI) and the antisense primer was 5′-GCCGGATCCCTAATCGATGTACTGTGCCAT-3′ (BamHI). The KChIP1-ECFP, pcDNA3-KChIP1, ECFP-Kv4.2, NCS-1-ECFP, and pcDNA3-αSNAP(L294A) constructs were made as described previously (Barnard et al., 1997; O'Callaghan et al., 2003a). The EGFP-P23 and EGFP-P24 plasmids were provided by F.A. Barr (MPI of Biochemistry, Munich, Germany), and the ts045 VSVG-GFP plasmid (Presley et al., 1997) was provided by J. Lippincott-Schwartz (National Institutes of Health, Bethesda, Maryland). The EYFP-tagged KChIP1 triple EF-hand mutant KChIP1(2–4)-EYFP was made by using the QuickChange site-directed mutagenesis kit (Stratagene Europe) and by changing the Asp and Gly residues at positions 1 and 6, respectively, to Ala within EF-hands 2, 3, and 4 of KChIP1 (pKChIP1(D99A, G104A, D135A, G140A, D183A, G188A)-EYFP), as described previously (An et al., 2000). The sequences of these constructs were confirmed by automated sequencing by Oswel or DBS Genomics. Plasmid encoding ARF1(Q71L) with a COOH-terminal HA tag was described previously (Haynes et al., 2005). The ECFP-ER plasmid encoding ECFP linked to the calreticulin ER targeting sequence and the KDEL retrieval sequence to allow localized expression in the ER lumen, and the EYFP-Golgi plasmid encoding EYFP linked to the NH_2 terminus of β 1,4-galactosyltransferase for trans- and medial-Golgi targeting were obtained from CLONTECH Laboratories, Inc. For the Golgi-ECFP plasmid, the EYFP was substituted with EFCP by using digestion with Age I and BrsGI and subsequent ligation.

Culture and transfection of HeLa cells

HeLa cells were grown in DME (GIBCO BRL) containing 5% FCS (GIBCO BRL) and 1% nonessential amino acids (GIBCO BRL) at 37°C in an atmosphere of 5% CO_2. 4–16 h before transfection, the cells were plated onto glass coverslips in a six-well plate at ~100,000 cells/well. The transfection reaction mixture contained 95 μl DME (GIBCO BRL), 3 μl Fugene (Roche), and 2 μl of plasmid DNA (500 μg/μl). This was incubated at room temperature for 30 min before being added drop-wise to the cells. The cells were maintained for 16–66 h before being used in experiments. Similar conditions were used for the culture and transfection of COS7 cells.

Culture and transfection of hippocampal neurons

The hippocampi from 1–2-d-old male Wister rats were removed and incubated in trypsin at 37°C for 10 min. The cells were allowed to settle to the bottom of the tube and the trypsin was removed. The cells were resuspended in 1 ml of media (MEM [GIBCO-BRL], 10% horse serum [Sigma-Aldrich], insulin at 0.005 mg/ml [Sigma-Aldrich], and gentamicin at 25 μg/ml [Sigma-Aldrich]) and this wash step was repeated twice. The cells were passed through three graded pipettes, counted, and diluted in fresh media to allow plating at a density of ~30,000 cells/ml. The cells were aliquoted onto coverslips that were precoated with poly-l-ornithine (Sigma-Aldrich), and were incubated at 37°C for 2 h. The excess media was removed, and a fresh 2 ml of media was added to each well. The neurons were left to mature for 14–21 d before transfection, with 25% of the me-

dia changed at 4-d intervals. For transfection, calcium phosphate precipitation (Promega) was used, and each well was incubated with 6 μg of each plasmid following the manufacturer's protocol. The neurons were incubated for an additional 48–72 h before imaging.

Immunocytochemistry

Cells attached to coverslips were washed twice in phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4) and fixed in PBS containing 4% formaldehyde for 30 min. The cells were washed twice in PBS and incubated in PBT (PBS, 0.1% Triton X-100, 0.3% BSA) for 30 min. The PBT was removed and the primary antibody (rabbit [rb] or mouse [m]) was added at the appropriate dilution in PBT (anti-Kv4.2 [rb] 1 in 500 [Exalpha Biologicals, Inc.], anti-KChIP1 [m] 1 in 10 [J.S. Trimmer, University of California, Davis], anti-calnexin [rb] 1 in 100 [Sigma-Aldrich], anti-Sec13 [rb] 1 in 100 [W. Balch]; anti-ERGIC-53 [m] 1 in 1000 [H.-P. Hauri, University of Basel, Basel, Switzerland]; anti-β-COP [rb] 1 in 500 [Affinity BioReagents, Inc.]; anti-Sec23 [rb] 1 in 330 [Affinity BioReagents, Inc.]; anti-αSNAP [rb] 1 in 100 [Santa Cruz Biotechnology, Inc.]; anti-HA [m] 1 in 100 [BAbCO]). The primary antibody was incubated for 2 h, removed, and the cells washed three times in PBT. The cells were incubated for an additional hour with the appropriate biotinylated secondary antibody (GE Healthcare) diluted to 1 in 100 with PBT. The cells were washed three times in PBT, and then incubated in streptavidin-Texas red (GE Healthcare) diluted 1 in 50 with PBT for 30 min. The cells were washed three times with PBT. The coverslips were dried and mounted in antifade Vectashield (Vector Laboratories). For electron microscopy, transfected HeLa cells were fixed for 1 h, washed with 0.2 M phosphate buffer, scraped from the culture dish, and pelleted at 8,000 rpm in a microfuge. The cells were resuspended in warm gelatin (10% in phosphate buffer), and repelleted at maximum speed in the microfuge. After cooling, the gelatin-embedded cells were infiltrated with polyvinylpyrrolidone-sucrose overnight at 4°C, and then processed for frozen sectioning as described previously (Liou et al., 1996). Ultrathin frozen sections (50–70 nm) were immunolabeled using goat anti–rabbit 15-nm gold and goat anti–mouse 10-nm gold (British Biocell International), stained, and viewed on a 120-kV FEI Tecnai Spirit microscope.

Confocal laser scanning microscopy

For confocal laser scanning microscopy, transfected cells were examined with a Leica TCS-SP-MP microscope or a Leica TCS-SP2-AOBS microscope using a 22-μm pinhole and a 63× water immersion objective with a 1.2 numerical aperture. Live cells were bathed in a Krebs-Ringer solution (145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes; pH 7.4). For optimal imaging of ECFP-tagged proteins, the cells were excited at 405 nm and light was collected at 450–500 nm. EGFP was imaged using excitation at 488 nm and light collection at 500–550 nm, EYFP was excited at 514 nm and emission collected at 530–585 nm, and Texas red and HcRed were imaged by excitation at 594 nm with light collected at 610–700 nm. All images were compiled in CorelDraw.

Online supplemental material

Fig. S1 shows a comparison of the localization of tagged and untagged KChIP1 and Kv4.2. Fig. S2 shows evidence that Kv4.2 expressed alone in HeLa cells reaches the Golgi complex. Fig. S3 shows the localization of Kv4.2 in cells expressing the EF-hand mutant of KChIP1 and its relationship to secretory pathway markers. Fig. S4 shows that localization of KChIP1 and NCS-1 is expressed in hippocampal neurons. Fig. S5 shows the localization of a tetramerization mutant of Kv4.2 and the failure of KChIP1 to stimulate its traffic to the cell surface. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506006/DC1.

Plasmid constructs were provided by F.A. Barr, W.E. Balch, and J. Lippincott-Schwartz. Antisera were provided by H-P. Hauri, W.E. Balch, and J.S. Trimmer.

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