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# Identification of gene structure and subcellular localization of human centaurin α2, and p42$^{IP4}$, a family of two highly homologous, Ins 1,3,4,5-$P_4$-/PtdIns 3,4,5-$P_3$-binding, adapter proteins

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**Abstract**

Proteins which recognize the two messengers phosphatidylinositol 3,4,5-trisphosphate (PtdIns$P_3$), a membrane lipid, and inositol 1,3,4,5-tetrakisphosphate (Ins$P_4$), a water-soluble ligand, play important roles by integrating external stimuli, which lead to differentiation, cell death or survival. p42$^{IP4}$, a PtdIns$P_3$/Ins$P_4$-binding protein, is predominantly expressed in brain. The recently described centaurin α2 of similar molecular mass which is 58% identical and 75% homologous to the human p42$^{IP4}$ orthologue, is expressed rather ubiquitously in many tissues. Here, elucidating the gene structure for both proteins, we found the human gene for centaurin α2 located on chromosome 17, position 17q11.2, near to the NF1 locus, and human p42$^{IP4}$ on chromosome 7, position 7p22.3. The two isoforms, which both have 11 exons and conserved exon/intron transitions, seem to result from gene duplication. Furthermore, we studied binding of the two second messengers, PtdIns$P_3$ and Ins$P_4$, and subcellular localization of the two proteins. Using recombinant baculovirus we expressed centaurin α2 and p42$^{IP4}$ in Sf9 cells and purified the proteins to homogeneity. Recombinant centaurin α2 bound both Ins$P_4$ and PtdIns$P_3$ equally well *in vitro*. Furthermore, fusion proteins of centaurin α2 and p42$^{IP4}$, respectively, with the green fluorescent protein (GFP) were expressed in HEK 293 cells to visualize subcellular distribution. In contrast to p42$^{IP4}$, which was distributed throughout the cell, centaurin α2 was concentrated at the plasma membrane already in unstimulated cells. The protein centaurin α2 was released from the membrane upon addition of wortmannin, which inhibits PI3-kinase. p42$^{IP4}$, however, translocated to plasma membrane upon growth factor stimulation. Thus, in spite of the high homology between centaurin α2 and p42$^{IP4}$ and comparable affinities for Ins$P_4$ and PtdIns$P_3$, both proteins showed clear differences in subcellular distribution. We suggest a model, which is based on the difference in phosphoinositide binding stoichiometry of the two proteins, to account for the difference in subcellular localization.

**Keywords:** human genome, inositol tetrakisphosphate, lipid second messenger, membrane translocation, phosphatidylinositol trisphosphate, signal transduction.

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The phosphatidylinositol 3,4,5-trisphosphate (PtdIns$P_3$) and inositol 1,3,4,5-tetrakisphosphate (Ins$P_4$)-binding protein p42$^{IP4}$ is predominantly expressed in brain and occurs both as membrane-associated and cytosolic protein (Stricker *et al*. 1997; Sedehizade *et al*. 2002). By immunohistochemistry analysis of human brain, we detected p42$^{IP4}$ highly expressed in neurones from amygdala, hippocampus and hypothalamus (Sedehizade *et al*. 2002). Similar results had been found before for the rat brain (Kreutz *et al*. 1997a). The p42$^{IP4}$ protein was also detected in retinal neurones of different mammalia (Kreutz *et al*. 1997b). We have cloned the cDNA for p42$^{IP4}$ from human, rat and pig (Stricker *et al*. 1997; Aggensteiner *et al*. 1998; Sedehizade *et al*. 2002). The

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*Abbreviations used:* Arf, ADP-ribosylation factor; CLSM, confocal laser scanning microscope; DAG, diacyl glycerol; EGF, epidermal growth factor; FCS, fetal calf serum; GAP, GTPase activating protein; GEF, guanine nucleotide-exchange factor; GFP, green fluorescent protein; GSH, glutathione; GST, glutathione-S-transferase; Ins$P_3$, inositol 1,4,5-trisphosphate; Ins$P_4$, inositol 1,3,4,5-tetrakisphosphate; NF1, neurofibromatosis 1; NLS, nuclear localization signal; PH, domain, pleckstrin homologuey domain; PLC, phospholipase C; PtdIns, phosphatidylinositol; PtdIns$P_3$, phosphatidylinositol 3,4,5-trisphosphate; PtdIns$P_2$, phosphatidylinositol 4,5-bisphosphate; RTK, receptor tyrosine kinase; SH2, Src homologuey domain 2.

  

human form has also been described as centaurin α1 (Venkateswarlu and Cullen 1999). The bovine orthologue has been designated PIP3BP (Tanaka *et al*. 1997). The cDNAs are coding for a protein with 374 amino acid residues. Recently, indications for the existence of a highly conserved isoform, centaurin α2, have been found for human (Jenne *et al*. 2001), rat (Whitley *et al*. 2002) and mouse (Hanck and Reiser, unpublished). Centaurin α2, however, has not yet been characterized in detail.

The p42$^{IP4}$ protein has already been studied intensively (Stricker *et al*. 1997; Hanck *et al*. 1999). Recombinant purified p42$^{IP4}$ shows binding affinities similar to those of the native p42$^{IP4}$ isolated either from pig cerebellum or from rat brain. The $K_d$ for $InsP_4$ was 3.9 nM and 2.2 nM for the recombinant and native pig protein, respectively. Moreover, we could show that p42$^{IP4}$ specifically binds $PtdInsP_3$, since the recombinant protein associates with lipid vesicle membranes containing $PtdInsP_3$. This ligand binding is inhibited by $InsP_4$ (Stricker *et al*. 1997, 1999; Hanck *et al*. 1999). Furthermore, we showed that p42$^{IP4}$ could be induced to dissociate from cerebellar membranes by incubation with $InsP_4$. This release was concentration-dependent (> 100 nM), detectable within a few minutes, ligand-specific and reversible. We demonstrated that, when $InsP_4$ was degraded by a membrane-associated 5-phosphatase to Ins 1,3,4-$P_3$, p42$^{IP4}$ re-bound to the membranes, from which it could again be released by renewed addition of $InsP_4$ (Stricker *et al*. 1999).

The properties of p42$^{IP4}$ found *in vitro* could be confirmed by our *in vivo* studies when we expressed the p42$^{IP4}$ as a fusion protein with green fluorescent protein (GFP) in cultured mammalian cells. The GFP-p42$^{IP4}$ fusion protein shows a diffuse subcellular distribution (Sedehizade *et al*. 2002). Therefore, the association/dissociation of p42$^{IP4}$ to membranes and intracellular organelles can be tightly controlled by the following enzymes: (i) PI 3-kinases, raising the level of $PtdInsP_3$; (ii) $InsP_3$ 3-kinase, raising the intracellular amount of $InsP_4$; (iii) SH2-containing inositol 5-phosphatase (SHIP) (Phee *et al*. 2000; Rohrschneider *et al*. 2000) or the dual specificity phosphatase PTEN (phosphatase and tensin homologue) (Das *et al*. 2003), reducing the level of $PtdInsP_3$; and (iv) membrane-associated 5-phosphatase (Majerus *et al*. 1999), degrading $InsP_4$ to Ins 1,3,4-$P_3$.

As the second messenger $PtdInsP_3$ is of central importance in controlling cell proliferation, survival and secretion (Rameh and Cantley 1999), $PtdInsP_3$-binding proteins also play critical roles in brain plasticity during differentiation, and in pathogenesis such as tumour development and neurodegenerative processes, as suggested for p42$^{IP4}$ in Alzheimer pathology (Reiser and Bernstein 2002). For the p42$^{IP4}$ protein, we proposed a model which puts p42$^{IP4}$ downstream of several signal transduction pathways (Stricker *et al*. 1999). However, the role of the centaurin α2 protein in signal transduction pathways still has to be elucidated.

Structural analysis of p42$^{IP4}$ (and centaurin α2) revealed a zinc finger motif with high homology to Arf-GAP (ADP-ribosylation factors-GTPase activating proteins), followed by two PH (pleckstrin homology) domains. Therefore, the physiological roles of p42$^{IP4}$ and centaurin α2 are expected to involve cellular functions of Arfs and their regulators. Arfs and their regulator proteins, which also contain PH domains, are important in vesicular trafficking, cytoskeletal organization, differentiation and development. Arfs are regulated by GAPs and guanine nucleotide-exchange factors (GEFs) (Jackson *et al*. 2000), which are both also linked to PI 3-kinase pathways (Jackson *et al*. 2000; Suzuki *et al*. 2002). Interestingly, the three highly homologous isoforms of Arf-GEFs, which are designated ARNO, Cytohesin and GRP1 (Kolanus *et al*. 1996; Knorr *et al*. 2000; Hernandez-Deviez *et al*. 2002), apparently have different functions, in spite of equally binding $PtdInsP_3$.

An important question is whether the two proteins, p42$^{IP4}$ and centaurin α2, which share a conserved structure, are members of a distinct family of $InsP_4$- and $PtdInsP_3$- binding proteins. Here, by analysis and comparison of the two isoforms human p42$^{IP4}$ and centaurin α2, we demonstrate that both proteins evolved from a common ancestor gene but diverged, yielding proteins with different subcellular localization, ligand binding stoichiometry and pattern of tissue expression.

## Materials and methods

### Cloning and sequencing of human centaurin α2

For the human centaurin α2 there was no full length cDNA clone available (Jenne *et al*. 2000; D. Jenne, personal communication). Analysis of the database gave homology to the I.M.A.G.E. clone (Integrated Molecular Analysis of Genomes and their Expression) No. 685913, available as IMAGp998I181676 from the RZPD (Resource Center/Primary Database, Germany). The nucleic acid extract, prepared from this I.M.A.G.E. clone, was tested by polymerase chain reaction (PCR) using primer CA2-forward (5′-CCCCCGGGCCGGCCATGGGCG-3′) and primer CA2-reverse (5′- GAATTCTCACCTGCTGCTGCGGCCACTCTC-3′). Competent *E. coli* K12 cells were transformed with the nucleic acid extract, and three independent clones were fully sequenced in both directions with vector-specific and internal sequencing primers on an ABI310 automated sequencer with the BigDye terminator cycle sequencing kit 1.0 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The derived nucleotide sequence of 1802 base pairs (bp) coded for a protein (cds: 280–1482) with 381 amino acids and high homology to the human p42$^{IP4}$ protein (Sedehizade *et al*. 2002). The sequence from position 180 to 1786 was 100% identical to the sequence of centaurin α2 (accession EMBL AJ272195), and the sequence from position 1 to 1787 was identical to another sequence of centaurin α2 (accession EMBL AJ272195) except that our sequence had a 'C' to 'G' transversion at position 1496. The derived amino acid

sequence was 100% identical to all other amino acid sequences for the human centaurin α2 found in protein databases.

The coding sequence of centaurin α2 was amplified by PCR using primer CA2-forward and primer CA2-reverse. The *Sma*I/*Eco*RI-DNA fragment was subcloned to pAcG3X transfer vector and pEGFPC1 expression vector (BD Biosciences Clontech, Palo Alto, CA, USA). All constructs were verified with vector-specific and internal sequencing primers on an ABI310 automated sequencer, as described above.

All database search and biocomputing was carried out using either the software program Heidelberg Unix Sequence Analysis Resources (HUSAR 4.0) based on GCG, Genetic Computer Group Inc. Madison, Wisconsin, USA, Baylor College of Medicine Search Launcher, or the facilities from the National Center for Biotechnology Information (NCBI Conserved Domain Search v1.58).

**Production, purification and analysis of recombinant proteins**

For transfection, 2 μg transfer vector-DNA (pAcG3X-centaurin α2) were mixed with 2.5 μg BaculoGold-DNA (BD Biosciences Pharmingen, San Diego, CA, USA), end volume 12 μL. The DNA mixture was mixed with 8 μL lipofectin (Invitrogen, Karlsruhe, Germany) and 4 μL $H_2O$. With the transfection mixture, $3 \times 10^6$ Sf9 cells in 5 mL IPL41-medium (Gibco/BRL) were transfected. Recombinant baculovirus was amplified and tested for production of recombinant protein.

The production and purification of the recombinant centaurin α2 was similar to the procedure described earlier for $p42^{IP4}$ (Hanck *et al.* 1999). The protein cleaved from the glutathione-S-transferase (GST) tag was further purified on a Hi Trap Heparin Sepharose column (Amersham Biosciences, Frieburg, Germany). The column was equilibrated with protease buffer [50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, 0.5% (w/v) lubrol-PX] without protease. The protein solution with centaurin α2 was loaded onto the column at 0.5 mL/min. The column was washed first with 15 bed volumes of protease buffer without protease, then with 15 bed volumes buffer 1 (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 200 mM NaCl), 15 bed volumes of buffer 2 (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 400 mM NaCl) and 5 bed volumes of buffer 3 (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 600 mM NaCl). Finally, the centaurin α2 was eluted with 5 bed volumes of buffer 4 (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 800 mM NaCl). The protein solution with centaurin α2 was set to a concentration of 100 μg/mL and was stable over several weeks at 4°C.

The glutathione (GSH) beads were resuspended in 200 μL sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and denatured for 5 min at 100°C. Solutions with non-immobilized proteins were mixed 1 : 1 with 2 × sample buffer for SDS-PAGE and denatured for 5 min at 100°C. The proteins were analysed on 10% polyacrylamide (30 : 0.8) gels according to Laemmli (1970) and stained with Coomassie Blue R250.

**Cell culture, transfection and confocal microscopy**

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Hams'F12 1 : 1 (Biochrom, Berlin, Germany) nutrient mixture supplemented with 10% fetal calf serum (FCS; Biochrom) and 0.5% penicillin/streptomycin in a humidified $CO_2$ atmosphere at 37°C.

The transfection was carried out using DOTAP-Kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions. The cells were transiently transfected with plasmid DNAs (1.5 μg DNA) from the constructs pEGFPC1-centaurin α2 or pEGFPC2-$p42^{IP4}$ respectively, (Sedehizade and Reiser, unpublished). For transient transfection, the cells were grown on Poly D-Lysine-coated cover slips (2 cm diameter) to approximately 70% confluency. The transfected cells were kept for 8–10 h in serum- and antibiotic-free medium. After 24–48 h under normal culture conditions, the cells were fixed in ice-cold methanol for 20 min at −20°C, stained with 0.2 mg/mL propidium iodide (Molecular Probes, Eugene, OR, USA) for 5 min at room temperature and mounted on glass slides with Aquatex (Merck, Darmstadt, Germany). Fluorescence imaging was performed with a Zeiss (Carl Zeiss, Jena, Germany) inverted LSM 510 confocal laser scanning microscope (CLSM) equipped with an Achroplan 40× objective. Fluorescence of GFP was excited using a 488 nm argon/krypton laser and the emitted fluorescence was detected with a 505–530 band pass filter. For propidium iodide detection, a 543 nm helium/neon laser was used for excitation and the fluorescence was detected with a 560 nm long pass filter.

For EGF (Calbiochem, San Diego, CA, USA) stimulation, the transiently-transfected cells were serum-starved post-transfection for 12–16 h. For wortmannin (Calbiochem) treatment, transfected cells were maintained in serum-containing culture medium. Life imaging experiments were performed in Na-HBS buffer (145 mM NaCl, 5.5 mM KCL, 1.8 mM $CaCl_2 \times 2H_2O$, 1 mM $MgCl_2 \times 6H_2O$, 25 mM glucose, 20 mM HEPES; pH 7.4) from selected cells with a CLSM 510 controlled with Release 2.5 software (Zeiss).

**Binding assay**

Inositol phosphates were obtained from the following sources: [$^3$H]Ins 1,3,4,5-$P_4$ (777 GBq/mmol, NEN Life Science Products, Kötn, Germany), D-Ins 1,3,4,5-$P_4$ (Roche Molecular Biochemicals, Indianapolis, IN, USA) and D-Ins 1,4,5-$P_3$ (Biomol; Hamburg, Germany); the water-soluble di$C_8$PtdIns 3,4,5-$P_3$ and di$C_8$PtdIns 4,5-$P_2$ were a gift from Dr J. R. Falck (Dallas, Texas).

For human centaurin α2, recombinant protein (100–200 ng/assay) was incubated with about 1.1 nM D-[$^3$H]Ins 1,3,4,5-$P_4$ in binding buffer (10 mM HEPES/KOH, pH 7.0, 100 mM KCl, 20 mM NaCl, 1 mM EDTA) and varying concentrations of unlabelled D-Ins 1,3,4,5-$P_4$ or the other compounds tested in a total volume of 200 μL. After incubation for 20 min on ice, bound ligand was separated from free ligand by addition of 50 μL γ-globulin (10 mg/mL, horse; Sigma, St Louis, MO, USA) and 250 μL 30% PEG-4000 (Merck), further incubation for 15 min and centrifugation (14 000 *g*, 15 min, 4°C). Supernatant fluids were removed and pellets rinsed with 200 μL binding buffer, solubilized in 2% SDS, and radioactivity measured in Ultima Gold (Packard Instruments, Meriden, CT, USA) with a liquid scintillation counter. Non-specific binding was determined in the presence of 10 μM D-Ins 1,3,4,5-$P_4$. The ligand displacement curves are shown in Fig. 3(a).

For comparison, displacement curves using recombinant pig $p42^{IP4}$ are shown in Fig. 3(b). They were derived by including some of the datapoints published previously (Hanck *et al.* 1999) and the binding assays were performed as described therein. In brief, purified recombinant protein (70–500 ng) was incubated for 20 min on ice in a total volume of 280 μL of 25 mM sodium

acetate/25 mM potassium phosphate, pH 5.0, 0.05% Brij58, 1 mM EDTA supplemented with approximately 1 nM [$^3$H]Ins 1,3,4,5-$P_4$ and varying concentrations of unlabelled D-Ins 1,3,4,5-$P_4$ or other inositol phosphates or phosphoinositides. Bound ligand was separated from free ligand by centrifugation through columns containing 1.2 mL packed resin of BioGel P-4 (Bio-Rad) equilibrated with binding buffer. Non-specific binding was determined in the presence of 1 μM D-Ins 1,3,4,5-$P_4$. All measurements were made in duplicate or triplicate and were repeated at least twice.

Analysis of binding was carried out as follows. Data are presented as specific binding, which is plotted as percentage of maximal specific binding (in the absence of competitor) corrected for non-specific binding, against the molar concentration of competitor. $IC_{50}$ values and slope factors were determined by fitting the resulting displacement curves in SIGMAPLOT (SPSS Inc., Chicago, IL, USA) to the following equation: % specific binding$_x$ = % specific binding$_0$/$(1 + X/IC_{50})^A$, where X is the molar concentration of competitor and A the slope factor. $K_d$ values and the number of binding sites were estimated using the KELL for Windows data-analysis computer program (Version 5; BIOSOFT, Cambridge, UK). The data were analysed for the presence of one and two classes of binding sites.

## Results

### Homology between centaurin α2 and p42$^{IP4}$

The cDNA clone of human centaurin α2 (1802 bp) codes for a protein with 381 amino acids and high homology to the human p42$^{IP4}$ protein (Sedehizade *et al*. 2002). We compared the nucleotide and amino acid sequences for human centaurin α2 and p42$^{IP4}$. The cDNAs share 67% similarity in their coding region. Both proteins are highly homologous; they are 58% identical (212 amino acid residues) and 75% similar (277 amino acid residues). They also show a conserved domain structure. At the amino terminus there is a zinc finger motif with high homology to Arf-GAP, followed by two PH domains. The amino acid residues conserved in both proteins in the consensus domain sequences are shown in Fig. 1(a). In each domain there is high homology, which is not found in the linking pieces. There is 62% identity and 81% similarity for the zinc finger motif, and 73% identity and 79% similarity for the N-terminal PH domain. However, the C-terminal PH domain shows only 51% identity and 75% similarity (Fig. 1a). The

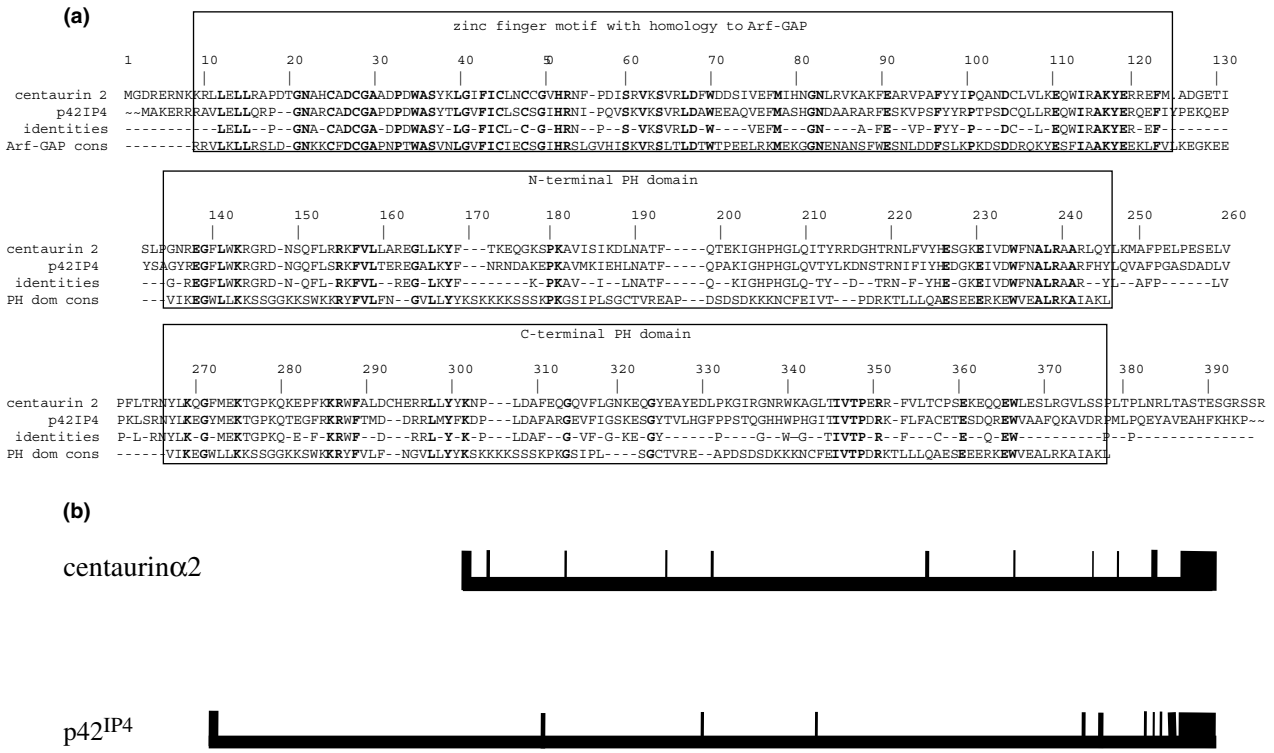


**Fig. 1** Sequence analysis and gene structure of human centaurin α2 and p42$^{IP4}$. (a) Homology alignment of domains of both proteins. The amino acid sequences of human centaurin α2 and p42$^{IP4}$ were aligned using the software program at Heidelberg Unix Sequence Analysis Resources. The consensus sequence in line 3 (identities) shows only identical amino acid residues of centaurin α2 and p42$^{IP4}$. The Arf-GAP and PH domain consensus sequences were assembled manually according to the output from the NCBI Conserved Domain Search. The amino acid residues conserved in the two proteins centaurin α2 and p42$^{IP4}$ and in the consensus domain sequences are given in bold characters. (b) Overall genomic structure of human centaurin α2 and p42$^{IP4}$. The length of the horizontal bar indicates the approximate size of the genes, excluding the promoter region. Vertical bars represent exons and their thickness indicates the length of exons. The space between the vertical bars corresponds to the length of the intron.

$p42^{IP4}$ protein has a nuclear localization signal (NLS) at its N-terminus (MA**KERRR**AV) that is not found at the N-terminus of centaurin α2.

No other human protein with a similar structure could be identified in database analysis. Proteins with partial homologies, which are restricted either to the zinc finger domain or to one of the PH domains, have also been designated centaurins (Jackson *et al*. 2000). However, the two proteins centaurin α2 and $p42^{IP4}$/centaurin α1 are the only isoforms of a distinct family for which, so far, no other proteins exist. These two forms have both been identified in human and rat. For mouse proteins, our analysis, which is not yet complete, suggests comparable structures.

Differences in tissue-specific expression of both genes indicate distinct roles for these two very similar proteins. These differences were found using northern blot analysis by Whitley *et al*. (2002) for centaurin α2 and by (Venkateswarlu and Cullen, 1999) for centaurin α1. We confirmed these results using human multiple tissue northern blots which were hybridized with radioactively-labelled cDNA probes of either centaurin α2 or $p42^{IP4}$. For centaurin α2, we obtained the strongest signals in placenta, spleen, kidney, skeletal muscle and adrenal gland, weak signals in thyroid, liver, heart, lung, small intestine, peripheral blood leucocytes, and no signals in spinal cord, brain, lymph node and bone marrow, stomach, trachea or colon (data not shown). In agreement with our previous experiments in which we could show that $p42^{IP4}$ is highly expressed in neuronal cells (Kreutz *et al*. 1997a; Stricker *et al*. 1997; Sedehizade *et al*. 2002), we found a strong signal for $p42^{IP4}$ in brain, spinal cord and peripheral blood, a weak signal in kidney, but no signals in all other tissues (data not shown). Such clear differences in expression between these two proteins stimulated us to investigate genomic structure and subcellular localization of these two proteins further.

### Genomic structures of the human centaurin α2 and $p42^{IP4}$

Our determination of the nucleotide sequences of the cDNAs of centaurin α2 and $p42^{IP4}$, and data from the human genome analysis, made it possible to identify their chromosomal localizations and, moreover, to delineate the genomic structure of both genes. The human gene for centaurin α2 is located at position 17q11.2, region 29 512 842–29 550 556 of human chromosome 17 (build 28). This is in the vicinity of the neurofibromatosis 1 (NF1) gene. The NF1 gene is located at position 17q11.2 (build 28) of human chromosome 17. The centaurin α2 gene has 37 674 bp, with 11 exons that give a cDNA of 2747 bp. In the genomic sequence, we identified the polyadenylation signal (AATAAA), position 29 550 534–29 550 539, that is 17 bp before the last cytosine residue present in the cDNA sequence before the polyA-tail. For the transcription start site, we propose the guanosine residue at position 29 512 842.

The human gene for $p42^{IP4}$ was also analysed. It is located on a different chromosome, i.e. human chromosome 7, at position 7p22.3 region, 564 582–621 309. The human $p42^{IP4}$ gene has 56 765 bp. As in the centaurin α2 gene, there are also 11 exons for the $p42^{IP4}$ gene, which give a cDNA with 2334 bp. In the genomic sequence, we identified the polyadenylation signal (AATAAA), position 564 566–564 557, that is 19 bp before the last thymidine residue present in the cDNA sequence before the polyA-tail. For the transcription start site, we propose the guanosine residue at position 621 309.

The intron/exon structure is remarkably conserved for both genes. First, both genes have 11 exons; secondly, the amino acids at the intron/exon boundaries are partly conserved and thirdly, even the nucleotide sequences at the boundaries are largely conserved. The data for exact chromosomal localization, lengths of exons and introns are summarized in Table 1. The overall scheme of the genomic structure for both genes is shown in Fig. 1(b). The differences between both genes are analysed in detail in the discussion.

### Heterologous expression of the human centaurin α2 in Sf9 cells and protein purification

In further experiments, we characterized centaurin α2 biochemically. We investigated whether centaurin α2 codes for a protein with ligand specificity similar to that of $p42^{IP4}$. The coding sequence of centaurin α2 was subcloned into pAcG3X transfer vector. We generated a recombinant baculovirus to express a fusion protein of centaurin α2 with glutathione-S-transferase (GST).

We overexpressed the fusion proteins of $p42^{IP4}$ or centaurin α2, respectively, in Sf9 cells. The results for overexpression of both proteins in Sf9 cells and their purification to homogeneity are presented in Fig. 2. The cleared lysates of Sf9 cells, infected either with recombinant viruses expressing GST-$p42^{IP4}$ or GST-centaurin α2, were compared with the cleared lysate of non-infected Sf9 cells (lane 2, 3 vs. lane 1 in Fig. 2). The arrowheads indicate the clearly visible bands of overexpressed fusion proteins. The fusion proteins were affinity-purified on glutathione beads from cell lysate of transfected Sf9 cells in a one-step procedure. The affinity-purified fusion proteins of GST-$p42^{IP4}$ and GST-centaurin α2, respectively, are shown in Fig. 2 lanes 5 and 6. The fusion proteins were then cleaved from GST in a two-phase reaction. The solid phase with the fusion protein bound via glutathione to the beads, whereas the liquid phase contained the protease factor Xa that releases the protein. The $p42^{IP4}$ and centaurin α2, respectively, were then further chromatographed on a Hi Trap Heparin Sepharose column. Both proteins adsorbed completely at the column and eluted at 800 mM NaCl (Fig. 2, lanes 8 and 9). The yield was approximately 5 mg/L culture ($2.5 \times 10^9$ cells). The purified centaurin α2 allowed us to characterize the protein biochemically and to compare the

**Table 1** Data of genomic structures of human centaurin α2 and p42$^{IP4}$. Comparison of chromosomal localization, region and orientation of human centaurin α2 and p42$^{IP4}$ protein. Contig indicates the accession under which the sequence data can be found. The length of the whole gene, exon 1 and exon 11 were calculated from the putative transcription start point and the final nucleotide after the polyadenylation signal sequence. Beginning and end of introns are marked by the nucleotide sequences of the exon/intron transitions. The first and last three nucleotides (bold, italic characters) before and after the sequence symbolized by the dashes indicate the introns

| Gene | Centaurin α2 | p42$^{IP4}$ |
|---|---|---|
| Chromosome | 17 | 7 |
| Cytogenetic marker | 17q11.2 | 7p22.3 |
| Region | 29 512,842–29 550 556 | 564 582–621 309 |
| Contig | NT-077766.1 | NT-007819.12 |
| Orientation | (–) – strand | (–) – strand |
| Total Length | 37 674 bp | 56 765 bp |
| Exon 1 | 373 bp | 273 bp |
| Intron 1 | 832 bp | 18 890 bp |
| Exon 2 | 131 bp | 131 bp |
| Intron 2 | 3757 bp | 8730 bp |
| Exon 3 | 92 bp | 92 bp |
| Intron 3 | 4971 bp | 6501 bp |
| Exon 4 | 80 bp | 83 bp |
| Intron 4 | 2217 bp | 14 795 bp |
| Exon 5 | 113 bp | 113 bp |
| Intron 5 | 10 603 bp | 787 bp |
| Exon 6 | 147 bp | 147 bp |
| Intron 6 | 4235 bp | 3509 bp |
| Exon 7 | 84 bp | 84 bp |
| Intron 7 | 3918 bp | 368 bp |
| Exon 8 | 63 bp | 63 bp |
| Intron 8 | 1128 bp | 611 bp |
| Exon 9 | 78 bp | 72 bp |
| Intron 9 | 1686 bp | 157 bp |
| Exon 10 | 229 bp | 229 bp |
| Intron 10 | 1366 bp | 83 bp |
| Exon 11 | 1357 bp | 1047 bp |
| cDNA | 2747 bp | 2334 bp |
| | Transitions of exon-intron-exon | |
| Intron 1 | GCAG***GTA***——***CAG***ATCC | CCGG***GTA***——***CAG***ATCC |
| Intron 2 | GGAG***GTA***——***CAG***TTTA | GGAG***GTA***——***CAG***TTCA |
| Intron 3 | GCCT***GTG***——***TAG***GGTC | GCCA***GTG***——***CAG***GCTC |
| Intron 4 | CCAG***GTA***——***AAG***GTAA | GCAG***GTG***——***CAG***GGTA |
| Intron 5 | ACAG***GTA***——***CAG***GGTA | TGAT***GTG***——***CAG***GCCA |
| Intron 6 | GAAG***GTG***——***CAG***GAGA | GAAG***GTG***——***TAG***GAGA |
| Intron 7 | TGAG***GTG***——***CAG***CTCG | AGAT***GTG***——***TAG***CTGG |
| Intron 8 | AAAG***GTA***——***CAG***CAGA | CAAG***GTG***——***CAG***CAAA |
| Intron 9 | ACTG***GTA***——***CAG***GATG | CCTG***GTA***——***CAG***GACG |
| Intron 10 | CTTA***GTA***——***CAG***CTGC | GCAG***GTG***——***CAG***TGGA |

results with those obtained for native and recombinant p42$^{IP4}$ protein.

### *In vitro* [$^3$H]Ins 1,3,4,5-$P_4$ binding to recombinant human centaurin α2 and p42$^{IP4}$

We have previously shown that the binding affinities of human, porcine and rat p42$^{IP4}$ protein for the second messenger molecules PtdIns$P_3$ and Ins$P_4$ are comparable (Stricker *et al*. 1997). Due to the high sequence homology between human p42$^{IP4}$ and centaurin α2, we expected that the latter would also bind both second messenger molecules. Therefore, we performed a binding study of D-[$^3$H]Ins$P_4$ with the purified centaurin α2 protein *in vitro*.

As shown in Fig. 3(a), D-[$^3$H]Ins$P_4$ binding to centaurin α2 was inhibited by Ins$P_4$ and PtdIns$P_3$, but was not at all affected by Ins$P_3$ or PtdIns$P_2$. Here, we used water-soluble di$C_8$-substituted lipid analogues. The slope factors of 0.97 for Ins$P_4$ and 0.96 for PtdIns$P_3$, derived from the data used in Fig. 3(a), indicate that there is only one class of binding sites present. From the displacement curves, we calculated the $K_D$ values for Ins$P_4$ and PtdIns$P_3$. The binding affinity is given by a $K_D$ of 28.7 nM (± 5.7; $n = 7$) for Ins$P_4$ and a $K_D$ of 30.4 nM (± 5.7; $n = 3$) for PtdIns$P_3$. Analysis of the data for the presence of one or two classes of binding sites was carried out as explained in Methods. This analysis revealed that a model for two different classes of binding sites did not

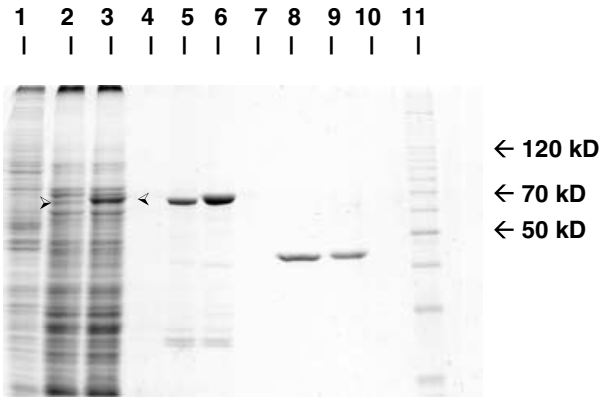


**Fig. 2** Heterologous expression and purification of p42$^{IP4}$ and centaurin α2 in Sf9 cells with recombinant baculoviruses. Sf9 cells were transfected with recombinant viruses. The proteins were analysed on a 10% polyacrylamide (30 : 0.8) gel and stained with Coomassie Blue R250. Lane 1: cleared cell lysate of non-infected Sf9 cells (5 μL); lane 2: cleared cell lysate of Sf9 cells infected with a recombinant baculovirus expressing GST-p42$^{IP4}$ (5 μL); lane 3: cleared cell lysate of Sf9 cells infected with a recombinant baculovirus expressing GST-centaurin α2 (5 μL); lane 4: empty; lane 5: GST-p42$^{IP4}$, affinity-purified on GSH beads (2,5 μL); lane 6: GST-centaurin α2, affinity-purified on GSH beads (2,5 μL); lane 7: empty; lane 8: p42$^{IP4}$ fraction eluted with 800 mM NaCl-containing buffer from Heparin-Sepharose column (15 μL); lane 9: centaurin α2 fraction eluted with 800 mM NaCl-containing buffer from Heparin-Sepharose column (15 μL); lane 10: empty; lane 11: 10 kDa ladder (Gibco/BRL, 5 μL). The arrowheads indicate the overexpressed GST fusion proteins in the cleared cell lysates.

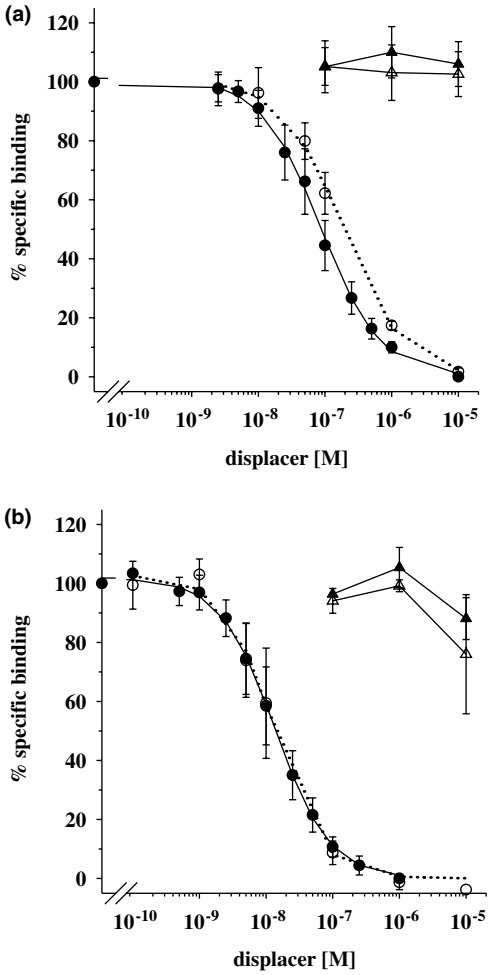


**Fig. 3** Ligand binding of centaurin α2 (a) and p42$^{IP4}$ (b), expressed in Sf9 cells. Purified centaurin α2 protein (human) and p42$^{IP4}$ protein (pig) were incubated with D-[$^3$H]Ins 1,3,4,5-$P_4$ in binding buffer and appropriate displacer (D-Ins 1,3,4,5-$P_4$, filled circles; D-Ins 1,4,5-$P_3$, filled triangles; di$C_8$PtdIns 3,4,5-$P_3$, open circles; di$C_8$PtdIns 4,5-$P_2$, open triangles, as described under Materials and methods. Datapoints shown are means and SD from 2 to 10 experiments done in duplicate or triplicate. The curves displayed for D-Ins 1,3,4,5-$P_4$ (solid line) and di$C_8$PtdIns 3,4,5-$P_3$ (dotted line) were fitted to the data by non-linear regression.

fit the data significantly better than a model with one class of binding sites.

The calculated $B_{Max}$ value derived from the binding experiments was 37.9 (± 4.0) nmol/mg of protein. As 1 mg of centaurin α2 protein corresponds to 23 nmol, an important result of this biochemical analysis of centaurin α2 is the finding that the $B_{Max}$ value of 37.9 nmol/mg of protein suggests that human centaurin α2 has a stoichiometry of two binding sites, which display identical affinity for the ligand $InsP_4$.

For recombinant p42$^{IP4}$ expressed in Sf9 cells, displacement curves are shown in Fig. 3(b). The displacement curves obtained for $InsP_4$ and $PtdInsP_3$ indicate the presence of one class of binding site with $IC_{50}$ values of 13.5 nM and 12.5 nM, and slope factors of 1.06 and 1.19, respectively. As with centaurin-α2, $InsP_3$ and $PtdInsP_2$ did not displace $InsP_4$ at concentrations up to 10 μM. Analysis of the data gave a $K_D$ of 3.9 nM (± 1.5; $n = 10$) and a $B_{Max}$ of 11.3 (± 6.3) nmol/mg of protein, and the data were best described by a model using one class of binding sites.

### Subcellular localization of the human centaurin α2 and p42$^{IP4}$ protein

To compare the subcellular distributions of both proteins, we transiently transfected HEK-293 cells either with an expression vector in which centaurin α2 was tagged to the C-terminus of GFP, or a corresponding expression vector with p42$^{IP4}$ fused to GFP. Interestingly, compared with p42$^{IP4}$, centaurin α2 showed a completely different subcellular localization. The p42$^{IP4}$ protein was localized diffusely in cytosol and nucleus (Fig. 4a), as shown previously for NG-108 cells (Sedehizade *et al.* 2002). In contrast, centaurin α2 was localized exclusively at the plasma membrane in cells grown in the presence of serum (Fig. 4d). Also, in the absence of serum, centaurin α2 was located similarly at the plasma membrane. Centaurin α2 was excluded from the nucleus (Fig. 4f), whereas p42$^{IP4}$ was found in the nucleus

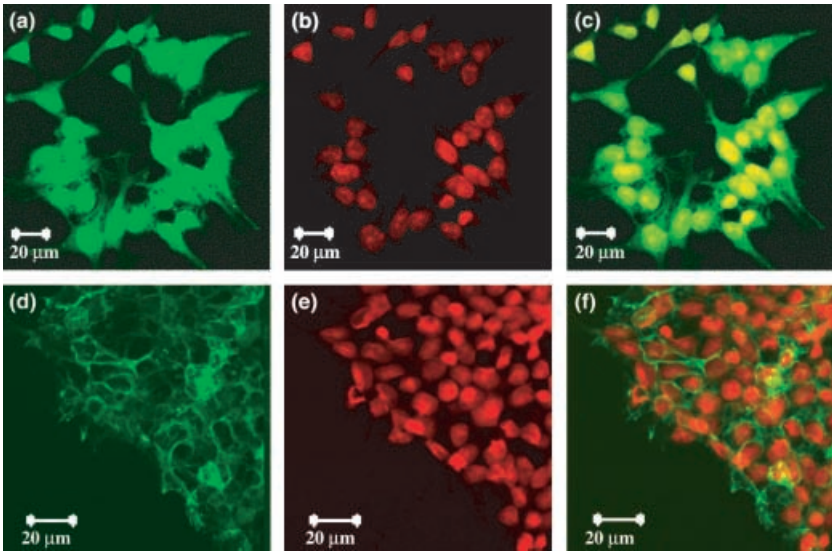


**Fig. 4** Subcellular localization of GFP-p42$^{IP4}$ and GFP-centaurin α2. HEK-293 cells were transiently-transfected with GFP-p42$^{IP4}$ (a–c) and GFP-centaurin α2 (d–f) and both were grown in the presence of serum. Cells were fixed, the nuclei were stained with propidium iodide (b and e), and images were taken by confocal laser scanning microscope (CLSM). Green colour shows the subcellular localization of GFP-p42$^{IP4}$ in (a) and of GFP-centaurin α2 in (d), nuclei are visualized in red (b and e); the overlay (c and f) indicates overlap of both the protein and the nucleus, given as yellow in c, but no overlap in f. Bars represent 20 μm.

(Fig. 4c), as shown by the superposition of GFP fluorescence and nuclear staining.

The subcellular localization of the GFP-centaurin α2 was investigated further. Using CLSM *in vivo* imaging, we observed that incubation of the transfected cells with wortmannin at a concentration that specifically inhibits PI 3-kinase (100 nM) caused dissociation of the membrane-bound GFP-centaurin α2 into the cytosol. Within about 2 min, the dissociation started (Fig. 5a), and 14 min after exposure of the cells to wortmannin, centaurin α2 had almost completely disappeared from the plasma membrane (Fig. 5a). Centaurin α2 was then found in the cytoplasm but was still excluded from the nucleus, and there was no redistribution to the plasma membrane within 20 min (Fig. 5a).

In contrast, GFP-p42$^{IP4}$ had a widespread distribution in the cell (Fig. 4a–c). Since we have already shown specific binding of human p42$^{IP4}$ to Ins 1,3,4,5-$P_4$ and PtdIns 3,4,5-$P_3$ [Fig. 3 and Sedehizade *et al*. (2002)], we decided to investigate the effect of EGF, which leads to PI3-kinase activation and, in consequence, to the generation of the lipid PtdIns 3,4,5-$P_3$ (Cantley *et al*. 1991). As shown in Fig. 5(b), EGF stimulation of the transfected HEK-293 cells resulted in a redistribution of GFP-p42$^{IP4}$ from cytosol to the periphery (plasma membrane) and the nucleus. However, as expected, stimulation with EGF had no effect on the subcellular distribution in GFP-centaurin α2-expressing cells, which was analysed in 10 cells from different cultures (data not shown).

## Discussion

The human centaurin α2 protein is 58% identical and 75% similar to the human p42$^{IP4}$. These two proteins form a protein family, which is, until now, restricted to vertebrates. We found orthologues of centaurin α2 in rat, mouse and puffer fish (fugu rubripes). No orthologues of this protein family could be identified in *Drosophila melanogaster* or *Caenorhabditis elegans*. Other proteins found in nucleotide and protein databases were also named centaurins, types beta, gamma or delta. However, these proteins have substantial differences in structure and have only partial homology which is restricted either to the zinc finger domain or to one of the two PH domains. Therefore, this nomenclature is very confusing because only the two proteins, p42$^{IP4}$/centaurin α1 and centaurin α2, are a distinct family. This family, which consists only of these two proteins, has been identified in human, rat and mouse.

The zinc finger domain of centaurin α2 and p42$^{IP4}$/centaurin α1 shows identity with the catalytic domain of Arf-GAPs. The Arf-GAP domain is found in a large variety of proteins of all eukaryotic species, including plants and fungi. For p42$^{IP4}$/centaurin α1, no Arf-GAP activity has been detected, but it was found to substitute for the loss of yeast Gsc1p (Venkateswarlu *et al*. 1998), which has similarities to the zinc finger domain (Jackson *et al*. 2000). Therefore, p42$^{IP4}$/centaurin α1 either requires another Arf/Arl partner, additional cofactors, or does not possess GAP activity. Experiments concerning the possible Arf-GAP activity of centaurin α2 have not yet been reported.

The nucleotide sequence of human p42$^{IP4}$ cDNA (Sedehizade *et al*. 2002) and of the human centaurin α2 cDNA (Whitley *et al*. 2002) allowed us to identify the chromosomal localization of both genes. The human gene for centaurin α2 is located on chromosome 17, in close proximity to the neurofibromatosis type 1 (NF1) gene. In 5–20% of patients with NF1, gross deletions resulting in the loss of at least 11 functional genes are found. Patients with the NF1 large-deletion syndrome have a significantly increased risk of neurofibroma development and mental retardation. The deleted region around the neurofibromin locus includes the

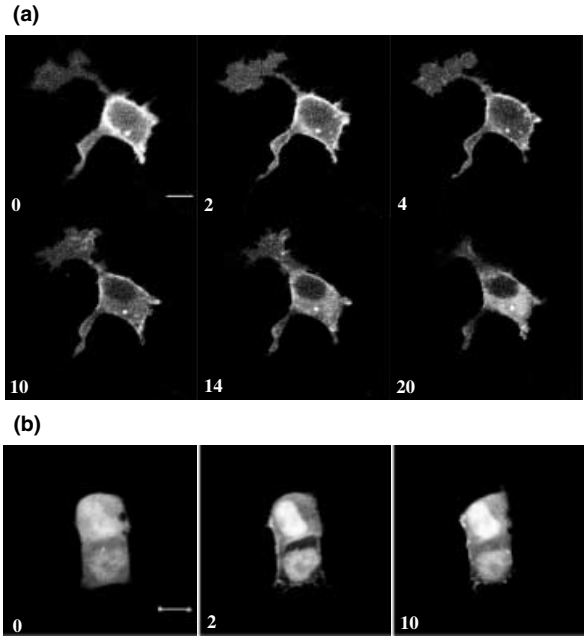


**Fig. 5** Changes in the subcellular distribution of GFP-centaurin α2 (a) and GFP-p42$^{IP4}$ (b) in cells treated with wortmannin and EGF, respectively. HEK-293 cells were transiently-transfected either with GFP-centaurin α2 (a) or with GFP-p42$^{IP4}$ (b). Post-transfection cells expressing GFP-centaurin α2 were maintained in serum-containing culture medium and cells expressing GFP-p42$^{IP4}$ were serum-starved for 12–16 h. Stimulation of the cells was performed in Na-HBS buffer (see Methods). Life images by CLSM were taken from selected cells at 10 s intervals. (a) HEK-293 cells expressing GFP-centaurin α2 were treated with wortmannin (final concentration 100 nM). This dissociation was clearly detectable in 45 out of 71 cells examined. Neither the incubation buffer nor the solvent DMSO used for wortmannin had any effect on the association of the membrane-bound GFP-centaurin α2. (b) HEK-293 cells expressing GFP-p42$^{IP4}$ were stimulated with EGF (final concentration 300 ng/mL). From 120 cells tested, 100 responded similarly. The numbers in the figures indicate elapsed time in minutes. The bars represent 10 μm.

centaurin α2 gene. This may contribute to the severe phenotype in these patients (Jenne *et al*. 2000). The role of centaurin α2 in this context is still unclear. The human gene for p42$^{IP4}$ is located on chromosome 7, but no correlation to inherited diseases has been documented.

Although only 58% of the amino acids of both proteins, p42$^{IP4}$ and centaurin α2, are identical, we found that the two proteins display the following striking similarities. They have 11 similar exons, the amino acids of the intron/exon boundaries are conserved, and also the lengths of the exons are conserved. Only exons 1 and 11 differ due to the 5′- and 3′-untranslated regions. Exon 1 of centaurin α2 codes for four additional amino acids, and exons 9 and 11 for two additional amino acids. In exon 4, p42$^{IP4}$ has one additional amino acid. The differences due to additional amino acids in exons 1 and 4 are restricted to the beginning or the end of the zinc finger motif (Fig. 1a). The additional amino acids in exon 9 are located at the C-terminal PH domain (Fig. 1a) and might play a role in determining differences in ligand binding.

On the other hand, the intron sizes of both genes are remarkably different (Table 1). The scheme in Fig. 1(b) shows that the gene for centaurin α2 has a rather uniformly spaced distribution of the 11 exons over the locus, whereas exons 5–11 of the human p42$^{IP4}$ gene are concentrated at the end of the gene locus, as depicted in the scheme in Fig. 1(b). The genomic organization of p42$^{IP4}$/centaurin α1 and centaurin α2 in other species is under investigation. The mouse genes are located on chromosomes 11 (p42$^{IP4}$) and 5 (centaurin α2), and the rat genes on chromosomes 12 (p42$^{IP4}$) and 10 (centaurin α2). Detailed analysis of both the mouse and rat genomic organization will be a prerequisite for further functional studies of these two genes.

All members of this protein family identified up to now have a conserved structure except for one rat homologue of p42$^{IP4}$ which had been designated centaurin α (Hammonds-Odie *et al*. 1996). Centaurin α has a remarkable difference in protein sequence in the N-terminal PH domain, corresponding to exon 5 of human p42$^{IP4}$, and an extended carboxy terminus (exon 11). Analysis of the nucleotide sequence of human intron 4, exon 5 and exon 11 showed that such differences could not result from differential splicing. However, the rat homologue of p42$^{IP4}$ that has been cloned and characterized in our laboratory (Aggensteiner *et al*. 1998) has an almost identical amino acid sequence to the human p42$^{IP4}$. Therefore, our previous suggestion (Aggensteiner *et al*. 1998) that these peculiarities in the centaurin α sequence might be due to artifacts of cDNA, DNA library or sequencing is confirmed by the present genome analysis.

Heterologous expression of the GST-centaurin α2 fusion protein in Sf9 cells with a recombinant baculovirus was very effective and resulted in functionally active protein. The amount of purified protein was even slightly higher than the amount obtained for p42$^{IP4}$ (Fig. 2, lanes 2 and 3) and both proteins could be further purified on heparin sepharose, as previously described for p42$^{IP4}$ (Hanck *et al*. 1999). The centaurin α2 protein has a calculated molecular mass of 44.4 kDa, whereas the p42$^{IP4}$ protein has a calculated molecular mass of 43.4 kDa. This difference in molecular mass seems to be detectable in Fig. 2, lanes 8 and 9. Centaurin α2 and p42$^{IP4}$ bind D-Ins 1,3,4,5-$P_4$ and PtdIns 3,4,5-$P_3$ *in vitro* but have different $K_D$ and $B_{Max}$ values. For the recombinant centaurin α2 protein, the $K_D$ for D-Ins 1,3,4,5-$P_4$ was 28.7 nM, compared with 3.9 nM for p42$^{IP4}$. The $B_{max}$ of 37.9 nM/mg protein for centaurin α2 with 23 nmol per mg of protein indicates that centaurin α2 has two $InsP_4$ binding sites with identical affinities. This is in contrast to p42$^{IP4}$ where we could find a maximum of one binding site per protein (Hanck *et al*. 1999; Stricker *et al*. 1999).

The most striking difference which we found between the two isoforms, p42$^{IP4}$ and centaurin α2, was their subcellular localization. Whereas centaurin α2 was at the plasma membrane, p42$^{IP4}$ was located diffusely in the cytosol. The ligand binding data presented here, comparing centaurin α2 and p42$^{IP4}$, could provide an explanation for this difference. This explanation is based on the model of regulated avidity proposed by Lemmon and Ferguson (2000). Their interesting hypothesis suggests that PH domains, even when they have rather low affinity for phosphoinositides, might bind increasingly to membranes when the PH domains form oligomers. This phenomenon has been denoted as the avidity effect. Already, formation of dimers, which doubles binding enthalpies, can enhance the apparent affinity (equal to avidity) by a factor of 1000. Regulated avidity could be employed in signal-dependent membrane recruitment of signalling molecules. In their work, Lemmon and Ferguson state that there is hardly any experimental evidence to support (or refute) this model of regulated avidity. Centaurin α2, which, in contrast to p42$^{IP4}$/centaurin α1 despite large sequence homology, tightly binds to the plasma membrane, seems to be a clear physiological example supporting the avidity hypothesis. We extend the model of Lemmon and Ferguson to molecules with two binding sites with virtually identical affinity towards the same ligand. Centaurin α2 displays two independent binding sites with identical affinity, whereas p42$^{IP4}$/centaurin α1 with a very similar sequence binds only one ligand molecule per protein. A similar effect on apparent affinity might be applicable for FYVE domains, which, in the form of two tandem domains, were a much better indicator of PtdIns 3-phosphate in membranes than a single FYVE domain (Gillooly *et al*. 2000).

Upon PtdIns 3-kinase activation, p42$^{IP4}$ migrates to the plasma membrane and to the nucleus. This translocation occurs very rapidly, since at 120 s after stimulation of the cells with EGF, translocation is completed (Fig. 5). Centaurin α2 behaves completely differently. It is localized at the plasma membrane already under resting conditions and is excluded from the nucleus. The latter difference can easily be explained by the finding that p42$^{IP4}$ has a NLS at its N-terminus that is not found at the N-terminus of centaurin α2. For nuclear localization of p42$^{IP4}$, a functional significance became evident from the recent report that this protein displays interaction with the nuclear protein nucleolin (Dubois *et al*. 2003). This protein association was found to occur *in vitro* and *in vivo*. The possibility that centaurin α2 merely interacts with other proteins, which are bound to or are part of the plasma membrane, seems to be ruled out by our experiments using wortmannin, where the centaurin α2 protein was released from the plasma membrane to the cytoplasm (Fig. 5a).

In conclusion, centaurin α2 and p42$^{IP4}$, which form a distinct protein family, have high homology and the same overall protein structure. Both proteins specifically bind $InsP_4$ and $PtdInsP_3$, but they do not interact with $InsP_3$ or $PtdInsP_2$. Both proteins when overexpressed and purified to homogeneity displayed binding activity. For p42$^{IP4}$/centaurin α1, the possible function as adapter protein linking other proteins to membranes or subcellular structures was elucidated by specific interaction with the nuclear protein nucleolin (Dubois *et al*. 2003) and also with some isoforms of casein kinase I (Dubois *et al*. 2001). The difference in subcellular distribution that we showed for both proteins is, on the one hand, due to the NLS which is found exclusively in p42$^{IP4}$/centaurin α1, and on the other hand, to difference in ligand-specific stoichiometry of binding. Further experiments are required to determine whether protein interactions are regulated differently for centaurin α2 and p42$^{IP4}$, and to elucidate their possible roles of adapter proteins.

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