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RESEARCH COMMUNICATION

Identification of centaurin-α_1 as a potential *in vivo* phosphatidylinositol 3,4,5-trisphosphate-binding protein that is functionally homologous to the yeast ADP-ribosylation factor (ARF) GTPase-activating protein, Gcs1

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Centaurin-α is a 46 kDa *in vitro* binding protein for the lipid second messenger PtdIns(3,4,5)P_3. In this report we have addressed whether centaurin-α_1, a human homologue of centaurin-α, binds PtdIns(3,4,5)P_3 *in vivo* and furthermore, identified a potential physiological function for centaurin-α_1. Using confocal microscopy of live PC12 cells, transiently transfected with a chimera of green fluorescent protein (GFP) fused to the N-terminus of centaurin-α_1 (GFP-centaurin-α_1), we demonstrated the rapid plasma membrane recruitment of cytosolic GFP-centaurin-α_1 following stimulation with either nerve growth factor or epidermal growth factor. This recruitment was dependent on the centaurin-α_1 pleckstrin homology domains and was blocked by the PtdIns(4,5)P_2 3-kinase (PI 3-kinase) inhibitors wortmannin (100 nM) and LY294002 (50 μM), and also by co-expression with a dominant negative p85. Functionally, we demonstrated that centaurin-α_1 could complement a yeast strain deficient in the ADP-ribosylation factor (ARF) GTPase-activating protein Gcs1; a complementation that was blocked by mutagenesis of conserved cysteine residues within the ARF GTPase-activating protein analogous domain of centaurin-α_1. Taken together, our data demonstrated that centaurin-α_1 could potentially function as an ARF GTPase-activating protein that, on agonist stimulation, was recruited to the plasma membrane possibly through an ability to interact with PtdIns(3,4,5)P_3.

Key words: inositol 1,3,4,5-tetrakisphosphate, pleckstrin homology domain, zinc finger.

INTRODUCTION

One of the most exciting recent developments in inositol signalling has been the identification of the 3-phosphorylated inositol lipid family [1,2]. These lipids are formed from the phosphorylation of PtdIns(4,5)P_2 by a family of receptor-regulated PtdIns(4,5)P_2 3-kinases (PI 3-kinases) to initially produce PtdIns(3,4,5)P_3 which is subsequently converted into PtdIns(3,4)P_2 by the actions of a 5-phosphatase [1,2]. Of these inositol lipids the production of PtdIns(3,4,5)P_3 has been linked to the triggering of a diverse array of cellular responses, including mitogenesis, glucose transport, superoxide production, vesicle transport, membrane ruffling, actin cytoskeletal rearrangements, stimulation of p70 S6-kinase and neurite outgrowth (reviewed in [2]). A number of putative PtdIns(3,4,5)P_3 receptors have been identified [2] including members of the centaurin family [3–6].

Centaurin-α was originally purified from rat brain as a 46 kDa protein based on its ability to bind an affinity matrix composed of the inositol head group of PtdIns(3,4,5)P_3, Ins(1,3,4,5)P_4 [3]. Subsequently, the centaurin family has been extended following the purification and cloning of highly related proteins from pig and rat brain (termed p42^{IP4}) [4,5] and bovine brain (termed PtdIns(3,4,5)P_3-binding protein) [6]. Structurally, centaurins contain an N-terminal zinc finger motif that shows similarity with the catalytic domain of the rat liver ADP-ribosylation factor (ARF)1 GTPase-activating protein [7] and the yeast ARF GTPase-activating protein Gcs1 [8], and two pleckstrin homology (PH) domains (designated PH-N and PH-C). Currently, however, no physiological function has been assigned to any member of the centaurin family.

Here we report the characterization of a human homologue of p42^{IP4} and PtdIns(3,4,5)P_3-binding protein, which we have called centaurin-α_1. In particular, we demonstrated that when expressed in PC12 cells, a green fluorescent protein (GFP)-tagged centaurin-α_1 chimera (GFP-centaurin-α_1) was rapidly recruited to the plasma membrane following stimulation with agonists that elevated plasma membrane PtdIns(3,4,5)P_3. In addition, we showed that centaurin-α_1 can functionally complement a Δ*gcs1* yeast strain thereby demonstrating that centaurin-α_1 may function as an ARF GTPase-activating protein.

MATERIALS AND METHODS

cDNA cloning of human centaurin-α_1

Two oligonucleotide primers (sense: 5′-CCAGGGGCCAGCG-ACGCAGATCTGGTGCCA-3′; antisense: 5′-GCGGTCATC-CATGGTGAACCAGCGCTTCCG-3′) were used in the PCR to obtain a 129-bp fragment from a human blood cDNA library (Strategene no. 938202). The PCR product was used to screen approximately 1×10^6 clones of the blood cDNA library, essentially as described previously [9]. This yielded, after four rounds of screening, 10 independent clones of which four encoded for full length cDNAs. These were independently sequenced on an Applied Biosystems Automated DNA sequencer.

Abbreviations used: ARF, ADP-ribosylation factor; EGF, epidermal growth factor; GFP, green fluorescent protein; PH, pleckstrin homology; PI 3-kinase, PtdIns(4,5)P_2 3-kinase.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AJ006422.

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Cloning of centaurin-α_1 into pEGFP-C1

Primers (sense 5′-CG*GAATTC*CATGGCCAAGGAGCGGCG-CAGG-3′ and antisense 5′-CGC*GTCGAC*CTAAGGTTTATG-CTTGAAGTG-3′ containing the *Eco*RI and *Sal*I restriction sites) were designed to amplify the entire coding sequence of centaurin-α_1 by PCR (30 cycles with each cycle being 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C). The PCR product was digested with *Eco*RI/*Sal*I and cloned into the corresponding restriction sites of pEGFP-C1 (Clontech). The construct was fully sequenced prior to use.

Functional complementation of the Δgcs1 yeast strain PP444

The yeast strain PP444 (a generous gift from Professor Gerald Johnston, Dalhousie University, Halifax, Nova Scotia, Canada), containing a deletion of the Gcs1 gene [8] was transformed using the lithium acetate method with cDNA cloned into the yeast vector YEp352. YEp352-centaurin-α_1 was constructed by subcloning the *Eco*RI/*Sal*I fragment from pEGFP-C1-centaurin-α_1 into the corresponding sites within YEp352. Transformants were selected by uracil prototrophy and the resultant transformants were assayed for fluoride sensitivity by streaking on to YEPD agar [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] with or without 40 mM NaF followed by a 2 day incubation at 30 °C.

Site-directed mutagenesis

This was performed using the Transformer™ kit (Clontech) and the following primers (C21A, 5′-GCCGGGGAACGCGCGC-GCCGCGGACTGCGGCGCC-3′; C24A, 5′-CGCGCGCTGC-GCGGACGCCGGCGCCCCGGATCC-3′; R149C, 5′-GGGC-AGTTTTTGAGCTGCAAGTTTGTGCTGACAG-3′; R273C, 5′-GAAGGCTTCCGGAAGTGCTGGTTCACCATGG-3′). The double mutant (R149C/R273C) was generated by replacing the 0.4 kb *Bgl*II/*Sal*I fragment of the R149C mutant with the corresponding fragment from the R273C mutant. Cloning of these mutated centaurin-α_1 cDNAs into pEGFP-C1 and YEp352 was as described above.

Cell cultures, transfections and confocal microscopy

Cells were cultured and transfected as described previously [10,11]. Fluorescence imaging was performed with a Leica DM IRBE inverted confocal microscope controlled with TCS-NT4 software (Leica) as described previously [10,11].

RESULTS AND DISCUSSION

Cloning of centaurin-α_1

A BLAST search of the expressed sequence tagged data base (dbEST) identified one human EST sequence (accession no. D79989) that showed substantial similarity with rat centaurin-α. Using this information we designed two oligonuclotide primers that were used in the PCR to obtain a 129-bp fragment from a human blood cDNA library. The PCR product was used to probe the human blood cDNA library. Of 10 independent clones the largest, 1562 bp, was sequenced in both directions. The open reading frame encoded for a protein with a calculated molecular mass of 43429 Da which shared 90.1, 93.9, 92.0 and 84.7 % sequence identity with pig and rat brain p42[IP4] [4,5], bovine brain PtdIns(3,4,5)P_3-binding protein [6] and rat brain centaurin-α respectively [3]. This emphasized that the clone described above was the human orthologue of pig and rat p42[IP4] and bovine PtdIns(3,4,5)P_3-binding protein, but was distinct from rat brain centaurin-α, which has a unique 45 amino-acid extension at the C-terminus [3]. To signify this distinction we have designated our clone, centaurin-α_1.

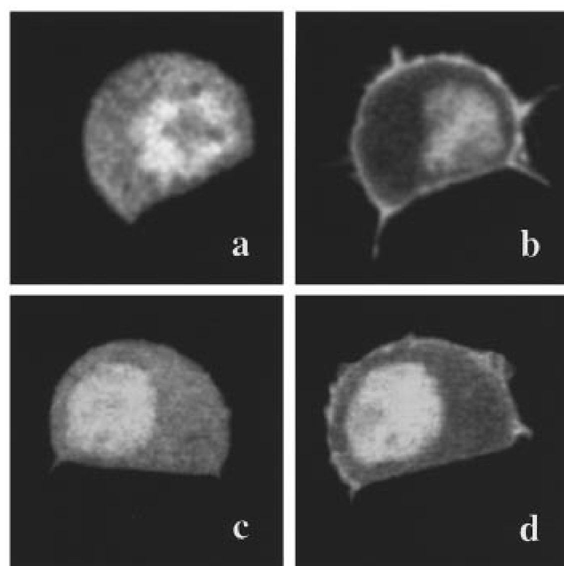

Figure 1 Agonist-dependent translocation of GFP-centaurin-α_1 to the plasma membrane of PC12 cells

PC12 cells were transfected with pEGFP-centaurin-α_1. After 24 h, cells were starved of serum and imaged by laser-scanning confocal microscopy. Images are of PC12 cells in the absence (**a**,**c**) or presence of (**b**) EGF (100 ng/ml) and (**d**) nerve growth factor (100 ng/ml). In all cases images were taken 1 min after agonist stimulation.

Identification of centaurin-α_1 as a potential *in vivo* PtdIns(3,4,5)P_3-binding protein

Analysis of [^{32}P]Ins(1,3,4,5)P_4-binding to a recombinant GST-centaurin-α_1 demonstrated that, as has previously been shown for p42[IP4] [4], centaurin-α_1 bound Ins(1,3,4,5)P_4 with nanomolar affinity (K_d of 139.7 ± 10.5 nM) and had a high degree of inositol phosphate specificity [K_d values for Ins(1,3,4,5,6)P_5, Ins(1,3,4)P_3, Ins(1,4,5)P_3, and InsP_6 of 676.7 ± 77.6, > 10000, > 10000 and > 10000 nM respectively (n = 3 for each inositol phosphate)]. Of particular interest is that esterification of the 1-phosphate of Ins(1,3,4,5)P_4 with either glycerol or diacetylglycerol (to form *GroP*Ins(3,4,5)P_3 and diacetyl *GroP*Ins(3,4,5)P_3 respectively) did not significantly alter the ability of Ins(1,3,4,5)P_4 to interact with centaurin-α_1 (K_d values of 296.7 ± 20.5 and 85.5 ± 5.6 nM respectively). Together this specificity highlighted that centaurin-α_1, by not only binding Ins(1,3,4,5)P_4 with a 100-fold higher affinity than either the inositol head group of PtdIns(4,5)P_2 or PtdIns(3,4)P_2 but also by accommodating glycerol substitution on the 1-phosphate, displayed the characteristics of a potential PtdIns(3,4,5)P_3 receptor.

To address this issue more directly we transiently transfected PC12 cells with a chimera of GFP fused to the N-terminus of centaurin-α_1. Using laser scanning confocal microscopy we studied the resultant subcellular localization of GFP-centaurin-α_1 prior to and during stimulation with epidermal growth factor (EGF), an agonist that induced an increase in plasma membrane PtdIns(3,4,5)P_3 within these cells [12]. In control unstimulated PC12 cells, GFP-centaurin-α_1 was localized in the cytoplasm as well as the nucleus (Figure 1a). Stimulation with EGF (100 ng/ml), however, resulted in an almost complete trans-

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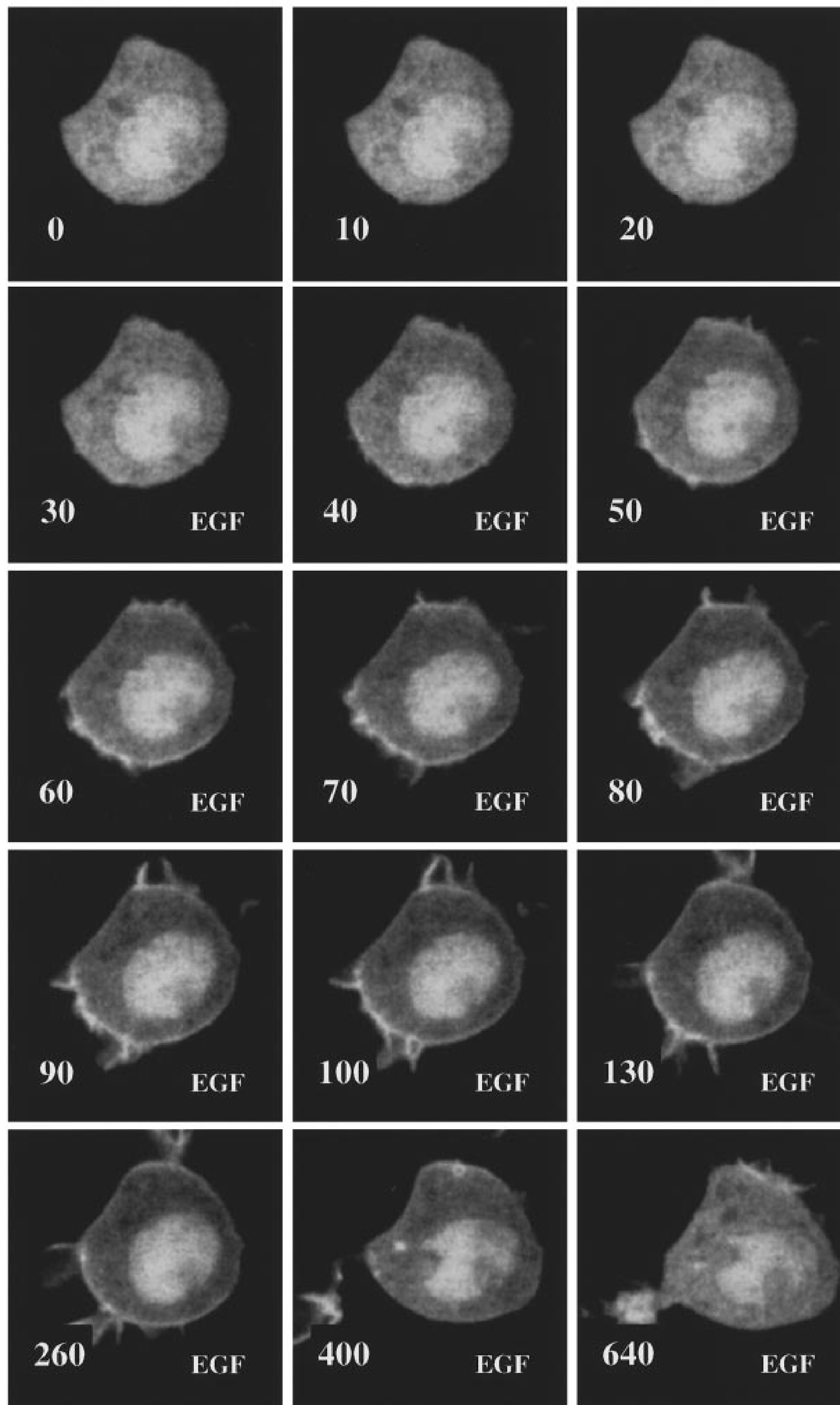

Figure 2 Time-lapse confocal imaging of the effect of EGF stimulation on the subcellular localization of GFP-centaurin-α_1 in PC12 cells

PC12 cells were transfected with pEGFP-centaurin-α_1 and a selected cell was imaged 24 h later; the number in each panel indicates the time (in seconds) at which each image was captured. Images were taken at 10 s intervals for the first 100 s and then at the times stated. EGF (100 ng/ml) was added immediately after collecting the 20 s image. Similar data were obtained in nine other imaged cells. This Figure can be viewed as a time-lapse movie sequence (http://www.BiochemJ.org/bj/340/bj3400359add.htm).

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Table 1 Inhibition of the EGF-stimulated plasma membrane recruitment of GFP-centaurin-α_1 by site-directed mutagenesis of the PH-N and PH-C domains

PC12 cells, transfected with wild-type pEGFP-centaurin-α_1 or the —(R149C), —(R273C) and —(R149C/R273C) mutants, were starved of serum prior to stimulation with EGF (100 ng/ml). Following 1.5 min of stimulation cells were fixed with paraformaldehyde and imaged as in Figure 1. Cells showing plasma membrane localization of centaurin-α_1, as depicted in Figure 1(b) (compare with Figure 1a), were scored as having translocated.

GFP-centaurin-α_1 mutant	Cells showing GFP-centaurin-α_1 localized to the plasma membrane (%)
Wild-type	87.5 ($n = 169$)
—(R149C)	51.8 ($n = 143$)
—(R273C)	27.1 ($n = 151$)
—(R149C/R273C)	4.8 ($n = 125$)

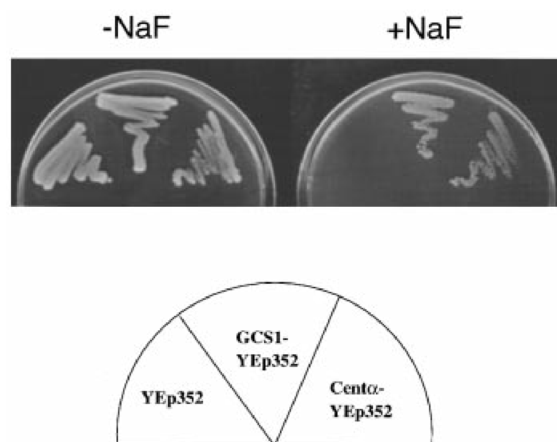

Figure 3 Functional complementaion of the *Δgcs1* yeast strain by centaurin-α_1

The Δ*gcs1* yeast strain was transformed with YEp352 control plasmid, YEp352-Gcs1 or YEp352-centaurin-α_1. Transformed yeast were streaked on to YEPD agar with or without 40 mM NaF prior to incubation at 30 °C for 2 days. Identical results were obtained in two separate sets of transformations.

location of cytosolic GFP-centaurin-α_1 to the plasma membrane (Figure 1b). A similar translocation was also observed following stimulation of PC12 cells with nerve growth factor (100 ng/ml) (Figures 1c and 1d). To examine whether the recruitment of GFP-centaurin-α_1 required the EGF-dependent activation of PI 3-kinase, we preincubated transiently transfected PC12 cells with the chemically unrelated PI 3-kinase inhibitors wortmannin and LY294002. In the absence of inhibitors EGF caused a translocation of GFP-centaurin-α_1 in 85.7 % of cells ($n = 147$), whereas in the presence of 100 nM wortmannin, the EGF-dependent translocation occurred in only 5.4 % of cells ($n = 129$). Similarly, with 50 μM LY294002 EGF-induced translocation occurred in only 6.7 % of cells ($n = 135$). Finally, EGF-induced translocation occurred in 4.4 % of cells ($n = 67$) when we co-expressed a dominant negative p85 (Δp85), which blocked the elevation in PtdIns(3,4,5)P_3 by uncoupling the ability of activated tyrosine-kinase receptors to stimulate PI 3-kinase.

Using time-lapse confocal microscopy on live cells, we examined the kinetics of the translocation of GFP-centaurin-α_1. An initial appearance of GFP-centaurin-α_1 at the plasma membrane occurred within 30 s of the addition of EGF, and was completed within 60 s, by which time most of the cytosolic GFP-centaurin-α_1 had translocated (Figure 2). The EGF-stimulated translocation of GFP-centaurin-α_1 was transient in nature, having almost completely returned to the cytosol after approx. 10 min of stimulation, a time course consistent with the transient nature by which EGF stimulates the generation of PtdIns(3,4,5)P_3 in this cell type [12].

An interesting characteristic of centaurin-α_1 is the presence of two PH domains (PH-N and PH-C) that both share a high degree of similarity with the PtdIns(3,4,5)P_3-binding PH domain from Bruton's tyrosine kinase [13]. To address the role of these domains in the EGF-stimulated recruitment of centaurin-α_1 we converted, in each PH domain, the highly conserved arginine residue that is required for PtdIns(3,4,5)P_3-binding to the Bruton's tyrosine kinase PH domain [12] [$Arg^{149} \rightarrow$ Cys (R149C) in PH-N and $Arg^{273} \rightarrow$ Cys (R273C) in PH-C]. As shown in Table 1, although the EGF-stimulated recruitment of the GFP-centaurin-α_1(R149C) mutant was reduced compared with wild-type, a more dramatic inhibition was observed with the R273C mutation. However, the mutation of both arginine residues [GFP-centaurin-α_1(R149C/R273C)] was required in order to completely inhibit the plasma membrane recruitment of GFP-centaurin-α_1.

Centaurin-α_1 functionally complements the yeast strain *Δgcs1*

As noted above, the similarity of centaurin-α_1 with the catalytic domains of mammalian ARF1 GTPase-activating protein [7] and the yeast ARF1 GTPase-activating protein Gcs1 [8] has led to the suggestion that centaurin-α_1 may function as an ARF GTPase-activating protein. To address this possibility we have made use of a Gcs1 deleted yeast strain Δ*gcs1*, which is unable to grow on fluoride containing growth media. The inhibition results from fluoride reducing the rate of ARF-GTP hydrolysis that, when coupled with the absence of the ARF GTPase-activating protein Gcs1, leads to an elevation in the level of GTP-bound ARF which impairs vesicle transport and hence inhibits growth [8,14]. As can been seen in Figure 3, transformation of the Δ*gcs1* yeast with either Gcs1 or centaurin-α_1 rescued growth on the NaF containing medium. Furthermore, mutagenesis of the conserved cysteine residues required for ARF GTPase-activating protein activity in mammalian ARF1 GTPase-activating protein [7] (residues Cys^{21} and Cys^{24} in centaurin-α_1), resulted in a complete loss of centaurin-α_1 complementation (Figure 4). However, as expected the centaurin-α_1 PH-N and PH-C mutants were still capable of functional complementation (Figure 4). These results demonstrate that centaurin-α_1 functionally complements the Δ*gcs1* yeast strain via its highly conserved ARF GTPase-activating protein analogous domain. Thus centaurin-α_1 can indeed function as an ARF GTPase-activating protein, although it should not be overlooked that a recent study has identified a number of ARF GTPase-activating protein related proteins, including Gcs1, as suppressers of an ARF loss of function mutation [15]. Therefore centaurin-α_1 may also be viewed, not simply as negative regulator of ARF signalling, but as a potential ARF effector in which the GTPase-activating protein analogous region provides a binding site for GTP-bound ARF [15]. Our inability to observe any ARF1 GTPase-activating protein activity *in vitro* for recombinant centaurin-α_1 in the presence or absence of PtdIns(3,4,5)P_3 favours this latter possibility (results not shown; see also [6]). Irrespective of these conclusions, our data clearly demonstrate a role for centaurin-α_1 in ARF signalling.

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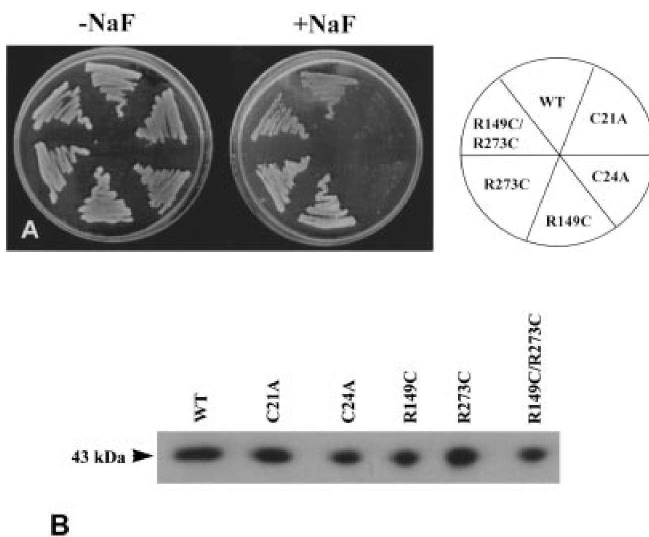

Figure 4 Mutagenesis of conserved cysteine residues within the ARF GTPase-activating protein analogous domain of centaurin-α_1 inhibits the ability to functionally complement the *Δgcs1* yeast strain

The Δ*gcs1* yeast strain was transformed with YEp352-centaurin-α_1(C21A), YEp352-centaurin-α_1(C24A), YEp352-centaurin-α_1(R149C), YEp352-centaurin-α_1(R273C), YEp352-centaurin-α_1(R149C/R273C) or YEp352-centaurin-α_1. Transformed yeast were streaked on to YEPD agar with or without 40 mM NaF and incubated at 30 °C for 2 days (**A**). Expression levels of the various centaurin-α_1 mutants, as detected by Western blotting with an anti-centaurin-α antipeptide antibody, are shown in (**B**). Identical results were obtained in two separate sets of transformations.

In light of the recent demonstration of a nuclear PI 3-kinase activity [16,17], the nuclear localization of centaurin-α_1 is an intriguing observation. This localization does not result from the GFP-tagging, since in PC12 cells haemagglutinin-tagged centaurin-α_1 and endogenous centaurin-α both retain a nuclear, as well as a cytosolic, localization (results not shown). Interestingly, as shown in Figure 2, prolonged EGF stimulation does not appear to induce the translocation of GFP-centaurin-α_1 out of the nucleus. Together therefore, these data suggest a role for centaurin-α_1 in PI 3-kinase signalling not only at the plasma membrane but also potentially within the nucleus. While this paper was under review Tanaka et al. [18] published the nuclear localization of endogenous centaurin-α_1 (termed PtdIns(3,4,5)P_3-binding protein) in rat brain.

In summary, the data described above imply that centaurin-α_1 binds PtdIns(3,4,5)P_3 *in vivo*, which, together with the observation of its ability to functionally complement the yeast ARF GTPase-activating protein Gcs1, clearly highlights a potential role for centaurin-α_1 in PtdIns(3,4,5)P_3 regulated ARF signalling.

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