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Kinesin-1 plays a role in transport of SNAP-25 to the plasma membrane

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ABSTRACT

The cellular molecular motor kinesin-1 mediates the microtubule-dependent transport of a range of cargo. We have previously identified an interaction between the cargo-binding domain of kinesin-1 heavy chain KIF5B and the membrane-associated SNARE proteins SNAP-25 and SNAP-23. In this study we further defined the minimal SNAP-25 binding domain in KIF5B to residues 874–894. Overexpression of a fragment of KIF5B (residues 594–910) resulted in significant colocalization with SNAP-25 with resulting blockage of the trafficking of SNAP-25 to the periphery of cells. This indicates that kinesin-1 facilitates the transport of SNAP-25 containing vesicles as a prerequisite to SNAP-25 driven membrane fusion events.

Introduction

The kinesin-1 family, a member of the kinesin superfamily (KIF), belongs to the N-kinesins with a highly conserved N-terminal motor domain which binds microtubules [1]. Kinesin-1 is a tetrameric protein consisting of two identical 120 kDa heavy chains and two identical 64 kDa light chains which mediates plus-end directed microtubule-dependent transport of cargo [1]. The mammalian genome contains three kinesin-1 heavy chain (KHC) genes (*KIF5A*, *KIF5B* and *KIF5C*) [2–4] and three kinesin-1 light chain (KLC) genes (*KLC1*, *KLC2*, *KLC3*) [5,6]. KIF5A and KIF5C are expressed only in neurons while KIF5B is ubiquitously expressed [4]. Both KLC1 and KLC2 have been shown to interact with both KIF5A and KIF5B [6] although KLC appears to always be present as a homodimer in native tetrameric kinesin-1 [7]. KHC contains three domains including the microtubule-binding ATPase motor/head, the stalk and the tail. The stalk consists of heavy chain homodimers [8] as well as heavy and light chain heterodimers formed *via* heptad-repeat regions in the N-terminal half of the light chain and the C-terminal end of the stalk domain of heavy chain [9–11]. The C-terminus KHC tail domain contains both a heptad-repeat region and a globular domain. This globular domain, along with KLC, is responsible for autoinhibition of kinesin, due to formation of a folded conformation where the tail interacts with the motor domain and inhibits motor activity [12–14].

It is becoming apparent that both KHC and KLC are capable of interacting with multiple cargo typically *via* adaptor or scaffolding proteins [15]. In the case of KHC the C-terminal stalk/tail domain was initially implicated in binding to cargo [16]. Subsequently, studies with *Neurospora* KHC have demonstrated cargo-binding, in the absence of KLC, which maps to a region (KIF5B residues 825–910) in the heptad-repeat region of the C-terminal KHC tail domain [13]. A number of cellular proteins have now been shown to directly bind to this KHC tail domain [1,15]. While KLC is not essential for motor activity, it does play an essential role in the function of kinesin-1 both in the binding and targeting of cargo [17]. The various isoforms of KLC1, generated as a result of alternative splicing in the C-terminal region, have been shown to play a role in targeting of cargo to cellular compartments during transport [18]. In addition, several cellular proteins have now been identified which bind directly to the C-terminal tetratricopeptide repeat (TPR) region of KLC [15].

The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which include at least 38 members in mammals, are essential for intracellular membrane fusion [19,20]. SNARE proteins contain at least one coiled-coil domain consisting of heptad repeats referred to as the SNARE motif (further classified as Qa, Qb, Qc or R-SNAREs) and usually a transmembrane domain [19,20]. An exception is the Qbc-SNARE subfamily which includes the synaptosomal-associated proteins (SNAP) of 23, 25, 29 and 47 kDa referred to as SNAP-23, SNAP-25, SNAP-29 and SNAP-47 [19,20]. This subfamily lacks a transmembrane domain and contains two SNARE domains, Qb and Qc, with an intervening linker that in the case of SNAP-23 and SNAP-25, contain

Abbreviations: GRIP1, α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor subunit-GluR2-interacting protein; HSV-1, herpes simplex virus type 1; KHC, kinesin-1 heavy chain; KIF, kinesin superfamily; KLC, kinesin-1 light chain; RanBP2, Ran-binding protein 2; SNAP-23, synaptosomal-associated protein (SNAP) of 23 kDa; SNAP-25, SNAP of 25 kDa; SNARE, *N*-ethylmaleimide-sensitive factor attachment protein receptor; TPR, tetratricopeptide repeat.

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multiple cysteine residues which are palmitoylated to direct association with membranes. All members of this SNARE subfamily have been identified on purified synaptic vesicles but are also present on other intracellular membrane pools [21].

Prior to intracellular membrane fusion events there is a key requirement for trafficking of vesicles containing components necessary for membrane fusion. Transport of vesicles from the trans-*Golgi* network or the endocytic recycling compartment to the plasma membrane depends particularly on kinesin-1 and kinesin-2, another N-kinesin [22–24]. It has also been proposed that components of the nerve terminal, including synaptic vesicles and membranes containing presynaptic membrane proteins, are either transported separately or are pre-assembled in the cell body prior to kinesin-dependent transport down axons [25]. Furthermore, KIF5A and KIF5B have been detected on purified synaptic vesicles [21]. A direct interaction of KIF5B with the synaptic vesicle protein SNAP-25 (as well as the homologue SNAP-23) has been reported in our laboratory [26]. In addition, KIF5B has also been shown to directly interact with syntabulin to mediate anterograde axonal transport of syntaxin-1 containing synaptic vesicles [27] and mitochondria [28]. In the case of transport of synaptic vesicles syntabulin acts as a link between syntaxin-1 and KIF5B [27]. These transported syntaxin-1 containing vesicles also contained SNAP-25 raising the possibility of multiple links with KIF5B [28].

In this study we have investigated further the role of the interaction of SNAP-25 and KIF5B. The minimal binding domain in KIF5B for SNAP-25 was delineated using an *in vitro* pulldown assay. Subsequent coexpression in HeLa cells of SNAP-25 and an inhibitory fragment of KIF5B, encompassing the minimal SNAP-25 binding domain, resulted in marked colocalization with and blockage of SNAP-25 transport to the plasma membrane.

Materials and methods

Expression constructs. All DNA fragments were amplified by PCR using a Geneamp® XL PCR Kit (PE Applied Biosystems). DNA fragments encoding KHC KIF5B amino acids 594–824, 594–859, 594–873, 594–894 and 594–910 were generated from pGEX-2T/KIF5B 555-963 (provided by Dr. Ron Vale, UCSF) [9] using 5′ primers that contained an EcoRI site and 3′ primers that contained a XhoI site. KHC KIF5A amino acid fragment 591–908 was made from full-length KIF5A in pBSK (pWBC7; kindly provided by Dr. Ron Vale, UCSF) [2] using a 5′ primer that contained an EcoRI site and 3′ primer that contained a SalI site. KHC PCR products were then cloned between the EcoRI and SalI sites of pCOLADuet-1 (Novagen) or pEYFP-C1 (Clontech) to generate in-frame N-terminal His_6 or EYFP tags, respectively. Full-length SNAP-25 (amino acids 1–206) was generated from pGEX-2T/SNAP-25 [26] using a 5′ primer containing a BglII site and a 3′ primer containing a XhoI site (with or without a stop codon). SNAP-25 PCR product containing a stop codon was cloned between the BglII and XhoI sites of pCOLADuet-1, with and without a KHC insert, to generate untagged SNAP-25. SNAP-25 PCR product without a stop codon was cloned between the BglII and SalI sites of pECFP-N1 (Clontech) to generate an in-frame C-terminal ECFP tag.

In vitro pulldown assay. The coexpression plasmid pCOLADuet-1 containing KHC fragments and/or SNAP-25 was introduced into *E. coli* strain BL21 (DE3). Proteins were expressed, harvested and lysed as described previously [26]. The conditions for binding and elution from nickel-activated beads were as previously described [26]. Protein complexes were separated by SDS–PAGE and proteins identified by Coomassie staining as previously described [9].

Cell culture, transfection and virus infections. HeLa cells were grown to ~80% confluency in DMEM (Invitrogen) supplemented with 9% (v/v) FCS and 1% (v/v) penicillin/streptomycin (Sigma) on glass coverslips in 24-well tissue culture plates prior to transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were processed for confocal microscopy at 48 h post-transfection. In some cases, at 24 h post-transfection with pEYFP-KIF5B 594–910, HeLa cells were infected at a multiplicity of infection of 5 pfu/cell with herpes simplex virus type 1 (HSV-1) strain 17 (provided by Dr. Frazer Rixon, MRC Glasgow) and then processed for confocal microscopy at 16 h post-infection.

Immunofluorescence confocal microscopy. Cultured cells on coverslips were fixed, permeabilised and immunolabelled at room temperature. The buffer used for fixation and antibody dilutions was a combination of PBS/BSA/cold water fish gelatin (Sigma) with 10% (v/v) and 1.5% goat serum (v/v), respectively. Cells were fixed for 20 min with 4% (v/v) paraformaldehyde and then permeabilised for 10 min with 0.1% (v/v) Triton X-100. Non-specific antibody binding was blocked by preincubation for 15 min in 10% (v/v) goat serum. Primary antibody incubations were for 45 min while secondary antibody incubations were for 1 h. The primary antibodies used included mouse monoclonal against α-tubulin (Invitrogen) and rabbit polyclonal against HSV-1 glycoprotein gD (Abcam). Secondary antibodies were AlexaFluor 633-conjugated goat anti-mouse IgG and AlexaFluor 633 goat anti-rabbit IgG (Invitrogen). Fluorescent images were captured, and overlayed using a Leica TCS SP2 confocal microscope and Leica confocal software version 2.61, build 1537.

Results

Definition of the minimal SNAP-25 binding site in KIF5B

Previously our laboratory identified an interaction between SNAP-25 (and SNAP-23) and the cargo-binding domain (residues 825–910) of KHC KIF5B [26]. This interaction was confirmed using a combination of yeast two-hybrid and *in vitro* pulldown assays.

In this study we expanded these previous observations to define a minimal SNAP-25 binding domain in KIF5B. Fragments of KIF5B spanning the stalk domain (containing the KLC-binding site [9,26]) and including C-terminal truncations of the cargo-binding domain were expressed with an N-terminal His_6 tag (Fig. 1A). Each of the KHC constructs begin near the C-terminal end of the hinge region which spans amino acids 505–605 of the stalk domain of KIF5B [14]. In addition, the homologous cargo-binding domain of KIF5A (residues 591–908) was also expressed to establish whether SNAP-25 binds selectively to KIF5B. From the same constructs untagged full-length SNAP-25 was also expressed. All the KIF5 fragments were expressed at similar levels in bacterial lysates, except for KIF5A 591–908 (Fig. 1B). In addition, expression of SNAP-25 was the same in the presence or absence of KHC (Fig. 1B). Complexes were then purified using nickel-activated beads (Fig. 1C). SNAP-25 was found to coelute with KIF5B 594–910 and 594–894 (Fig. 1C). Further truncation of the cargo-binding site essentially abrogated binding of SNAP-25 (Fig. 1C). Untagged SNAP-25, expressed in the absence of KIF5B, did not bind to nickel beads and the SNAP-25 band was not present when KIF5B was expressed alone (Fig. 1B and C). This indicates that the major binding site for SNAP-25 in KIF5B maps to residues 874–894. Attempts to ascertain whether the equivalent region in KIF5A also binds to SNAP-25 were inconclusive due to low expression levels of KIF5A 591–908 (Fig. 1B).

Characterization of an inhibitory fragment of KIF5B

Based on the initial mapping studies, KIF5B 594–910 (contains the minimal SNAP-25 binding domain) and KIF5B 594–824 (lacks

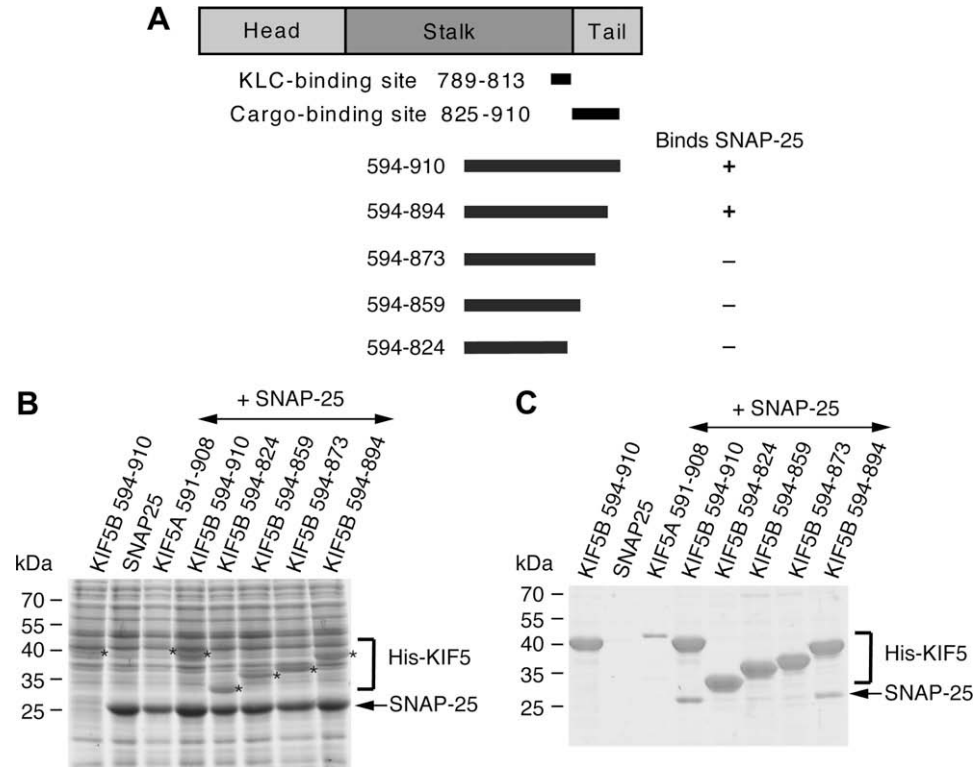

Fig. 1. Mapping of the SNAP-25 binding site in kinesin-1 heavy chain (KHC) KIF5B. (A) Diagram of the domain structure of KHC showing the head, stalk and tail domains [3]. The known kinesin-1 light chain (KLC) [9,26] and cargo-binding sites [13] are highlighted. The fragments generated in this study and ability to bind to SNAP-25 are indicated. (B) Bacterial lysates containing coexpressed His_6-tagged KIF5 fragments and untagged full-length SNAP-25. The position of His-KIF5 fragments are indicated (∗). (C) Protein complexes were purified using nickel-activated beads. SNAP-25 bound specifically to KIF5B 594–910 and KIF5B 594–894, indicating the minimal binding domain for SNAP-25 maps to KIF5B amino acids 874–894. Proteins were separated by 14% SDS–PAGE.

the SNAP-25 binding domain) (Fig. 1A) were tagged at the N-terminus with EYFP. KIF5B 594–824 spans the C-terminal end of the stalk domain while KIF5B 594–910 also includes the N-terminal region of the tail domain which contains the cargo-binding domain (Fig. 1A). In addition, full-length SNAP-25 was tagged at the C-terminus with ECFP. The distribution of each fusion construct was initially assessed in HeLa cells (Fig. 2). Both EYFP and ECFP only were distributed throughout the entirety of transfected HeLa cells

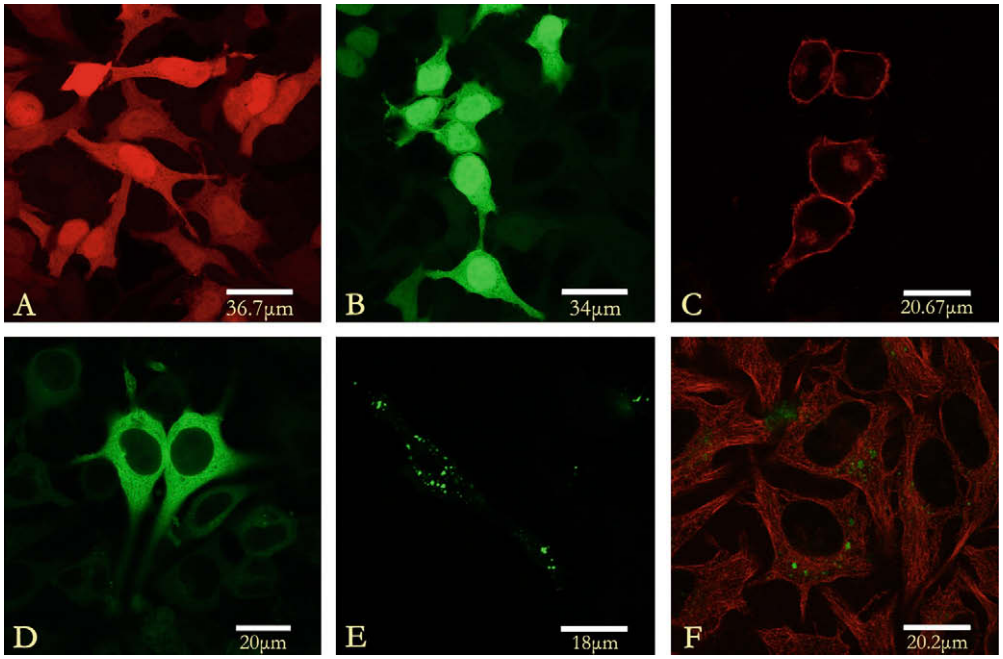

Fig. 2. Distribution in HeLa cells of exogenously expressed KIF5B fragments and SNAP-25. Cells were imaged at 48 h post-transfection. (A) ECFP alone. (B) EYFP alone. (C) SNAP-25-ECFP. (D) EYFP-KIF5B 594–824. (E) EYFP-KIF5B 594–910. (F) EYFP-KIF5B 594–910 (green) does not colocalize with α-tubulin (red).

(Fig. 2A and B). SNAP-25-ECFP was found to distribute mainly to the plasma membrane, as well as to a perinuclear compartment (Fig. 2C) which is a similar distribution previously reported for both endogenous and GFP-tagged SNAP-25 [29]. This study also confirms the previous observation that SNAP-25 does not target to the plasma membrane as efficiently in non-neuronal neurosecretion-incompetent HeLa cells and in addition, membrane targeting does not require syntaxin-1A, which is absent from HeLa cells [29]. EYFP-KIF5B 594–824 was diffusely distributed throughout the cytoplasm, with no localization within the nucleus (Fig. 2D). A similar distribution has been reported for expression of KIF5B stalk domain fragment 511–766 [3]. In contrast, EYFP-KIF5B 594–910 demonstrated a punctate, vesicular distribution throughout the cytoplasm which presumably reflects the presence of the cargo-binding domain within this fragment (Fig. 2E). Furthermore, the distribution of EYFP-KIF5B 594–910, which lacks the microtubule-binding KHC motor domain (Fig. 1A), as expected did not colocalize with microtubules (Fig. 2F). A number of studies have used fragments of KHC which include some or all of the globular region of the tail domain (amino acids 911–963) [3,30,31] which contains a cryptic microtubule-binding site [3,32]. The use of dominant/negative motorless KHC fragments which still bind microtubules can potentially result in cytotoxic effects due to modification of the microtubule network [33]. This was not the case with KIF5B 594–910, which had no apparent effect on the distribution of the microtubule network in comparison to untransfected HeLa cells (Fig. 2F). Therefore, KIF5B 594–910 represents a novel tool to specifically interfere with kinesin-1 dependent transport *via* direct competition with endogenous kinesin without the side effects of morphological changes to the microtubule cytoskeleton.

Inhibition of SNAP-25 transport to the plasma membrane

The effect of overexpression of KIF5B 594–910 and 594–824 on the distribution of SNAP-25, tagged with C-terminal ECFP, was then assessed in HeLa cells. In the presence of EYFP-KIF5B 594–824, SNAP-25-ECFP was found to distribute to the plasma membrane as observed for SNAP-25-ECFP only (Fig. 3A–C). The fact that the transport of SNAP-25 to the cell membrane was not inhibited supports the *in vitro* mapping studies which showed that the 594–824 fragment of KIF5B was insufficient to interact with SNAP-25 (Fig. 1). In contrast, in the presence of EYFP-KIF5B 594–910, SNAP-25-ECFP distribution was no longer at the plasma membrane but concentrated predominantly around the nucleus in a punctate vesicular pattern which colocalized with EYFP-KIF5B 594–910 (Fig. 3D–F). However,

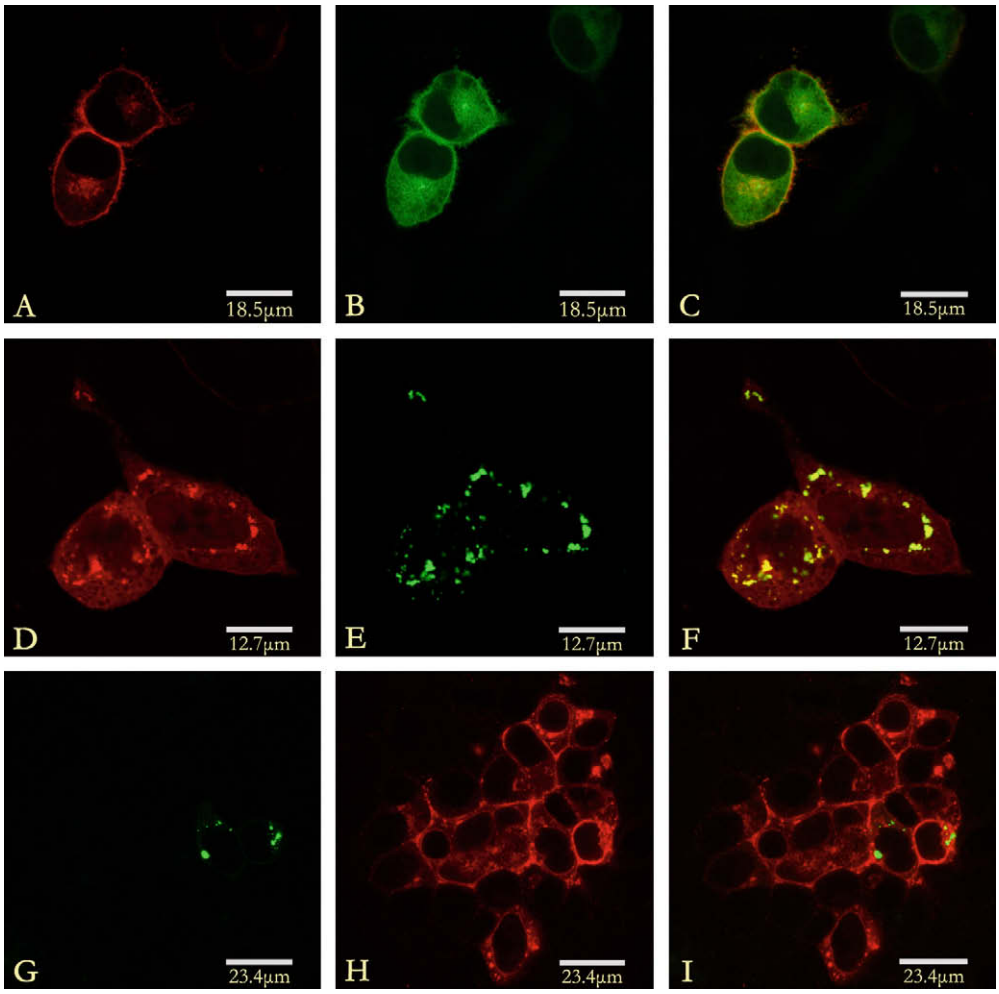

Fig. 3. KIF5B 594–910 colocalizes with and blocks transport of SNAP-25 to the plasma membrane of HeLa cells. Coexpression of (A) SNAP-25-ECFP (red) with (B) EYFP-KIF5B 594–824 (green) had no effect on SNAP-25 distribution to the plasma membrane and no significant colocalization was observed in overlays (C). Coexpression of (D) SNAP-25-ECFP (red) with (E) EYFP-KIF5B 594–910 (green) blocks transport of SNAP-25 to the plasma membrane and significant colocalization was observed in overlays (F). Coexpression of (G) EYFP-KIF5B 594–910 (green) and (H) HSV-1 transmembrane glycoprotein gD (red) had no effect on the plasma membrane distribution of gD and no colocalization was observed in overlays (I). For cotransfections (A–F), cells were imaged at 48 h post-transfection. For transfection and then infection with HSV-1 (G–I), cells were imaged at 16 h post-infection.

transport of SNAP-25 was not completely eliminated, with some of the protein clearly observable in reduced amounts at the cellular membrane, as well as elsewhere in the cytoplasm. To establish that overexpression of EYFP-KIF5B 594–910 does not nonspecifically block membrane trafficking we also transfected HeLa cells with pEYFP-KIF5B 594–910 and then infected with HSV-1. The distribution of the HSV-1 transmembrane glycoprotein gD was found to be identical in untransfected and transfected HeLa cells (Fig. 3G–I).

Discussion

Kinesin-1 has been shown to bind a range of cellular and pathogen cargo and to mediate microtubule-dependent transport of this bound cargo [1,15]. It is now generally accepted that both the heavy chain of kinesin-1 and associated light chain directly contribute to the binding of this cargo [1,15]. We have previously shown, using yeast two-hybrid and *in vitro* pulldown assays, that KHC KIF5B directly interacts with the SNARE proteins SNAP-25 and SNAP-23 [26]. Additional studies have shown that synaptic vesicles contain both KIF5B and SNAP-25 [21,34,35] supporting a proposed role for the KIF5B/SNAP-25 interaction in the axonal transport of presynaptic membrane precursors [1]. The axonal transport of syntaxin-1 containing presynaptic membrane precursors has also been shown to require the interaction of KIF5B and syntabulin [27,34].

In this study we initially developed a novel *in vitro* pulldown assay based on coexpression of His_6-tagged fragments of KIF5B and untagged full-length SNAP-25 in bacteria. This facilitated a one step pulldown of KIF5B/SNAP-25 complexes on nickel-activated beads and resolved the SNAP-25 binding site in KIF5B to amino acids 874–894. This is similar to the binding site in KIF5B (residues 867–894) we previously determined for the HSV-1 protein pUS11 [36]. This overlaps with the Ran-binding protein 2 (RanBP2)-binding site in KIF5B and KIF5C (residues 826–936) [31,37] and the α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor subunit-GluR2-interacting protein (GRIP1)-binding site in KIF5A, KIF5B and KIF5C (residues 753–987) [38]. Whether SNAP-25 is capable of binding all KIF5 heavy chains requires further determination.

Subsequent overexpression of KIF5B 594–910 encompassing the minimal SNAP-25 binding site resulted in significant colocalization with SNAP-25. In addition, the distribution of SNAP-25 in the presence of KIF5B 594–910 changed markedly from the plasma membrane to the cytoplasmic punctate KIF5B 594–910 containing compartments. The nature of this presumably vesicular compartment targeted by the cargo-binding domain within the KIF5B 594–910 fragment requires further identification. Kinesin-1 has been shown to be involved in trafficking between a number of organelle compartments and in particular away from the Golgi towards the ER or plasma membrane [24].

In summary, our results suggest that the KIF5B fragment 594–910 is sufficient to bind SNAP-25 *in vivo* and indicates that kinesin-1 plays an important role in trafficking of SNAP-25 containing vesicles to the cellular periphery. Future studies will involve identifying the nature of the vesicle compartment containing colocalized KIF5B 594–910/SNAP-25 and to elucidating the role of the KIF5B/SNAP-25 interaction in neurons.

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