Mammalian casein kinases I (CKI) belong to a family of serine/threonine protein kinases involved in diverse cellular processes including cell cycle progression, membrane trafficking, circadian rhythms, and Wnt signaling. Here we show that CKIα co-purifies with centaurin-α1 in brain and that they interact in vitro and form a complex in cells. In addition, we show that the association is direct and occurs through the kinase domain of CKI within a loop comprising residues 217–233. These residues are well conserved in all members of the CKI family, and we show that centaurin-α1 associates in vitro with all mammalian CKI isoforms. To date, CKIα represents the first protein partner identified for centaurin-α1. However, our data suggest that centaurin-α1 is not a substrate for CKIα and has no effect on CKIα activity. Centaurin-α1 has been identified as a phosphatidylinositol 3,4,5-trisphosphate-binding protein. Centaurin-α1 contains a cysteine-rich domain that is shared by members of a newly identified family of ADP-ribosylation factor guanosine triphosphatase-activating proteins. These proteins are involved in membrane trafficking and actin cytoskeleton rearrangement, thus supporting a role for CKIα in these biological events.

The casein kinase I (CKI) family of serine/threonine kinases is ubiquitously expressed in a range of eukaryotes including yeast and humans as well as in plants (reviewed in Ref. 1). Seven isoforms from distinct genes are expressed in mammals (CKIα, β, γ1, γ2, γ3, δ, and ε), four in Saccharomyces cerevisiae (Hrr25, Yck1, Yck2, and Yck3), and five in Schizosaccharomyces pombe (Cki1, Cki2, Cki3, Hhp1, and Hhp2). The CKI family is characterized by a conserved core kinase domain and variable amino- and carboxyl-terminal tails.

Yeast CKI isoforms are involved in DNA repair (2–4). Recently, many reports (5–12) indicate that they also play a role in cytokinesis and in vesicle trafficking especially in endocytosis. The functions of the mammalian isoforms are less well understood, but based on high homology with their yeast counterparts, they may have similar biological functions. CKIβ and CKIδ play a role in the regulation of p53 (13, 14). CKIε has also been implicated in circadian rhythms in Drosophila (15, 16) and in development by transducing the Wnt pathway (17, 18). CKIγ might play a role in cytokinesis and in membrane trafficking (19). CKIα has been shown to play a role in cell cycle progression (20) and in membrane trafficking (21, 22). Recently, CKIα has been shown to be implicated in regulating the nucleocytoplasmic localization of some substrates (23, 24).

Several substrates, including nuclear and cytosolic proteins and membrane receptors, have been reported to be phosphorylated at least in vitro by a CKI activity (reviewed in Ref. 1). CKI isoforms are thought to be constitutively active and second messenger-independent. However, it has been shown that CKIβ and CKIε are regulated by autophosphorylation (25–28). CKIα is also autophosphorylated, but whether this has an effect on its activity is not well defined. CKIα is negatively regulated by PtdIns(4,5)P2 (21). Moreover, CKI isoforms have been reported to phosphorylate some of their substrates only if they were previously phosphorylated by another kinase two or three residues carboxyl-terminal to the CKI phosphorylation site. In this way, the effect of CKI is dependent on other kinases. CKIε is present in cells in different spliced forms (1, 29) exhibiting different substrate specificities and differences in their protein-protein interactions.

Although the yeast CKI isoforms have been well characterized, the functions of the mammalian CKI isoforms are much less known. Therefore, the identification of mammalian CKI substrates and CKI-binding proteins should help to clarify their cellular function(s). CKIα interacts with NF-AT4 (23), the paired helical filament proteins (30), G-protein-coupled receptors (31), and the AP-3 complex (22). CKIα also forms a complex with certain splicing factors but these interactions may be indirect (32).
In the present study, we have shown that CKIα interacts with centaurin-α. Centaurin-α is a PtdIns(3,4,5)P3-binding protein containing two PH domains (33–35) and a zinc finger motif similar to the one found in a newly identified family of ADP-ribosylation factor (ARF) guanine triphosphatase-activating proteins (GAP) (reviewed in Refs. 36–39). The yeast protein that shows the highest homology to centaurin-α, Gsc1, also contains a zinc finger motif that confers its ARF-GAP activity (40). Members of this family are involved in membrane trafficking and in actin cytoskeleton rearrangement. Our results suggest that CKIα plays a role in membrane trafficking and/or actin cytoskeleton rearrangement, thus confirming previous reports (21, 22).

MATERIALS AND METHODS

cDNA Cloning—The cDNA corresponding to rabbit muscle CKIα was originally cloned in the pET 3 vector (Novagen) (41). CKIα cDNA from this clone was amplified by the polymerase chain reaction (PCR) using two oligonucleotides (5′-ggtagctatgggacagggctccg-3′) and (5′-gggacatttggaggtggtttgcc-3′) in order to create a 5′ NotI site and a 3′ BamHI site (both are underlined in sequences). Amplified cDNA was inserted in a pET-16b vector (Novagen) at NotI/BamHI restriction sites to express CKI as a histidine-tagged protein. The CKIα cDNA was inserted in a pDNA3 vector (Invitrogen) from the original clone after being amplified by PCR using two oligonucleotides (5′-gggatccggctatggctagctgctgctggtggtggttgggggtttgggc-3′ and 5′-gggacatttggaggtggtttgccgatatgaagctttcacttgagatggtcattggggtttgggc-3′) in order to create a 5′ BamHI site and a 3′ NotI site (both are underlined in sequences). The Kozak sequence is shown in italics and the HA-tagged sequence in bold. CKIα mutants were generated by PCR and cloned in the pSP72 vector (Promega). The EcoRI and BamHI sites downstream of the T7 promoter. The oligonucleotides (the EcoRI and BamHI sites are underlined) were used:

- (5′-gggggatccgcgaagatcggggtttgggacagggctccg-3′) and (5′-gggacatttggaggtggtttgccgatatgaagctttcacttgagatggtcattggggtttgggc-3′).

The construction of GST-centaurin-α was described previously (42). Centaurin-α cDNA from that vector was recovered after digestion with EcoRI and XbaI and subcloned in the FLAG-env2 vector. HA-tagged CKIα (pET8c) in pDNA3 is a gift from F. McKeon (23). FLAG-centaurin-α is from Trevor R. Jackson.

For the experiment performed in Fig. 7B, pET19c CKIα (41), pET8c CKIα (25), pET8c CKIαΔ317 (25), pET8c CKIα (19), pSV2Xe CKIα (from Louise Larose), pET8c CKIα (19), and pSP72 CKIα were used. pSP72 CKIα construct was subcloned using human CKIα plasmid (pV405) provided by Dr. David Virshup (27). CKIα cDNA was amplified by PCR using two oligonucleotides (5′-gggatccggctatggctagctgctgctggtggtggttgggggtttgggc-3′ and 5′-gggacatttggaggtggtttgccgatatgaagctttcacttgagatggtcattggggtttgggc-3′) in order to create a 5′ BamHI site and a 3′ HindIII site and inserted into pSP72 vector.

Identification of Centaurin-α by Mass Spectrometry—After the final chromatography step during the purification of CKIα as a 14-3-3 kinase from brain (Sephadryl S-100 gel exclusion), fractions containing this kinase activity were pooled and loaded on 12.5% SDS-PAGE (43). Gels were stained for 5–10 min and then destained for the minimum time. The stained proteins were cut out of the gel and destained, and the peptide mass data were used in the PeptideSearch program.

Recombinant Protein Purification—Escherichia coli carrying GST-centaurin-α, GST-centaurin-α, or histidine-tagged CKIα plasmid were grown overnight at 37 °C in Liquid Broth medium containing 50 μg/ml ampicillin and were diluted at the following day (1/10) in the same medium. Culture was then continued until the absorbance (600 nm) of the bacterial growth reached 0.6. Expression of the tagged proteins was induced with 0.5 μM isopropyl β-D-thiogalactopyranoside for 3–5 h at 25 °C. The fusion proteins were purified by affinity chromatography on glutathione-Sepharose beads or nickel columns (Amersham Pharmacia Biotech). Proteins were further purified on a MonoS column using the

AKTA purifier (Amersham Pharmacia Biotech) and stored in 20 ml Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, and 50% glycerol at −20 °C. Recombinant 14-3-3γ was purified as described previously (43).

In Vitro binding between Purified CKIα and Members of the Centaurin-α Family—GST, GST-centaurin-α, and GST-centaurin-α (0.2 μM final concentration) were incubated with histidine-tagged CKIα in binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1% Iodoacetamide, 0.1% Nonidet P-40) for 2 h at 4 °C. Glutathione-Sepharose beads were added and incubated for a further 1 h. Bead precipitates were then washed 4 times with binding buffer and once with kinase buffer (50 mM Hepes, pH 7.0, 10 mM MgCl2, 100 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, and 100 μM cold ATP). The washed beads were incubated with kinase buffer (without NaCl and 0.1% Nonidet P-40) containing 40 μM of a CKI-specific phosphopeptide substrate (New England Biolabs) and [γ-32P]ATP (2 μCi/point) (Amersham Pharmacia Biotech) in a final volume of 30 μl. Reactions were performed at room temperature for 30 min. After centrifugation, 20 μl was spotted on PV1 paper squares (Whatman) and washed four times with 1% phosphoric acid. Radioactivity retained on the papers was quantified by liquid scintillation counting.

In Vitro Kinase Assays—One μg of purified GST, GST-centaurin-α, GST-centaurin-α, and GST-14-3-3ζ were subjected to an in vitro kinase assay using purified histidine-tagged CKIα as described previously (43). Proteins were analyzed on 10% SDS-PAGE. Gels were stained with Coomassie Blue, dried, and autoradiographed. To study the effect of centaurin-α on CKIα activity, different amounts of centaurin-α (0.1, 1, or 10 μg) were preincubated with histidine-tagged CKIα for 15 min at room temperature. The reaction was initiated by the addition of ATP (50 μM final, 1 μCi/point) and 14-3-3ζ or GST-14-3-3ζ or phosvitin (0.3 μg/point) as CKI substrates. After 30 min at room temperature, the reactions were stopped and analyzed on 10% SDS-PAGE. Gels were stained with Coomassie Blue, dried, and autoradiographed.

In Vitro Translation and Transfection—CKIα and GST-14-3-3ζ were expressed in vitro using a T7 TNT-coupled transcription/translation reticulocyte lysate assay (Promega Corp., Madison, WI). The reactions (50 μl) were performed following the manufacturer’s instructions using [35S]methionine (Amersham Pharmacia Biotech) for 90 min at 30 °C. The reactions were then diluted 4-fold with binding buffer (containing 0.1 or 1% Nonidet P-40 as indicated in figure legends) and split in two for incubation (as indicated in the figure legends) with 1 or 0 μg of GST, GST-centaurin-α, or GST-centaurin-α at 30 °C for 15 min. Glutathione-Sepharose beads and binding buffer (300 μl) were added to the reactions and incubated at room temperature for an additional 1 h. The beads were washed 5 times with 1 ml of binding buffer and electrophoresed on SDS-PAGE. After staining/destaining, the gels were incubated with AmplifyTM (Amersham Pharmacia Biotech), dried, and exposed to film.

Cell Culture and Transfection—COS-7 cells were obtained from the European Collection of Cell Cultures. They were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf

![FIG. 1. Co-purification of a 45-kDa protein with CKIα. CKIα was purified from pig brain after four chromatography steps (43). The fractions containing CKI activity were electrophoresed on 10% SDS-PAGE (lane 2), and the gel was stained with Coomassie Blue. CKIα was previously identified by mass spectrometry (43). A total of seven other proteins co-purified with CKIα (indicated by arrows), suggesting that they may form protein complexes. The major co-purifying band migrates at 45 kDa.](http://www.jbc.org/content/18578/2/4957/F1.large.jpg)
serum (Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.) at 37 °C in a humidified chamber with 5% CO₂. Cells were transfected using Fugene (Roche Molecular Biochemicals) for 24–36 h in 60-mm diameter Petri dishes with HA-tagged CKIα (D136N) and/or FLAG-tagged centaurin-α/centaurin-α1 and/or empty vectors (4 μg of total DNA).

Co-immunoprecipitation—Cells were lysed with 1 ml of lysis buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA) containing a mixture of protease inhibitors (Roche Molecular Biochemicals), 1 mM sodium fluoride, 10 μM sodium orthovanadate, and 10 mM sodium pyrophosphate. Lysates were pre-cleared with Pansorbin cells (Roche Molecular Biochemicals) and centrifuged for 20 min at 15,000 g at 4 °C. Mouse anti-FLAG M2 antibodies (Sigma) were added to the lysates for 2 h. Protein-A/G coupled to Sepharose (Amersham Pharmacia Biotech) was then added for an additional 1 h of incubation. The beads were washed 4 times with 1 ml of lysis buffer, and the proteins associated with the beads were resolved on 10% SDS-PAGE. Proteins were transferred onto nitrocellulose (Bio-Rad), and the presence of HA-CKIα was detected by Western blotting with a rat anti-HA (Roche Molecular Biochemicals) antibody and ECL detection (Amersham Pharmacia Biotech).

Affinity Chromatography with the CKI Peptide—Two rat brains were homogenized in 20 ml of lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 2 mM DTT) containing a mixture of protease inhibitors (Roche Molecular Biochemicals). Nonidet P-40 was then added to a final concentration of 0.1%, and the mixture was incubated at 4 °C for 2 h with constant agitation and subsequently clarified by centrifugation at 15,000 g for 30 min, followed by ultracentrifugation at 100,000 g for 1 h. The resulting high speed supernatant was loaded onto a 1-ml Sulfo-Link (Pierce) column to which 1 mg of a peptide corresponding to residues 214–233 of CKIα was coupled (according to the manufacturer’s instructions). A cysteine was added at the amino terminus to allow efficient coupling to Sulfo-Link. Brain extract was also loaded onto a control column. The columns were washed with 50 ml of lysis buffer containing 0.1% Nonidet P-40 and with 50 ml of phosphate-buffered saline. Bound proteins were eluted with 10 ml of 50 mM Tris, pH 7.5, 10% glycerol, 1 μg EDTA, and 2 mM DTT. Eluted fractions and the last 5-ml washes were concentrated on Centricon-10 (Amicon) and analyzed by immunoblotting. The presence of 14-3-3 proteins was detected using a rabbit polyclonal 14-3-3 Pan antibody (KK 1106) from our laboratory. Centaurin-α1 was detected using a rabbit polyclonal anti-
RESULTS

A Protein of 45-kDa Co-purifies with CKIα in Brain—We have previously reported that 14-3-3ζ was phosphorylated on a novel site (44) and identified the kinase as CKIα after four conventional chromatography steps (43). A total of seven proteins with molecular masses ranging from 25 to 80 kDa were shown to co-purify with CKIα (Fig. 1), and we postulated that they may form protein complexes in brain. A protein migrating at 45 kDa represented the most abundant co-purifying protein as judged by Coomassie Blue staining (Fig. 1). The putative association between CKIα and the 45-kDa protein would appear to be of high affinity because the complex was observed after elution with 0.5 M NaCl during the two cationic exchange chromatography steps and after elution with 0.6–0.8 M NaCl from the Affi-Gel blue column (the chromatography steps are described in Ref. 43). Experiments were performed to identify the 45-kDa protein and to elucidate whether it associates with CKIα.

Identification of the 45-kDa Protein as Centaurin-α1—The 45-kDa protein band was subjected to in-gel trypsin digestion, and the mass of each peptide was measured by electrospray mass spectrometry. Analysis of the peptide mass map using the “PeptideSearch” program identified the 45-kDa protein as a PtdIns(3,4,5)P3-binding protein. We identified nine peptides that matched with centaurin-α1 and eight peptides that matched with centaurin-α (Fig. 2A). Centaurin-α1 shares high homology to centaurin-α. Centaurin-α contains an extended carboxyl-terminal tail and only one PH domain compared with centaurin-α (Fig. 2B). Therefore, peptide mass analysis differentiated these two proteins and identified the 45-kDa protein unequivocally as centaurin-α1 (42). Centaurin-α1 has been given different names in the literature including p42IP4 (34) and PIP3BP (35). However, for clarity in the rest of this paper, we refer to it as centaurin-α1.

CKIα Associates Physically with Centaurin-α1—We tested the potential interaction between CKIα and centaurin-α1 using purified recombinant proteins. We show that CKIα associates with GST-centaurin-α1 and not with GST (Fig. 3A). In addition, CKIα also binds directly to centaurin-α (Fig. 3A).
Interaction between CKI and Centaurin-α₁

In order to confirm this interaction, [35S]methionine-labeled CKIα was synthesized using a cell-free coupled transcription/translation system and incubated with GST, GST-centaurin-α₁, or GST-centaurin-α₂ (Fig. 3B). The results show that CKIα associates with centaurin-α₁ and with centaurin-α₂.

Therefore, two distinct experiments showed that CKIα interacts physically with both centaurins. The levels of CKIα that associated with centaurin-α₁ remained the same in the presence of 0.1–1% Nonidet P-40 (data not shown).

In order to verify the specificity of this interaction, [35S]methionine-labeled CKIα and 14-3-3z were incubated with either GST or GST-centaurin-α₁ (Fig. 3C). By using higher stringency (1% detergent), centaurin-α₁ indeed associates with CKIα but not with 14-3-3z even though the latter is expressed at much higher levels than CKIα (Fig. 3C, panel Input), thus showing the specificity of the interaction between centaurin-α₁ and CKIα. In conclusion, centaurin-α and centaurin-α₁ associate specifically and directly with CKIα. As rat centaurin-α (except its unique carboxyl-terminal tail) shares 94% identity with rat centaurin-α₁ (Fig. 2, A and B), one could imagine that they bind to CKIα via a common domain, thus eliminating the importance of the first PH domain of centaurin-α₁ and the carboxyl-terminal domain of centaurin-α.

**CKIα Associates in Cells with Centaurin-α and Centaurin-α₁**—We tested whether CKIα associates in cells with centaurin-α₁. For that purpose, we transiently co-transfected different cell lines in order to check the expression of ectopically expressed CKIα. However, the expression of CKIα was not detectable in cell lysates of PC12 and NIH3T3 cells, was low in HEK 293 cells, and only really detectable in COS-7 cells (data not shown). Therefore, we have used COS-7 cells for the transient transfection experiments as the other cell lines were not suitable for co-immunoprecipitation experiments due to the low expression level of CKIα. COS-7 cells were co-transfected with HA-tagged CKIα and with FLAG-tagged centaurin-α₁ or centaurin-α₂. FLAG-tagged proteins were immunoprecipitated, and CKIα in the immunoprecipitates was revealed by Western blot using HA antibodies. This experiment shows that CKIα is pulled down in centaurin-α₁ (Fig. 4, 3rd lane) and centaurin-α₁

**Fig. 4.** CKIα associates in vitro with centaurin-α and centaurin-α₁. COS-7 cells were co-transfected with plasmids expressing the proteins indicated at the top of the figure or empty vectors. After 36 h, cells were harvested in lysis buffer containing 1% Nonidet P-40. Centaurins were immunoprecipitated using mouse monoclonal antibodies (mAb) M2 anti-FLAG antibodies (Ab), and the presence of CKIα was detected by Western blotting with a rat anti-HA monoclonal antibody (clone 3F10).

**Fig. 5.** Centaurins are not phosphorylated by CKIα and have no effect on CKIα kinase activity. A, 1 μg of purified GST, GST-centaurin-α₁ (GST-α₁), and GST-centaurin-α₂ (GST-α₂) were assessed for in vitro phosphorylation by purified histidine-tagged CKIα as described under “Materials and Methods.” Reactions were stopped and electrophoresed on 10% SDS-PAGE. The gel was dried and autoradiographed. 14-3-3z was phosphorylated by CKIα as a positive control (lane 14-3-3z). B, 0.1, 1, or 10 μg of recombinant centaurin-α₁ or buffer (b) was preincubated for 15 min with CKIα. The reaction was initiated with the addition (“+”) of the CKI substrate 14-3-3z. No substrate was added in (“−”) as a control in case some degraded forms of centaurin-α₁ would be phosphorylated by CKIα. C, 0, 0.1, 1, or 10 μg of recombinant centaurin-α₁ or the corresponding volume of buffer (b) was incubated with CKIα as described above. The reaction was initiated with the addition of GST-14-3-3z or phosvitin.

Centaurins Are Not Phosphorylated by CKIα—We tested whether CKIα phosphorylates centaurin-α₁ and centaurin-α₂ in vitro using recombinant proteins. Fig. 5A shows that GST-centaurin-α₁ and GST-centaurin-α₂ were not substrates for CKIα in vitro. As a positive control, 14-3-3z, a CKI substrate (43), was shown to be phosphorylated (Fig. 5A, lane 14-3-3z). In addition, recombinant centaurin-α₁ is not phosphorylated by CKIα indicating that the GST tag itself does not confer conformational restraint and has an effect on the result (data not shown). However, CKI isoforms have been shown to phosphorylate some substrates only if they have been previously phosphorylated by another kinase two or three residues carboxy-terminal to the CKI site. Therefore, our in vitro results do not completely eliminate the possibility that centaurins are not substrates for CKIα. To investigate this possibility, FLAG-tagged centaurin-α₁ and centaurin-α₂ immunoprecipitated from COS-7 cells were subjected to an in vitro kinase assay using purified histidine-tagged CKIα. However, immunoprecipitated proteins were not phosphorylated by purified CKIα (data not shown). Therefore, we conclude from our experiments that centaurin-α₁ and centaurin-α₂, either purified or immunoprecipitated from COS-7 cells are not substrates for CKIα.
**Interaction between CKI and Centaurin-α₁**

**Centaurin-α₃ Does Not Affect CKIα Activity**—As centaurin-α₁ does not represent a substrate for CKIα, we have tested whether centaurin-α₁ affects the kinase activity of CKIα. We have shown that different amounts of recombinant centaurin-α₁ (0.1, 1, or 10 μg) have no effect on CKI activity using 14-3-3ζ (Fig. 5B), GST-14-3-3ζ, or phosphovitin (Fig. 5C) as CKI substrates.

**Centaurin-α₁ Interacts with Residues 217–233 of CKIα**—Mammalian CKIα belongs to a family of seven isoforms that show a high degree of homology in their kinase domains and have variable amino- and carboxyl-terminal tails. Therefore, it would be interesting to identify the centaurin-α₁-binding site in CKIα in order to elucidate whether centaurin-α₁ associates in a region specific to CKIα or one that is also present in other CKI isoforms. To address this question, we constructed a set of CKIα deletion mutants (Fig. 6A). These mutants were synthesized and labeled with [35S]methionine in a cell-free coupled transcription/translation system (Fig. 6B) and incubated with GST or GST-centaurin-α₁ (Fig. 6C). The different mutants were expressed in similar amounts apart from mutants 217–325 and 233–325 that were less well synthesized (Fig. 6B). The results show that the amino- and carboxyl-terminal domains (mutants 17–325 and 17–287) of CKIα are not required for the association. In addition, centaurin-α₁ binds with high efficiency to all mutants apart from mutant 233–325. Although the levels of expression of mutants 217–325 and 233–325 in cell lysates were the same, mutant 217–325 bound to centaurin-α₁ whereas mutant 233–325 did not (Fig. 6, C and D), demonstrating that the binding occurs between residues 217 and 233 within the kinase domain of CKIα. These residues belong to a loop between helices E and F of CKIα that has been suggested to be the target region for protein-protein interactions (46). In order to confirm that residues 217–233 represent the site of interaction with centaurin-α₁, a brain extract was loaded onto a column containing this peptide or a control column (Fig. 6D). A longer exposure of the experiment shown above is presented for the mutants 217–325 and 233–325 because of their lower expression compared with the other mutants, thus providing means of a better interpretation of the results. E. brain extracts were loaded onto a 1-ml peptide affinity column to which a peptide corresponding to residues 214–233 (C₁₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋_-14-3-3_ζ (Fig. 5B), GST-14-3-3_ζ, or phosphovitin (Fig. 5C) as CKI substrates.

In conclusion, our results mapped biochemically the centaurin-α₁-interacting site on a single loop of CKIα that had been proposed previously from the three-dimensional x-ray structure to be the site of interaction for CKI partners.

**Centaurin-α₁ Associates with all Members of the CKI Family**—Residues 217–233 of CKIα are well conserved in all CKI isoforms as indicated in Fig. 7A. Therefore, it was interesting to determine whether other CKI isoforms were able to associate with centaurin-α₁. To that purpose, all mammalian CKI isoforms were synthesized and labeled with [35S]methionine in a cell-free coupled transcription/translation system and incubated with GST or GST-centaurin-α₁ (Fig. 7B). All mammalian CKI isoforms were capable of associating with GST-centaurin-α₁ and not with GST alone (Fig. 7B). Interestingly, a...
mutant of CKIδ deleted of its carboxyl-terminal domain (CKIδΔ317) binds as well as wild type CKIδ to centaurin-α1 (Fig. 7B). This result is in agreement with data from Fig. 6C that show that the kinase domain of CKIα is sufficient for its association to centaurin-α1. These data have led us to strongly believe that centaurin-α1 interacts with residues 217–233 of CKIα that are well conserved in all CKI isoforms.

**DISCUSSION**

In this report we have identified centaurin-α1 as a novel CKIα partner based on the following evidence: (a) they co-purified from brain after elution from four chromatography steps; (b) centaurin-α1 associates in vitro with CKIα indicating that the binding is direct; (c) the binding is specific as centaurin-α1 does not interact in vitro with 14-3-3 ζ under the same conditions; (d) they form a protein complex in COS-7 cells as shown in immunoprecipitation experiments; (e) centaurin-α1 interacts with residues 217–233 of CKIα using deletion mutants of CKIα; and (f) centaurin-α1 elutes from a peptide affinity chromatography column containing residues 214–233 of CKIα.

CKI isoforms are characterized by a conserved core kinase domain and by variable amino- and carboxyl-terminal tails. We report here that centaurin-α1 interacts with the kinase domain and not with the unique tails of CKIα. Moreover, a mutant of CKIδ deleted of its carboxyl-terminal domain binds to centaurin-α1, as well as does CKIδ, suggesting that the kinase domain of CKIδ represents the centaurin-binding site. In addition, the site of interaction within the kinase domain (residues 217–233) is present in a loop between two helices which has been proposed to represent an interaction domain for CKI targets (46). The residues within that loop are well conserved among the CKI family. Indeed, we have shown that all mammalian CKI isoforms are able to associate with centaurin-α1 in vitro. This suggests that the same loop, present in all CKI isoforms, is responsible for the interaction with centaurin-α1.

Centaurin-α1 and centaurin-α have been identified as PtdIns(3,4,5)P3-binding proteins (33–35). Phosphatidylinositol 3-kinase 3-kinase is mainly responsible for the synthesis of PtdIns(3,4,5)P3 by phosphorylating PtdIns(4,5)P2 at the 3-OH position (47). Phosphatidylinositol 3-kinase is involved in regulating various biological processes including membrane ruffling, membrane trafficking, and actin cytoskeleton regulation (48–50). It is interesting to note that CKIα has recently been shown to interact with the clathrin adaptor AP-3 (22), another target that is involved in membrane ruffling and actin cytoskeleton rearrangement. Therefore, our data support a role for CKIα in these biological events, in agreement with previous reports (21, 22). Indeed, CKIα interacts with and phosphorylates the clathrin adaptor AP-3 (22), which is involved in endocytosis. It is interesting to note that a genetic interaction between yeast CKI1 and AP-3 was identified previously (8). Moreover, CKIα has been found to co-localize in neurons with synaptic vesicle markers and phosphorylates some vesicle synaptic associated proteins (21). Interestingly, centaurin-α1 has been shown to associate with presynaptic vesicular structures (53). An actin-associated protein kinase shown to be a member of the CKI family phosphorylates actin in vitro (54). The molecular mass of the kinase (37 kDa) suggests that it could be CKIα, and we have shown that recombinant CKIα indeed phosphorylates actin.2 In addition, the protein DAH (Discontinuous Actin Hexagon) that interacts with the actin cytoskeleton has been shown to be phosphorylated by CKIα in vitro (55).

Members of the ARF-GAP family contain several domains for protein-protein interactions, and they have been shown to be associated with a number of different proteins. This suggests that ARF-GAP proteins may act as scaffold proteins in addition to their function as GAP proteins. Whether other ARF-GAP proteins interact with CKI is not known. The ARF-GAP proteins Git1 and Git2 have been reported to regulate the internalization of some G-protein-coupled receptors (56–58). CKIα has been shown to interact with and phosphorylate these G-protein-coupled receptors (31, 59). In addition, most of the identified ARF-GAP proteins are involved in the Pak signaling pathway (reviewed in Ref. 36). Intriguingly, CKI1 has been shown to interact with the adaptor molecule Nck (60) in a complex with Pak1 (61), thus raising the possibility that CKI may associate with other ARF-GAP proteins. Therefore, it would be important to investigate whether ARF-GAP proteins interact with CKI isoforms.

Gcs1, the budding yeast homologue of centaurin-α1, also contains a cysteine-rich domain that is necessary for its ARF-GAP activity (40). As yet, no ARF-GAP activity has been reported for centaurin-α1, but it is able of rescuing a Gcs1 strain mutant indicating that centaurin-α1 and Gcs1 may have similar function(s) (62). Gcs1 has been shown to be necessary for

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2 T. Dubois, S. K. Maciver, and A. Aitken, unpublished data.
the resumption of cell proliferation from stationary phase (63) and is involved in endocytosis (7). Gcs1 also plays a role in actin cytoskeleton regulation in vivo and binds to actin in vitro (64). As vesicle trafficking is closely associated to actin organization in yeast, Gcs1 might link vesicle trafficking and the actin cytoskeleton (64). Yeast CKIs (Yck1 and Yck2) were shown to suppress the Gcs1 blockage effects on cell proliferation and endocytosis (7). The membrane association of Yck2 was necessary for this effect (7). Yck1/2 is involved in cytosis, in bud development (5, 6), and regulation of the actin cytoskeleton as yck<sup>m</sup> mutants fail to depolarize the actin cytoskeleton during mitosis (5). Another link between Gcs1 and CKI is the ankyrin repeat protein Akr1p. Gcs1 has been shown to interact with Akr1p in yeast two-hybrid experiments (65). Akr1p and Yck1/2 regulate yeast endocytosis, and Akr1p regulates the plasma membrane localization of Yck1/2 (11). These authors proposed that the Yck1/2 membrane localization may involve other proteins such as Gcs1 (65).

Our data suggest that CKIα does not phosphorylate centaurin-α and centaurin-α<sub>1</sub>. In addition, centaurin-α<sub>1</sub> has no effect on CKI activity. Therefore, what is the functional relevance of the interaction between CKIα and these PtdIns(3,4,5)P<sub>3</sub>-binding proteins? As CKIα does not contain a lipid binding domain, it may associate with membranes through interaction with other proteins. Centaurin-α<sub>1</sub> may represent one of these proteins, as has been proposed for its yeast counterpart (see above and Ref. 65). CKIα may also represent a downstream target for centaurin-α<sub>1</sub> as suggested by the results in budding yeast showing that CKIα suppresses Gcs1 mutant phenotypes.

In conclusion, we have shown an interaction between CKIα and centaurin-α<sub>1</sub>, a member of the ARF-GAP protein family that is involved in membrane trafficking and actin cytoskeleton regulation. Our present results are in agreement with data reported previously suggesting a role for CKI in membrane trafficking and/or regulation of the actin cytoskeleton. Our findings are further supported by evidence of a genetic link between CKIα and Gcs1 in budding yeast.

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Casein Kinase I Associates with Members of the Centaurin-α Family of Phosphatidylinositol 3,4,5-Trisphosphate-binding Proteins

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