PI-3-kinase-dependent membrane recruitment of centaurin-α_2 is essential for its effect on ARF6-mediated actin cytoskeleton reorganisation

Kanamarlapudi Venkateswarlu*, Kevin G. Brandom and Hongruo Yun

Department of Pharmacology, The University of Bristol, Bristol, BS8 1TD, UK

*Author for correspondence (e-mail: k.venkateswalru@bristol.ac.uk)

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Summary

GTPase activating proteins (GAPs) of the centaurin family regulate the actin cytoskeleton and vesicle trafficking through inactivation of the ADP-ribosylation factor (ARF) family of small GTP-binding proteins. We report the functional characterisation of centaurin-α_2, which is structurally related to the centaurin-α_1 ARF6 GAP. Centaurin-α_2 contains an N-terminal GAP domain followed by two pleckstrin homology (PH) domains (N-PH and C-PH). In vitro, GFP-centaurin-α_2 specifically binds the phosphatidylinositol (PI) 3-kinase lipid products, PI 3,4-P_2 and PI 3,4,5-P_3 (PIP_3), through its C-terminal PH domain. In agreement with this observation, GFP-centaurin-α_2 was recruited to the plasma membrane from the cytosol in EGF-stimulated cells in a PI-3-kinase-dependent manner. Moreover, the C-PH domain is sufficient and necessary for membrane recruitment of centaurin-α_2. Centaurin-α_2 shows sustained kinetics of PI-3-kinase-mediated membrane recruitment in EGF-stimulated cells, owing to its binding to PI 3,4-P_2. Centaurin-α_2 prevents ARF6 translocation to, and cortical actin formation at, the plasma membrane, which are phenotypic indications for ARF6 activation in EGF-stimulated cells. Moreover, the constitutively active mutant of ARF6 reverses the effect of centaurin-α_2 on cortical actin formation. The membrane targeted centaurin-α_2 is constitutively active. Together, these studies indicate that centaurin-α_2 is recruited in a sustained manner to the plasma membrane through binding to PI 3,4-P_2 and thereby regulates actin reorganisation via ARF6.

Key words: Centaurin-α_2, PI 3-kinase, PIP_3, PI 3,4-P_2, ARF6, Actin, GAP

Introduction

Phosphatidylinositol (PI) 3-kinase phosphorylates the 3′ position of the inositol ring of PI and its derivatives to generate the 3-phosphorylated PI lipids, including PI 3-P, PI 3,4-P_2 and PI 3,4,5-P_3 (PIP_3) (Vanhaesebroeck et al., 2001). PI 3,4-P_2 is also produced from dephosphorylation of PIP_3 by 5-phosphatases such as the inositol polyphosphate 5-phosphatase (PIPP) (Ooms et al., 2006). For this reason, PIP_3 production in stimulated platelets is immediate and transient whereas PI 3,4-P_2 accumulation is slightly delayed and significantly more sustained (Franke et al., 1997). Both PIP_3 and PI 3,4-P_2 play major roles as second messengers in many cellular functions such as cell migration and vesicle budding. These lipid second messengers function mainly by acting as site-specific ligands for the recruitment and/or activation of intracellular proteins required for the formation of signalling complexes at the plasma membrane. A large number of downstream targets, including ARF regulators, such as cytohesins and centaurins, have been identified for these lipids (Rameh and Cantley, 1999). Most of these proteins contain modules, typically pleckstrin homology (PH) domains, that are capable of binding to the inositol head group of PIP_3 and/or PI 3,4-P_2.

ADP-ribosylation factors (ARFs) are a family of monomeric small GTPases that are ubiquitously expressed and play important roles in the regulation of cellular processes such as membrane trafficking and cytoskeletal actin reorganisation, which are vital for cell adhesion, cell migration and cell polarity (D'Souza-Schorey and Chavrier, 2006). They do so by shuttling between an inactive GDP-bound and an active GTP-bound form. To date, six members of this family (ARFs1-6) have been identified in mammalian cells (Kahn et al., 2006; Moss and Vaughan, 1998). ARF1 and ARF6 are the least related and also the best studied of the ARF family. ARF1 cycles between the cytosol and the Golgi depending on the bound nucleotide status, localising to the cytosol in the GDP-bound form and attaching to the Golgi membrane through the N-terminal myristoylation signal in the GTP-bound form (Donaldson et al., 2005). By contrast, ARF6 localises to endosomes in its GDP-bound form and to the plasma membrane in its GTP-bound form (Donaldson, 2003). ARF1 mainly functions in the perinuclear areas, mediating the recruitment of cytosolic coat proteins such as COPI and AP1 onto the membranes required for vesicle budding at the Golgi (Donaldson et al., 2005). By contrast, ARF6 primarily functions at the cell periphery, mediating membrane and cell surface receptor endocytosis as well as actin cytoskeleton rearrangements beneath the plasma membrane (D'Souza-Schorey and Chavrier, 2006; Donaldson, 2003).

The activation-inactivation cycle of ARFs is regulated by GTP-exchange factors (GEFs) and GTPase activating proteins (GAPs) (Moss and Vaughan, 1998; Randazzo et al., 2000). GEFs activate ARFs by catalysing the exchange of ARF bound

GDP for GTP. In the active GTP-bound state, ARFs transmit the signal downstream by interacting with specific effector proteins such as the GGA coat protein family and PIP kinases (D'Souza-Schorey and Chavrier, 2006; Nie et al., 2003). GAPs inactivate ARFs by stimulating hydrolysis of the ARF bound GTP to GDP. Although ARF function requires the regulated shuttling between an active GTP-bound and an inactive GDP-bound form, very little is known about the regulation of ARF GTPases by extracellular stimuli. Recent identification of ARF GEFs, such as cytohesins (cytohesins 1-3), and ARF GAPs, such as centaurin-α_1 and ARAP3, as PIP_3-binding proteins has highlighted their potential to serve as signal transducers that link upstream PI 3-kinase to the regulation of ARF GTPases (Cullen and Venkateswarlu, 1999; Hawadle et al., 2002; Jackson et al., 2000).

Centaurin proteins are a family of GAPs for the ARF family of small G proteins (Randazzo and Hirsch, 2004). Centaurin-α_1 (also known as PIP3BP/p42^{IP4}) was originally identified as a PIP_3 or its inositol head group, inositol 1,3,4,5-tetrakisphosphate (IP_4), binding protein (Hammonds-Odie et al., 1996; Stricker et al., 1997; Tanaka et al., 1999). This protein acts as a PIP_3-dependent ARF6 GAP that regulates GPCR internalisation and actin reorganisation (Lawrence et al., 2005; Venkateswarlu et al., 1999a; Venkateswarlu et al., 2004; Venkateswarlu et al., 2005). Centaurin-α_1 contains an N-terminal ARF GAP domain and two adjacent PH domains [one in the middle (N-PH) and the other at the C terminus (C-PH)] that are required for binding to PIP_3 (Venkateswarlu et al., 1999a). Centaurin-α_1 localises to the cytosol and nucleus, and contains a nuclear localisation signal at the N terminus (Tanaka et al., 1999; Venkateswarlu and Cullen, 1999). It associates with and is phosphorylated by PKC isoforms (Zemlickova et al., 2003). Centaurin-α_1 has a restricted tissue distribution and is expressed particularly at high levels in rat brain, indicating that it is important for neuronal functions (Aggensteiner and Reiser, 2003; Hammonds-Odie et al., 1996; Venkateswarlu and Cullen, 1999). Centaurin-α_1 expression is increased in neurons of patients with Alzheimer's disease (AD) (Reiser and Bernstein, 2002). Moreover, it interacts with casein kinase Iα, which hyperphosphorylates the β-amyloid precursor protein in the brains of AD patients (Dubois et al., 2001). To date, the significance of the increase in centaurin-α_1 protein expression in terms of AD pathology is unknown.

Whitley et al. (Whitley et al., 2002) identified centaurin-α_2 as a novel 43 kDa protein by screening a rat adipocyte cDNA library. Centaurin-α_2 shares 58% amino acid identity with centaurin-α_1 and contains the same domain structure as centaurin-α_1, an N-terminal GAP domain followed by two PH domains (N-PH and C-PH). However, there are marked differences between centaurin-α_1 and centaurin-α_2 in terms of the chromosomal localisation, tissue distribution and phosphorylation sites. The gene for human centaurin-α_2 is located on chromosome 17 near to the neurofibromatosis type 1 (NF1) gene, whereas the centaurin-α_1 gene is located on human chromosome 7 (Hanck et al., 2004). In contrast to centaurin-α_1, which is expressed mainly in brain, centaurin-α_2 is expressed in many tissues such as liver and heart but not in brain (Hanck et al., 2004; Whitley et al., 2002). Centaurin-α_1 contains a nuclear localisation signal at its N terminus, which centaurin-α_2 lacks. Similarly centaurin-α_1 contains two PKC phosphorylation sites (one in the GAP domain, S87, and the other in the C-PH domain, T276), which are absent in centaurin-α_2 (Zemlickova et al., 2003). Moreover, the function of centaurin-α_2 is not known. Therefore, in this study we sought to determine the PI binding and ARF GAP activity of centaurin-α_2 in intact cells in order to understand its cellular functions and regulation. We demonstrate that centaurin-α_2 is a target for activated PI 3-kinase that is recruited, in a sustained manner, to the plasma membrane and thereby negatively regulates ARF6-mediated cytoskeleton actin reorganisation.

Results

Centaurin-α_2 interacts with PIP_3 and PI 3,4-P_2 via the C-terminal PH domain in vitro

Since centaurin-α_2 is structurally related to PIP3 binding centaurin-α_1, we hypothesised that centaurin-α_2 may bind to PIP_3 or other PI 3-kinase lipid products. To assess whether centaurin-α_2 associates directly with PI 3-kinase lipid products, we incubated the lysate of cells expressing either GFP or GFP-centaurin-α_2 with avidin beads alone (control beads) or coupled to biotinylated PI 3-kinase lipid products (PIP_3, PI 3,4-P_2 and PI 3-P) or PI 4,5-P_2. The GFP-centaurin-α_2 selectively bound to the avidin beads containing either PI 3,4-P_2 or PIP_3 but not to the control beads or the beads that contained either PI 3-P or PI 4,5-P_2 (Fig. 1A). The amount of GFP-centaurin-α_2 bound to PI 3,4-P_2 beads was relatively higher than that bound to PIP_3 beads. By contrast, GFP alone failed to bind any of the PIs tested under these conditions. This data suggests that centaurin-α_2 specifically interacts with PIP_3 and PI 3,4-P_2, albeit with slightly higher affinity to PI 3,4-P_2.

We subsequently examined the sensitivity of centaurin-α_2 interaction with the PI 3-kinase products by competition binding experiments (Fig. 1B). PI 3-P failed to block centaurin-α_2 binding to PI 3,4-P_2 beads whereas PI 4,5-P_2 partially blocked the binding but only at a very high concentration (10 μM). However, PI 3,4-P_2 and PIP_3 blocked centaurin-α_2 binding to PI 3,4-P_2 beads, the former being more effective. The concentration required to block 50% of the binding (EC_{50}) of PI 3,4-P_2, PIP_3, PI 4,5-P_2 and PI 3-P is calculated from the displacement curve (Fig. 1B) as 20 nM, 87.6 nM, >10 μM and >10 μM, respectively. As centaurin-α_2 contains two PH domains that are known to bind PIs, we generated GFP-tagged centaurin-α_2 PH domain mutants by converting the conserved arginine residue to cysteine in either the N-PH (R152C), or the C-PH (R276C) alone or in both the PH domains [R152C/R276C; double PH mutant (DM)]. We then analysed their ability to bind PIs to determine which PH domain is required for the binding to PI 3,4-P_2 and PIP_3 (Fig. 1C). Binding of the R152C mutant to PI 3,4-P_2 and PIP_3 beads was identical to that of the wild-type (WT) protein whereas the R276C and DM mutants of centaurin-α_2 failed to bind to PI 3,4-P_2 and PIP_3 beads under the same conditions. These results indicate that centaurin-α_2 interacts with PI 3,4-P_2 and/or PIP_3 through the C-PH domain.

Centaurin-α_2 is recruited to the plasma membrane in EGF-stimulated PC12 cells

Centaurin-α_1 is recruited to the plasma membrane from the cytosol in PC12 cells after stimulation with EGF (Venkateswarlu et al., 1999a). Since centaurin-α_2 is structurally related to centaurin-α_1, we tested whether centaurin-α_2 is also

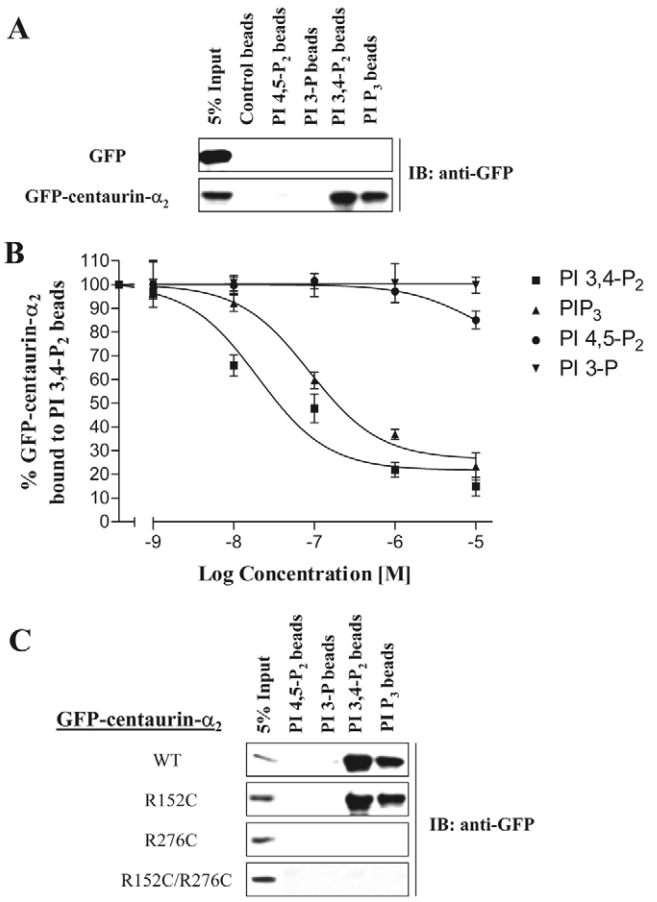

Fig. 1. Centaurin-α_2 interacts with PI 3,4-P_2 and PIP_3 via the C-terminal PH domain in vitro. COS7 cells were transiently transfected with GFP, GFP-centaurin-α_2 or its mutants [the N-PH mutant (R152C), the C-PH mutant (R276C) and the double mutant (R152C/R276C)]. After 2 days, the cells were lysed and the lysates were incubated with the indicated biotinylated PI lipids coupled to avidin beads either in the presence (B) or absence (A,C) of water soluble non-biotinylated PI lipids for 2 hours at 4°C. Protein that remained bound to the PI beads after washing with 0.5% NP-40 was analysed by immunoblotting with an anti-GFP antibody.

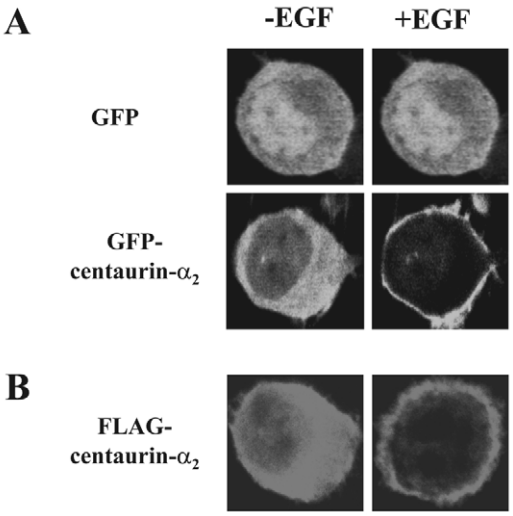

Fig. 2. Centaurin-α_2 is recruited to the plasma membrane in EGF-stimulated PC12 cells. PC12 cells were transiently transfected with the indicated expression vector for 2 days. (A) The cells expressing either GFP or GFP-centaurin-α_2 were serum starved and live cells were imaged by confocal microscopy in the absence or presence of 0.1 μg/ml EGF (images were captured 2 minutes after EGF addition). Similar data were obtained from five other imaged cells. (B) The cells expressing FLAG-centaurin-α_2 were serum starved and stimulated with or without EGF (0.1 μg/ml) for 5 minutes. The cells were then fixed, immunostained with an anti-FLAG antibody and imaged using a confocal microscope. Images are representative of 100-150 transfected cells from three experiments.

recruited to the plasma membrane in EGF-stimulated cells. For this purpose, we expressed GFP-centaurin-α_2 in PC12 cells and analysed subcellular localisation before and after stimulation with EGF by live-cell imaging. Before stimulation, this protein showed a diffuse cytoplasmic localisation. After stimulation with EGF-, GFP-centaurin-α_2, but not GFP alone, showed a clear relocation to the plasma membrane within 2 minutes (Fig. 2A). Similar results were obtained with FLAG-tagged centaurin-α_2 (Fig. 2B), indicating that the large GFP tag had no affect on subcellular localisation of centaurin-α_2.

The membrane recruitment of centaurin-α_2 is mediated through PI 3-kinase activation

Since EGF activates PI 3-kinase and centaurin-α_2 binds PI 3-kinase products in vitro, we tested whether the redistribution of centaurin-α_2 is dependent on activation of PI 3-kinase. This was done by using two chemically distinct PI 3-kinase inhibitors, wortmannin and LY294002. We found that both PI 3-kinase inhibitors can completely block the EGF-stimulated redistribution of centaurin-α_2 to the plasma membrane (Fig. 3A). Moreover, when centaurin-α_2 is co-expressed with Δp85 (a dominant negative mutant of the PI 3-kinase regulatory subunit), the EGF-stimulated membrane recruitment of centaurin-α_2 upon was also prevented (Fig. 3A). We then quantified the effect of PI 3-kinase inhibitors on the EGF-induced membrane recruitment of centaurin-α_2 (Fig. 3C). 97.5%, 1.3%, 2.5% and 1.5% of transfected cells showed membrane localisation of centaurin-α_2 when stimulated with EGF in the presence of DMSO solvent, wortmannin, LY294002 and Δp85, respectively.

To determine whether activation of PI 3-kinase is sufficient for the recruitment of centaurin-α_2 to the plasma membrane, PC12 cells were co-transfected with GFP-centaurin-α_2, and either a membrane-bound, constitutively active, PI 3-kinase (membPI3K) or a control membrane-bound kinase-dead PI 3-kinase (membPI3K-KD), and the subcellular localisation of GFP-centaurin-α_2 was analysed in the absence of EGF. Centaurin-α_2 was recruited to the membrane in cells co-transfected with membPI3K but not membPI3K-KD (Fig. 3B). We found that 96% cells co-transfected with membPI3K, compared to 6% of cells co-expressing membPI3K-KD, showed GFP-centaurin-α_2 localisation at the plasma membrane (Fig. 3C). These results suggest that the activation of PI 3-kinase is responsible for the membrane recruitment of centaurin-α_2.

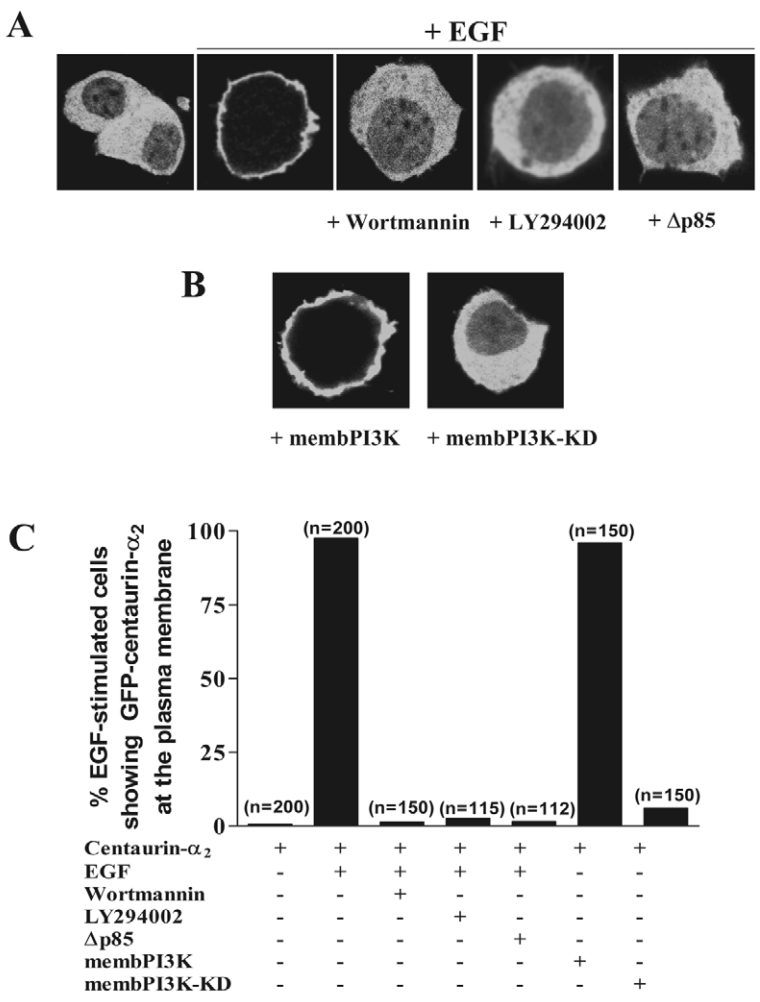

Fig. 3. The membrane recruitment of centaurin-α_2 is mediated through PI 3-kinase activation. (A) PC12 cells transiently transfected with GFP-centaurin-α_2 alone or with the PI 3-kinase regulatory subunit (Δp85). After 2 days, the cells were serum starved and stimulated with 0.1 μg/ml EGF for 5 minutes. The cells expressing GFP-centaurin-α_2 alone were preincubated with PI 3-kinase inhibitors (0.1 μM wortmannin or 50 μM LY294002) prior to EGF-stimulation. The cells were fixed and imaged by confocal microscopy. (B) PC12 cells were co-transfected with GFP-centaurin-α_2 and Myc-tagged membrane-targeted PI 3-kinase (membPI3K) or its inactive form (kinase dead; membPI3K-KD). After 2 days, cells were serum starved, fixed, immunostained with an anti-Myc antibody (not shown) and imaged using a confocal microscope. (C) The EGF-stimulated cells expressing GFP-centaurin-α_2 were scored for the plasma membrane localisation of centaurin-α_2. The number of cells (n) counted for each condition are shown in brackets in the graph. Data are from three independent experiments.

The C-terminal PH domain is sufficient and necessary for membrane recruitment of centaurin-α_2

We next determined which PH domain of centaurin-α_2 is required for EGF-stimulated membrane recruitment. When the GFP-tagged PH mutants (Fig. 4A) were expressed in PC12 cells, we found that the N-PH mutant (R152C), but not the C-PH mutant (R276C) or the double PH mutant (DM), was recruited to the plasma membrane after stimulation (Fig. 4B). In addition, the mutation of the highly conserved arginine residue (corresponding to the arginine in centaurin-α_1 required for the GAP activity) in the GAP domain to cysteine (R53C) had no affect on the EGF-stimulated membrane recruitment of centaurin-α_2. Consistent with this, R276C and DM, but not R152C, failed to localise at the plasma membrane when co-expressed with membPI3K (data not shown). 97%, 96%, 92%, 2% and 1% of cells transfected with wild-type (WT) centaurin-α_2, R53C, R152C, R276C and DM, respectively, were found to show EGF-induced recruitment to the membrane (Fig. 4C). Consistent with the in vitro PI binding data, this result suggests that the C-PH domain appears to be solely responsible for the PI 3-kinase-dependent membrane recruitment of centaurin-α_2.

Centaurin-α_2 shows sustained recruitment to the membrane in EGF-stimulated cells

We have also examined the kinetics of membrane recruitment of centaurin-α_2 in single cells using the live-cell imaging approach (Fig. 5A). In EGF-stimulated PC12 cells, the membrane recruitment of GFP-centaurin-α_2 became visible within 30 seconds after addition of the stimulus, was completed within 90-120 seconds and was sustained (visible up to 60 minutes after stimulation). Centaurin-α_2 binds to PIP_3 and PI 3,4-P_2 in vitro with high affinity. Since PIP_3 production is transient whereas PI 3,4-P_2 accumulation is more sustained in agonist-stimulated cells, we reasoned that centaurin-α_2 is retained at the plasma membrane in a sustained fashion through binding to PI 3,4-P_2. To investigate this possibility, the kinetics of centaurin-α_2 recruitment were compared with the EGF-stimulated redistribution of GFP-tagged cytohesin 3, TAPP1 and PLCδ1-PH, which have been used as biosensors in intact cells to detect PIP_3, PI 3,4-P_2 and PI 4,5-P_2, respectively (Oatey et al., 1999; Ooms et al., 2006). The EGF-stimulated membrane recruitment of cytohesin 3 occurred rapidly (within 30 seconds) and transiently, with visible membrane localisation disappearing by 16 minutes after stimulation. By contrast, the kinetics of EGF-induced plasma membrane redistribution of TAPP1 (sustained) were similar to those of the centaurin-α_2. The PLCδ1-PH domain binds to the constitutively present PI 4,5-P_2 and therefore, as expected, showed constitutive association with the plasma membrane.

The above results suggest that the sustained recruitment of centaurin-α_2 to the membrane in EGF-stimulated cells is likely to be due to its binding to PI 3,4-P_2. To investigate this we used two inositol polyphosphate phosphatases, PIPP, which hydrolyses PIP_3 to PI 3,4-P_2, and type 1α inositol polyphosphate 4-phosphatase (4-phosphatase), which specifically hydrolyses PI 3,4-P_2 to PI 3-P (Ivetac et al., 2005; Ooms et al., 2006). We co-expressed either GFP-tagged centaurin-α_2, cytohesin 3 or TAPP1 with control empty vector, HA-PIPP or FLAG-4-phosphatase in PC12 cells and assessed the plasma membrane recruitment of GFP-tagged proteins after stimulation with EGF (Fig. 5B). Under basal conditions, none of the proteins associated with the plasma membrane in cells

expressing control empty vector, PIPP or 4-phosphatase (data not shown). Upon stimulation with EGF, recruitment of cytohesin 3 to the membrane was observed in control empty vector and 4-phosphatase-expressing cells whereas TAPP1 and centaurin-α_2 were seen at the plasma membrane in control empty vector and PIPP-expressing cells. To analyse the data more quantitatively, we determined the percentage of transfected cells in which the GFP-tagged proteins were recruited to the plasma membrane in response to EGF-stimulation (Fig. 5C). Plasma membrane localisation of GFP-cytohesin 3 was seen in 88% of cells expressing control empty vector compared to 21% and 91% of cells exogenously expressing PIPP and 4-phosphatase, respectively. This suggests that the decrease in PIP_3 accumulation at the plasma membrane was due to overexpression of PIPP. By contrast, TAPP1 associated with the membrane in 83%, 79% and 19% of cells expressing control empty vector, PIPP or 4-phosphatase, respectively, indicating a reduction in the plasma membrane PI 3,4-P_2 levels after ectopic expression of 4-phosphatase. Similar to TAPP1, centaurin-α_2 was detected at the plasma membrane in 86%, 88% and 17% of cells expressing control empty vector, PIPP or 4-phosphatase, respectively. The inhibition of recruitment of centaurin-α_2 to the membrane with 4-phosphatase overexpression indicates that sustained recruitment of centaurin-α_2 to the membrane is indeed a result of its binding to plasma membrane PI 3,4-P_2.

Centaurin-α_2 prevents ARF6 translocation to and cortical actin formation at the plasma membrane

Centaurin-α_1 inhibits the redistribution of ARF6 to the plasma membrane and cortical actin formation in the EGF-stimulated cells by acting as a PIP_3-dependent GAP for ARF6 in vivo (Venkateswarlu et al., 2004). Since centaurin-α_2 contains a GAP domain similar to that of centaurin-α_1 and is recruited to the plasma membrane, we examined whether centaurin-α_2 would also inhibit the recruitment of ARF6 to the plasma membrane and cortical actin formation by acting as an in vivo ARF6 GAP. In unstimulated cells, ARF6 showed a punctate distribution, indicative of endosomal localisation, when co-expressed with either control GFP or GFP-centaurin-α_2, suggesting that centaurin-α_2 does not affect ARF6 localisation under basal conditions (Fig. 6). In EGF-stimulated cells co-expressing ARF6-HA and GFP, ARF6 localised to the plasma membrane. However, EGF-stimulated cells co-transfected with GFP-centaurin-α_2 (WT) and ARF6-HA showed the recruitment of centaurin-α_2 to the plasma membrane whereas ARF6 was not. Neither the GAP inactive mutant (R53C), which is able to bind PIP_3 and/or PI 3,4-P_2, or the GAP active DM, which is unable to bind PIP_3 and/or PI 3,4-P_2, of centaurin-α_2 were able to inhibit ARF6 redistribution in EGF-stimulated cells. This suggests that centaurin-α_2 requires both its ARF GAP activity and association with the plasma membrane to inhibit the ARF6 activation.

We then examined cortical actin formation in EGF-stimulated cells expressing GFP-centaurin-α_2 as a means of studying the effect of centaurin-α_2 on the endogenous ARF6 activation (Fig. 6). In the unstimulated condition, the actin network in the control GFP-transfected cells was similar to that in cells transfected with GFP-centaurin-α_2, indicating that centaurin-α_2 had no effect on the actin network. EGF-induced cortical actin formation was observed in control GFP-expressing cells, but not in the GFP-centaurin-α_2-expressing cells, suggesting that centaurin-α_2 prevented cortical actin formation in EGF-stimulated cells. However, the GAP (R53C) and DM mutants of centaurin-α_2 were unable to prevent cortical actin formation in EGF-stimulated cells. This analysis also suggests that the inhibition of EGF-induced cortical actin formation was dependent on both the plasma membrane recruitment and the GAP activity of centaurin-α_2.

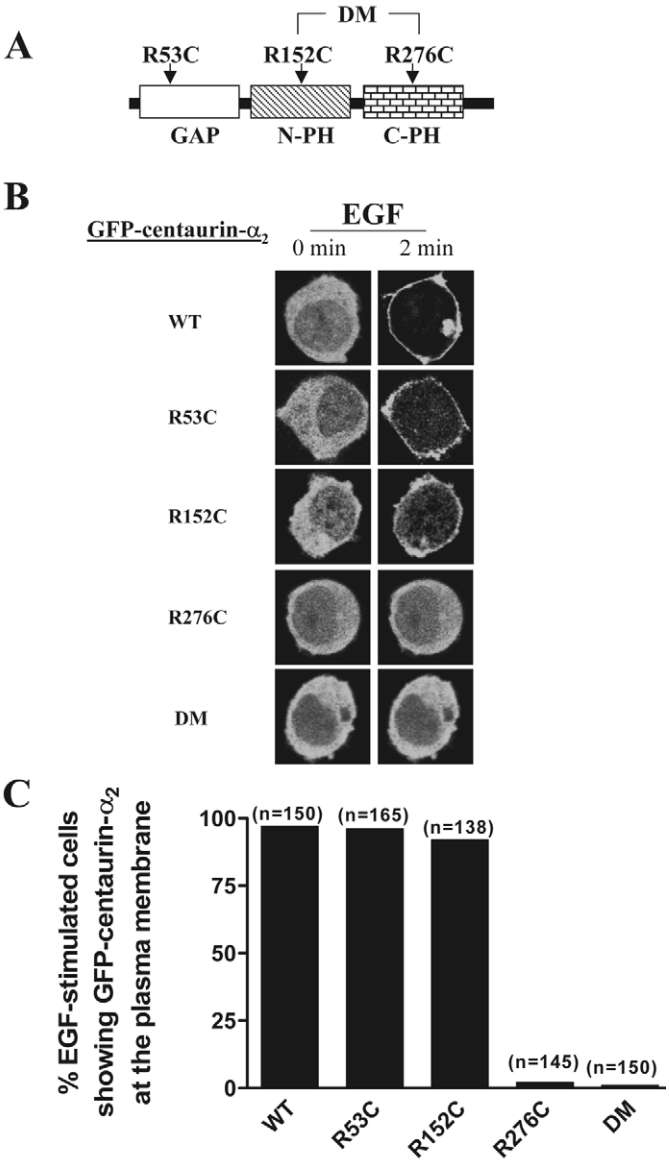

Fig. 4. Centaurin-α_2 interacts with the PI 3-kinase lipids products via the C-terminal PH domain in vivo. (A) Schematic of the mutant domains. (B) PC12 cells were transiently transfected with GFP, GFP-tagged centaurin-α_2 (wild type; WT) or its mutants {R53C, the GAP mutant; R152C, the N-PH mutant; R276C, the C-PH mutant; R152C/R276C, the double PH domains mutant (DM)]. After 2 days, the cells were serum starved and live cells were imaged by confocal microscopy in the absence or presence of 0.1 μg/ml EGF. Images were captured 2 minutes after EGF addition. (C) The cells expressing GFP-tagged proteins were scored for the plasma membrane localisation. The number of cells (n) counted for each condition are shown in brackets in the graph. Data are from three experiments.

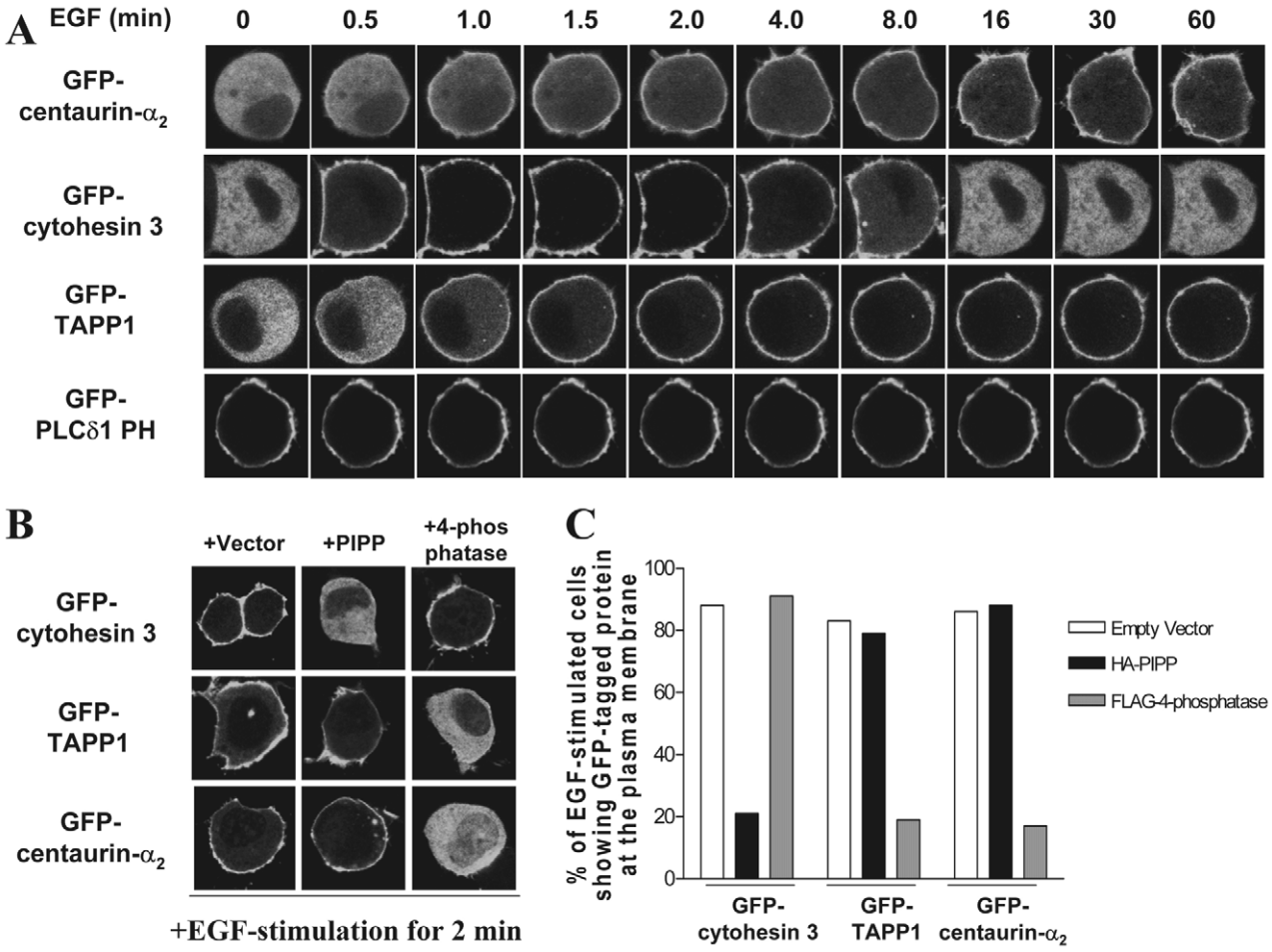

Fig. 5. Centaurin-α_2 shows sustained kinetics of membrane recruitment after stimulation with EGF. (A) PC12 cells transiently transfected with either GFP-centaurin-α_2, GFP-cytohesin 3, GFP-TAPP1 or GFP-PLCδ1 PH expression plasmid for 2 days were serum starved and live cells were imaged by confocal microscopy in the presence of 0.1 μg/ml EGF. Time points (top) indicate minutes of EGF stimulation. (B) PC12 cells were transiently transfected with expression plasmids encoding GFP-cytohesin 3, GFP-TAPP1 or GFP-centaurin-α_2 with HA alone (vector), HA-PIPP or FLAG-4-phosphatase. After 2 days, cells were serum starved and stimulated with 0.1 μg/ml EGF for 2 minutes, fixed, immunostained with an anti-HA or an anti-FLAG antibody (not shown) and imaged using confocal microscopy. (C) The transfected cells, as shown in B, were scored for GFP-tagged protein as an indicator of plasma membrane localisation. A minimum of 200 transfected cells from three independent experiments were scored for each condition.

The constitutively active mutant of ARF6 (ARF6$_{Q67L}$) reverses the inhibitory effect of centaurin-α_2 on cortical actin formation

We next analysed whether centaurin-α_2 could affect EGF-stimulated cortical actin formation by inhibiting ARF6 activation using ARF6$_{Q67}$, which acts as a dominant negative mutant for centaurin-α_2. ARF6$_{Q67L}$, but not the constitutively active mutant of ARF1 (ARF1$_{Q71L}$), reversed the inhibition of cortical actin formation by centaurin-α_2 in EGF-stimulated cells (Fig. 7). This indicates that centaurin-α_2 prevents EGF-stimulated cortical actin formation by inhibiting ARF6 activation.

The membrane targeted centaurin-α_2 is constitutively active

To study whether PIP_3 and/or PI 3,4-P_2 are required just for the membrane recruitment of centaurin-α_2 or for both the membrane recruitment and activation of centaurin-α_2, we artificially targeted centaurin-α_2 (WT) and its GAP (R53C) and DM mutants to the plasma membrane by attaching the CAAX motif of K-Ras to the C terminus and analysed their effect on the redistribution of ARF6 to the plasma membrane by cytohesin 2$_{CAAX}$, the plasma membrane-targeted ARF6 GEF that activates ARF6 constitutively (Venkateswarlu et al., 2004). GFP-centaurin-α_{2CAAX} WT and DM, but not R53C, inhibited the constitutive re-distribution of ARF6 to the plasma membrane (Fig. 8). This result suggests that centaurin-α_2 requires PIP_3 and/or PI 3,4-P_2 for recruitment to the plasma membrane but not for its activity.

Discussion

Many cytosolic proteins are reversibly recruited to the plasma membrane to reach their substrate and/or to become activated. The membrane recruitment is often accomplished by protein-lipid or protein-protein interactions, which can be mediated by PH and phosphotyrosine binding (PTB) domains. PH domains

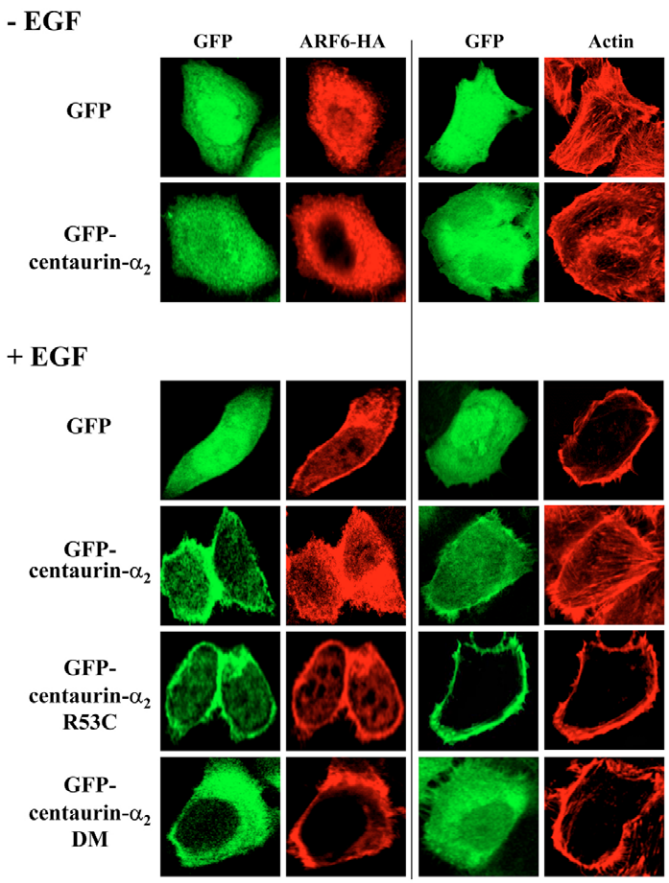

Fig. 6. Effect of centaurin-α_2 and its mutants on ARF6 localisation and cortical actin formation in EGF-stimulated cells. HeLa cells were transiently transfected with GFP, GFP-centaurin-α_2 or its mutants (R53C; DM), with or without ARF6-HA. After 2 days, the cells were serum starved and stimulated with EGF (0.1 μg/ml). The cells were then fixed, immunostained with an anti-HA antibody or Rhodamine-conjugated phalloidin (for actin) and imaged using a confocal microscope. The images are representative of 175-180 transfected cells from three experiments.

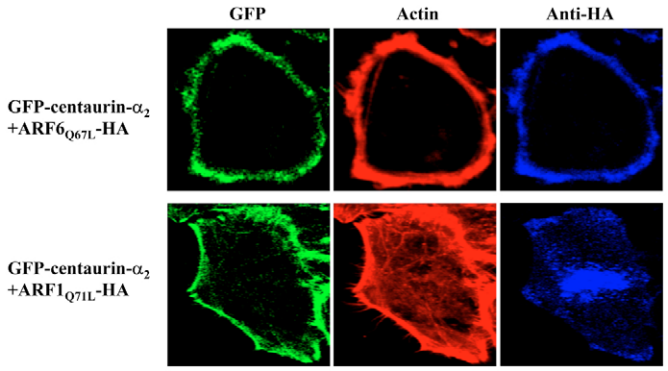

Fig. 7. The constitutively active mutant of ARF6, $ARF6_{Q67L}$, prevents inhibition of cortical actin formation by centaurin-α_2 in EGF-stimulated cells. HeLa cells were transiently transfected with either GFP or GFP-centaurin-α_2 with $ARF6_{Q67L}$-HA or $ARF1_{Q71L}$-HA. After 2 days, the cells were serum starved before stimulation with EGF. The cells were then fixed, immunostained with an anti-HA antibody and Rhodamine-conjugated phalloidin (for actin), and imaged using a confocal microscope. The images are representative of 90-95 transfected cells from three experiments.

generally bind PIs, as in the case of centaurin-α_1 (Venkateswarlu et al., 1999a), whereas PTB domains interact with phosphotyrosine residues of plasma membrane-localised receptors, as in the case of Shc (Ferguson et al., 2000). Centaurin-α_2 contains two PH domains, suggesting that this protein may be recruited to the membranes by binding to PI via the PH domains. We therefore first analysed its in vitro PI binding specificity. This analysis indicates that centaurin-α_2 binds preferentially to PI 3,4-P_2 and PIP_3, which are produced by PI 3-kinase, by its C-PH domain. Consistent with the in vitro PI binding data, we found that centaurin-α_2 shows growth factor-stimulated and wortmannin (a PI 3-kinase inhibitor)-sensitive translocation from the cytosol to the plasma membrane. Furthermore, we have shown that active PI 3-kinase is sufficient for the recruitment of centaurin-α_2 to plasma membrane through the C-PH domain. A recent study, however, has shown that centaurin-α_2 binds to PIP_3 in vitro and it localises constitutively to the plasma membrane in a wortmannin-sensitive manner (Hanck et al., 2004). In this study, the transfected cells were not serum starved before analysing centaurin-α_2 subcellular location. We also found that centaurin-α_2 shows membrane localisation in various cell lines (PC12, COS7, HeLa and HEK293) when the transfected cells were not serum starved, indicating that centaurin-α_2 retains at the membrane probably by binding to the serum-activated PI 3-kinase products (data not shown).

TAPP 1 and 2 specifically bind PI 3,4-P_2 and therefore have recently been used as biosensors to detect this phospholipid (Ooms et al., 2006). TAPP 1 and 2 show sustained kinetics of PI 3-kinase-mediated membrane recruitment in activated B lymphocytes (Marshall et al., 2002). Like TAPP 1 and 2, centaurin-α_2 shows sustained PI 3-kinase-mediated recruitment to the plasma membrane in growth factor-stimulated cells. Moreover, overexpression of 4-phosphatase inhibits the membrane retention of centaurin-α_2 in growth factor-stimulated cells. Together, these studies indicate that centaurin-α_2 is also retained at the membrane by binding to PI 3,4-P_2. Consistent with this, Franke et al. (Franke et al., 1997) found in platelets that the time course of the activation of PKB, which binds both PIP_3 and PI 3,4-P_2, reflects the more sustained accumulation of PI 3,4-P_2 than the transient appearance of PIP_3. We have shown that centaurin-α_1 binds PIP_3 and is recruited to the plasma membrane, where it functions as an in vivo GAP for ARF6 (Venkateswarlu et al., 2004). Similar to centaurin-α_1, centaurin-α_2 is localised to the plasma membrane in a PI 3-kinase-dependent manner. However, there is a difference in how these two proteins are recruited to the plasma membrane. Recruitment of centaurin-α_1 to the membrane is transient whereas centaurin-α_2 shows sustained membrane localisation in growth factor-stimulated cells, indicating that they are regulated differently.

Centaurin-α_2 shows high amino acid sequence similarity to centaurin-α_1, particularly in the GAP domain (81% similarity), suggesting that it may also act as an ARF6 GAP. In order to address this, we looked at the effect of centaurin-α_2 on ARF6 localisation and cortical actin formation as a read-out for cytoskeletal actin rearrangements, one of the well established

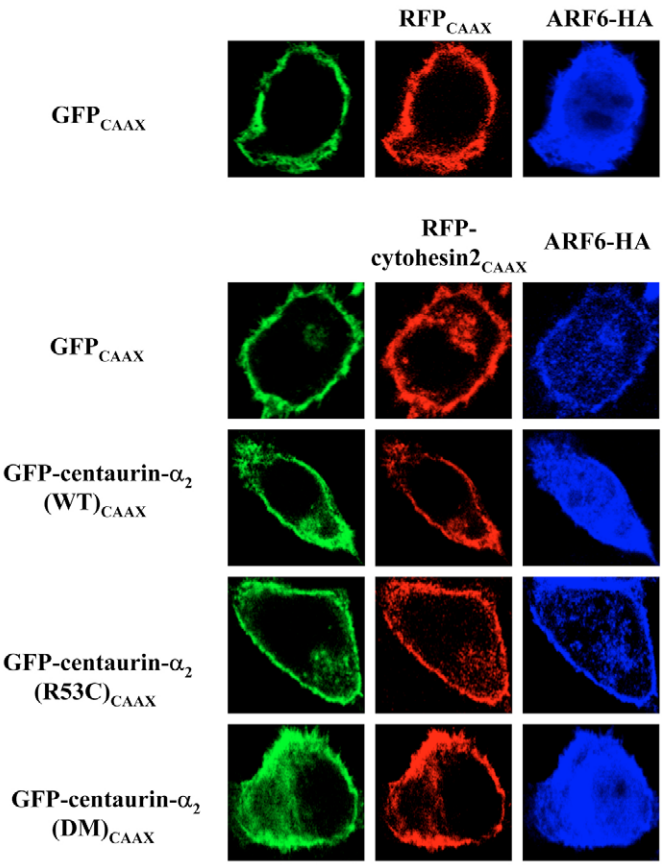

Fig. 8. Effect of membrane targeted centaurin-α_2 and its mutants on ARF6 activation by cytohesin 2$_{CAAX}$. HeLa cells were transiently transfected with the indicated expression vectors. After 2 days, the cells were serum starved, fixed, immunostained with an anti-HA antibody and imaged using a confocal microscope. Images are representative of 90-95 transfected cells from three experiments.

ARF6 cellular functions, in EGF-stimulated cells. Our studies demonstrate that centaurin-α_2 also displays GAP activity towards ARF6. Moreover, like centaurin-α_1, the GAP activity is mediated via the GAP domain and is specific for ARF6 with the constitutively active mutant of ARF6, ARF6$_{Q67L}$, but not that of ARF1, preventing inhibition of cortical actin formation in agonist-stimulated cells. In addition, we have shown that the distribution of centaurin-α_2 in intact cells is predominantly cytosolic. However, unlike centaurin-α_1, centaurin-α_2 has no association with the nucleus. Since centaurin-α_1 has a very narrow tissue distribution and centaurin-α_2 shows a much broader tissue distribution, it is likely that centaurin-α_2 acts as an ARF6 GAP in non neuronal cells whereas centaurin-α_1 may well be important in neurological signalling. There is already evidence to suggest that centaurin-α_1 may be involved in Alzheimer's disease (Reiser and Bernstein, 2002; Reiser and Bernstein, 2004). Interestingly, there have been reports of centaurin-α_1 interacting with the nuclear protein nucleolin (Dubois et al., 2003; Reiser and Bernstein, 2004) suggesting that centaurin-α_1 may well also play a role in nuclear signalling. Centaurin-α_2 gene deletion has been found in a majority of neurofibromatosis type I patients who show an earlier onset of cutaneous neurofibromas dysmorphic facial features, and lower IQ values, indicating that this protein may contribute to the severe phenotype of these patients (Jenne et al., 2000).

In conclusion, we have shown that, like centaurin-α_1, the PI 3-kinase-dependent plasma membrane association of centaurin-α_2 is required for its involvement in ARF6-mediated actin cytoskeleton remodelling. However, the recruitment of centaurin-α_1 to the membrane is transient whereas that of centaurin-α_2 is sustained. This study should help us in understanding the function of the centaurin-α subfamily GAPs in cellular events requiring actin rearrangements, such as regulated exocytosis and cell motility, which are all known to be regulated by ARF6.

Materials and Methods

Plasmid constructs

The full-length human centaurin-α_2 cDNA was amplified from mammalian gene collection clone 45054 (MRC-UK gene service) by PCR, using High Fidelity Taq DNA polymerase (Roche Applied Science) and sequence-specific primers containing *Eco*RI (sense) and *Sal*I (antisense) restriction sites. The cDNA was digested with *Eco*RI and *Sal*I, and cloned into the same sites of pCMV-Tag2b (Stratagene) for expression as a FLAG-tagged fusion protein and pEGFPC2 vector (Clontech) for expression as a green fluorescent protein (GFP)-tagged fusion protein in mammalian cells. The centaurin-α_2 R53C, R152C and R276C mutants were generated using the Quickchange II Site-Directed Mutagenesis kit (Stratagene). The double mutant (DM; R152C/R276C) of centaurin-α_2 in pEGFPC2 was generated by replacing the 0.4 kb *Sac*I-*Sal*I fragment of the R152C mutant with the corresponding fragment from the R276C mutant. pEGFP-centaurin-α_2 and its mutants were membrane targeted by attaching a C-terminal CAAX motif using PCR and 3′-primer containing the coding sequence for the CAAX motif from K-Ras (KDGKKKKKKSKTKCVIM) as described previously (Venkateswarlu and Cullen, 2000; Venkateswarlu et al., 2004). The ARF6-HA/pXS, ARF6$_{Q67L}$-HA/pXS and ARF1$_{Q71L}$-HA/pXS constructs, kindly provided by Julie Donaldson (National Institute of Health), were used to express ARF proteins with a C-terminal hemagglutinin (HA) epitope tag in mammalian cells. Cytohesin 3-pEGFPC1 and Δp85 constructs were described previously (Venkateswarlu et al., 1998b; Venkateswarlu et al., 1999b). TAPP1-pEGFPC1 was kindly provided by Dario Alessi (Dundee University, UK), PLCδ1-PH/pEGFPC1 was a gift from Tamas Balla (NIH), HA-PIPP and FLAG-4-phosphatase vectors were kindly provided by Christina Mitchell (Monash University, Australia), membPI3K and membPI3K-KD were from Doreen Cantrell (Dundee University, UK).

Cell culture and transient transfection

HeLa and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin sulphate and 10% foetal calf serum (full-serum medium) under 5% CO_2 at 37°C. PC12 cells were maintained in the full-serum medium containing 5% horse serum. For transient transfections, the cells were transfected at approximately 70% confluency, with plasmid DNA using Genejuice (Novagen) according to the manufacturer's instructions.

In vitro PI binding assay

This assay was performed as described previously (Rao et al., 1999). Briefly, COS7 cells transfected with GFP or GFP-tagged centaurin-α_2 or its mutants for 2 days were lysed in lysis buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol) containing 1% protease inhibitors mix (Sigma). The cell lysates were incubated with avidin beads (Pierce) or avidin beads coupled to biotinylated PI (Cell Signals, USA) for 2 hours at 4°C. For competition binding experiments, biotinylated PI 3,4-P_2 beads were incubated with the cell lysate in the presence of 1 nM to 10 μM nonbiotinylated water soluble (diC8) PIs (Cell Signals). The beads were washed twice with lysis buffer and the bound proteins were eluted by boiling the beads in sodium dodecyl sulphate (SDS) sample buffer. The proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Whatman). The blots were analysed by immunoblotting using an anti-GFP polyclonal antibody (Santa Cruz Biotechnology) and scanned using Scion Image™ (Scion Corporation) to ascertain relative intensities of the bands (Venkateswarlu, 2003).

Live cell imaging of PC12 cells

This was carried out as described previously with a few modifications (Venkateswarlu et al., 1998a; Venkateswarlu et al., 1998b). Briefly, PC12 cells plated onto polylysine (0.1 mg/ml; Sigma)-coated 22 mm coverslips were

transfected with 2 μg of GFP-tagged protein expression plasmid. After 2 days transfection, cells were serum starved overnight and washed three times with Krebs-Ringer phosphate buffer (K-R buffer; 136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$ 1.25 mM $CaCl_2$, 5 mM $NaPO_4$, 2 mM $NaHCO_3$ and 25 mM Hepes, pH 7.4), and then imaged using a Leica confocal microscope. Basal images were taken before adding 0.1 μg/ml of epidermal growth factor (EGF; Sigma). Images were then taken every 10 seconds for 2 minutes, then every 2 minutes for 18 minutes and finally for every 10 minutes for 40 minutes to follow any movement of the GFP-tagged protein with a 100× oil immersion lens. All images presented are single sections in the z plane.

Fluorescence imaging of fixed cells

PC12 cells were transfected with expression plasmids encoding GFP-tagged protein or GFP-tagged protein and Δp85. After 2 days, cells were serum starved overnight, and stimulated for 5 minutes at 37°C in K-R buffer alone or K-R buffer containing 0.1 μg/ml EGF. The transfected cells were incubated with 0.1 μM wortmannin (Tocris, Bristol, UK) or 50 μM LY294002 (Tocris) for 15 minutes at 37°C prior to stimulation with EGF, where indicated. Cells were then fixed with 4% paraformaldehyde, washed three times with phosphate-buffered saline (PBS) and mounted on glass slides using mounting solution [0.1 M Tris-HCl, pH 8.5, 10% Mowiol (Calbiochem) and 50% glycerol, containing 2.5% DABCO (Sigma)]. Confocal images were obtained using a Leica confocal microscope with a 100× oil immersion lens.

Immunofluorescent staining

HeLa or PC12 cells, plated onto 13 mm coverslips, were transiently transfected with the indicated expression plasmids. After 2 days, cells were serum starved overnight and stimulated with or without 0.1 μg/ml EGF for 5 minutes. After the stimulation, cells were fixed with 4% paraformaldehyde, permeabilised with 0.2% Triton X-100 in PBS, and then blocked with blocking buffer, 1% BSA in wash buffer (PBS+0.1% Triton X-100). The cells were incubated with anti-FLAG monoclonal M2 (Sigma), anti-HA monoclonal HA11 (Covance) or anti-Myc monoclonal 9E10 (Santa Cruz) primary antibody for 1 hour, and washed three times with wash buffer. Cells were then incubated with either a Rhodamine-conjugated anti-mouse secondary antibody (Jackson Laboratories), or a Rhodamine-conjugated phalloidin (Sigma) when staining actin, in blocking buffer for 1 hour, and washed three times with wash buffer. The cells were mounted on slides with mounting solution. Immunofluorescent staining was visualised using a Leica confocal microscope with a 100× oil immersion lens.

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