Biochemical and Biophysical Research Communications **262,** 237–244 (1999)
Article ID bbrc.1999.1065, available online at http://www.idealibrary.com on IDEAL®

Molecular Cloning and Functional Characterization of a Human Homologue of Centaurin-α[1]

Kanamarlapudi Venkateswarlu and Peter J. Cullen[2]
Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom

Received June 21, 1999

We report here the molecular cloning, expression, and characterisation of a human homologue of rat centaurin-α, which we have termed centaurin-α_1. The cDNA contains a single open reading frame, which encodes a 373-amino-acid protein with a calculated molecular weight of 43,429 Daltons. Centaurin-α_1 shows high identity at the amino acid level with the other centaurin-α homologues, p42^{IP4} and PIP$_3$BP. Northern analysis revealed that centaurin-α_1 expresses as a single 2.5-kb transcript, mainly in the brain. Recombinant centaurin-α_1 binds the inositol head group of PtdIns(3,4,5)P$_3$ and Ins(1,3,4,5)P$_4$, with high affinity (K_d 139.7 ± 10.5 nM) and inositol phosphate specificity, consistent with it functioning as a putative PtdIns(3,4,5)P$_3$ receptor. In keeping with this conclusion, we have shown that GFP-tagged centaurin-α_1 recruits to the plasma membrane in a PI 3-kinase-dependent manner and the recruitment is inhibited by the PI 3-kinase inhibitor wortmannin. These results suggest that centaurin-α_1 can function as an *in vivo* PtdIns(3,4,5)P$_3$ receptor. © 1999 Academic Press

The importance of 3-phosphorylated inositol lipids in mediating cellular responses to a number of hormones and growth factors has become evident in recent years (1, 2). These lipids are formed from the phosphorylation of phosphatidylinositol 4,5-bisphosphate (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 to PtdIns(3,4)P$_2$ by the actions of a 5-phosphatase (3). PI 3-kinases can be activated either through tyrosine kinase coupled or G-protein linked receptors (3, 4). Of these 3-phosphorylated inositol lipids, PtdIns(3,4,5)P$_3$ has aroused the most interest. The level of PtdIns(3,4,5)P$_3$ *in vivo* increases 10 to 100-fold within seconds to minutes following stimulation with agonist and has been linked to the triggering of a diverse array of cellular responses, which include mitogenesis, metabolic processes (such as glucose transport), inflammatory responses (such as superoxide production), vesicle transport, cellular motility (including membrane ruffling and cytoskeletal rearrangements) and apoptosis (reviewed in 5, 6). A number of proteins bind PtdIns(3,4,5)P$_3$ *in vitro,* these include protein kinase B, PtdIns(3,4,5)P$_3$-dependent protein kinase 1 (PDK1), Bruton's tyrosine kinase (Btk), various PKC isoforms, synaptotagmin, proteins that regulate the ADP ribosylation factor (ARF) family of small GTP-binding proteins and centaurin-α (5, 6).

The ARF family of GTPases were first identified as cofactors required for the cholera toxin-catalysed ADP-ribosylation of the heterotrimeric G protein G_s (7, 8). More recently, however, increasing evidence has suggested that ARFs are key regulators of vesicle trafficking in eukaryotic cells (9, 10). For instance ARF1, the most extensively studied of the six mammalian ARFs, functions to recruit coat proteins to membranes of the Golgi apparatus (11), whereas ARF6 appears to regulate some aspect of endocytosis (12). The ability of ARFs to regulate such membrane fusion events may in part be due to their ability to alter membrane phospholipid metabolism through the activation of phospholipase D (13, 14).

Like other low molecular weight GTPases, ARFs function as molecular switches by cycling between inactive GDP- and active GTP-bound states. This GTP/GDP cycle is regulated by interactions with GTPase-activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). A family of ARF GEFs has recently been identified, which includes cytohesin-1 (15, 16), ARNO (ARF nucleotide-binding site opener) (17, 18) and GRP1 (General receptor for phosphoinositides 1) (19). All of these proteins are structurally related in having an N-terminal coiled-coil domain, a central Sec7 catalytic domain, and a C-terminal pleckstrin homology (PH) domain. This family of ARF GEFs has been shown to display characteristics predicted of

[1] The complete cDNA and deduced amino acid sequences for human homologue of centaurin-α reported in this paper have been deposited in the EMBL/Gene Bank Database with Accession No. AJ006422.

[2] Author for correspondence. Fax: +44 117 9288274. E-mail: Pete.Cullen@bris.ac.uk.

0006-291X/99 $30.00

putative PtdIns(3,4,5)P_3 receptors i.e. *in vitro* they specifically bind either PtdIns(3,4,5)P_3 or the inositol head group of PtdIns(3,4,5)P_3, Ins(1,3,4,5)P_4 (20–23). Indeed we have recently shown that in either insulin-stimulated 3T3 L1 adipocytes or EGF-stimulated PC12 cells ARNO (22), cytohesin-1 (23) and GRP1 (24) all translocate to the plasma membrane in response to an *in vivo* elevation of PtdIns(3,4,5)P_3, raising the intriguing possibility that ARF associated cellular responses may be regulated by PtdIns(3,4,5)P_3. This possibility is further strengthened by the recent identification of centaurin-α as a PtdIns(3,4,5)P_3-binding protein, which shows a high degree of homology with known ARF GAPs.

Centaurin-α was purified from rat brain as a 46 kDa protein by using an Ins(1,3,4,5)P_4 affinity column (25). Subsequent cloning characterised rat brain centaurin-α as a novel protein consisting of a number of ankyrin-like repeats flanked by a C-terminal PH domain and an N-terminal zinc-finger motif which shows a high degree of homology to the catalytic region of rat ARF1 GAP and yeast ARF GAP, Gcs1 (26, 27). Homologues of centaurin-α have recently been purified and cloned from pig brain (termed p42^{IP4}) and bovine brain (termed PIP$_3$BP) (28, 29). We report here the cloning and functional characterisation of a human homologue of centaurin-α, which appears identical to p42^{IP4} and PIP$_3$BP; we have termed this protein centaurin-α_1. Furthermore we demonstrate that human centaurin-α_1 displays the *in vitro* pharmacological characteristics predicted of a putative PtdIns(3,4,5)P_3 receptor and that centaurin-α_1 recruits to the plasma membrane by interacting with PtdIns(3,4,5)P_3 generated in the intact cells.

MATERIALS AND METHODS

Isolation of centaurin-α_1 cDNA. The expressed sequence tagged data base (dbEST) at the National Centre for Biotechnology Information (NCBI) was searched using the BLASTN program to identify human DNA sequences homologous to rat centaurin-α. Out of many EST hits, one EST sequence (accession number T09325; EST07218), which showed substantial homology with rat centaurin-α at the amino acid level, was selected to use as a probe for screening a human blood cDNA library (oligodT and randomly primed; Stratagene). The EST07218 sequence was obtained as a 129-base pair DNA fragment from the human blood cDNA library by polymerase chain reaction (PCR) using sequence specific primers (sense: 5′-CCA GGG GCC AGC GAC GCA GAT CTG GTG CCA-3′; antisense: 5′-GCG GTC ATC CAT GGT GAA CCA GCG CTT CCG-3′). The PCR amplification was carried out for 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C with High Fidelity Taq DNA polymerase (Boehringer-Mannheim). The PCR product, after sequencing, was radiolabelled with [α-^{32}P]dCTP by using a random priming kit (Promega). The radiolabelled product was used as a probe to screen the above mentioned cDNA library. Approximately 1×10^6 plaque forming units of the cDNA library were blotted onto nitrocellulose filters, lysed with NaOH, neutralised and cross-linked by UV irradiation. The nitrocellulose filters were prehybridised at 65°C for 60 min in QuikHyb (Stratagene). Hybridisation was performed in QuikHyb containing the radiolabelled probe (1×10^7 dpm per ml) for 12 hours at 68°C. The hybridised filters were washed three times with 2 × SSC/0.1% SDS followed by three times with 0.2 × SSC/0.1% SDS, each wash was carried out for 15 min at 65°C. The washed blots were exposed to X-ray film overnight at −80°C to pick positive plaques. After carrying secondary and tertiary screening under identical conditions, the phagemid was excised from 4 positive plaques and the nucleotide sequence of both strands of the insert was determined using internal oligonucleotide primers on an Applied Biosystems Automated DNA Sequencer.

Northern analysis. Human multiple tissue northern blots (Clontech) were prehybridised in QuikHyb for 30 min and then hybridised for 2 hours with [α-^{32}P]dCTP labelled full length human centaurin-α_1 in QuikHyb (2×10^6 dpm per ml). The hybridised Blots were washed with 3 × 50 ml of 2 × SSC/0.1% SDS followed by 3 × 50 ml washes with 0.2 × SSC/0.1% SDS, each wash was carried out for 20 min. All incubations and washes were performed at 68°C. Radioactivity retained on the blots was visualised by phosphoimaging.

Construction of expression vectors and site-directed mutagenesis described in this study. The coding sequence of centaurin-α_1 was isolated from the original clone by PCR using High Fidelity *Taq* Polymerase and sequence specific primers (sense 5′-CGGAATTC-CATGGCCAAGGAGCGGCGCAGG-3′ and antisense 5′-CGCGTCGAC-CTAAGGTTTATGCTTGAAGTG-3′) containing *Eco*RI and *Sal*I restriction sites respectively (underlined). The resulting PCR product was sub-cloned into *Eco*RI/*Sal*I sites of pGEX (Pharmacia) and pEGFP (Clontech) vectors to express as a GST-fusion protein and GFP-fusion protein, respectively. An haemagglutinin-tag (HA-tag) coding sequence with an initiation codon (5′-ATGTACCCTTAC-GATGTGCCTGATTACGCT-3′) was attached at the 5′ end of the centaurin-α_1 coding sequence by PCR and cloned into pCDNA3 (Invitrogen) to express centaurin-α_1 as a HA-tagged protein. The PCR comprised 30 cycles with each cycle being 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. The constructs were fully sequenced prior to use. The expression vector for constitutively active PI 3-kinase (p110^{CAAX}) was described elsewhere (30). Site-directed mutagenesis was performed using the sequence specific primers (Arg149→Cys [R149C], 5′-GGGCAGTTTTTGAGCTGCAAGTTTGTGCTGACAG-3′; Arg273→Cys [R273C], 5′-GAAGGCTTCCGGAAGTGCTGGTTCA-CCATGG-3′) and Transformer Site-directed Mutagenesis kit (Clontech). The double mutant (R149C/R273C) was generated by replacing the 0.4 Kb *Bgl*II/*Sal*I fragment of the R149C with that of the R273C mutant.

Expression and purification of GST-centaurin-α_1 in E. coli. Human centaurin-α_1 was expressed as a GST-fusion protein in *E. coli* strain *BL21(DE3)* and purified by using glutathione sepharose (Pharmacia) as described previously (24).

[^{32}P]Ins(1,3,4,5)P_4-binding assays. These were performed as described previously (31, 32).

Cell culture, transfections and confocal microscopy. CHO.T cells were grown in Ham's F12 medium (GibcoBRL) supplemented with 5% Foetal Calf Serum, 10 mM HEPES (pH-7.4), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in the presence of 5% CO_2 until approximately 80% confluent. The cells grown on the cover slips (22-mm diameter) were transiently transfected for 2 hours with 1-2 μg of expression plasmid in serum free medium using Tfx-50 (Promega). After 48 hours, the cells were serum starved for 2 hours and washed twice with PBS. The cells were then fixed with 4% paraformaldehyde for 15 min and mounted onto glass slides with Mowiol containing 2.5% DABCO (Sigma). For immunofluorescence, the paraformaldehyde fixed cells were washed 3 times with PBS and permeabilised with 0.2% Triton-X 100 for 10 min. To prevent non-specific binding of antibodies, the cells were incubated with PBS containing 0.1% Triton-X 100 and 3% BSA (blocking buffer) for 15 min and then primary antibodies (HA11 monoclonal antibodies, BabCo; 1:1000 dilution in blocking buffer) were applied for 60 min. After 3 rinses in PBS/0.1% Triton-X 100 for

5 min each, secondary antibodies coupled with fluorescein (FITC, Sigma; 1:200 dilution in blocking buffer) were applied for 30 min. Finally the cells were washed 3 times with PBS/0.1% Triton-X 100 for 5 min each and mounted onto glass slides as described above. All incubations were carried out at room temperature. In some experiments, cells were incubated with 100 nM wortmannin for 10 min at 37°C prior to fixation. Fluorescence imaging was performed with a Leica DM IRBE confocal microscope controlled with TCS-NT4 software (Leica).

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of Centaurin-α_1

Centaurin-α_1 cDNA was isolated by screening a human blood cDNA library using the region of EST07218 which predicted amino acid sequence share 100% homology with rat centaurin-α as a probe. After secondary and tertiary screening, 10 clones were found to be positive among 10^6 plaques screened. Sequencing of the largest cDNA revealed a single open reading frame of 1125 bp, extending from nucleotide 203 to 1327. The open reading frame predicted to encode a protein of 373 amino acids with a calculated molecular mass of 43,429 Da and a pI of 9.0. The entire nucleotide sequence of centaurin-α_1 is shown in Fig. 1 along with the deduced amino acid sequence. Sequence comparison revealed the predicted protein to be highly similar to the other centaurin-α homologues, having 90.1, 93.9, 92.0 and 86.7% identity with p42^{IP4} of pig (28) and rat (33), bovine PIP_3BP (29) and rat centaurin-α (25) respectively (Fig. 2). The human protein is similar in length to p42^{IP4} and PIP_3BP but it is 45 amino acid shorter at the C-terminal end compared to rat centaurin-α. Therefore we termed this protein centaurin-α_1.

Centaurin-α_1 contains, like p42^{IP4} and PIP_3BP, two PH domains (one at the N-terminus, residues 130 to 231, and the other at the C-terminus, residues 253 to 357) and an N-terminal zinc finger motif (residues 1 to 117). The C-terminal PH domain of centaurin-α_1 has high sequence homology with the corresponding domain from the ARF GEFs such as ARNO, GRP1 and cytohesin-1 (Fig. 3B). The zinc finger, co-ordinated around the two pairs of cysteine residues with the consensus $CX_2CX_{16}CX_2C$, shows highest homology with the catalytic region of *C. elegans* predicted protein W09D10.1, *Drosophila melanogaster* and rat liver ARF1 GAP and the yeast ARF GAP, Gcs1 (Fig. 3A).

Centaurin-α_1 mRNA Expression in Human Tissues

Northern blots of human multiple tissues were probed with the coding sequence of the cDNA to determine centaurin-α_1 mRNA expression in different tissues. This analysis revealed that centaurin-α_1 encoding sequence hybridises to a 2.5 kb transcript expressed in brain and peripheral blood leukocytes with a relatively high level in the brain compared to leukocytes (Fig. 4). The size of transcript and the tissue-specific expression of human centaurin-α_1 are in agreement with that reported previously for rat centaurin-α (25). Although in rat brain the presence of further minor transcripts has been reported (25), we were unable to visualise any such transcripts in human brain upon longer exposure (data not shown). Tissue-specific distribution of p42^{IP4} and PIP_3BP has not yet been reported, however they have been purified and cloned from brain indicating that these proteins are also express predominantly in brain tissues (28, 29). The expression of centaurin-α_1 is confined mainly to brain tissues suggests that it might have some brain specific function(s).

```
GGCACGAGGCGACCGCGGCGGAGACACGAGCGGCGGCGGCGGAGCCCAGAGGGCGGC 57
GGGAATGCGGAACCGGCGCGCGGGCTGAGGCCGCCCGGGATGGCGCAGGGGCGGCGG 114
CGGCGGGCGGCCTGCGCGGGGCCCTAGCGAGCCGGGCTGACGCTCCCGGCCCCGGCC 171
CCGGCATCGGCATCGCGGGCGCATCGCGGCCATGGCCAAGGAGCGGCGCAGGGCGGT 228
                                M  A  K  E  R  R  A  V
CCTGGAGCTGCTGCAGCGGCCGGGGAACGCGCGCTGCGCGGACTGCGGCGCCCCGGA 285
  L  E  L  L  Q  R  P  G  N  A  R  C  A  D  C  G  A  P  D
TCCCGACTGGGCCTCCTACACTCTGGGCGTCTTCATCTGCCTGAGCTGCTCGGGAAT 342
  P  D  W  A  S  Y  T  L  G  V  F  I  C  L  S  C  S  G  I
CCACCGGAATATCCCCCAGGTCAGCAAGGTGAAGTCCGTCCGCCTGGACGCCTGGGA 399
  H  R  N  I  P  Q  V  S  K  V  K  S  V  R  L  D  A  W  E
GGAGGCCCAAGTGGAGTTCATGGCCTCCCACGGGAACGACGCCGCGAGAGCCAGGTT 456
  E  A  Q  V  E  F  M  A  S  H  G  N  D  A  A  R  A  R  F
TGAGTCCAAAGTACCCTCCTTCTACTACCGGCCCACGCCCTCCGACTGCCAGCTCCT 513
  E  S  K  V  P  S  F  Y  Y  R  P  T  P  S  D  C  Q  L  L
TCGAGAGCAGTGGATCCGGGCCAAGTACGAGCGACAGGAGTTCATCTACCCGGAGAA 570
  R  E  Q  W  I  R  A  K  Y  E  R  Q  E  F  I  Y  P  E  K
GCAGGAGCCCTACTCGGCAGGGTACCGTGAGGGTTTTCTCTGGAAGCGTGGCCGGGA 627
  Q  E  P  Y  S  A  G  Y  R  E  G  F  L  W  K  R  G  R  D
CAACGGGCAGTTTTTGAGCCGGAAGTTTGTGCTGACAGAACGAGAGGGTGCTCTGAA 684
  N  G  Q  F  L  S  R  K  F  V  L  T  E  R  E  G  A  L  K
GTATTTCAACAGAAATGATGCCAAGGAGCCCAAGGCCGTGATGAAGATCGAGCACCT 741
  Y  F  N  R  N  D  A  K  E  P  K  A  V  M  K  I  E  H  L
GAACGCCACCTTCCAGCCGGCCAAGATCGGCCACCCCCACGGCCTGCAGGTCACCTA 798
  N  A  T  F  Q  P  A  K  I  G  H  P  H  G  L  Q  V  T  Y
CCTGAAGGACAACAGCACCCGTAACATCTTCATCTACCATGAGGACGGGAAGGAGAT 855
  L  K  D  N  S  T  R  N  I  F  I  Y  H  E  D  G  K  E  I
TGTGGACTGGTTCAATGCACTCCGAGCTGCTCGCTTCCACTACCTGCAGGTGGCATT 912
  V  D  W  F  N  A  L  R  A  A  R  F  H  Y  L  Q  V  A  F
CCCAGGGGCCAGCGACGCAGATCTGGTGCCAAAGCTCTCCAGGAACTACCTGAAGGA 969
  P  G  A  S  D  A  D  L  V  P  K  L  S  R  N  Y  L  K  E
AGGCTACATGGAGAAGACGGGGCCCAAGCAAACGGAAGGCTTCCGGAAGCGCTGGTT 1026
  G  Y  M  E  K  T  G  P  K  Q  T  E  G  F  R  K  R  W  F
CACCATGGATGACCGCAGGCTCATGTACTTCAAAGACCCCCTGGACGCCTTCGCCCG 1083
  T  M  D  D  R  R  L  M  Y  F  K  D  P  L  D  A  F  A  R
AGGGGAAGTCTTCATTGGCAGCAAGGAGAGTGGCTACACGGTGCTGCATGGGTTCCC 1140
  G  E  V  F  I  G  S  K  E  S  G  Y  T  V  L  H  G  F  P
GCCGTCCACCCAGGGCCACCACTGGCCACATGGCATCACCATCGTCACGCCCGACCG 1197
  P  S  T  Q  G  H  H  W  P  H  G  I  T  I  V  T  P  D  R
CAAGTTTCTGTTTGCCTGCGAGACGGAGTCCGACCAGAGGGAGTGGGTGGCGGCCTT 1254
  K  F  L  F  A  C  E  T  E  S  D  Q  R  E  W  V  A  A  F
CCAGAAGGCGGTGGACAGGCCCATGCTGCCCCAGGAGTACGCAGTGGAGGCGCACTT 1311
  Q  K  A  V  D  R  P  M  L  P  Q  E  Y  A  V  E  A  H  F
CAAGCATAAACCTTAGCGAGTGCGGCTGGAGGACCACGGACATTGGACTCACTGTGG 1368
  K  H  K  P  *
CTGGACGGAGGGGACCCGTGGATGGGGGGGCTCTGGCGTCCTGAAGCCACCTGGCCC 1425
CACCTGCTCCCTCAAGGCAGCCCGGCGCGGGCAAGGTAGGGCCCGAGCTTCAGCTTC 1482
CAAGATGCTTCTCTGGAACCTCAAGGCAAGCAACCCAAGGCCCTGGGCCTGATCTCC 1539
TAACCCCGTCATGCTGCTGCTGA                                   1562
```

FIG. 1. The cDNA sequence and derived amino acid sequence of human centaurin-α_1. The corresponding amino acids are listed below the nucleotide sequence.

[^{32}P]Ins(1,3,4,5)P_4-Binding to Expressed Human Centaurin-α_1

As can clearly been seen from Fig. 5, [^{32}P]-Ins(1,3,4,5)P_4 bound to the recombinant centaurin-α_1 with nanomolar affinity and a high degree of inositol

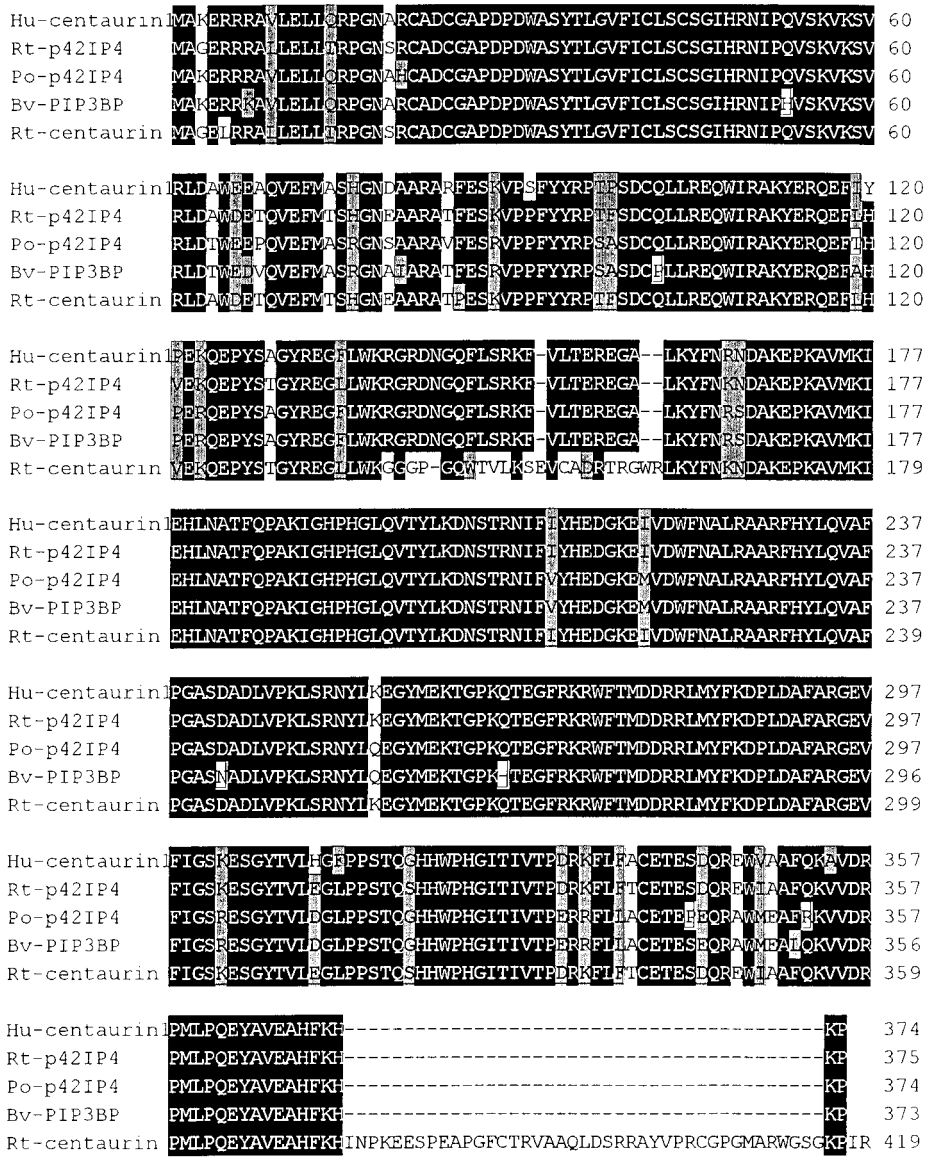

FIG. 2. Sequence alignment of human centaurin-α_1 (Hu-centaurin1) with bovine PIP_3BP (Bv-PIP3BP; accession number D89940), pig $p42^{IP4}$ (Po-p42IP4; accession number U88363) rat $p42^{IP4}$ (Rt-p42IP4; accession number AJ007422) and rat centaurin-α (Rt-centaurin; accession number U51013). Residues identical to human centaurin-α_1 are shaded in black whereas conservative substitutions are shaded in grey and alignment gaps in the sequences are represented by '–'.

phosphate specificity. The K_d value for Ins(1,3,4,5)P_4 {*i.e.* the concentration required to compete for 50% of bound [^{32}P]Ins(1,3,4,5)P_4} was 139.7 ± 10.5 nM with the other inositol phosphates tested namely Ins(1,3,4,5,6)P_5, Ins(1,3,4)P_3, Ins(1,4,5)P_3 and InsP_6 having K_d's of 676.7 ± 77.6, >10,000, >10,000 and >10,000 nM respectively. Detailed Scatchard transformation of the Ins(1,3,4,5)P_4 competition curve (using 4-5 points per log unit) resolved a single class of binding sites (data not shown).

As stated in the introduction, the inositol head group of PtdIns(3,4,5)P_3 is identical to Ins(1,3,4,5)P_4 except that its 1-phosphate is attached via glycerol to the fatty acids. This has led us to previously predict that a PtdIns(3,4,5)P_3 receptor may in fact bind Ins(1,3,4,5)P_4 *in vitro* (34). It would be expected, however, that esterification of the 1-phosphate of Ins(1,3,4,5)P_4, particularly with glycerol or diacetyl glycerol (to form *GroP*Ins(3,4,5)P_3 and diacetyl *GroP*Ins(3,4,5)P_3 respectively), would result in a decrease in binding affinity to an Ins(1,3,4,5)P_4 receptor, but would not effect, or may even increase, the binding affinity to a PtdIns(3,4,5)P_3 receptor. *GroP*Ins(3,4,5)P_3 and diacetyl *GroP*Ins(3,4,5)P_3 competed for [^{32}P]Ins(1,3,4,5)P_4-binding to centaurin-α_1 with a K_d of 296.7 ± 20.5 and 85.5 ± 5.6 nM respectively. These results suggest that centaurin-α_1 does indeed

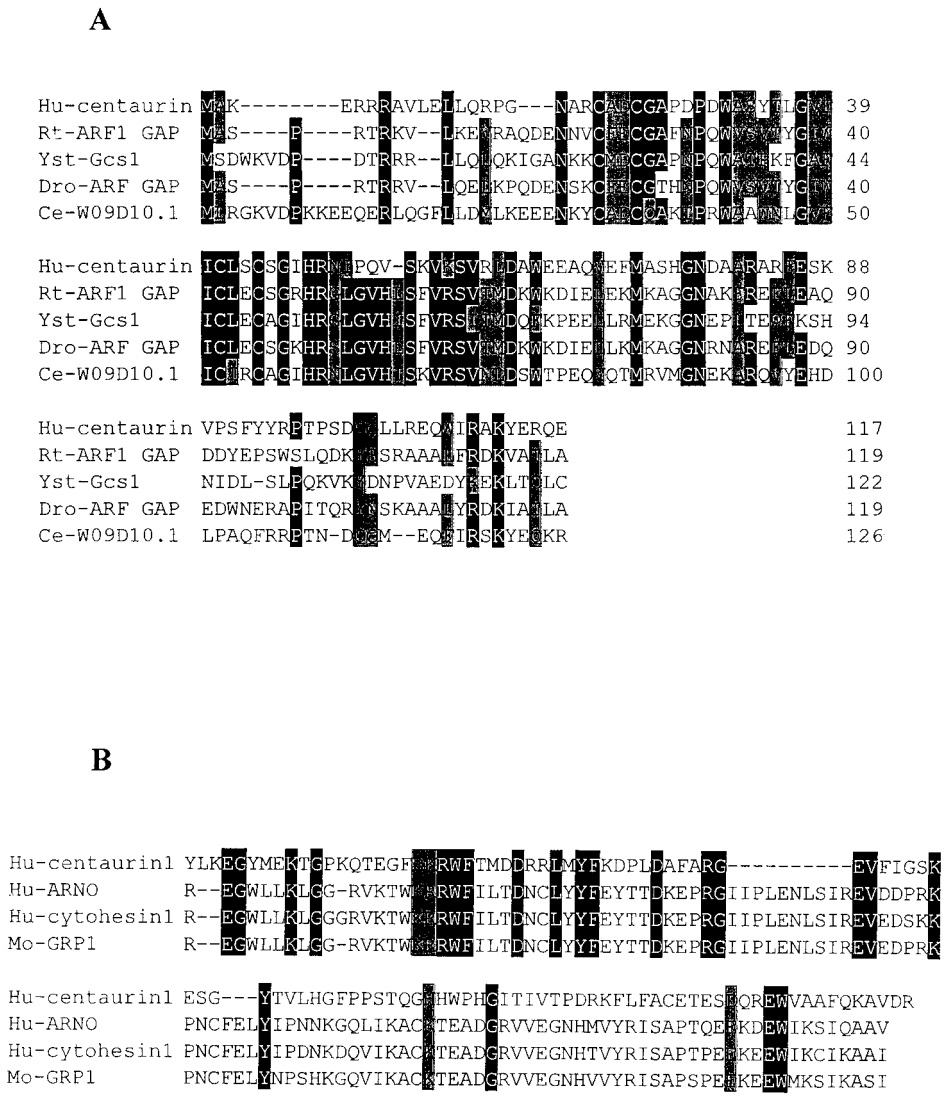

FIG. 3. (A) Alignment of the N-terminal region of centaurin-α_1 showing homology with the rat ARF1 GAP (Rt-ARF1 GAP; accession number U35776), yeast Gcs1 (yst-Gcs1; accession number P35197), *Drosophila* ARF GAP (Dro-ARF GAP; accession number AF011427). *C. elegans* W09D10.1 hypothetical protein (Ce-W09D10.1; accession number Z93785). (B) Alignment of the C-terminal PH domain of centaurin-α_1 (Hu-centaurin1, residues 253–357) with the PH-domains of known PtdIns(3,4,5)P_3 binding proteins: human ARNO (Hu-ARNO, residues 262–375; accession number X99753), mouse GRP1 (Mo-GRP1, residues 267–379; accession number AF001871) and human cytohesin-1 (Hu-cytohesin1, residues 263–378; accession number M85169). Residues identical to human centaurin-α_1 and conservative substitutions are shaded in black and grey respectively. Alignment gaps in the sequences are represented by '–'.

constitute a putative PtdIns(3,4,5)P_3 receptor, in that *in vitro* it specifically recognises the 1,3,4,5-phosphate configuration on the inositol ring in preference to the 1,4,5 or 1,3,4 configuration of PtdIns(4,5)P_2 and PtdIns(3,4)P_2 respectively. Moreover, unlike the putative Ins(1,3,4,5)P_4 receptor GAP1^{IP4BP} (35), centaurin-α_1 can accommodate a glycerol substitution on the 1-phosphate.

Demonstration of Centaurin-α_1 as a Cellular PtdIns(3,4,5)P_3 Binding Protein

In order to demonstrate the interaction of centaurin-α_1 with PtdIns(3,4,5)P_3 *in vivo,* we have transiently transfected CHO.T cells with constructs expressing GFP fused to the N-terminus of centaurin-α_1 (GFP-centaurin-α_1) and constitutively active, membrane targeted, p110 catalytic subunit of PI 3-kinase (p110^{CAAX}), and studied the subcellular localisation of GFP-centaurin-α_1 using confocal microscopy. Since, expression of p110^{CAAX} results in accumulation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in the plasma membrane (30), we assumed interaction of centaurin-α_1 with these 3-phosphorylated inositol lipids might produce a redistribution of centaurin-α_1. In the absence of p110^{CAAX}, GFP-centaurin-α_1 was localised to the cytosol and the

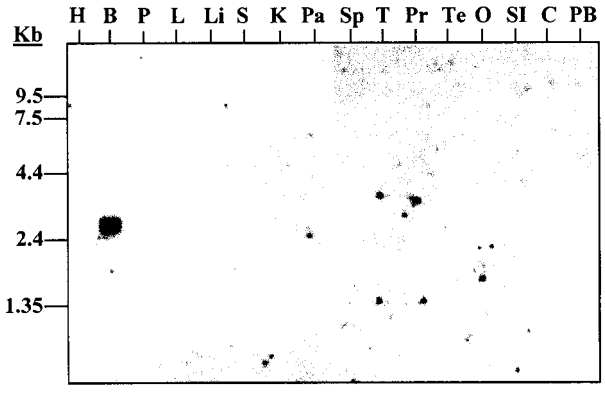

FIG. 4. Northern analysis of centaurin-α_1 expression in human tissues. The multiple human tissue northern blots containing approximately 2 μg of purified poly A^+ RNA isolated from each tissue were probed with the centaurin-α_1 as described in Materials and Methods. The same blots were re-hybridised with β-actin to ensure an uniform RNA concentration in each lane (data not shown). Size markers are as indicated on the left of panel. H, heart; B, brain; P, placenta; L, lung; Li, liver; S, skeletal muscle; K, kidney; Pa, pancreas; Sp, spleen; T, thymus; Pr, prostrate; Te, testis; O, ovary; SI, small intestine; C, colon (mucosal lining); PB, peripheral blood leukocytes.

nucleus (Fig. 6A). However, a clear wortmannin (PI 3-kinase inhibitor) sensitive recruitment of GFP-centaurin-α_1 to the plasma membrane was observed when co-expressed with $p110^{CAAX}$ (Fig. 6A), suggesting that the resulting elevation in 3-phosphorylated inositol lipids induces the recruitment of GFP-centaurin-α_1 to the plasma membrane. Taken together, the *in vitro* binding and *in vivo* localisation studies demonstrate that PtdIns(3,4,5)P_3 functions as a specific ligand for centaurin-α_1 at the plasma membrane. The PtdIns(3,4,5)P_3-binding site probably resides within the PH domains of centaurin-α_1 since these, in particular the C-terminal PH domain, show a high degree of homology with the PtdIns(3,4,5)P_3-binding PH domain of ARNO, cytohesin-1 and GRP1. Furthermore, mutation of highly conserved arginine residue within the both PH domains of PIP_3BP completely inhibited PtdIns(3,4,5)P_3 binding (29). To address the role of the PH domains in the membrane recruitment of centaurin-α_1, we generated three mutants, two single mutants (R149C and R273C) and a double mutant (R149C/R273C), by mutating the conserved arginine residue in both the PH domains and analysed their localisation by co-expressing with $p110^{CAAX}$. The membrane recruitment of GFP-centaurin-α_1 (R149C) and GFP-centaurin-α_1 (R279C) was reduced, but to different levels, compared with that of GFP-centaurin-α_1 (Table 1). However, the double mutant, GFP-centaurin-α_1 (R149C/R273C), completely lost the ability to recruit to the membrane (Table 1). These results clearly suggest that both of the PH domains are required for the membrane recruitment of centaurin-α_1 in a PI 3-kinase dependent manner.

In order to determine whether nuclear localisation of centaurin-α_1 is due to GFP-tagging, we have expressed centaurin-α_1 as a HA-tagged protein and studied subcellular localisation using immunofluorescence. The HA-centaurin-α_1 is also localised to nucleus as well as to cytosol and recruited to the plasma membrane when co-expressed with $p110^{CAAX}$ (Fig. 6B). The plasma mem-

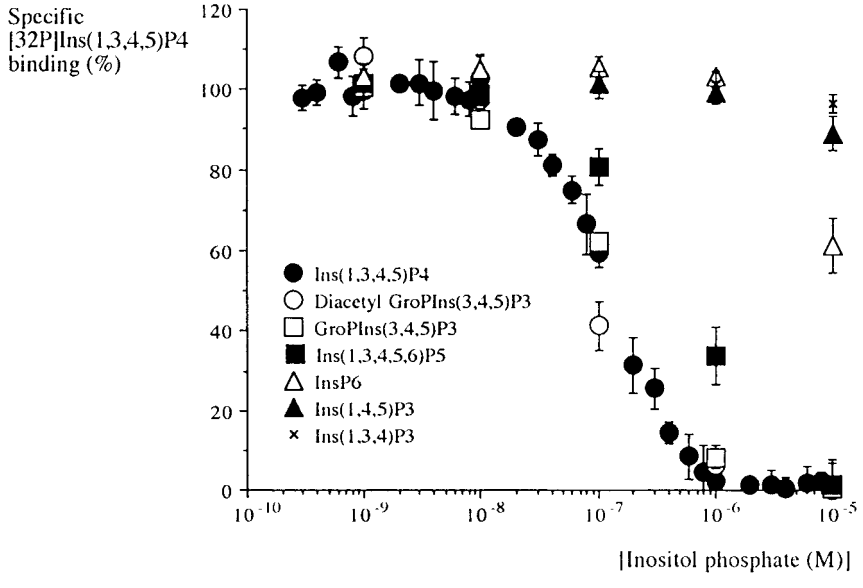

FIG. 5. Displacement of [^{32}P]Ins(1,3,4,5)P_4 from human centaurin-α_1. Characterisation of Ins (1,3,4,5)P_4-binding to a fusion protein of glutathione-S-transferase (GST) and full length centaurin-α_1. Binding was performed as described in Materials and Methods. Specificity of the binding site is shown using the various inositol phosphate isomers. Non-specific binding was defined as the binding remaining in the presence of 10 μM Ins(1,3,4,5)P_4. Data are means ± S.E.M. of values from three individual determinations.

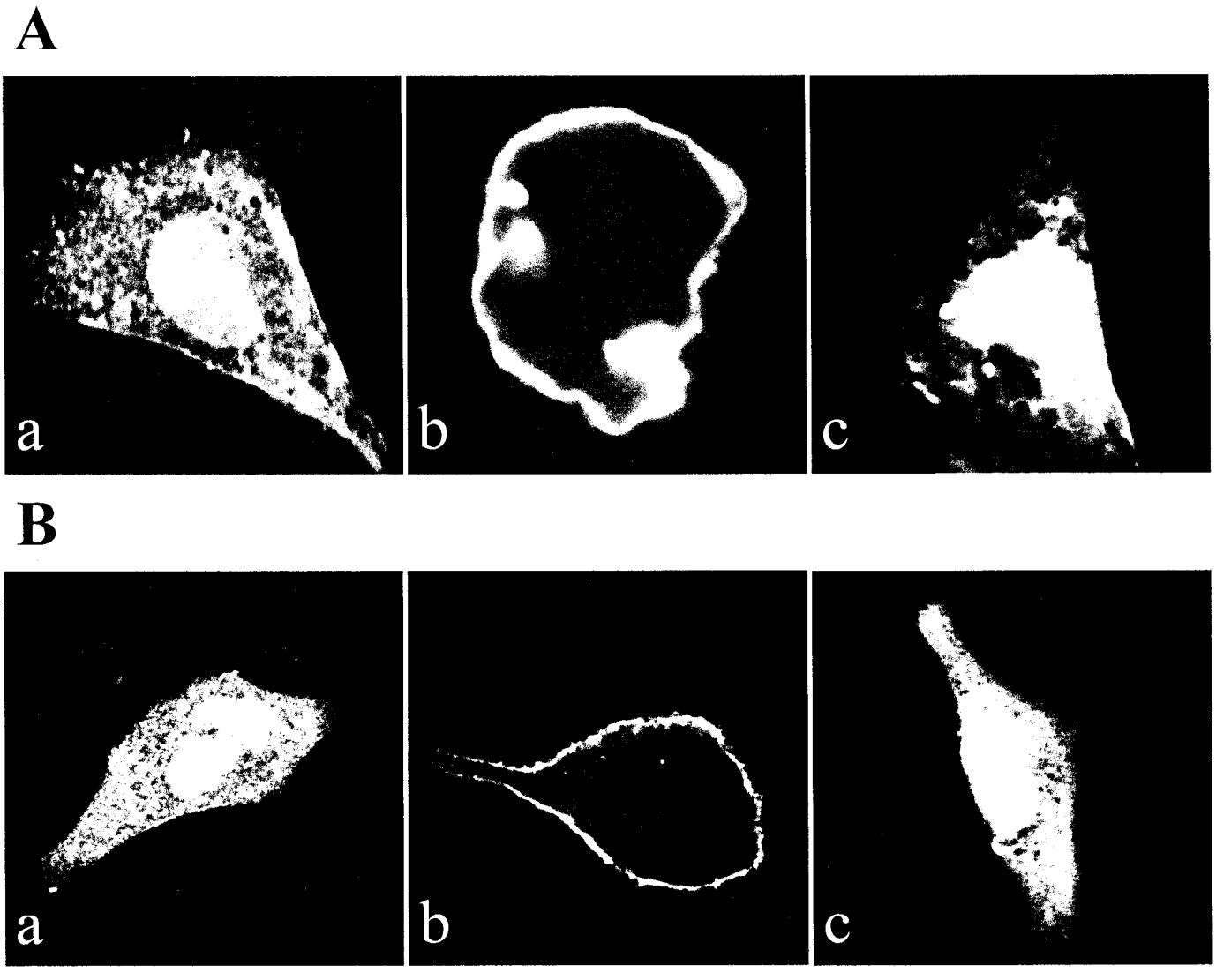

FIG. 6. Recruitment of GFP- or HA-centaurin-α_1 to the plasma membrane of CHO.T cells expressing $p110^{CAAX}$. CHO.T cells were co-transfected with pEGFPC1- (A) or pCDNA3-centuarin-α_1 (B) and either pSG5 control plasmid (a) or pSG5-$p110^{CAAX}$ (b,c). After 48 hours, cells were serum starved for 2 hours and incubated for 10 minutes at 37°C in the absence (a,b) or presence of 100 nM wortmannin (c). Cells were fixed, immunostained (in the case of HA-tagged centaurin-α_1 expressing cells) and imaged by laser scanning confocal microscopy. Similar data was obtained from over 50 imaged cells.

brane recruitment was blocked by wortmannin. These results indicate that nuclear localisation is not an artifact of GFP-tagging. Consistent with this, Tanka *et al.* have recently reported the localisation of PIP_3BP expressed in COS-7 cells to the nucleus and furthermore have suggested the presence of a putative nuclear localisation signal in the N-terminal end of PIP_3BP (36). They have also showed translocation of PI 3-kinase to the nucleus and production of PtdIns(3,4,5)P_3 in the nucleus in agonist stimulated cells (36). It is tempting to speculate, therefore, that nuclear centaurin-α_1 may play a specific role in PtdIns(3,4,5)P_3 signalling within the nucleus.

The physiological function of centaurin-α_1 and its homologues is not yet known. Since centaurin-α_1 shows

TABLE 1

Effect of Site-Directed Mutagenesis of the PH Domains on the PI 3-Kinase-Dependent Membrane Localization of Centaurin-α_1

GFP-centaurin-α_1 mutant	Cells showing the membrane localisation of GFP-centaurin-α_1 (%)
Wild-type	89.5 (n = 210)
−(R149C)	50.1 (n = 198)
−(R273C)	26.8 (n = 224)
−(R149C/R273C)	3.2 (n = 217)

Note. CHO.T cells were co-transfected with $p110^{CAAX}$ and GFP-centaurin-α_1 or −(R149C) or −(R273C) or (R149C/R273C). After 48 hours, the cells were serum starved for 2 hours, fixed, and imaged (n = number of cells imaged).

significant homology with the catalytic domains of recently cloned mammalian ARF1 GAP (26) and yeast ARF GAP, Gcs1 (27), one physiological role of centaurin-α_1 may be to function as an ARF GAP. At present, however, we have been unable to demonstrate any functional ARF1 GAP activity for recombinant centaurin-α_1 either in presence or absence of PtdIns(3,4,5)P_3 containing liposomes (data not shown). It remain possible that centaurin-α_1 possesses GAP activity towards another member of the ARF or ARF-like protein families, a possibility which is currently under investigation. However, a recent study has identified a number of ARF GAP related proteins, including the yeast homologue of centaurin-α_1 (Gcs1), as suppressors of the loss of ARF function leading to the suggestion that these proteins should be viewed as an effectors rather than down regulators of ARF signalling (37). Thus the ARF GAP homology region in centaurin-α_1 may provide a binding site for GTP-bound ARF without exerting a GTPase-activating function.

In conclusion, we have described the cloning and initial characterisation of human centaurin-α_1 as a potential *in vivo* PtdIns(3,4,5)P_3 receptor. The exact physiological function of centaurin-α_1, and its roles in ARF regulation and nuclear PtdIns(3,4,5)P_3 signalling remain to be determined.

ACKNOWLEDGMENTS

We thank the Medical Research Council for providing an Infrastructure Award (G4500006) to establish the School of Medical Sciences Cell Imaging Facility and Mark Jepson and Alan Leard for their assistance. We thank the other members of this laboratory for their support and Barry Potter and Andrew Riley for their kind gift of *D*-Ins(1,3,4)P_3. This work is funded by the Biotechnology and Biological Sciences Research Council. P.J.C. is a Lister Fellow.

REFERENCES

1. Stephen, L. R., Jackson, T. R., and Hawkins, P. T. (1993) *Biochim. Biophys. Acta* **1179,** 27–75.
2. Martin, T. F. J. (1998) *Annu. Rev. Cell Dev. Biol.* **14,** 231–264.
3. Vanhaesbroeck, B., Leevers, S. J., Panayotoy, G., and Waterfield, M. D. (1997) *Trends Biochem. Sci.* **22,** 276–272.
4. Hawkins, P. T., Welch, H., McGregor, A., Eguinoa, A., Gobert, S., Krugmann, S., Anderson, K., Stokoe, D., and Stephens, L. R. (1997) *Biochem. Soc. Trans.* **25,** 1147–1151.
5. Toker, A., and Cantley, L. C. (1997) *Nature* **387,** 673–677.
6. Rameh, L. E., and Cantley, L. C. (1999) *J. Biol. Chem.* **274,** 8347–8350.
7. Kahn, R. A., and Gilman, A. G. (1984) *J. Biol. Chem.* **259,** 6228–6232.
8. Kahn, R. A., and Gilman, A. G. (1986) *J. Biol. Chem.* **261,** 7906–7911.
9. Donaldson, J. G., and Klausner, R. D. (1994) *Curr. Opin. Cell Biol.* **6,** 527–537.
10. Moss, J., and Vaughan, M. (1998) *J. Biol. Chem.* **273,** 21431–21434.
11. Donaldson, J. G., Gassel, D., Kahn, R. A., and Klausner, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6408–6412.
12. Song, J., Kachikian, Z., Radhakrishan, H., and Donaldson, J. G. (1998) *J. Cell Sci.* **111,** 2257–2267.
13. Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunnigham, E., Gout, I., Hiles, I., Totty, N. F., Troung, O., and Hsuan, J. J. (1994) *Science* **263,** 523–526.
14. Hammond, S. M., Altshuller, Y. M., Sung, T. C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) *J. Biol. Chem.* **270,** 29640–29643.
15. Kolanus, W., Nagel, W., Schiller, B., Zeitlmann, L., Godar, S., Strockinger, H., and Seed, B. (1996) *Cell* **86,** 233–242.
16. Meacci, E., Tsai, S-C., Adamik, R., Moss, J., and Vaughan, M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1745–1748.
17. Chardin, P., Paris, S., Antonny, B., Robineau, S., Beraud-Dufour, S., and Jackson, C. L. (1996) *Nature* **384,** 481–484.
18. Frank, S., Upender, S., Hansen, S. H., and Casanova, J. E. (1998) *J. Biol. Chem.* **273,** 23–27.
19. Klarlund, J. K., Guilherme, A., Holik, J. J., Virbasius, J., Chawla, A., and Czech, M. P. (1997) *Science* **275,** 1927–1930.
20. Klarlund, J. K., Rameh, L. E., Cantley, L. C., Buxton, J. M., Holik, J. J., Sakelis, C., Patki, V., Corvera, S., and Czech, M. P. (1998) *J. Biol. Chem.* **273,** 1859–1862.
21. Nagel, W., Zeitlmann, L., Schilcher, P., Geiger, C., Kolanus, J., and Kolanus, W. (1998) *J. Biol. Chem.* **273,** 14853–14861.
22. Venkateswarlu, K., Oatey, P. B., Tavare, J. M., and Cullen, P. J. (1998) *Curr. Biol.* **8,** 463–466.
23. Venkateswarlu, K., Gunn-Moore, F., Tavare, J. M., and Cullen, P. J. (1999) *J. Cell Sci.* **112,** 1957–1965.
24. Venkateswarlu, K., Gunn-Moore, F., Oatey, P. B., Tavare, J. M., and Cullen, P. J. (1998) *Biochem. J.* **335,** 139–146.
25. Hammonds-Ode, L. P., Jackson, T. R., Profit, A. A., Blader, I. J., Turck, C. W., Prestwich, G. D., and Theibert, A. B. (1996) *J. Biol. Chem.* **271,** 18859–18868.
26. Cukierman, E., Huber, I., Rotman, M., and Cassel, D. (1995) *Science* **270,** 1999–2002.
27. Poon, P. P., Wang, X., Rotman, M., Huber, I., Cukierman, E., Cassel, D., Singer, R. A., and Johnston, G. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10074–10077.
28. Striker, R., Hulser, E., Fischer, J., Jarchau, Th., Walter, U., Lottspeich, F., and Reiser, G. (1997) *FEBS Letts.* **405,** 229–236.
29. Tanaka, K., Imahoh-Ohmi, S., Sawada, T., Shirai, R., Hashimoto, Y., Iwasaki, S., Kaibuchi, K., Kanaho, Y., Shirai, T., Terada, Y., Kimura, K., Nagata, S., and Fukui, Y. (1997) *Eur. J. Biochem.* **245,** 512–519.
30. Khawaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. *EMBO J.* **16,** 2783–2793.
31. Cullen, P. J., Dawson, A. P., and Irvine, R. F. (1995) *Biochem. J.* **305,** 139–143.
32. Cullen, P. J., Patel, Y., Kakkar, V. V., Irvine, R. F., and Authi, K. S. (1994) *Biochem. J.* **298,** 739–742.
33. Aggensteiner, M., Stricker, R., and Reiser, G. (1998) *Biochim. Biophys. Acta* **1387,** 117–128.
34. Cullen, P. J., and Irvine, R. F. (1996) *Curr. Biol.* **6,** 537–540.
35. Cullen, P. J., Hsuan, J. J., Troung, O., Letcher, A. J., Jackson, T. R., Dawson, A. P., and Irvine, R. F. (1995) *Nature* **376,** 527–530.
36. Tanaka, K., Horiguchi, K., Yoshida, T., Takeda, M., Fujisawa, H., Takeuchi, K., Umeda, M., Kato, S., Ihara, S., Nagata, S., and Fukui, Y. (1999) *J. Biol. Chem.* **274,** 3919–3922.
37. Zhang, C. -J., Cavenagh, M. M., and Kahn, R. A. (1998) *J. Biol. Chem.* **273,** 19792–19796.