

Centaurin- α_1 interacts directly with kinesin motor protein KIF13B

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Summary

Centaurin- α_1 is a phosphatidylinositol 3,4,5-trisphosphate binding protein as well as a GTPase activating protein (GAP) for the ADP-ribosylation factor (ARF) family of small GTPases. To further understand its cellular function, we screened a rat brain cDNA library using centaurin- α_1 as bait to identify centaurin- α_1 interacting proteins. The yeast two-hybrid screen identified a novel kinesin motor protein as a centaurin- α_1 binding partner. The motor protein, termed KIF13B, encoded by a single ~9.5-kb transcript, is widely expressed with high levels observed in brain and kidney. Yeast two-hybrid and GST pull-down assays showed that the interaction between centaurin- α_1 and KIF13B is direct and mediated by the GAP domain of centaurin- α_1 and the stalk domain of KIF13B. Centaurin- α_1 and KIF13B form a complex in vivo and the KIF13B

interaction appears to be specific to centaurin- α_1 as other members of the ARF GAP family did not show any binding activity. We also show that KIF13B and centaurin- α_1 colocalize at the leading edges of the cell periphery whereas a deletion mutant of centaurin- α_1 that lacks the KIF13B binding site, failed to colocalize with KIF13B in vivo. Finally, we demonstrate that KIF13B binding suppresses the ARF6 GAP activity of centaurin- α_1 in intact cells. Together, our data suggest a mechanism where direct binding between centaurin- α_1 and KIF13B could concentrate centaurin- α_1 at the leading edges of cells, thus modulating ARF6 function.

Key words: Centaurin- α_1 , KIF13B, PI 3-kinase, PtdIns(3,4,5) P_3 , ARF6, Trafficking

Introduction

The ARFs are Ras-related small GTPases that regulate intracellular vesicle trafficking by shuttling between an inactive GDP-bound and an active GTP-bound form (Moss and Vaughan, 1998; Randazzo et al., 2000). ARFs in mammalian cells are divided into three classes: the class 1 ARFs (ARFs 1-3) function in protein trafficking between Golgi and endoplasmic reticulum, the less well studied class 2 ARFs (ARFs 4 and 5) and the class 3 ARFs (ARF6) play critical roles in endocytosis, exocytosis and cell spreading by regulating membrane ruffling and cytoskeletal actin dynamics near the cell surface (Moss and Vaughan, 1998; Randazzo et al., 2000; Donaldson, 2003; Sabe, 2003). ARFs are regulated by guanine-nucleotide exchange factors (GEFs), which activate ARFs by catalyzing the exchange of bound GDP with GTP, and the GTPase activating proteins (GAPs), which inactivate ARFs by stimulating hydrolysis of bound GTP to GDP (Donaldson and Jackson, 2000). It has been shown recently that the cytohesin family of ARF GEFs is recruited to the plasma membrane in agonist-stimulated cells by binding phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) P_3], thus leading to the activation of ARF6 (Cullen and Venkateswarlu, 1999; Hawadle et al., 2002). This observation suggests a possible involvement of PtdIns(3,4,5) P_3 in ARF6-associated cellular responses, a view further strengthened by the identification of ARF GAPs such as centaurin- α_1 and ARAP3 (ARF GAP, Rho GAP, ankyrin repeat, PH protein 3) as PtdIns(3,4,5) P_3 binding proteins

(Hammonds-Odie et al., 1996; Krugmann et al., 2002). PtdIns(3,4,5) P_3 is a ubiquitous lipid second messenger produced by agonist-stimulated phosphoinositide (PI) 3-kinase and plays an important role in many cellular functions such as glucose uptake, vesicle trafficking, cell adhesion and cell secretion by acting as a site-specific signal for recruitment and/or activation of cytosolic proteins required for the formation of functional complexes at the plasma membrane (Rameh and Cantley, 1999; Vanhaesebroeck et al., 2001).

Centaurin- α_1 (also known as p42IP₄BP or PIP₃BP) is a protein that is highly abundant in the brain. It was originally purified using PtdIns(3,4,5) P_3 and its inositol head group, inositol 1,3,4,5-tetrakisphosphate [IP₄] affinity columns, and characterized as a PtdIns(3,4,5) P_3 /IP₄ binding protein (Hammonds-Odie et al., 1996; Tanaka et al., 1997; Striker et al., 1997). We have previously shown that the green fluorescent protein (GFP)-tagged centaurin- α_1 translocates in a PtdIns(3,4,5) P_3 -dependent manner from the cytosol to the plasma membrane of cells in response to epidermal growth factor (EGF) stimulation, indicating that centaurin- α_1 can function as a PtdIns(3,4,5) P_3 binding protein in intact cells (Venkateswarlu et al., 1999). Centaurin- α_1 has an N-terminal zinc-finger motif containing the ARF GAP domain followed by two PH domains (one in the middle of the protein, N-PH, and one at the C-terminus, C-PH) that are required for PtdIns(3,4,5) P_3 binding (Tanaka et al., 1997; Venkateswarlu et al., 1999). Centaurin- α_1 localizes to the cytosol and nucleus

(Venkateswarlu and Cullen, 1999) presumably because the N-terminus half of the zinc-finger motif contains a nuclear targeting signal (Tanaka et al., 1999). Although in vitro evidence for centaurin- α_1 ARF GAP activity is lacking at present, we have shown that centaurin- α_1 can functionally complement the yeast ARF GAP, Gcs1, suggesting that it may function as an ARF GAP in vivo (Venkateswarlu et al., 1999). More recently, we have provided evidence suggesting that the centaurin- α_1 specifically acts as a GAP for ARF6 in intact cells (Venkateswarlu et al., 2004).

Centaurin- α_1 is upregulated in neurons of patients with Alzheimer's disease (AD) (Reiser and Bernstein, 2002). However, the functional significance of centaurin- α_1 amplification in AD pathology is presently unknown. Notwithstanding the potential role of centaurin- α_1 as an ARF6 GAP in vivo, the physiological function of centaurin- α_1 remains largely unknown. Therefore, identification of the binding partners of centaurin- α_1 and their localization within the cell might provide clues to the cellular functions of centaurin- α_1 in vivo. For example, the casein kinase 1 α (CK1 α) and protein kinase C (PKC) isoforms have recently been shown to interact with centaurin- α_1 ; however, the functional consequences of these interactions have not been fully characterized (Dubois et al., 2001; Zemlickova et al., 2003). In this manuscript, we present evidence that centaurin- α_1 binds to a kinesin motor protein termed KIF13B. The biochemical interaction between endogenous centaurin- α_1 and KIF13B was confirmed by co-immunoprecipitation and the GAP domain of centaurin- α_1 bound directly to the stalk domain of KIF13B. Importantly, binding with KIF13B was essential for the localization of centaurin- α_1 to the leading edges of the cell and the regulation of ARF6 GAP activity of centaurin- α_1 in vivo.

Materials and Methods

Antibodies and reagents

The anti-Myc monoclonal antibody 9E10, anti-haemagglutinin (HA) monoclonal antibody HA11 and an anti-HA polyclonal, an anti-GFP polyclonal and anti-FLAG monoclonal antibody M2 were purchased from Roche Applied Science, Covance, Santa Cruz Biotechnology and Sigma, respectively. Rabbit polyclonal anti-KIF13B C-terminus antiserum and an affinity-purified rabbit polyclonal antibody against the N-terminus KIF13B were described previously (Hanada et al., 2000). A mouse monoclonal anti-centaurin- α_1 was obtained from HyTest. Rabbit polyclonal anti-centaurin- α_1 antiserum was raised against a synthetic peptide derived from the C-terminus of centaurin- α_1 as described previously (Striker et al., 1995) and affinity purified using glutathione S-transferase (GST)-centaurin- α_1 coupled to glutathione beads followed by the adsorption with glutathione beads coupled with GST alone. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse immunoglobulin IgG, and glutathione-Sepharose beads were purchased from Amersham Biosciences. Cyanine (Cy)3-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG secondary antibodies were obtained from Jackson Immuno Research Laboratories. DNA restriction enzymes were from Roche Applied Science. All other chemicals were obtained from Sigma unless otherwise specified.

DNA constructs

The full-length centaurin- α_1 and its deletion mutants cDNA sequences were amplified by PCR using centaurin- α_1 /pGEX4T1 plasmid as a

template (Venkateswarlu and Cullen, 1999), *Pfu* DNA polymerase (Stratagene), and the following sets of primers containing *EcoRI* (sense, underlined) and *SalI* (antisense, underlined) restriction sites. FL (the full-length human centaurin- α_1 , aa 1-374): sense primer, 5'-CGCGAATTCATGGCCAAGGAGCGGCGCAGG-3' and antisense primer, 5'-CGCGTTCGACCTAAGGTTTATGCTTGAAGTG-3'; Δ C133 (centaurin- α_1 deletion mutant without the C-PH domain, aa 1-241): FL sense primer and antisense primer, 5'-CGCGTTCGACCTAG-GCCCCTGGGAATGCCACCTG-3'; Δ C248 (centaurin- α_1 ARF GAP domain, aa 1-126): FL sense primer and antisense primer, 5'-CGCGTTCGACCTACTCTGCTTCTCCGGGTAGATG-3'; Δ N125 (centaurin- α_1 deletion mutant without the ARF GAP domain, aa 126-374): sense primer, 5'-CGCGAATTCATGCCCTACTCG-GCAGGGTACCGTG-3' and FL antisense primer. The resulting cDNAs were digested with *EcoRI* and *SalI*, and cloned into the same sites of a bait plasmid pBTM116 (Vojtek et al., 1993) for expression as LexA DNA binding domain fusion proteins in yeast and pEGFPC2 vector (Clontech) for expression as GFP-tagged fusion proteins in mammalian cells. The centaurin- α_1 FL and Δ N125 cDNAs were ligated to pCMV-tag 2b vector (Stratagene) for expression as FLAG-tagged fusion proteins in mammalian cells. The full-length KIF13B released from KIF13B/pGEMT (see below) was cloned into pEGFP-C1 and pCMV-Myc (Clontech) for expression as GFP-tagged and Myc-tagged fusion proteins, respectively, in mammalian cells. The KIF13B Δ C cDNA released from KIF13B/pEGFPC1 by *EcoRI* digestion was subcloned into the same site of pET32b to prepare radiolabelled KIF13B Δ C (aa 1-991) using in vitro translation system. The KIF13A cDNA (aa 389-649; isolated by yeast two-hybrid screening, see below) was subcloned into the *EcoRI* site of pGEX4T1 (Amersham Biosciences), pEGFP-C2 and pCMV-tag 3b (Stratagene) for expression as GST-tagged fusion protein in bacteria and for expression as GFP-tagged and Myc-tagged fusion proteins in mammalian cells respectively. The KIF13B Δ MD (KIF13B without the motor domain; aa 355-1767) and KIF13B Δ CG (KIF13B lacking the CAP-GLY domain; aa 1-1660) were generated by PCR amplification and cloned into pEGFPC1 for expression as GFP-tagged fusion proteins in mammalian cells. GIT1-GFP/pBKA (23), kindly provided by Richard Premont (Duke University Medical Centre, Durham, NC), was used for expressing GFP-tagged G-protein-coupled receptor kinase interacting target (GIT)1 fusion protein in mammalian cells. Centaurin- β_1 , centaurin- δ_2 and ARF1GAP cDNAs amplified from KIAA0050, KIAA0782 (kindly provided by T. Nagase, Kazusa DNA Research Institute, Japan) and MGC (mammalian gene collection) 39924 clones, respectively, by PCR using the sequence specific primers were subcloned into *BglIII-SalI* sites of pEGFP-C2 vector for expression as GFP-fusion proteins in mammalian cells. ARF6-HA/pXS construct, kindly provided by J. Donaldson (NIH, USA), was used to express ARF6 with a C-terminal HA epitope-tag in mammalian cells. MT2A/pGEX4T1 was used to express the full-length metallothioneine 2A (MT2A) as GST-fusion protein in *E. coli* (Venkateswarlu, 2003). PXGH5 (Nichols Institute Diagnostics, USA) was used to express human growth hormone (hGH) in mammalian cells. Cytohesin 2/pBTM116 bait vector and interaction protein for cytohesin exchange factors (IPCEF)-1 and IPCEF-2 cloned into pGAD424 (prey vector; Clontech) for expression as GAL4 activation domain fusion proteins in yeast cells were described previously (Venkateswarlu, 2003). The authenticity of all constructs was verified by nucleotide sequencing using an ABI Prism 211 automated sequencer (Perkin Elmer). All bait constructs tested negative for autoactivation of reporter gene activity in the yeast two-hybrid reporter strain L40 (containing histidine 3 and β -galactosidase reporter genes) (Vojtek et al., 1993).

Yeast two-hybrid screening

The yeast two-hybrid screening was carried out essentially as described previously (Venkateswarlu, 2003). Briefly, the yeast strain

L40 was transformed with the pBTM116-centaurin- α_1 bait vector using the lithium chloride method and the transformants were selected for growth on synthetic solid media lacking tryptophan (Stephens and Banting, 1999). L40 transformants carrying the bait vector were subsequently transformed with a rat brain pGAD10 (prey vector) cDNA library (Clontech). The transformation mixture was grown overnight at 30°C in synthetic media lacking Trp and Leu to select the transformants carrying the bait as well as the prey vectors. A total of ten million transformants were assayed for growth on synthetic drop-out medium in the absence of His, Leu and Trp. His-positive colonies were further tested for β -galactosidase activity. β -Galactosidase filter assays were carried out as described (Gietz et al., 1997). The pGAD plasmids were recovered from the transformed yeast colonies using *E. coli* strain HB101 as a recipient strain and selecting on M9 minimal medium. After a second round of assaying for growth on synthetic drop-out medium lacking His (-His plate) and β -galactosidase activity using lamin/pBTM116 bait vector as a negative control (Stephens and Banting, 1999), the cDNA inserts of the recovered plasmids were sequenced and identified by the BLAST algorithm. The screening resulted in obtaining a partial sequence of kinesin 13B (KIF13B) motor protein, KIF13B Δ (aa 399-661). The full-length KIF13B cDNA was amplified by PCR using rat brain Marathon Ready cDNA (Clontech) as a template, KOD DNA polymerase (Novagen) and the gene specific primers (sense, 5'-ATGAAAACCATTCAGATGAAAGGCCTGCTTC-3' and antisense, 5'-CTAGCTGGCCCAGGACTTCCGGTTCTCAGG-3') designed based on the cDNA sequence predicted from the genomic sequence (GenBank accession numbers XP_224287 and XM_224288). The PCR product was cloned into pGEMT vector (Promega) and sequenced as described.

Northern blotting

A cDNA fragment of 1159-1947 base pairs of the KIF13B coding region (the KIF13B Δ cDNA isolated by two-hybrid screening) was labelled with 32 P using Random Primer DNA labelling kit (Roche Applied Science) and then used to probe the rat multiple tissue Northern blot (Clontech), as described previously (Venkateswarlu and Cullen, 1999).

GST-fusion protein expression and purification

This was performed as described previously (Venkateswarlu et al., 1998). Briefly, GST-fusion protein was expressed in BL21(DE3) strain of *E. coli* (Stratagene) by induction with 0.2 mM isopropyl β -D-thiogalactoside (IPTG) for 3 hours at 30°C. Cells were harvested by centrifugation and resuspended in ice-cold buffer A [PBS containing 1 mM EDTA, 1 mM EGTA, 1 mM β -mercaptoethanol and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)]. After incubation for 10 minutes on ice, cells were lysed by sonication and lysate was cleared by centrifugation. The supernatant was incubated with glutathione-Sepharose beads at 4°C overnight with constant mixing. The beads were washed five times with ice-cold buffer A and stored in buffer A containing 50% glycerol at -20°C. Centaurin- α_1 was cleaved from the GST-tag by incubating GST-centaurin- α_1 coupled to glutathione beads with thrombin in PBS at 4°C for 12 hours and was stored at -80°C in 50% glycerol. The purity of protein either bound to the beads or eluted by thrombin digestion was analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining. Protein content of the purified recombinant centaurin- α_1 was estimated by the Bradford method using BSA as a standard.

In vitro protein-protein interaction assay

This assay was performed as described (Asaba et al., 2003). Briefly, 35 S-radiolabelled KIF13B Δ C (aa 1-991) was prepared using the STP3 in vitro translation system (Novagen) and KIF13B Δ C/pET32a

expression vector in presence of [35 S]methionine. After completion of protein synthesis, the lysate was diluted tenfold in binding buffer (PBS with 1% Triton X-100) and incubated either with GST alone or GST-centaurin- α_1 coupled to glutathione beads for 2 hours at 4°C. A direct binding assay was performed by incubation of purified recombinant centaurin- α_1 with glutathione beads containing either GST or GST-KIF13B Δ in the binding buffer for 2 hours. After washing beads with the binding buffer, protein was recovered from beads by boiling in 1 \times SDS-PAGE sample buffer for 5 minutes. The proteins in the samples were separated by SDS-PAGE and visualized by autoradiography, Coomassie Blue staining or immunostaining using an anti-centaurin- α_1 antibody.

Cell culture and transfection

HeLa, COS, Madin-Darby canine kidney (MDCK) and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin sulphate and 10% fetal calf serum (FCS) (full-serum medium) under 5% CO₂ at 37°C. Jurkat cells were maintained in RPMI-1640 medium supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml) and 10% FCS. PC12 cells were maintained in the full-serum medium containing 5% horse serum. For transfections, the cells were transfected at approximately 70% confluence with plasmid DNA using FuGene6 (Roche) according to the manufacturer's instructions. We routinely achieve about 30-40%, 15-20% and 5-10% transfection efficiency when we transfect cells with one plasmid, two plasmids and three plasmids, respectively.

GST-fusion protein pull-down assay

After 2 days of transfection, cells were washed twice in cold 1 \times PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40) supplemented with 1% complete protease inhibitors mix (Sigma). The cell lysates were precleared by centrifugation at 16,000 g for 15 minutes and incubated with either GST or GST-fused proteins coupled to glutathione beads for 2 hours at 4°C. In some experiments, the cell lysates were incubated with GST coupled to glutathione beads for 2 hours at 4°C prior to incubation with glutathione beads harboring GST fused proteins. The beads were washed three times with PBS containing 0.1% Triton X-100 and boiled in the sample buffer. The proteins in the samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Whatman). The blots were analysed by immunoblotting using an anti-GFP polyclonal antibody.

Immunoprecipitation

COS cells were transiently transfected with GFP or GFP-KIF13B and empty FLAG or FLAG-tagged centaurin- α_1 expression plasmids and after 2 days the cells were lysed as described above. The cell extracts were incubated first with 10 μ l anti-GFP polyclonal antibody for 30 minutes at 4°C and then with 30 μ l protein A Sepharose beads (Novagen) for 4 hours at 4°C. The beads were washed five times with lysis buffer and the bound proteins were detected by immunoblotting as described above using anti-FLAG M2 monoclonal antibody. To examine the interaction between endogenous centaurin- α_1 and KIF13B, rat brains were homogenized in four volumes of lysis buffer and incubated the extracts with 10 μ l pre-immune serum, an anti-KIF13B or an anti-centaurin- α_1 for 30 minutes at 4°C. Immunoprecipitation followed by immunoblotting using affinity-purified anti-KIF13B polyclonal and anti-centaurin- α_1 monoclonal antibodies was performed as described above. To study the interaction between KIF13B, centaurin- α_1 and ARF6_{GTP}, COS cells were transiently transfected with HA-ARF6_{Q67L} and GFP-KIF13B or

FLAG-centaurin- α_1 or both. After 2 days, the cells were lysed as described above and the cell extracts were incubated with an anti-HA polyclonal antibody for 30 minutes at 4°C. Immunoprecipitation and then immunoblotting were done as described above using anti-HA (HA11), anti-GFP and anti-FLAG (M2) monoclonal antibodies.

Immunostaining

HeLa cells were seeded onto 13 mm coverslips in 24-well plates and were transfected at 60–70% confluence with the indicated plasmids. After two days, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes. The cells were permeabilized with 0.2% Triton X-100 for 10 minutes and then blocked with blocking buffer (1% BSA, 0.1% Triton X-100 in PBS) for 30 minutes. The cells were incubated with anti-FLAG M2 antibody (10 $\mu\text{g}/\text{ml}$) in blocking buffer for 1 hour and then incubated with a 1:500 dilution of goat Cy3-conjugated anti-mouse antibody in blocking buffer for 1 hour, and mounted on slides with mounting solution [0.1 M Tris-HCl, pH 8.5, 10% Mowiol (Calbiochem), 50% glycerol], containing 2.5% DABCO [1,4-Diazabicyclo(2.2.2)octane]. The transfected cells were incubated with 3.5 μM nocadazole for 1 hour at 37°C prior to the fixation where indicated. Immunofluorescence staining was visualized using a Leica TCS-NT confocal microscope equipped with Kr/Ar laser. All images presented are single sections in the z -plane.

In vivo ARF6 translocation assay

HeLa cells seeded on 13 mm coverslips were transiently transfected with the indicated plasmids. After 48 hours, cells were serum starved for 2 hours. The cells were then incubated with or without 200 ng/ml of EGF (Sigma) for 5 minutes and immunostained as described above using an anti-HA monoclonal and an anti-Myc polyclonal primary antibody, and a Cy3-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG secondary antibodies.

In vivo ARF6 activation assay

This assay was performed as described elsewhere (Venkateswarlu, 2003; Schweitzer and D'Souza-Schorey, 2002). COS cells transfected with ARF6-HA, FLAG-empty or FLAG-centaurin- α_1 and GFP or GFP-tagged KIF13 Δ (3:1:1 ratio; 10 μg total DNA) were serum starved for 2 hours. The cells were then incubated for 5 minutes with or without 200 ng/ml EGF and lysed in 0.5 ml of lysis buffer B (50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 0.1% protease inhibitor mixture). The lysates were clarified by centrifugation at 16,000 g for 10 minutes at 4°C. Approximately 90% of the cell extract was incubated with GST-MT2A coupled to glutathione beads in presence of 2 mM ZnCl_2 . After 1 hour of mixing at 4°C, the beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM MgCl_2 , 1% Triton X-100, 2 mM ZnCl_2 and 0.1% protease inhibitors), boiled in SDS-PAGE sample buffer and analysed by immunoblotting using a monoclonal anti-HA antibody. The cell lysates (input) were also immunoblotted using anti-HA, anti-FLAG, and anti-GFP antibodies to determine HA-ARF6, FLAG-centaurin- α_1 and GFP-KIF13 Δ levels, respectively. Immunoblots were scanned and the amount of ARF6 precipitated with GST-MT2A was normalized to total ARF levels in the lysates to compare ARF_{GTP} levels in cells transformed with the indicated constructs. The GST-MT2A fusion protein was expressed in BL21(DE3) strain of *E. coli* and coupled to glutathione beads as described (Schweitzer and D'Souza-Schorey, 2002).

hGH secretion assay

This assay was performed as described previously (Rajebhosale et al., 2003). Briefly, PC12 cells seeded into a collagen-coated six-well

tissue culture plate were transiently transfected at approximately 80% confluency with pXGH5 and the indicated plasmids (1 μg each) as described above. 2 days after transfection, cells were stimulated with 1 ml Locke's buffer (5 mM HEPES, pH 6.8, 156 mM NaCl, 5.6 mM KCl, 0.2 mM EGTA, 3.6 mM NaHCO_3 and 5.6 mM glucose) or Locke's buffer containing 0.3 mM ATP for 10 minutes. After removing the assay medium, PC12 cells in the dish were lysed in 1 ml PBS containing 1 mM EDTA by five cycles of rapid freezing and thawing. The cell extracts and the assay medium were cleared by centrifugation at 16,000 g for 10 minutes at 4°C. The amount of hGH secreted into the assay medium and the amount of unsecreted hGH in the cell lysate were estimated using an enzyme-linked immunosorbent (ELISA) assay kit (Roche Applied Science) according to the manufacturer's instructions and calculated hGH secretion as a percentage of the total hGH.

Results

Identification of KIF13B as a centaurin- α_1 binding partner

To identify potential centaurin- α_1 interacting proteins, we used full-length centaurin- α_1 fused to the LexA DNA binding domain as bait in a yeast two-hybrid screening of a rat brain cDNA library. By screening a total of ten million independent colonies, ten clones positive for both histidine autotrophy and β -galactosidase activity were obtained. Five of the positive clones yielded an identical sequence encoding a part of the kinesin (KIF)13B gene, KIF13B Δ (aa 387–649) (Fig. 1A). The full-length KIF13B cDNA was isolated by PCR from rat brain total cDNA using forward and reverse primers derived from the genomic sequence, sequenced and deposited into GenBank under accession number AJ605719. The KIF13B gene is located on chromosome 15. The deduced amino acid sequence and the domain structure of KIF13B are shown in Fig. 1. KIF13B has an N-terminal motor domain (aa 56–354) and a large stalk domain (aa 366–1660), which are conserved domains among most KIF family proteins. In addition, KIF13B has a putative microtubule-interacting sequence known as the CAP-Gly (cytoskeleton-associated protein) domain (aa 1661–1703) at the C-terminus. Rat KIF13B is highly homologous to that of the human form; it shares overall 83% amino acid identity (98% identity in the motor domain, 93% identity in the stalk domain and 79% identity in the CAP-Gly domain) with the human protein. Moreover, this protein shows about 62% amino acid identity to rat KIF13A. Human KIF13B was originally identified as a protein interacting with the human homologue of *Drosophila* discs large tumour (hDlg) suppressor protein and hence named guanylate kinase-associated kinesin (GAKIN) (Hanada et al., 2000). Dlg is a member of the membrane-associated guanylate kinase (MAGUK) family of scaffold proteins. The kinesin motor domain of KIF13B contains an ATP-binding motif and the C-terminal half of the domain is involved in microtubule binding. A segment of the human KIF13B stalk domain interacts with hDlg, and this interaction is needed to maintain the localization of hDlg at cell junctions in MDCK cells (Asaba et al., 2003). The centaurin- α_1 interacting region, KIF13B Δ (aa 387–649) does not overlap with the hDlg binding region in the stalk domain of KIF13B, suggesting that the stalk domain of KIF13B contains more than one protein-protein binding site.

We performed northern blot analysis to determine the tissue distribution of KIF13B mRNA. A rat multiple tissue Northern

blot was hybridized with a cDNA fragment of KIF13B (nucleotides 1159-1947) isolated by the yeast two-hybrid screening (Fig. 2), which corresponds to the N-terminus of the stalk domain of KIF13B. A single band of approximately 9.5 kb was detected. The expression level of KIF13B was the highest in brain and modest in the kidney. KIF13B expression was also detected as a very faint band in the testis. It has been shown previously by northern blot analysis that centaurin- α_1 is expressed as a 2.5 kb transcript predominantly in rat brain and at very low but detectable levels in lung, kidney and spleen (Hammonds-Odie et al., 1996). The expression profile of KIF13B is similar to that of centaurin- α_1 , indicating the possibility of a functionally conserved interaction between KIF13B and centaurin- α_1 . To confirm the expression of KIF13B in mammalian cultured cells and rat brain, western blot analysis of cultured cell and rat brain lysates was performed using an affinity-purified anti-KIF13B polyclonal antibody. A major protein band of approximately 200 kDa was detected at high levels in rat brain, with moderate levels in Jurkat and HEK293 cells (Fig. 2B).

The specificity of the interaction between KIF13B and centaurin- α_1 was confirmed by additional yeast two-hybrid analysis. To accomplish this goal, KIF13B Δ , IPCEF-1 or IPCEF-2 prey constructs were transformed together with either centaurin- α_1 or cytohesin 2 bait vector into the yeast strain L40, and we subsequently analysed the transformants for histidine autotrophy and β -galactosidase activity. Cytohesin 2 is a GEF for ARF6 and contains a PH domain similar to the C-PH domain of centaurin- α_1 whereas IPCEF-1 interacts with cytohesin 2 and modulates its activity (Venkateswarlu, 2003). As shown in Fig. 3, only yeast transformants harbouring both centaurin- α_1 and KIF13B Δ or cytohesin 2 and IPCEF-1/IPCEF-2 were able to grow on -His plates and expressed β -galactosidase. The interaction between centaurin- α_1 and KIF13B is specific, as centaurin- α_1 and cytohesin 2 did not support yeast growth on -His plates or β -galactosidase expression under the same conditions when co-transformed with IPCEF-1/IPCEF-2 or KIF13B Δ , respectively.

To verify the biochemical interaction between centaurin- α_1 and KIF13B, in vitro translated [35 S]Met-labelled KIF13BAC (aa 1-991) was incubated with GST-alone or GST-centaurin- α_1 bound to glutathione-Sepharose beads. The protein complex was resolved by SDS-PAGE and visualized by autoradiography (Fig. 4A). [35 S]Met-labelled KIF13BAC binds to GST-centaurin- α_1 , but not to GST alone. We also analysed the

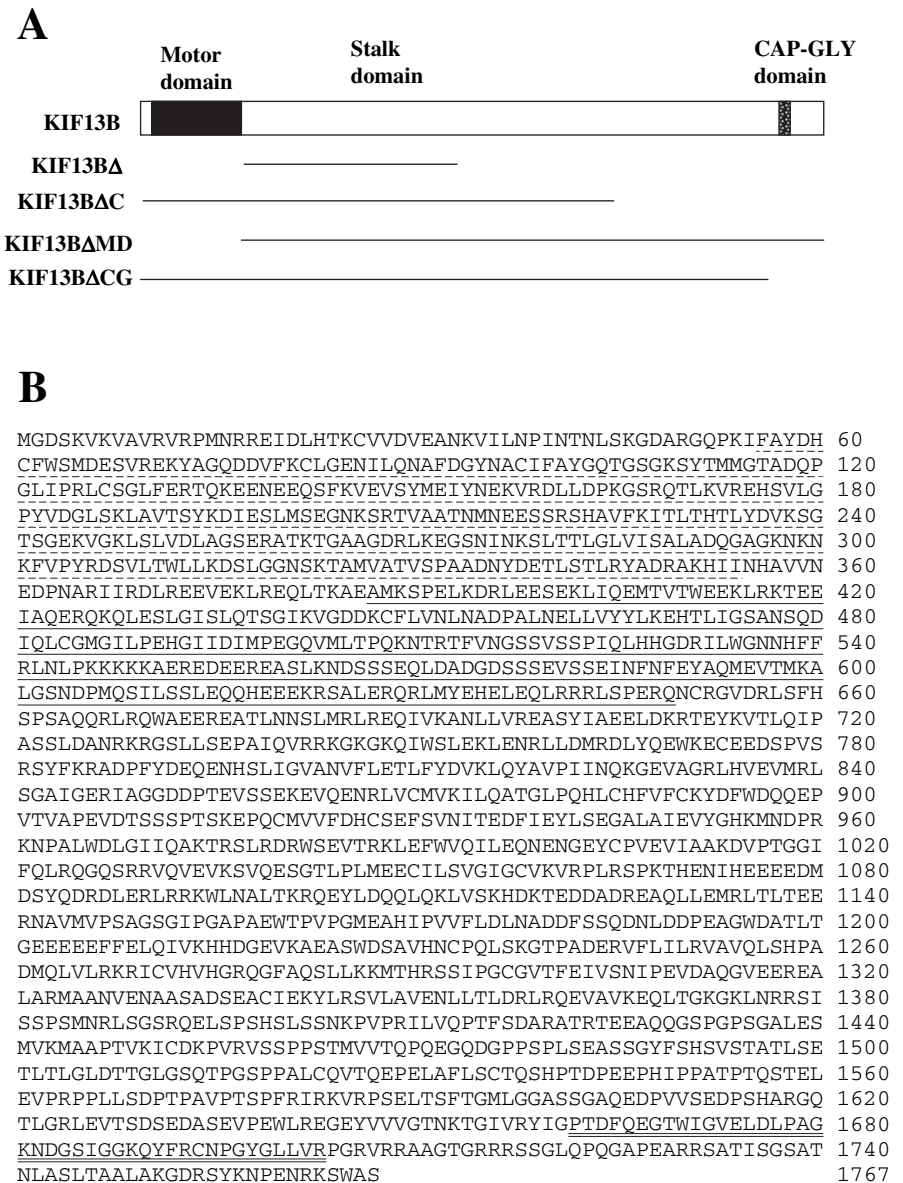


Fig. 1. Schematic diagram of the domain structure of KIF13B and its deduced amino acid sequence. (A) KIF13B contains an N-terminal motor domain (aa 56-354), a middle stalk domain and a C-terminal CAP-GLY domain (aa 1661-1703). Amino acid residues are numbered from the first Met of KIF13B. KIF13B Δ (aa 387-649) represents the cDNA clone isolated by two-hybrid screening. KIF13BAC (aa 1-998) was used to study the interaction between KIF13B and centaurin- α_1 in vitro. KIF13BAMD (aa 355-1767) and KIF13BACG (aa 1-1660) were used to study the importance of the motor domain and the CAP-GLY domain for the intracellular localization of KIF13B. (B) Predicted amino acid sequence of KIF13B (GenBank/EMBL/DBJ accession number AJ605719) is shown. The motor domain, KIF13B sequence and the CAP-GLY are underlined with dashed line, solid line and double solid line, respectively.

interaction between centaurin- α_1 and KIF13B using purified proteins. Purified recombinant centaurin- α_1 associated with GST-KIF13B Δ and not with GST alone (Fig. 4B,C). Together, these results indicate that centaurin- α_1 interacts directly with KIF13B.

To confirm the association of centaurin- α_1 with KIF13B in vivo, co-immunoprecipitation experiments were performed. Either GFP or GFP-KIF13B was co-transfected with empty

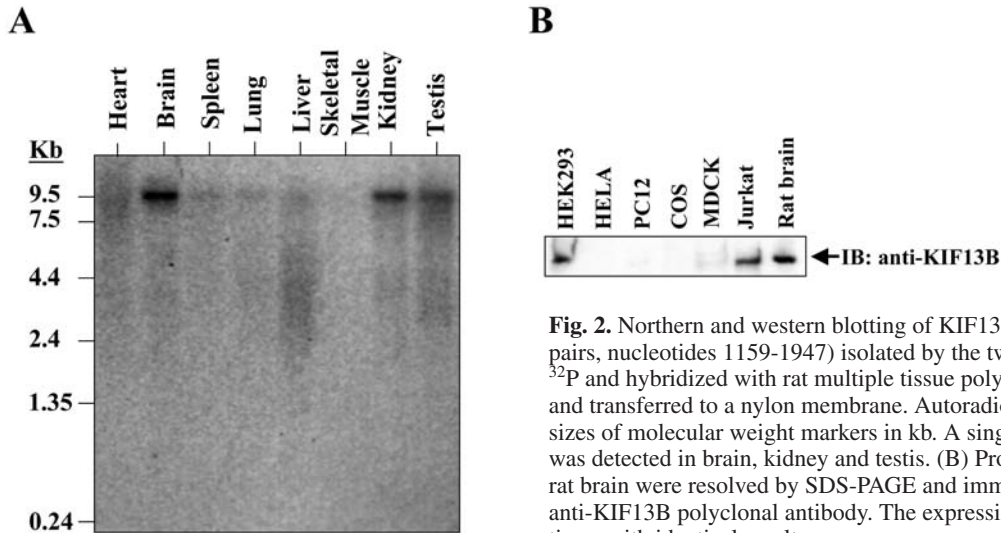


Fig. 2. Northern and western blotting of KIF13B. The KIF13BΔ cDNA (789 base pairs, nucleotides 1159-1947) isolated by the two-hybrid screening was labelled with ^{32}P and hybridized with rat multiple tissue poly(A) $^+$ RNA, resolved by electrophoresis and transferred to a nylon membrane. Autoradiography of the blot is shown with the sizes of molecular weight markers in kb. A single band of KIF13B mRNA (9.5 kb) was detected in brain, kidney and testis. (B) Protein samples of various cell lines and rat brain were resolved by SDS-PAGE and immunoblotted using an affinity-purified anti-KIF13B polyclonal antibody. The expression analysis was repeated two further times with identical results.

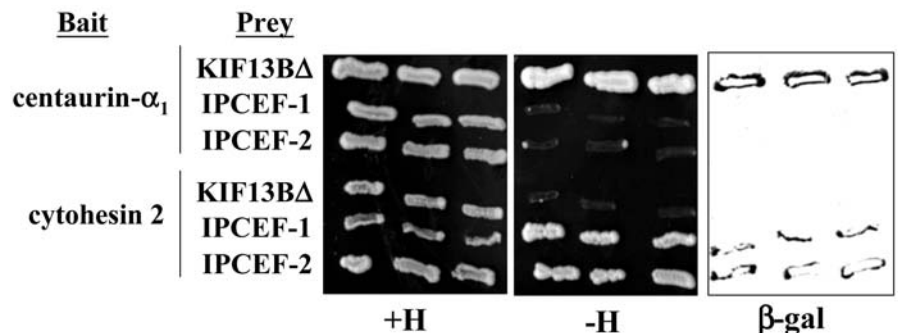
FLAG-vector or FLAG-tagged centaurin- α_1 into COS cells and the cell lysates were immunoprecipitated using an anti-GFP antibody. The proteins in the immunoprecipitates were visualized by immunoblotting using an anti-FLAG antibody. As shown in Fig. 5A, FLAG-centaurin- α_1 was co-precipitated with GFP-KIF13B but not with GFP-alone. To verify that the interaction of centaurin- α_1 with KIF13B occurs at endogenous levels of expression, rat brain lysates were immunoprecipitated with anti-KIF13B polyclonal antibody, and endogenous centaurin- α_1 in the precipitate was visualized by immunoblotting using an anti-centaurin- α_1 monoclonal antibody (Fig. 5B). Conversely, KIF13B was detected in anti-centaurin- α_1 immunoprecipitates of brain lysate by Western blot using an anti-KIF13B antibody. Together, these results indicate that centaurin- α_1 interacts with KIF13B *in vivo*.

KIF13B interacts with the ARF GAP domain of centaurin- α_1

To determine the region of the KIF13B binding site within centaurin- α_1 , various deletion mutants of centaurin- α_1 fused with the LexA DNA-binding domain were generated (Fig. 6A), and analysed for their ability to interact with KIF13BΔ fused to the activation-domain of GAL4 using the yeast two-hybrid system. Transformants containing either the full-length

centaurin- α_1 or its deletion mutants (ΔC133 and ΔC248) that contain the N-terminal ARF GAP domain showed histidine autotrophy and β -galactosidase activity suggesting that centaurin- α_1 binds to KIF13B through its zinc-finger motif-containing ARF GAP domain (Fig. 6B). To further confirm the interaction of KIF13B with centaurin- α_1 *in vitro*, full-length centaurin- α_1 and various deletion mutants of centaurin- α_1 tagged with GFP were expressed at comparable levels in COS cells; KIF13BΔ was expressed as a GST-fusion protein in bacteria. The GST-fusion KIF13BΔ was affinity purified using glutathione-Sepharose and incubated with the lysates of COS cells expressing either GFP or GFP-tagged centaurin- α_1 or its deletion mutants. The bound proteins were analysed by immunoblotting using an anti-GFP antibody (Fig. 7A). Consistent with the yeast two-hybrid results, full-length centaurin- α_1 and deletion mutants (ΔC133 and ΔC248) of centaurin- α_1 , which all contain the ARF GAP domain, were able to interact with GST-KIF13BΔ. In contrast, neither the centaurin- α_1 deletion mutant lacking the ARF GAP domain (ΔN125) nor GFP alone bound to GST-KIF13BΔ. Furthermore, GST alone showed no interaction with either GFP alone or GFP-tagged centaurin- α_1 or its deletion mutants. Together, these results suggest that KIF13B binds to centaurin- α_1 and that the ARF GAP domain of centaurin- α_1 is required for this interaction.

Fig. 3. Analysis of the specificity of interaction between centaurin- α_1 and KIF13B by yeast two-hybrid assay. Centaurin- α_1 or cytohesin 2 fused with the DNA binding domain of LexA (Bait) were co-transformed with KIF13BΔ/IPCEF-1/IPCEF-2 fused to the activation domain of Gal4 (Prey) into yeast (L40) and then we analysed the ability of the transformants to grow on solid synthetic drop-out medium lacking histidine, and to produce β -galactosidase as assessed by conversion of X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) into a blue-coloured product. The panels left to right show three independent clones of each transformant grown in the presence (+H) and absence (-H) of histidine, and the filter β -galactosidase assay (β -gal). This analysis was performed three times with three different yeast transformants, with identical results.



This analysis was performed three times with three different yeast transformants, with identical results.

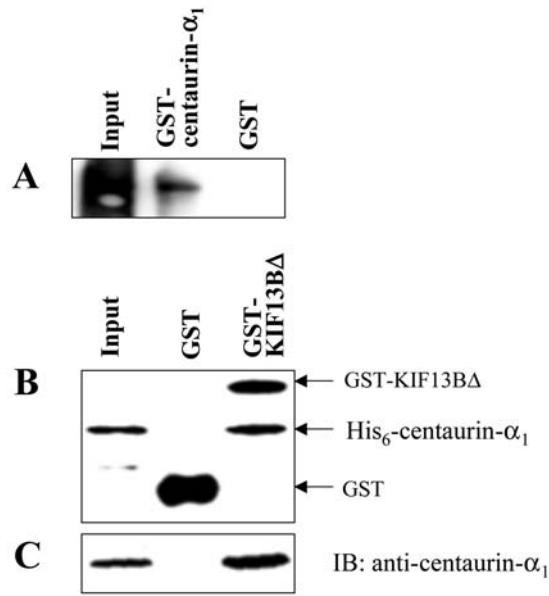


Fig. 4. Centaurin- α_1 directly binds KIF13B in vitro. (A) ^{35}S -radiolabelled KIF13B ΔC (aa 1-991) was expressed using an in vitro translation system and incubated with glutathione beads immobilized with either GST or GST-centaurin- α_1 . The beads were washed and bound protein was resolved by SDS-PAGE and detected by fluorography. (B,C) Purified recombinant centaurin- α_1 was incubated with beads linked to GST or GST-KIF13B ΔA . After washing the beads, protein bound to the beads was resolved by SDS-PAGE and analysed by Coomassie Blue staining (B) or immunostaining using an anti-centaurin- α_1 antibody (C). The experiment was repeated two further times with identical results.

To confirm the centaurin- α_1 binding site in KIF13B in vitro, GFP or GFP-tagged KIF13B or KIF13 ΔA were expressed in COS cells. The cell lysates were incubated with either GST alone or GST-tagged centaurin- α_1 , and the bound proteins visualized by western blotting using an anti-GFP antibody (Fig. 7B). This analysis revealed that centaurin- α_1 interacts with KIF13B or KIF13 ΔA but not GFP. Similar results were also obtained using a GST-tagged centaurin- α_1 deletion mutant that contains the KIF13B binding site ($\Delta\text{C}248$), but not the deletion mutant that lacks the KIF13B binding site ($\Delta\text{N}125$), in place of GST-centaurin- α_1 (data not shown). These results confirm that the N-terminal ARF GAP domain of centaurin- α_1 binds to KIF13B ΔA .

KIF13B does not associate with other members of the ARF GAP family

As KIF13B interacts with the ARF GAP domain of centaurin- α_1 , we tested whether KIF13B interacts with other members of the ARF GAP family such as ARF1 GAP, centaurin- β_1 /ACAP1, centaurin- δ_2 /ARAP1 and GIT1 by GST-fusion protein pull-down assay using GST-KIF13B ΔA . The first two of these proteins act as GAPs for ARF1 whereas the latter two are GAPs for ARF6. For this purpose, we expressed these ARF GAPs along with centaurin- α_1 (positive control) as GFP-tagged fusion proteins in COS cells. The cell lysates were incubated with GST-tagged KIF13 ΔA and the bound protein detected by immunoblotting using an anti-GFP antibody. GST-

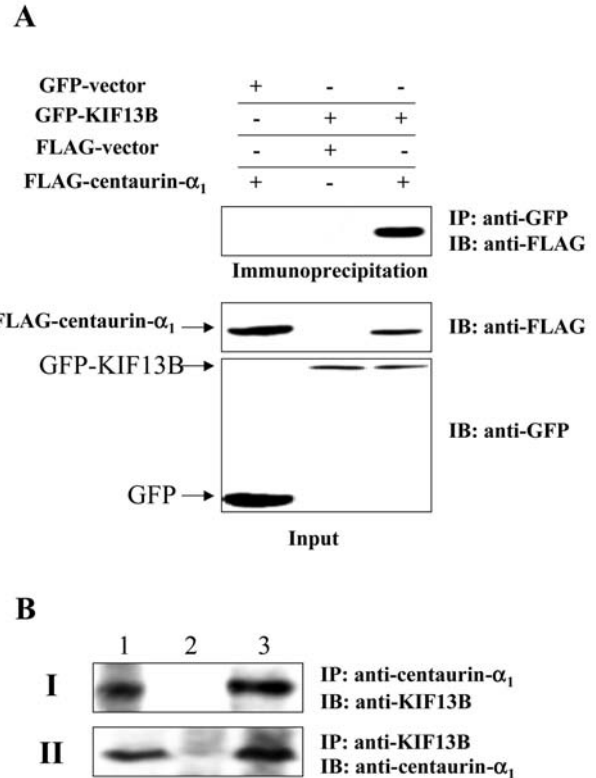


Fig. 5. In vivo interaction between centaurin- α_1 and KIF13B. (A) Co-immunoprecipitation of FLAG-centaurin- α_1 with GFP-KIF13B. COS cells transfected with the indicated expression vectors were lysed after 2 days and immunoprecipitated (IP) with an anti-GFP antibody. After washing, immunoprecipitates were resolved on SDS-PAGE, blotted onto PVDF membranes and probed with a monoclonal anti-FLAG antibody (IB) to detect FLAG-tagged centaurin- α_1 . One-twentieth of the input (cell lysates) was also immunoblotted (IB) with anti-GFP (lower panel) and anti-FLAG (upper panel) antibodies to ensure that GFP-KIF13B and FLAG-centaurin- α_1 , respectively, were expressed. (B) Co-immunoprecipitation of endogenous centaurin- α_1 and KIF13B. The lysates of brain were immunoprecipitated (IP) with control pre-immune serum (lane 2) or an anti-centaurin- α_1 antiserum (lane 3) and endogenous KIF13B visualized in the precipitate and cell lysate (lane 1) by immunoblotting with an anti-KIF13B antibody (I). The lysates of brain were also immunoprecipitated (IP) with control pre-immune serum (lane 2) or an anti-KIF13B antiserum (lane 3) and endogenous centaurin- α_1 detected in the precipitate and cell lysate (lane 1) by immunoblot (IB) using an anti-centaurin- α_1 antibody (II). These experiments were repeated a further two times with similar results.

KIF13B ΔA precipitated centaurin- α_1 but not the other ARF GAPs from COS cell extracts (Fig. 8).

Subcellular localization of centaurin- α_1 and KIF13B

To determine whether centaurin- α_1 and KIF13B colocalize in cultured mammalian cells, GFP-tagged KIF13B or its deletion mutants (ΔMD , no motor domain, and ΔCG , without the CAP-GLY domain) and FLAG-tagged centaurin- α_1 or centaurin- $\alpha_1\Delta\text{N}125$ (the deletion mutant lacking the GAP domain that contains the zinc-finger motif and binds KIF13B) were transiently expressed either alone or together in HeLa cells.

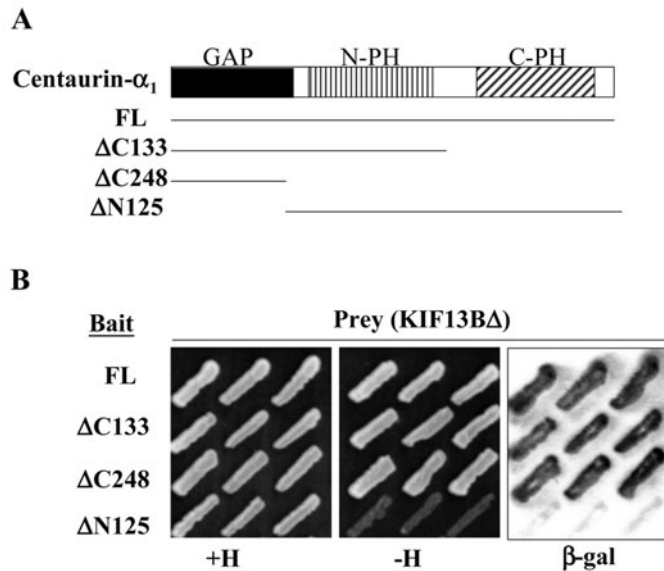


Fig. 6. Mapping of the KIF13B binding site within centaurin- α_1 using yeast two-hybrid analysis. (A) Schematic representation of the domain structure (GAP domain, aa 1-126; N-PH, aa 130-230; C-PH, aa 253-356) and various deletion mutants of centaurin- α_1 . (B) Interaction of KIF13B with the full-length (FL) and deletion constructs of centaurin- α_1 . The full-length and deletion mutants of centaurin- α_1 fused to the DNA binding domain of LexA (bait) pBTM, were co-transformed with KIF13B Δ fused to the activation domain of Gal4 (prey) into the L40 yeast strain, and the transformants analysed for their ability to grow on medium lacking histidine, and to metabolise X-gal by β -galactosidase. Panels, left to right, show three clones of each transformant grown on medium with (+H) and without (-H) histidine, and the filter β -galactosidase assay (β -gal). The experiment was performed three times with three different yeast transformations with identical results.

The subcellular localization of GFP-tagged KIF13B or its deletion mutants (Δ MD and Δ CG) was determined by GFP fluorescence and of FLAG-tagged centaurin- α_1 or its deletion mutant (Δ N125) by immunofluorescence using a monoclonal anti-FLAG primary antibody and Cy3-labelled (red) secondary antibody. GFP-tagged KIF13B was localized to the leading edges of cells (Fig. 9) and this specific localization of KIF13B disappeared upon treatment with nocadazole, an antimitotic agent that disrupts microtubules, or by deleting the motor domain (Δ MD). This result further confirms that KIF13B is a microtubule-dependent protein (Asaba et al., 2003). However, the deletion mutant of KIF13B (Δ CG) showed a localization identical to that of the wild-type protein, suggesting that the CAP-GLY domain, which is required for KIF13B to bind actin, is not essential for the localization of KIF13B to the leading edges of cells. We also stained untransfected and GFP-KIF13B transfected HeLa cells with α -tubulin antibody (microtubules) and phalloidin (F-actin) to determine whether exogenous expression of KIF13B has any effect on microtubule and actin cytoskeleton structures. The microtubule tubulin and F-actin staining in the control untransfected HeLa cells was similar to that in the cells expressing KIF13B, indicating that KIF13B expression had no effect on microtubule tubulin and F-actin structures (data not shown).

FLAG-tagged centaurin- α_1 showed localization to the

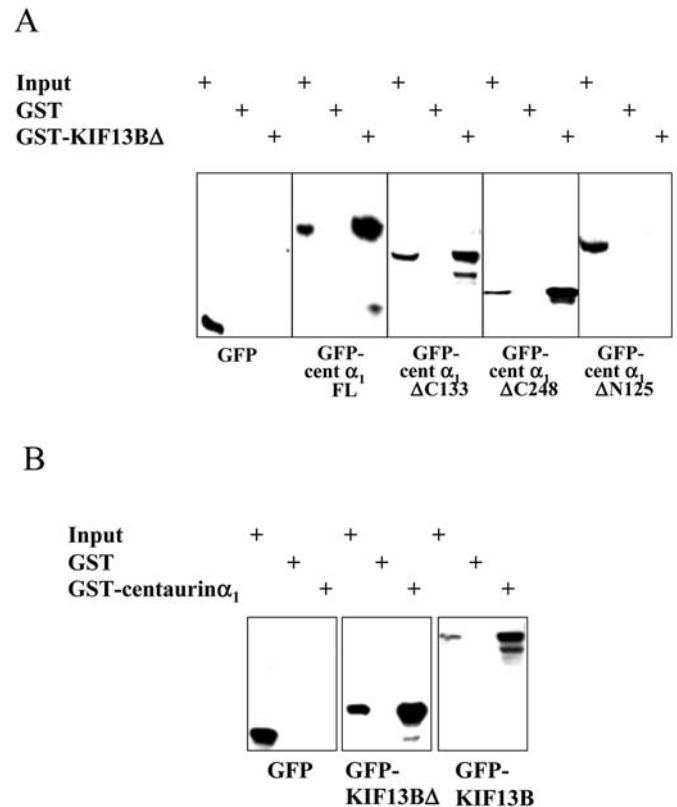


Fig. 7. Mapping of the centaurin- α_1 and KIF13B binding sites using a GST pull-down assay. (A) Lysates of COS cells expressing GFP or GFP-tagged centaurin- α_1 (cent α_1) or its deletion mutants were incubated with either GST or GST-KIF13B coupled to glutathione beads. (B) The lysates of COS cells expressing GFP-tagged KIF13B or KIF13B Δ were incubated with either GST or GST-centaurin- α_1 coupled to glutathione-beads. After washing the beads, the bound proteins were separated on SDS-PAGE, blotted onto PVDF membranes, and probed with an anti-GFP antibody. 5% of the inputs (cell lysates) are also shown for each of the sets of experiments. These experiments were repeated three times with similar results.

cytosol and nucleus (Fig. 9), consistent with the observation that GFP- or HA-tagged exogenously expressed or endogenous centaurin- α_1 localizes to the cytosol and nucleus (Venkateswarlu and Cullen, 1999). Centaurin- α_1 Δ N125 mutant lost its ability to localize to the nucleus, confirming that the zinc-finger motif of centaurin- α_1 GAP domain contains a nuclear targeting signal (Tanaka et al., 1999). Centaurin- α_1 colocalized with KIF13B at the leading edges of cells when co-expressed with GFP-tagged KIF13B. However, nocadazole treatment or deletion of the motor domain of KIF13B (Δ MD) prevented colocalization of centaurin- α_1 and KIF13B at the leading edges of cells. Moreover, the deletion mutant of centaurin- α_1 (Δ N125), lacking the KIF13B binding domain, failed to colocalize with KIF13B. Together, these results suggest that KIF13B binds and transports centaurin- α_1 to the leading edges of cell in a motor domain dependent manner.

KIF13B affects *in vivo* ARF GAP activity of centaurin- α_1
To explore the functional significance of the KIF13B-

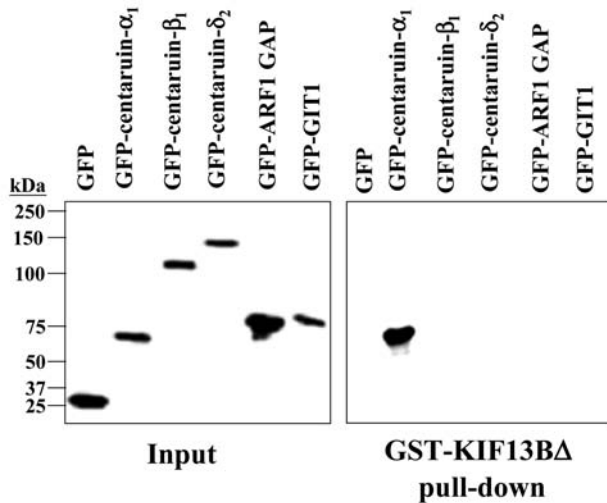


Fig. 8. Interaction with KIF13B is not conserved among ARF GAPs. COS cells were transiently transfected with the indicated expression vectors. After 2 days, cells were lysed and incubated with GST-tagged KIF13BA coupled to glutathione beads. After washing the beads, the bound proteins were analysed by immunoblotting using an anti-GFP antibody (right-hand panel). 5% of the input (cell lysates) was also immunoblotted using an anti-GFP antibody to ensure that the indicated GFP-tagged ARF GAPs were expressed (left-hand panel). Positions of molecular mass standards (kDa) are shown. This analysis was performed three times with identical results.

centaurin- α_1 interaction, we examined whether KIF13B affects the ARF GAP activity of centaurin- α_1 . ARF6 associates with a tubular endosomal compartment in its inactive GDP-bound state and localizes to the plasma membrane in its active GTP-bound state. We have shown recently that centaurin- α_1 specifically inhibits GTP loading of ARF6 in vivo and redistribution of ARF6 from the endosomal compartment to the plasma membrane in EGF-stimulated cells by acting as a PtdIns(3,4,5) P_3 -dependent GAP for ARF6 (Venkateswarlu et al., 2004). To study the effect of KIF13B on the inhibition of ARF6 redistribution by centaurin- α_1 , we expressed HA-tagged ARF6, GFP or GFP-tagged centaurin- α_1 and Myc-tagged KIF13BA or KIF13B in HeLa cells, and assessed ARF6 activation following EGF stimulation by immunostaining for Myc- and HA-tagged proteins (Fig. 10). As observed previously, ARF6 showed punctuate localization when co-expressed with either control GFP or GFP-centaurin- α_1 and redistributed to the plasma membrane in GFP, but not in GFP-centaurin- α_1 expressing cells upon stimulation with EGF (Venkateswarlu et al., 2004). However, centaurin- α_1 was ineffective in preventing ARF6 redistribution in the presence of either KIF or KIF13BA, indicating that KIF13B may inhibit the ARF6 GAP activity of centaurin- α_1 in intact cells. Moreover, KIF13B, but not KIF13BA that lacks motor domain, recruited ARF to the leading edges of the cell in presence of centaurin- α_1 in EGF-stimulated cells, suggesting that KIF13B may interact with ARF_{GTP} via centaurin- α_1 . To confirm that KIF13B affects the centaurin- α_1 ARF6 GAP activity, we analysed the biochemical effect of KIF13B on the in vivo GAP activity of centaurin- α_1 . As with other small GTPases, ARF6 has also been shown to interact specifically with its effectors such as MT2A when it is in the active GTP-bound form

(Schweitzer and D'Souza-Schorey, 2002). By making use of this interaction as a readout for the ARF6 activation, a GST effector pull-down assay has been developed to study ARF6 activation in vivo (Schweitzer and D'Souza-Schorey, 2002). This assay is useful to correlate the intracellular distribution of ARF with its nucleotide bound status. For this in vivo analysis of ARF6 activation, HA-tagged ARF6 was co-expressed with FLAG-empty vector or FLAG-centaurin- α_1 GFP and GFP-KIF13BA in COS cells. Following EGF stimulation, activated ARF6 was precipitated from the cell lysates using GST-MT2A coupled to the glutathione resin. ARF6 activation in EGF-treated cells was reduced to basal levels by centaurin- α_1 and the reduction was prevented by KIF13BA (Fig. 11). However, KIF13BA had no effect on the ARF6 activation in the absence of centaurin- α_1 . Identical results were obtained when KIF13B was used in place of KIF13BA in the assay (data not shown). Furthermore, KIF13B increased EGF-stimulated ARF6 activation in PC12 cells, which express centaurin- α_1 but not KIF13B endogenously (see Fig. 2B), indicating that it inhibits the ARF6 GAP activity of endogenous centaurin- α_1 (data not shown). To further confirm the functional consequences of the interaction between centaurin- α_1 and KIF13B, we analysed the effect of centaurin- α_1 and KIF13B on exocytosis. Using hGH secretion as a readout assay for the exocytosis, the requirement of ARF6 activation for the exocytosis of dense core vesicles in PC12 cells has recently been shown (Vitale et al., 2002; Aikawa and Martin, 2003). As centaurin- α_1 functions as a GAP for ARF6 and KIF13B inhibits the GAP activity of centaurin- α_1 , we hypothesized that centaurin- α_1 may inhibit exocytosis by inactivating ARF6, and KIF13B would prevent this inhibition. To test this hypothesis, we co-expressed hGH with FLAG-empty vector or FLAG-centaurin- α_1 and GFP or GFP-KIF13B in PC12 cells and assessed hGH secretion under basal and ATP-stimulated conditions (Fig. 12). Consistent with the previous report (Rajebhosale et al., 2003), ATP-stimulation induced a three- to fourfold increase in hGH secretion in PC12 cells. Centaurin- α_1 significantly inhibited ATP-stimulated hGH secretion. However, the GAP mutant (R49C), and the double PH mutant (R149C/R273C), which is defective in binding to PtdIns(3,4,5) P_3 , of centaurin- α_1 (Venkateswarlu et al., 2004) were unable to inhibit growth hormone secretion in ATP-stimulated cells, suggesting that centaurin- α_1 requires both its GAP activity and association with the plasma membrane by binding to PtdIns(3,4,5) P_3 , to inhibit the growth hormone secretion (our unpublished data). The inhibitory effect of centaurin- α_1 on hGH secretion was completely abolished when we co-expressed centaurin- α_1 with KIF13B. However, KIF13B had increased ATP-stimulated hGH secretion in PC12 cells when expressed alone, supporting the notion that KIF13B activates ARF6 by inhibiting endogenous centaurin- α_1 (see above). Together, these studies clearly suggest that KIF13B regulates ARF6 activation by binding to centaurin- α_1 .

As immunofluorescence analysis suggested that KIF13B might interact with ARF6_{GTP} through centaurin- α_1 , we next used immunoprecipitation as a direct approach to determine whether KIF13B forms a ternary complex with ARF6_{GTP} and centaurin- α_1 . For this purpose, we co-expressed HA-ARF6_{Q67L} (the constitutively active GTP-bound mutant) with FLAG-centaurin- α_1 or GFP-KIF13B, or both, in COS cells and immunoprecipitated with an anti-HA antibody (Fig. 13). GFP-

KIF13B did not coprecipitate with HA-ARF_{6GTP} in the absence of FLAG-centaurin- α_1 . However, it coprecipitated with HA-ARF_{6GTP} when FLAG-centaurin- α_1 , which contains the binding sites for both KIF13B and ARF6, was co-

expressed. This analysis clearly indicates that ARF_{6GTP} and KIF13B interact with centaurin- α_1 but not to each other.

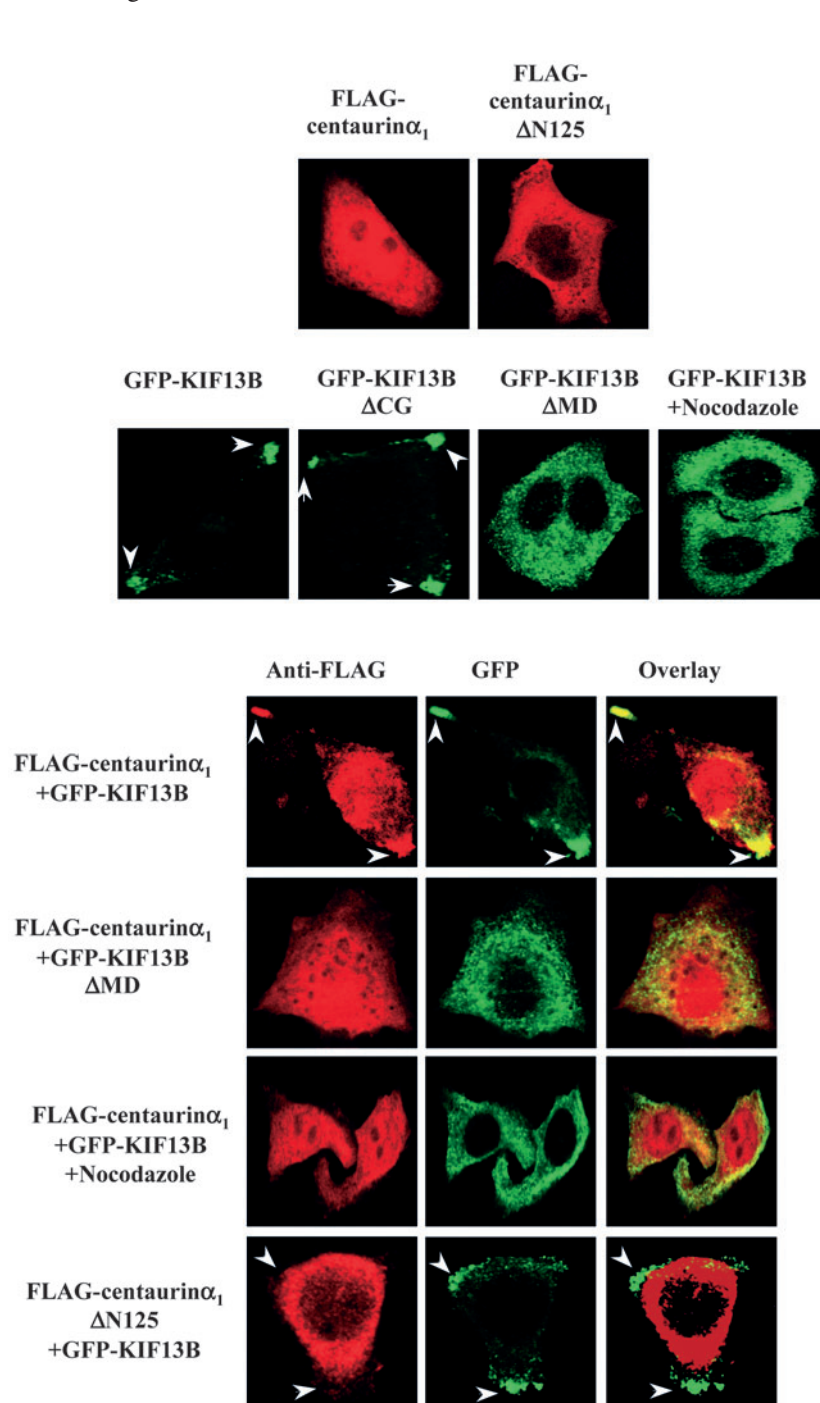


Fig. 9. Centaurin- α_1 colocalizes with KIF13B at the leading edges of the cell. HeLa cells were transiently transfected with either FLAG-centaurin- α_1 or GFP-KIF13B, or both. After 2 days, the cells were fixed with paraformaldehyde and then immunostained with an anti-FLAG primary antibody and a Cy3-labelled secondary antibody, mounted onto glass slides and imaged using a confocal microscope. Where indicated, the transfected cells were incubated with 3.5 μ M nocodazole for 60 minutes at 37°C prior to the fixation. The images are representative of 85-90% of 90-110 transfected cells from five different cell preparations. Arrows indicate regions within the cell where KIF13B is localised. Magnification 100 \times .

Discussion

Members of the ARF family of small GTPases regulate membrane trafficking at multiple sites within the cell (Moss and Vaughan, 1998; Randazzo et al., 2000). As the ARF GEFs and ARF GAPs outnumber ARFs, an emerging theme supports the notion that these proteins contribute to the site-specific regulation of ARFs (Bonifacino and Jackson, 2003). Moreover, the GEFs and GAPs themselves are recruited to specific intracellular locations by binding to lipids and proteins. We and others have recently characterized the phosphoinositide lipids and ARF GEF binding proteins that are involved in the recruitment and regulation of the ARF GEFs (Cullen and Venkateswarlu, 1999; Hawadle et al., 2002). Phosphoinositides such as PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3 play vital roles by regulating the recruitment and activity of ARF GAPs such as centaurin- α_1 , ARAPs 1-3, GIT1 and centaurin- β isoforms (Venkateswarlu et al., 1999; Vitale et al., 2000; Jackson et al., 2000; Krugmann et al., 2002; Miura et al., 2002). The protein interactors for ARF GAPs are also important for the regulation and recruitment of ARF GAPs to specific sites within the cell. For example, the KDEL (lysine-asparatate-glutamate-leucine) receptor, ERD2 (endoplasmic reticulum retention-defective complementation group 2), interacts with the GAP for ARF1 thereby regulating the recruitment of cytosolic ARF1GAP to the Golgi complex (Aoe et al., 1997). Centaurin- β_3 /ASAP1 (ARF GAP containing SH3, ankyrin repeats and PH domain 1) and centaurin- β_4 /KIAA0400 are recruited to focal contacts and regulate cell movement by associating with Pyk2 (proline-rich tyrosine kinase 2) and focal adhesion kinase (FAK) tyrosine kinases, and the focal adhesion adaptor protein paxillin (Andreev et al., 1999; Kondo et al., 2000; Liu et al., 2002). Intriguingly, GIT1 regulates cell adhesion by its recruitment to focal contacts through interaction with β PIX (a Rho GEF), FAK and paxillin (Bagrodia et al., 1999; Turner et al., 1999; Zhao et al., 2000). In neuronal cells, GIT1 plays an important role in the organization of cytoskeletal actin at active zones, which have been implicated in defining neurotransmitter release sites, by forming a complex with piccolo, β PIX FAK and paxillin (Kim et al., 2003). GIT1 also interacts with G-protein coupled receptor (GPCR) kinase 2 and plays a role in GPCR internalization at the cell surface (Premont et al., 1998).

In this report, we used a yeast two-hybrid based cDNA library screening approach to identify proteins that interact with centaurin- α_1 .

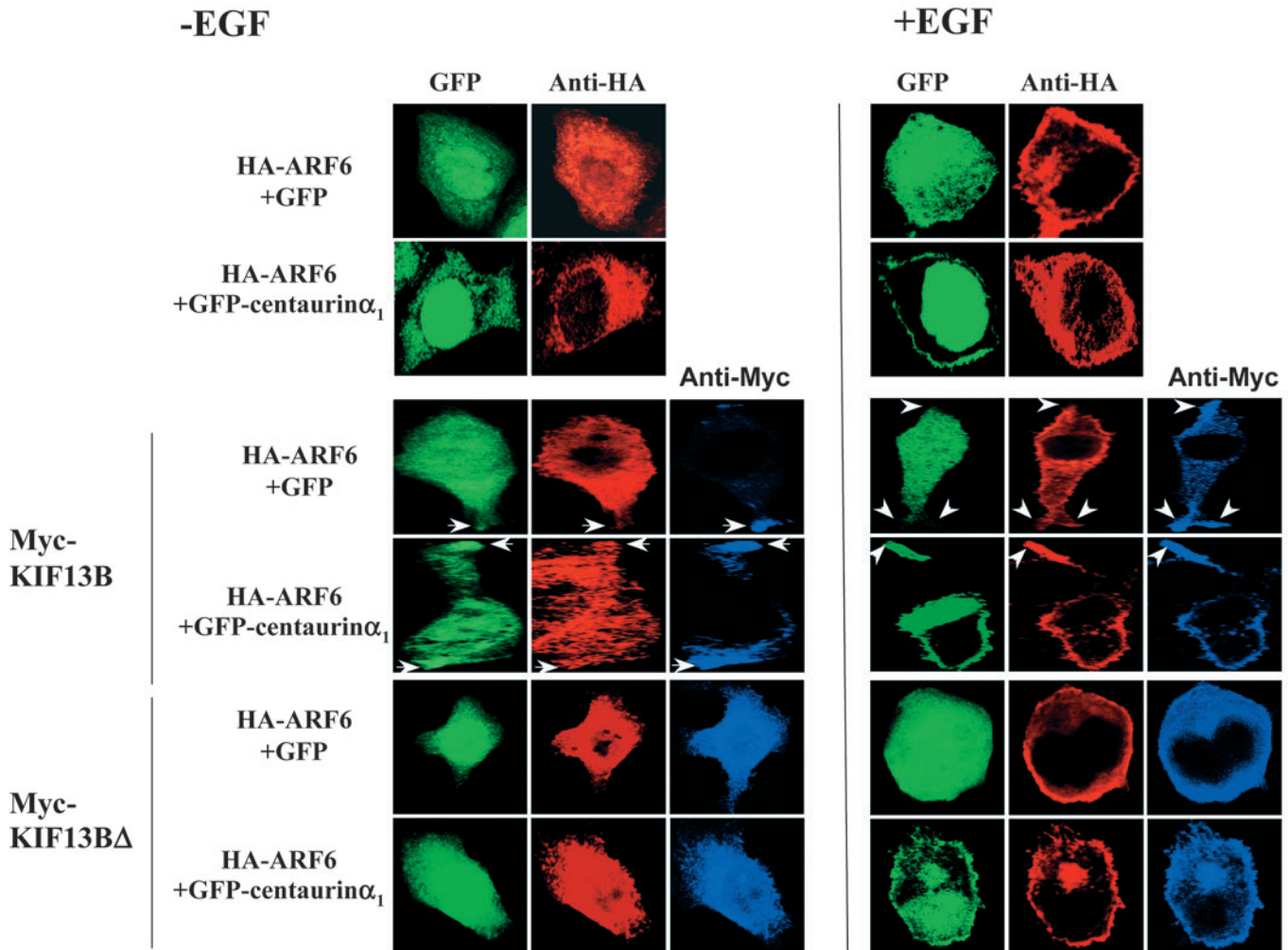


Fig. 10. KIF13B inhibits the ARF6 GAP activity of centaurin- α_1 . HeLa cells were transfected with HA-ARF6, GFP or GFP-centaurin- α_1 , and Myc-KIF13B or Myc-KIF13B Δ . After 2 days, the cells were serum starved for 2 hours, incubated with or without EGF (200 ng/ml) for 5 minutes and fixed with paraformaldehyde. The fixed cells were then immunostained using anti-HA monoclonal and anti-Myc polyclonal primary antibodies, and Cy3- and Cy5-labelled secondary antibodies. The immunostained cells were imaged by confocal microscopy. The images are representative of 80-90% of 40-50 transfected cells from five similar experiments. Arrows indicate regions within the cell where KIF13B is localised. Magnification 100 \times .

This screen identified a novel kinesin motor protein termed KIF13B as a potential binding partner. We have demonstrated the interaction between centaurin- α_1 and KIF13B *in vitro*, *in vivo* and in intact mammalian cells based on the following experiments: (1) The binding of KIF13B to centaurin- α_1 is specific, as KIF13B does not interact with other members of the ARF GAP family in GST pull-down assays under identical conditions; (2) purified centaurin- α_1 associates with the purified KIF13B *in vitro*, indicating that the interaction is direct; (3) immunoprecipitation experiments show that KIF13B and centaurin- α_1 form a complex *in vivo* and also when expressed heterologously; (4) centaurin- α_1 interacts with the KIF13B stalk domain through its ARF GAP domain; (5) KIF13B recruits centaurin- α_1 to the leading edges of the cell surface; (6) KIF13B inhibits ARF6 GAP activity of centaurin- α_1 and by doing so it increases the ARF6-mediated hGH secretion from PC12 cells; (7) Centaurin- α_1 forms a ternary complex with KIF13B and ARF6_{GTP}.

The kinesins (KIF) are a superfamily of microtubule-associated motor proteins that mediate intracellular vesicle and organelle transport, and cell division (Vale and Milligan, 2000). These proteins utilize the energy generated from ATP hydrolysis to transport their cargo along microtubules. KIFs possess a conserved motor domain of approximately 350 amino acids, which is responsible for microtubule binding and ATP hydrolysis. These motor proteins are ubiquitously expressed and are subject to spatial and temporal regulation. Although the mechanism underlying the selective targeting and specific recognition of the cargo by KIFs is not yet clear, it has been hypothesized that KIFs recognize their cargo by binding to proteins localized on the surface of the cargo vesicles (Klopfenstein et al., 2000). These motor proteins are classified into several families based on domain organization, sequence similarity, motility properties, and cellular functions (Miki et al., 2001). There are currently two known KIF13 family members, KIF13A and KIF13B. KIF13A is a microtubule plus

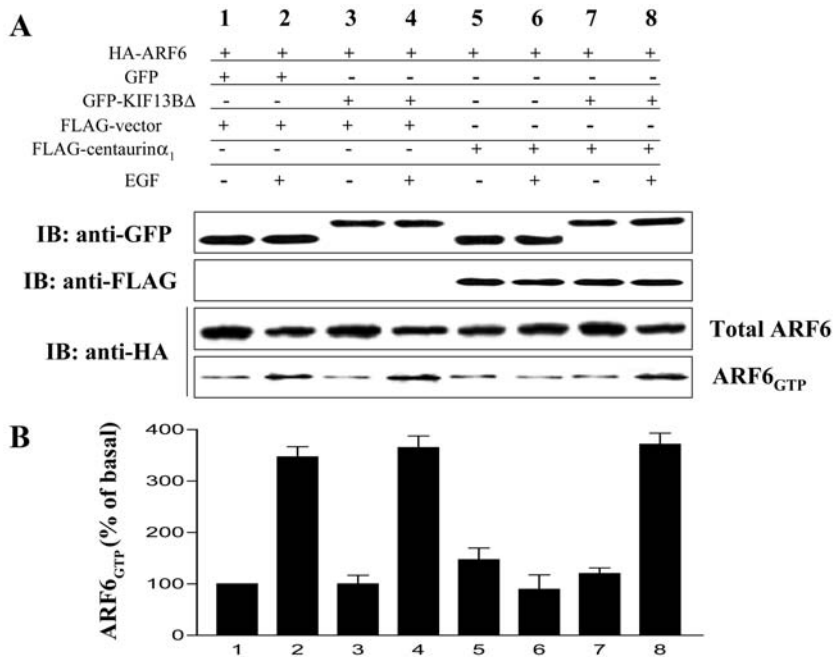


Fig. 11. Biochemical confirmation of KIF13B inhibition of ARF6 GAP activity of centaurin- α_1 . (A) COS cells were transfected with the indicated expression plasmids. 2 days later cells were serum starved, incubated with or without EGF (200 ng/ml). The cells were then lysed and ARF6_{GTP} precipitated using the GST-MT2A coupled to glutathione beads. The precipitates were then immunoblotted (IB) with an anti-HA antibody. The cell lysates (input) were also immunoblotted using anti-HA antibody, anti-GFP and anti-FLAG antibodies to determine HA-ARF6, FLAG-centaurin α_1 and GFP-KIF13BΔ levels respectively. (B) Quantification of data obtained from three similar experiments as shown in A above. Data are the means \pm s.e.

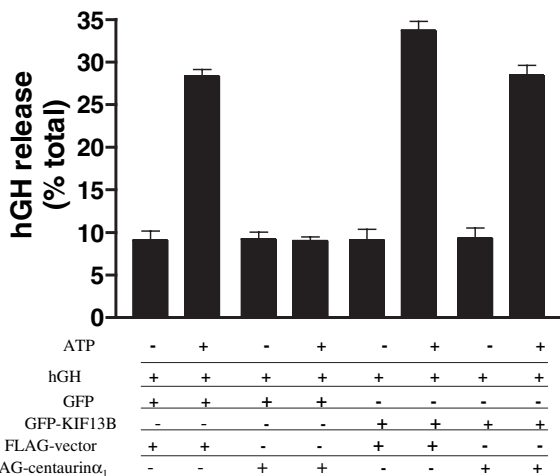


Fig. 12. Effect of centaurin- α_1 and KIF13B on hGH secretion. PC12 cells were co-transfected with pXGH5 and the indicated test plasmids. After 2 days, the cells were incubated under basal or stimulatory (0.3 mM ATP) conditions for 10 minutes. The amount of secreted hGH in the assay medium as well as unsecreted hGH in the cells was determined by ELISA, and the percentage of total hGH secreted was calculated. Data are means \pm s.e. of three independent experiments performed in triplicate.

end-directed motor protein and therefore it carries cargo in the anterograde direction, from cell centre to the cell periphery. KIF13A carries cargo vesicles containing the adaptor protein-1 (AP-1) complex and the mannose-6-phosphate receptor (M6PR) from the trans-Golgi network to the plasma membrane by associating with β 1-adaptin subunit of the AP-1 complex, which in turn interacts with M6PR (Nakagawa et al., 2000). KIF13B interacts with MAGUK family proteins, including hDlg, postsynaptic density (PSD)-95 and centaurin- α_1 via the non-overlapping regions of the N-terminal regions of stalk domain (Hanada et al., 2000). In MDCK cells, overexpression of KIF13B has been shown to induce long process structures where the motor accumulates together with endogenous hDlg particularly at the tip of these projections (Asaba et al., 2003).

We demonstrate, in this report, that exogenously expressed KIF13B interacts with centaurin- α_1 and recruits it to the leading edges at the cell surface in HeLa cells. The specific localization of exogenously expressed KIF13B in HeLa and MDCK cells is similar to that observed for heterologously expressed microtubule plus end-directed kinesins such as KIF13A (Nakagawa et al., 2000). Moreover, microtubules stimulate the *in vitro* ATPase activity of KIF13B (Asaba et al., 2003). Together, these observations indicate that KIF13B is a microtubule plus-end directed kinesin motor protein. What might be the role of a centaurin- α_1 -KIF13B interaction in cellular functions? KIF13B might simply be regulating the ARF6 GAP activity and recruitment of centaurin- α_1 to the specific sites. Alternatively, as centaurin- α_1 also interacts with the second

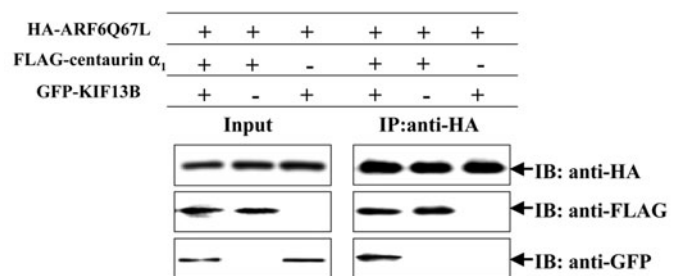


Fig. 13. Co-immunoprecipitation of GFP-KIF13B and FLAG-centaurin- α_1 with HA-ARF6_{GTP}. COS cells transiently transfected with the indicated expression constructs were lysed after 2 days of transfection and immunoprecipitated (IP) with an anti-HA antibody polyclonal antibody. The precipitates were then immunoblotted (IB) with anti-HA, anti-FLAG and anti-GFP monoclonal antibodies to detect HA-ARF6, FLAG-centaurin- α_1 and GFP-KIF13B, respectively. One-twentieth of the cell lysates (Input) were also immunoblotted (IB) with the anti-HA, anti-FLAG and anti-GFP antibodies to ensure that HA-ARF6_{GTP}, FLAG-centaurin- α_1 and GFP-KIF13B, respectively, were expressed. The experiment was repeated two further times with identical results.

messenger PtdIns(3,4,5) P_3 and with ARF6_{GTP} (Venkateswarlu et al., 1999; Venkateswarlu et al., 2004), KIF13B may transport vesicles containing PtdIns(3,4,5) P_3 and/or ARF6 by using centaurin- α_1 as bait. Dictyostelium KIF1A/Unc104, closely related to KIF13B, transports PtdIns(4,5) P_2 -containing lipid vesicles along microtubules in vitro by binding to PtdIns(4,5) P_2 via its PH domain, suggesting that it could also recognize and transport PtdIns(4,5) P_2 -containing vesicles in vivo (Klopfenstein et al., 2002). However, KIF13B interacts with neither PtdIns(3,4,5) P_3 nor ARF6_{GTP} on its own (our unpublished data). Although PtdIns(3,4,5) P_3 is thought to localize to the inner leaflet of the plasma membrane, it has recently been demonstrated that the production of intracellular PtdIns(3,4,5) P_3 is triggered by receptor endocytosis (Sato et al., 2003). Taken together, these observations suggest that the interaction between centaurin- α_1 and KIF13B represents an important connection between ARF6- and/or PtdIns(3,4,5) P_3 -associated membrane trafficking pathways and a kinesin motor protein. Although PIs, small GTPases such as ARF6, and kinesin motor proteins are vital for membrane trafficking, the molecular mechanisms linking these three components have not been characterized. Furthermore, this study is another indication of the importance of motor proteins in selective recruitment of small GTPases and their regulators within the cell. Recent studies have described the binding of ARFs to mitotic kinesin-like protein (MKLP)1, Rab6 small GTPase to the kinesin-related protein rabkinesin, Rab27 to myosin Va, Rab11a to myosin Vb and Rab7 to dynein motor, and the importance of these interactions in membrane trafficking (Boman et al., 1999; Echard et al., 1998; Hammer III and Wu, 2002; Jordens et al., 2001).

As KIF13B inhibits the ARF6 GAP activity of centaurin- α_1 , a specific regulatory mechanism such as phosphorylation after reaching the target site could dissociate centaurin- α_1 from KIF13B, allowing centaurin- α_1 to inactivate ARF6. Centaurin- α_1 interacts with both CK1 α and PKC but is only phosphorylated by PKC (Dubois et al., 2001; Zemlickova et al., 2003) whereas KIF13B is phosphorylated by several unidentified kinases (Hanada et al., 2000). In this respect, it is worth noting here that PtdIns(3,4,5) P_3 , ARF6 and PKC isoforms have been shown to concentrate at the leading edges of the cell surface in order to regulate cell adhesion and migration (Sabe, 2003; Ridley et al., 2003). These cellular events are essential for a wide variety of physiological and pathological processes, including embryogenesis, wound healing and tumour metastasis (Ridley et al., 2003). We are currently investigating whether the centaurin- α_1 -KIF13B interaction could regulate ARF6- and/or PtdIns(3,4,5) P_3 -associated membrane trafficking and if this interaction could be controlled by phosphorylation. In summary, our results indicate that KIF13B directly interacts with and recruits centaurin- α_1 to specialized membrane sites at the cell surface, and regulates its ARF6 GAP activity.

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