CPI-17 and centaurin-α_1 signalling complexes

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To my parents and my brother Petr

DECLARATION

I declare that the thesis has been composed by me and the work presented is my own work, unless stated otherwise. This work has not been submitted for any other degree or professional qualification except as specified.

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ABSTRACT

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 and Wnt signalling. CKIα was identified by our laboratory as the protein kinase from brain which phosphorylates 14-3-3ζ on residue 233. Phosphorylation of this residue *in vivo* negatively regulates 14-3-3ζ binding to c-Raf and may be important in Raf-mediated signal transduction. The protein kinase C-potentiated myosin light chain phosphatase (MLCP) inhibitor, CPI-17 and the phosphatidylinositol binding protein, centaurin-α_1,were two out of six proteins which co-purified with CKIα from brain after 4 chromatography steps, suggesting they might be forming a complex. The aim of this study was to characterise the interaction between CPI-17 and CKIα further and to identify new binding proteins for CPI-17 and centaurin-α_1 by affinity chromatography and subsequent Western blotting and/or mass spectrometry analysis to increase the understanding of the physiological functions CPI-17 and centaurin-α_1.

PKC isoforms from all PKC classes, casein kinase I (CKI) isoforms (CKIα and CKIε) and cyclic AMP dependent kinase (PKA) were eluted from the CPI-17 affinity column. The site of interaction was mapped to the cysteine rich domain of PKC and to the kinase domain of CKI. The interaction site of CPI-17 with these two kinases was mapped to the first 120 residues. CPI-17 was shown to be phosphorylated by PKC isoforms from all PKC classes, but not by CKI. However, CPI-17 inhibited CKI activity. Novel potential binding proteins of CPI-17 were identified by mass spectrometry and included cytoskeletal proteins and proteins involved in vesicular trafficking. These results indicate that CPI-17 occurs in a multiprotein complex, suggesting that CPI-17 may have roles other than MLCP inhibition during smooth muscle contraction.

Novel centaurin-α_1 binding proteins were identified, amongst them PKC isoforms from all PKC classes, which all phosphorylated centaurin-α_1. The site of interaction was mapped to the cysteine rich domain of PKC. The nucleolar protein, nucleolin was identified as another centaurin-α_1 binding protein and the interaction was shown to be RNA dependent. A number of potential novel centaurin-α_1 binding proteins

was identified by mass spectrometry and included proteins involved in ribosome biogenesis and RNA processing. This suggested a role for centaurin-α_1 and possible involvement in other nuclear events. In summary, novel CPI-17 and centaurin-α_1 binding proteins were identified and some of these interactions characterised, opening up new potential roles for CPI-17 and centaurin-α_1 in cellular processes ranging from signal transduction to vesicle trafficking and ribosome biogenesis.

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ABBREVIATIONS

AKAP	A kinase anchoring protein
ADP	Adenosine-5'-diphosphate
Arf	ADP ribosylation factor
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
cpm	Counts per minute
CaM	Calmodulin
CamKII	$Ca^{2+/}$calmodulin-dependant protein kinase II
CDK	Cyclin dependent kinase
CKI	Casein kinase I
CKII	Casein kinase II
CPI-17	Protein kinase C-potentiated inhibitor of 17 kDa
DAG	Diacylglycerol
DARPP-32	Dopamine-and cAMP-regulated phophoprotein of 32 kDa
DMEM	Dulbecco's modification of Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide trisphosphate
DTT	Dithiothreitol
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
EDTA	Diaminoethane-tetra-acetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid
FBS	Foetal bovine serum
GAP	GTPase-activating protein
GDP	Guanosine 5' diphosphate

GEF	Guanine nucleotide exchange factor
G-protein	Guanine nucleotide binding regulatory protein
GST	Glutathione-S-transferase
GTP	Guanosine 5' triphosphate
HEK293	Human embryonic kidney 293 cells
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMG1	High-mobility group 1
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
LB	Luria-Bertani
LIMK-2	LIM Kinase-2
MLCP	Myosin light chain phosphatase
mRNA	messenger RNA
MW	Molecular weight
NGF	Nuclear growth factor
OD_{600}	Optical density (e.g. at 600 nm)
O/N	Over night
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK	PtdIns-(3,4,5)-P_3-dependent protein kinase
PH domain	Pleckstrin homology domain
PI 3-Kinase	Phosphatidylinositol 3-kinase
PKA	cAMP dependent protein kinase, protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP1	Protein phosphatase 1
PRK1/PKN	PKC related kinase 1/Protein kinase N
PtdIns	Phosphatidylinositol

PtdIns-(3)-P	Phosphatidylinositol-3 monophosphate
PtdIns-(3,4)-P_2	Phosphatidylinositol-3,4-bisphosphate
PtdIns-(3,4,5)-P_3	Phosphatidylinositol-3,4,5-trisphosphate
Ins-(1,3,4,5)-P_4	Inositol-1,3,4,5-tetrakisphosphate
RBD	RNA binding domain
RNA	Ribonucleic acid
ROCK	Rho-associated coiled-coil forming protein kinase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SR	Sarcoplasmic reticulum
TAE	Tris-acetate/EDTA
TBS	Tris buffered saline
TE	Tris/EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Triton X-100	Tris(hydroxymethyl)methylamine-hydrochloric acid
UV	Ultraviolet
VAMP	Vesicle associated membrane protein
W/V	Weight (mass) for volume

CHAPTER 1

Introduction

1. Introduction

1.1. Preamble

The introduction gives a general overview of casein kinase I (CKI), protein kinase C-potentiated inhibitor of 17 kDa, (CPI-17), protein kinase C (PKC), centaurin-α_1 and nucleolin. The objective of this study was to identify novel CPI-17 and centaurin-α_1 binding proteins in order to increase the understanding of the physiological roles of these proteins. Our laboratory has identified CPI-17 and centaurin-α_1 as two proteins co-purifying with CKIα from brain after four chromatography steps suggesting they form a complex. Originally, CPI-17 was identified as a MLCP inhibitor and the inhibition by CPI-17 is strongly potentiated by phosphosphorylation of Thr-38 *in vivo.* Centaurin-α_1 has been identified as a phosphatidylinositol interacting protein, whose function remains unknown.

Data presented in this thesis demonstrate that PKC interacts with CPI-17 and centaurin-α_1 and phosphorylates both proteins. CKI was identified as a novel CPI-17 binding protein. Furthermore, a number of novel potential CPI-17 and centaurin-α_1 binding proteins were identified, and a possible role for CPI-17 in cytoskeletal re-arrangement and vesicular trafficking and for centaurin-α_1 in nuclear processes such as ribosome biogenesis is proposed.

1.2. CKI

The casein kinase I (CKI) family of serine/threonine kinase is highly conserved and ubiquitously expressed in every eukaryotic organism tested so far, from yeast to human (reviewed in Gross and Anderson, 1998). Until now, seven isoforms from distinct genes have been identified in mammals, designed α, β, γ_1, γ_2, γ_3, δ and ε. Four isoforms are expressed in S*accharomyces cerevisiae* (Hrr25, Yck1, Yck2 and Yck3) and five in *Schizosaccaromyces pombe* (Cki1, Cki2, CKi3, Hhp1 and Hhp2).

The CKI family is characterised by a conserved kinase domain (more than 50% identity) and variable amino- and carboxyl-terminal tails. The diverse amino and carboxyl termini may provide each isoform with distinct functions and regulation mechanisms in cells (Gross and Anderson, 1998). The carboxyl-terminal extensions of different kinase family members have been shown to be important for mediating down-regulation of kinase activity through autophosphorylation (Cegielska et al., 1998; Graves and Roach, 1995), for directing subcellular localisation (Wang et al., 1992) and potentially for interaction with protein substrates (Sakanaka et al., 1999). It has been shown that when phosphorylated, a number of the residues within their respective carboxyl termini would generate a phosphorylated region which could act as a pseudosubstrate and inhibit kinase activity (Gross and Anderson, 1998). A schematic representation of mammalian CKI isoforms is shown in Figure 1.1 which has been adapted from Gross and Anderson (1998).

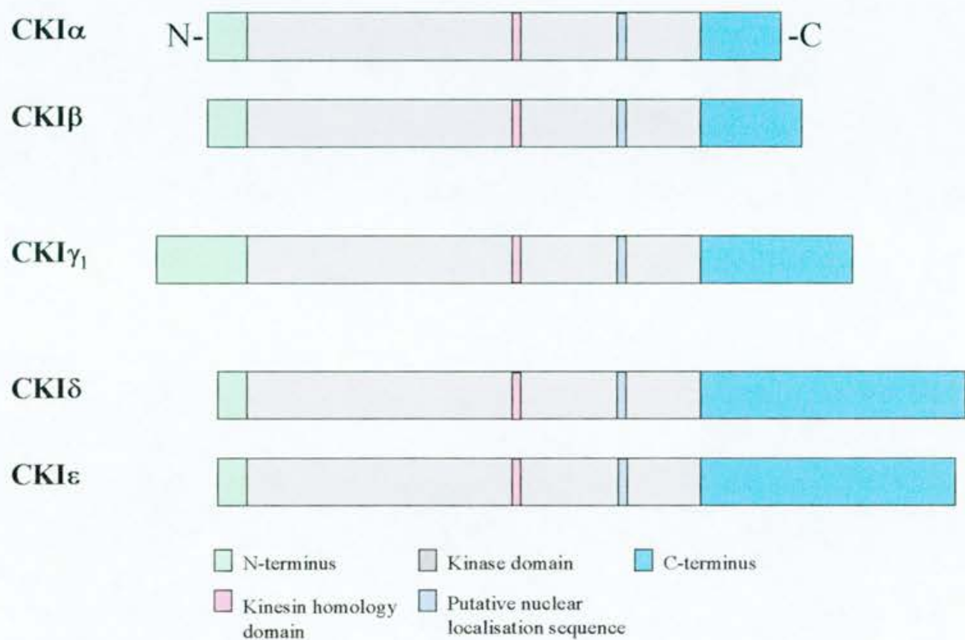


Figure 1.1 Schematic representation of the mammalian CKI isoforms.
Grey regions represent the conserved kinase domains, green and blue delineate the variable regions in the amino and carboxyl termini, respectively. The kinesin homology domain (pink) and nuclear localisation signal (purple) are also shown.

CKI isoforms possess a near-consensus SV40 T-antigen putative nuclear localisation sequence and they also contain a kinesin homology domain, that is conserved among a number of kinesin homologues (Gross and Anderson, 1998). In kinesins, which are microtubule associated motor proteins involved in a number of cellular events including vesicular trafficking and mitotic spindle assembly, this domain is thought to be involved in microtubule interactions and it has been proposed that certain CKI isoforms could be involved in similar cellular events. The molecular weight and tissue distribution of mammalian CKI isoforms are summarised in the table below (Gross and Anderson, 1998).

Table 1.1 Molecular weights and tissue distribution of CKI isoforms

CKI isoform	Molecular weight (kDa)	Tissue distribution
α	38	All tissues
β	39	Brain Other tissues?
γ_1	43	Testis
γ_2	45	Testis
γ_3	50	All tissues, except heart and spleen
δ	49	Pre-dominantly testis
ε	47	Number of cell lines Tissue distribution unknown

1.2.1. CKI substrates

CKI proteins phosphorylate substrates with a negatively charged region (i.e. acidic amino acids like aspartate) amino-terminal to the site of phosphosphorylation (reviewed in Vielhaber and Virshup, 2001). Some CKI isoforms have been shown to phosphorylate tyrosine as well as serine/threonine (Braun et al., 1984; Hoekstra et al., 1994; Pulgar et al., 1996), but it is not yet known which CKI isoforms are dual-

specificity kinases and what the *in vivo* implications could be (Gross and Anderson, 1998). The consensus phosphorylation sequence for CKI has been defined as S/T/Y(P)X_{1-2}S/TY or D_n-X_{1-2}S/T/Y where S/T/Y(P) stands for any phosphorylated serine, threonine or tyrosine residue, X is any amino acid and D is aspartic acid (Vielhaber and Virshup, 2001). CKI isoforms have been shown to phosphorylate a large number of different proteins from all cellular compartments *in vitro*. CKI substrates include insulin growth factor (Rapuano and Rosen, 1991; Tuazon et al., 1985), tumour necrosis factor α (TNFα) (Beyaert et al., 1995), β-adrenergic receptor (Tobin et al., 1997), cyclin-dependent kinase 5 (cdk5; Sharma et al., 1999), cytoskeletal proteins such as spectrin (Manno et al., 1995; Simkowski and Tao, 1980; Tuazon et al., 1985), a regulatory subunit (phosphatase inhibitor-2) of protein phosphatase 1 (Agostinis et al., 1992), translation factors (Haas and Hagedorn, 1991; Tuazon et al., 1989) and glycogen synthase (Flotow and Roach, 1989). Furthermore, CKIα has been shown to phosphorylate a subset of vesicle proteins including the synaptic vesicle-specific protein SV2 (Gross et al., 1995). Finally, CKI phosphorylates a number of nuclear substrates such as polymerase I and II (Dahmus, 1981), transcription factor NF-AT4 (Zhu et al., 1998), SV40 large T antigen (Cegielska and Virshup, 1993; Grasser et al., 1988) and p53 (Knippschild et al., 1997). However, only little evidence exists to link phosphorylation by CKI with modulation of substrate function (Vielhaber and Virshup, 2001).

1.2.2. Function of CKI

Yeast CKI isoforms are involved in DNA repair (Dhillon and Hoekstra, 1994; Ho et al., 1997; Hoekstra et al., 1991) and have been linked with a role in cytokinesis (Robinson et al., 1993 and 1999) and in vesicle trafficking, especially in endocytosis (Feng and Davis, 2000; Friant et al., 2000; Hicke et al., 1998; Marchal et al., 2002; Panek et al., 1997; Wang et al., 1996). The functions of the mammalian isoforms are less well understood, but based on high homology with their yeast counterparts they may have similar functions (Gross and Anderson, 1998). Amongst others CKI has been

shown to down regulate the activity of glycogen synthase (Flotow and Roach, 1989) and the tyrosine activity of platelet derived growth factor (PDGF) receptor (Bioukar et al., 1999). Furthermore, CKI phosphorylates serine 174 of phosphatase inhibitor-2, leading to inhibition of the phosphatase (Agostinis et al., 1987 and 1992). CKI has also been shown to modify DARPP-32 (dopamine-and cAMP-regulated phophoprotein of 32 kDa), an inhibitor of calcineurin, a Ca^{2+}/calmodulin dependent catalytic subunit of PP1 (PP1-C; Desdouits et al., 1995a and b). Phosphorylation of DARPP-32 by CKI results in activation of the inhibitor and down-regulation of calcineurin activity (Desdouits et al., 1995b).

CKIε and CKIδ play a role in regulating the tumour suppressor protein p53 (Behrend et al., 2000a; Dumaz et al., 1999; Knippschild et al., 1997). CKIε has been implicated in circadian rhythms in *Drosophila melanogaster* and in development by transducing the Wnt (Wingless (Wg) in *Drosophila*)/β-catenin pathway (Liu et al., 2002; Peters et al., 1999; Sakanaka et al., 1999). The Wnt/β-catenin pathway regulates cell growth, developmental and oncogenic processes (reviewed in Polakis, 2000). Recently, Mckay and co-workers have reported that the CKI isoforms α, β, γ and δ could also activate the Wnt pathway in *Xenopus laevis* embryos (McKay et al., 2001).

CKIγ has been proposed to play a role in cytokinesis and/or membrane trafficking, as it has been shown that CKIγ_1 and CKIγ_3 complement a yeast strain devoid of the yeast CKI homologues, YCK1 and YCK2 (Zhai et al., 1995), which are essential for vegetative growth, normal bud morphogenesis, cytokinesis and vesicular trafficking (Gross and Anderson, 1998).

CKIα has been implicated in cell cycle progression in mouse oocytes (Gross et al., 1997) and in membrane trafficking by associating with synaptic vesicles and phosphorylating a subset of vesicle proteins including the synaptic vesicle-specific protein SV-2 and adaptor protein AP-3 which has been implicated in the formation of synanptic vesicles (Faundez and Kelly, 2000; Gross et al., 1995). SV-2 is a glycoprotein which is required for normal neurotransmission and has been shown to modulate the size of the readily releasable pool of secretory vesicles (Xu and Bajjalieh, 2001).

Furthermore, CKI seems to be involved in the regulation of one or more steps of mRNA metabolism by co-localising with factors involved in pre-mRNA splicing and associating with a complex containing splicing factors (Gross et al., 1999).

In addition, CKIα phosphorylates 14-3-3τ and ζ isoforms on residue 233. 14-3-3 proteins are a family of conserved regulatory dimeric molecules expressed in all eukaryotic cells. 14-3-3 proteins play a role in diverse cellular processes including neuronal development, cell cycle control, cell growth control and programmed cell death (reviewed in Aitken et al., 2002). *In vivo* phosphorylation of 14-3-3ζ at the CKIα site (Thr-233) negatively regulates its binding to c-Raf, and may be important for Raf-mediated signal transduction (Dubois et al., 1997).

Furthermore, we have demonstrated that CKIα associates with the phosphatidylinositol 3,4,5-trisphosphate-binding protein (Dubois et al., 2001), centaurin-α_1 suggesting an involvement of CKIα in membrane trafficking, actin cytoskeletal re-arrangement and possibly in phosphatidylinositol signalling. In addition, our laboratory has shown that CKIα phosphorylates syntaxin-1A (Dubois et al., 2002a), a protein involved in exocytosis (reviewed in Lin and Sheller, 2000). This could indicate a role for CKIα in synaptic vesicle exocytosis.

Finally, our laboratory has identified novel CKIα co-purifying proteins from brain which could represent novel CKIα binding partners. These proteins include regulator of chromosome condensation 1 (RCC1), high mobility group protein 1 and 2 (HMG1 and HMG2), Erf (Ets2 repressor factor), centaurin-α_1, synaptotagmin IX and CPI-17 (Figure 1.2, from Dubois et al., 2002b). The seven proteins co-purified with CKIα from brain after four chromatography steps, including a FF SP-Sepharose column, Affi-Gel-Blue column, a Mono S column and a Sephacryl S-100 column. This suggested that these proteins formed a tight protein complex with CKIα. However, it should be stressed that this was not enough evidence to prove that all seven proteins directly associated with CKIα, which remained to be confirmed by further experiments.

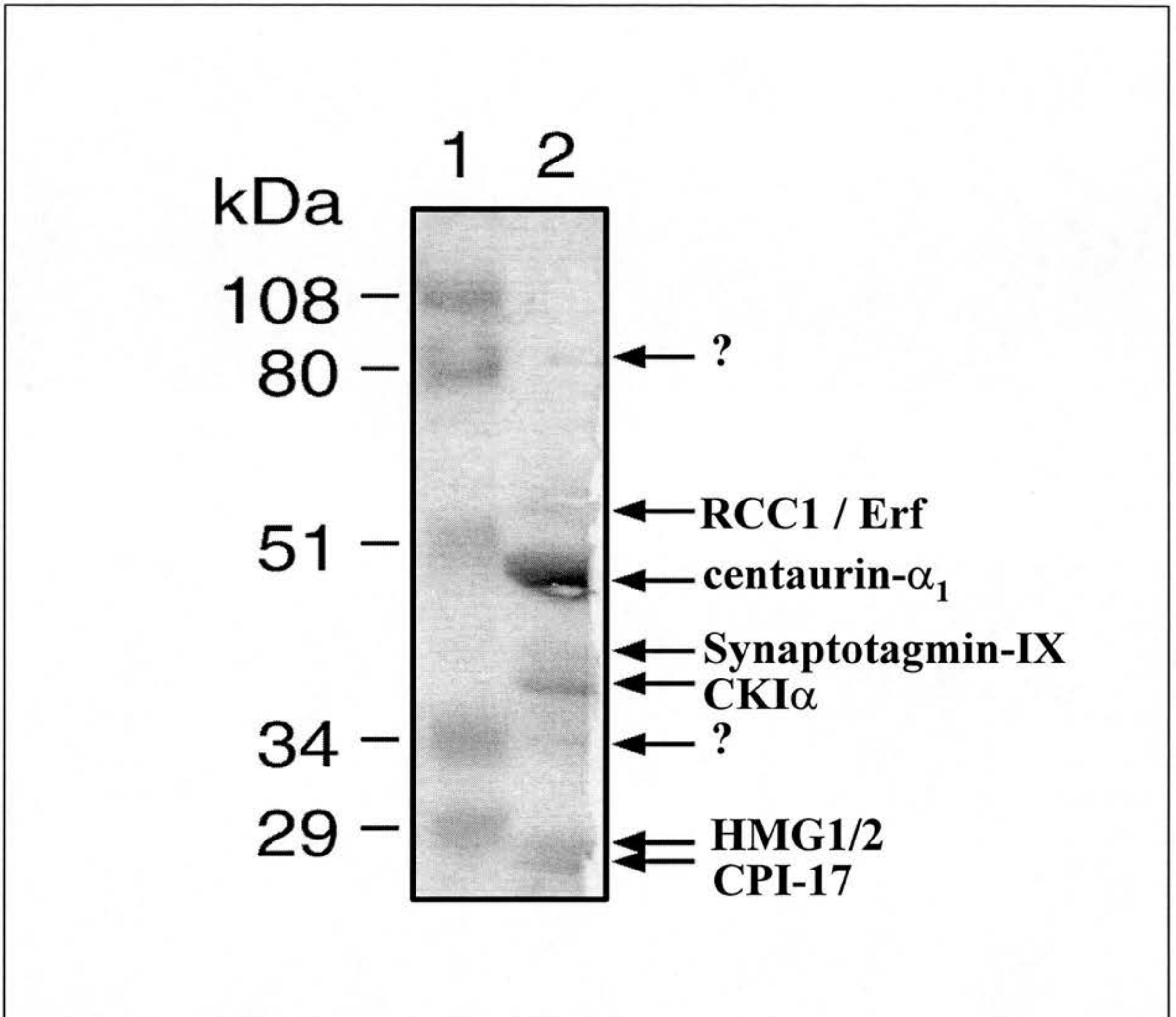


Figure 1.2 Co-purification of proteins with CKIα.
CKIα was purified from pig brain after four chromatography steps. The fractions containing CKI activity were electrophoresed on 10% SDS-PAGE (lane 2) and the gel was stained with Coomassie blue. A total of seven other proteins co-purified with CKIα (indicated by arrows), suggesting that they may form protein complex(es). The molecular weight markers (lane 1) are shown in kDa.

In addition, we showed that RCC1 associated with CKIα in COS-7 cells (Dubois et al., 2002b). Some of the functions of the CKIα co-purifying proteins are summarised in Table 1.2. Interestingly, some of the proteins co-purifying with CKIα have previously been implicated in CKI functions. For instance, HMG1, an architectural DNA binding protein (Muller et al., 2001; Thomas and Travers, 2001), has recently been shown to be phosphorylated by CKI (Okano et al., 2001). Erf is a member of the Ets family (Mavrothalassitis and Ghysdael, 2000) and Ets1 has been used as *in vitro* CKI substrate (Cegielska et al., 1998). Synaptotagmin IX has been shown to associate with a mRNA binding protein (Mizutani et al., 2000), and the association of synaptotagmin IX with CKIα is particularly interesting in the light of evidence which suggests that CKIα may regulate certain steps of mRNA metabolism (Gross et al., 1999).

Table 1.2 Identification and function of novel CKIα co-purifying proteins from brain

CKIα co-purifying proteins	Function	Reference
HMG1/HMG2	DNA binding proteins	(Muller et al., 2001; Thomas and Travers, 2001)
RCC1	Mitotic spindle formation, nucleo-cytoplasmic shuttle	(Melchior, 2001; Dasso, 2001; Kahana and Cleveland, 2001)
Synaptotagmin IX	Associates with mRNA binding proteins; Ca^{2+} sensor in exocytosis	(Mizutani et al., 2000) (Fukuda et al., 2002)
Erf	Transcription factor of the Ets family (repressor)	(Mavrothalassitis and Ghysdael, 2000)
CPI-17	Protein phosphatase 1 inhibitor	(Eto et al., 1995)
Centaurin-α_1	Membrane trafficking?	See chapter 4 and (Dubois et al., 2001; Donaldson et al., 1998)

In addition a number of proteins were found to associate with the region of CKIα comprising residues 217-233. These included: Importin-α_1/β, a protein with similar roles to RCC1, involved in both nuclear transport and spindle assembly (reviewed in Dasso,

2001); protein phosphatase 2A (PP2A), tubulin, HMG1 and actin (Dubois et al., 2002b). Interestingly, phosphorylation/dephosphorylation events by the CKI/PP2A complex have been shown to be important in endocytosis and actin cytoskeleton organisation in yeast (Friant et al., 2000). These data further support a role of CKIα in multiple cellular events possibly including membrane trafficking, mRNA metabolism, mitotic spindle assembly and actin cytoskeleton re-arrangement.

As the last point it should be added that CKI isoforms have been associated with the pathology of Alzheimer's disease (reviewed in Vielhaber and Virshup, 2001). This progressive, neurodegenerative disease is characterised by accumulation of a hyperphosphorylated form of the microtubule–associated protein *tau* into filamentous and granulovacuolar lesions. CKI is one of the kinases which phosphorylates *tau*, inducing it to adopt an Alzheimer's like conformation. The levels of a number of CKI isoforms (α, δ, ε) have been found to be elevated several fold in this disease with the levels of CKIδ increased >30 fold. These results suggest that CKI does not only participate in hyperphosphorylation of *tau*, but its overexpression could play a key role in Alzheimer's disease progression (Vielhaber and Virshup, 2001).

1.2.3. Regulation of CKI

The presence of CKI in a number of cellular compartments including cytosolic vesicles and distinct nuclear structures suggests that these kinases are highly regulated. So far little is known about the regulation of CKI activity. Although CKI isoforms appeared to be constitutively active and insensitive to second messengers, a number of regulatory mechanisms have been put forward in recent years (Gross and Anderson, 1998). To date, two CKI isoforms, CKIδ and CKIε have been shown to be inhibited by autophosphorylation, however, recent data indicate that *in vivo*, cellular phosphatases continually dephosphorylate these CKI isoforms, keeping them constitutively active (Cegielska et al., 1998; Graves and Roach, 1995; Rivers et al., 1998). Since the CKI autophosphorylation decreases kinase activity, this dynamic autophosphorylation/dephosphorylation cycle provides a mechanism for kinase

regulation *in vivo* (Rivers et al., 1998). The association of CKIα with native membranes and its catalytic activity are regulated by phosphatidylinositol 4,5-bisphosphate (PtdIns–(4,5)-P_2; Brockman and Anderson, 1991). In this study the authors show that small increases in the membrane content of PtdIns 4,5 P_2 result in CKIα inhibition on native membranes.

Furthermore, the CKIα homologue in *Drosophila melanogaster,* dmCKIα, has been shown to be activated by γ-irradiation (Santos et al., 1996). Moreover, CKI isoforms have been reported to phosphorylate some of their substrates only if they were previously phosphorylated by another kinase (Gross and Anderson, 1998). In this way, the effect of CKI is dependent on other kinases and phosphatases which in many cases are regulated by second messengers or other components of signal-transduction pathways. Furthermore, certain CKI isoforms are also regulated at the level of alternative mRNA splicing. CKIα and CKIγ_3 are alternatively spliced (Gross and Anderson, 1998), and differently spliced CKIα isoforms have been shown to exhibit different substrate specificities and differences in their protein-protein interactions (Zhang et al., 1996). One of the splicing variants, CKIαL contains an insert of 28 amino acids within the kinase domain (Rowles et al., 1991) which has been shown to alter the substrate specificity, possibly by changing the tertiary structure of the catalytic cleft (Zhang et al., 1996). Finally, there is evidence that the subcellular localisation of CKI defines both function and regulation. CKIα for example has been shown to possess a cell-cycle-dependent subcellular distribution, including association with cytosolic vesicles (in interphase), the centrosome (in prophase and telophase), the mitotic spindle (in mitosis), and distinct nuclear structures (in anaphase) (Brockman et al., 1992; Gross et al., 1997). The association of CKIα with distinct structures throughout the cell might therefore restrict CKI function by defining substrate access.

1.3. CPI-17

The protein kinase C-potentiated inhibitor of 17 kDa, CPI-17, was previously identified by our laboratory as one out of six proteins co-purifying with CKIα as part of a protein complex from mammalian brain (Dubois et al., 2002b) as described in section 1.2.2. In this section known properties and functions of CPI-17 will be reviewed.

1.3.1. CPI-17 as a myosin light chain phosphatase inhibitor

CPI-17 was shown to be a protein phosphatase 1 (PP1) inhibitor and specifically to inhibit myosin light chain phosphatase (MLCP), however it should be noted that CPI-17 is also an inhibitor of the free catalytic subunit of protein phosphatase 1 (PP1-C) *in vitro* (Eto et al., 1995 and 1997).

MLCP is a trimeric protein complex consisting of a 110-130 kDa regulatory or myosin targeting subunit (M 110-130/MYPT), a ~37 KDa catalytic (PP1-C) subunit and a 20 kDa subunit of unknown function (reviewed in Somlyo and Somlyo, 2000). MLCP plays a pivotal role in smooth muscle force regulation. Contractile activity in smooth muscle is linked to an increase of intracellular [Ca^{2+}]. Ca^{2+} combines with calmodulin and the Ca^{2+}-calmodulin (CaM) complex activates MLCK which phosphorylates the 20 kDa myosin light chain (MLC20) on Ser-19 resulting in contraction (reviewed in Pfitzer, 2001). The level of phosphorylated MLC20 is governed by a balance of the activity of two counteracting enzymes: a Ca^{2+}-calmodulin-dependent myosin light chain kinase and the above described MLCP (reviewed in Brozovich, 2002). However, a membrane potential (and therefore intracellular [Ca^{2+}]) independent agonist induced mechanisms can also lead to contraction. This mechanism produces Ca^{2+} sensitisation, or an increase in contraction force at a constant [Ca^{2+}] and correlates with an inhibition of MLCP (reviewed in Brozovich, 2002; Richards et al., 2002). The inhibition of MLCP is thought to occur via the small GTPase RhoA and one of its downstream targets, Rho-associated kinase (ROK/ROCK/Rho-kinase). RhoA can bind both GDP and GTP, and is active in the GTP bound status and inactive in the GDP bound form. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP and GTPase-activating

proteins (GAPs) act as negative regulators by accelerating the intrinsic GTPase activity of RhoA and converting it back to the inactive GDP-RhoA (reviewed in Fukata et al., 2001; Nieuw Amerongen and van Hinsbergh, 2001). Binding of Rho-associated kinase to RhoA-GTP enhances the activity of Rho-associated kinase (Fukata et al., 2001). Rho-associated kinase has been shown to phosphorylate MYPT at Thr-695 (Feng et al., 1999). Phosphorylation at this site decreases MLCP activity and leads to an increase in both MLC20 phosphorylation and contractile force (Brozovich, 2002). A HeLa zipper-interacting protein (ZIP)-like kinase and an integrin-linked kinase (ILK) have also been shown to phosphorylate MYPT on residue 695, decreasing MLCP activity (Borman et al., 2002; MacDonald et al., 2001a; Muranyi et al., 2002). Another mechanism for Ca^{2+} sensitisation is dissociation of the subunit of MLCP by arachidonic acid (Brozovich, 2002). Activation of guanine nucleotide-binding proteins (G proteins), increases in intracellular [Ca^{2+}] and activation of kinases such as mitogen activated protein kinase (MAPK) leads to an increase in the activity of phospholipase A_2 and production of arachidonic acid from membrane glycerophospholipids (reviewed in Hirabayashi and Shimizu, 2000), which binds to and dissociates MLCP to reduce phosphatase activity (Brozovich, 2002). A third mechanism for Ca^{2+} sensitisation involves CPI-17. As described in more detail below, CPI-17 phosphorylated by PKC (Eto et al., 1995), Rho-associated kinase (Koyama et al., 2000), protein kinase N (PKN) (Hamaguchi et al., 2000) or by ILK (Deng et al., 2002), has been shown to inhibit MLCP activity. A simplified diagram summarising the possible involvement of CPI-17 in a pathway which could lead to cellular events mediated by MLC20 phosphorylation/dephosphorylation is shown in Figure 1.3.

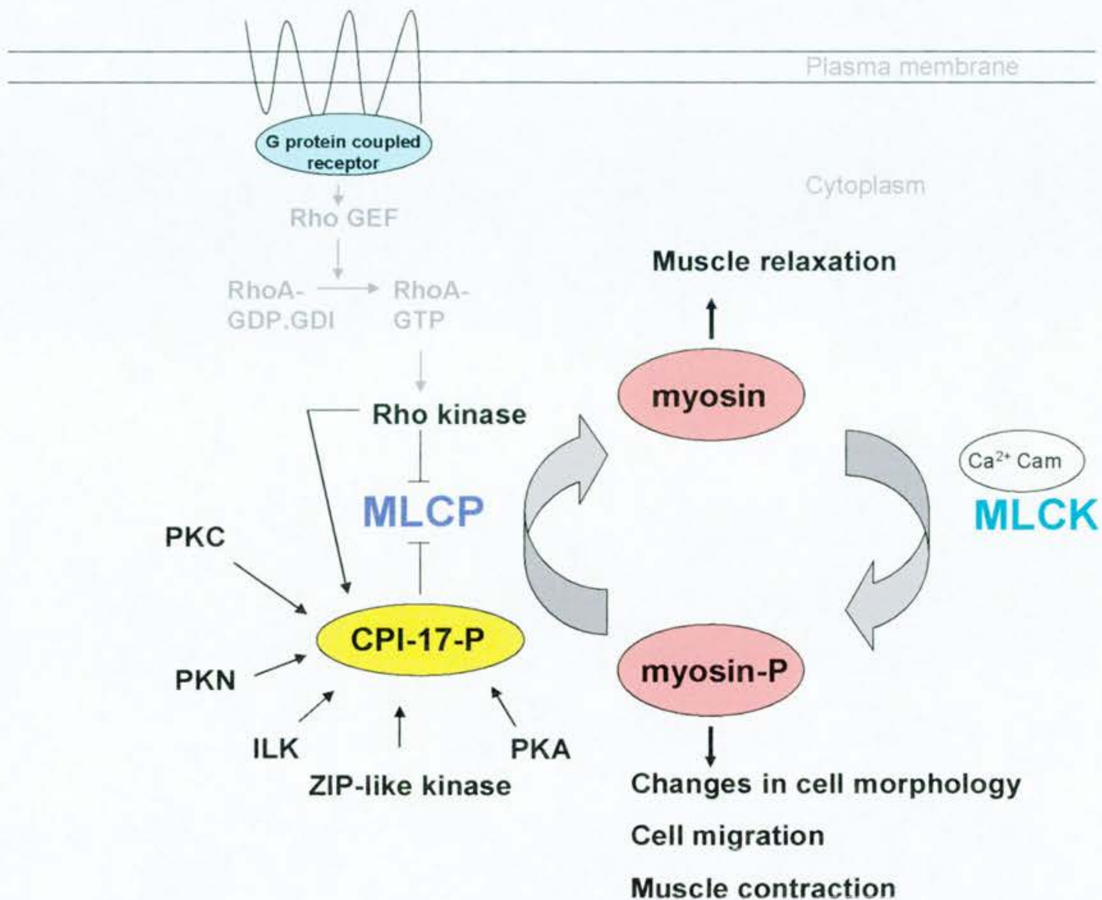


Figure 1.3 Involvement of CPI-17 in MLCP regulation.
Agonist binding to heterotrimeric G-protein leads to RhoA activation via linked guanine nucleotide exchange factors (GEF). RhoA activates Rho-associated kinase which then phosphorylates the regulatory subunit of MLCP and inhibits MLCP activity. Phosphorylation of CPI-17 on Thr-38 by PKC, PKN, PKA, Rho-kinase, ZIP-like kinase or ILK also leads to inhibition of MLCP. MLCK activated by a Ca^{2+}/calmodulin complex phosphorylates myosin resulting in changes in cell morphology, cell migration and leading to muscle contraction. Dephosphorylation of myosin by MLCP results in muscle relaxation (based on diagram in Somlyo and Somlyo, 2000).

MLCP is a serine/threonine phosphatase involved in many cellular functions such as muscle contraction, cell migration, metastasis and cytokinesis (reviewed in Matsumara et al., 2001; Somlyo and Somlyo, 2000) . It should be noted that MLCP is also expressed in non-muscle cells including human prostate cancer cells (Somlyo and Somlyo, 2000) and therefore the identification and characterisation of proteins which regulate its activity is of major importance.

Eto and co-workers have shown that the inhibition of myosin light chain phosphatase by CPI-17 alters microfilament organisation and retards cell spreading in fibroblasts (Eto et al., 2000). Originally, CPI-17 was thought to be smooth muscle specific, but it is also expressed in human platelets and has been implicated in playing a role in human platelet secretion by inhibiting the myosin light chain phosphatase (Watanabe et al., 2001). Our laboratory has found it in brain (Dubois et al., 2002b) and those results are supported by Eto and co-workers, who have found weak mRNA expression of CPI-17 in porcine brain (Eto et al., 1997).

1.3.2. Phosphorylation of CPI-17

CPI-17 is phosphorylated on Thr-38 *in vivo*, which increases its ability to inhibit PP1-C by 1000-fold (Eto et al., 1995). CPI-17 was originally shown to be phosphorylated by PKC *in vitro* (Eto et al., 1995). However, many other kinases were found to phosphorylate CPI-17 on Thr-38, including the PKC-related kinase 1, PRK1 or PKN (Hamaguchi et al., 2000), Rho-associated kinase (Koyama et al., 2000), HeLa zipper-interacting protein (ZIP)-like kinase (MacDonald et al., 2001b) and the very recently identified integrin-linked kinase, ILK (Deng et al., 2002). A further phosphorylation site has been identified by MacDonald and co-workers on Ser-12. This site has been shown to be phosphorylated by a ZIP-like kinase (MacDonald et al., 2001b). Furthermore, our laboratory has shown that protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) phosphorylate CPI-17 *in vitro* on residues Thr-38 and Ser-130, respectively. In addition, a novel CPI-17 *in vivo* phosphorylation site on Ser-128 was identified by our laboratory, however, the kinase

which phosphorylates this residue remains unknown. A schematic representation of known CPI-17 phosphorylation sites and the respective kinases is shown in Figure 1.4.

It is interesting to note that residues 31-120 of CPI-17 constitute a novel domain that is also present in the phosphatase holoenzyme inhibitor, PHI-1 (Eto et al., 1999) and in the serine/threonine kinase, LIM kinase-2 (LIMK-2) as our database searches revealed. LIMK-2 is involved in stress-fibre and focal adhesion formation (Amano et al., 2001). Interestingly, the region around Thr-38 on CPI-17 is conserved in LIMK-2 (Thr-596), as our database searches revealed and in PHI-1 (Thr-57) (Eto et al., 1999). The equivalent residue in PHI-1, Thr-57 has been shown to be phosphorylated by PKC and this results in an increase in the ability of PHI-1 to inhibit PP1 (Eto et al., 1999). A simplified schematic representation of the conserved domains present in CPI-17, PHI-1 and LIMK-2 is shown in Figure 1.5.

Agonist stimulation of vascular smooth muscle leads to CPI-17 phosphorylation on Thr-38 resulting in increased vascular smooth muscle contractility (Kitazawa et al., 2000). By contrast, CPI-17 dephosphorylation in arterial smooth muscle occurs during nitric oxide induced relaxation (Etter et al., 2001). Recombinant CPI-17 phosphorylated on Thr-38 induced MLC phosphorylation and potentiated PKC-induced contraction of arterial smooth muscle strips (Kitazawa et al., 1999; Li et al., 1998). In addition, Eto and co-workers showed that histamine-induced vasoconstriction involves phosphorylation of CPI-17 and PKCα and δ, indicating that PKC may be the kinase which phosphorylates CPI-17 on Thr-38 *in vivo,* since ROCK and PKN do not seem play an important role in this process, which was shown by experiments based on sensitivity to different kinase inhibitors (Eto et al., 2001). This is supported by the finding that phosphorylation of CPI-17 on Thr-38 by PKC leads to MLCP inhibition in human platelets (Watanabe et al., 2001).

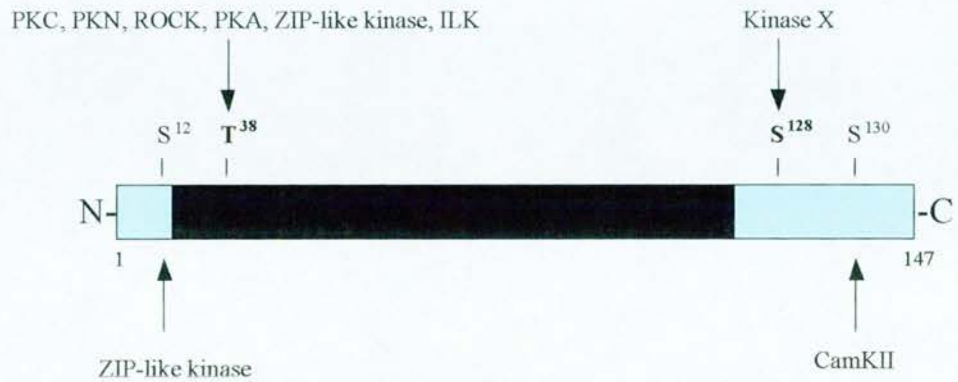


Figure 1.4 Schematic representation of known CPI-17 phosphorylation sites. Schematic representation of CPI-17 with the *in vivo* phosphorylation sites indicated in bold (T38 and S128). The *in vitro* phosphorylation sites are also shown (S12 and S130). The kinases known to phosphorylate the *in vivo* sites are indicated at the top of the figure and those that phosphorylate the *in vitro* sites, at the bottom of the diagram. The black shading represents the domain which is also present in PHI-1 and LIMK-2.

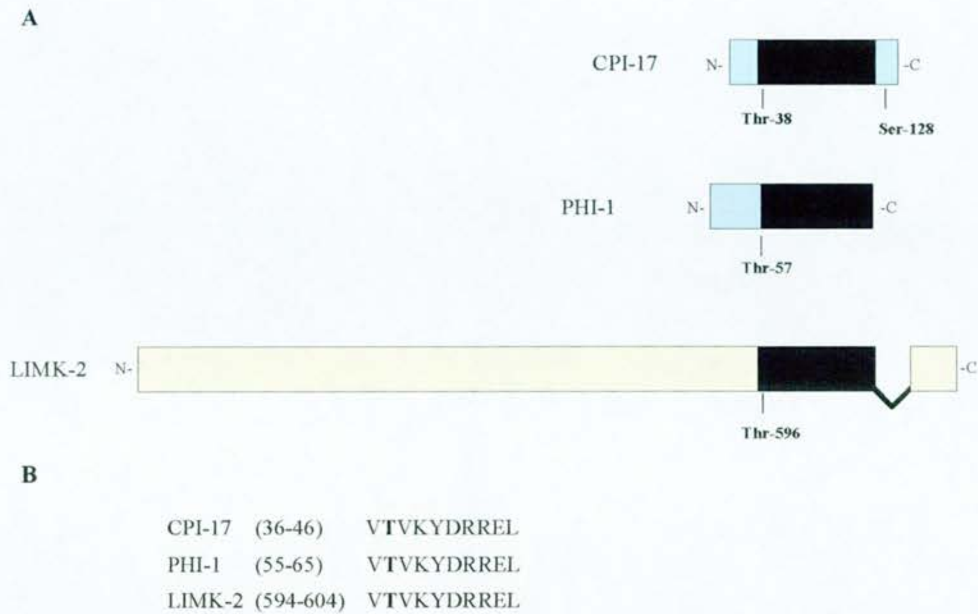


Figure 1.5 Sequence conservation between CPI-17, PHI-1 and LIMK-2.
A, Schematic representation of the conserved domains present in CPI-17, PHI-1 and LIMK-2. CPI-17 is composed mainly of a domain (black box, called "ProD domain 25745" from ProDom 2000.1 programme at http://protein.toulose.inra.fr) which is also found in the LIMK-2 and the PP1 inhibitor PHI-1. The positions of the phosphorylated residues are indicated. Thr-38 which is known to be phosphorylated *in vivo* on CPI-17 is conserved in PHI-1 (Thr-57) and LIMK-2 (Thr-596). The corresponding residue in PHI-1 (Thr-57) is phosphorylated by PKC (Eto et al., 1999). The C-terminal tail of CPI-17 contains the serine that is phosphorylated *in vivo* (Ser-128).
B, The region containing Thr-38 in CPI-17 is conserved in PHI-1 and LIMK-2. The residues 36-46 of CPI-17 containing the *in vivo* phosphorylation site Thr-38 (in bold) is conserved in PHI-1 (residues 55-65) and LIMK-2 (residues 594-604).

1.4. PKC

PKCs comprise a family of serine/threonine kinases which play an important role in a large number of signal transduction events elicited by various extracellular stimuli such as growth factors, hormones and neurotransmitters (reviewed in Mochly-Rosen and Gordon, 1998). Up to 12 distinct family members have been identified in mammalian cells and have been classified into different groups according to their structural features and activation parameters. PKC plays fundamental roles in mitogenesis and proliferation of cells, apoptosis, platelet activation, neurotransmission and remodelling of the actin cytoskeleton (reviewed in Jaken and Parker, 2000; Toker 1998).

1.4.1. Structure of PKC

The structure of PKC isozymes includes conserved domains referred to as C1-C4 (reviewed in Liu and Heckman, 1998) (see Figure 1.6). These domains are interrupted by variable regions V1-V5 whose function is not yet fully understood. However these regions seem to mediate protein-protein interactions and regulate spatiotemporal localisation of some PKC isoforms (Sanchez et al., 1998; Tabellini et al., 2002; Vallentin et al., 2000). PKC can be divided into two functionally distinct domains: the amino-terminal regulatory domain (C1-V3) regions, of which the C1 domain binds 1,2-sn-diacylglycerol (DAG) or phorbol esters and is immediately preceded by an autoinhibitory pseudosubstrate sequence (not in PKCμ/PKD); the C2 domain which binds calcium and acidic phospholipids (and in the case of PKCμ a pleckstrin homology (PH) domain) and the carboxyl terminal catalytic domain (C3-V5), where C3 and C4 domains form the ATP-binding and substrate binding lobes of the kinase core (reviewed in Newton, 1995). The regulatory and catalytic domains are separated by a hinge region that can be proteolytically cleaved when the enzyme is membrane-bound (for example in response to apoptotic stimuli; Emoto, et al., 1995) resulting in the release of the kinase domain (sometimes referred to as protein kinase M) from inhibition by the pseudosubstrate domain. Once released this way PKC is constitutively active. However,

the free catalytic subunit is unstable and degrades quickly in cells (Murray, et al., 1987).

1.4.2. The PKC superfamily

The mammalian isoforms of PKC have been divided into four distinct subfamilies if PKCμ/PKD is considered as a subfamily on its own. Several non-mammalian PKCs have also been identified (Toker, 1998). A schematic representation of the primary structures of mammalian PKC subfamilies is outlined in Figure 1.6.

The Ca^{2+}-dependent or conventional PKC (cPKC) isozymes (α, γ, and the alternatively spliced βI and βII) contain four conserved domains, namely, the C1 domain (which contains a tandem cysteine-rich motif, C1A and C1B), the C2 domain and the catalytic C3 and C4 domains interspaced with variable domains (reviewed in Newton, 1995 and 2001). Conventional PKC isozymes are activated by Ca^{2+}, DAG and phosphatidylserine (PS), a membrane phospholipid (Liu and Heckman, 1998).

The Ca^{2+}-independent novel PKC (nPKC) isozymes (δ, ε, η, and θ) contain a C2-like N-terminal domain and are also activated by DAG and require PS as a cofactor, but do not require calcium (Newton 2001; Toker, 1998).

The third PKC class, atypical PKC (aPKC) (ζ, ι/λ) (ι and λ are the human and mouse homologues of the same isoform), lack the C2 domain and only contain one C1 domain. Atypical PKCs are insensitive to calcium or DAG, but require PS as cofactor (Liu and Heckman, 1998; Toker, 1998).

In addition, PKCμ/PKD1 is considered by some to constitute a fourth PKC class and by others to comprise a distinct family called protein kinase D (PKD). Other PKD members include PKD2 and PKCν/PKD3 (reviewed in Van Lint et al., 2002; Newton, 2001). This protein kinase contains in addition to the catalytic and C1 domains, a pleckstrin homology (PH) domain and a putative signal peptide (SP) and a transmembrane (TM) domain (Toker, 1998; Van Lint et al, 2002). This kinase does not require calcium, but is activated by DAG and PS (Newton, 2001; Van Lint et al., 2002). The kinase domain exhibits some similarity with members of the PKC family, but is more related to the kinase domain of the myosin light chain kinase of *Dictyostelium* and Ca^{2+}/calmodulin-dependent kinase II (Van Lint et al., 2002). A summary of the PKC

family is outlined in Table 1.3 which is adapted from Liu (1996) and Newton (2001).

Table 1.3 The PKC isoforms

Subfamily	PKC isoform	Molecular weight (kDa)	Activators
Conventional PKC (cPKC)	PKCα PKCβI PKCβII PKCγ	76.8 76.8 76.9 77.5	Ca^{2+}, DAG and phosphatidylserine
Novel PKC (nPKC)	PKCδ PKCε PKCη PKCθ	77.5 83.5 78.0 81.6	DAG and phosphatidylserine
Atypical PKC (aPKC)	PKCζ PKCι/λ	67.7 67.2	phosphatidylserine
	PKD/PKCμ	115.0	DAG and phosphatidylserine

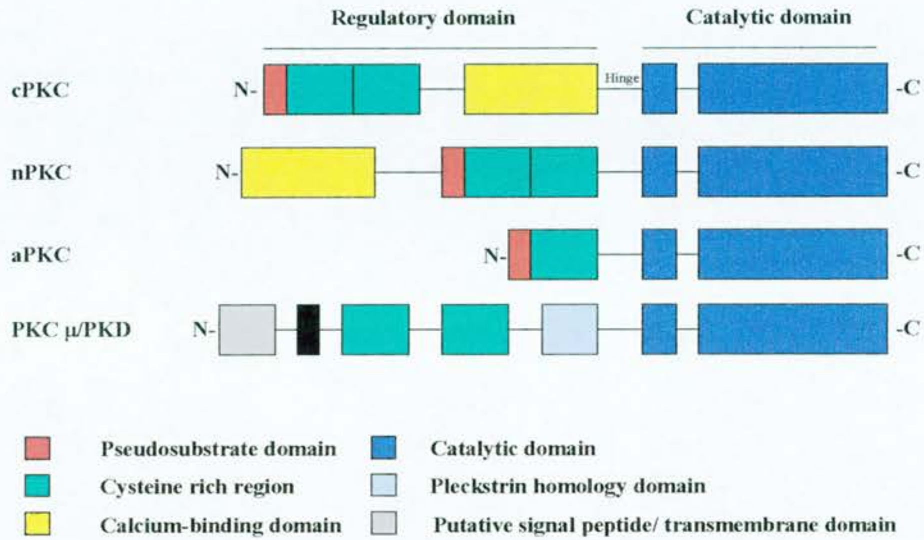


Figure 1.6 Schematic representation of primary structures of mammalian PKC family members.
The catalytic domains, pseudosubstrate domains (PS), the cysteine rich domains, the pleckstrin homology domains (PH), the calcium-binding domain and the putative signal peptide(SP)/transmembrane domain (TM) in PKCμ/PKD are shown.

1.4.3. Tissue distribution and subcellular localisation

Multiple PKC isozymes are found in any one tissue as shown in Table 1.4. PKCα, βI, βII, δ, ε, λ and ζ seem to be ubiquitous or expressed in most tissues. PKCγ is mainly restricted to the central nervous system and spinal cord (reviewed in Liu, 1996; Rennecke et al., 1996) . However, the expression levels of each isozyme differ significantly (Rennecke et al., 1996).

Table 1.4 PKC isozymes in mammalian tissue

PKC isozymes	Tissue Expression
α/βI/βII/δ/ε/λ/μ/ζ	Ubiquitous/most tissues
γ	Neural
η	Neural, epithelium
θ	Ovary, skeletal muscle, platelets

PKC isozymes localise to distinct subcellular compartments including plasma membrane, cytoskeletal elements and nuclei and are expressed differently among various cell types (reviewed in Jaken and Parker, 2000; Liu and Heckman, 1998; Mochly-Rosen and Gordon, 1998). PKC isoforms have different tissue distribution, are localised to specific cellular structures (as will be described in section 1.4.4) and have different substrate and cofactor specificity. This suggests that different PKC isoforms control distinct cellular processes.

1.4.4. Regulation of PKC

PKC activity must be under tight structural and spatial regulation as these enzymes are implicated in a multitude of physiological processes.

In response to extracellular stimuli, PKC isozymes undergo translocation from one subcellular compartment or location to another. Agonist-stimulated activation of phospholipase type C (PLC) results in the hydrolysis of membrane inositol phospholipids. This leads to the generation of 1,2-sn-diacylglycerol (DAG) and soluble inositol phosphates, including inositol-1,4,5-trisphosphate (Ins-(1,4,5)-P_3), which is

responsible for stimulating release of calcium from intracellular stores such as the sarcoplasmic reticulum (SR) (Toker, 1998). The dogma has been that binding of DAG and PS results in the recruitment of most PKC isoforms from the cytosol where they are maintained in an inactive conformation, to the plasma membrane, where they become allosterically activated (Newton, 2001). However, there is increasing evidence that suggests that individual PKC isoforms can translocate to subcellular locations other than the plasma membrane, including membrane vesicles, nuclear structures and cytoskeletal components (reviewed in Keenan and Kelleher, 1998).

In addition to allosteric activation by binding to second messengers, phosphorylation has emerged as an important mechanism of regulation of all PKCs and plays a crucial role in the activation of PKC (Newton, 2001). PKC has been shown to be phosphorylated *in vivo* at three key residues in the catalytic (C4) domain (Keranen et al., 1995; Tsutakawa et al., 1995). Initially, PKC is phosphorylated by the phosphoinositide-dependent kinase PDK-1 followed by subsequent autophosphorylation (Cenni et al., 2002; Chou et al., 1998; Dutil et al., 1998; Le Good et al., 1998). Interestingly the PKCμ isoform has been shown to be activated via a PKCε or PKCη dependent pathway which could provide another regulatory mechanism (Rey et al., 2001; Zugaza et al., 1997). In addition to phosphorylation as a regulatory mechanism of PKC, there is increasing evidence which suggests a role for protein phosphatases in controlling the phosphorylation state of PKC (Newton, 2001).

The correct subcellular localisation of specific PKC isoforms is essential for their biological function(s) and constitutes the third major regulatory mechanism of PKC activity (Newton, 2001). It has been shown that many anchoring/scaffold proteins bind PKC, target it to various cellular microdomains and position PKC isoenzymes near their substrate or near regulators of activity such as phosphatases and kinases. One example is the family of proteins called RACKs (Receptors for Activated C Kinase) which anchor the active conformation of phosphorylated protein kinase C at specific cellular locations (reviewed in Mochly-Rosen and Gordon, 1998). Another example are members of the family of AKAPs (A Kinase Anchoring Proteins) which position phosphorylated, but

inactive PKC near relevant substrates (Newton, 2001). Furthermore, it has been suggested that anchoring proteins for inactive PKCs exist. It has been shown that there are likely δPKC-specific binding proteins that anchor inactive δPKC at the Golgi structures of NG108-15 cells (reviewed in Mochly-Rosen and Gordon, 1998). These proteins were collectively termed RICKs (Receptors for Inactive C Kinase), however, to date, the hypothetical RICKs have not been identified (reviewed in Dorn and Mochly-Rosen, 2002). These examples demonstrate how specific PKC isoforms can be localised to various subcellular domains and how their activity can be spatially regulated.

1.5. Centaurin-α_1

Centaurin-α_1 was one out of six proteins co-purifying with CKIα from brain after four chromatography steps (Dubois et al., 2002b) as described in section 1.2.2.

Originally, centaurin-α_1 was identified as a phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns-(3,4,5)-P_3) and inositol-(1,3,4,5)-tetrakisphosphate (Ins-(1,3,4,5)-P_4) binding protein (Hammonds-Odie et al., 1996; Rao et al., 1999; Stricker et al., 1997; Tanaka et al., 1999). Ins-(1,3,4,5)-P_4 is the soluble head group of PtdIns-(3,4,5)-P_3. Inositol phosphates and phosphoinositides, generated by the receptor-regulated phosphoinositide 3-kinases (PI 3-Ks), play a pivotal role as second messengers in a number of cellular processes including vesicle transport, membrane ruffling, proliferation, regulation of cytosolic Ca^{2+} and cell survival (reviewed in Rameh and Cantley, 1999).

1.5.1. Structure of centaurin-α_1

Centaurin-α_1 is a member of the centaurin family, which is comprised of several members and includes amongst others centaurin $\beta_{1\text{-}4}$, $\delta_{1\text{-}2}$, $\gamma_{1\text{-}2}$, ASAP1 (Arf GAP with SH3 ankyrin repeat and PH domains) and PAP (protein tyrosine kinase (Pyk2) C terminus-associated protein; reviewed in Jackson et al., 2000). Centaurins contain a pleckstrin homology (PH) domain, ankyrin repeats and a N-terminal zinc finger motif. The PH domains have been shown to be responsible for the binding to PtdIns-(3,4,5)-P_3 and were indispensable for the PI 3-K dependent translocation of centaurin-α_1 to the plasma membrane (Tanaka et al., 1999; Venkateswarlu and Cullen, 1999; Venkateswarlu et al., 1999). The zinc finger motif comprises part of an ADP-ribosylation factor (ARF) GTPase-activating proteins (GAP) domain originally identified in ARF GAP1, a member of the ARF GAP protein family (reviewed in Donaldson, 2000; Jackson et al., 2000). Figure 1.7 shows a schematic representation of the primary structure of centaurin-α_1.

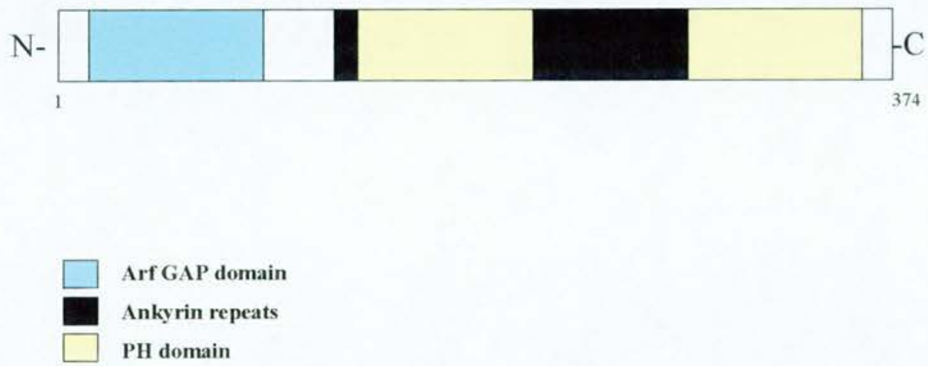


Figure 1.7 Schematic representation of the primary structure of centaurin-α_1.
The Arf GAP domain, the ankyrin repeats and the PH domain are shown.

1.5.2. Centaurin-α_1 as an ARF GAP?

ADP-ribosylation factors (Arfs) function in cells to regulate vesicular membrane transport and cytoskeleton (Jackson et al., 2000). Arfs are members of the Ras GTPase superfamily and require guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) for nucleotide cycling (Jackson et al., 2000). The Arf GAP domain has been found in several novel proteins playing a role in cytoskeleton re-arrangement and membrane trafficking including the phosphatidylinositol 4,5-biphosphate–dependent Arfl GTPase-activating protein, called ASAP1 (Randazzo et al., 2000; Brown et al., 1998) and the protein tyrosine kinase (Pyk2) C terminus-associated protein, PAP (Andreev et al., 1999; Jackson et al., 2000). Centaurin-α_1 has been shown to complement a yeast strain devoid in the Arf GAP, Gcs1, which suggested that centaurin-α_1 could have an Arf GAP activity, however, such an activity has not been reported to date (Venkateswarlu et al., 1999), indicating that centaurin-α_1 either requires another co-factor or Arf partner than tested or that it is not an Arf GAP (Jackson et al., 2000). Gcs1 has been shown to be necessary for the resumption of cell proliferation from stationary phase (Ireland et al., 1994) and to be involved in endocytosis (Wang et al., 1996). Gcs1 is also involved in the regulation of the actin cytoskeleton *in vivo* and binds to actin *in vitro* (Blader et al., 1999). As vesicle trafficking is closely associated with actin organisation in yeast, it has been proposed that Gcs1 may provide a link between vesicle trafficking and the actin cytoskeleton (Blader et al., 1999). Whether centaurin-α_1 has similar roles in mammalian cells remains to be established.

1.5.3. Tissue expression and subcellular localisation of centaurin-α_1

Centaurin-α_1 is ubiquitously expressed, with especially high levels of expression in brain (Hammonds-Odie et al., 1996) with main localisation in the neurons of hippocampus, cortex, cerebellum and in the hypothalamus (Kreutz et al., 1997).

Centaurin-α_1 has been shown to associate with membranes and vesicular presynaptic structures (Dubois et al., 2002c; Kreutz et al., 1997). Recently, centaurin-α_1 was found to be localised in the nucleus of different cell types, including the neuronal

PC12 cells (Venkateswarlu et al., 1999) and our laboratory has shown that centaurin-α_1 localises in the nucleus of human embryonic kidney HEK293 cells (Dubois et al., 2002c).

1.5.4. Association of centaurin-α_1 with CKI

As mentioned previously we found that centaurin-α_1 co-purified with CKIα from brain as part of a protein complex (Dubois et al., 2001). Furthermore, our laboratory has shown that the binding between CKI isoforms and centaurin-α_1 is direct and CKI was the first centaurin-α_1 binding protein to be identified (Dubois et al., 2001). The binding site was mapped to residues 217-233 within the kinase domain of CKI. However, centaurin-α_1 was not phosphorylated by CKIα and did not have an effect on CKIα activity.

Interestingly, a CKIα-like kinase has been shown to interact with another PtdIns-(3,4,5)-P_3 binding protein, the adaptor protein AP-3 (Faundez and Kelly, 2000). A further link between CKI and the phosphoinositol pathway has also been demonstrated in *S. pombe*. Vancurova and co-workers show that yeast CKI homologue Cki1, phosphorylates and inhibits phosphatidylinositol 4-phosphatase 5-kinase (Vancurova et al., 1999). The association of centaurin-α_1 and CKI isoforms has been further confirmed by affinity chromatography experiments described in chapter 3, section 3.4.2 of this study. In this section it was shown that CKIα and CKIε associated with the GST-centaurin-α_1 column (Figure 3.15).

1.6. Nucleolin

Nucleolin was identified as one of the proteins associated with the GST-centaurin-α_1 affinity chromatography column as described in section 4.2.1 of this study.

Nucleolin is an abundant nucleolar phosphoprotein in exponentially growing cells and may represent as much as 10% of total nucleolar proteins. Nucleolar proteins structurally related to nucleolin are found in a large number of organisms, including plants, yeasts and mammals.

Nucleolin is a nucleic acid binding protein (Herrera and Olson, 1986; Olson et al., 1983) and has been shown to be involved in chromatin structure modulation (Erard et al., 1988 and 1990), rDNA transcription (Borer et al., 1989; Bouche et al., 1987; Caizergues-Ferrer et al., 1989; Herrera and Olson, 1986), rRNA maturation (Abadia et al., 1998; Ginisty et al., 1998), ribosome assembly (reviewed in Ginisty et al., 1999), nucleogenesis (Caizergues-Ferrer et al., 1989) and nucleocytoplasmic transport (Borer et al., 1989). Recently, nucleolin has been implicated in genotoxis stress (DNA damage) responses (Yang et al., 2002a), in early events in the HIV entry process (Nisole et al., 2002a) and a role for nucleolin in mitosis and Alzheimer's disease has been proposed (Dranovsky et al., 2001). In addition nucleolin is a calcium-binding protein and a role in chromatin regulation, possibly during apoptosis has been proposed (Gilchrist et al., 2002). Finally, nucleolin has been shown to repress RNA polymerase I transcription by acting directly on the transcription machinery or on the rDNA promoter sequence and not, as previously thought, through interaction with the nascent pre-rRNA (Roger et al., 2002).

Nucleolin is target for regulation by proteolysis, methylation, ADP-ribosylation (reviewed in Srivastava and Pollard, 1999), and phosphorylation by casein kinase II (CKII; Schneider and Issinger, 1989), cell-division cycle (cdc) 2 kinase (Peter et al., 1990), cell-surface protein kinase (ecto-PK; Jordan et al., 1994) and PKCζ (Zhou et al., 1997). The N-terminal domain contains sites for modification by phosphorylation and proteolysis, whereas the C-terminal domain has sites for methylation and proteolysis. For example the idea that nucleolin function is linked to growth control by

phosphorylation is supported by the fact that active rRNA transcription is positively correlated with highly phosphorylated nucleolin. In addition, the phosphorylation of nucleolin by CK2 is highly regulated during the cell cycle. Methylation of a peptide corresponding to residues 676-692 of human nucleolin was shown to modulate its interaction with nucleic acids (Raman et al., 2001). The exact roles of nucleolin regulation by proteolysis and ADP-ribosylation have yet to be determined.

1.6.1. Structure of Nucleolin

Nucleolin consists of three functional domains, the amino-terminal domain, a RNA binding domain (RBD) and a carboxyl-terminal domain (Figure 1.8); adapted from Ginisty et al., (1999).

The N-terminal domain contains highly acidic regions separated by basic sequences (Ginisty et al., 1999). It has been suggested that the acidic domains bind histone H1 which would lead to chromatin decondensation (Erard et al., 1988). In addition this domain has been shown to be involved in a number of protein-protein interactions (Ginisty et al., 1999). It is the N-terminal domain of nucleolin that is highly phosphorylated by the kinases listed above. In addition, the N-terminal domain contains a nuclear localisation signal (NLS), which is necessary for nuclear localisation, however, it alone is not sufficient for the nucleolus localisation (Schmidt-Zachmann et al., 1993).

The central domain, the RNA binding domain (RBD), contains four RNA binding motifs and has been shown to interact with pre-ribosomal RNA (pre-rRNA) through the first two RNA binding motifs (Allain et al., 2000). Interestingly, a novel nucleolin-binding ribonucleoprotein complex has been characterised recently (Yanagida et al., 2001). Over sixty proteins were identified, out of which forty were ribosomal proteins (Yanagida et al., 2001). Yanagida and co-workers demonstrated that the RBD of nucleolin was sufficient to hold the entire nucleolin-binding ribonucleoprotein complex and that nucleolin holds the ribonucleoprotein complex mainly by RNA-protein

interaction and not by protein-protein interaction (Yanagida et al., 2001). This confirms a suggestion that the presence of several RBDs in nucleolin enables nucleolin to interact with multiple RNA targets (Ginisty et al., 1999).

The C-terminal domain or Arg-Gly-Gly (RGG) domain is rich in glycine, arginine and phenylalanine (Ginisty et al., 1999). The RGG motif seems particularly frequent in nucleolar proteins. This domain has been shown to interact non-specifically with RNA, leading to unstacking and unfolding of this RNA, thus it has been proposed that this domain facilitates the interaction of nucleolin RBD domains with targets located within large and complex RNA. Furthermore, it has been proposed that this domain might be involved in controlling nucleolar localisation (Srivastava and Pollard, 1999). Finally, the RGG domain also seems to be involved in specific protein-protein interactions including the binding of ribosomal proteins (Ginisty et al., 1999).

N- domain Central domain C- domain

N- -C

1 707

Acidic residues
NLS
RNA binding domains
RGG domain

Figure 1.8 Schematic representation of the primary structure of nucleolin.
Nucleolin contains three different domains, the N-domain, the central domain and the C-domain. The acidic residues, the nuclear localisation signal (NLS), the RNA binding domains and the Arg-Gly-Gly (RGG) domain are shown.

1.6.2. Subcellular localisation of nucleolin

Although nucleolin is predominantly localised in the nucleolus, it has been shown that nucleolin shuttles between the nucleus and cytoplasm, suggesting a role for nucleolin in nuclear transport (Borer et al., 1989; Schmidt-Zachmann et al., 1993). In addition, it was demonstrated that nucleolin is localised in a phosphorylated form on the cell surface of different cells (Pfeifle and Anderer, 1983). Hovanessian and co-workers have shown that cytoplasmic nucleolin is found in small vesicles that appear to translocate nucleolin to the cell surface, suggesting a role for nucleolin as a cell surface receptor for various ligands (Hovanessian et al., 2000). This proposal was later confirmed: cell surface-localised nucleolin has been shown to be a receptor amongst others for the bacterial membrane protein, intimin-γ (Sinclair and O'Brien, 2002). In addition, nucleolin has been implicated as a cell-surface receptor for HIV particles (Callebaut et al., 1998; Nisole et al., 1999). Interestingly, the anti-HIV pentameric pseudopeptide HB-19 and the cytokine midkine, have been shown to bind to cell-surface expressed nucleolin, thus preventing anchorage of HIV particles in the plasma membrane (Said et al., 2002; Nisole et al., 2002b). Finally, nucleolin has been shown to relocalise from the nucleolus to the nucleoplasm in response to heat shock and ionizing radiation (Daniely and Borowiec, 2000; Daniely et al., 2002) .

AIMS

This study aimed to identify novel CPI-17 and centaurin-α_1 binding proteins and to characterise these interactions to increase the understanding of the physiological roles of CPI-17 and centaurin-α_1. For this purpose, affinity chromatography followed by Western blotting and/or mass spectrometry was used. CPI-17 has previously been shown to be phosphorylated by PKC and to co-purify with CKIα from brain and therefore the interaction between CPI-17 and these two kinases was characterised. In addition, novel potential CPI-17 binding proteins were identified.

Centaurin-α_1 was also shown to be phosphorylated by PKC and therefore a possible interaction between centaurin-α_1 and PKC was investigated. Furthermore, novel centaurin-α_1 binding proteins were identified one of them being nucleolin. The interaction between centaurin-α_1 and nucleolin was further characterised. Finally, novel potential centaurin-α_1 binding proteins were identified.

The identification of novel CPI-17 and centaurin-α_1 binding partners opens up new potential functions for these proteins in a number of important cellular processes.

CHAPTER 2

Materials and methods

2. Materials and methods

Throughout the following methods the names of the companies used for the purchase of specific reagents or materials are given in parentheses. Where no company is mentioned, the reagent was purchased from Sigma-Aldrich. All reagents were of analytical grade.

CELL CULTURE TECHNIQUES

2.1. Cell culture

All cells were routinely cultured in 75 cm^2 flasks at 37°C in 95% air/5% CO_2 in a humidified incubator. The sera used to culture these cells were obtained from Gibco BRL (UK).

2.1.1. COS-1 and COS-7 cells

The monkey kidney cell lines, COS-1 and COS-7 were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum containing 2 mM L-Glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin (Gibco BRL, UK). The adherent cells were passaged when they were~90% confluent and were split 1:6.

2.1.2. Transient transfection of COS-1/COS-7 cells with plasmid DNA

Table 2.1 DNA amount and cell numbers used for transient transfections with plasmid DNA

Method	surface area	cell number	plasmid DNA amount (μg)
FuGENE	100 mm	0.25×10^6	2.5-5
Electroporation	100 mm	2×10^6	5-10

2.1.2.1. Transfection of COS-1/COS-7 cells by electroporation

Table 2.2 Buffer used for transfection of COS-1/COS-7 cells by electroporation

HeBS	6 mM	Glucose D^+
	20 mM	HEPES pH 7.05
	137 mM	NaCl
	5 mM	KCl
	0.7 mM	Na_2HPO_4

Table 2.3 Clones used for transfection

FLAG-centaurin-α_1(FLAG-cmv2)	Thierry Dubois
FLAG-CPI-17	Thierry Dubois
HA-CKIα (pcDNA3) HA-CKIα ΔKD (pcDNA3)	Frank McKeon

COS cells (COS-1 or COS-7) were used for transfection when they were ~ 90% confluent. The cells were harvested by trypsinisation, washed twice with HEPES buffered saline (HeBS) and counted using a haemocytometer grid. 2 x 10^6 cells were resuspended in 250 μl HeBS containing 5 μg plasmid DNA and 50 μg sonicated herring sperm carrier DNA. The mixture was transferred into a 0.4 μm Bio-Rad electroporation cuvette and pulsed at 250 V/125 μF using a Bio-Rad Gene Pulser. The cells were allowed to recover for 10 min at room temperature before seeding into 100 mm tissue culture dishes in 10 ml complete medium. The cells were incubated in a humidified incubator for 48 h before they were used for immunoprecipitation as described under 2.17.

2.1.2.2. Transfection of COS-7 cells using FuGENE

COS-7 cells were transiently transfected using FuGENE 6 Transfection Reagent. FuGENE 6 is a non-liposomal formulation. In general, 9x10^5 cells were seeded onto 100 mm tissue culture dishes and grown under the same conditions as described under 2.1.1 for 24-48 hours prior to transfection. Immediately before transfection the culture medium was replaced with 10 ml fresh complete growth media.

For a 100 mm plate, 15 μl of FuGENE were added carefully to 85 μl DMEM

mixed and incubated for 5 min. 2.5-5 μg of plasmid DNA were added into a separate Eppendorf tube and the FuGENE/DMEM mixture was added drop by drop. This mixture was then incubated for 15 min at room temperature to allow DNA-FuGENE complexes to form. The solution was then added into the plates which were then gently rocked to mix the transfection solution with the growth media. The cells were incubated at 37°C for 24-48 h until they were assayed for transient expression.

MOLECULAR TECHNIQUES

2.2. Agarose gel electrophoresis of DNA

Table 2.4 Buffers used for the separation of DNA by agarose gel electrophoresis

TAE running buffer	40 mM 20 mM 1 mM	Tris-HCl, pH8.0 Acetic acid EDTA
Agarose gel (1%) in TAE buffer	1% (w/v) 0.5 μg/ ml	Agarose Ethidium bromide TAE buffer

DNA was separated and visualised by electrophoresis on 1% agarose gels in the presence of ethidium bromide. The agarose gel mix was prepared as described above. Appropriate volumes of 6x blue/orange loading dye (Promega, UK) were added to the DNA samples to give a final concentration of 1x. These samples were applied to the gel and separated by electrophoresis in TAE running buffer at 100V. The presence of the intercalating dye, ethidium bromide, allowed the visualisation of the separated DNA bands under UV transillumination. The DNA fragments were sized by comparison to the molecular weight standard XIV (Roche, UK), which contained fragments of the following sizes: 2642, 1500, 1000, 500, 400, 300, 200 and 100 base pairs (bp).

2.3. Transformation of *Escherichia coli* with plasmid DNA

2.3.1. Preparation of heat-shock competent *E. coli*

Table 2.5 Media and buffers used for the preparation of heat-shock competent *E. coli*

LB liquid media	10 g/l	Select Peptone
	5 g/l	Bacto-yeast
	10 mM	NaCl
Transformation buffer	10 mM	Pipes
	55 mM	$MnCl_2$
	15 mM	$CaCl_2$
	250 mM	KCl

To make Luria-Bertani (LB) broth, all the components were mixed and the mixture was autoclaved. To make transformation buffer (TB), all the components except for $MnCl_2$ were added and the pH adjusted to 6.7 with KOH. After that the $MnCl_2$ was added and the buffer was sterilised by filtration through a 0.45 μm filter and stored at 4°C (Inoue et al., 1990). To prepare 'heat-shock' transformation-competent *E. coli*, an aliquot of frozen DH5α cells was thawed and streaked on LB agar plates and grown overnight at 37°C. Around 15 colonies were isolated with an inoculation needle, and grown in 250 ml LB in a 2l flask at 18°C with vigorous shaking until the absorbance at 600 nm (OD_{600}) reached 0.6. The flask was placed on ice for 10 min and the culture was transferred into a 1l centrifugation bottle and centrifuged at 5000g for 10 min. The supernatant was removed and the pellet was resuspended in 80 ml of ice-cold TB, incubated on ice for further 10 min, and spun down as described previously. The pellet was resuspended in 20 ml ice-cold TB and DMSO was added to a final concentration of 7%. The mixture was incubated on ice for another 10 min and the cell mixture was aliquated by 500 μl into cryogenic vials and immediately snap frozen by immersion into an ethanol/dry ice bath and stored at -80°C.

2.3.2. Heat-shock transformation of competent *E. coli.*

Table 2.6 Media and buffers used for the transformation of competent *E. coli*

LB liquid media	10 g/l 5 g/l 10 mM	Select Peptone (Gibco) Bacto-yeast (Gibco) NaCl
LB agar plates (containing ampicillin or kanamycin)	10 g/l 5 g/l 10 mM 2% (w/v) 100 μg/ml	Select Peptone Bacto-yeast NaCl Agar Ampicillin or kanamycin

Competent *E. coli* were transformed with plasmid DNA using the heat shock method described in (Sambrook et al., 1989). A sample of the plasmid DNA (3 μl) was gently mixed with an aliquot (200 μl) of the heat-shock competent cells prepared as described in the previous section. This mixture was incubated on ice for 15 min. It was then heat-shocked at 42°C for 1 min 15 sec and returned on ice for a further 5 min. LB buffer (800 μl) was then added to the cells and they were shaken at 200 rpm for 1 h at 37°C. The cells were then spread onto a LB agar plate containing 100 μg/ml ampicillin or kanamycin and incubated overnight at 37°C in order to obtain ampicillin or kanamycin resistant colonies (NB all the plasmids used in this study contained either ampicillin or kanamycin resistance gene for selection).

2.4. Preparation of plasmid DNA

Plasmid DNA was prepared from transformed *E. coli* cultures using the QIAGEN DNA purification system.

2.4.1. Small scale plasmid DNA preparation

QIAprep Miniprep kits (QIAGEN) were used for small-scale preparations of plasmid DNA. DNA purifications were carried out according to the instructions provided by the manufacturers. Briefly, an overnight *E.coli* culture in LB (3 ml) was centrifuged and the pellet was resuspended in 250 μl buffer P1. In the next step the

bacteria were lysed under alkaline conditions for 5 min by adding 250 μl buffer P2 and gentle mixing. 300 μl Buffer N3 was added to neutralise the alkaline conditions. The lysate was cleared by centrifugation and the DNA was adsorbed to the QIAprep membrane. The membrane was washed and the DNA eluted with 50 μl Elution Buffer (10 mM Tris-HCl, pH 8.5).

2.4.2. Large scale plasmid DNA preparation

QIAfilter Plasmid Maxi Kits (QIAGEN) were used for large-scale preparations of plasmid DNA. DNA purifications were carried out according to the instructions provided by the manufacturers. Briefly, an overnight *E.coli* culture in LB (100 ml) was centrifuged and the pellet was resuspended in 10 ml buffer P1. In the next step the bacteria were lysed under alkaline conditions for 5 min by adding 10 ml buffer P2 and gentle mixing. The addition of 10 ml buffer P3 neutralised the alkaline conditions. The lysate was incubated in the barrel of the QIAfilter Cartridge for 10 min. In the next step the lysate was cleared by filtration through the QIAfilter Cartridge and the DNA was bound to the QIAGEN Resin of the QIAGEN-tip. The QIAGEN-tip was washed with 2 x 30 ml buffer QC and the DNA was eluted with 15 ml buffer QF. The DNA was precipitated by the addition of 10.5 ml isopropanol and centrifuged. After that the DNA pellet was washed with 70% ethanol, air-dried for 15 min and re-dissolved in TE (pH 8.0).

2.5. DNA manipulation

2.5.1. Cloning of CPI-17 1-120 mutant

Human CPI-17 cloned into pGEX-4T1 vector (Pharmacia Biotech) was obtained from Dr Thierry Dubois. It was cloned as described (Dubois et al., 2002d).The expression vector pGEX-4T1 produces a glutathione S-transferase fusion protein expressed from a tac promoter. The vector has a thrombin protease recognition site for cleaving the protein from the fusion product.

GST-CPI-17 mutant comprising residues 1-120 was created by amplifying the

CPI-17 cDNA by polymerase chain reaction (PCR) using two oligonucleotides (5-'GCAGGATCCATGGCAGCTCAGCGGCTGGGC-3') to create a 5' *Bam*HI site and (5'-CACGAATTCTCACCTGTGGAGGCCTTGAAGCTTTC-3') to create a 3' *Eco*RI site (both are underlined in sequences). Amplified cDNA was inserted into pGEX-4T1 vector (Pharmacia Biotech).

2.5.1.1. Polymerase chain reaction

Table 2.7 Buffers and solutions used for PCR

Pfu DNA Polymerase 10x Reaction Buffer with $MgSO_4$ (PromegaCorporation)	200 mM	Tris-HCl, pH 8.8
	100 mM	KCl
	100 mM	$(NH_4)_2SO_4$
	20 mM	$MgSO_4$
	1 mg/ml	nuclease-free BSA
	1%	Triton X-100
dNTP mix (Promega Corporation)	10 mM	dATP
	10 mM	dCTP
	10 mM	dGTP
	10 mM	dTTP

Two microlitres GST-CPI-17 template (250 ng) was mixed with 64 μl nuclease free H_2O, 10 μl *Pfu* DNA Polymerase, 10x buffer, 2 μl dNTP mix, 50 pmol of each primer and 1 μl of *Pfu* DNA Polymerase. The reaction was started by initial denaturation at 95°C for 1 min. After that each cycle included denaturation at 95°C for 1 min, annealing of DNA primers at 55°C for 30 sec and DNA extension at 72°C for 1 min. A total of 30 cycles were carried out on Hybaid Thermal Cycler. This was followed by a final extension at 72°C for 5 min. The product was electrophoresed on a 1% TAE agarose gel, visualised under ultra-violet illumination and purified using the QIAquick PCR purification kit (QIAGEN). The purified DNA was subsequently subjected to restriction enzyme digestion.

2.5.1.2. Restriction enzyme digestion

Twenty microlitres PCR product were mixed with 2 μl *Bam*H1 (10 U/μl, Roche Diagnostics), 2 μl *Eco*R1(10 U/μl, Roche Diagnostics), 4 μl of 10 x SuRE/Cut Buffer B (100 mM Tris-HCl, 50 mM $MgCl_2$, 1M NaCl and 10 mM 2-Mercaptoethanol, pH 8.0 at 37°C) and 12 μl of ddH_20. Two microlitres pGEX-4T1 (2 μg) were mixed with 1 μl *Bam*H1, 1 μl *Eco*R1, 2 μl of 10 x SuRE/Cut Buffer B and 14 μl of ddH_20. Samples were incubated at 37°C for 2 h and purified by running the samples on a 1% TAE agarose gel, excising the bands and using the QIAquick gel exctraction kit (QIAGEN).

2.5.1.3. Ligation

Two microlitres pGEX-4T1 DNA (50 ng) were mixed with 6 μl CPI-17 1-120 (200 ng), 1.5 μl 10x T4 DNA buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM DTT and 10 mM ATP, pH 7.5), 1 μl T4 DNA ligase (1 U/μl, Roche Diagnostics) and 4.5 μl ddH_20. The ligation reaction was incubated O/N at 22°C.

2.5.1.4. Transformation

Fifteen microlitres of the ligation product was added to 200 μl of competent *E.coli* DH5α cells and a transformation was performed as described under 2.3.2. 200 μl of transformed *E.coli* were plated onto L-agar plates containing 100 μg/ml ampicillin.

2.5.1.5. Plasmid purification

Plasmids were purified using the QIAprep Miniprep Kit (QIAGEN) as described under 2.4.1. To screen for positive clones, plasmids were treated with *Eco*RI and *Bam*H1, analysed on 1% TAE agarose gel and sent for sequencing to Cytomix, Cambridge.

2.5.2. Site-directed mutagenesis

Site-directed mutagenesis was used to introduce point mutations into CPI-17 in order to study the effects of specific amino acids on the binding properties of CPI-17 to other protein binding partners. The point mutations were also used to study the effect of specific residues on CKI activity. Mutants of CPI-17 were made using the QuikChange^{TM} site directed mutagenesis kit (Stratagene).

Table 2.8 Primers and restriction enzymes used to create CPI-17 point mutations

Mutation	Primers 5'-3'	Primers 3'-5'	Restriction Enzymes
CPI-17 T38A	5'GAAGCGGCACGC GCGAGTCGCCGTC AAGTATGACC3'	3'CTTCGCCGTGCGC GCTCAGCGGCAGTT CATACTGG5'	*Bss*H1 site removed.
CPI-17 T38D	5'GCACGCGCGCGT CGACGTCAAGTAT GACCGGCG3'	3'CGTGCGCGCGCA GCTGCAGTTCATAC TGGCCGC5'	*Sal*I site created.
CPI-17 T128D	5'CAGGCAGCCCGG GCTCCGCCAGCCA GACCCCTCCCACGA CG3'	5'GTCCGTCGGGCC CGAGGCGGTCGGT CTGGGGAGGGTGC TGC3'	*Sma*I site created.

The pGEX-4T1-CPI-17 plasmid was used as DNA template. The oligonucleotide primers, each complementary to opposite strands of the vector were extended during temperature cycling by means of *Pfu*Turbo DNA polymerase. Incorporation of the oligonucleotide primer generated a mutated plasmid. Following temperature cycling the product was treated with *Dpn*I, an endonuclease which is specific for methylated and was used to digest the parental DNA (Nelson and McClelland, 1992). DNA isolated from almost all *E.coli* strains is methylated and therefore, susceptible to *Dpn*I digestion. The DNA containing the desired mutation was then transformed into *E.coli* (using modification of the Stratagene site-directed mutagenesis kit data sheet). To select for mutant CPI-17 plasmids a specific restriction enzyme site was introduced into the oligonucleotide primer containing the desired mutation

2.5.2.1. Polymerase chain reaction

Three microlitres of pGEX-4T1-CPI-17 plasmid (20 ng) were mixed with the two respective primers (125 ng each), 1 μl dNTP mix (2.5 mM each dNTP), 5 μl 10x reaction buffer (same as described in Table 2.7) and water to a final volume of 50 μl. The reaction was started by an initial denaturation step at 95°C for 30 sec. Following temperature cycling (15 cycles each including denaturation at 95°C for 30 sec, annealing of DNA primers at 55°C for 1 min and extension at 68°C for 10 min) the PCR product was treated with 1 μl of the *Dpn*I restriction enzyme (10 U/μl) and incubated at 37°C for 1 h. 5 μl of *Dpn*I treated PCR product were transformed into *E.coli* DH5α cells using the heat-shock method described under 2.3.2. The DNA was purified using the QIAprep Miniprep kit as described under 2.4.1. To screen for positive clones, plasmids were treated with the respective restriction enzyme, analysed on 1% TAE agarose gel and sent for sequencing to Cytomix, Cambridge.

2.6. Over-expression and protein purification

2.6.1. Over-expression of GST-fusion proteins expressed in *E. coli*

Table 2.9 Clones used for protein over-expression in *E. coli*

GST (pGEX-4TI)	Pharmacia Biotech
GST-centaurin-α_1 (pGEX-4TI)	Kanamarlapudi Venkateswarlu
GST-CPI-17 (pGEX-4TI)	Thierry Dubois
GST-CPI-17 S128A (pGEX-4TI)	Thierry Dubois
GST-14-3-3ζ (pGEX-4TI)	Thierry Dubois

Table 2.10 Media and buffers used for the over-expression of GST-fusion proteins

Isopropyl β-D-thiogalactopyranoside (IPTG)	1M	stock solution (stored at –20 °C)
LB liquid media	10 g/l 5 g/l 10 mM	Select Peptone Bacto-yeast NaCl

Table 2.11 Growth and induction conditions used to over-express various GST-fusion proteins

Protein	Growth temperature	Concentration of IPTG for induction	Time of induction
GST	37ºC	1 mM	4 h
GST-14-3-3 ζ	37ºC	1 mM	4 h
GST-CPI-17 wild-type and mutants*	37ºC	1 mM	4 h
GST-centaurin α_1	25ºC	0.5 mM	4 h

* GST-CPI-17 mutants: T38A, T38D, S128A, S128D and GST-CPI-17 1-120.

E. coli (BL21 strain, Stratagene) was transformed with the respective recombinant protein (GST-tagged) and grown overnight. *E. coli* containing the transformed plasmid were grown overnight in 200 ml LB supplemented with 100 μg/ml ampicillin at 37º C or 25ºC. The cultures were diluted 1/10 in the same medium and growth was continued at 37ºC or 25ºC until the optical density reached 0.6 (at 600 nm). Expression of the recombinant protein was induced by adding 0.5-1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h. The bacteria were harvested by centrifugation for 15 min at 5000 g and stored at -80ºC.

2.6.2. Purification of GST fusion proteins expressed in *E.coli*

Table 2.12 Buffers and materials used for GST fusion protein purification

Sonication buffer	50 mM	Tris pH 7.4
	100 mM	NaCl
	1 mM	EDTA
	1 mM	EGTA
	10%	Glycerol
	1 mM	DTT
	1/50 ml	Complete TM EDTA-free protease inhibitor cocktail (Roche Diagnostics, UK)
Elution buffer	10 mM	reduced Glutathione
	50 mM	Tris-HCl, pH 8.0
PBS	137 mM	NaCl
	2.7 mM	KCl
	4 mM	Na_2HPO_4
	0.15 mM	NaH_2PO_4 (pH 7.4)
Glutathione Sepharose 4B		Amersham Pharmacia Biotech,
Dialysis buffer	20 mM	Tris pH 7.5
	100 mM	NaCl
	1 mM	EDTA
	10 mM	β-mercaptoethanol
	10%	Glycerol

All the following procedures were carried out at 4°C. The pellet was resuspended in sonication buffer. Bacteria were incubated with 0.5 mg/ml of lysozyme for 30 min. This was followed by sonication of the bacteria 6 times for 20 sec at 5-10 μA. Bacterial debris was then pelleted by centrifugation for 20 min at 14 000g at 4°C. The supernatant was transferred to a fresh tube and filtered through a 0.45 μm filter.

Glutathione-Sepharose matrix was equilibrated by washing twice with 10 bed volumes of ice cold sonication buffer. The filtered bacterial supernatant was loaded onto the glutathione Sepharose matrix. The glutathione-Sepharose affinity support bound the GST fusion protein. Unbound material was passed through the column a further two times to increase the yield of protein bound to the column. To remove all non-specific

binding, the matrix was washed 2x with 10 bed volumes sonication buffer containing 600 mM NaCl. To remove the NaCl the matrix was then washed 3 times with 10 bed volumes PBS. The protein was eluted with 10 bed volumes elution buffer. Purified fusion proteins were dialysed against 20 mM Tris and 1 mM EDTA overnight at 4°C. Protein concentration was estimated by using the Bio-Rad protein assay and protein purity was determined by analysis on 12.5% SDS-PAGE gel.

2.6.3. Purification of recombinant 14-3-3ζ and CPI-17 WT and mutants T38A, T38D, S128A, S128D and CPI-17 1-120

Table 2.13 Buffers and materials used for 14-3-3ζ WT/T233A and CPI-17 WT/mutants purification

Equilibration buffer	50 mM	Tris-HCl pH8
	150 mM	NaCl
	2.5 mM	$CaCl_2$
Thrombin		Sigma
Elution buffer	20 mM	Tris-HCl pH8
	500 mM	NaCl
Benzamidine Sepharose		Amersham Pharmacia, UK

Similar procedures for 14-3-3ζ and CPI-17 purification were followed as described above. Instead of eluting the proteins however, the columns were washed with 3 column volumes equilibration buffer. GST was removed by digestion with thrombin (100 U/2 ml column volumes) for 30 min at room temperature. Proteins were eluted by washing the columns with 2 column volumes elution buffer. Fractions containing recombinant protein were loaded onto a benzamidine Sepharose matrix (1 ml) to remove thrombin contamination. The purity of 14-3-3ζ and CPI-17 was determined by analysis on 12.5% or 15% SDS-PAGE, respectively.

2.7. Protein assay

Protein was routinely measured by the method of Bradford (Bradford, 1976). Protein assay dye concentrate (Bio-Rad) was diluted 1:4 with water to produce the protein assay dye solution. Samples were diluted with water and 1 volume of diluted sample was incubated with 4 volumes of protein assay dye solution for 5 min at room temperature. The absorbance was then measured at 595 nm (A_{595}). Bovine serum albumin (BSA) was used as a standard to construct a curve of A_{595} versus protein concentration.

2.8. SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Table 2.14 Buffers and solutions for SDS-PAGE

Resolving gel (10-15%)	10-15%(w/v) 0.375 M 0.1% 0.1% 0.04%	29:1 acrylamide:N,N'-methylenebisacrylamide* Tris-HCl, pH8.8 SDS Ammonium persulphate TEMED
Stacking gel (10%)	10% (w/v) 0.125 M 0.1% 0.1% 0.1%	29:1 acrylamide:N,N'-methylenebisacrylamide Tris-HCl (pH6.8) SDS Ammonium persulphate TEMED
Tris-glycine running buffer	0.2 M 25 mM 0.1% (w/v)	Glycine Tris SDS
3X SDS loading buffer (Laemmli buffer)	0.15 M 30% 9% 15% 0.007%	Tris-HCl (pH6.8) Glycerol SDS β-mercaptoethanol Bromophenol blue
Coomassie blue stain	10% (v/v) 50% (v/v) 0.2% (w/v)	Glacial acetic acid Methanol Coomassie brilliant blue
Destain	10% (v/v) 30% (v/v)	Glacial acetic acid Methanol

*Referred to as acrylamide in the text

Proteins were separated according to their size by Tris–glycine SDS PAGE, following the method of Laemmli (Laemmli, 1970). Laemmli buffer (3x) was added to the samples to give a final concentration of 1x. These samples were boiled for 5 min prior to storage and/or separation. Samples were separated by downward migration through a vertical two-phase gel consisting of a stacking gel (top) and a resolving gel

(bottom). The gel apparatus was obtained from Bio-Rad. The gel was suspended between two separate reservoirs of Tris-glycine running buffer. A cathode and anode were placed in the top and bottom reservoirs respectively. Proteins were separated at 40 mA constant current for 1 h (5 ml resolving gel). A 10% (w/v) acrylamide resolving gel was used for the separation of 60-100 kDa proteins, whereas a 12.5% (w/v) acrylamide resolving gel was used for the separation of 30-60 kDa proteins and a 15% (w/v) acrylamide resolving gel was used to separate 10-30 kDa proteins (Sambrook et al., 1989). The pre-stained, broad range protein molecular weight markers used were obtained from Bio-Rad. Separated proteins were visualised by Coomassie blue staining (20 min stain followed by 30 min destain) or were transferred onto nitrocellose membrane for analysis by Western blotting.

2.9. Western blotting

Table 2.15 Buffers for Western blotting

Transfer buffer	192 mM	Glycine
	25 mM	Tris
	0.1%	SDS
	20%	Methanol
TBS-Tween	137 mM	NaCl
	20 mM	Tris-HCl, pH 7.6
	0.1%	Tween-20

Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes (Bio-Rad) by electroblotting in transfer buffer at 180 mA constant current for 90 min. The nitrocellulose membranes were blocked with 10% skimmed milk in TBS-Tween for 1 h shaking at room temperature. They were then probed with primary antibody, diluted in 10% skimmed milk in TBS-Tween, either shaking at room temperature for 2 h or at 4°C overnight. The antibody dilutions that were used are shown in Table 2.16. After six 5 min washes in TBS-Tween, the nitrocellulose membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody, diluted in 10% skimmed milk in TBS-Tween, for 1 h shaking at room temperature. The membranes were washed

in TBS-Tween as before and subjected to enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions. Immediately after treating the membranes with ECL reagent, they were exposed to Kodak X-ray film for various lengths of time (10 sec-30 min).

Table 2.16 List of antibodies and the dilutions used for Western blotting or immunoprecipitation

Specific for:	Name	Company	Primary or secondary	Monoclonal/ Polyclonal	Dilution for Western blotting
CamKII	CamKII	Transduction Laboratories	Primary	Monoclonal	1/1000
CKIα (C-terminus)	CKIα (C-19)	Santa Cruz	Primary	Polyclonal	1/250
CKI δ	CKIδ (R-19)	Santa Cruz	Primary	Polyclonal	1/250
CKIε	CKIε	Transduction Laboratories	Primary	Monoclonal	1/250
CKI	CK1	StressGen	Primary	Polyclonal	1/1000
HMG1	Anti-HMG1	Pharmingen	Primary	Polyclonal	1/1000
Hsp70	Hsp70	Transduction Laboratories	Primary	Monoclonal	1/1000
Nucleolin	C23 (MS-3)	Santa Cruz	Primary	Polyclonal	1/250
PKA catalytic subunit	PKA_C	Transduction Laboratories	Primary	Monoclonal	1/1000
PKCα	PKCα	Transduction Laboratories	Primary	Monoclonal	1/1000
PKCβ	PKCβ	Transduction Laboratories	Primary	Monoclonal	1/1000
PKCγ	PKCγ	Transduction Laboratories	Primary	Monoclonal	1/1000
PKCλ	PKCλ	Transduction Laboratories	Primary	Monoclonal	1/1000

Specific for:	**Name**	**Company**	**Primary or secondary**	**Monoclonal/ Polyclonal**	**Dilution for Western blotting**
PKCμ	PKCμ (C-20)	Santa Cruz	Primary	Polyclonal	1/250
PKCζ	PKCζ	van Blitterswijk	Primary	Polyclonal	1/1000
PRK1/PKN	PRK1	Transduction Laboratories	Primary	Monoclonal	1/1000
ROCK	ROKα	Transduction Laboratories	Primary	Monoclonal	1/1000
14-3-3ζ	14-3-3ζ	Alastair Aitken and Harry Martin	Primary	Polyclonal	1/1000
Flag-tagged proteins	Anti-flag M2	Sigma	Primary	Monoclonal	1/1000
HA-tagged proteins	Anti-HA HA-7	Sigma	Primary	Monoclonal	1/1000
HA-tagged proteins	Anti-HA High Affinity	Roche Diagnostics	Primary	Monoclonal	1/1000
Myc-tagged proteins	c-Myc (A-14)	Santa Cruz	Primary	Polyclonal	1/1000
Flag-tagged proteins	Anti-flag M2-peroxidase conjugate	Sigma	Primary and Secondary	Monoclonal	1/250
Goat IgG	Anti-Goat IgG	SAPU	Secondary (HRP conjugated)		1:2000
Rat IgG	Anti-Rat IgG	Amersham Pharmacia Biotech	Sedondary (HRP conjugated)		1/1000

Specific for:	Name	Company	Primary or secondary	Monoclonal/ Polyclonal	Dilution for Western blotting
Mouse IgG	Anti-Mouse IgG	Amersham Pharmacia Biotech	Secondary (HRP conjugated)		1:5000
Rabbit IgG	Anti-Rabbit IgG	Santa Cruz	Secondary (HRP conjugated)		1:2000

2.10. Generation of a polyclonal antiserum to CPI-17

2.10.1. Immunisation of the sheep

The following procedures were carried out by Diagnostics Scotland. For the primary immunisation, 1.25 mg of purified CPI-17 protein were mixed with Freund's Complete Adjuvant (FCA), an emulsifying agent containing mycobacteria which enhance the immune response to the immunogen. The three subsequent booster injections contained 1.25 mg of CPI-17 protein in Freund's Incomplete Adjuvant (FIA), an adjuvant where the mycobacteria are not included. Donations are up to 1 l of whole blood, which yields 200-400 ml of serum. The animal was injected every 28 days and was not exsanguinated at the termination of procedure.

2.10.2. Affinity purification of anti-CPI-17

Table 2.17 Buffers used for affinity purification of anti-CPI-17

Buffer A	20 mM	Tris-HCl, pH 7.5
Buffer B	20 mM 500 mM	Tris-HCl, pH 7.5 NaCl
Elution Buffer	3.5 M	$MgCl_2$

Recombinant CPI-17 protein (2 mg) was covalently bound to SulfoLink Coupling Gel column (2 ml column volume) following the manufacturer's instructions (Pierce). The column and buffers were at room temperature for the following procedures. The column was equilibrated with 10 column volumes. The serum (10 ml) was dialysed against buffer A, filtered on 0.45 μm and passed through the column. The flow-through was collected and passed through the column three more times. The column was washed with 5 column volumes buffer A and then with 5 column volumes Buffer B. The antibody was eluted with 5 column volumes elution buffer and dialysed against PBS O/N. The antibody was concentrated to a final concentration of 0.5 mg/ml and 2 mg/ml BSA were added for storage. The antibody was aliquoted and stored at -20°C. It was used at a dilution 1/100 in Western blotting.

2.11. Chemical crosslinking of CPI-17

Table 2.18 Chemical crosslinkers (Pierce)

Crosslinker	Crosslinker Type	Spacer Arm Length
DMS (Dimethyl suberimidate.2 HCl)	Imidoester Crosslinker	11Å
DTBP (Dimethyl 3,3'-dithiobispropionimidate. HCl)	Imidoester Crosslinker	11.9Å

DMS and DTBP are water soluble homobifunctional imidoesters and possess two identical groups which can react with primary amine groups to form stable covalent bonds (Pierce). To determine whether CPI-17 is a dimer, crosslinking experiments with the two crosslinkers were carried out, and 14-3-3τ was used as a positive control. CPI-17 (50 μM) or 14-3-3τ (50 μM) were mixed with a freshly prepared solution of the respective crosslinker (5 mM) and the reaction was made up to 15 μl with PBS. The reactions were incubated at room temperature for 2 h. The reactions were stopped by the addition of Tris-HCl, pH 7.4 to a final concentration of 500 mM. Reactions were analysed on 15% SDS-PAGE and protein bands were either visualised by staining with Coomassie blue or analysed by Western blotting with an anti-CPI-17 antibody or 14-3-3ζ antibody.

2.12. Affinity chromatography of proteins that interact with CPI-17 and centaurin-α_1

Table 2.19 Buffers used for affinity chromatography

Homogenisation buffer	50 mM	Tris-HCl pH 7.4
	100 mM	NaCl
	1 mM	EDTA
	1 mM	EGTA
	1 mM	DTT
	10%	Glycerol
	2/50 ml	Protease inhibitor tablets
Elution buffer	Same as above +	
	1 M	NaCl

Glutathione-Sepharose affinity columns with ~ 25mg of GST, GST-CPI-17 or GST-centaurin-α_1 were constructed as described under 2.6.2, however, the proteins were not eluted and the buffer was equilibrated with homogenisation buffer. Sheep brain (~ 80 g) (or 10 rat brains, ~ 2 g each) were homogenised in homogenisation buffer. Triton X-100 was then added to a final concentration of 0.5% and the mixture was incubated at 4°C for 2 h with constant agitation, and clarified by centrifugation at 14000 g for 30 min at 4°C. This was followed by ultracentrifugation at 100000 g for 1 h at 4°C. The supernatant was filtered through a 0.45μm filter and incubated with GST for 2 h at 4°C with constant agitation. A centrifugation at 4000 g for 5 min at 4°C followed. The supernatant was incubated by batch with the GST, GST-CPI-17 or GST-centaurin-α_1 column overnight at 4°C. Beads were then left to settle and the columns were washed with lysis buffer containing 0.5% Triton. The columns were subsequently washed with 4 x 15 ml of homogenisation buffer. The proteins were eluted with homogenisation buffer containing 1M NaCl, and concentrated on Centricon 10 (Millipore). Protein concentration was determined using the Bio-Rad protein assay. Samples were analysed by 12.5% or 10% SDS-PAGE, transferred onto nitrocellulose and Western blotting using CamKII, CKIα, CKIε, HMG1, PKA_C, PKCα, PKCε, PKCλ, PKCμ, PKCζ, PKN (PRK1) and ROCK (ROKα) antibodies was performed.

2.13. *In vitro* binding between purified PKC isoforms and CPI-17/centaurin-α_1

Table 2.20 Buffer used for *in vitro* binding studies between purified PKC isoforms and CPI-17/centaurin-α_1

Binding buffer	20 mM	Tris-HCl, pH 7.4
	100 mM	NaCl
	1 mM	DTT
	1%	Nonidet-P40 (Fisher)
	10%	Glycerol
	0.1%	Bovine Serum Albumin (BSA)

GST, GST-14-3-3 ζ, GST-CPI-17 or GST-centaurin-α_1 (5 μg) were incubated with human recombinant PKCα, ε, μ, or ζ (1 U) (Calbiochem) in binding buffer for 2 h at 4°C with constant rotation. Glutathione-Sepharose beads were then added and incubated for a further 1 h. Bead precipitates were washed 4 times with binding buffer and bound proteins were eluted using SDS sample buffer. The samples were analysed by 10% SDS-PAGE, transferred onto nitrocellulose and Western blotted using PKC antibodies.

2.14. *In vitro* CKI assays

2.14.1. *In vitro* binding between purified CKI and CPI-17

Table 2.21 Buffers used for *in vitro* binding study between purified CKI and CPI-17

Binding buffer	20 mM	Tris-HCl, pH 7.4
	100 mM	NaCl
	1 mM	DTT
	1%	Nonidet-P40 (Fisher)
	10%	Glycerol
	0.1%	Bovine Serum Albumin (BSA)
Kinase buffer	25 mM	HEPES, pH7.0
	10 mM	$MgCl_2$
	1 mM	DTT

GST, GST-centaurin-α_1 and GST-CPI-17 (5 μg) were incubated with recombinant CKI (0.2 μg) (*S.pombe ski1*, Upstate Biotechnology) in binding buffer for 2 h at 4°C. Glutathione-Sepharose beads were then added and incubated for a further 1 h. Bead precipitates were then washed 4 times with binding buffer and once with kinase buffer. The washed beads were incubated with kinase buffer (without NaCl) containing 40 μM of a CKI-specific phosphopeptide (CKRRALS(p)VASLPGL, where S(p) is a phosphoserine; Cali Hyde, National Institute for Medical Research, Mill Hill, London) substrate and 50 μM ATP including [γ-^{32}P]-ATP (Amersham) in a final volume of 60 μl. Samples were incubated at 30°C for 30 min, centrifuged (10000 g) and 20 μl of the reaction mixture was spotted in duplicate on P81 paper squares (Whatman). The papers were washed 5 times with 1% aqueous phosphoric acid and radioactivity retained on the papers was quantified by liquid scintillation counting.

2.14.2. Phosphorylation of 14-3-3ζ wt by a kinase eluted from the CPI-17 column

Table 2.22 Buffers used for phosphorylation of 14-3-3ζ by a kinase eluted from the CPI-17 column

Buffer	Concentration	Component
Binding buffer	20 mM	Tris-HCl, pH 7.4
	100 mM	NaCl
	1 mM	DTT
	1%	Nonidet-P40 (Fisher)
	10%	Glycerol
	20 mM	EDTA
Kinase buffer	25 mM	HEPES, pH7.0
	10 mM	$MgCl_2$
	1 mM	DTT

Five micrograms of GST, GST-CPI-17 or GST-centaurin-α_1 column eluate was incubated with 5 μg of GST, GST-14-3-3ζ or GST-14-3-3ζ T233A mutant (from Thierry Dubois) in kinase buffer (as described in 2.14.1) in a final volume of 40 μl. Reactions were performed at 30°C for 30 min. The reaction was stopped with 1 ml of

binding buffer. 20 μl of glutathione Sepharose beads were added and the samples were incubated for 1 h at room temperature. The beads were washed 3 times with binding buffer and incubated with SDS sample buffer. Samples were analysed by 12.5% SDS-PAGE. Gels were stained with Coomassie Blue and autoradiographed.

2.14.3. Phosphorylation of CPI-17 by a kinase eluted from the CPI-17 column

Five micrograms of GST or GST-CPI-17 column eluate was incubated with 5 μg of GST or GST-CPI-17 in kinase buffer (as described in 2.14.1) in a final volume of 40 μl. Reactions were performed at 30°C for 30 min. The reaction was stopped with 1 ml of binding buffer. 20 μl of glutathione Sepharose beads were added and the samples were incubated for 1 h at room temperature. The beads were washed 3 times with binding buffer and incubated with SDS sample buffer. Samples were analysed by 15% SDS-PAGE. Gels were stained with Coomassie Blue and autoradiographed.

2.14.4. Kinase assay to test the effect of CPI-17 on CKI activity

CPI-17 wt, CPI-17 T38A, CPI-17 T38D, CPI-17 S128A, CPI-17 S128D and CPI–17 1-120 (7.5 μg) were pre-incubated with recombinant CKIα (from Dr Thierry Dubois) (Dubois et al., 1997) for 15 min at 30°C. To test the effect of CPI-17 on CKI, casein (5 μg) or CKI-specific phosphopeptide substrate (40 μM) were added with the kinase buffer (as described in section 2.14.1). The reaction was started by addition of 50 μM ATP including [γ-^{32}P]-ATP. The reaction was carried out in a final volume of 60 μl. Reactions were performed at 30°C for 30 min, 20 μl of the reaction were spotted in duplicates on P81 paper squares (Whatman) and the papers were washed 5 times with 1% TCA. Radioactivity retained on the papers was quantified by liquid scintillation counting. In order to measure the half-maximum inhibition/activation, recombinant CKIα was pre-incubated with different amounts of CPI-17 S128A or CPI-17 wt/S128D (14 nM-11 mM) for 15 min at 30°C. CKI specific phosphopeptide (40 μM) was used as substrate and the reactions were carried out as described above.

2.14.5. Kinase assay to test the effect of CPI-17 on CKII activity

Table 2.23 Buffer used for CKII *in vitro* assay

Kinase buffer	20 mM	Tris-HCl, pH 7.5
	50 mM	KCl
	10 mM	$MgCl_2$

CPI-17 wt, CPI-17 T38A, CPI-17 T38D, CPI-17 S128A and CPI-17 S128D (5 μg) were pre-incubated with CKII (Upstate Biotechnology). After 15 min at 30°C, casein (5 μg) in kinase buffer. 50 μM ATP including [γ-^{32}P]-ATP were added. The reaction was performed in a final volume of 60 μl at 30°C for 30 min. An aliquot of the reaction (20 μl) was spotted in duplicates on P81 paper squares (Whatman). The papers were washed 5 times with 1% TCA and radioactivity retained on the papers was quantified by liquid scintillation.

2.15. Phosphorylation of CPI-17 by PKC and CKIα

Table 2.24 Buffers used for PKC assay

Kinase buffer (for PKCε, μ and ζ)	40 mM	HEPES, pH 7.4
	2 mM	EGTA
	20 mM	$MgCl_2$
Kinase buffer (for PKCα)	Same as above +	
	3 mM	$CaCl_2$

CPI-17 (5 μg) was incubated with 1 U of PKCα, PKCε, PKCμ or PKCζ in kinase buffer. 0.03 mg/ml phosphatidylserine, 8 μg/ml diacylglycerol and 50 μM ATP including [γ-^{32}P]-ATP, Amersham were added to the reactions. The reaction was performed at 30°C for 15 min in a final volume of 40 μl. The reactions were stopped by the addition of SDS sample buffer and analysed on 15% SDS-PAGE. Gels were stained with Coomassie Blue and autoradiographed.

Phosphorylation of CPI-17 (5 μg) by CKIα was performed by incubating CPI-17 with CKIα in kinase buffer (as described in 2.14.1) at 30°C for 30 min in a final volume of 40 μl. The reactions were stopped by the addition of SDS sample buffer and analysed

on 15% SDS-PAGE. Gels were stained with Coomassie Blue and autoradiographed.

2.16. *In vitro* transcription and translation (IVTT) and GST pull-down assays

Table 2.25 Buffer used for IVTT

Binding buffer	20 mM	Tris-HCl, pH 7.4
	100 mM	NaCl
	1 mM	DTT
	1%	Nonidet-P40 (Fisher)
	10%	Glycerol

Table 2.26 Clones used for IVTT

Clone	**From**
HA-CKIα (pcDNA3)	Frank McKeon
HA-CKIα 17-325 HA-CKIα 164-325 HA-CKIα 189-325 HA-CKIα 217-325 HA-CKIα 233-325 HA-CKIα 17-287 HA-CKIα 164-287 HA-CKIα 189-287 (all cloned in pcDNA3)	Thierry Dubois
CKIδ CKIδΔ317 (deleted of the carboxyl terminus) CKIγ_1 CKIγ_3 (all cloned in pET8c)	Peter Roach
CKIγ_2 (pSV2Zeo)	Louise Larose
CKIε (pV405)	David Virshup
PKCα (pCO2)	Peter Parker
PKCλ (pcDNA3)	Terje Johansen
PKCμ wt PKCμ 1-340 PKCμ ΔAC PKCμ ΔPH (all in pcDNA3)	Franz-Josef Johannes
PKCζ (pcDNA3.1)	Feng Liu
Nucleolin (deleted of last 171 C-terminus residues)	Josee Golay

CKIα, CKIδ, CKIε, CKIγ_1 CKIγ_2 CKIγ_3, PKC α, λ, μ, ζ and nucleolin were expressed *in vitro* using a T7 TNT coupled transcription/translation reticulocyte lysate (Promega Corp., Madison, WI). The reactions (50 μl) were performed following the manufacturer's instructions using [^{35}S] methionine (Amersham) for 90 min at 30°C. Samples were then diluted 3 fold with binding buffer and incubated for 15 min at 30°C with 5 μg of GST, GST-centaurin-α_1, GST-CPI-17, GST-CPI-17 1-120, GST-14-3-3ζ or GST-VAMP. When ribonuclease (RNase) treatment was performed, 2, 4, or 8 μl of RNase ONE™ ribonuclease (5-10 units/ml) (Promega Corp.) were added to the reaction before addition of the recombinant proteins and incubated for 15 min at 30°C. BSA was added to a final volume of 0.1% to remove non-specific binding. Glutathione-Sepharose beads (Amersham Pharmacia) and binding buffer (300 μl) were added to the reactions and incubated at room temperature for a further 1 h. The beads were washed 5 times with 1 ml of binding buffer, boiled in sample buffer for 5 min and electrophoresed on 12.5% SDS-PAGE. After staining/destaining, the gels were incubated for 30 min with Amplify™ (Amersham Pharmacia), dried and exposed to film.

2.17. Co-transfection/immunoprecipitation of CPI-17 and CKI

Table 2.27 Buffer used for immunoprecipitation

Lysis buffer	20 mM	Tris-HCl, pH 7.4
	100 mM	NaCl
	1 mM	EDTA
	1 mM	EGTA
	1 mM	DTT
	1%	Nonidet-P40 (Fisher)
	10%	Glycerol
	10 mM	NaF
	1 mM	$C_3H_7O_6PNa_2$
	1 mM	Na_3VO_4
	1/50 ml	protease inhibitor tablets

COS-1 or COS-7 cell were co-transfected with 2 μg flag-CPI-17/flag-centaurin-α_1 (flag2B vector) and 4 μg HA-CKIαWT/KD (pcDNA3.1) using electroporation or FuGENE (as described under 2.1.2.1 and 2.1.2.2). After transfection the cells were seeded into 100 mM tissue culture dishes in complete medium and cultured for 48 h. The cells were lysed in 1 ml of lysis buffer. Lysates were incubated with Pansorbin cells (Calbiochem) with constant agitation (final volume 10%) for 30 min at 4°C and centrifuged at 10000g for 30 min. The supernatants were incubated for 2 h with constant agitation at 4°C with anti-HA antibody (3 μg) (Sigma) to allow binding to the transfected HA-tagged CKIα. 25 μl protein A/G Sepharose (Pharmacia Amersham) were added and the immunoprecipitates were incubated for a further 1 h at 4°C and washed 3 times with lysis buffer. Bound proteins were eluted from the protein-A/G Sepharose beads with 20 μl 1x sample buffer and analysed by SDS-PAGE gel. Western blot analysis was carried out using anti-flag (1/250) (Sigma).

2.18. Mass spectrometry

To identify new protein binding partners, CPI-17 and centaurin-α_1 affinity columns were constructed, and brain lysate was passed through as described in 2.12. For CPI-17 the experiment was also repeated using rat brain lysate. 40 μg of CPI-17 and centaurin-α_1 column eluate was separated on 12.5% SDS-PAGE and stained with GelCODE. MALDI-TOF (Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry) setup was used to identify the proteins. The protein bands were excised and digested with trypsin. Samples were analysed by 0.5 ml sample digest with 0.5 ml matrix (α-Cyano-4-hydroxycinnamic acid (CHCA), Sigma) onto a 100 well MALDI plate. Mass Spectrometry analysis was carried out using an Applied Biosystems Voyager DE (delayed extraction) STR MALDI-TOF instrument. The instrument was set up as follows:

Table 2.28 MALDI-TOF instrument set up

Mode	Positive Ion Reflector
Grid Voltage	66%
Accelerating Voltage	20000V
Low Mass Gate	500Da
Delay time	150nsec
Laser energy	Variable from 1830-2080
Laser shots	400 per acquisition

All samples were spotted in duplicate to account for irregularities in crystallising. This is variable from spot to spot. Where poor spectra were obtained, Zip Tipping of the digest solution was carried out. Zip Tips (Milipore) are generally used to desalt or concentrate samples up. The final spectra were calibrated using known trypsin peaks visible within each spectrum. From this a list of peaks (peptide masses) was copied into Protein Prospector http://prospector.ucsf.edu/ucsfhtml4.0u/msfit.htm or Matrix Science http://matrixscience.com/cgi/master.

A number of different databases can be searched using the search engines MS-Fit (Protein Prospector) or Mascot (Matrix Science): NCBI, SwissProt, Owl, pbEST,Genpept. In this case the NCBI database was used. The search engine matches experimental peak mass data with theoretical peptide masses from hypothetical digests of sequenced proteins. From this, a MOWSE and/or percent coverage score is given. This MOWSE scoring, represents a number providing a probability for a potential hit. Typically, the higher the MOWSE score the better (For Mascot Search, scores greater than 65 are considered to be significant). The percentage coverage represents the matched peptides as a percentage of the total protein. For proteins under 100kDa a minimum of 20% is generally required. A minimum of 4 peptides had to be matched before a score was given. Again, the more matches the better. The mass spectrometry analysis, including the trypsin digestion were carried out by Dr Andy Cronshaw and Hannah Florance (University of Edinburgh, Wellcome trust, Edinburgh Protein Interaction Centre, (EPIC)) for identification of novel CPI-17 binding partners. Dr Steven Howell (National Institute for Medical Research, Mill Hill, London) carried out the identification of centaurin-α_1 protein partners, apart from the identification of

nucleolin which was also done by Dr Andy Cronshaw. The experimental procedures description is based on a summary of Mass Spectrometry Method written and developed by Dr Andy Cronshaw.

CHAPTER 3

Identification of novel CPI-17 binding proteins

3. Identification of novel CPI-17 binding proteins

INTRODUCTION

CKIα was identified as the kinase from brain which phosphorylates 14-3-3ζ on residue 233 (Dubois et al., 1997). The protein kinase C-potentiated inhibitor, CPI-17, was one of seven proteins which co-purified with CKIα from brain after four chromatography steps suggesting that they might be forming a complex (Dubois et al., 2002b). The aim was to characterise the interaction between CPI-17 and CKIα further and to identify new protein binding partners for CPI-17 by affinity chromatography to increase our understanding of the functions that CPI-17 might have in cells. In this chapter dimerisation experiments with CPI-17 and the generation of a CPI-17 antibody (sections 3.2.1 and 3.2.2) will be discussed. The identification of new CPI-17 binding proteins will be described and will focus on the characterisation of the interaction between PKC and CPI-17 (section 3.3), because it has been shown that phosphorylation of CPI-17 on Thr-38 by PKC increases its potency to inhibit the catalytic subunit of PP1 (PP1-C) by 1000-fold. The second part of this chapter (section 3.4) will focus on the characterisation of the interaction between CPI-17 and CKI since our laboratory identified CPI-17 as one of the proteins co-purifying with CKIα from brain as mentioned above. In the last part of this chapter the identification of novel potential CPI-17 binding proteins by mass spectrometry will be discussed and some of the potential functions CPI-17 might have in cells in the light of these newly identified binding partners will be proposed (section 3.5). The diagram below shows a summary of the three major parts of this chapter which will be discussed (Figure 3.1).

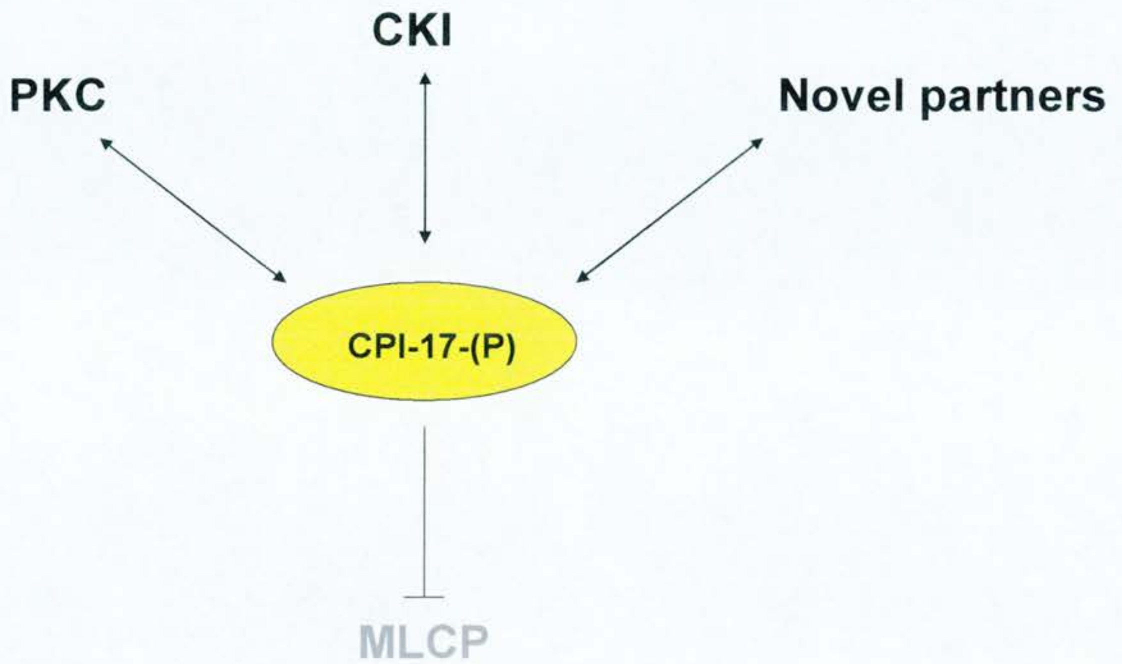


Figure 3.1 Interactions with CPI-17 described in chapter 3.
CPI-17 associates with PKC (section 3.3), CKI (section 3.4) and novel binding partners (section 3.5); these interactions are indicated by double arrows. When phosphorylated CPI-17 is also a myosin light chain phosphatase (MLCP) inhibitor indicated by a bar.

RESULTS AND DISCUSSION

3.1. DNA manipulation and purification of recombinant proteins

GST-CPI-17 cloned in pGEX-4TI was from Thierry Dubois. GST-CPI-17 1-120 was cloned using the PCR and sequenced to verify the nucleotide sequence of human CPI-17 1-120 as described in the Methods section 2.5.1. GST-CPI-17 T38A, T38D, S128A and S128D were cloned using the site-directed mutagenesis kit as described in the Methods section 2.5.2. GST-14-3-3ζ and GST-centaurin-α_1 cloned in pGEX-4TI were from Dr Thierry Dubois. The recombinant fusion proteins were expressed in *E. coli* as described in the Methods section 2.6. To obtain the GST fused protein, standard procedures were used (Figure 3.2A, C and D). Recombinant CPI-17, was obtained by cleavage with thrombin (Figure 3.2B). Fractions containing CPI-17 were typically 2 mg/ml as measured by Bio-Rad protein assay and stored at -20°C.

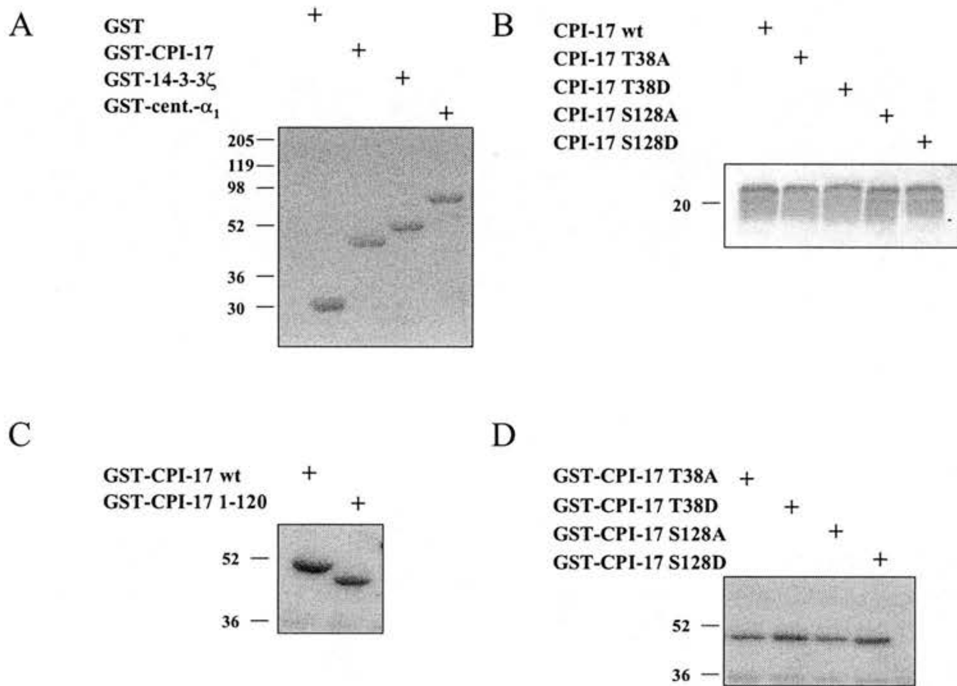


Figure 3.2 Purified recombinant proteins.
A, Coomassie blue-stained 12.5% SDS-PAGE of 5 μg each of recombinant protein (GST-tagged). Molecular weight markers in kDa are shown on the left.
B, Coomassie blue-stained 15% SDS-PAGE of 5 μg each of recombinant CPI-17 protein. Molecular weight markers in kDa are shown on the left.
C and D, Coomassie blue-stained 12.5% SDS-PAGE of 5 μg of recombinant CPI-17 proteins (GST-tagged). Molecular weight markers in kDa are shown on the left.

3.2. CPI-17

3.2.1. Generation of anti-CPI-17

Antiserum was raised against purified human recombinant CPI-17 in one sheep as described in Methods section 2.10. To test the antiserum produced, 1 μg of CPI-17 and GST-CPI-17 were separated by SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose membrane was then immunoblotted with the antiserum, or pre-immunised serum, as primary antibody. The two sera were diluted 1:100. Both CPI-17 and GST-CPI-17 were recognised by the antiserum (Figure 3.3). However, the antiserum only recognised a minimum of 0.1-0.5 μg of recombinant protein (data not shown). The anti-serum only recognised 1 band and therefore the specificity appears to be good. The pre-immune serum did not recognise CPI-17 or GST-CPI-17.

3.2.2. CPI-17 dimerisation

To test whether CPI-17 forms dimers, cross-linking experiments were performed as described in Methods section 2.11. 14-3-3ζ was used as a positive control. The results show that CPI-17 is a dimer (Figure 3.4). To the best of my knowledge this is the first time this has been demonstrated. These results were further confirmed by light scattering experiments carried out by Yuan De Yang (Institute of Cell and Molecular Biology (ICMB), University of Edinburgh; data not shown). CPI-17 dimerisation might be a prerequisite for interactions with certain proteins. Interestingly, it has been suggested that the PP1 inhibitor, PHI-1 is either a dimer of 46kDa or contains an extended polypeptide that exhibits an anomalous size in solution (Eto et al., 1999).

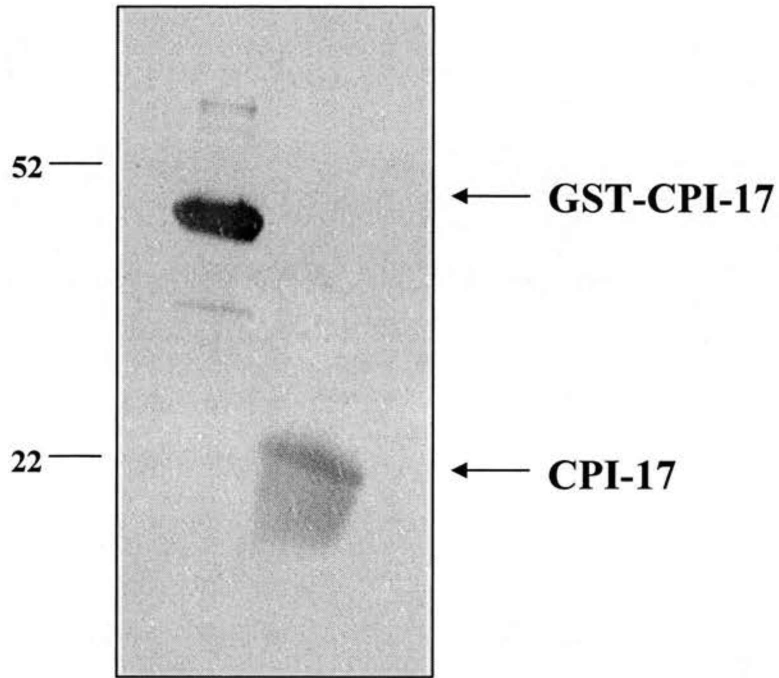


Figure 3.3 Generation of anti-CPI-17.
This figure shows that the sheep had raised antibodies to CPI-17 just 28 days after the first injection with CPI-17. The antiserum detected a band at approximately 20kDa which is the molecular weight at which CPI-17 migrates on 15% SDS-PAGE (Eto et al., 1995).

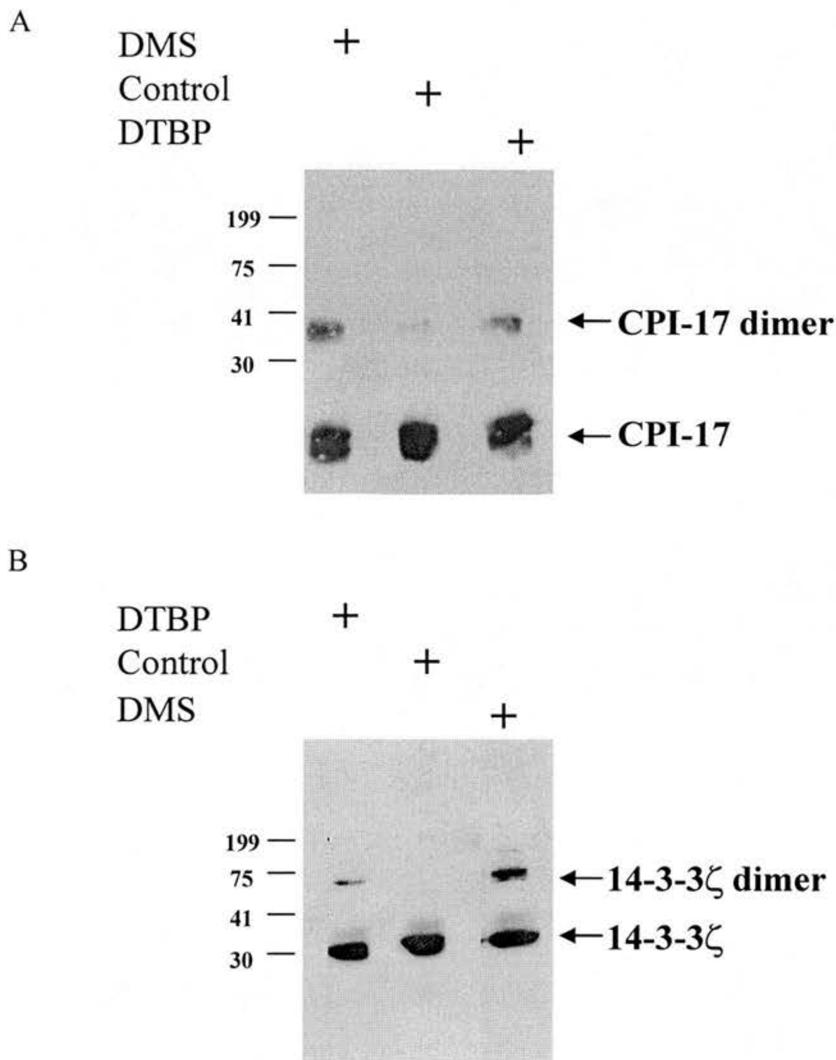


Figure 3.4 CPI-17 dimerisation.
A, CPI-17 (50 μM) was incubated in the presence (+) or absence (-) of DMS or DTBP chemical cross-linking agents. The presence of monomeric and dimeric forms of CPI-17 was determined by Western blotting using CPI-17 antibodies.
B, As a positive control, 14-3-3ζ (50 μM) was incubated in the presence (+) or absence (-) of DMS or DTBP chemical cross-linking agents . The presence of monomeric and dimeric forms of 14-3-3ζ was determined by Western blotting using 14-3-3ζ antibodies.

3.3. Characterisation of the association between CPI-17 and PKC

The next part of this chapter will focus on the identification of novel CPI-17 binding partners and particularly focus on the interaction between CPI-17 with PKC and CKI. To investigate the function(s) of CPI-17, novel CPI-17 protein partners were identified by affinity chromatography as described in more detail in section 3.5.

CPI-17 is phosphorylated on Thr-38 *in vitro* by a number of kinases such as PKC (Eto et al., 1995), PKA (Dubois et al., 2002d), ROCK (Koyama et al., 2000), PKN (Hamaguchi et al., 2000), a ZIP-like kinase (MacDonald et al., 2001b) and ILK (Deng et al., 2002), however the *in vivo* kinase that phosphorylates this residue remains unknown. Phosphorylation of Thr-38 plays a key role in regulating CPI-17 activity (Eto et al., 1995), therefore the interaction of some of these kinases with CPI-17 was tested, with the ultimate aim to gather evidence which would lead to the identification of the *in vivo* kinase.

3.3.1. A kinase which phosphorylates CPI-17 associates with the CPI-17 column

To determine whether a kinase associated with the GST-CPI-17 column which could phosphorylate CPI-17 wt, 5 μg of CPI-17 wt were incubated with 5 μl CPI-17 column eluate or GST column eluate in the presence of CKI kinase buffer. Casein kinase buffer was used because I wanted to test whether CPI-17 wt could be phosphorylated by CKI which could be present in the CPI-17 column eluate. The results show that CPI-17 wt was phosphorylated by a kinase present in the CPI-17 column eluate (Figure 3.5), however the identity of this kinase remains unknown. It should be pointed out that it is very unlikely that the kinase is CKI, because in chapter 3.4.6 it will be shown that CPI-17 is only a very weak substrate for CKI. It is possible that a number of kinases are present in the CPI-17 eluate which phosphorylate CPI-17 *in vitro* on multiple phosphorylation sites.

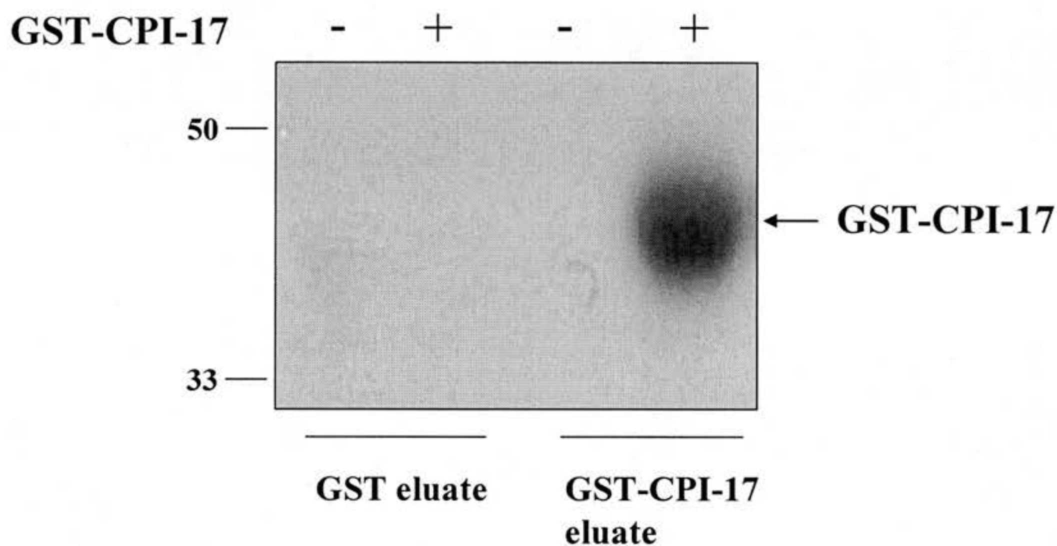


Figure 3.5 A kinase associates with the GST-CPI-17 column which phosphorylates CPI-17.
GST ("GST-eluate") or GST-CPI-17 ("GST-CPI-17 eluate") (5 μg) column eluate were incubated for 30 min at 30°C with 5 μg of GST or GST-CPI-17 in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. A GST pull-down assay was performed and the samples were analysed by 15% SDS-PAGE and autoradiography. The positions of the molecular weight markers (kDa) are indicated, as is the position of the phosphorylated GST-CPI-17.

3.3.2. PKC isoforms from all PKC classes bind to the CPI-17 column

PKC has been shown to phosphorylate CPI-17 on Thr-38 (Eto et al., 1995) and therefore the association of PKC isoforms with the CPI-17 column was tested. Proteins eluted from the GST[1] and GST-CPI-17 column (10 μg) were separated on a 10% SDS-PAGE gel, transferred onto nitrocellulose and Western blotted with anti-PKCα, ε, λ, μ and ζ antibodies (for PKCλ and ζ 5 μg of GST-CPI-17 column eluate and GST-centaurin-α_1 column eluate were loaded). No PKC isoforms were detected in the eluate from the control GST column. PKCα (conventional PKC), PKCε (novel PKC), PKCλ and ζ (atypical PKCs) and PKCμ all associated with the GST-CPI-17 column, and PKCλ and PKCζ also associated with the centaurin-α_1 column (Figure 3.6). The PKCζ antibody seems to recognise a lower molecular weight band in the brain lysate and in the GST-centaurin-α_1 column eluate compared to the CPI-17 column eluate. This could be explained by the fact that the PKCζ isoform present in the CPI-17 eluate could be phosphorylated and therefore migrates higher than the unphosphorylated form which might be present in the brain lysate and GST-centaurin-α_1 column eluate. The other possibility could be that the antibody against PKCζ is of low quality and might not be very specific. One cannot conclude whether PKCα, ε and μ also bound to the GST-centaurin-α_1 column as not enough material was available. The kinases ROCK, PKN and PKA were also shown to phosphorylate CPI-17 on Thr-38 *in vitro* (Dubois, et al., 2002d; Hamaguchi et al, 2000; Koyama et al., 2000). Therefore it was tested whether ROCK, PKN and the catalytic unit of PKA (PKA_C) associated with the GST-CPI-17 column (Figure 3.7). Out of these kinases only PKA_C associated with the CPI-17 column. Because it was not tested whether a regulatory subunit of PKA associated with the CPI-17 column, it was difficult to conclude whether the PKA_C detected was a free, active subunit from the cytosol or part of the inactive complex containing regulatory

[1] Note that due the low concentration of proteins in the GST column eluate, it was not possible to load the same amount of protein i.e. 5-10 μg and therefore 20 μl of the GST column eluate were loaded.

subunits and possibly anchored via an AKAP to a specific subcellular domain. The association of CamKII, which phosphorylates CPI-17 on Ser-130 (Dubois et al., 2002d), with the CPI-17 column was tested, however, no binding could be detected (Figure 3.7). The results from the CPI-17 affinity chromatography indicated that PKC isoforms from all PKC classes (α, ε, λ, μ and ζ) and PKA_C associated with the GST-CPI-17 column. However, one cannot conclude from this experiment whether the binding of the kinases was directly to CPI-17 or whether it was mediated by other proteins associated with the GST-CPI-17 column.

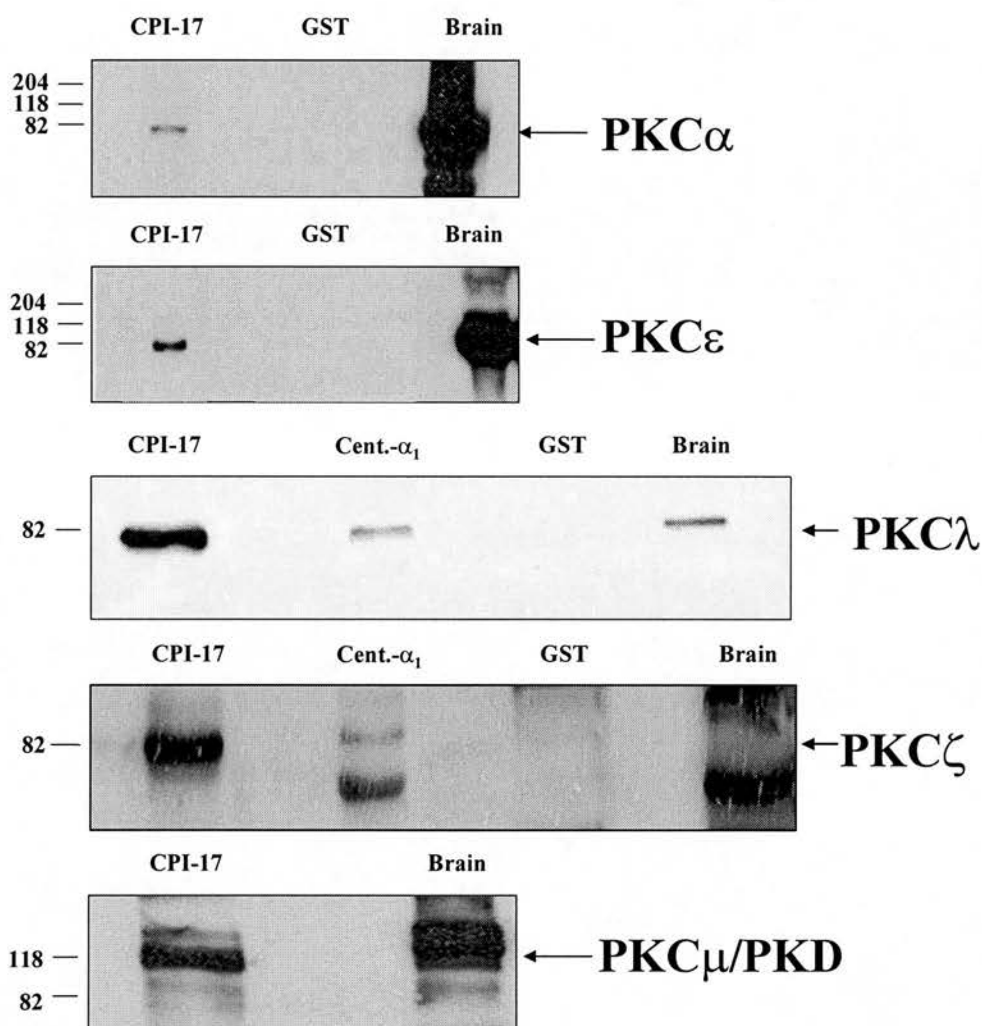


Figure 3.6 PKCs associate with the CPI-17 column.
All PKC classes elute from the GST-CPI-17 column. Protein (5 μg or 10 μg) eluted from the GST-CPI-17 ("CPI-17"), GST-centaurin-α_1 ("Cent.-α_1") or GST column was separated on 10% SDS-PAGE, transferred onto nitrocellulose and Western blotted with PKCλ and ζ antibodies (for GST-CPI-17 and GST column eluate also with PKCα and PKCε antibodies; for GST-CPI-17 column eluate only also with PKCμ antibodies). A brain extract was used as a positive control for the antibodies. The positions of the molecular weight markers (kDa) are indicated.

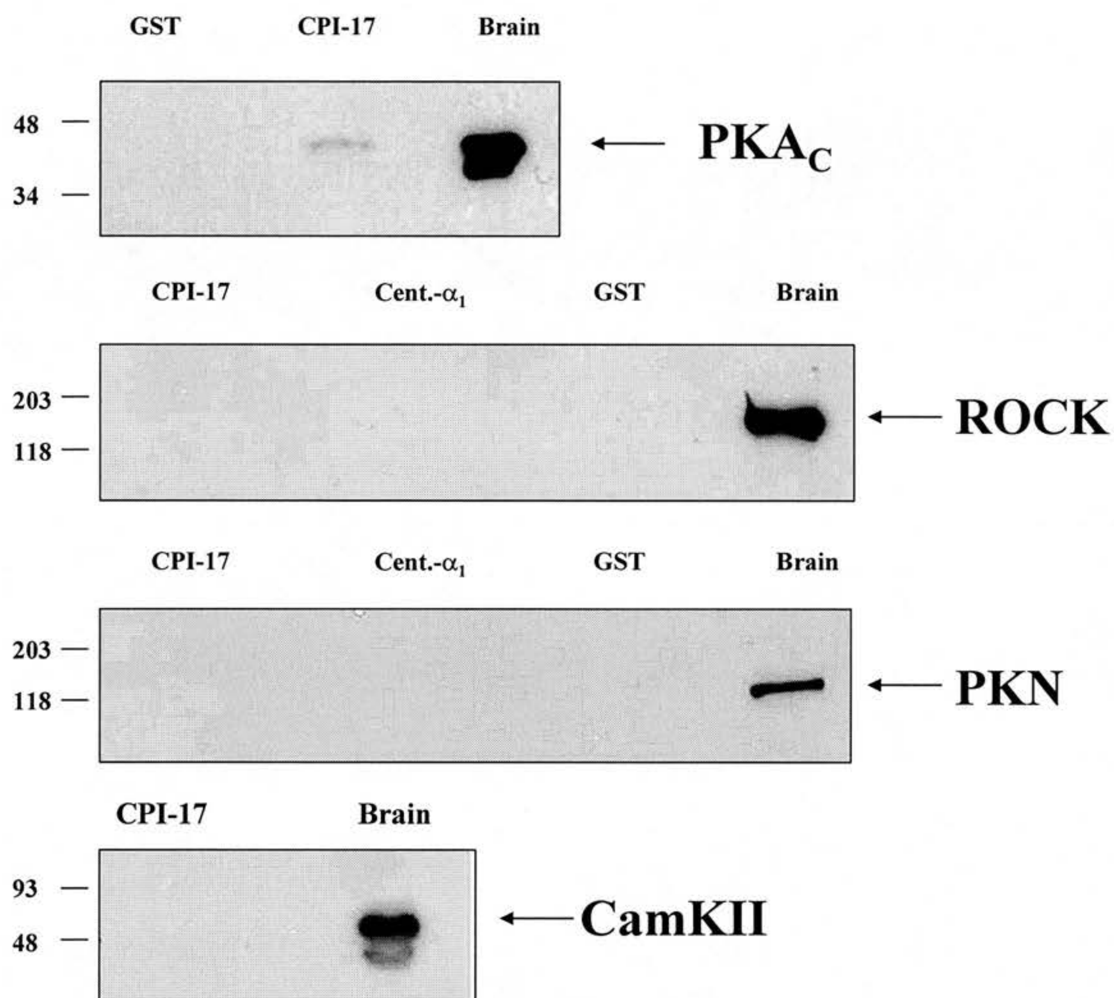


Figure 3.7 PKA$_C$ associates with the GST-CPI-17 column, but ROCK, PKN and CamKII do not.
As described for Figure 3.6, but Western blot analysis was performed with PKA$_C$, ROCK, PKN and CamKII antibodies.

3.3.3. *In vitro* binding of all PKC isoforms to CPI-17

To further investigate whether the association of PKCs with CPI-17 was selective, two different *in vitro* experiments were carried out.

Firstly, the binding between CPI-17 and PKCs from all classes was investigated using the *in vitro* transcription/translation assay (IVTT). PKCα, λ, μ and ζ were expressed and labelled with [^{35}S] methionine in a reticulocyte lysate, and incubated with GST, GST-14-3-3ζ or GST-CPI-17. All these PKC isoforms were associated with GST-CPI-17 and not with GST (Figure 3.8). GST-14-3-3ζ did not associate with PKCα, λ, and μ but interacted very weakly with PKCζ (Figure 3.8). Our data show that all PKC isoforms tested bind to CPI-17. To confirm the selectivity of the interaction between PKC and CPI-17, PKCμ and 14-3-3ζ were expressed and labelled with [^{35}S] methionine and incubated with GST and GST-CPI-17. Only PKCμ and not 14-3-3ζ bound to GST-CPI-17 (Figure 3.9). Neither PKCμ nor 14-3-3ζ bound to GST (negative control). Results from Figure 3.8 and Figure 3.9 indicated that the binding between CPI-17 and PKC was selective.

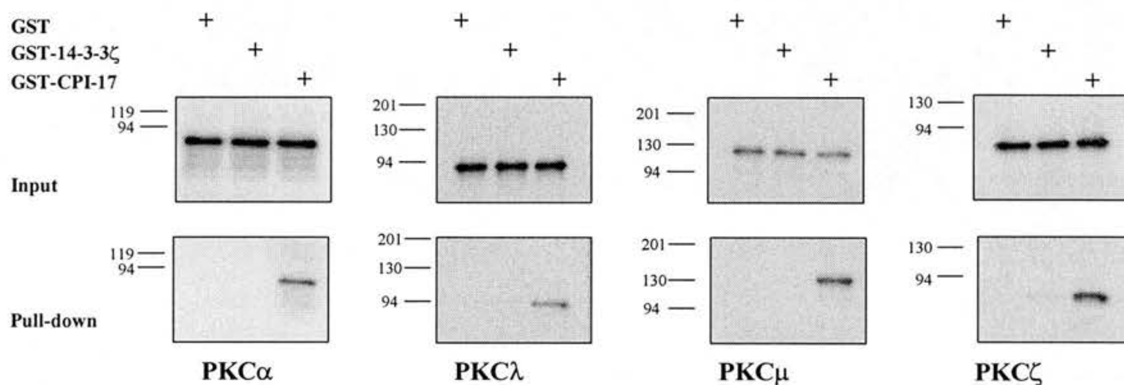


Figure 3.8 CPI-17 associates with all PKC isoforms tested.
PKCα, λ, μ and ζ were expressed and labelled with [^{35}S] methionine in a reticulocyte lysate and incubated for 15 min at 30°C with 5 μg GST, GST-14-3-3ζ and GST-CPI-17 in the presence of 1% Nonidet P-40. Glutathione-Sepharose beads were added and incubated at room temperature for 1 h. Beads were washed and samples analysed by 10% SDS-PAGE followed by autoradiography (panel "Pull-down"). An aliquot of the lysate was loaded on the gel to visualise expressed PKCs (panel "Input"). The positions of the molecular weight markers (kDa) are indicated.

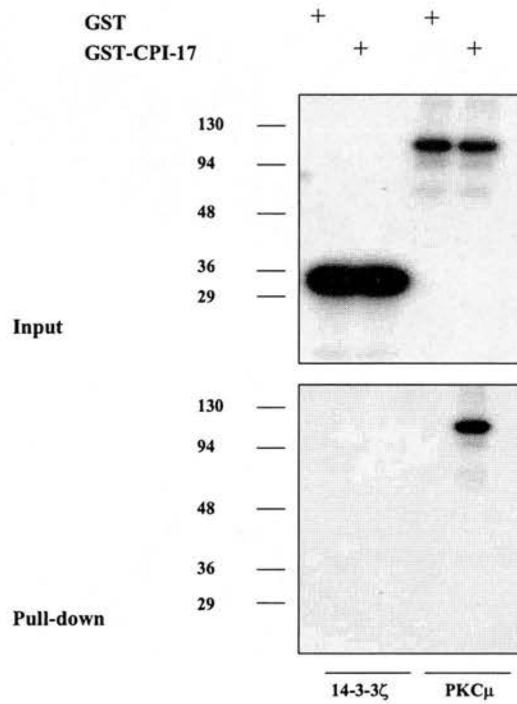


Figure 3.9 CPI-17 interacts selectively with PKC.
PKCμ and 14-3-3ζ expressed in a reticulocyte lysate, were incubated with 5 μg GST and GST-CPI-17, pulled-down with glutathione Sepharose beads and analysed as described in Figure 3.8. The positions of the molecular weight markers (kDa) are indicated.

To determine whether the binding between CPI-17 and PKC was direct, GST, GST-14-3-3ζ, GST-centaurin-α_1 and GST-CPI-17 were incubated with recombinant PKCα, PKCε, PKCμ and PKCζ, pulled-down with glutathione Sepharose beads and the samples were analysed on a 10% SDS-PAGE gel, transferred onto nitrocellulose and Western blotted with the respective PKC antibodies. The results showed that GST-CPI-17, but not GST, directly bound to all PKC isoforms tested (Figure 3.10). GST-centaurin-α_1 also bound to all PKC isoforms (Figure 3.10). In addition, 14-3-3ζ directly associated with PKCε and PKCμ, but not with PKCα nor PKCζ (Figure 3.10). These results were in contrast to those presented in Figure 3.8, where we could not detect any binding between 14-3-3ζ and PKCμ. This might be due to the presence of other proteins in the reticulocyte lysate that may compete with PKC for binding to 14-3-3ζ. However, the results in Figure 3.10 are in agreement with a reported interaction between 14-3-3ζ and PKCμ (Hausser et al., 1999) and the binding between 14-3-3ζ and PKCε has also been confirmed (Gannon-Murakami and Murakami, 2002). The results of the binding of 14-3-3ζ and PKCζ have been conflicting: van der Hoeven and co-workers show that PKCζ does not co-immunoprecipitate with 14-3-3ζ in COS cells (van der Houven van Oordt et al., 2000) whereas Gannon-Murakami and co-workers suggest constitutive binding between 14-3-3ζ and PKCζ in PC12 cells (Gannon-Murakami and Murakami, 2002). However, these authors also provided evidence that the binding between PKCα and 14-3-3ζ in PC12 cells is differentiation dependent. One possibility might be that PKCα and 14-3-3ζ need to be localised to specific subcellular compartments in differentiated cells in order to bind. It is also possible that another factor (e.g. agonist or protein partner) that is only present in differentiated cells is required which would allow the two proteins to interact. This could offer an explanation why no interaction between PKCα and 14-3-3ζ could be detected in the direct binding experiments as mentioned above. It is important to point out that purified proteins were used for this direct binding experiment and the reaction was *in vitro*, therefore, it is difficult to compare these results to experiments conducted in cells. Using two different methods, it was shown that the association between CPI-17 and PKC was selective and direct. This suggested that CPI-

17 associated with a domain common to all PKC isoforms, i.e. the cysteine rich domain (C1) or the kinase domain (see Figure 1.6 for schematic representation of primary structures of mammalian PKC family members).

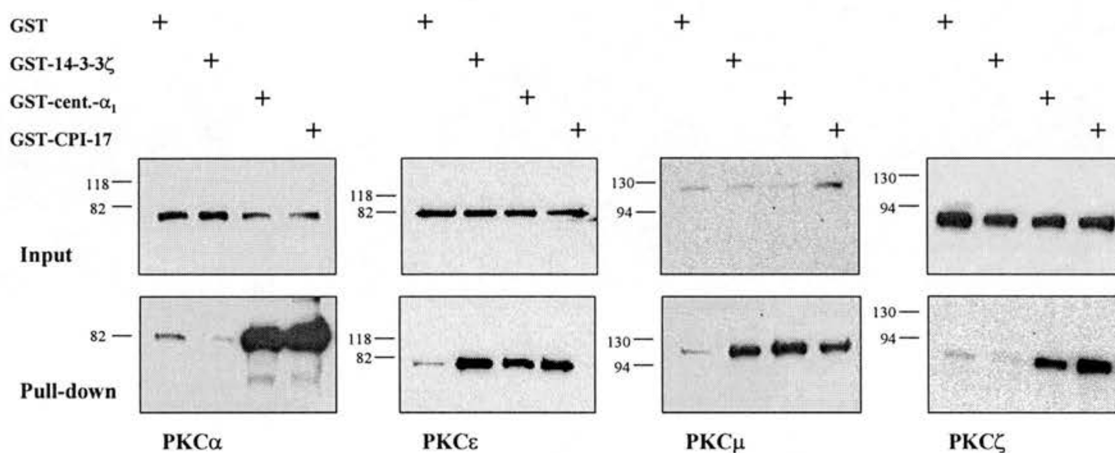


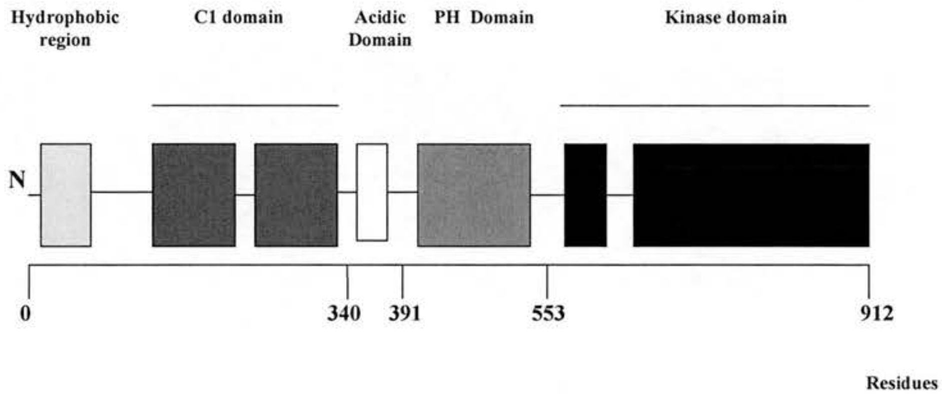
Figure 3.10 CPI-17 directly associates with PKC isoforms from all sub-families.
5 μg each of GST, GST-14-3-3ζ, GST-centaurin-α_1 and GST-CPI-17 were incubated with 0.1 μg of human recombinant PKCα, PKCε, PKCμ and PKCζ. GST pull-down assays were analysed on 10% SDS-PAGE, transferred onto nitrocellulose and Western blotted with PKCα, ε, μ and ζ antibodies as indicated. Top panels represent an aliquot of the assays to visualise the PKCs (panels "Input"). Bottom panels represent the GST pull-down assays (panel "Pull-down"). The positions of the molecular weight markers (kDa) are indicated.

3.3.4. Binding occurs via C1 domain of PKC

PKC isoforms from all PKC classes bind directly to CPI-17 (section 3.3.3) which suggests that the binding occurs via a common domain. The PKCμ isoform was used to map the CPI-17 binding site. PKCμ wt and PKCμ deletion mutants Δ1-340 (ΔC1, Cysteine Rich Domain), Δ336-391 (ΔAD, Acidic Domain) and Δ417-553 (ΔPH domain) (see Figure 3.11A for schematic diagram) were expressed, laboratory labelled with [^{35}S] methionine and incubated with GST and GST-CPI-17. The results from Figure 3.11B showed that PKCμ wt bound to GST-CPI-17. Deletion of the acidic domain or the PH domain of PKCμ did not affect the binding to CPI-17. However, no binding was observed between CPI-17 and PKCμ Δ1-340 (Figure 3.11B). Therefore, amino acids 1-340 of PKCμ were necessary for the association with CPI-17, thus suggesting that the C1 domain of PKCμ represented the target region for binding with CPI-17. To further test whether the C1 domain of all PKC isoforms is involved in the binding to CPI-17, deletion mutants of the different PKC isoforms containing only the C1 domain could have been made and used in *in vitro* transcription/translation experiments as described above. If the C1 domain was sufficient for the binding, all the deletion mutants of the different PKC isoforms should associate with CPI-17.

Since all PKC isoforms have a least one C1 domain (reviewed in Toker, 1998), these results complement those from Figure 3.6 and Figure 3.10 showing that all PKC isoforms associated *in vitro* with CPI-17. These results are in agreement with previously published data showing that a number of proteins bind to the C1 domain of PKCμ, including 14-3-3τ (Hausser et al., 1999) and Bruton's tyrosine kinase (BTK; Johannes et al., 1999). An interaction between 14-3-3ζ with PKCζ and with PKCε was also found in agreement with their association in differentiated PC12 cells (Gannon-Murakami and Murakami, 2002). It is interesting to note that the cysteine fingers in the C1 region of PKC provide a binding site for lipid second messengers as well as for regulatory proteins affecting protein kinase activity (Toker, 1998). Therefore, it would be interesting to investigate whether CPI-17 affects PKC activity.

A



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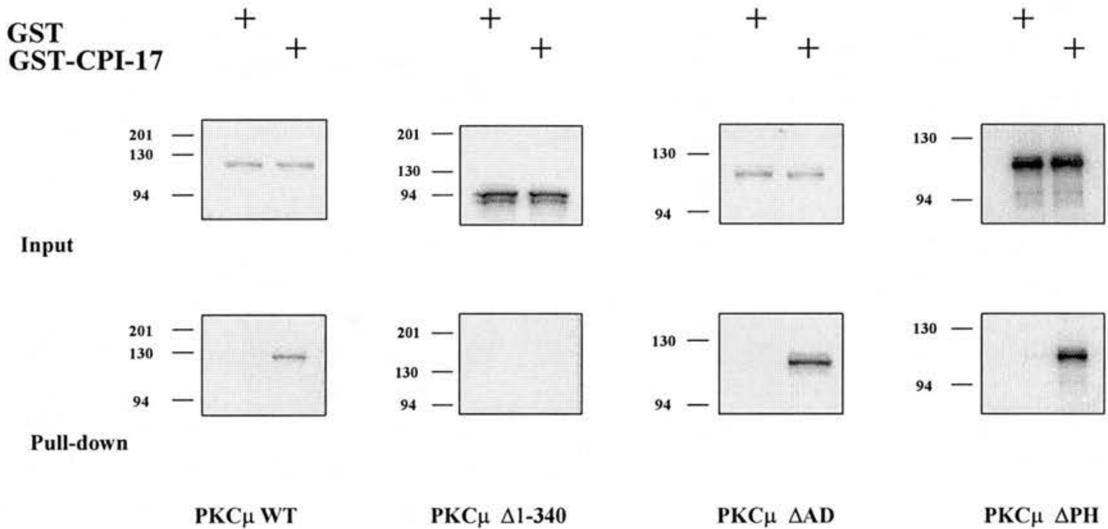

Figure 3.11 CPI-17 associates with the C1 domain of PKC.
A, Schematic representation of the known functional domains of PKCμ.
B, CPI-17 binds to the C1 domain of PKCμ. PKCμ wt and PKCμ deletion mutants (Δ1-340, ΔAD and ΔPH) were expressed in a reticulocyte lysate, and incubated with 5 μg each of GST and GST-CPI-17 as described in Figure 3.8. GST pull-down assays were analysed by SDS-PAGE and autoradiographed (panel "Pull-down"). An aliquot of the lysate was loaded on the gel (panel "Input"). The positions of the molecular weight markers (kDa) are indicated.

3.3.5. Residues 121-147 of CPI-17 are not involved in the binding to PKC

As mentioned in the Introduction (section 1.3), CPI-17 is mainly composed of a domain which is also present in PHI-1 (Eto et al., 1999) and LIM kinase-2 (LIMK-2). However, residues 121-147 are specific to CPI-17 and contain Ser-128, a residue that our laboratory has shown to be heavily phosphorylated *in vivo* (Dubois et al., 2002d). To determine whether residues 121-147 were involved in the interaction with PKC, PKCμ wt was expressed, labelled with [^{35}S] methionine and incubated with GST, GST-CPI-17 wt or GST-CPI-17 1-120. The results showed that both GST-CPI-17 wt and GST-CPI-17 1-120 bound to the same extent to PKCμ (Figure 3.12), indicating that the region 121-147 unique to CPI-17 was not involved in the interaction with PKC. This result suggests that PHI-1 and LIMK-2 might also be possible binding partners for PKCμ and possibly for other PKC isoforms. No interaction between PHI-1 and PKC has been reported so far, however, PKC has been shown to phosphorylate PHI-1 on Thr-57 (Eto et al., 1999). To the best of my knowledge there has also not been a reported interaction between LIMK-2 with PKC to date.

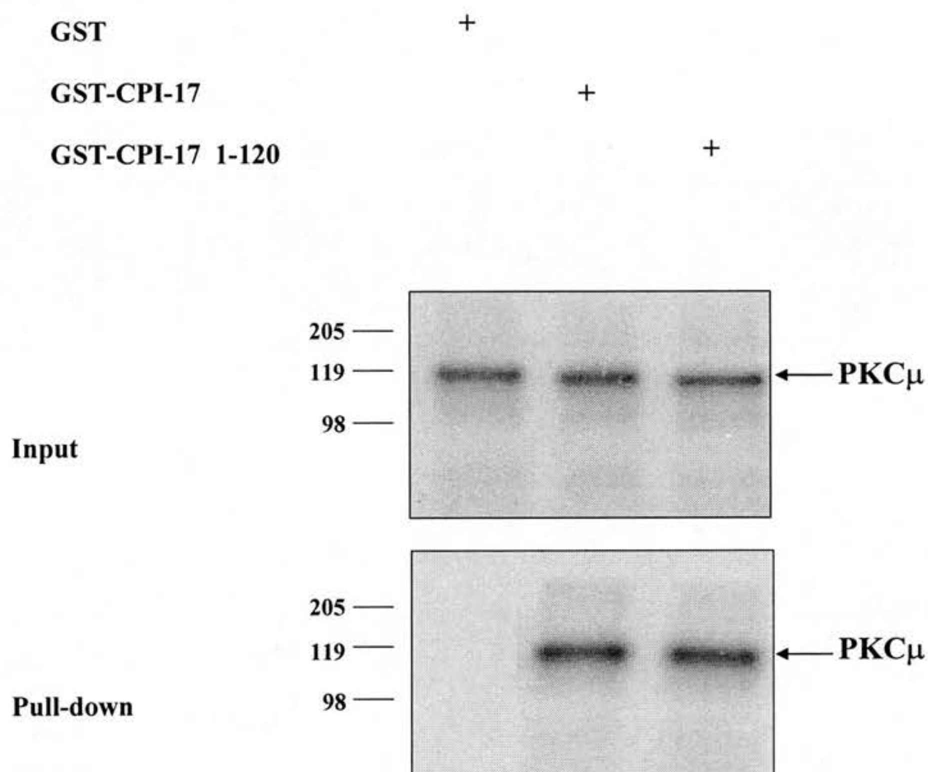

Figure 3.12 Residues 121-147 of CPI-17 are not involved in binding to PKC.
PKCμ was incubated with 5 μg of GST, GST-CPI-17 or GST-CPI-17 1-120 as described in Figure 3.8. PKC associated with the beads was analysed by SDS-PAGE and autoradiography (panel "Pull-down"). The panel "Input" represents an aliquot of the lysate. The positions of the molecular weight markers (kDa) are indicated.

3.3.6. All PKC isoforms phosphorylate CPI-17

To test whether CPI-17 was phosphorylated by different PKC isoforms, 1 U of PKCα, ε, μ and ζ were incubated with 5 μg of CPI-17 in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. The results indicated that all PKC isoforms were able to phosphorylate CPI-17 *in vitro* (Figure 3.13). From the results shown in Figure 3.13 there would appear to be a phosphorylation preference: PKCα seems to phosphorylate CPI-17 to the greatest extent, followed by PKCε, then PKCζ and PKCμ. However, this can be explained by different PKC activities as judged by autophosphorylation and histone H III phosphorylation which was used as control (data not shown). The activity of PKCμ towards CPI-17 and histone H III was very low. The results shown here and in section 3.3.3 demonstrate for the first time that PKC isoforms from different sub-families bound to CPI-17 *in vitro* and that the PKC isoforms α, ε, μ and ζ all phosphorylated CPI-17. This suggests that CPI-17 may be a general substrate for all PKC isoforms, however the interaction between the two proteins may depend on their subcellular localisation. PKCs undergo translocation from one subcellular location to another in response to extracellular stimuli and individual PKC isoforms possess a degree of substrate specificity and sensitivity to different activators (reviewed in Sim and Scott, 1999). However, since most cells synthesise a range of PKC isoforms, cell specific expression and isotype specific activation represent only a part of the mechanism for generating specificity of PKC-mediated signalling (Sim and Scott, 1999). It has been shown that many anchoring/scaffold proteins bind PKC and target it to various cellular microdomains (reviewed in Newton, 2001). One example is the family of proteins called RACKs which anchor the active conformation of phosphorylated protein kinase C at specific cellular locations (Newton, 2001). Another example are members of the family of AKAPs (A Kinase Anchoring Proteins) which position phosphorylated, but inactive PKC near relevant substrates (Newton, 2001). Furthermore, it has been suggested that anchoring proteins for inactive PKCs exist. These proteins were collectively termed RICKs (Receptors for Inactive C Kinase), however, to date, the hypothetical RICKs have not been identified (reviewed in Dorn and Mochly-Rosen, 2002). These examples

demonstrate how specific PKC isoforms can be localised to various subcellular domain and how their interaction with substrates can be controlled. To date the subcellular distribution of CPI-17 has not been studied and therefore one cannot speculate how CPI-17 distribution in cells is regulated. However, this would be an interesting point to investigate. Having shown that PKC isoforms from different PKC classes phosphorylate residue Thr-38 in CPI-17, one can speculate that the equivalent residue within LIMK-2, which is Thr-596 may also be phosphorylated by different PKC isoforms. This is supported by the fact that the equivalent residue Thr-57 in PHI-1 is phosphorylated by PKC resulting in an increased ability of PHI-1 to inhibit PP1 (Eto et al., 1999).

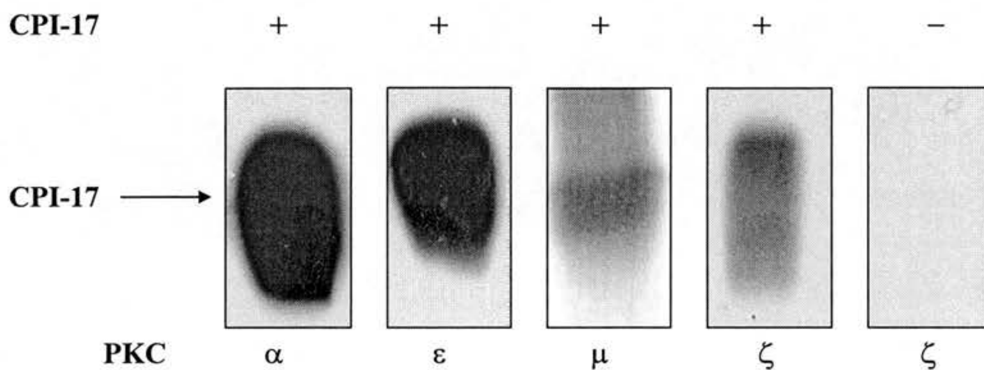

Figure 3.13 CPI-17 is phosphorylated by all classes of PKC.
One unit each of PKCα, ε, μ and ζ were incubated with 5 μg CPI-17 in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer for 15 min at 30°C. The samples were analysed on 15% SDS-PAGE followed by autoradiography. The fifth panel shows as an example the equivalent region of the gel after incubation of PKCζ in absence of CPI-17.

3.4. Characterisation of the association between CPI-17 and CKI

Our laboratory has shown that CPI-17 is one of the proteins which co-purifies with CKIα as part of a protein complex from brain (Dubois et al., 2002b). Therefore, in order to confirm this it was investigated whether CKI associated with the GST-CPI-17 column and also which CKI isoforms were associated with the GST-CPI-17 column.

3.4.1. Association of a kinase with CKIα activity with the GST-CPI-17 column

CKIα was shown to phosphorylate 14-3-3ζ and τ on residue 233 (Dubois et al., 1997). Therefore, an assay for CKI activity was devised by using 14-3-3ζ wt as a substrate and comparing its phosphorylation with a 14-3-3ζ T233A point mutant. To verify whether a kinase with CKIα-like activity eluted from the CPI-17 column, CPI-17 column eluate, GST column eluate and centaurin-α_1 column eluate were incubated with 14-3-3ζ wt or the 14-3-3ζ T233A mutant in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. The samples were then analysed on 12.5% SDS-PAGE and autoradiographed. Only 14-3-3ζ wt, but not the 14-3-3ζ T233A mutant, was phosphorylated by a kinase present in the GST-CPI-17 eluate (Figure 3.14). No phosphorylation of 14-3-3ζ wt or T233A mutant was detected when 14-3-3ζ wt or 14-3-3ζ T233A was incubated with the GST-centaurin-α_1 column eluate or GST column eluate (Figure 3.14). This result indicated that there was a protein kinase, specifically bound to CPI-17, which was able to phosphorylate 14-3-3ζ on the CKIα site, suggesting that CKIα from brain bound to the GST-CPI-17 column. One could expect that a kinase which phosphorylates 14-3-3ζ on the CKIα site would also elute from the GST-centaurin-α_1 column as a direct association between CKIα and centaurin-α_1 has been shown (Dubois et al., 2001). As described in 3.4.2, CKIα did associate with the centaurin-α_1 column, however, the amount of CKIα associated with the GST-centaurin-α_1 column was significantly lower than the amount associated with the GST-CPI-17 column and therefore the CKI assay used might not have been sensitive enough to detect any CKI activity associated with the GST-centaurin-α_1 column. Another explanation

might be that there are other proteins present in the centaurin-α_1 eluate which are absent from the CPI-17 column eluate and which inhibit CKIα activity.

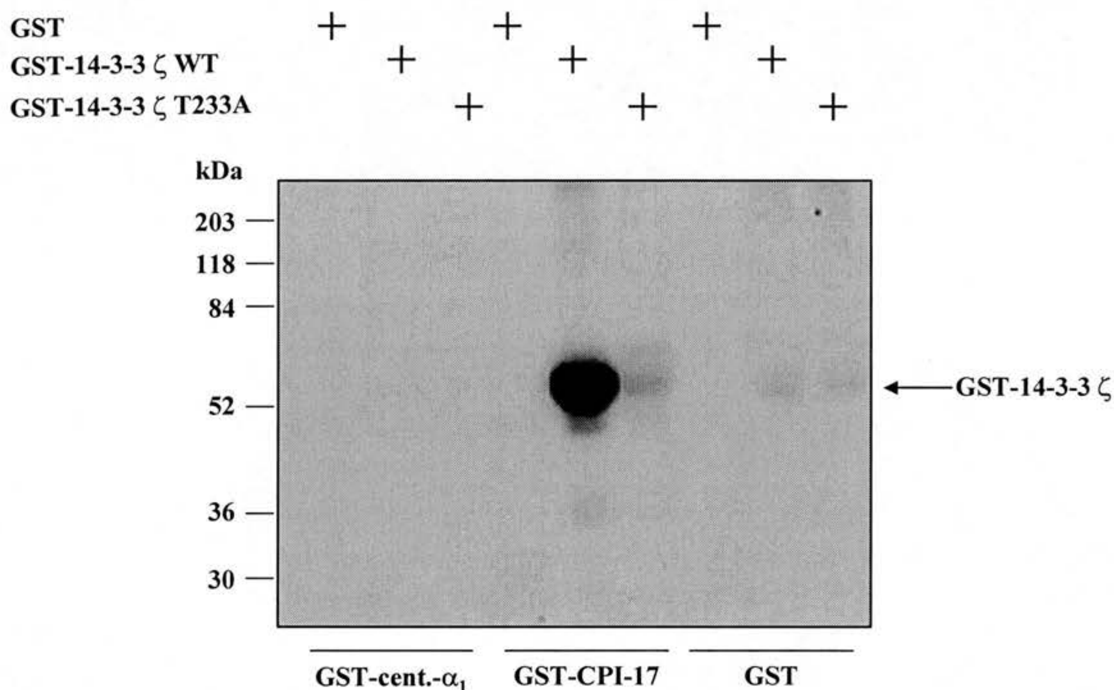

Figure 3.14 CKIα-like activity associates with the GST-CPI-17 column.
5 μg of protein eluted from the GST-centaurin-α_1 column (3 first lanes), GST-CPI-17 column (3 middle lanes) or from the GST column (3 last lanes) were incubated for 30 min at 30°C with 5 μg GST, GST-14-3-3ζ wt and GST-14-3-3ζ T233A in the presence of 50μM [γ-^{32}P] ATP and kinase buffer. A GST pull-down assay was performed and the samples were analysed by 12.5% SDS-PAGE and autoradiography. The positions of the molecular weight markers (kDa) are indicated, as is the position of the phosphorylated GST-14-3-3ζ.

3.4.2. CKIα and ε bind to the GST-CPI-17 column

To verify whether CKI bound to the GST-CPI-17 column, proteins eluted from the GST-CPI-17, GST and GST-centaurin-α_1 column were separated on a 12.5% SDS-PAGE gel, transferred onto nitrocellulose and Western blotted with anti-CKIα or anti-CKIε antibodies. Both CKIα and ε isoforms were eluted from the GST-CPI-17 and GST-centaurin-α_1 column, but not from the GST column (Figure 3.15). This supports the findings in 3.4.1 where it is shows that a kinase was present in the CPI-17 eluate which phosphorylates the CKIα site on 14-3-3ζ. It was also tested whether other CKI isoforms, including CKIγ_2 and CKIδ were present in the CPI-17 or centaurin-α_1 column eluate, however, the antibodies were not of good quality and were not able to specifically detect CKIγ_2 or δ from brain which was used as positive control (data not shown). The high-mobility group 1 (HMG1) protein, which also co-purified with CKIα from brain (Dubois et al., 2002b), did not associate with the GST-CPI-17 column (Figure 3.15), verifying the selectivity of the CKI association with CPI-17.

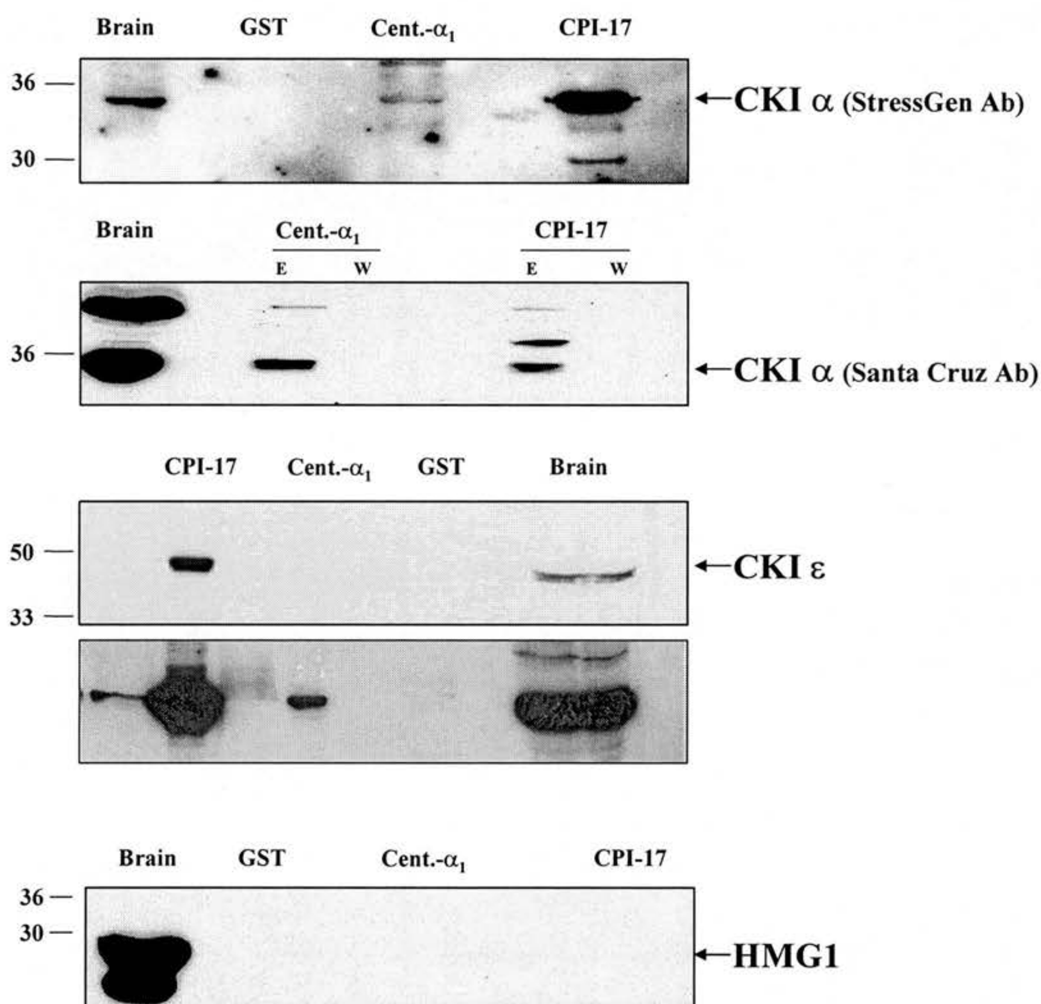

Figure 3.15 CKIα and CKIε were eluted from the GST-CPI-17 column.
5 μg of GST-CPI-17 column eluate ("CPI-17"), GST-centaurin-α_1 column eluate ("Cent.-α_1"), GST column eluate ("GST") and 1 μg of brain lysate ("Brain") were run on 12.5% SDS-PAGE, transferred onto nitrocellulose and Western blotted with CKIα (StressGen antibody and Santa Cruz antibody), CKIε (two different exposures) and HMG1 antibodies as indicated. The positions of the molecular weight markers (kDa) are indicated as are the positions of CKIα, CKIε and HMG1. E and W indicate column eluate and column wash, respectively.

3.4.3. *In vitro* binding of all CKI isoforms to CPI-17

Two different *in vitro* experiments were used to further investigate whether the association of CKI with CPI-17 was selective. CKIα, δ, ε and γ_1, γ_2 and γ_3 were expressed and labelled with [^{35}S] methionine, then incubated with GST or GST-CPI-17. All the CKI isoforms tested bound to CPI-17 (Figure 3.16). However, the association was stronger with CKIα, δ, and ε than with CKIγ_{1-3}. Therefore, it appeared that CPI-17 interacted preferentially with some CKI isoforms. This is in contrast to centaurin-α_1 that we found to interact to a similar extent with all CKI isoforms (Dubois et al., 2001). This suggests that CPI-17 may be a tissue specific substrate for different CKI isoforms and that the interaction between the two proteins may depend on their subcellular localisation. As mentioned in Results section 3.3.6, the subcellular localisation of CPI-17 is unknown. However, it has been suggested that the subcellular localisation of CKI isoforms is essential for their the function. Mammalian CKIα for example, is not distributed homogeneously throughout the cell, but associates with distinct structures and factors throughout the cell (Gross and Anderson, 1998): CKIα has been demonstrated to possess a cell-cycle-dependent subcellular distribution, including the binding to the centrosome, cytosolic vesicles (e.g. vesicles of the *trans*-Golgi network), the mitotic spindle and distinct nuclear structures. Another CKI isoform, CKIδ, has been shown to associate with different cellular targets in a cell-cycle dependent manner: CKIδ co-localises with the *trans*-Golgi network (TGN) and interacts with microtubules in interphase cells (Behrend et al., 2000b). During mitosis, CKIδ is evenly distributed throughout the cytoplasm, however, upon DNA damage CKIδ specifically associates with the mitotic spindle. These examples indicate that the function and regulation of CKI isoforms may result from defining its localisation spatially and temporally. Therefore, it is possible that if specific CKI isoforms and CPI-17 interact *in vivo*, this association might also be dependent on the subcellular localisation of both proteins and possibly also on the stage of the cell cycle. It is interesting to note that the expression level and localisation of another inhibitor, PP1inhibitor-2, fluctuate during different stages of the cell cycle (Brautigan et al., 1990; Kakinoki et al., 1997).

3.4.3.1. Binding selectivity

To investigate binding selectivity between CPI-17 and CKI, CKIα and 14-3-3ζ were expressed and labelled with [^{35}S] methionine and incubated with GST and GST-CPI-17. GST-CPI-17 bound to CKIα, but 14-3-3ζ did not associate with GST-CPI-17 (Figure 3.17A). CKIδ was the CKI isoforms which was particularly well expressed in a reticulocyte lysate and therefore it was used in the second binding selectivity experiment: CKIδ was expressed and labelled with [^{35}S] methionine and incubated with GST (negative control), GST-CPI-17, GST-14-3-3ζ (positive control), GST-VAMP (vesicle associated membrane protein involved in exocytosis; reviewed in Gerst, 1999) and GST-centaurin-α_1 as control (Figure 3.17B). Results in Figure 3.17B confirmed the interaction between CPI-17 with CKI. Centaurin-α_1 also associated with CKIδ confirming previous reports (Dubois et al., 2001). GST-14-3-3ζ also bound to CKIδ (Figure 3.17B). This is the first demonstration that CKI isoforms bind to 14-3-3 although it was previously shown by our laboratory that 14-3-3ζ is phosphorylated by CKIα (Dubois et al., 1997).

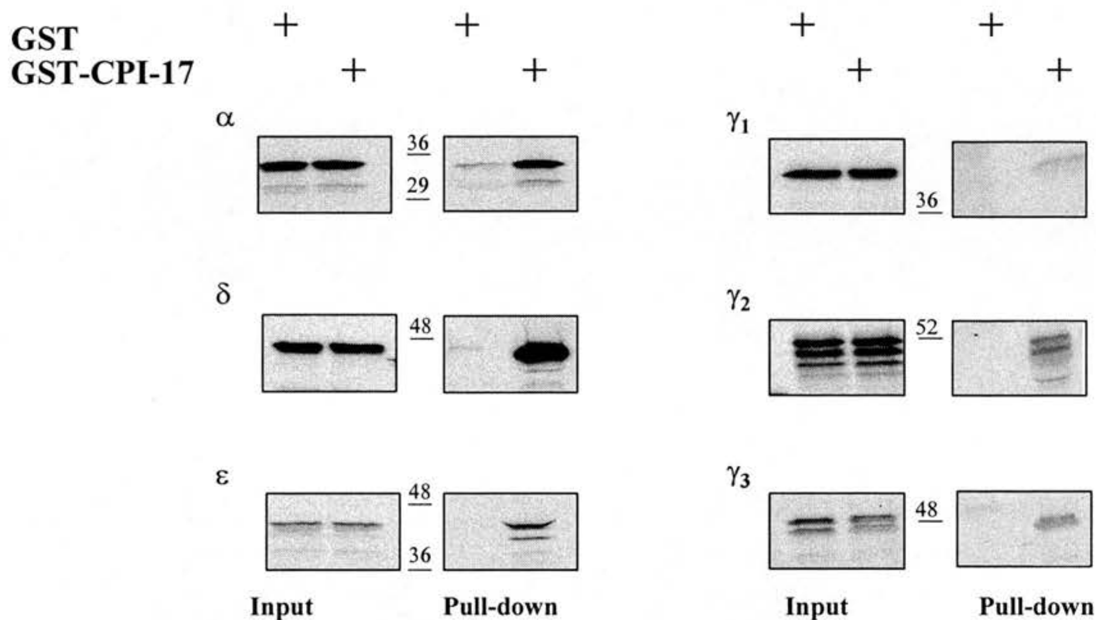

Figure 3.16 CPI-17 associates with all CKI isoforms.
CKIα, δ, ε and $\gamma_{1\text{-}3}$ isoforms (as indicated) were expressed in a reticulocyte lysate and incubated with 5 μg GST or GST-CPI-17 as described in Figure 3.8. GST-pull-down assays were analysed by 12.5% SDS-PAGE and autoradiography ("Pull-down"). An aliquot of the lysate was analysed ("Input"). The positions of the molecular weight markers (kDa) are indicated.

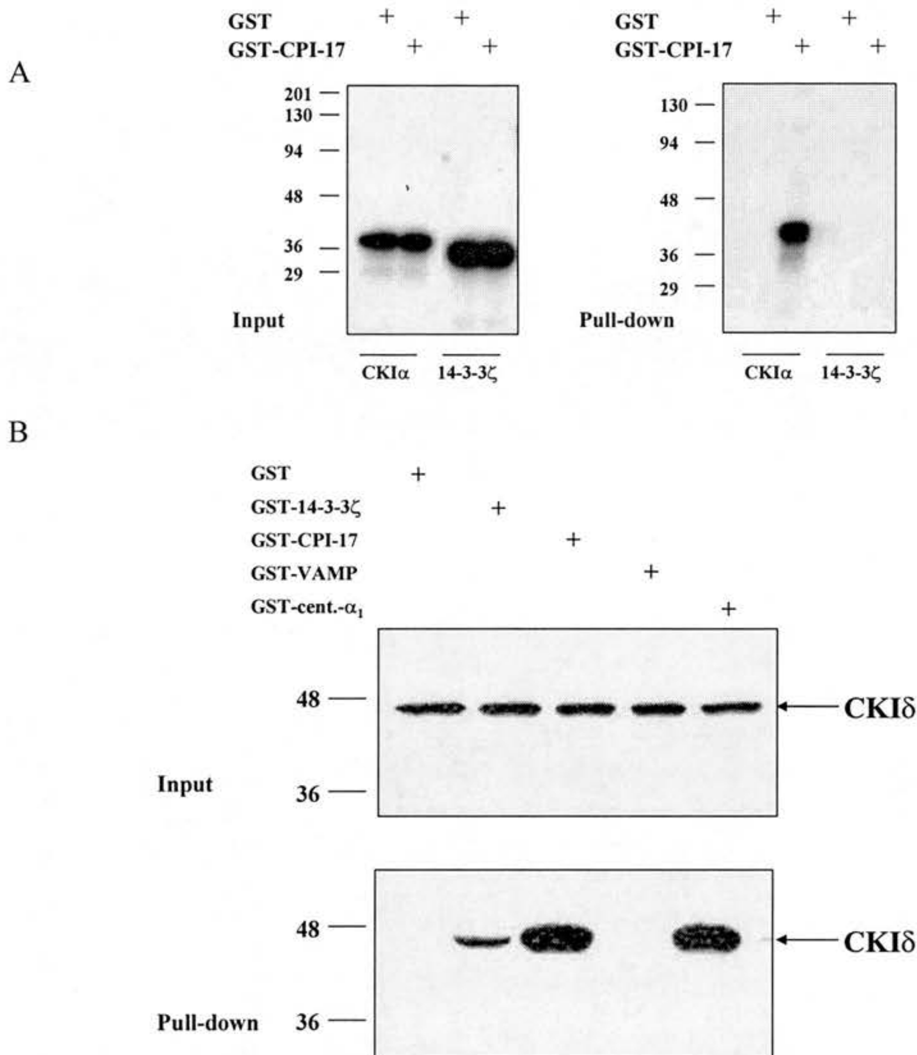

Figure 3.17 Binding selectivity between CKI and CPI-17.
A, CKI associates with CKIα but not with 14-3-3ζ.
CKIα and 14-3-3ζ were expressed in a reticulocyte lysate and incubated with 5 μg GST or GST-CPI-17 as described in Figure 3.8. The left panel "Input" shows an aliquot of the lysate, and the panel "Pull-down" shows the proteins bound to GST-CPI-17. The positions of the molecular weight markers (kDa) are indicated.
B, CKI associates selectively with CPI-17. CKIδ, expressed in a reticulocyte lysate was incubated with 5 μg GST, GST-14-3-3ζ, GST-CPI-17, GST-VAMP or GST-centaurin-α_1 as described Figure 3.8. The upper panel "Input" shows an aliquot of the lysate, and the panel "Pull-down" shows the proteins bound to the GST-fusion proteins. The positions of the 36 and 48kDa molecular weight markers are indicated on the left, and the position of CKIδ is shown on the right.

3.4.3.2. Direct binding between CKI and CPI-17

To determine whether the association between CKI and CPI-17 was direct or mediated through other proteins which might have been present in the reticulocyte lysate, recombinant CKI (*ski 1, S. pombe*) was incubated with purified GST, GST-CPI-17, and GST-centaurin-α_1 (used as a positive control; Dubois et al., 2001). The GST fusion proteins were pulled-down with glutathione Sepharose beads and a kinase assay was performed on the precipitated beads using the CKI phosphopeptide substrate. The results show that CKI directly associated with GST-centaurin-α_1, (confirming our previous results) and with GST-CPI-17 (Figure 3.18). These results also indicate that structural features necessary for this interaction exist when the proteins are expressed in bacteria. This may suggest that the interaction between CPI-17 and CKI is not dependent on post-translational modifications.

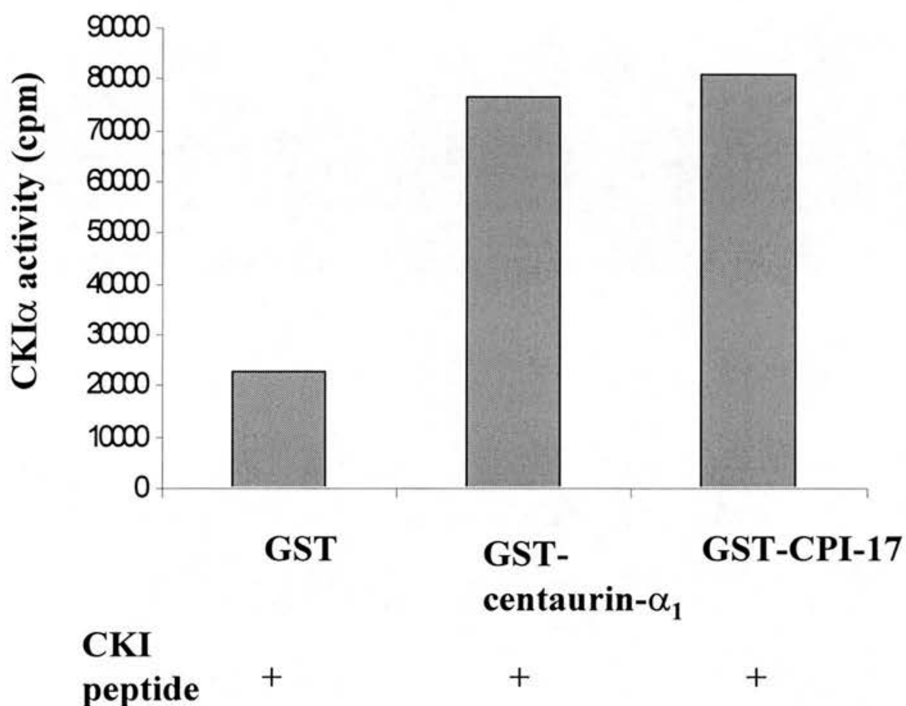

Figure 3.18 CPI-17 directly binds to CKI.
Recombinant CKI (0.2 μg) was incubated with 5 μg of GST, GST-centaurin-α_1 or GST-CPI-17 for 2 h at 4 °C. Glutathione-Sepharose beads were then added. Beads were washed and subjected to an *in vitro* kinase assay using the CKI-specific phosphopeptide substrate. The presence of ^{32}P incorporated into the peptide (cpm) was quantified by liquid scintillation counting and represents CKI activity associated with the beads.

3.4.4. CPI-17 binds to a region within the kinase domain of CKI

Since CPI-17 bound to all CKI isoforms tested (α, δ, ε, γ_1, γ_2 and γ_3), one can propose that CPI-17 interacted with a region common to all members of the CKI family. Therefore a range of CKIα deletion mutants were tested (see Figure 3.19 for schematic diagram) and CKIδ Δ317 (deleted of the C-terminal tail). CKIα wt, CKIδ Δ317 and a range of CKIα deletion mutants (see Figure 3.19A) all bound to CPI-17 (Figure 3.19B). These data demonstrated that the kinase domain of CKI is sufficient for binding to CPI-17. CKI isoforms are characterised by a conserved kinase domain and by variable amino-and carboxyl-terminal tails. CKI isoforms are more than 50% identical at the amino acid level within the catalytic domain; the catalytic domain of CKIε for example is more than 98% identical with the catalytic domain of CKIδ (Gross and Anderson, 1998). The binding decreased significantly between the CKIα 233-325 mutant and CPI-17. Therefore, the binding site between CKIα and CPI-17 can be narrowed down to CKIα residues 217-233. These results are in agreement with a previous report from our laboratory showing that centaurin-α_1 also binds to CKIα residues 217-233 (Dubois et al., 2001). To further confirm the association of CPI-17 with CKIα residues 217-233, a peptide column containing these CKIα residues could be constructed and the association of purified CPI-17 with this column could be tested.

Because all CKI isoforms tested bind to CPI-17 as shown in 3.4.3, it was assumed that the binding occurred through the kinase domain which is highly conserved within the CKI family. However, it was found that the binding between CPI-17 to CKIγ_{1-3} was weaker than to the other CKI isoforms. This could be due to the fact that CKIγ_{1-3} isoforms contain a longer amino terminal domain (Gross and Anderson, 1998) which could interfere with the binding to CPI-17. The residues corresponding to 217-233 in CKIα, which were mapped as the binding site to CPI-17 are highly conserved throughout all the CKI isoforms tested (see Figure 3.19C for schematic representation of this domain).

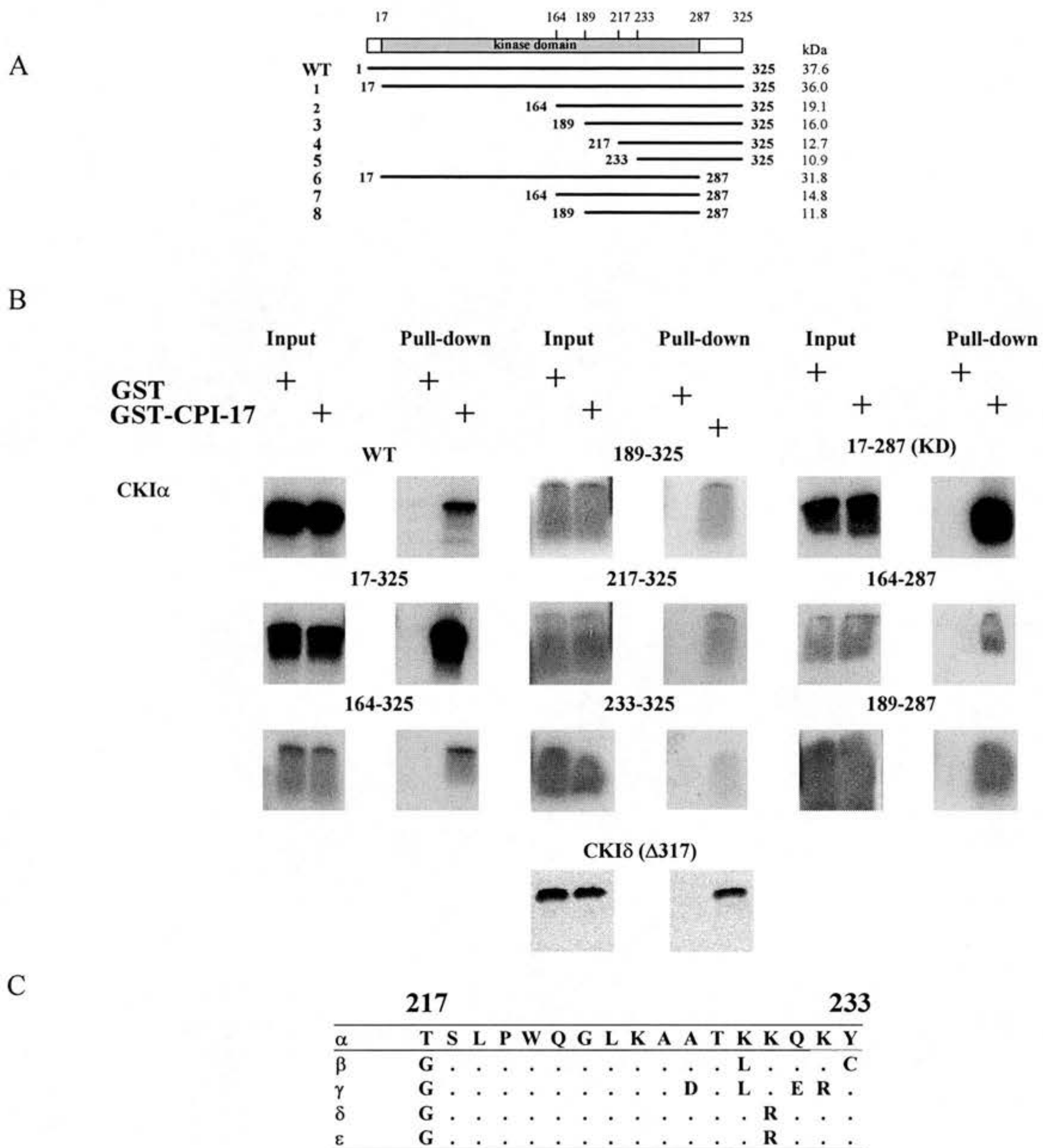

Figure 3.19 CPI-17 binds to a region within the kinase domain of CKI isoforms.
A, Schematic representation of CKIα deletion mutants.
B, CPI-17 binds to a region within the kinase domain of CKI. CKIα wt and CKIα mutants 17-325, 164-325, 189-325, 217-325, 233-325, 17-287, 164-287, 189-287 and CKIδ Δ317 were expressed in a reticulocyte lysate and incubated with 5 μg of GST or GST-CPI-17 as in Figure 3.8. An aliquot of the lysate ("Input") and the GST pull-down assays ("Pull-down") were analysed as in Figure 3.8.
C, Sequence alignment of human CKI isoforms of residues corresponding to residues 217-233 in CKIα.

3.4.5. Residues 121-147 of CPI-17 are not involved in the binding to CKI

As described in the Introduction (section 1.3), CPI-17 is mainly composed of a domain which is also present in PHI-1 (Eto et al., 1999) and LIM kinase-2. To test whether the residues 121-147 that are unique to CPI-17 were involved in CKI binding, the kinase domain of CKIα was expressed and labelled with [^{35}S] methionine and incubated with GST, GST-CPI-17 wt or GST-CPI-17 1-120. The results showed that both GST-CPI-17 wt and GST-CPI-17 1-120 bound to the kinase domain of CKIα (Figure 3.20). The association between CKIα with CPI-17 1-120 seems to be stronger compared to the association with GST-CPI-17 wt. This could indicate that the C-terminus of CPI-17 may possibly negatively regulate CKI binding to CPI-17. This result also demonstrated that the region unique to CPI-17 containing Ser-128, which is heavily phosphorylated *in vivo* (Dubois et al., 2002d), was not involved in the interaction with CKI. As described for PKCμ (section 3.3.5), this result further suggests that PHI-1 and LIMK-2 might also be possible binding partners for CKIα. Combining the results from 3.3.5, where it was shown that CPI-17 residues 121-147 are not necessary for the binding to PKCμ and the results described in this section, it appears that the binding of CPI-17 to its binding proteins occurs via the first 1-120 residues in CPI-17. Although residues 121-147 are not involved in binding to CKIα they may have a regulatory role conferring specificity on binding.

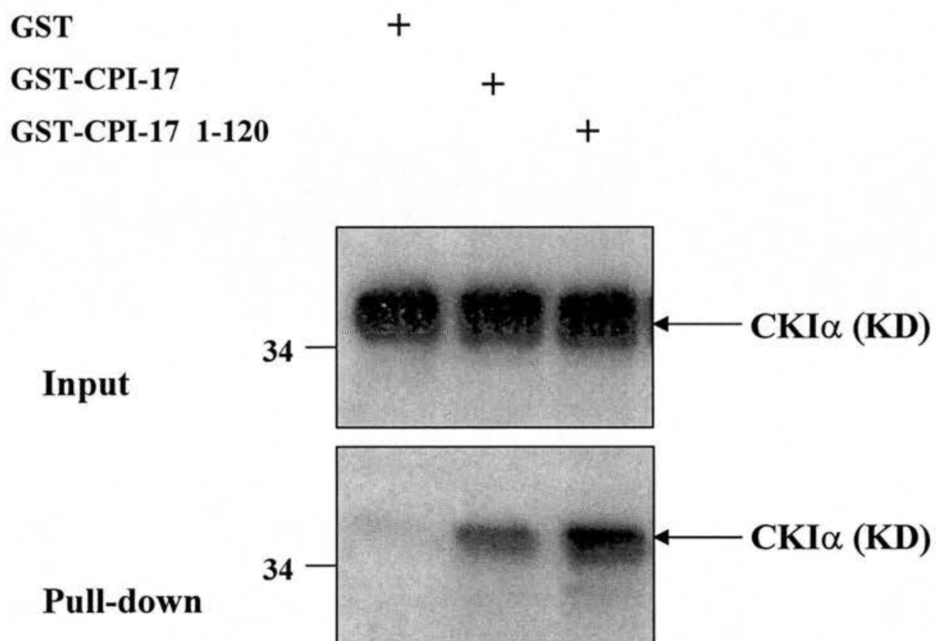

Figure 3.20 Residues 121-147 of CPI-17 are not involved in binding to CKI.
The kinase domain of CKIα (CKIα KD) was incubated with 5 μg of GST, GST-CPI-17 and GST-CPI-17 1-120 as described in Figure 3.8. An aliquot of the lysate ("Input") and the GST pull-down assays ("Pull down") were analysed as in Figure 3.8. The position of the molecular weight markers are indicated on the left, and the position of CKIα KD on the right.

3.4.6. CKI does not phosphorylate CPI-17 to any significant extent

Having shown the interaction between CPI-17 and CKI, the next step was to determine whether CPI-17 was a substrate for CKI. Recombinant 14-3-3τ (from Dr Thierry Dubois) and CPI-17 (5 μg each) were incubated with 0.1 μg recombinant CKI in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. The samples were analysed by SDS-PAGE followed by autoradiography. Figure 3.21 shows that the positive control, 14-3-3τ, was phosphorylated by CKI, but CPI-17 was not phosphorylated to any significant extent by CKI. From that it was concluded that CPI-17 is probably not a substrate for CKI *in vivo*. However, CKI isoforms have been shown to phosphorylate some substrates only if they have been previously phosphorylated by another kinase two or three residues C-terminal to the CKI site. The consensus phosphorylation sequence for CKI has been defined as S/T/Y(P)$X_{1\text{-}2}$S/TY or D_n-$X_{1\text{-}2}$S/T/Y where S/T/Y(P) stands for any phosphorylated serine, threonine or tyrosine residue, X is any amino acid and D is aspartic acid (Vielhaber and Virshup, 2001). Our laboratory has shown by HPLC/mass spectrometry that CKI does phosphorylate CPI-17 very weakly within the peptide comprising residues 126-141 (Q^{126}PSPSHDG<u>S</u>LSPLQDR^{141}). Residue 134 (underlined in sequence) represents the CKI consensus site. To test whether phosphorylation of residue 130 or 128 would create a better consensus site for CKI, synthetic peptides phosphorylated on these residues were used as substrates for CKI. The results indicated that these phosphorylations did not create a better consensus site for CKI (SpHDG<u>S</u>) (A. Aitken, S. Clokie and R. Wakefield, unpublished data) confirming the results that CPI-17 is not phosphorylated by CKI to any significant extend.

Centaurin-α_1, which also directly associates with CKIα has also been shown not to be a CKIα substrate (Dubois et al., 2001).

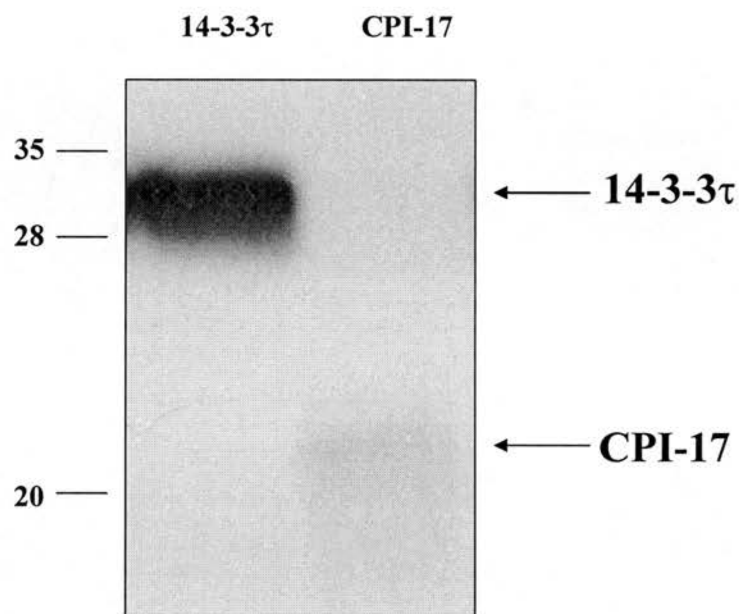

Figure 3.21 CPI-17 is not a significant substrate for CKI.
Recombinant CPI-17 and 14-3-3τ (5 μg) were incubated for 15 min at 30°C with 0.1 μg recombinant CKI in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. The samples were analysed on 15% SDS-PAGE followed by autoradiography. The positions of the molecular weight markers are indicated on the left, and those of CPI-17 and 14-3-3τ on the right.

3.4.7. CPI-17 regulates CKI activity

It was shown in section 3.4.6 that CPI-17 is not a substrate for CKIα. Therefore, it was tested whether CPI-17 had an effect on CKIα activity. For that purpose, CPI-17 wt, CPI-17 T38A, CPI-17 T38D, CPI-17 S128A, CPI-17 S128D and CPI-17 1-120 were incubated with CKIα in the presence of a CKI-specific phosphopeptide substrate. The Thr-38 mutants were used, because it has been shown that phosphorylation of this residue *in vivo* increases the ability of CPI-17 to inhibit PP1-C by 1000-fold (Eto et al., 1995) and it was important to test whether a mutation to aspartate, which through its negative charge mimicks the phosphorylated state, would have an effect on CKIα activity. The S128D mutant has been used, because our laboratory has identified residue S128 as a novel CPI-17 *in vivo* phosphorylation site (Dubois et al., 2002d) and again it was important to test whether a mutation of this residue would modulate CKIα activity.

The results show that CKIα was inhibited by CPI-17 wt by 37% and by CPI-17 S128D by 29% (Figure 3.22). The values for CPI-17 mutants CPI-17 T38A, T38D and CPI-17 1-120 were 103%, 99% and 104%, respectively (Figure 3.22). These mutants did not have an effect on CKIα activity (Figure 3.22). Interestingly, the CPI-17 S128A mutant seemed to activate CKIα by 29% (Figure 3.22). The p-values were calculated using the unpaired t-test calculator (GraphPad.com).

To see whether complete inhibition of CKIα could be achieved, the incubation time of CPI-17 wt and mutants with CKIα was increase from 15 min to 1 hour, but no difference in CKIα activity was observed (data not shown). Because the CPI-17 1-120 mutant had no effect on CKIα activity the inhibitory effect must be due to residues 121-147. Similar data were obtained when casein was used as a substrate (data not shown). Since the phosphorylation of both peptide and protein substrates were affected to a similar extent by the CPI-17 constructs, the inhibition of CKIα is clearly not simply due to restricted access of a relatively large protein substrate to the kinase active site. This is supported by our finding that residues 189-287 of CKIα are sufficient for binding CPI-17. This region comprises the C-terminal part of the kinase domain and excludes the

ATP binding site and the active site lysine residue. It was also tested whether the CPI-17 peptides containing residues 120-135 (KQPGFPQPSPSDDPSC, from Peter Fletcher, NIMR, Mill Hill, London), had an effect on CKIα activity. One of the peptides was unphosphorylated on residue S128, the other peptide was phosphorylated on S128 (underlined in the sequence). Neither of these two peptides were capable of modulating CKIα activity, using peptide concentrations from 0.1 μM to 10 μM (data not shown). From this it can be concluded that either the region containing residues 120-147, the full length protein or the region containing residues 135-147 is required to affect CKIα activity. One possibility might be that residues 1-120 are required to bind to CKIα first and that only then the C-terminus is capable of inhibition. However, this is only a hypothesis. It should be noted that a GST-fusion of CPI-17 wt or mutants did not modulate CKIα activity in any way (data not shown). This may be due to steric hindrance of the active site by the GST moiety which is fused to the N-terminal region of CPI-17. The lack of inhibition for the T38A and T38D mutants could be explained by possible structural changes of CPI-17. Ohki and co-workers have shown by solving the NMR structure of CPI-17 that phosphorylation of residue T38 by PKC, changes the conformation of CPI-17 responsible for activation and resulting in the formation of a specific interaction with the catalytic site of MLCP (Ohki et al., 2001). Therefore one can imagine that a mutation of that residue could have a similar effect. This could account for the loss of inhibitory activity. Furthermore, it has been shown that the phosphorylation of CPI-17 on residue T38, induces a conformational change, resulting in exposure of the nearby phosphorylation loop and in forming specific interactions with the catalytic site of the phosphatase (Ohki et al., 2001). Because the phosphorylation of T38 plays such an important role in regulating PP1-C (Eto et al., 1995), it was also tested whether CPI-17 phosphorylated on T38 by PKCα would have an effect on CKIα activity. The result was negative (data not shown) and therefore it can be concluded that T38 does not seem to be involved in modulating CKIα activity. This supports the hypothesis that residues 121-147 might be responsible for modulating CKIα activity. It should be noted that preliminary results show that CPI-17 S128D had no inhibitory

effect on PP1-C (data not shown; this work was carried out in Carol Mackintosh's laboratory, MRC Protein Phosphorylation Unit, Dundee). This is in agreement with data obtained by Hayashi and co-workers who show that residues 35-120 of CPI-17 are sufficient for PP1 inhibition (Hayashi et al., 2001).

Intriguingly, the presence of CPI-17 S128A in the kinase assay reaction leads to an increase in CKIα activity. One possibility might be that a mutation of residue 128 to an alanine changes the conformation of CPI-17 and makes it a substrate for CKI. The increased CKI activity could represent phosphorylation of CPI-17 S128A in addition to the CKI specific phosphopeptide because the assay method used did not distinguish between the two. This hypothesis was tested, by incubating 5 μg of CPI-17 wt, CPI-17 1-120 and CPI-17S128A with CKIα. However, no increased phosphorylation of CPI-17 S128A by CKIα was detected. Neither CPI-17 wt or the mutants were substrates for CKIα (data not shown). Therefore, this possibility does not offer an explanation for increased CKIα activity. The alternative option could be that CPI-17 S128A has a different conformation compared to the CPI-17 wt and the other mutants and stabilises CKIα, resulting in an increased concentration of active CKIα and increased substrate phosphorylation. The effect of CPI-17 on CKI activity was selective, because CPI-17 wt did not modulate CKII activity (Figure 3.24). The p-value for CPI-17 wt was 0.5837. However, it is not known whether CPI-17 associates with CKII and therefore it would have been useful to test the effect of CPI-17 on a kinase which is known to bind to CPI-17, for example PKC.

CPI-17 and CPI-17 S128D mutant inhibit CKIα activity by around 30%, whereas CPI-17 S128A increases the activity of CKIα by around 30%. The CPI-17 1-120 mutant has no effect on CKIα activity. This indicates that the CPI-17 residues 121-147 could have a regulatory role on CKIα activity as discussed earlier in section 3.4.5. CKI has previously been shown to associate with and phosphorylate the PP1 inhibitor DARPP-32, indicating that CKI could function as a modulator of protein phosphatase I via

DARPP-32 (Desdouits et al., 1995a and b). In this case, however, the reverse situation seems to apply in which the PP1 inhibitor, CPI-17, modulates CKIα activity.

In the next part of this study the CPI-17 concentrations which gave half-maximal inhibition/activation were calculated. Different concentrations (1 nM-10 μM) of CPI-17 wt, CPI-17 S128D and CPI-17 S128A were incubated with CKIα and a kinase assay was carried out using CKIα specific phosphopeptide. Prism 3 from GraphPad.com was used to calculate the half maximum inhibition/activation concentrations of CPI-17 wt, CPI-17 S128D or CPI-17 S128A. The values for CPI-17 wt and CPI-17 S128D were 494 nM and 311nM, respectively (Figure 3.23A and B). The concentration of CPI-17 S128A, which gave half maximal activation was 224 nM (Figure 3.23C). CPI-17 wt and CPI-17 S128D both show submicromolar levels of inhibition, however, *in vivo* inhibition has yet to be shown. The values which gave half-maximal inhibition were very similar for both CPI-17 wt and CPI-17 S128D, suggesting that the mutation S128D has no effect on CPI-17 conformation. One explanation why CPI-17 wt/S128D did not completely inhibit CKIα activity could be that only a certain pool of CKIα was blocked by CPI-17 wt/S128D and the rest was not. Therefore only a partial inhibitory effect of around 30% can be observed.

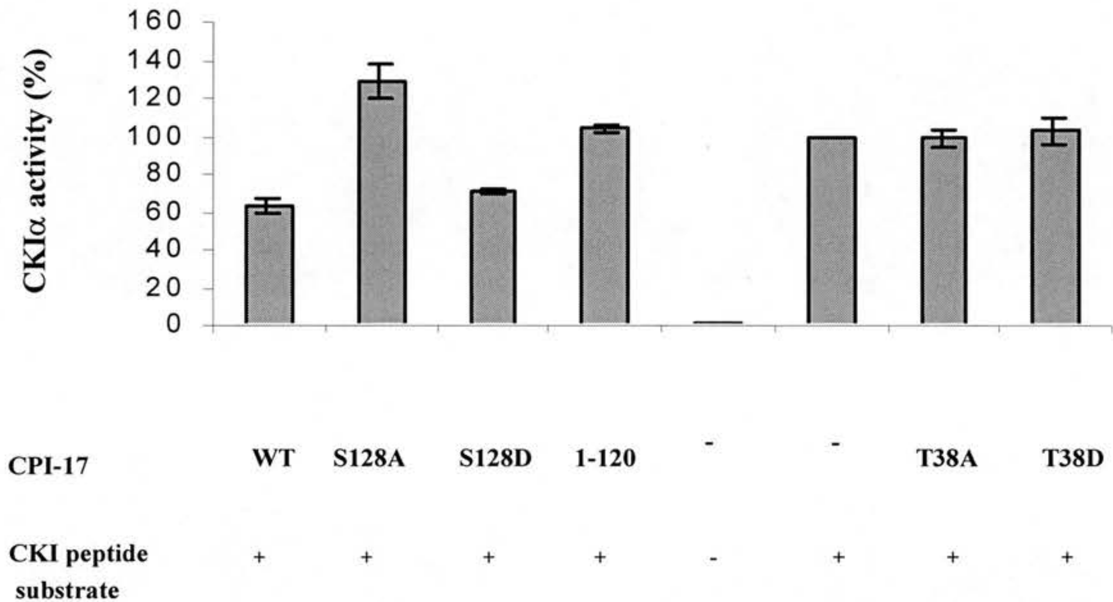

Figure 3.22 CPI-17 regulates CKIα activity.
CPI-17 regulates CKI activity. CPI-17 wt, CPI-17 T38A, CPI-17 T38D, CPI-17 S128A, CPI-17 S128D and CPI-17 1-120 (7.5 μg each) were incubated with purified histidine tagged CKIα for 15 min at 30°C. CKI-specific phosphopeptide substrate (40 μM) was then added and the reaction was incubated for 30 min at 30°C in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. ^{32}P incorporated into the peptide was quantified by liquid scintillation counting and represents CKI activity. CKIα was inhibited by CPI-17 wt by 37% (+/- 4 SEM (standard error of mean), n=6). The p-value was 0.0001, which is considered to be statistically significant. CKIα was inhibited by CPI-17 S128D by 29% (+/- 1 SEM, n=6). The p-value was 0.0001. The values for CPI-17 mutants CPI-17 T38A, T38D and CPI-17 1-120 were 103% (+/- 7 SEM, n=6), 99% (+/- 4 SEM, n=6) and 104% (+/- 1.8 SEM, n=6), respectively. The p-values were 0.885 for CPI-17 T38A, 0.7805 for CPI-17 T38D and 0.0941 for CPI-17 1-120, which is not considered to be statistically significant.

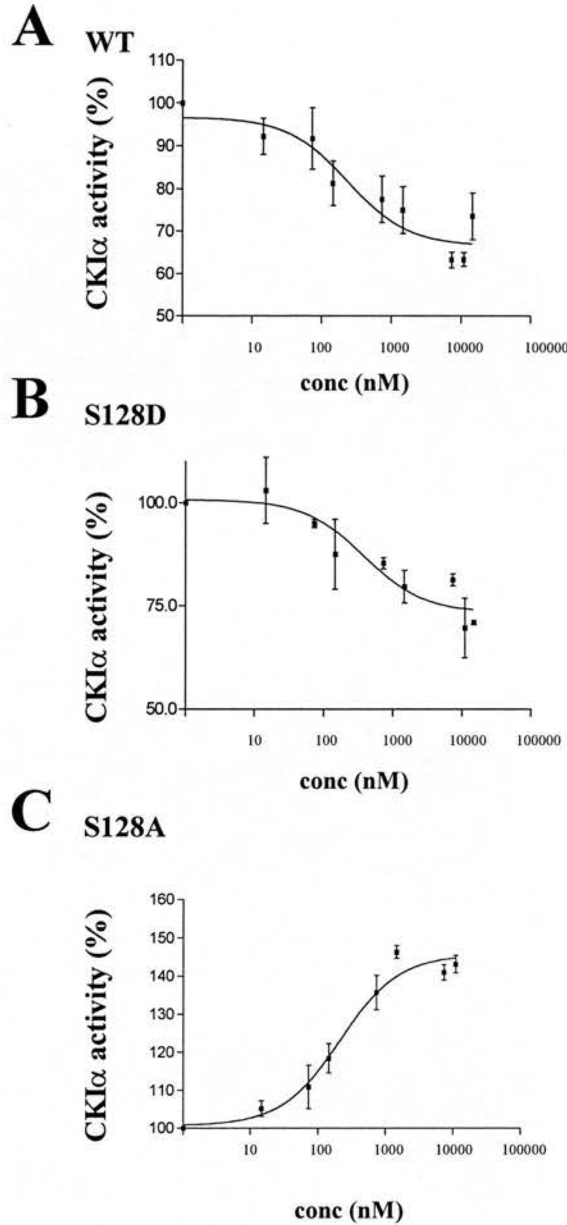

Figure 3.23A, B, C Determination of half-maximal inhibition by CPI-17.
CPI-17 wt, CPI-17 S128A and CPI-17 S128D (1 nM-10 μM) were incubated with purified histidine tagged CKIα and a kinase assay was carried out as described above. Prism 3 from GraphPad.com was used to calculate the half maximum inhibition/activation concentrations of CPI-17 wt, CPI-17 S128D and CPI-17 S128A. The values for CPI-17 wt and CPI-17 S128D were 494 nM (+/- 4.03 SEM, n=3) and 311 nM (+/- 4.099 SEM, n=3), respectively. The concentration of CPI-17 S128A, which gave half maximal activation was 224 nM (+/-4.035 SEM, n=3).

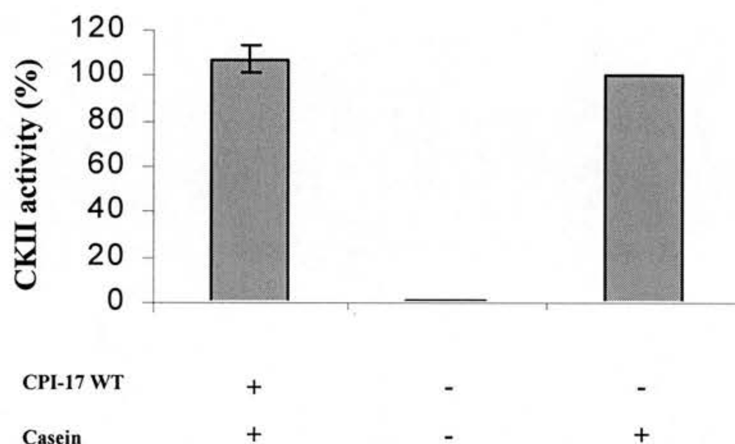

Figure 3.24 CPI-17 has no effect on CKII activity.
CPI-17 wt (5 μg) was incubated with purified CKII for 15 min at 30°C. Casein (5 μg) was then added and the reaction was incubated for 30 min at 30°C in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer. ^{32}P incorporated into casein was quantified by liquid scintillation counting and represents CKII activity. The p-value was calculated using the unpaired t-test calculator (GraphPad.com).

3.4.8. Binding between CPI-17 and CKI in COS cells

To investigate the possibility of an interaction between CPI-17 and CKI *in vivo*, flag-tagged CPI-17 and HA-tagged CKIα (wt) or CKIα (kd, kinase dead mutant) were used. CPI-17 was flag-tagged at the N-terminus as was HA-tagged CKIα (wt) and CKIα (kd) (from Dr Thierry Dubois). Transient transfection studies showed that flag-tagged CPI-17 was well expressed in mammalian COS-1/COS-7 cells, however, HA-CKIα (wt) was very poorly expressed as it could not be detected in detergent soluble cell lysates, nor when immunoprecipitated with the anti-HA antibody (data not shown). HA-CKIα (kd) was weakly expressed and detection was only possible when immunoprecipitated with the anti-HA antibody. The previous *in vitro* studies showed direct interaction between CPI-17 and CKIα and therefore the ability of CPI-17 to interact with CKIα in COS-7 cells was tested. Flag-CPI-17 and CKIα (wt) or CKIα (kd) were co-transfected into COS-7 cells. It should be noted that two different transfection methods were used: Electroporation and FuGENE as described in Method section 2.1.2. CKIα (wt) or CKIα (kd) were then immunoprecipitated using a HA-antibody and Western blot analysis was performed using anti-flag. In contrast to the *in vitro* studies it has not been possible to detect an interaction between CPI-17 and CKIα (wt) or CKIα (kd) (data not shown). This could be explained by the very low expression levels of CKIα.

3.5. New potential CPI-17 binding partners identified by mass spectrometry

To investigate the function of CPI-17, potential CPI-17 protein partners were identified by affinity chromatography and mass spectrometry analysis. Sheep or rat brain lysate, which had been pre-incubated with GST to remove non-specific binding, was loaded onto a GST, GST-CPI-17 or GST-centaurin-α_1 column. The columns were extensively washed and bound proteins eluted with 1M NaCl as described in the Methods chapter 2.12. Proteins eluted from the GST[2], GST-CPI-17 and GST-centaurin-α_1 column (40 μg) were separated on a 12.5% or 15% SDS-PAGE gel. More than 20 different proteins eluted from the GST-CPI-17 as judged by SDS-PAGE and GelCODE staining (Figure 3.25). More than 15 proteins eluted from the GST-centaurin-α_1 column and only around 5 proteins were recovered at low level from the GST column (Figure 3.25). The profiles from the 3 columns were completely different indicating that CPI-17 selectively bound to numerous proteins from mammalian brain that did not bind to GST or GST-centaurin-α_1 columns. The concentrations of the proteins eluted from the GST-CPI-17, GST-centaurin-α_1 and GST column were 2.7 μg/μl, 1.4 μg/μl and 0.2 μg/μl, respectively. It should be noted that from the same volume of brain lysate loaded on the columns, approximately 1% of the GST-CPI-17 column eluate, 2% of the GST-centaurin-α_1 eluate and 15% of the GST column eluate were loaded on the gel. The bands are difficult to see from Figure 3.26, but the arrows with sample number give an indication of the approximate molecular weight of the bands. The profiles of the proteins eluted from the GST-CPI-17 and GST columns (Figure 3.26) were very similar to the affinity chromatography results in Figure 3.25 when sheep brain lysate was used as judged from GelCODE staining (Figure 3.25). This further strengthened the point that the binding of the proteins to the GST-CPI-17 was selective.

[2] Note that due the low concentration of proteins in the GST column eluate, it was not possible to load the same amount of protein i.e. 40 μg and therefore 60 μl of the GST column eluate were loaded.

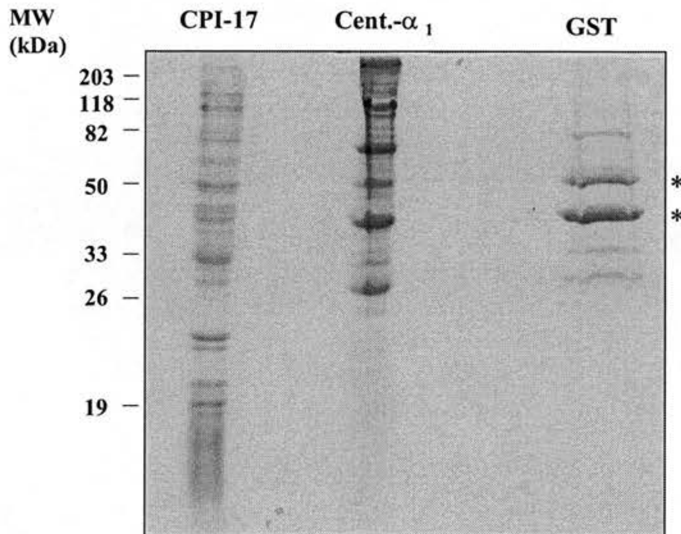

Figure 3.25 Mammalian brain proteins associate with CPI-17.
Sheep brain lysate was pre-incubated with GST in the presence of 0.5% Triton and subsequently loaded onto a GST-CPI-17 ("CPI-17"), GST-centaurin-α_1 ("Cent.-α_1") or GST column. Proteins bound to the columns were eluted with 1M NaCl and analysed on 12.5% SDS-PAGE and stained with GelCODE. The positions of the molecular weight markers (kDa) are indicated. * on a 15% SDS-PAGE gel these bands ran differently from the bands of similar apparent molecular weight on the GST-centaurin-α_1 column. In any case these 2 proteins were not identified (see Figure 4.8).

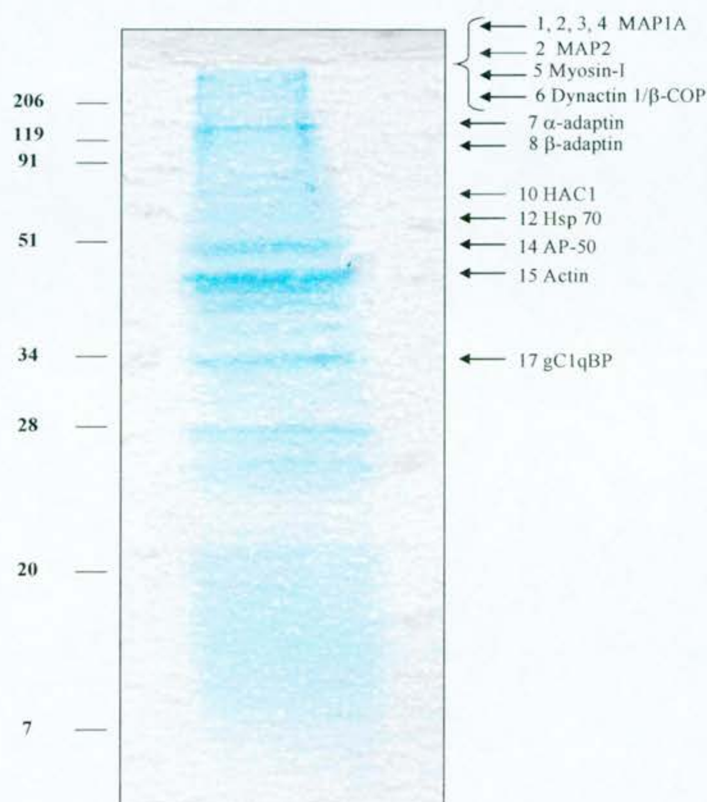

Figure 3.26 Rat brain proteins associate with CPI-17.
Rat brain lysate was pre-incubated with GST in the presence of 0.5% Triton, and subsequently loaded onto a GST-CPI-17. Proteins bound to the column were eluted with 1M NaCl, analysed on 15% SDS-PAGE, stained with GelCODE and analysed by mass spectrometry. The positions of the sample number (including sample name) are indicated with arrows on the right. The positions of the molecular weight markers (kDa) are indicated on the left.

A total of 19 protein bands from the GST-CPI-17 were excised from the gel in Figure 3.26 and digested with trypsin, which cleaves the peptide bond C-terminal to lysine and arginine residues. The proteins were identified using MALDI-TOF setup as described in the Methods chapter 2.18. Table 3.1 shows the proteins identified by mass spectrometry. Bands 9, 11, 13, 16, 18 and 19 were identified as contaminating *E.coli* proteins. The program MS-Fit was used to identify novel CPI-17 binding proteins.

Table 3.1 Proteins binding to the GST-CPI-17 column identified by mass spectrometry

Band	Identity	Mass (kDa)	% Coverage	Function
1	Microtubule associated protein 1A (MAP-1A)	156	32	Cytoskeletal protein (Sanchez et al., 2000)
2	MAP-2 MAP-1A	198 156	20 23	Cytoskeletal protein (Sanchez et al., 2000)
3	MAP-1A	156	26	Cytoskeletal protein
4	MAP-1A	156	19	Cytoskeletal protein
5	Myosin-I Myosin-I heavy chain	128 131	17 17	Cytoskeletal protein (Coluccio, 1997)
6	Dynactin 1 (150 kDa dynein-associated polypeptide/p150^{Glued})	141	12	Membrane skeleton component (Allan, 2000)
6	Coatomer β subunit (β coat protein, β–COP)	107	15	Protein transport (Roche, 1999)
7	Mouse α-adaptin (A)	107	42	Subunit of adaptor proteins (AP) (Boehm and Bonifacino, 2001)

Band	Identity	Mass (kDa)	% Coverage	Function
8	β-adaptin	104	26	Subunit of adaptor proteins (AP) (Boehm and Bonifacino, 2001)
10	Mouse RING finger protein HAC1	81	29	unknown
12	Heat shock protein (Hsp70)	71	46	Molecular chaperone (Hartl, 1996)
14	Assembly protein (AP 50)	50	30	Subunit of adaptor proteins (AP) (Boehm and Bonifacino, 2001)
15	Actin β and γ (many species)	42	59	Cytoskeletal protein (Machesky and Gould, 1999)
17	Mitochondrial precursor (glycoprotein gC1qBP)	31	41	unknown

From Table 3.1 it is clear that CPI-17 associates with numerous proteins and some of their functions will be briefly described in the following paragraphs. It should be pointed out however, that it has been not been investigated whether the association between CPI-17 and the proteins identified by mass spectrometry is direct. It is possible that only a few of the proteins directly bind to CPI-17 and that the binding of other proteins is indirect and occurs via protein complexes. Furthermore, it would be important to confirm the binding of the newly identified proteins to the GST-CPI-17 column by Western blotting. It should be pointed out that other proteins associated with the GST-CPI-17 column were not identified by mass spectrometry, among them the

kinases CKI and PKC. This could be explained by the fact that a large number of proteins were found to associate with the column and possibly not all the bands were cut out from the SDS-PAGE gel for mass spectrometry analysis. Furthermore, it is easier to identify more abundant proteins by this technique (for example cytoskeletal proteins) and any traces of CKI or PKC might not have been picked up by this method.

3.5.1. Binding of microtubule-associated proteins to the GST-CPI-17 column

Microtubule-associated proteins (MAPs) 1 (accession number in SWISS-PROT 56619) and 2 (accession number 56625) belong to the family of structural MAPs that bind microtubules and regulate cytoskeletal dynamics, the spacing between microtubules and dendritic elongation and oligodendrocyte process outgrowth in neurones (reviewed in Sanchez et al., 2000). MAP-1A and MAP-1B are distantly related heat-stable multimeric protein complexes containing one heavy and several light chains, present throughout the nerve cell (reviewed in Cassimeris and Spittle, 2001). Furthermore, they are thought to play a role in regulating the neuronal cytoskeleton, MAP-1B during neuritogenesis and MAP-1A in mature neurons. MAP-1A and MAP-1B bind to microtubules and microfilaments, suggesting that they are involved in mediating or regulating the interaction between axonal microtubules and actin filaments, which is believed to be essential for neuronal morphogenesis and function. Recently, it has been shown that the light chains of MAP-1A and MAP-1B directly associate with actin *in vivo* and *in vitro* (Noiges et al., 2002). One of the factors controlling MAP-1 activity is phosphorylation. However, very little is known to date about the phosphorylation of MAP-1A. So far it has only been shown that growth factor stimulation of NIH-3T3 cells results in MAP-1A phosphorylation (Erickson et al., 1990). MAP-1B on the other hand has been shown to be phosphorylated by proline directed kinases, likely candidates include Cdk5 and glycogen synthase kinase 3 β and casein kinase II. Dephosphorylation of MAP-1B is mainly accomplished by PP2a (Cassimeris and Spittle, 2001). The association of MAP-1A with the GST-CPI-17 column suggests a role for CPI-17 in neuronal cytoskeletal re-arrangement. It should be noted that the binding between CPI-

17 and MAP-1A might not be direct, but could be mediated by a protein complex containing actin for instance.

MAP-2 are heat-stable phosphoproteins predominantly expressed in neurons, and their expression is developmentally regulated. They serve as substrates for most of protein kinases and phosphatases present in neurons (reviewed in Sanchez et al., 2000). The kinases which phosphorylate MAP-2 include cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), PKC, extracellular signal-regulated kinases (ERKs), glycogen synthase kinase 3 (GSK3), cyclin dependent kinases (CDKs) and microtubule-affinity regulating kinases (MARKs). MAP-2 is dephosphorylated by PP1, PP2A, PP2B and PP2C. Phosphorylation of MAP-2 seems to control its association with the cytoskeleton, and a role for MAP-2 has been proposed to act as phosphorylation dependent cytoskeletal switch. MAP-2 may perform many functions including the nucleation and stabilisation of microtubules, the regulation of organelle transport within axons and dendrites, regulation of synaptic plasticity as well as the anchorage of regulatory proteins such as protein kinases which may be important for signal transduction. It is interesting to note that two of the kinases which associate with the GST-CPI-17 column, PKC and PKA, have been shown to phosphorylate MAP-2 as described above. MAP-2 has been shown to directly bind the regulatory subunit of PKA (Theurkauf and Vallee, 1982) and recently it has been shown that the catalytic subunit of PKA is localised to the actin and microtubule cytoskeletons in mouse hippocampal neurons, possibly via the AKAP, gravin, which can bind to PKA and F-actin simultaneously (Diviani and Scott, 2001; Sato et al., 2002). Therefore, it is possible that these two kinases associate with MAP-2, and that the binding of MAP-2 to the GST-CPI-17 column occurs via one or both of the kinases.

The association of MAP-2 with the GST-CPI-17 column suggests a potential role for CPI-17 in regulating PP1 activity in appropriate subcellular domains in neurons and to function as a molecular switch between phosphorylation and dephosphorylation and thereby leading to changes in the neuronal cytoskeleton. However, a possible direct association between CPI-17 with MAP-2 still remains to be established and as for MAP-1 it should be noted that the binding of MAP-2 to the GST-CPI-17 column could be

mediated via other proteins associated with the column.

3.5.2. Binding of adaptor protein subunits to the GST-CPI-17 column

Adaptins are subunits of adaptor proteins (AP) complexes involved in the formation of intracellular transport vesicles and in the selection of protein cargo for incorporation into the vesicles (reviewed in Boehm and Bonifacino, 2001). Four basic AP complexes have been described: AP-1-4. Each of these complexes is composed of two large adaptins (one each of γ/α/δ/ε and β1-4, respectively, 90-130kDa), one medium adaptin (μ1-4, ~ 50kDa) and one small adaptin (α1-4, ~ 20kDa). AP complexes are components of protein coats that associate with the cytoplasmic face of organelles of the secretory and endocytic pathways. AP-1, AP-3, and AP-4 mediate sorting events at the *trans*-Golgi network and/or endosomes while AP-2 mediates rapid endocytosis from the plasma membrane. Some of these processes function in conjunction with clathrin, while others are clathrin independent. One example of clathrin-mediated endocytosis takes place at the nerve terminal, where clathrin-coated vesicles are involved in the recycling of synaptic vesicles. There has been recent evidence which lends support to the idea that clathrin mediated endocytosis is controlled by cycles of phosphorylation/dephosphorylation (Joazeiro and Weissman, 2000; Lauritsen et al., 2000). The protein phosphatase PP2B, also known as calcineurin has been shown to be responsible for the essential dephosphorylation of key proteins involved in endocytosis (reviewed in Cousin and Robison, 2001; Yakel, et al., 1997).

The association of α-adaptin (accession number 49876), β-adaptin (accession number 203087) and clathrin coat assembly protein AP 50 (or the medium subunit of AP-2) (accession number 529580), with the GST-CPI-17 column, suggests that CPI-17 could be involved in endocytotic events in cells.

3.5.3. Binding of dynactin I and coatomer β subunit to the GST-CPI-17 column

One of the proteins identified in band six was the cytoskeletal protein dynactin I (also called 150 kDa dynein-associated polypeptide/DP-159/DAP-150/$p150^{Glued}$)

(accession number P28023). Dynactin I is a subunit of dynactin which consists of 11 different subunits: 8 or 9 x Arp1 (actin-related protein), 4 or 5 x dynamitin, 2 x p150^{Glued} (Dynactin I), 2xp24, one each of p62, conventional actin, Arp11, capping protein α and β, p27 and p25 (reviewed in Allan, 2000).

The dynactin complex is thought to mediate all dynein function in the cell, including cytoplasmic dynein-driven retrograde movement of vesicles and organelles along microtubules, axonal transport and the control of the microtubule cytoskeleton. For instance dynein-dynactin interaction is essential to keep microtubules associated with the centrosome and contributes to centrosome separation during cell division (reviewed in Karki and Holzbaur, 1999). The function(s) of dynactin are not well understood, but it is known that dynactin increases the distance that cytoplasmic dynein moves along microtubule before falling off. Dynactin also participates in cargo binding and the largest subunit p150^{Glued} has been shown to bind to both microtubules and the intermediate chain of dynein (Karki and Holzbaur, 1995; Muresan et al., 2001; Waterman-Storer et al., 1995 and 1997). The association of dynactin I with the GST-CPI-17 column could possibly be mediated via microtubule binding proteins, such as MAP-1A, and it could suggest a role for CPI-17 in microtubule skeleton regulation and vesicle trafficking.

Another protein in sample 6 was identified as the coatomer β subunit or β coat protein (β-COP) (accession number P23514). The β-COP is a subunit of the COP I (coat protein I) coatomer complex, which is involved in the generation and retrieval of vesicles from the Golgi to the endoplasmic reticulum (ER) compartment (reviewed in Roche, 1999). The COP I coat consists of coatomer, a stable 700-800 kDa complex and ADP ribosylation factor (Arf). The coatomer is a heptameric complex comprised of α, β, β', γ, δ, ε and ζ subunits (Roche, 1999). Interestingly, the β'-COP was shown to be a RACK for PKCε, suggesting a role for PKCε in vesicular trafficking (Csukai et al., 1997). In addition it has been demonstrated that the small GTPase Rab2, which initiates the recruitment of soluble components necessary for protein sorting and recycling from

pre-Golgi intermediates, requires PKCλ to recruit β-COP for vesicle formation (Tisdale, 2000). However, no direct interaction between PKCλ and β-COP was found. While the association of β-COP with the CPI-17 column strongly suggests that this could be mediated by a PKC isoform, a direct interaction between β-COP and CPI-17 should not be excluded.

3.5.4. Binding of gC1qBP protein to the GST-CPI-17 column

The gC1qBP (p33, p32, C1qR, TAP) is a widely expressed, multifunctional chaperone involved in numerous ligand-mediated cellular responses (reviewed in Ghebrehiwet et al., 2001). Originally, this 32 kDa protein has been described as a receptor of complement component C1q (Ghebrehiwet et al., 1994), the kinogen-binding protein p33 (Herwald et al., 1996) and splicing factor associated protein p32 (Krainer et al., 1991). Storz and co-workers have shown that association of PKCμ to p32 results in inhibition of the kinase. (Storz et al., 2000). A recent paper describes p32 as a general PKC-binding protein, but in contrast to Storz and co-workers, reports a stimulation of PKC activity (Robles-Flores et al., 2002). This discrepancy is explained by the fact that Storz and co-workers used a different construct from Robles-Flores and co-workers. In summary, p32 has been reported to bind and regulate PKC activity. Therefore, the binding of p32 (accession number 3334249) to the CPI-17 column could be mediated via PKC.

3.5.5. Binding of Hsp70 protein to the GST-CPI-17 column

To become functionally active, newly synthesized proteins must fold to correct three-dimensional structures. Chaperone proteins are involved in this process to avoid protein aggregation, misfolding or partial folding (reviewed in Hartl, 1996). One of these chaperone proteins is the heat shock protein (Hsp) 70, which amongst others, detects proteins that are incorrectly folded or denatured (reviewed in Bruemmer-Smith et al., 2001). Hsp70 then forms a complex with such proteins which can lead to correct folding, compartmentalisation in organelles, or to proteolytic degradation. Hsp70 has also been shown to play a role in protein unfolding, which is a key step in the import of some proteins into mitochondria and in the degradation of regulatory proteins by ATP-

dependent proteases (reviewed in Matouschek et al., 2000). The expression of heat shock proteins is particularly increased during stress conditions, such as heat shock, oxidative stress or inflammation (reviewed in Zylicz et al., 2001). During these conditions heat shock proteins increase cell survival by protecting and disaggregating stress-labile proteins, as well as the proteolysis of the damaged proteins. Because of its chaperone function, it is not surprising that Hsp70 (accession number 304516) was found to associate with the GST-CPI-17 column. The interaction of Hsp70 with the CPI-17 column has been further confirmed by Western blotting (Figure 3.27). It is possible that Hsp70 associated with a large number of proteins bound to the GST-CPI-17 column and therefore it is difficult to conclude whether there could be a direct interaction between CPI-17.

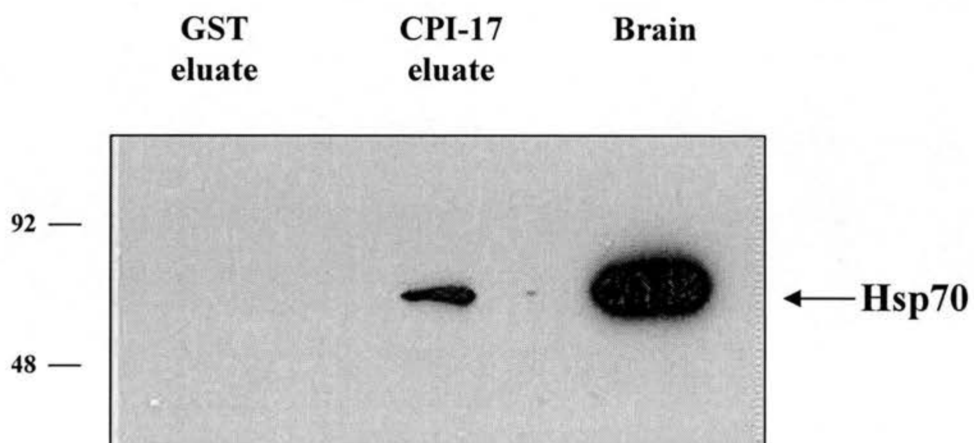

Figure 3.27 Association of Hsp70 with the GST-CPI-17 column.
Protein (5 μg) eluted from the GST-CPI-17 column ("CPI-17 eluate) and GST column ("GST eluate") was separated on 12.5% SDS-PAGE, transferred onto nitrocellulose and Western blotted with Hsp70 antibodies. A brain extract was used as a positive control for the antibody. The positions of the molecular weight markers (kDa) are indicated.

3.5.6. Binding of RING finger binding protein, HAC1, to the GST-CPI-17 column

HAC1 has been identified as a RING finger protein (Yanai K., Shimamoto Y., Hirota K. and Fukamizu A., unpublished results), however, nothing else is known about this protein to date. Hundreds of RING (Really Interesting New Gene) finger proteins have been reported so far and they are implicated in a diverse array of cellular processes, including development, oncogenesis and apoptosis (reviewed in Borden, 2000). Over the last few years evidence emerged that directs the role of the RING finger motif to the ubiquitin-ligase (E3) activity (reviewed in Joazeiro and Weissman, 2000). A large number of RING E3 has been identified so far, however it is not know whether all RING finger proteins are E3 (reviewed in Pickart 2001). Ubiquitination, a process in which target proteins are covalently modified by polyubiquitin and thus labelled for proteolysis, is understood as a major mechanism for cellular regulation (Pickart, 2001). Therefore, the elucidation of RING finger proteins functions and their regulation should be of great interest. The association of the RING finger protein HAC1 (accession number 5732193) with the GST-CPI-17 column, could indicate that HAC1 might also have an E3 activity and is therefore involved in cellular processes, by participating in controlling protein levels by proteolysis.

3.5.7. Binding of actin and myosin I to the GST-CPI-17 column

Actin and myosin I have been identified as two cytoskeletal proteins to associate with the GST-CPI-17 column. Myosin in connection with actin is responsible for a variety of cellular movements in eukaryotic cells. Chemical energy is transformed into directed movement by actin-activated ATP hydrolysis within myosin during the process (reviewed in Volkmann and Hanein, 2000). The actin cytoskeleton provides both the force and framework for much of cell motility, shape change and intracellular organisation (reviewed by Machesky and Gould, 1999). A large number of cellular processes including cytokinesis, endocytosis, exocytosis, chemotaxis, neurite outgrowth, signal transduction and mRNA transport are mediated by polymerisation of actin

filaments (reviewed in Carlier et al., 1999). There are three isoforms of actin in higher eukaryotes: α actin is found in muscle cells and β and γ actins are present in non-muscle cells (reviewed by Carpenter, 2000). Actin is often the most abundant protein in a cell and is present as a monomer and a polymer (Carpenter, 2000). The actin cytoskeleton is regulated by members of the Rho family of small GTPases (reviewed by Frame and Brunton, 2002). Interestingly, it has been shown that there is functional cooperation between the microtubule and actin cytoskeletons and MAPs were shown to be capable of cross-linking actin filaments and microtubules *in vitro* (reviewed by Goode et al., 2000). In addition, PKCβ and ε are known to bind directly to actin (reviewed in Jaken and Parker, 2000), and it has been demonstrated that PKC induces actin re-organisation in vascular smooth muscle cells (Brandt et al., 2002). Furthermore it should be mentioned that our laboratory identified actin as one of the proteins binding to the peptide column containing residues 214-233 of CKIα (Dubois et al., 2002b) and CKIα was able to phosphorylate actin *in vitro* (Dubois T., Maciver S.K. and Aitken A., unpublished results). From the above results it is clear that, the association of actin β and γ with the GST-CPI-17 column could be mediated via numerous proteins, including PKC, myosin I, CKIα, MAPs or other complexes, however it is also possible that actin associates directly with CPI-17.

Myosin I proteins, members of the unconventional myosin class, are single-headed, actin-binding, membrane associated proteins with heavy chains in the molecular range of 110-130 kDa (reviewed in Coluccio, 1997). Each heavy chain associates with 1-6 light chains. All members of the myosin I family, from yeast to man, have three structural domains: a catalytic head domain that binds ATP and actin; a tail domain thought to be involved in targeting the myosins to specific subcellular locations and a junction or neck domain that connects them and interacts with light chains (reviewed in Barylko et al., 2000). Myosin Is have been implicated in various motile processes, including cortical membrane tension (Dai et al., 1999), endocytosis and endocytotic trafficking (Geli and Riezman, 1996; Raposo et al., 1999) and membrane ruffling (Mezgueldi et al., 2002; Tang and Ostap, 2001). It is interesting to note that myosin Iα,

Iβ and Iγ can all be phosphorylated *in vitro* by PKC (reviewed in Barylko et al., 2000). The binding of myosin I to the GST-CPI-17 column could be mediated by protein complexes including actin or PKC, however, a possible direct interaction between CPI-17 and myosin I cannot be excluded.

The association of actin, myosin I, other cytoskeletal proteins, adaptor protein β-adaptin and the assembly protein AP 50 with the GST-CPI-17 column suggests a possible role for CPI-17 in the involvement of cytoskeletal regulation and/or vesicular trafficking.

CONCLUSION

In this chapter the identification of new CPI-17 binding proteins has been described. The focus has been particularly on the interaction of CPI-17 with PKC and CKI. PKC has been shown to phosphorylate CPI-17 on Thr-38 (Eto et al., 1995), however, to the best of my knowledge no evidence has been demonstrated that PKC binds to CPI-17. PKC isoforms from the 3 different PKC classes and PKCμ were shown in this study to directly bind to CPI-17 *in vitro*. The binding site has been mapped to the catalytic domain of PKCμ and to residues 1-120 of CPI-17. Furthermore, PKCs from the 3 different classes and PKCμ were shown to phosphorylate CPI-17 *in vitro*. The second part of this chapter demonstrated that CPI-17 associates with CKI isoforms and the site was mapped to the kinase domain of CKI and to residues 1-120 of CPI-17. Additionally, CPI-17 was shown not to be a substrate for CKI, but it appears to regulate CKIα activity. In the last part of this chapter the identification of new potential CPI-17 proteins by mass spectrometry was described. A number of proteins were shown to associate with the GST-CPI-17 column, including many cytoskeletal proteins and proteins involved in endocytosis/organelle transport. These results indicate that CPI-17 occurs as a multiprotein complex, suggesting that CPI-17 may have roles other than MLCP inhibition during smooth muscle contraction.

CHAPTER 4

Identification of novel centaurin-α_1 binding proteins

4. Identification of novel centaurin-α_1 binding proteins

INTRODUCTION

Centaurin-α_1 was identified as one out of six proteins co-purifying with CKIα from brain after four chromatography steps (Dubois et al., 2001) as described in section 1.2.2. Centaurin-α_1 (also called centaurin-α, PIP3BP and $p42^{IP4}$), a 43 kDa protein, has been originally described as a binding partner for inositol 1,3,4,5-tetrakisphosphate and phosphatidylinositol 3,4,5-trisphosphate (Hammonds-Odie et al., 1996; Rao et al., 1999; Stricker et al., 1997; Tanaka et al., 1999). In spite of the presence of a putative ADP-ribosylation factor (ARF) GTPase-activating protein domain, no ARF GAP activity has been attributed to centaurin-α_1 to date (reviewed in Jackson et al., 2000). Therefore, the function of this protein remains unknown. In order to elucidate potential intracellular roles for centaurin-α_1 the aim was to identify novel centaurin-α_1 binding partners. Using affinity chromatography followed by mass spectrometry and/or Western blot analysis, novel centaurin-α_1 binding partners were identified (see section 3.5).

In this chapter the evidence for the identification of PKC as a novel centaurin-α_1 protein partner and the characterisation of this interaction will be described. In the second part, the association of nucleolin, a nucleolar protein involved in RNA biosynthesis (reviewed in Ginisty et al., 1999), with centaurin-α_1 will be discussed. The final part of this chapter will focus on the identification of novel additional potential centaurin-α_1 binding proteins by mass spectrometry and some of the functions centaurin-α_1 might have in cell in the light of these newly identified binding partners will be proposed. An outline of this chapter is shown in Figure 4.1.

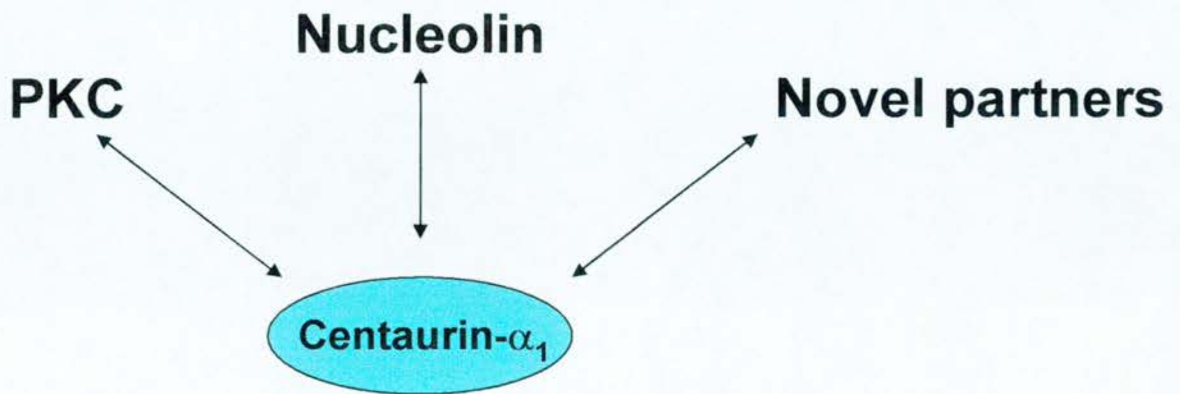

Figure 4.1 Interactions with centaurin-α_1 described in chapter 4.
Centaurin-α_1 binds to PKC (section 4.1), nucleolin (section 4.2) and novel protein partners (section 4.3). The protein interactions are indicated by double arrows.

RESULTS AND DISCUSSION

4.1. Centaurin-α_1 and PKC

4.1.1. PKCλ and ζ bind to the centaurin-α_1 column

Our laboratory has shown that centaurin-α_1 is a PKC substrate (Kerai P. and Dubois T., unpublished data). Therefore, it was important to test whether PKC associated with centaurin-α_1 and bound to the affinity chromatography column. Proteins eluted from the GST[3], GST-CPI-17 and GST-centaurin-α_1 column (5 μg) were separated on a 10% SDS-PAGE gel, transferred onto nitrocellulose and Western blotted with anti-PKCλ and ζ antibodies. PKCλ and ζ associated with the GST-centaurin-α_1 column and GST-CPI-17 column (Figure 3.6). No PKC isoforms were detected in the eluate from the control GST column (Figure 3.6). Further analysis to see whether PKC isoforms from other PKC classes bound to the GST-centaurin-α_1 column was not possible, as insufficient centaurin-α_1 eluate was available. However, these were the first results which indicated that members of the PKC family might be novel centaurin-α_1 protein partners. PKN and ROCK were not found to associate with the GST-centaurin-α_1 column (Figure 3.7)indicating that the binding of PKC isoforms to the GST-centaurin-α_1 column was selective.

[3] Note that due the low concentration of proteins in the GST column eluate, it was not possible to load the same amount of protein i.e. 5 μg and therefore 20 μl of the GST column eluate were loaded.

4.1.2. *In vitro* binding of all PKC isoforms to centaurin-α_1

In the previous section it is shown that atypical PKC isoforms associated with the GST-centaurin-α_1 and therefore it was interesting to test whether PKC isoforms from all subfamilies associated with centaurin-α_1. Furthermore, one cannot conclude whether association of the PKC isoforms with the GST-centaurin-α_1 column was direct or mediated via other proteins associated with centaurin-α_1. To investigate whether PKC isoforms from all classes were able to associate with centaurin-α_1 an *in vitro* transcription/translation assay was used. PKCα, λ, μ and ζ were expressed and labelled with [^{35}S] methionine in a reticulocyte lysate, and incubated with GST and GST-centaurin-α_1. All PKC isoforms tested were found to associate with GST-centaurin-α_1 but not with GST (Figure 4.2), indicating that the binding might occur via a domain present in all PKC isoforms. In order to determine whether the binding between centaurin-α_1 and PKC was direct, GST, GST-CPI-17, GST-14-3-3ζ and GST-centaurin-α_1 were incubated with recombinant PKCα, PKCε, PKCμ and PKCζ, pulled-down with glutathione Sepharose beads and Western blot analysis was performed with the respective PKC antibodies as described in chapter 3 section 3.3.3. The results showed that GST-centaurin-α_1, but not GST, directly bound to all PKC isoforms tested (Figure 3.10). CPI-17 directly bound to all PKC isoforms (positive control). In addition, 14-3-3ζ directly associated with PKCε and PKCμ, but not with PKCα nor PKCζ (Figure 3.10) as described in chapter 3 section 3.3.3. Using two different methods, the association between centaurin-α_1 and PKC was demonstrated. To the best of my knowledge this is the first time a member of the centaurin family has been shown to bind directly to PKC isoforms.

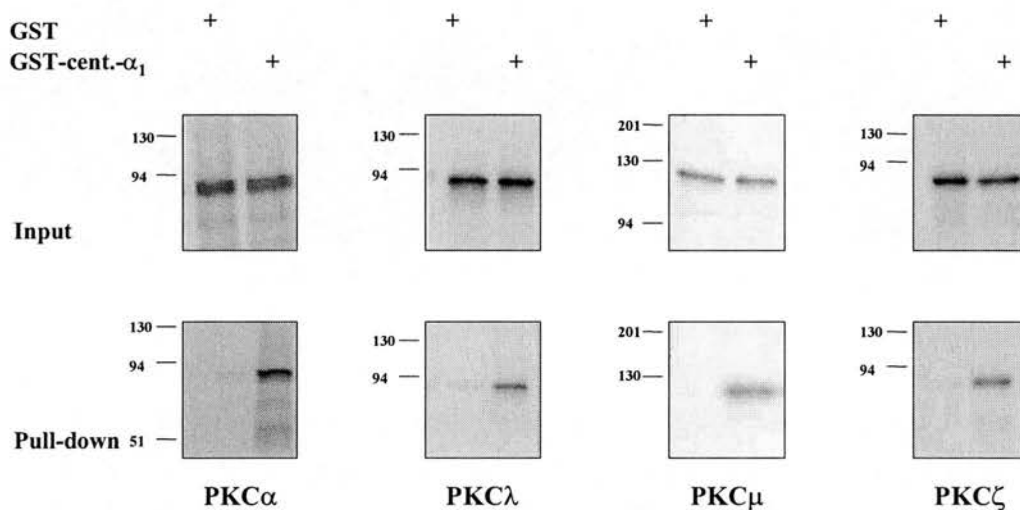

Figure 4.2 Centaurin-α_1 associates with all PKC isoforms tested.
PKCα, λ, μ and ζ were expressed and labelled with [^{35}S] methionine in a reticulocyte lysate and incubated for 15 min at 30°C with 5 μg GST and GST-centaurin-α_1 in the presence of 1% Nonidet P-40. Glutathione-Sepharose beads were added and incubated at room temperature for 1 h. Beads were washed and samples analysed by 10% SDS-PAGE followed by autoradiography (panel "Pull-down"). An aliquot of the lysate was loaded on the gel to visualise expressed PKCs (panel "Input"). The positions of the molecular weight markers (kDa) are indicated.

4.1.3. Binding occurs via the C1 domain of PKC

In section 4.1.2 it has been shown that PKC isoforms from all PKC classes bind directly to centaurin-α_1 which suggests that the binding occurs via a common domain. The PKCμ isoform was used to map the centaurin-α_1 binding site. PKCμ wt and PKCμ deletion mutants (Δ1-340 (ΔC1), Δ336-391 (ΔAD)) and Δ417-553 (ΔPH) were expressed, labelled with [^{35}S] methionine and incubated with GST and GST-centaurin-α_1. As shown in Figure 4.3 PKCμ wt bound to GST-centaurin-α_1. Deletion of the acidic domain (AD) or the pleckstrin homology (PH) domain of PKCμ did not affect the binding to centaurin-α_1. However, no binding was observed between centaurin-α_1 and PKCμ Δ1-340 (Figure 4.3). According to these data, amino acids 1-340 of PKCμ are necessary for the association with centaurin-α_1, indicating that the C1 domain of PKCμ represented the target region for binding to centaurin-α_1. This is not unexpected as several proteins have been described to be associated with the C1 region of PKCμ, including CPI-17 (positive control), 14-3-3ζ (Hausser et al., 1999) and Bruton's tyrosine kinase (BTK) (Johannes et al., 1999). Since all PKC isoforms have a least one C1 domain, these results complement those from Figure 4.2 showing that all PKC isoforms associated *in vitro* with centaurin-α_1.

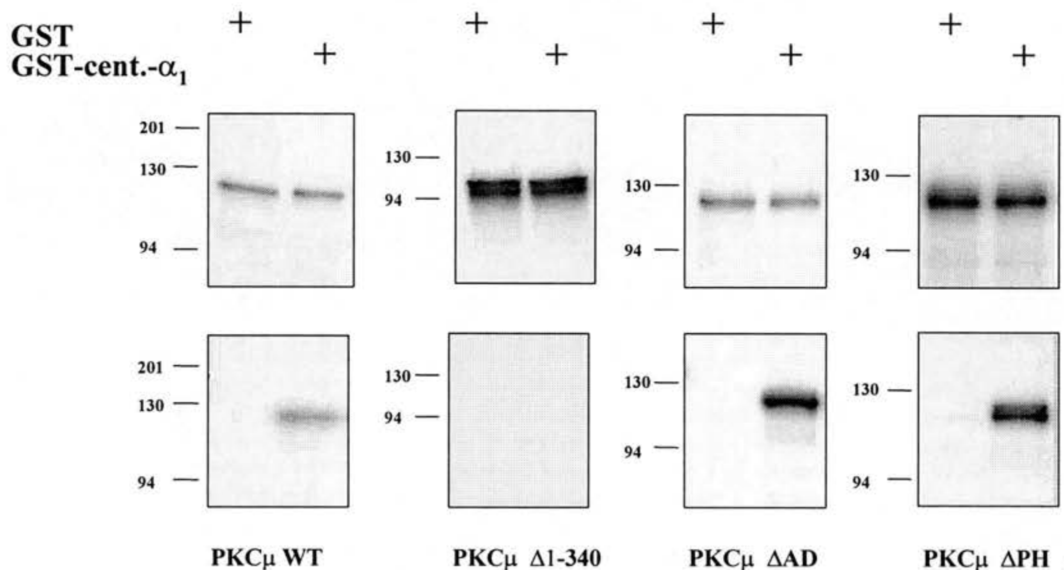

Figure 4.3 Centaurin-α_1 binds to the C1 domain of PKCμ.
PKCμ wt and PKCμ deletion mutants (Δ1-340, ΔAD and ΔPH) were expressed in a reticulocyte lysate, and incubated with 5 μg each of GST and GST-centaurin-α_1 as described in Figure 4.2. GST pull-down assays were analysed by SDS-PAGE and autoradiographed (panel "Pull-down"). An aliquot of the lysate was loaded on the gel (panel "Input"). The positions of the molecular weight markers (kDa) are indicated.

4.1.4. PKC phosphorylates centaurin-α_1

Since PKCs bind to centaurin-α_1 it was important to determine whether centaurin-α_1 served as a substrate for these kinases. Our laboratory has shown that PKC phosphorylates centaurin-α_1 (Kerai P. and Dubois T., unpublished data).We therefore tested which PKC isoforms were responsible. For that purpose, 1 U of PKCα, ε, μ and ζ was incubated with 5 μg of centaurin-α_1 in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer (kinase assay using PKCμ was carried out by Sam Clockie). The results indicated that all PKC isoforms tested were able to phosphorylate centaurin-α_1 *in vitro* (Figure 4.4A).

Although it may appear that PKCα is more potent in phosphorylation of centaurin-α_1 than PKCε, ζ and μ, this was not the case as there was a difference in activity between the different PKC isoforms as judged by the levels of autophosphorylation and of histone H III phosphorylation used as control (Figure 4.4B). This is the first centaurin family member that has been shown to be phosphorylated by PKC.

Phosphorylation of centaurin-α_1 by PKC could regulate centaurin-α_1 activity and therefore it would be important to clarify whether centaurin-α_1 has an Arf GAP activity and to test whether PKC phosphorylation has any effect on the regulation of centaurin-α_1. It is interesting to note that centaurin-α_1 has been shown to associate with CKIα, however, CKIα does not phosphorylate centaurin-α_1, (Dubois et al., 2001).

Having demonstrated for the first time that PKC isoforms from different sub-families bind to centaurin-α_1 *in vitro* and that the PKC isoforms α, ε, μ and ζ all phosphorylated centaurin-α_1 suggests that centaurin-α_1 may be a tissue specific substrate for different PKC isoforms and that the interaction between the two proteins may depend on their subcellular localisation.

At this point it should be noted that novel and atypical PKC isoforms have been shown to be activated by lipid second messengers including the PI 3-K products PtdIns-

(3,4)-P_2 and PtdIns-(3,4,5)-P_3 (reviewed in Toker, 1998). This could link PKCs to the phosphoinositide signalling pathway and also provide another link between PKC isoforms and centaurin-α_1. As mentioned previously in the Introduction (section 1.5.1), centaurin-α_1 contains two PH domains which comprise the PtdIns-(3,4,5)-P_3 binding site and are necessary for the PI 3-K dependent translocation of centaurin-α_1 from the nucleus and cytosol to the plasma membrane (Tanaka et al., 1999; Venkateswarlu and Cullen, 1999; Venkateswarlu et al., 1999). Therefore PKC and centaurin-α_1 could be regulated by PtdIns-(3,4,5)-P_3.

Centaurin-α_1 is ubiquitously expressed with especially high expression levels in brain (Hammonds-Odie et al., 1996) and PKC isoforms are also widely distributed in different tissues including brain (Liu, 1996). Furthermore, it has been demonstrated that centaurin-α_1 is mainly found in the nucleus of different cell lines, but it has also been shown to associate with the plasma membrane and to be present in the cytosol (Dubois et al., 2002c; Tanaka et al, 1999; Venkateswarlu and Cullen, 1999; Venkateswarlu et al., 1999). Centaurin-α_1 contains a putative nuclear localisation signal (NLS) at its N-terminal that was found to be critical for its targeting to the nucleus (Tanaka et al., 1999). Intriguingly, our laboratory showed that a mutant deleted of the first residues containing the putative NLS still localised in the nucleus to the same extent as centaurin-α_1 wild type (Dubois et al., 2002c). A recent report has shown the importance of the zinc finger motif for the nuclear localisation of centaurin-α_1 (Sedehizade et al., 2002). This could explain why a the centaurin-α_1 mutant deleted of the NLS still localised to the nucleus.

Most PKC isoforms have also been identified in the nucleus and increasing evidence has implicated a role for PKC in nuclear functions, such as cell proliferation, cell differentiation and apoptosis (reviewed in Martelli et al., 1999). As both centaurin-α_1 and PKC isoforms localise in the nucleus (as well as plasma membrane and cytosol) one can speculate that centaurin-α_1 could be involved in cellular processes which take place in the nucleus. In support of these data, centaurin-α_1 has been found to associate with the nucleolar protein, nucleolin, which will be discussed in the next part of this

chapter.

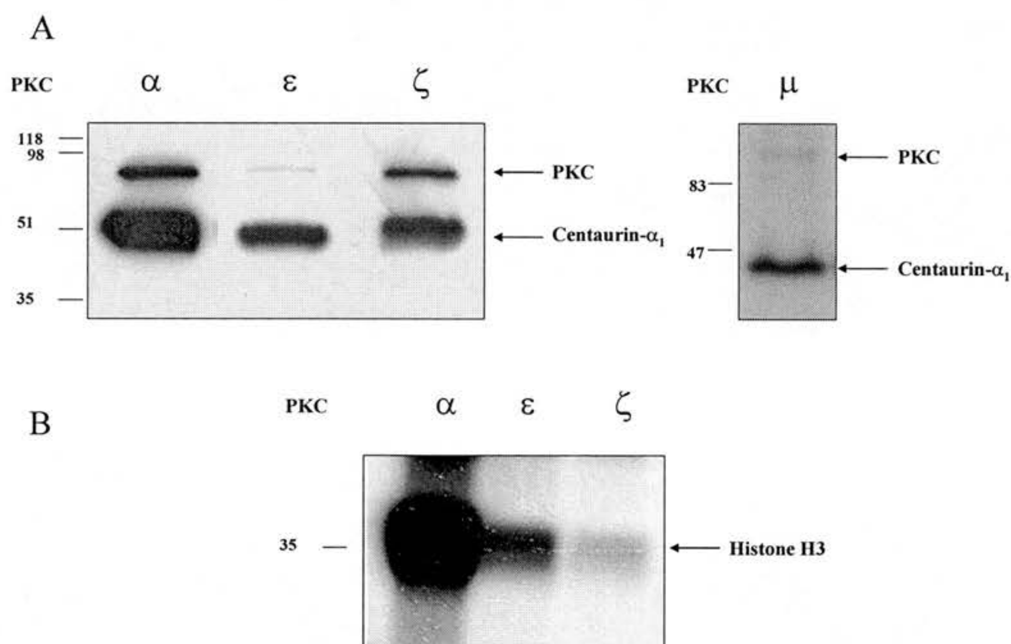

Figure 4.4 Centaurin-α_1 is phosphorylated by all classes of PKC.
A, One unit each of PKCα, ε, μ and ζ were incubated with 5 μg centaurin-α_1 in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer for 15 min at 30°C. The samples were analysed on 12.5% or 10% (for PKCμ phosphorylation) SDS-PAGE followed by autoradiography. The positions of phosphorylated centaurin-α_1 and autophosphorylated PKC isoforms are indicated on the right.
B, One unit each of PKCα, ε, and ζ were incubated with 5 μg histone H3 in the presence of 50 μM [γ-^{32}P] ATP and kinase buffer for 15 min at 30°C as a positive control for PKC activity. The samples were analysed on 15% SDS-PAGE followed by autoradiography. The position of phosphorylated histone H III is indicated on the right. The positions of the molecular weight markers (kDa) are indicated on the left.

4.2. Centaurin-α_1 and nucleolin

4.2.1. Nucleolin associates with the GST-centaurin-α_1 column

One of the proteins that associated with the GST-centaurin-α_1 column was nucleolin (SWISS-PROT accession number P19338, MW 76kDa) which is a phosphoprotein abundant in the nucleolus and can represent as much as 10% of total nucleolar protein (Ginisty et al., 1999). Nucleolin was identified as a centaurin-α_1 binding protein by mass spectrometry analysis and the band corresponding to nucleolin on a SDS-PAGE gel is indicated in Figure 4.5A. GST-centaurin-α_1 column eluate was separated by SDS-PAGE gel and stained with GelCODE and analysed as described in Method section 2.18 (Figure 4.5A). The results were confirmed using a nucleolin specific antibody (Figure 4.5B). It is evident from Figure 4.5A that nucleolin was one of the major proteins associated with the GST-centaurin-α_1 column. No nucleolin was identified in the GST-CPI-17 column eluate, confirming the selectivity of this interaction.

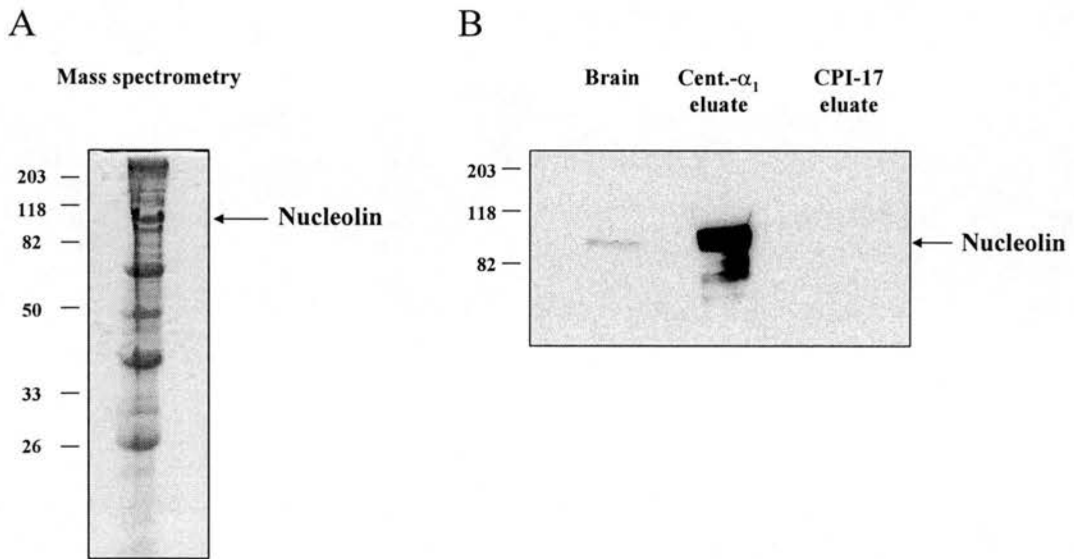

Figure 4.5 Nucleolin elutes from the GST-centaurin-α_1 column.
A, (as second column in Figure 3.25). Nucleolin associates with the GST-centaurin-α_1 column (detection by mass spectrometry). Sheep brain lysate was pre-incubated with GST in the presence of 0.5% Triton, and subsequently loaded onto a GST-centaurin-α_1 column. Proteins bound to the columns were eluted with 1M NaCl and analysed on 12.5% SDS-PAGE and stained with GelCODE. The band corresponding to nucleolin is indicated on the right and the positions of the molecular weight markers (kDa) are indicated on the left.
B, Nucleolin associates with the GST-centaurin-α_1 column (detection by Western blotting). Protein (5 μg) eluted from the GST-CPI-17 ("CPI-17 eluate") or GST-centaurin-α_1 ("Cent.-α_1 eluate") column was separated on 10% SDS-PAGE, transferred onto nitrocellulose and Western blotted with nucleolin antibodies. A brain extract was used as a positive control for the antibodies. The positions of the molecular weight markers (kDa) are indicated.

4.2.2. Nucleolin binds to centaurin-α_1 shown by IVTT

In section 4.2.1 it is shown that nucleolin associates with the GST-centaurin-α_1 column. To determine whether the binding between nucleolin and centaurin-α_1 is independent of other proteins binding to the GST-centaurin-α_1 column, an *in vitro* transcription/translation assay was used. Nucleolin was expressed and labelled with [^{35}S] methionine in a reticulocyte lysate, and incubated with GST, GST-CPI-17, GST-14-3-3ζ and GST-centaurin-α_1. Nucleolin associated with GST-centaurin-α_1, but not with GST, GST-CPI-17 or GST-14-3-3ζ. This confirmed that the binding between centaurin-α_1 and nucleolin is selective, since nucleolin was not found to bind to any other proteins tested. Furthermore, this result showed that the binding between centaurin-α_1 and nucleolin was not mediated via other proteins associated with the GST-centaurin-α_1 column. In addition the nucleolin construct used in the assay lacked the last 171 C-terminal residues of nucleolin which includes the RGG domain and the last RBD (Serin et al., 1997; Ying et al., 2000), demonstrating that this terminal region is not involved in the interaction. This interaction has been further confirmed in our laboratory by the association between centaurin-α_1 and endogenous nucleolin in HEK293 cells (Dubois et al., 2002c).

Nucleolin, belongs to a large family of RNA binding proteins. It contains 707 residues and its actual calculated mass is 77 kDa (reviewed in Srivastava and Pollard, 1999). However, nucleolin runs on SDS-PAGE gel with an apparent molecular mass of 105 kDa, which can be explained by the high content of negatively charged amino acids in the amino-terminal domain. Nucleolin has been shown to be involved in cellular functions such as ribosomal DNA (rDNA) transcription, chromatic decondensation, nucleocytoplasmic transport and ribosome biogenesis (Srivastava and Pollard, 1999). The association of centaurin-α_1 with nucleolin could reveal novel exciting roles for centaurin-α_1 in the nucleus.

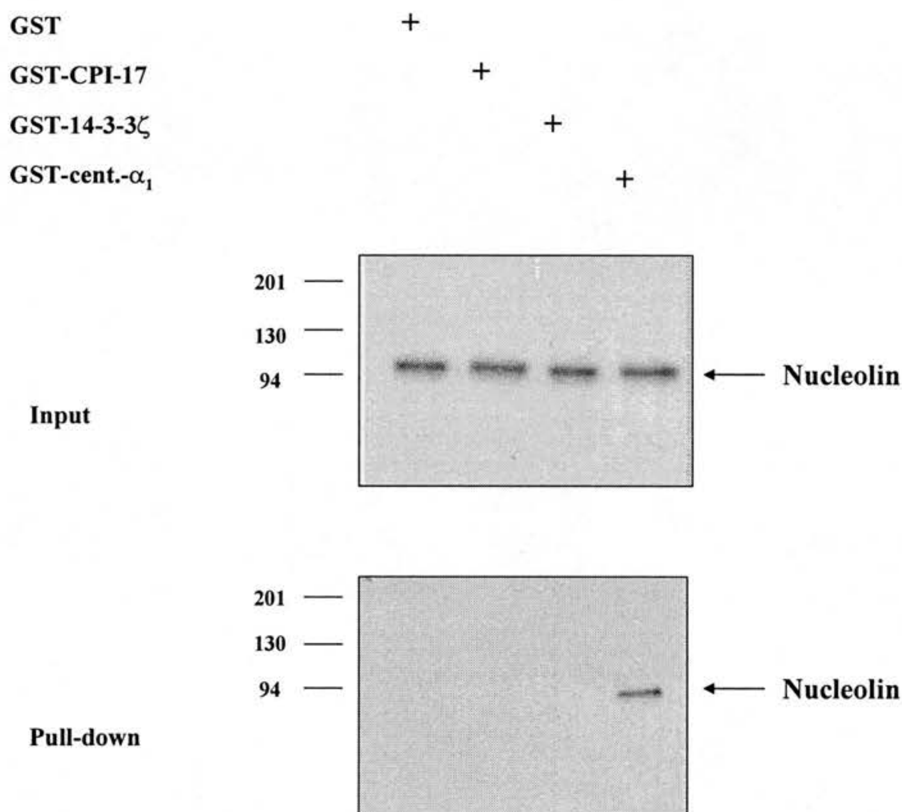

Figure 4.6 Nucleolin associates with centaurin-α_1.
Nucleolin was expressed in a reticulocyte lysate, and incubated with 5 μg each of GST, GST-CPI-17, GST-14-3-3ζ and GST-centaurin-α_1 as described in Figure 4.2. GST pull-down assays were analysed by SDS-PAGE and autoradiographed (panel "Pull-down"). An aliquot of the lysate was loaded on the gel (panel "Input"). The positions of the molecular weight markers (kDa) are indicated.

4.2.3. RNA is necessary for the binding of nucleolin to centaurin-α_1

Because nucleolin is a rRNA binding protein (Ginisty et al., 1999), and a smear was observed which could be due nucleic acids on a gel containing the proteins eluted from the GST-centaurin-α_1 column (see Figure 4.5), the effect of RNase treatment was tested on the association between centaurin-α_1 and nucleolin. It should be noted that we did not test whether nucleic acids, i.e. DNA or RNA were eluted from the GST-centaurin-α_1 column, which would be of interest for further studies.

Nucleolin was expressed and labelled with [^{35}S] methionine in a reticulocyte lysate, and incubated with different amounts of RNase as described in Methods section 2.16. This was followed by incubation with GST-centaurin-α_1. Interestingly, the binding between nucleolin and centaurin-α_1 decreased with increasing amounts of RNase (Figure 4.7), suggesting that RNA was involved in the formation of the complex between nucleolin and centaurin-α_1 and that they might be part of a ribonucleoprotein complex. These results were confirmed by demonstration that binding between nucleolin and centaurin-α_1 in HEK293 cells decreased upon treatment of cell lysate with RNase before centaurin-α_1 immunoprecipitation (Dubois et al., 2002c). As a control cell lysate treated with DNAse did not impair the binding between centaurin-α_1 and nucleolin, further supporting a role for RNA in the association between centaurin-α_1 and nucleolin (Dubois et al., 2002c). It is possible that the binding between nucleolin and centaurin-α_1 is mediated via RNA or that binding of RNA to nucleolin changes its conformation allowing it to bind centaurin-α_1.

Interestingly, a novel nucleolin-binding ribonucleoprotein complex has been characterised recently (Yanagida et al., 2001). Over sixty proteins were identified as part of the ribonucleoprotein which associated with nucleolin, out of which forty were ribosomal proteins. These authors demonstrated that the RBD of nucleolin was sufficient to hold the entire nucleolin-binding ribonucleoprotein complex and that nucleolin holds the ribonucleoprotein complex mainly by RNA-protein interactions and not by protein-protein interactions. This further strengthens the proposal that presence of RNA is necessary for the binding between nucleolin and centaurin-α_1.

Nucleolin has been shown to be involved in different steps of ribosome biogenesis including rRNA transcription, rRNA maturation and ribosome assembly (Ginisty et al., 1999). Interestingly, nucleolin is able to shuttle between the nucleus and the cytoplasm, which has raised the possibility that nucleolin could also be involved in the nuclear import of ribosomal components (for example ribosomal proteins), or in the nuclear export of the ribosomal subunits (Borer et al., 1989; Schmidt-Zachmann et al., 1993).

Another important discovery was that nucleolin is a substrate for PKCζ, which is required for nerve growth factor (NGF) induced differentiation in PC12 cells (Zhou et al., 1997). NGF treatment of PC12 cells resulted in translocation of PKCζ from the cytoplasm to the nucleus and in phosphorylation of nucleolin. Perander and co-workers showed that PKCζ contains a nuclear localisation signal (NLS) and a nuclear export signal (NES) which is responsible for the shuttling of PKCλ and PKCζ between cytoplasm and the nucleus (Perander et al., 2000). Interestingly, nucleolin was only phosphorylated by PKCζ when in the nucleus, but not in the cytoplasm (Zhou et al., 1997). From these results, it has been suggested that nucleolin serves to relay NGF signals from cell surface to the nucleus in PC12 cells. Phosphorylation of nucleolin by PKCζ provides a link between the three proteins, nucleolin, PKC and centaurin-α_1. Both nucleolin and centaurin-α_1 can serve as PKC substrates. However, *in vivo* nucleolin has been shown to be phosphorylated only by PKCζ, whereas data presented here show that centaurin-α_1 can be phosphorylated by PKC isoforms from all PKC classes (it should be noted that it remains to be established whether centaurin-α_1 is a substrate for PKC isoforms *in vivo*). This could indicate that centaurin-α_1 could be involved in signalling pathways mediated by different PKC isoforms, whereas nucleolin might only be involved in a very specific pathway signalling to the nucleus mediated by PKCζ. Because RNA was necessary for the binding between centaurin-α_1 and nucleolin, the findings described above suggest a possible role for centaurin-α_1 in ribosome biogenesis. However, as nucleolin is mainly expressed in the nucleolus (Ginisty et al., 1999), and centaurin-α_1 throughout the nucleus (Dubois et al., 2002c; Tanaka et al.,

1999), this suggests that centaurin-α_1 might have other binding partners in the nucleus and potentially other functions. The identification of novel, mainly nuclear, potential centaurin-α_1 protein partners will be discussed in the last part of this chapter.

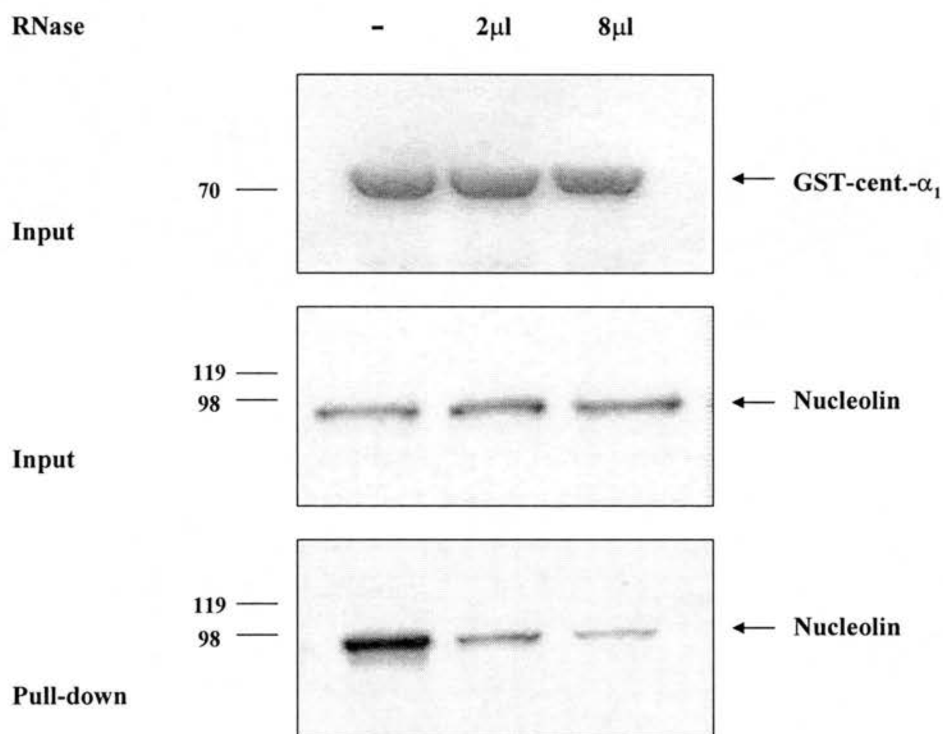

Figure 4.7 RNA is necessary for the binding between nucleolin and centaurin-α_1. Nucleolin was expressed in a reticulocyte lysate, and incubated for 15 min at 30°C with 5 μg GST, GST-CPI-17, GST-14-3-3ζ and GST-centaurin-α_1 in the presence of 1% Nonidet P-40 and RNase. Glutathione-Sepharose beads were added and incubated at room temperature for 1 h. Beads were washed and samples analysed by 10% SDS-PAGE followed by autoradiography (panel "Pull-down"). An aliquot of the lysate was loaded on the gel to visualise expressed PKCs (panel "Input"). The positions of the molecular weight markers (kDa) are indicated.

4.3. New potential centaurin-α_1 binding proteins identified by mass spectrometry

To investigate the function of centaurin-α_1, potential novel binding proteins were identified by affinity chromatography and mass spectrometry analysis. Sheep brain lysate, which had been pre-incubated with GST to remove non-specific binding, was loaded onto a GST, GST-CPI-17 or GST-CPI-17 column as described in chapter 3 section 3.5. The columns were extensively washed and bound proteins eluted with 1 M NaCl as described in the Methods chapter 2.12. More than 15 different proteins eluted from the GST-centaurin-α_1 as judged by SDS-PAGE and GelCODE staining (Figure 4.8). Only around 5 proteins were recovered at low level from the control GST-column and more than 20 proteins eluted from the GST-CPI-17 column. This further strengthened the point that the binding of the proteins to the GST-centaurin-α_1 was selective. A total of 10 proteins were identified by mass spectrometry as described in Method chapter 2.18. Table 4.1 shows their identification by mass spectrometry.

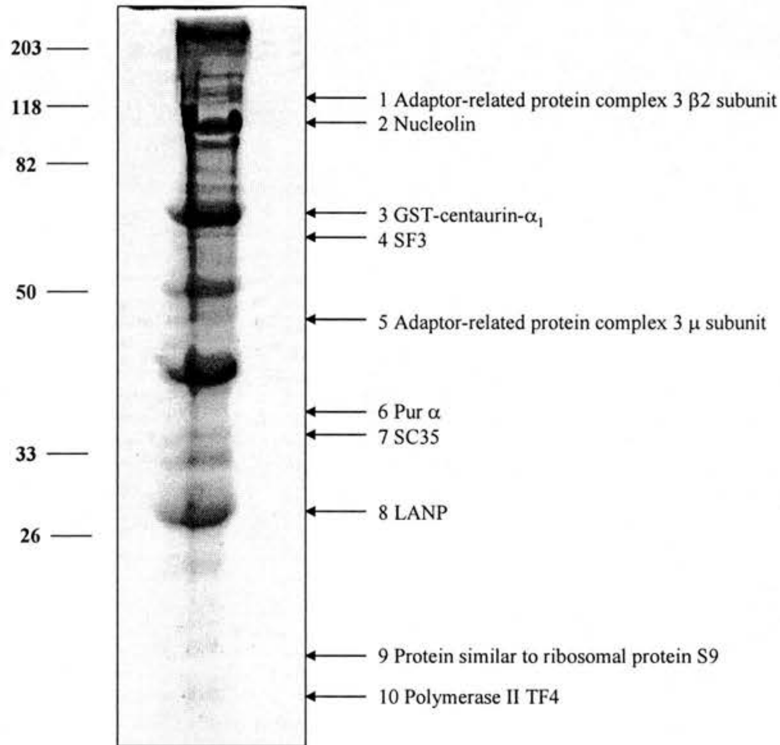

Figure 4.8 Mammalian brain proteins associate with centaurin-α_1.
Sheep brain lysate was pre-incubated with GST in the presence of 0.5% Triton, and subsequently loaded onto a GST-centaurin-α_1. Proteins bound to the column were eluted with 1M NaCl, analysed on 12.5% SDS-PAGE, stained with GelCODE and analysed by mass spectometry. The positions of the molecular weight markers (kDa) are indicated. The approximate positions of the identified proteins are indicated on the right.

Table 4.1 Proteins binding to the GST-centaurin-α_1 column identified by mass spectrometry

Sample number	Protein	MOWSE score	Molecular weight	Function	Reference
1	Adaptor-related protein complex 3 β2 subunit	82	119	formation of intracellular transport vesicles	(Boehm and Bonifacino, 2001)
2	Nucleolin	unknown	76	Ribosomal biogenesis	(Ginisty et al., 1999)
3	GST-Centaurin-α_1	243	43		
4	Pre-mRNA splicing factor SF3	144	59	pre-mRNA splicing	(Nesic and Kramer, 2001)
5	Adaptor-related protein complex 3 μ subunit	105	47	formation of intracellular transport vesicles	(Boehm and Bonifacino, 2001)
6	Pur α	204	34	transcriptional activation and repression; translation; cell growth; proliferation	(Gallia et al., 2000).
7	Splicing factor arginine/serine -rich 2 (SC35)	106	25	pre-mRNA splicing	(Hastings and Krainer, 2001)
8	Leucine-rich acidic nuclear protein LANP	183	19	unknown	
9	Protein similar to ribosomal protein S9	109	23	unknown	
10	RNA polymerase II transcription factor 4	89	14	Transcription activator	(Ge and Roeder, 1994)

From Table 4.1 it is clear that centaurin-α_1 associates with numerous proteins and some of their functions will be briefly described in the following paragraphs. It should be pointed out however, that the direct association between centaurin-α_1 and the proteins identified by mass spectrometry has not been investigated. It is possible that

only a few of the proteins directly bind to centaurin-α_1 and that the binding of other proteins is indirect and occurs via protein or RNA complexes. It would be important to confirm the newly identified proteins (apart from nucleolin) by Western blotting.

4.3.1. Centaurin-α_1 associates mainly with nuclear proteins

Centaurin-α_1 was mainly found to associate with nuclear proteins with the exception of the $\beta 2$ and μ subunits (or adaptins) of the adaptor-related protein complex 3 (accession number in SWISS-PROT: 4758760). Interestingly, CPI-17 was also found to associate with adaptins as mentioned in chapter 3 (section 3.5.2). As discussed previously, adaptins are subunits of adaptor proteins (AP) complexes involved in the formation of intracellular transport vesicles and in the selection of protein cargo for incorporation into the vesicles (reviewed in Boehm and Bonifacino, 2001; see also section 3.5.2). The association of centaurin-α_1 with adaptins suggests a role for centaurin-α_1 in vesicular trafficking. In addition, it has been shown that centaurin-α_1 is also found in cell fractions containing membranes (Dubois et al., 2002c) and the neuronal form of AP-3 has been associated with a role in synaptic vesicle formation (Blumstein et al., 2001), suggesting that centaurin-α_1 might also be involved in this cellular event. A CKIα –like activity has been shown to associate with AP-3, and therefore it is possible that the binding of AP-3 to the GST-centaurin-α_1 column could be mediated via CKIα. Furthermore, PI3-K and its product phosphatidylinositol-3-phosphate have been shown to be essential in early endosome fusion (de Renzis, et al., 2002; Zerial and McBride, 2001). This provides a further link between the phosphatidylinositol signalling pathway, centaurin-α_1 and vesicular trafficking.

Two other proteins which associated with the centaurin-α_1 column were, SC35 (accession number: 6755478) and SF3a (accession number : 5803167), both splicing factors involved in pre-mRNA splicing (reviewed in Hastings and Krainer, 2001; Nesic and Kramer, 2001). Pre-mRNA splicing, an essential step for the expression of most genes in higher eukaryotic cells, occurs in the nucleus in a large multicomponent ribonucleoprotein complex called the spliceosome, which is composed of small nuclear

ribonucleoprotein particles (snRNP; U1, U2, U4, U5 and U6) and many non-snRNP protein splicing factors (reviewed in Hastings and Krainer, 2001; Sureau et al., 2001; Wang et al., 2001). One group of non-snRNP proteins belongs to a conserved family of structurally and functionally related phosphoproteins called serine/arginine-rich (SR) proteins (Sureau et al., 2001). These proteins share a similar primary protein structure characterized by one or two RNA recognition motifs (RRMs) and an arginine/serine (RS) domain, which functions as a protein interaction domain (reviewed in Graveley, 2000). SR proteins are involved in multiple functions of the pre-mRNA splicing reaction, including early steps of spliceosome assembly, removal of spliced introns and regulation of alternative splicing. The latter is the formation of different mRNA isoforms from a single gene and a fundamental process controlling genetic expression in higher eukaryotes (Sureau et al., 2001).

The second splicing factor found to associate with the GST-centaurin-α_1 column was SF3a. The 17S U2 snRNP represents the active form of U2 snRNP that binds to the pre-mRNA during spliceosome assembly (Nesic and Kramer, 2001). U2 snRNP forms by association of the splicing factors SF3b and SF3a with the 12S U2 snRNP and it has been demonstrated that SF3a is required for the formation of the active 17S U2 and assembly of the prespliceosome. The association of proteins involved in mRNA splicing with the GST-centaurin-α_1 column gives rise to the possibility that centaurin-α_1 might be involved in mRNA splicing in the nucleus. However, it is also possible that the two splicing factors identified bind to the GST-centaurin-α_1 column via RNA or other proteins associated the GST-centaurin-α_1 column.

Another nucleic acid binding protein, Pur α (accession number 6679573) associated with the GST-centaurin-α_1 column. Pur α is a ubiquitous, sequence specific DNA and RNA-binding protein which is highly conserved in eukaryotes (reviewed in Gallia et al, 2000). This protein has been implicated in numerous cellular functions including transcriptional activation and repression, translation, cell growth and proliferation. Interestingly, RNA molecules have been shown to modulate the activity of Pur α. The association of Pur α with the GST-centaurin-α_1 column could therefore be

mediated by RNA molecules. However, an association with centaurin-α_1 or other proteins bound to the GST-centaurin-α_1 with Pur α should not be excluded.

A protein similar to ribosomal protein S9 (accession number 12654005) associated with the GST-centaurin-α_1 column. Ribosomes, the molecular machines that manufacture proteins, are composed of ribosomal RNA and ribosomal proteins (reviewed in Maguire and Zimmermann, 2001). They consist of a large and small subunit, which in *E. coli* are the S50 and S30 subunits, respectively. The large subunit contains the peptidyl transferase activity responsible for catalysing peptide bond formation during protein synthesis (reviewed in Doudna and Rath, 2002). The small subunit of the ribosome is responsible for binding and decoding messenger RNAs. Protein S9 is an *E.coli* 30S ribosomal protein (reviewed in Koc et al., 2001). It has been proposed that RNA molecules catalyse the crucial reactions on a ribosome, while the proteins play an active role in functions that may have evolved to streamline the process of protein synthesis (Maguire and Zimmermann, 2001). The association of a protein similar to ribosomal protein S9, with the GST-centaurin-α_1 column suggests that either a protein involved in the mRNA translation process is bound to the GST-centaurin-α_1 column and mediates this interaction or that the protein similar to ribosomal protein S9 associates via RNA molecules to the GST-centaurin-α_1 column. Another possibility is that centaurin-α_1 is indeed involved in ribosome biogenesis and binds to ribosome components.

Sample number 9, the leucine-rich acidic nuclear protein (LANP; accession number 5453880), was identified as another protein binding to the GST-centaurin-α_1 column. LANP has been identified as a cerebellar protein, particularly expressed in Purkinje cells and a role for LANP in a signal transduction pathway that directs differentiation of cerebellar neuron has been proposed, but not confirmed (Matsuoka et al., 1994). Leucine-rich domains appear to mediate protein-protein interactions (reviewed in Kobe and Kajava, 2001). Interestingly, LANP associates with the protein ataxin-1, the SCA1 gene product, which has been implicated in the pathology of

spinocerebellar ataxia type 1 (SCA1; Matilla et al., 1997). The features of the interaction between ataxin-1 and LANP, led these authors to propose a role for LANP in SCA1 pathogenesis. However, the precise function of this nuclear protein remains unknown. Association of LANP with the GST-centaurin-α_1 column further supports the fact that mainly nuclear proteins seem to associate with centaurin-α_1. To the best of my knowledge no other binding partners of LANP apart from ataxin-1, have been identified and therefore it is difficult to speculate through which protein the interaction between LANP and the GST-centaurin-α_1 column could be mediated, if the binding between LANP and centaurin-α_1 proves to be indirect.

The last protein identified which associated with the GST-centaurin-α_1 column was identified as the RNA polymerase II transcription cofactor 4 (also called PC4; accession number: 5729968). In eukaryotic cells the essential components of the transcription apparatus include RNA polymerase II and a set of general factors which are responsible for initiating promoter-dependent transcription (reviewed in Woychik and Hampsey, 2002). In addition, activator-dependent transcription in mammalian cells requires upstream stimulatory activity (USA)-derived cofactors including positive cofactors (PCs) (reviewed in Ge and Roeder, 1994; Ge et al., 1994; Kretzschmar et al., 1994). One of these positive cofactors, PC4 has been identified as a 127 residue, phosphorylation-regulatable DNA binding protein, that mediates functional interactions between upstream activators and the transcriptional machinery and enhances activator-dependent transcription. It was later shown that PC4 could act as a repressor of basal (activator-independent) transcription (Malik et al., 1998; Werten et al., 1998). Interestingly, PC4 also inhibits cyclin-dependent protein kinase (cdk) mediated phosphorylation of the largest subunit of RNA polymerase II (RNAPII) which correlated with its transcriptional inhibitory activity (Schang et al., 2000). Furthermore, Calvo and co-workers have provided evidence that links PC4 with mRNA termination (Calvo and Manley, 2001). The identification of PC4 in the GST-centaurin-α_1 eluate, suggests that this protein either bound directly to GST-centaurin-α_1 or that the association was

mediated by other proteins linked to RNA transcription or by nucleic acids possibly present on the GST-centaurin-α_1 column.

From the above described results it is evident that mainly nuclear proteins involved in ribosome biogenesis (nucleolin and a protein similar to ribosomal protein S9), in mRNA transcription (Pur α and PC4), mRNA splicing (splicing factors SF3 and SC35) and mRNA translation (Pur α and protein similar to ribosomal protein S9) associated with the GST-centaurin-α_1 column.

Most of the newly identified proteins binding to the GST-centaurin-α_1 column have one thing in common: They bind RNA or components involved in RNA processing. Therefore, it remains unclear whether these proteins constitute real centaurin-α_1 binding partners or whether their binding to the GST-centaurin-α_1 column was mediated via RNA/DNA or other proteins associated with the column. In support of the second option, RNA was shown to be necessary for the binding between nucleolin and GST-centaurin-α_1 and therefore a role for centaurin-α_1 in cellular events involving RNA is plausible. In addition, Yanagida and co-workers characterised a novel nucleolin-binding ribonucleoprotein complex. In their study, they identified over sixty proteins, out of which forty were ribosomal proteins, as part of the ribonucleoprotein which associated with nucleolin (Yanagida et al., 2001). They demonstrated that the RBD of nucleolin was sufficient to hold the entire nucleolin-binding ribonucleoprotein complex and that nucleolin holds the ribonucleoprotein complex mainly by RNA-protein interaction and not by protein-protein interaction. Interestingly, none of the proteins that were identified as part of the nucleolin-binding ribonucleoprotein complex were identical to proteins which eluted from the GST-centaurin-α_1 column. This suggests that some of the proteins bound to the GST-centaurin-α_1 column, might in fact be true centaurin-α_1 binding proteins, however, it is also possible that proteins eluted from the GST-centaurin-α_1 column are nucleolin or RNA binding proteins which have not been previously identified by the study of Yanagida et al., (2001).

It remains to be confirmed by other experiments whether centaurin-α_1 is a RNA

binding protein and if so, which domains within centaurin-α_1 could function as RNA binding domains.

CONCLUSION

In this chapter the identification of new centaurin-α_1 binding partners has been described. This focused particularly on the interaction of centaurin-α_1 with PKC and nucleolin. However, no interaction between centaurin-α_1 and PKC had been demonstrated. PKC isoforms from the 3 different PKC classes and PKCμ directly bound to centaurin-α_1 *in vitro* and the binding site was mapped to the catalytic domain of PKCμ/PKD. In addition, PKCs from the 3 different classes and PKCμ have been shown to phosphorylate centaurin-α_1 *in vitro*. The interaction between centaurin-α_1 and the nucleolar protein nucleolin has been characterised in the second part of this chapter. The binding between these two proteins is probably independent of other proteins. However RNA seems to be necessary for this interaction. The final part of this chapter focused on the identification of novel potential centaurin-α_1 binding proteins identified by affinity chromatography and subsequent mass spectrometry analysis, including many proteins involved in ribosome biogenesis and RNA processing. This suggests a novel role for centaurin-α_1 in these nuclear events.

CHAPTER 5

General discussion

5. General discussion

The aim of this investigation was to identify novel CPI-17 and centaurin-α_1 binding partners in order to increase our understanding of the functions of these two proteins in cells. Affinity chromatography in combination with Western blotting and mass spectrometry were used for this purpose. Affinity chromatography is a useful technique to identify potential protein partners very quickly. However, this technique does not allow one to distinguish between proteins that interact directly with the protein of interest and proteins which associate with the column through complex formation. Therefore, it is necessary to confirm potential protein-protein interactions by other techniques. Furthermore, proteins which are more abundant in the specific tissue lysate used, are generally the ones to be identified, therefore it is easy to overlook other potential protein partners. The 1 M NaCl should elute most proteins associated with the affinity chromatography column, however, to check whether any proteins remained bound to the column, the glutathione-Sepharose beads can be boiled with sample buffer, run on a SDS-PAGE gel and stained with Coomassie Blue.

In chapter 3 the identification of novel CPI-17 partners was discussed. PKC isoforms from all PKC classes were shown to interact with CPI-17, probably via their C1 domain. Furthermore, it was shown that CPI-17 is a substrate for PKCα, ε, ζ and μ. In the second part of that chapter the characterisation of the interaction between CPI-17 and CKI isoforms was discussed. CPI-17 was shown to bind to all CKI isoforms, via the kinase domain. However, CPI-17 was not shown to be a significant CKIα substrate but instead appeared to modulate CKI activity. The final part of chapter 3 focused on the identification of novel CPI-17 partners by mass spectrometry. A number of proteins were shown to associate with the CPI-17 column, including many cytoskeletal proteins and proteins involved in endocytosis/organelle transport. Therefore, in the next part of this discussion, evidence will be discussed which supports a novel role of CPI-17 in cytoskeletal re-arrangement and vesicular trafficking, by outlining the involvement of CPI-17 binding partners in these cellular events.

CPI-17 associates with PKC and is phosphorylated by PKC isoforms from all classes. Members of the PKC family have been shown to be involved in a large number of cellular processes, including roles in cytoskeletal re-organisation and vesicular trafficking: PKCβ and myristoylated alanine-rich C kinase substrate (MARCKS) have been shown to be essential for vesicular trafficking in brain neurons (Yang et al., 2002b); Berglund and co-workers have demonstrated that secretion from neurons is increased by activation of PKC and results from an increase in the pool size of releasable synaptic vesicles (Berglund et al., 2002); in chromaffin cells PKC enhances exocytosis both by increasing the number of readily releasable vesicles and by shifting vesicles to a highly Ca^{2+} sensitive state, enabling exocytosis at sites relatively distant from Ca^{2+} channels (Yang, et al., 2002); phosphorylation of synaptosome-associated protein (SNAP) of 25 kDa at Ser-187 by PKC has been shown to potentiate vesicle recruitment (Nagy, et al., 2002); in brain nerve terminals PKC is involved in a kiss-and–run like mechanism of synaptic vesicle recycling (Cousin and Robinson, 2001); PKCε has been shown to associate with actin and myosin IIA, suggesting a role in cell spreading (England et al., 2002); PKCι/λ has been implicated in microtubule dynamics in the early secretory pathway by phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tisdale, 2002) and PKCμ has been shown to regulate the fission of cell surface destined transport carriers from the *trans*-Golgi network (Liljedahl et al., 2001).

Amongst the many functions of CKI, there is evidence that members of the CKI family are involved in endocytotic trafficking and neurotransmitter release. CKIα has been shown to associate with cytosolic vesicles of the *trans*-Golgi network including small synaptic vesicles and to be capable of phosphorylating certain synaptic vesicle-associated proteins (Gross et al., 1995). In addition, it has been demonstrated that CKIδ is mainly cytoplasmic and particularly enriched within Golgi and ER-Golgi transport vesicles supporting a role in vesicular trafficking (Milne et al., 2001). A recent report links CKI to the control of a late step in the endocytotic trafficking of yeast uracil permease (Marchal et al., 2002).

Interestingly, it has been shown that CKI phosphorylates the phosphatase 1 modulator subunit (inhibitor-2, from rabbit skeletal muscle), resulting in activation of the inhibitor and inhibition of the phosphatase (Agostinis et al., 1992). CKI has also been shown to associate with and phosphorylate the PP1 inhibitor DARPP-32, indicating that CKI could function as a modulator of protein phosphatase I via DARPP-32 (Desdouits et al., 1995a and b).

The association of CKI, PKC, numerous cytoskeletal proteins and proteins such as β-COP, dynactin and AP-2 which are all involved in endocytotic trafficking or cytoskeletal re-arrangement, with the GST-CPI-17 column, suggests that CPI-17 could be involved in cytoskeletal re-arrangement, vesicle transport, endocytosis and/or exocytosis in brain and that its role might not be limited to regulating MLCP activity during smooth muscle contraction. In support of these findings a role for PP1 in synaptic transmission, actin cytoskeleton regulation and protein synthesis has been proposed (reviewed in Cohen, 2002). In addition, Eto and co-workers demonstrated a role for CPI-17 in microfilament organization and cell spreading via the inhibition of myosin phosphatase (Eto et al., 2000). Furthermore, it should be pointed out that the phosphorylation of myosin regulatory chain in smooth and non-muscle cells leads to contraction, cell migration and metastasis (reviewed in Somlyo and Somlyo, 2000). Therefore, it is of major importance to investigate proteins which are involved in the phosphorylation and dephosphorylation process of the myosin regulatory light chain. For example, inhibition of MLCP by CPI-17, blocks (at least partially) the dephosphorylation of the myosin regulatory light chain (Somlyo and Somlyo, 2000). Therefore, if an inhibitor specific for CPI-17 could be identified it could have potential implications in controlling cell migration and metastasis of malignant cells with highly important pathological consequences in diseases like cancer. However, one should remember that inhibition of MLCP by CPI-17 plays only a partial role in controlling this phosphatase (Somlyo and Somlyo, 2000). At this point it should be again stressed that CPI-17 inhibits the myosin-bound PP1 complex as well as the free PP1 catalytic subunit (PP1-C) *in vitro* (Eto et al., 1997). Therefore it is possible that CPI-17 could function as a general PP1-C inhibitor during the above suggested cellular events, leading to an

enhanced effect of kinases such as PKC and CKI.

Furthermore, in support of data presented here, our laboratory has shown that CKIα associates with cytoskeletal protein, including actin and tubulin (Dubois et al., 2002b), indicating that CKIα could also play a role in cytoskeletal re-arrangement, thus further supporting a possible involvement of CPI-17 in these cellular events.

In chapter 4 the identification of new centaurin-α_1 binding partners was described. This chapter was particularly focused on the interaction of centaurin-α_1 with PKC and nucleolin. PKC isoforms from the conventional, novel, atypical PKC subfamilies and PKCμ were found to directly bind to centaurin-α_1 *in vitro* and the binding site was mapped to the catalytic domain of PKCμ. In addition, PKCs from the 3 different classes and PKCμ/PKC have been shown to phosphorylate centaurin-α_1 *in vitro*. The interaction between centaurin-α_1 and the nucleolar protein nucleolin was discussed in the second part of this chapter. The binding between these two proteins appeared to be RNA dependent suggesting a role for centaurin-α_1 in a number of cellular processes involving RNA molecules e. g. ribosome biogenesis.

The final part of chapter 4 focused on the identification of novel potential centaurin-α_1 binding proteins by affinity chromatography and subsequent mass spectrometry analysis. This analysis identified many proteins, including proteins involved in ribosome biogenesis, RNA processing, but also in vesicular trafficking. Thus, in the following discussion a role for centaurin-α_1 in these cellular events will be proposed, by examining the involvement of the newly identified centaurin-α_1 partners in a number of cellular reactions including ribosome genesis and RNA processing.

Interestingly, novel and atypical PKC isoforms have been shown to be activated by lipid second messengers including the PI 3-K products PtdIns-(3,4)-P_2 and PtdIns-(3,4,5)-P_3 (reviewed in Toker, 1998). This could link PKCs to the phosphoinositide

signalling pathway and also provide a link between PKC isoforms and centaurin-α_1. As both centaurin-α_1 and PKC isoforms localise in the nucleus (as well as plasma membrane and cytosol) one can speculate that centaurin-α_1 could be involved in cellular processes which take place in the nucleus and involve PKC isoforms. However, it should be noted that both proteins have also been shown to be localised in the cytosol and to the plasma membrane, which suggests a role for centaurin-α_1 in signalling events between the plasma membrane and the nucleus, possibly mediated by phosphatidylinositol phosphates. Interestingly, nucleolin has been shown to interact with the regulatory subunit p85 of PI 3-K, thus linking nucleolin with the phosphatidylinositol pathway (Barel et al., 2001). Figure 5.1 shows a simplified diagram of how centaurin-α_1, CKI, PKC and nucleolin could all be linked by the phosphatidylinositol pathway. This figure is modified from Rameh and Cantey (1999).

Figure 5.1 Phosphatidylinositol pathway linking centaurin-α_1, PKC, CKI and nucleolin.

The phosphatidylinositol lipids are indicated. Nucleolin associates with PI 3-K. CKI is negatively regulated by PtdIns-(4,5)-P_2. Centaurin-α_1 binds PtdIns-(3,4,5)-P_3 and Ins-(1,3,4,5)-P_4. Novel and atypical PKC isoforms are activated by PtdIns-(3,4)-P_2 and PtdIns-(3,4,5)-P_3 (indicated by blue arrows). CPI-17 associates with and is phosphorylated by PKC (indicated by a grey arrow). In addition, CPI-17 binds to CKI and leads to inhibition of CKI activity (indicated by a grey bar). PLC hydrolyses PtdIns-(4,5)-P_2 to produce DAG and Ins-(1,4,5)-P_3 (indicated by black arrows). DAG activates PKC (indicated by a black arrow). PTEN (phosphatase and Tensin homology deleted on chromosome Ten) dephosphorylates PtdIns-3,4,5-P_3.

In addition, members of the Arf-GAP family are involved in membrane trafficking and actin cytoskeletal re-arrangement (reviewed in Donaldson, 2000; Jackson et al., 2000). These are cellular events in which PKC plays a role (reviewed in Keenan and Kelleher, 1998; Toker 1998) and therefore it is possible that centaurin-α_1 is also involved as it is a member of the Arf-GAP family, however with no shown Arf-GAP activity to date (reviewed in Donaldson, 2000). In support of this possibility, centaurin-α_1 has been shown to associate with CKIα (Dubois et al., 2001) which has been implicated in membrane trafficking (Faundez and Kelly, 2000; Gross et al., 1995) and actin *in vitro* phosphorylation (Dubois T., Maciver S.K. and Aitken A., unpublished results). Furthermore, the β2 and μ subunits of the AP-3 complex bind to the GST-centaurin-α_1 column. AP complexes are involved in the formation of intracellular transport vesicles and in the selection of protein cargo for incorporation into the vesicles (reviewed in Boehm and Bonifacino, 2001). The interaction between AP-3 subunits and centaurin-α_1 remains to be confirmed by other methods. However, if confirmed, it would further support a role for centaurin-α_1 in membrane trafficking.

In the next part the interaction between centaurin-α_1 and novel, mainly nuclear proteins which bind to the GST-centaurin-α_1 column was discussed. The interaction between nucleolin and centaurin-α_1 was selective and probably RNA dependent. Therefore, the association of nucleolin with the GST-centaurin-α_1 column might not be direct but could be mediated via RNA. A number of additional RNA/DNA binding proteins were identified in the GST-centaurin-α_1 column eluate, suggesting that the interaction between these proteins and the GST-centaurin-α_1 column could indeed be mediated via RNA/DNA and that a large protein complex might be present. The presence of numerous RNA binding proteins on the GST-centaurin-α_1 column and the fact that RNA seems to be necessary for the binding between nucleolin and centaurin-α_1 suggests that centaurin-α_1 could be a RNA binding protein involved in nuclear events such as ribosome biogenesis, mRNA transcription, mRNA splicing and translation.

However, these potential roles for centaurin-α_1 in the nucleus remain to be confirmed. On the other hand it is also possible that centaurin-α_1 does not bind RNA, but that association of RNA with nucleolin changes the conformation of nucleolin allowing it to bind centaurin-α_1 as mentioned previously.

The identification of potential novel centaurin-α_1 binding partners suggests multiple roles for this protein in different cellular compartments, ranging from membrane trafficking in the cytosol and plasma membrane to ribosome biogenesis in the nucleus. The direct interaction between these proteins and centaurin-α_1 remains to be confirmed. However, if centaurin-α_1 proves to be a RNA binding protein, it will clearly be involved in fundamental cellular processes and further investigation of its potential roles should be of major interest.

CONCLUSION

In conclusion, novel potential CPI-17 and centaurin-α_1 binding proteins have been identified. Most of the newly identified proteins were selective for CPI-17 and centaurin-α_1 respectively, suggesting distinct roles for both proteins in cells. However, some proteins associated with CPI-17 as well as with centaurin-α_1, thus the possibility exists that some of the functions of the two proteins could converge in specific cellular compartments and both proteins might for instance be involved in vesicular trafficking.

CHAPTER 6

Future work

6. Future work

The present study has identified novel CPI-17 and centaurin-α_1 binding proteins and has proposed potential roles for these proteins in cells. However, a number of questions remain to be answered. The association of CPI-17 with CKI and PKC *in vivo*, should be subject of further study to provide clues to the physiological relevance of these interactions. Co-transfection of COS-7 cells with PKC and CPI-17 could be used to study potential *in vivo* binding between these two proteins.

Novel potential CPI-17 have been identified, however their identity should first be confirmed by Western blotting. Once confirmed, one should test whether these proteins bind directly to CPI-17 or whether their interaction with the GST-CPI-17 column is mediated by protein complexes. This would provide a more direct link to the potential physiological functions of CPI-17.

The identification of the kinase which phosphorylates CPI-17 on S128 *in vivo* could provide more answers in understanding the functions of CPI-17.

It would be of major interest to identify the subcellular localisation of CPI-17 by immunofluorescence, and perform co-localisation studies with CKI and PKC, respectively. It would be interesting to test whether activation of PKC leads to changes in localisation of CPI-17, which could provide further clues for potential roles of CPI-17. To narrow down the binding site between CPI-17 and PKC or CKI, further deletion mutants of CPI-17 could be created and the binding to the two kinases could be tested. To understand the mechanism of binding to CPI-17 it would be of great interest to obtain the crystal structure of CPI-17.

To further investigate the role of the association between centaurin-α and PKC, the *in vivo* association between centaurin-α_1 and PKC should be investigated. The subcellular localisation of centaurin-α_1 has been investigated, however, it would be interesting to study whether centaurin-α_1 co-localises with PKC and if changes in

localisation of centaurin-α can be observed upon stimulation of PKC.

It would be of great interest to identify the phosphorylation site of centaurin-α_1 by PKC, an investigation which is underway. The exact roles of centaurin-α_1 remain to be established, however, once identified it would be interesting to see whether phosphorylation by PKC modulates the functions of this protein. Furthermore, it remains to be elucidated what role the binding between centaurin-α_1 and nucleolin has in cells. An important study would be to determine whether centaurin-α_1 is a RNA binding protein, which could for instance be investigated by Northwestern blotting.

As for CPI-17, novel potential centaurin-α_1 binding proteins have been identified by mass spectrometry, but their identity remains to be confirmed by Western blotting. The next study would test whether the proteins associated with centaurin-α_1 directly or whether the binding was mediated by protein complexes or via ribonucleoprotein complexes. Finally, it would be of great importance to study the interaction of centaurin-α_1 and these novel binding proteins.

7. References

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APPENDIX

Published papers:

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14-3-3 Proteins in Cell Regulation

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Specificity of 14-3-3 isoform dimer interactions and phosphorylation

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Abstract

Proteins that interact with 14-3-3 isoforms are involved in regulation of the cell cycle, intracellular trafficking/targeting, signal transduction, cytoskeletal structure and transcription. Recent novel roles for 14-3-3 isoforms include nuclear trafficking the direct interaction with cruciform DNA and with a number of receptors, small G-proteins and their regulators. Recent findings also show that the mechanism of interaction is also more complex than the initial finding of the novel phosphoserine/threonine motif. Non-phosphorylated binding motifs that can also be of high affinity may show a more isoform-dependent interaction and binding of a protein through two distinct binding motifs to a dimeric 14-3-3 may also be essential for full interaction. Phosphorylation of specific 14-3-3 isoforms can also regulate interactions. In many cases, they show a distinct preference for a particular isoform(s) of 14-3-3. A specific repertoire of dimer formation may influence which of the 14-3-3-interacting proteins could be brought together. Mammalian and yeast 14-3-3 isoforms show a preference for dimerization with specific partners *in vivo*.

Introduction

The name 14-3-3 was given to an abundant mammalian brain protein family due to its particular migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis [1]. The first function ascribed to this family of proteins was activation of tyrosine and tryptophan hydroxylases, the rate-limiting enzymes involved in catecholamine and serotonin biosynthesis, essential for the synthesis of dopamine and other neurotransmitters [2]. Subsequently we showed that 14-3-3 could regulate (inhibit) activity of protein kinase C (PKC) [3,4]. 14-3-3 was then implicated as a novel type of chaperone protein that modulates interactions between components of signal-transduction pathways [5]. A large number of publications began to appear in the mid-1990s showing that 14-3-3 proteins could interact with a range of protein kinases, phosphatases and other signalling proteins. This implied a role for the 14-3-3 family of proteins to mediate the formation of protein complexes involved in signal transduction, trafficking and secretion, perhaps to bind to different signalling proteins on each subunit of the dimer, as a novel type of 'adapter protein'. It is now clear that 14-3-3 isoforms are involved in many other cell functions and this is only one of their many roles. A number of intrinsic enzymic activities have been ascribed to 14-3-3, but none have been substantiated in the literature.

The five major mammalian brain 14-3-3 isoforms are named α–η in order of their respective elution positions on HPLC [2,6] and we showed that α and δ are the phosphoforms of β and ζ respectively [7]. Two other isoforms, τ and σ, are expressed in T-cells and epithelial cells respectively, although the former is also widely expressed in other tissues, including brain.

Muslin and co-workers [8] showed that target-protein phosphorylation is important for

Key words: phosphorylation, protein-interaction motif, signalling.
Abbreviations used: PKC, protein kinase C; AANAT, arylalkylamine N-acetyltransferase.
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Table 1

14-3-3-interacting sequences in mammalian systems

pS, phosphoserine; pT, phosphothreonine; GM-CSF, granulocyte/macrophage colony-stimulating factor; TNF, tumour necrosis factor.

Target protein and motifs	Sequence	Reference
(a) Protein kinases		
Breakpoint cluster region protein (Bcr)	$RSQSTPS^{303}EQ$	[33]
Ca^{2+}/calmodulin-dep myosin light-chain kinase, skeletal	$HSPpS^{161}CP$	[35]
PCTAIRE-1, protein kinase	$KRLpS^{119}LP$	[38]
PKCμ (PKD)	$RLpS^{205}NVS^{208}$, $RTSpS^{219}AELpS^{223}$	[39]
PKCζ	$RHDMpS^{186}YMP$	[30]
c-Raf-1 kinase	$RSTpS^{259}TP$, $RSApS^{621}EP$	[40]
B-Raf	$RSApS^{728}EP$	[41]
Testicular protein kinase (TESK1)	$RCRpS^{439}LP$	[42]
Wee1 cell-cycle Y kinase	$RSVpS^{642}LT$	[43]
(b) Phosphatases		
PTPH1, tyrosine phosphatase	$RSLpS^{359}VE$, $RVDpS^{853}EP$	[44]
Cdc25A, cell-cycle dual-specificity phosphatase	$RSPpS^{290}MP$	[45]
Cdc25C, cell-cycle phosphatase	$RSPpS^{216}MP$	[46,47]
(c) Receptors, G-proteins and related proteins		
α-Chain of interleukin 9 receptor (IL-9R)	$RpS^{519}WpT^{521}F$	[48]
β-Chain of GM-CSF, interleukin-3 and -5 receptors	$HSRS^{585}LP$	[49]
CLIC4 (p64H1), chloride channel	$R^{219}YLpTNAYpS$	[50]
Exoenzyme-S, ADP-ribosylation	$DALDL^{428}$	[51]
GPIbα subunit of platelet membrane glycoprotein Ib-IX-V	$RLpS^{166}LTDP$, $RYSGHSL^{610}{}_{-COOH}$	[52]
Insulin growth factor I receptor	$S\ VPLDPSASSSpS^{1283}LP$	[53]
lip35, major histocompatibility complex-associated	$RSRpS^{8}CR$	[54]
Insulin receptor substrate 1 (IRS-1)	$RSKpS^{270}QS$, $HSRpS^{374}IP$, $KSVpS^{641}AP$	[53]
Na^+/H^+ exchanger isoform-1 (NEH1)	$RIGpS^{703}DP$	[55]
Nicotinic acetylcholine receptor α 4 subunit	$RSLS^{441}VQ$	[56]
Nuclear receptor (Nur77)	$RLPpS^{350}KP$	[57]
Phosducin (photoreceptor G$\beta\gamma$-binding protein)	$RQMpS^{54}SP$, $RKpS^{73}IQ$	[58]
RAS effector protein RIN1	$RSMpS^{351}AA$	[59]

Regulators of G-protein signalling, RGS3, RGS7 (and others)	EKDpS^{496}YP, KSDpS^{434}YP	[60]
p190RhoGEF, guanine nucleotide exchange factor	I^{1370}QAIQNL	[61]
(d) Apoptosis-regulating proteins		
A20, zinc finger protein, inhibitor of TNF-induced apoptosis	RSKpS DP	[62]
Apoptosis signal-regulating kinase 1 (ASK1)	RSIpS^{967}LP	[63]
BAD, apoptosis-regulating	RHSpS^{112}YP, RSRpS^{136}AP, RRMpS^{155}DFE	[64,65]
(e) Adaptor proteins		
KSR (kinase suppressor of Ras)	RSKpS^{297}HE, RTEpS^{392}VP	[65a]
Cbl	RHpS^{619}LPFpS^{623}, RLGpS^{639}TFpS^{642}	[66]
(f) Transcription factors and nuclear proteins		
Forkhead transcription factor (FKHRL1)	RPRSCpT^{32}WP, RRRAVpS^{253}MD	[67,68]
Histone deacetylase, HDAC4	RKTApS^{246}EP, RTQpS^{467}AP, RAQpS^{632}SP	[69]
Histone deacetylase, HDAC5	RKTApS^{259}EP, RTQpS^{498}SP	[70]
Histone deacetylase, HDAC7	RKTVpS^{178}EP, RTRpS^{344}EP, RAQpS^{479}SP	[71]
Transcriptional co-activator with PDZ-binding domain (TAZ)	RSHpS^{89}SP	[72]
Nuclear factor of activated T-cells (NFAT3)	RRYpS^{272}pSpS, RRGpS^{289}	[73]
p53 tumour-suppressor/transcription factor	KGQSTpS^{378}RH/G	[10]
(g) Enzymes and others		
43 kDa inositol polyphosphate 5-phosphatase	ELVLRSESEEKVV^{371}	[74]
K1F1C, kinesin-like protein	RRQRpS^{1092}AP	[75]
Keratin 18 cytoskeletal component	RPVpSSAApS^{33}	[76]
Middle T antigen, polyoma virus	RSHpS^{257}YP	[77]
Serotonin N-acetyltransferase	RRHpT^{31}LP	[20]
Tyrosine hydroxylase	RRAVpS^{19}ELD	[78]

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14-3-3 binding via a novel phosphoserine sequence motif. It has subsequently been shown that while many 14-3-3-interacting proteins contain this motif, many others do not, indicating that additional sequences and modes of interaction/contacts also allow 14-3-3 binding. Cocrystal structures with non-phosphorylated motifs suggest that the same binding pocket or 'groove' is involved [9]. Not only can phosphorylation of the motif be an important regulatory component of the interaction but dephosphorylation can also lead to creation of an interaction motif. Waterman et al. [10] showed that ionizing radiation led to dephosphorylation of Ser-376 in the sequence KGQS^{376}TS^{378}RH/G in the p53 tumour-suppressor protein (see Table 1), creating a consensus binding site for 14-3-3 proteins which led to association of p53 with 14-3-3. This in turn increased the affinity of p53 for sequence-specific DNA.

14-3-3 isoforms and sequence conservation

The 14-3-3 family is highly conserved over a wide range of mammalian species, where the individual isoforms β, ε, η, γ, τ (also called θ), ζ and σ are either identical or contain a few conservative substitutions. Homologues of 14-3-3 proteins have also been found in a broad range of eukaryotic organisms and are probably ubiquitous (reviewed in [11,12]). In every organism studied, at least two isoforms of 14-3-3 have been observed.

The chromosomal location of 14-3-3 isoforms has been deduced from the human genome database. The haemopoietic ε sequence variant, ... VEL<u>D</u>VE ..., where the underlined D replaces T in this most highly conserved 14-3-3, is on chromosome 2 in the current draft genome sequence. No variants in the amino acid sequence of ε are otherwise known in any mammalian species. We have identified this haemopoietic tissue ε variant in human, ovine, bovine and rodent ε 14-3-3, in the same individual animal ([13] and A. Aitken, A. Toker and Y. Soneji, unpublished results). This form may also be present at low levels in keratinocytes. Structure analysis of the variation on the outer surface of ε 14-3-3 suggests this may have an important effect on interaction with other proteins. The most striking finding in the genome analysis is a large number of sequences matching the ζ isoform; at least nine protein translations in a number of chromosomes, most of which are presumably pseudogenes.

14-3-3 proteins are quite distinct in sequence and structural topology from other protein families in the database. Exceptions may be tertiary structure similarity with tetratricopeptide repeat ('TPR') helices [14] and primary structure similarity with α-synuclein in particular regions [15].

14-3-3 dimers, binding motifs and their interactions

Crystal structures of both the τ and ζ isoforms of 14-3-3 [16,17] show that they are highly helical, dimeric proteins. Each monomer is composed of nine anti-parallel α-helices, organized into an N-terminal and a C-terminal domain. The dimer creates a large negatively charged channel. Those regions of the 14-3-3 protein which are invariant throughout all the isoforms are mainly found lining the interior of this channel, while the variable residues are located on the surface of the protein.

This channel would recognize common features of target proteins, so the specificity of interaction of 14-3-3 isoforms with diverse target proteins may involve the outer surface of the protein. The N-terminal residues of all 14-3-3 homologues are variable, and as these residues are important for dimer formation there may be a limit to the number of possible homo- or hetero-dimer combinations. Residues involved in dimerization are 5–21 in the A-helix of one subunit and residues 58–89 of the C and D helices of the other (see Figure 1).

After the demonstration by Muslin and coworkers [8] that a novel phosphoserine-containing motif initially identified in Raf kinase was important for interaction, the motif was further refined into two subtypes and the structural details of the interaction with 14-3-3 were elucidated [18,19]. The binding site for the phosphoserine consists of a basic pocket composed of Lys-49, Arg-56 and Arg-127, as well as Tyr-128, within the C and E helices (see the underlined residues in Figure 1).

The proline residues are in different conformations in the two classes of phosphopeptide consensus motif. In the mode 1 phosphopeptide [18] the proline is in a *cis*-conformation while in the mode 2 phosphopeptide [19] the proline has a *trans*-conformation. Nevertheless, both proline conformations result in a sharp alteration in chain direction, allowing the peptide to exit the binding groove. This is also clear in the recent deter-

mination of the arylalkylamine N-acetyltransferase (AANAT)/14-3-3 co-crystal structure [20].

The ligand-binding groove runs in opposite directions in the two subunits of the dimer and a highly stable and isoform-specific interaction may result, distinct from the involvement of 14-3-3 dimer in binding two distinct target proteins together in a chaperone-like role.

Many of the 14-3-3-interacting proteins contain either one or both of two consensus motifs, R(S/Ar)(+)pSXP and RX(Ar)(+)pSXP [18] where pS is phosphoserine, Ar is an aromatic residue (particularly Tyr or Phe in mode 2) and + is a basic amino acid. Residue X following the phosphoserine is commonly Leu, Glu, Ala or Met. The phosphorylated residue may also be a threonine. Although most motifs contain Ser some well-characterized ones, e.g. AANAT, have Thr and there seem to be no structural reasons why this residue could not fit equally well into the cleft. Other motifs that are listed in Table 1 may be categorized as RSXpS motifs, $RX_{1-2}SX_{2-3}S$ motifs and non-phosphorylated motifs.

When phosphopeptide libraries were tested on six of the 14-3-3 isoforms and the yeast BMH1 and BMH2, all the isoforms were found to have very similar preferences for the individual residues within each consensus motif. There are seven mammalian isoforms of 14-3-3 that are all highly conserved and one supposes that each isoform would have a distinct function or subset of functions. For example, several isoforms of 14-3-3 are involved in the G_2/M cell-cycle checkpoint: 14-3-3 σ is responsible for sequestering the Cdc2–cyclin B1 complex in the cytoplasm, while the β and ε isoforms bind Cdc25C [21].

The complete sequence conservation in the observed ligand-binding regions of 14-3-3 would support the hypothesis that there may be little isoform specificity in the interaction between 14-3-3 and protein ligands; therefore many isoform-specific functions of 14-3-3 observed *in vivo* may result either from subcellular localization or transcriptional regulation of particular isotypes rather than from inherent differences in their ability to bind to particular ligands. However, the finding that phosphorylated 14-3-3 negatively regulates the interaction with the Raf N-terminal domain [22] suggests that additional interactions may occur on the outer surface of 14-3-3 or its dimer. A number of publications in the literature confirm recently that for full functionality in at least some cases the dimer of 14-3-3 is essential [23].

Figure 1

Human ζ 14-3-3 phosphorylation sites and motifs

The positions of the known phosphorylation sites on various isoforms are shown in large type and the phosphopeptide-binding residues are underlined. Ac denotes the N-terminus of the monomer (this is *N*-acetylmethionine, residue 1 in most isoforms). The numbering is that of the alignment of the correctly processed longer members of the family (i.e. ε and longer form of β that commence at residue 1) and the introduction of gaps of two residues maximize the alignment.

5 21
Ac-MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGA
dimerisation
AKT/SDK1/PKC PKC
59 64
RRSSWRVVSSIEQKTEGAEKKQQMAREYREKIETELRDICNDVL
58 89
RSX^{P}SXP motif binding
SLLEKFLIPNASQAESKVFYLKMKGDYYRYLAEVAAGDDKKGIVDQSQQAYQEAFEIS

185
KKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKTAFDEAIAELDTLSE

233
ESYKDSTLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN
CK1α

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Recent findings relating to the structures and mechanism of interaction of 14-3-3 with its partners strengthen the evidence that interaction is not simply mediated by the canonical phosphoserine-containing motif. Some well-characterized interacting proteins such as Raf kinase have been shown to have additional binding site(s) for 14-3-3 on their cysteine-rich regions. Bcr and Ksr also bind via serine-rich regions. The interaction between 14-3-3 and Raf is complex and the exact role of 14-3-3 remains controversial. The fact that Raf contains a third interaction domain in the cysteine-rich region would add further complexity. This could also confer isoform specificity. It has also been suggested that due to the very common occurrence of two binding sites or motifs on one polypeptide chain of an interacting protein, the synergy between the two may also lead to isoform preference of interaction. Interaction motifs that are near the optimum, as determined by surface plasmon resonance spectroscopy [18], show no specificity but less optimal ones do. The binding of a protein in both (lower) affinity sites may greatly increase target-protein specificity of recognition and affinity. This suggests a dual site-recognition mechanism in which, for example, a 14-3-3 ζ dimer interacts with both glycoprotein I (GPI)bα(unphosphorylated motif) and GPIbβ (with a phosphorylation-dependent binding site), resulting in high-affinity binding (see Table 1).

The 14-3-3 phosphoserine-binding motif is also a common feature in plant and other eukaryotic species [24]. The plant plasma membrane H^+-ATPase motif, QQYpT^{947}V-$_{COOH}$, is noteworthy in that it has so far been found only in plant proteins [25].

Around 100 proteins have been shown to interact with 14-3-3. The well-characterized 14-3-3-interacting proteins and the motif(s) that have been identified are listed in Table 1. Some target proteins contain more than one motif that has been shown to be involved in 14-3-3 binding and they are listed only once. In many cases the actual residue numbers will be different in mammalian species other than the one(s) that were studied, although the site will be equivalent; for example, Ser-178 is the most important in mouse HDAC7. The equivalent residues in the human sequence are Ser-156, Ser-318 and Ser-446 in the motif RAQS^{446}SP.

In addition to those listed in Table 1, mammalian proteins that have been shown to interact include the following. (a) Protein kinases; CK1α, Chk1 kinase, MEKK1, -2 and -3, type I PKC (α,β,γ), PKCε, PKCθ and PKUα. (b) Phosphatase Cdc25B; see also (g). (c) Receptors; α2-adrenergic, γ-aminobutyric acid (GABA) (B), glucocorticoid, adrenergic, α and β oestrogen, nuclear receptor co-repressor RIP140 and small G-proteins and their regulators, Rad and Rem. (d) Proteins involved in apoptosis including α-synuclein and the neurotrophin receptor (p75NTR)-associated cell death executor NADE. (e) Adaptor/scaffolding molecules p130Cas, Grb2 and Shc (p66). (f) Transcription factors and other proteins involved in transcriptional control, TATA box-binding proteins TBP and TFIIB, histone acetyltransferase 1, Msn2p and 4p, other forkhead family members, the co-activator YAP (Yes-associated protein), myocyte enhancer binding factor 2 (MEF2D) proteins in the MADS [MCM1, AGAMOUS, DEFICIENS and SRF (where SRF is serum response factor)] box family of transcription factors, FKBP12-rapamycin-associated protein (FRAP), primary response gene BRF1, PHDfinger-HD, topoisomerase IIα, initiation factor EIF2α, integrin CD18 chain, TLX-2 homeodomain transcription factor and histones. (g) Enzymes and others including tryptophan hydroxylase, catalytic subunit (p110) of phosphoinositide 3-kinase, calmodulin, mitochondrial uncoupling protein (UCP) and CMP-NeuAc:GM1 α-2,3-sialyltransferase; structural and cytoskeletal proteins including keratin K8, Kif1C, Tau and vimentin; proteins involved in cell-cycle control including cell-cycle phosphatase Cdc25B and telomerase catalytic subunit (TERT).

14-3-3-binding motif kinases

Kinases that have been shown to phosphorylate the phosphoserine or threonine in the phosphorylated binding motifs include protein kinase B (also called Akt), cAMP-dependent protein kinase, p21-activated protein kinase 1 (PAK), Ras-mitogen-activated protein kinase (RSK1, also known as MAPKAP-K1), MAP kinase-activated protein kinase-2 (MAPKAP-K2) and PKC, as would be expected from their substrate specificity. In most cases the physiological kinases(s) have not been demonstrated but those that co-localize in the cell would be prime candidates; e.g. Cdc25C has been reported to be phosphorylated by a number of kinases such as Cds-1/Chk2, Chk1 and Cdc25C-associated kinase 1 (C-TAK1). The first two are nuclear-located. In addition, Ca^{2+}-calmodulin-dependent kinase II (CAM kinase II), p90 ribosomal S6K, protein kinase D (also known

as PKCμ) and casein kinase II (CKII) have also been implicated in phosphorylation of the motifs.

14-3-3 isoform phosphorylation

Our sequence analysis of brain 14-3-3 separated by reversed-phase HPLC failed to show any differences between α and β on the one hand and δ and ζ on the other [26]. We subsequently showed by mass spectrometric analysis that α and δ were the phosphorylated forms of β and ζ respectively and we identified the site as Ser-185 [7] using the numbering of the generic mammalian 14-3-3. The acetylation at the N-terminus of 14-3-3 isoforms, with or without removal of the initiator methionine, has been verified by mass spectrometry for all isoforms except σ [26].

β 14-3-3 is expressed at alternate start sites, 40% of which is co-translationally processed, resulting in an additional threonine residue at the N-terminus. There is no evidence that this would affect dimer formation.

This phosphorylation of Ser-185 may not 'turnover', as high levels of the phospho-forms are recovered without precautions taken to inhibit phosphatases. Although the ε and σ isoforms contain a serine residue in equivalent positions (SPDR and SPEE respectively), they are not known to be phosphorylated at this site. The ε isoform is particularly abundant in brain; therefore it would have been identified by mass spectrometric analysis when we characterized the α and δ isoforms. We have found no evidence for α and δ phospho-forms in a wide range of tissue types and cell lines other than brain. Inspection of the many two-dimensional gel electrophoretograms that are in the literature lend support to the idea that this phosphorylation may be specific to brain 14-3-3.

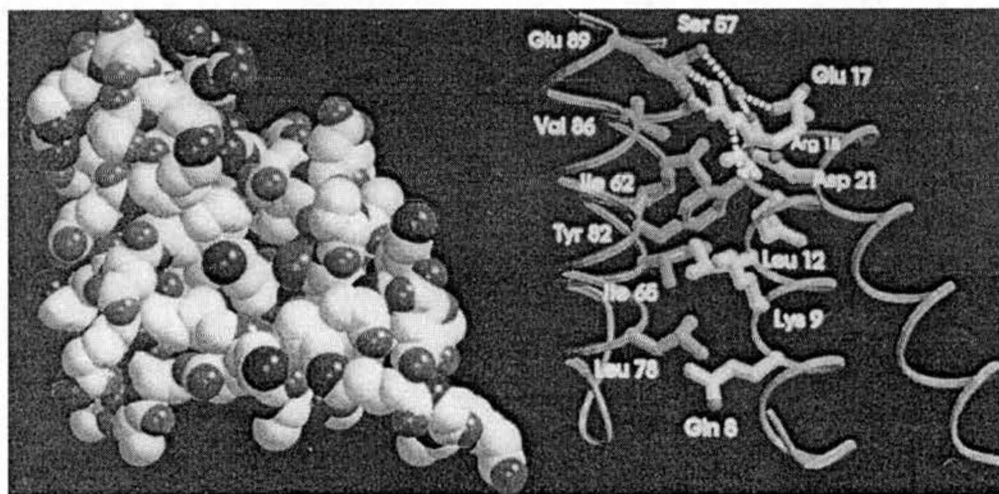
We identified casein kinase 1α (CKIα) as the brain kinase that phosphorylated 14-3-3 ζ and τ isoforms on Thr- and Ser-233 respectively. 14-3-3 ζ is phosphorylated on Thr-233 in HEK-293 cells [22]. Residue 233 is located within a region involved in the association of 14-3-3 with target proteins. *In vivo* phosphorylation of 14-3-3 ζ at this site negatively regulates its binding to c-Raf, and may be important in Raf-mediated signal transduction. We have also shown (S. Mackie, T. Dubois and A. Aitken, unpublished work) that CK1α binds to 14-3-3 isoforms *in vitro*.

Some isoforms of 14-3-3 have been shown to be phosphorylated by a sphingosine-dependent kinase [27] and PKB/Akt [27a]. The site these authors determined, Ser-59, is masked in the dimer interface [16]. If phosphorylation occurs, it could be a mechanism for regulation of dimer formation. However, it might be difficult under physiological conditions for a kinase to gain access, since the 14-3-3 dimer is very stable under normal conditions (see Figure 2). Our results have shown that while brain PKC did phosphorylate some 14-3-3 isoforms on an adjacent residue, Ser-64, the phosphorylation did not appear to occur to any physiologically significant extent [6], although there is an excellent motif in this region. In contrast, in our deletion mutants that exposed

Figure 2

Structure of the τ 14-3-3 dimer interface

The interface between the monomers is mainly hydrophobic, comprising 70% of the total 2150 Å^2 of inaccessible surface [16].



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this site [28] there are very high levels of phosphorylation. We also showed that large synthetic peptides are also stoichiometrically phosphorylated at Ser-59 and there were low levels of phosphorylation at Ser-64 [29].

Van Der Hoeven et al. [30] showed that 14-3-3 β and τ were substrates for PKC-ζ (sites not determined) and Autieri and Carbone [31] showed phosphorylation of human 14-3-3 γ by PKC isoforms on undetermined site(s). However, their cDNA sequence was quite different from previous γ sequences (including that in the human genome) and included changes in residues known to be invariant.

Reuther and co-workers [32] showed that 14-3-3 τ interacted with c-Bcr and with Bcr/Abl. Their results indicated that 14-3-3 τ was a substrate for the Bcr serine/threonine kinase and was also phosphorylated on tyrosine by Brc/Abl but not by c-Abl. On attempting to identify this site(s) of phosphorylation on these and other 14-3-3 isoforms we have evidence (S. Clokie, S. Mackie and A. Aitken, unpublished work) that the phosphorylation is due to a co-immunoprecipitating kinase at a known site of phosphorylation.

Dimerization of mammalian and yeast 14-3-3 isoforms *in vivo*

In many cases, interaction between 14-3-3 and other signalling proteins shows a distinct preference for particular isoform(s) of 14-3-3. The existence of particular combinations of heterodimeric 14-3-3 isoforms *in vivo* has important implications for function as an adaptor protein in signalling. This may allow the interaction between signalling proteins that do not directly associate with each other. Bcr and Raf, for example, do not associate directly but form a complex mediated through 14-3-3 [33]. β and τ 14-3-3 associate with Bcr while the ζ and β 14-3-3 isoforms interact with Raf. We have shown that isoforms of 14-3-3 can form homo- and hetero-dimers *in vivo* and *in vitro* and a specific repertoire of dimer formation may influence which of the 14-3-3-interacting proteins could in turn be brought together [34]. Since the residues involved in dimerization exhibit some isoform variation, this may limit the possible homo- or hetero-dimer combinations and may confer specificity on 14-3-3 function.

The dimers of 14-3-3 are stable and do not readily exchange unless they are denatured and renatured [34]. It should be noted that a single recombinant 14-3-3 isoform will form homodimers regardless of the actual preference *in vivo* since a monomeric form would be thermodynamically unstable. 14-3-3 proteins also have a slow rate of turnover.

We analysed the pattern of dimer formation for two of the most abundant isoforms of 14-3-3 ε and γ, following their stable expression in the neuronal pheochromocytoma PC12 cell line (M. Chaudhri and A. Aitken, unpublished work). This revealed a distinct preference for particular dimer combinations that is largely independent of cellular conditions. γ 14-3-3 formed homo- and hetero-dimers, mainly with ε. In turn, the ε isoform formed heterodimers with all the 14-3-3 isoforms tested (β, η, γ and ζ), but no homodimers were detected. This suggests that the observed dimer combinations may be due to structural properties of the dimer interface of the individual isoforms. This pattern may be generally applicable since the dimerization patterns of γ and ε isoforms in transiently transfected COS cells were also similar overall. The pattern of dimer formation was not simply a reflection of the amounts of available 14-3-3 isoform present in the cell or in a particular subcellular location. Analysis of the two 14-3-3 homologues, BMH1 and BMH2, from the budding yeast *Saccharomyces cerevisiae* also revealed that between 65 and 80 % are heterodimers (M. Chaudhri and A. Aitken, unpublished work).

Homo- and hetero-dimers of 14-3-3 proteins may play different roles. It is possible that homodimers of a particular isoform will function to sequester or chaperone a protein, while heterodimers are more likely to act as adaptor proteins, modulating the interaction of two distinct proteins, each of which specifically associates with one of the isoforms of the heterodimer. The γ isoform may be more involved in sequestration functions, while the ε isoform is more likely to function as an adaptor protein in signal-transduction events. Indeed, γ 14-3-3 has been found to be the major isoform in Golgi and is implicated in secretion and protein trafficking while the ε isoform is more generally found in association with proteins involved in signal transduction.

In conclusion, association with 14-3-3 isoforms has led to the activation or stabilization of some proteins, and the inactivation of others, while for many proteins 14-3-3 isoforms have played an organizational role as a 'scaffold'. In the complex multistep process of Raf activation, 14-3-3 appears to play several of these roles. It is likely that its ability to form homo- and various heterodimeric combinations is crucial and the analysis of the exact combinations of homo- and hetero-dimers of

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14-3-3 isoforms that are present within cell compartment(s) and that are involved in interactions with particular proteins will be important.

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Survival-promoting functions of 14-3-3 proteins

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Abstract

The 14-3-3 proteins are a family of phosphoserine/phosphothreonine-binding molecules that control the function of a wide array of cellular proteins. We suggest that one function of 14-3-3 is to support cell survival. 14-3-3 proteins promote survival in part by antagonizing the activity of associated proapoptotic proteins, including Bad and apoptosis signal-regulating kinase 1 (ASK1). Indeed, expression of 14-3-3 inhibitor peptides in cells is sufficient to induce apoptosis. Interestingly, these 14-3-3 antagonist peptides can sensitize cells for effective killing by anticancer agents such as cisplatin. Thus, 14-3-3 may be part of the cellular machinery that maintains cell survival, and targeting 14-3-3–ligand interactions may be a useful strategy to enhance the efficacy of conventional anticancer agents.

Key words: apoptosis, ASK1, Bad, difopein.
Abbreviations used: PKB, protein kinase B; ASK1, apoptosis signal-regulating kinase 1; EYFP, enhanced yellow fluorescent protein.
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Introduction

14-3-3 proteins can bind a variety of proteins that are critical mediators of intracellular signalling

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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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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-α_1 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-α_1 associates *in vitro* with all mammalian CKI isoforms. To date, CKIα represents the first protein partner identified for centaurin-α_1. However, our data suggest that centaurin-α_1 is not a substrate for CKIα and has no effect on CKIα activity. Centaurin-α_1 has been identified as a phosphatidylinositol 3,4,5-trisphosphate-binding protein. Centaurin-α_1 contains a cysteine-rich domain that is shared by members of a newly identified family of ADP-ribosylation factor guanosine trisphosphatase-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)[1] 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 (CKI α, 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. CKIε 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/or in membrane trafficking (19). CKIα has been shown to play a role in cell cycle progression (20) and in membrane trafficking (21, 22). Recently, CKIs have 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 CKIδ 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)P_2 (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. CKIα 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 filaments (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).

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[1] The abbreviations used are: CKI(s), casein kinase(s) I; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; ARF, ADP-ribosylation factor; GAP, guanosine trisphosphatase-activating protein; PH, pleckstrin homology; HA, hemagglutinin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

In the present study, we have shown that CKIα interacts with centaurin-α_1. Centaurin-α_1 is a PtdIns(3,4,5)P_3-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) guanosine trisphosphatase-activating proteins (GAP) (reviewed in Refs. 36–39). The yeast protein that shows the highest homology to centaurin-α_1, Gcs1, 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'-gggcatatggcgagcagcag-cggctccaagg-3') and (5'-gggggatccttagaaacctgtgggggtttgggc-3') in order to create a 5' *Nde*I site and a 3' *Bam*HI site (both are underlined in sequences). Amplified cDNA was inserted in a pET-16b vector (Novagen) at *NdeI/Bam*HI restriction sites to express CKIα as a histidine-tagged protein. The CKIα cDNA was inserted in a pcDNA3 vector (Invitrogen) from the original clone after being amplified by PCR using two oligonucleotides (5'-gggggatccg*ccacc***atggcctacccctacgacgtgcccgactacgcc**cccgcgagcagcagcggctcc-3') and (5'-gcggcggccgcttagaaacctgtgggggtttgggc-3') in order to create a 5' *Bam*HI site and a 3' *Not*I 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) at the *Eco*RI and *Bam*HI sites downstream of the T7 promoter. The oligonucleotides (the *Eco*RI and *Bam*HI sites are underlined) used are 5'-ggggaattcgccaccatgtacaaactggtacggaagatcgg-3'; 5'-ggggaattcgccaccatgtacagagacaacagaacaaggc-3'; 5'-ggggaattcgccaccatgagcatcaatgcacatcttgg-3'; 5'-ggggaattcgccaccatgaccagcctgccgtggcaagg3'; 5'-ggggaattcgccaccatgtacgagaagattagcgaaaagaagatgtcc-3'; 5'-gggggatccttaggtcctgaaaaggatgcgg-3'; 5'-gggggatccttagaaacctgtgggggtttgggc-3'. The construction of GST-centaurin-α_1 has been described previously (42). Centaurin-α_1 cDNA from that vector was recovered after digestion with *Eco*RI and *Xba*I and subcloned in a FLAG-cmv2 vector. HA-tagged CKIα(D136N) in pcDNA3 is a gift from F. McKeon (23). FLAG-centaurin-α is from Trevor R. Jackson.

For the experiment performed in Fig. 7*B*, pET3c CKIα (41), pET8c CKIδ (25), pET8c CKIδ Δ317 (25), pET8c CKIγ1 (19), pSV2Zeo CKIγ2 (from Louise Larose), pET8c CKIγ3 (19), and pSP72 CKIϵ were used. pSP72 CKIϵ construct was subcloned using human CKIϵ plasmid (pV405) provided by Dr. David Virshup (27). CKIϵ cDNA was amplified by PCR using two oligonucleotides (5'-ggaagatctatggagctacgtgtggggaacaag-3') and (5'-ggaaaagctttcacttcccgagatggtcaaatgg-3') in order to create a 5' *Bgl*II site and a 3' *Hin*dIII site and inserted into pSP72 vector.

Identification of Centaurin-α_1 by Mass Spectrometry—After the final chromatography step during the purification of CKIα as a 14-3-3 kinase from brain (Sephacryl 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 45-kDa band was excised and subjected to trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, Worthington) digestion. The extracts were then dried in a Speedvac vacuum centrifuge and made up to the injection volume with water for on-line liquid chromatography mass spectrometry. Electrospray mass spectrometry of in-gel digested protein was carried out as described (43), and the peptide map data were used in the PeptideSearch program.

Recombinant Protein Purification—*Escherichia coli* carrying GST-centaurin-α_1, 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 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 mM 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 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, and 50% glycerol at −20 °C. Recombinant 14-3-3 ζ was purified as described previously (43).

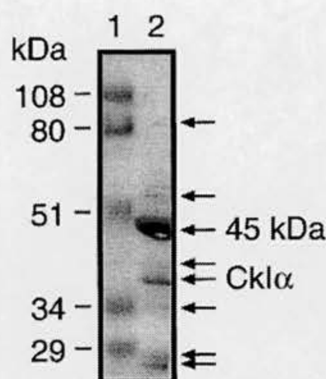


FIG. 1. **Co-purification of a 45-kDa protein with CKIα.** CKIα was purified from pig brain after four chromatography steps (43). The fractions containing CKI activity were electrophoresed on 10% SDS-PAGE (*lane 2*), and the gel was stained with Coomassie Blue. CKIα was previously identified by mass spectrometry (43). A total of seven other proteins co-purified with CKIα (indicated by *arrows*), suggesting that they may form protein complex(es). The major co-purifying band migrates at 45 kDa. Molecular mass standards (*lane 1*) are shown in kDa.

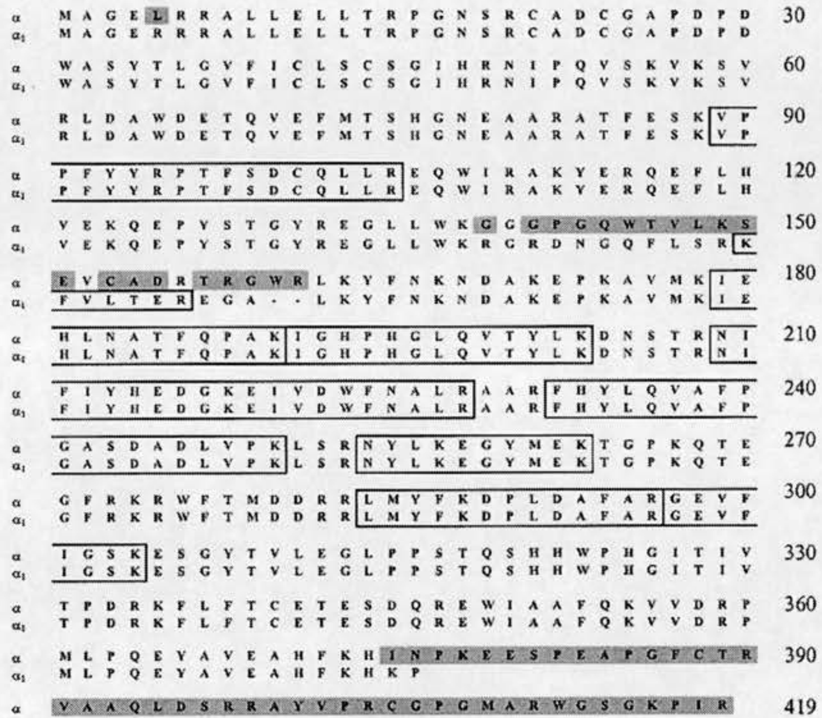
In Vitro binding between Purified CKIα and Members of the Centaurin-α Family—GST, GST-centaurin-α_1, or GST-centaurin-α (0.2 μM final concentration) were incubated with histidine-tagged CKIα in binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM DTT, 0.1% Nonidet P-40) for 2 h at 4 °C. 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 $MgCl_2$, 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-^{32}P]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 P81 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_1, and GST-14-3-3 ζ 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_1 on CKIα activity, different amounts of centaurin-α_1 (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 14-3-3 ζ or GST-14-3-3 ζ 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 Transcription and Translation—CKIα and 14-3-3 ζ subcloned in pcDNA3 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 [^{35}S]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 10 μg of GST, GST-centaurin-α, or GST-centaurin-α_1 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 Amplify™ (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

A



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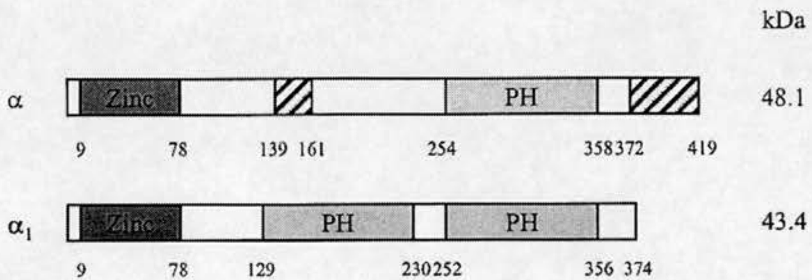

FIG. 2. **Identification of the 45-kDa protein as centaurin-α_1, a PtdIns-(3,4,5)P_3-binding protein.** *A*, the band corresponding to the 45-kDa protein (Fig. 1) was excised and subjected to trypsin digestion and electrospray mass spectrometry. Nine peptides were recovered (shown in *boxes*). By using the "PeptideSearch" program, they all matched with centaurin-α_1 and eight of them with centaurin-α. Both aligned sequences are from rat (33, 45). Note that the first peptide is not identical in sequence to the one that we found (from pig brain), but for easier interpretation of the figure, we aligned the sequence of centaurin-α and -α_1 from the same species (rat). The 45-kDa protein was purified from pig brain, and the actual sequence of the peptide is VPPFYYRPSASDCQLLR (the different amino acids are underlined). This peptide is identical in the pig centaurin-α_1 (34). *B*, a schematic representation of centaurin-α and centaurin-α_1. Both these proteins contain a zinc finger motif (*Zinc*) related to the one found in ARF-GAP proteins. Centaurin-α differs from centaurin-α_1 by the presence of only one PH domain (*PH*) rather than two and by a 43-amino acid extension at the carboxyl-terminal end (the differences are shown by *dashed boxes*). The mass spectrometric analysis was able to differentiate between these two proteins. Indeed, one peptide (KFVLTER) is only present in centaurin-α_1 (in its first PH domain) and not in centaurin-α.

serum (Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.) at 37 °C in a humidified chamber with 5% CO_2. 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-α_1 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 DTT) containing a mixture of protease inhibitors (Roche Molecular Biochemicals), 1 mM sodium fluoride, 1 mM 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 (C-^{214}FNRTSLPWQGLKAATKKQKY^{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, 1 M NaCl, 10% glycerol, 1 mM 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-α_1 was detected using a rabbit polyclonal anti-

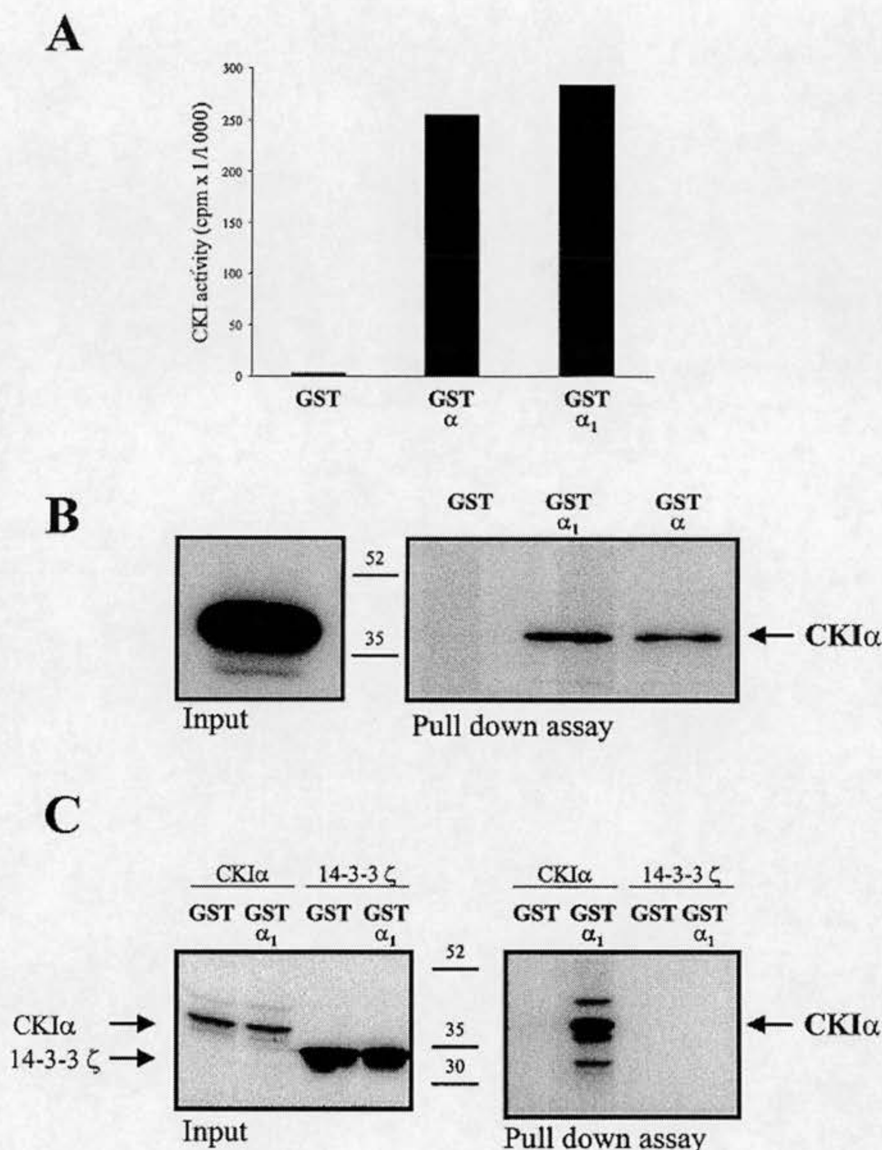

FIG. 3. **CKIα associates physically with centaurin-α and centaurin-α_1.** *A*, GST, GST-centaurin-α (*GST-α*), and GST-centaurin-α_1 (*GST-α_1*) were incubated for 2 h at 4 °C with purified histidine-tagged CKIα. Glutathione-Sepharose beads were then added. Beads were washed and subjected to an *in vitro* kinase assay using a CKI-specific phosphopeptide substrate. The presence of ^{32}P incorporated into the peptide was quantified by liquid scintillation counting and represents CKIα activity associated with the beads. *B*, CKIα was expressed and ^{35}S-labeled using a T7 TNT-coupled transcription/translation reticulocyte cell-free system and incubated with 1 μg of GST, GST-centaurin-α_1 (*GST-α_1*) or GST-centaurin-α (*GST-α*) for 15 min at 30 °C in the presence of 0.1% Nonidet P-40. Glutathione-Sepharose beads were added and incubated at room temperature for an additional 1 h. Beads were washed, and samples were analyzed by 10% SDS-PAGE followed by autoradiography (*panel Pull down assay*). An aliquot of the lysate from the *in vitro* transcription/translation assay was loaded on the gel (*panel Input*). *C*, CKIα and 14-3-3 ζ were synthesized as described above (*panel Input*). They were incubated with 10 μg of GST or GST-centaurin-α_1 (*GST-α_1*) for 15 min at 30 °C in the presence of 1% Nonidet P-40. Beads were washed, and samples were analyzed by 10% SDS-PAGE followed by autoradiography (*panel Pull down assay*).

body (J49) that was raised against an amino-terminal peptide of centaurin-α_1 (33).

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 complex(es) 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-α_1, a PtdIns(3,4,5)P_3-binding Protein—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)P_3 binding protein. We identified nine peptides that matched with centaurin-α_1 and eight peptides that matched with centaurin-α (Fig. 2*A*). Centaurin-α_1 shares high homology to centaurin-α. Centaurin-α contains an extended carboxyl-terminal tail and only one PH domain compared with centaurin-α_1 (Fig. 2*B*). Therefore, peptide mass analysis differentiated these two proteins and identified the 45-kDa protein unequivocally as centaurin-α_1 (42). Centaurin-α_1 has been given different names in the literature including p42^{IP4} (34) and PIP3BP (35). However, for clarity in the rest of this paper, we refer to it as centaurin-α_1.

CKIα Associates Physically with Centaurin-α_1—We tested the potential interaction between CKIα and centaurin-α_1 using purified recombinant proteins. We show that CKIα associates with GST-centaurin-α_1 and not with GST (Fig. 3*A*). In addition, CKIα also binds directly to centaurin-α (Fig. 3*A*).

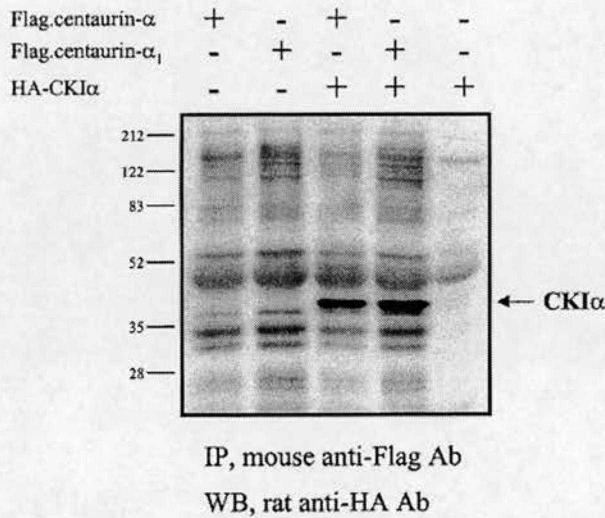

FIG. 4. **CKIα associates *in vivo* with centaurin-α and centaurin-α_1.** COS-7 cells were co-transfected with plasmids expressing the proteins indicated at the *top* of the figure or empty vectors. After 36 h, cells were harvested in lysis buffer containing 1% Nonidet P-40. Centaurins were immunoprecipitated using mouse monoclonal antibody (*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).

In order to confirm this interaction, [^{35}S]methionine-labeled CKIα was synthesized using a cell-free coupled transcription/translation system and incubated with GST, GST-centaurin-α, or GST-centaurin-α_1 (Fig. 3*B*). The results show that CKIα associates with centaurin-α and with centaurin-α_1.

Therefore, two distinct experiments showed that CKIα interacts physically with both centaurins. The levels of CKIα that associated with centaurin-α_1 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, [^{35}S]methionine-labeled CKIα and 14-3-3 ζ were incubated with either GST or GST-centaurin-α_1 (Fig. 3*C*). By using higher stringency (1% detergent), centaurin-α_1 indeed associates with CKIα but not with 14-3-3 ζ even though the latter is expressed at much higher levels than CKIα (Fig. 3*C*, *panel Input*), thus showing the specificity of the interaction between centaurin-α_1 and CKIα. In conclusion, centaurin-α and centaurin-α_1 associate specifically and directly with CKIα. As rat centaurin-α (except its unique carboxyl-terminal tail) shares 94% identity with rat centaurin-α_1 (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-α_1 and the carboxyl-terminal domain of centaurin-α.

CKIα Associates in Cells with Centaurin-α and Centaurin-α_1—We tested whether CKIα associates in cells with centaurin-α_1. For that purpose, we transiently 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-α or centaurin-α_1. 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-α (Fig. 4, *3rd lane*) and centaurin-α_1 (Fig. 4, *4th lane*) immunoprecipitates and not in the controls (Fig. 4, *1st, 2nd* and *5th lanes*). Therefore, the experiment demonstrates that CKIα associates with both centaurins in COS-7 cells.

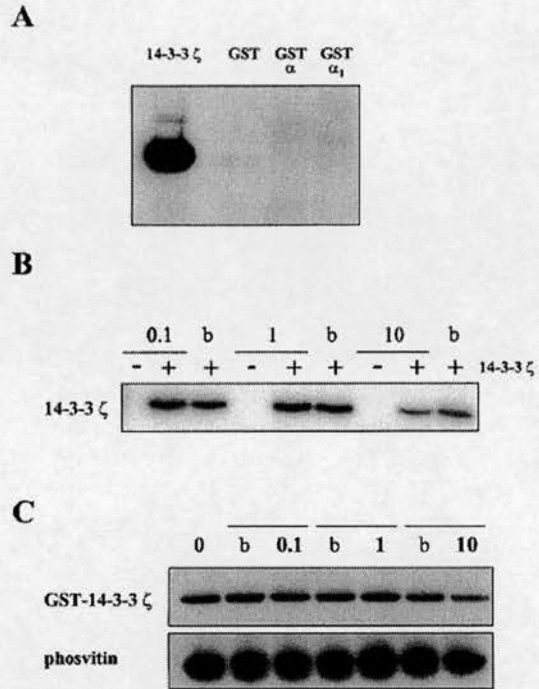

FIG. 5. **Centaurins are not phosphorylated by CKIα and have no effect on CKIα kinase activity.** *A*, 1 μg of purified GST, GST-centaurin-α (*GST-α*), and GST-centaurin-α_1 (*GST-α_1*) were assessed for *in vitro* phosphorylation by purified histidine-tagged CKIα as described under "Materials and Methods." Reactions were stopped and electrophoresed on 10% SDS-PAGE. The gel was dried and autoradiographed. 14-3-3 ζ was phosphorylated by CKIα as a positive control (*lane 14-3-3*). *B*, 0.1, 1, or 10 μg of recombinant centaurin-α_1 or buffer (*b*) was preincubated for 15 min with CKIα. The reaction was initiated with the addition ("+") of the CKI substrate 14-3-3 ζ. No substrate was added in ("−") as a control in case some degraded forms of centaurin-α_1 would be phosphorylated by CKIα. *C*, 0, 0.1, 1, or 10 μg of recombinant centaurin-α_1 or the corresponding volume of buffer (*b*) was incubated with CKIα as described above. The reaction was initiated with the addition of GST-14-3-3 ζ or phosvitin.

Centaurins Are Not Phosphorylated by CKIα—We tested whether CKIα phosphorylates centaurin-α and centaurin-α_1 *in vitro* using recombinant proteins. Fig. 5*A* shows that GST-centaurin-α and GST-centaurin-α_1 were not substrates for CKIα *in vitro.* As a positive control, 14-3-3 ζ, a CKI substrate (43), was shown to be phosphorylated (Fig. 5*A*, *lane 14-3-3 ζ*). In addition, recombinant centaurin-α_1 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 carboxyl-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-α and centaurin-α_1 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-α and centaurin-α_1 either purified or immunoprecipitated from COS-7 cells are not substrates for CKIα.

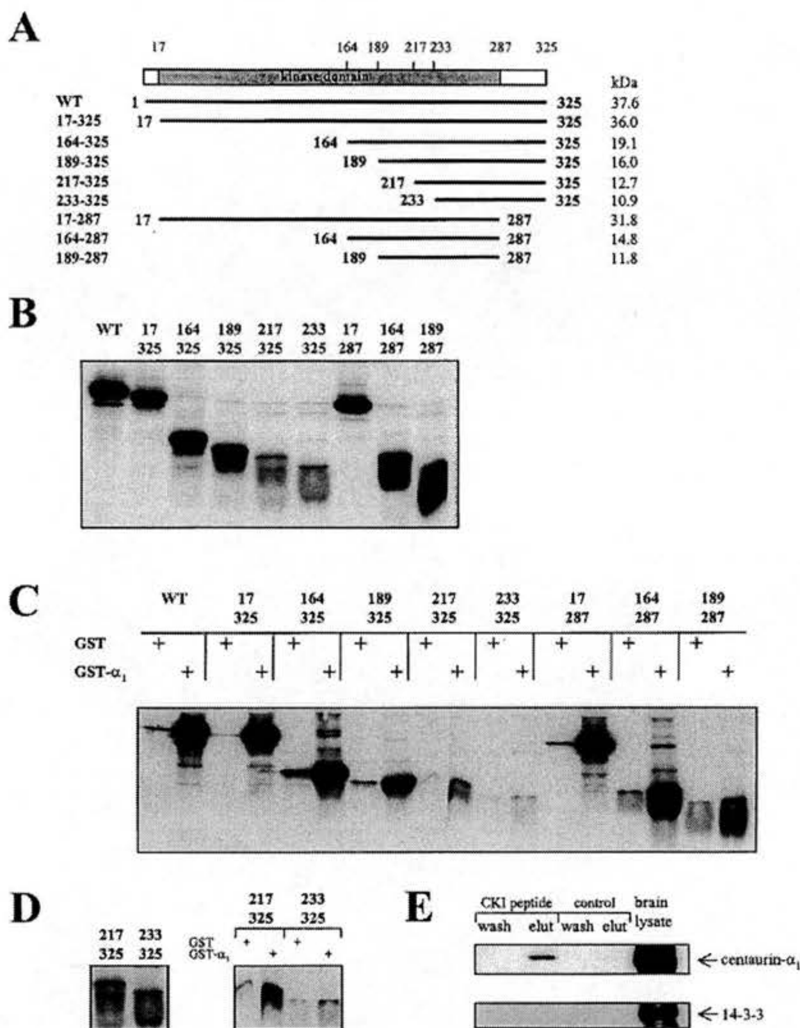

FIG. 6. **Centaurin-α_1 associates with the residues 217–233 of CKIα.** *A*, schematic representation of a series of deletion mutants of CKIα (*WT*, wild type). Residues 17–287 constitute the kinase domain. Residues 164–189 represent a loop containing a kinesin homology domain (1). Residues 217–233 are found in a loop that has been proposed to be responsible for the interaction of CKIα with other proteins (46). The calculated molecular masses of wild type and deletion mutants of CKIα are shown in kDa. *B*, wild type and mutant CKIα were synthesized using an *in vitro* transcription/translation assay, and an aliquot of the reaction (0.5% input) was analyzed on 15% SDS-PAGE. *C*, the rest of the reaction was incubated with 10 μg of GST or GST-centaurin-α_1 using the same protocol as described in Fig. 3*B*. The binding of CKIα was determined by analysis on 15% SDS-PAGE and autoradiography. *D*, 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 (C-[214]FNRTSLPWQGLKAATKKQKY[233]) of CKIα was coupled (*CKI peptide*) or a control column (*control*). The columns were washed, and bound proteins were eluted with buffer containing 1 M NaCl. Eluted fractions (*elut*) and the last washes (*wash*) were subjected to SDS-PAGE and analyzed by immunoblotting using centaurin-α_1 or 14-3-3 antibodies. A brain extract was also analyzed as a positive control for the antibodies (*brain lysate*).

Centaurin-α_1 Does Not Affect CKIα Activity—As centaurin-α_1 does not represent a substrate for CKIα, we have tested whether centaurin-α_1 affects the kinase activity of CKIα. We have shown that different amounts of recombinant centaurin-α_1 (0.1, 1, or 10 μg) have no effect on CKI activity using 14-3-3 ζ (Fig. 5*B*), GST-14-3-3 ζ, or phosvitin (Fig. 5*C*) as CKI substrates.

Centaurin-α_1 Interacts with Residues 217–233 of CKIα—Mammalian CKIα belongs to a family of seven isotypes 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-α_1-binding site in CKIα in order to elucidate whether centaurin-α_1 associates in a region specific to CKIα or one that is also present in other CKI isotypes. To address this question, we constructed a set of CKIα deletion mutants (Fig. 6*A*). These mutants were synthesized and labeled with [^{35}S]methionine in a cell-free coupled transcription/translation system (Fig. 6*B*) and incubated with GST or GST-centaurin-α_1 (Fig. 6*C*). The different mutants were expressed in similar amounts apart from mutants 217–325 and 233–325 that were less well synthesized (Fig. 6*B*). 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-α_1 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-α_1 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-α_1, a brain extract was loaded onto a column containing this peptide or a control column. Centaurin-α_1 was found to associate to the peptide column (Fig. 6*E*). This association is highly specific as we did not detect the presence of 14-3-3 proteins that represent 1% of total brain proteins.

In conclusion, our results mapped biochemically the centaurin-α_1-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-α_1 Associates with all Members of the CKI Family—Residues 217–233 of CKIα are well conserved in all CKI isoforms as indicated in Fig. 7*A*. Therefore, it was interesting to determine whether other CKI isoforms were able to associate with centaurin-α_1. To that purpose, all mammalian CKI isoforms were synthesized and labeled with [^{35}S]methionine in a cell-free coupled transcription/translation system and incubated with GST or GST-centaurin-α_1 (Fig. 7*B*). All mammalian CKI isoforms were capable of associating with GST-centaurin-α_1 and not with GST alone (Fig. 7*B*). Interestingly, a

mutant of CKIδ deleted of its carboxyl-terminal domain (CKIδ Δ317) binds as well as wild type CKIδ to centaurin-α_1 (Fig. 7*B*). 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-α_1. These data have led us to strongly believe that centaurin-α_1 interacts with residues 217–233 of CKIα that are well conserved in all CKI isoforms.

DISCUSSION

In this report we have identified centaurin-α_1 as a novel CKIα partner based on the following evidence: (*a*) they co-purified from brain after elution from four chromatography steps; (*b*) centaurin-α_1 associates *in vitro* with CKIα indicating that the binding is direct; (*c*) the binding is specific as centaurin-α_1 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-α_1 interacts with residues 217–233 of CKIα using deletion mutants of CKIα; and (*f*) centaurin-α_1 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-α_1 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-α_1 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-α_1 *in vitro*. This suggests that the same loop, present in all CKI isoforms, is responsible for the interaction with centaurin-α_1.

Centaurin-α_1 and centaurin-α have been identified as PtdIns(3,4,5)P_3-binding proteins (33–35). Phosphatidylinositol 3-kinase is mainly responsible for the synthesis of PtdIns(3,4,5)P_3 by phosphorylating PtdIns(4,5)P_2 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 PtdIns(3,4,5)P_3-binding protein (51). PtdIns(4,5)P_2 has been shown to inhibit CKIα activity *in vitro* (21). However, the physiological relevance of the inhibition of CKIα by these two phospholipids remains to be demonstrated. Another link between CKI and the phosphoinositide pathway has been reported in *S. pombe*. The authors showed that a yeast CKI homologue, Cki1, phosphorylates and inhibits PtdIns(4)P 5-kinase (52).

Centaurin-α_1 belongs to a newly identified family of ARF-GAP proteins (reviewed in Refs. 36–39). Members of this family share a cysteine-rich GAP domain and contain several other domains such as PH domains, SH3 domains, and ankyrin repeats. These proteins are involved in vesicle trafficking and in 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 CKI and AP-3 was identified previously (8). Moreover, CKIα has been found to co-localize in neurones with synaptic vesicle markers and phosphorylates some vesicle synaptic associated proteins (21). Interestingly, centaurin-α_1 has been shown to associate with presynaptic vesicular structures (53). An actin-associated protein kinase

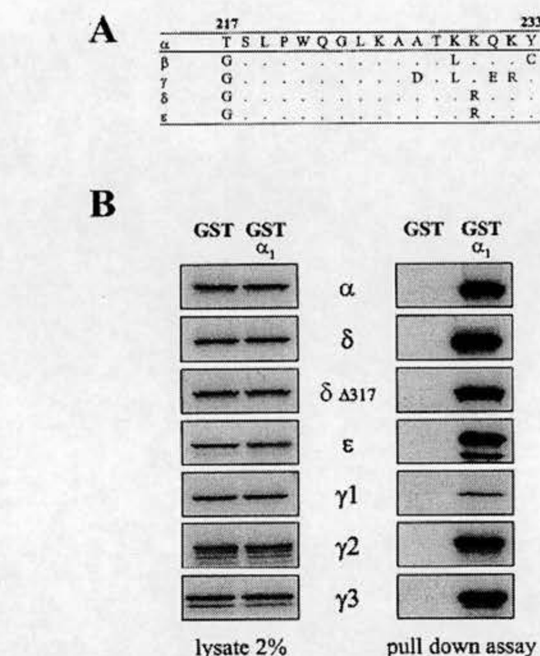

FIG. 7. **Centaurin-α_1 associates with all members of the CKI family.** *A*, sequence alignment of residues 217–233 of CKIα with those corresponding to the other CKI isoforms (β–ε). *B*, CKI isoforms (*α, δ, δ Δ317, ε, γ1, γ2,* and *γ3*) were synthesized as described in Fig. 3*B*. They were incubated with 10 μg of GST or GST-centaurin-α_1 (*GST-α_1*) as described in Fig. 3*C* in the presence of 1% Nonidet P-40 and 0.1% bovine serum albumin. Beads were washed, and samples were analyzed by 12.5% SDS-PAGE followed by autoradiography (*pull down assay*). Aliquots representing 2% of total lysate were analyzed as a control (*lysate 2%*).

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 CKI *in vitro* (55).

Members of the ARF-GAP family contain several domains for protein-protein interactions, and they have been shown to associate 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, CKIγ2 has been found 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-α_1, 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-α_1, but it is able of rescuing a ΔGcs1 strain mutant indicating that centaurin-α_1 and Gcs1 may have similar function(s) (62). Gcs1 has been shown to be necessary for

[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 may 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 cytokinesis, in bud development (5, 6), and regulation of the actin cytoskeleton as yck^{ts} 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_1. In addition centaurin-α_1 has no effect on CKI activity. Therefore, what is the functional relevance of the interaction between CKIα and these PtdIns(3,4,5)P_3-binding proteins? As CKIα does not contain a lipid binding domain, it may associate with membranes through interaction with other proteins. Centaurin-α_1 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_1 as suggested by the results in budding yeast showing that CKIs suppress Gcs1 mutant phenotypes.

In conclusion, we have shown an interaction between CKIα and centaurin-α_1, 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 CKI and Gcs1 in budding yeast.

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Identification of casein kinase Iα interacting protein partners

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Abstract **Casein kinase Iα (CKIα) belongs to a family of serine/threonine protein kinases involved in membrane trafficking, RNA processing, mitotic spindle formation and cell cycle progression. In this report, we identified several CKIα interacting proteins including RCC1, high mobility group proteins 1 and 2 (HMG1, HMG2), Erf, centaurin-α_1, synaptotagmin IX and CPI-17 that were isolated from brain as CKIα co-purifying proteins. Actin, importin-α_1, importin-β, PP2Ac, centaurin-α_1, and HMG1 were identified by affinity chromatography using a peptide column comprising residues 214–233 of CKIα. We have previously shown that centaurin-α_1 represents a CKIα partner both in vitro and in vivo. The nuclear protein regulator of chromosome condensation 1 (RCC1) is a guanosine nucleotide exchange factor for Ran which is involved in nuclear transport and mitotic spindle formation. Here we show that CKIα and RCC1 interact in brain and in cultured cells. However, the interaction does not involve residues 217–233 of CKIα which are proposed from X-ray structures to represent an anchoring site for CKI partners. Formation of the RCC1/CKIα complex is consistent with the association of the kinase with mitotic spindles. In conclusion, we have identified a number of novel CKIα protein partners and their relations to CKI are discussed.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Casein kinase Iα; Regulator of chromosome condensation 1; Mitotic spindle formation

1. Introduction

Members of the casein kinase I (CKI) family are serine/threonine protein kinases which are widely expressed in a number of species ranging from plants to mammals (reviewed in [1]). Seven isoforms from distinct genes are expressed in mammals (CKIα, β, δ, ε, γ_1, γ_2, γ_3). However, many more CKI forms are generated as a result of alternative splicing. CKI isoforms contain a highly conserved kinase domain and variable amino- and carboxy-terminal tails. CKIs appear to be constitutively active and are not regulated by second messengers. However, CKIδ/ε activities are modulated by autophosphorylation [2–5]. CKI isoforms preferentially phosphorylate proteins on residues that occur two to three residues downstream of an acidic or a phosphorylated residue.

Mammalian CKI isoforms are involved in various biological functions. For instance, CKIε and CKIδ play a role in the regulation of p53 [6,7]. CKIε has also been implicated in circadian rhythms [8] and in the Wnt signalling pathway [9,10]. CKIδ/ε are also implicated in membrane trafficking, in the regulation of centrosome and in spindle formation [11–13]. CKIγ_2 appears to play a role in actin cytoskeleton organisation [14,15]. CKIα has been shown to play a role in cell cycle progression [16], in membrane trafficking [17–19] and in RNA processing [20]. CKIs have also been shown to regulate the nucleo-cytoplasmic localisation of some of their substrates [21,22].

CKI isoforms contain a putative nuclear localisation signal (NLS), a kinesin homology domain and exhibit a high degree of variation in their subcellular localisations [1]. CKIα has been shown to localise to synaptic vesicles, to the centrosomes, with spindle microtubules at mitosis and also to nuclear structures such as speckles [17,20,23]. CKIγ_2 is present at the plasma membrane where it associates with membrane receptors, Pak1 and Nck [14,15,24]. CKIδ is associated with post-Golgi structures, microtubules and the spindle apparatus [11–13].

Although the yeast CKI isoforms have been well characterised, the functions of the mammalian CKI isoforms are much less known. Therefore, the identification of mammalian CKI substrates and binding proteins should help us to clarify their cellular function(s). CKIα has been shown to interact with the transcription factor NF-AT4 [21], G protein-coupled receptors [25], the AP3 complex [18], DARPP32 [26] and centaurin-α_1 [19]. In this report, we have identified numerous novel CKIα protein partners. One of these CKIα interacting proteins is the regulator of chromosome condensation 1 (RCC1). RCC1 is a guanosine exchange factor for the small G protein Ran which plays a role in nuclear transport and in mitotic spindle formation (reviewed in [27–29]). We show that RCC1 co-purifies with CKIα in brain. RCC1 associates with a CKI activity in brain and in cultured cells. Moreover, RCC1 interacts with ectopically expressed CKIα in COS-7 cells. Our data suggest a role for CKIα in mitotic spindle formation which is in agreement with its association with mitotic spindles [23].

2. Materials and methods

2.1. *Identification of the CKIα co-purifying proteins from brain lysate by mass spectrometry*

We have previously identified from brain CKIα as a kinase which phosphorylates 14-3-3 τ and ζ [30] and we found that several proteins co-purified with CKIα [19,30]. The identification of these CKIα co-purifying proteins was performed by electrospray mass spectrometry after trypsin digestion as described previously [19].

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2.2. Cell culture

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% foetal calf serum (Life Technologies, Inc.) and 1% penicillin/streptomycin (Life Technologies, Inc.) at 37°C in a humidified chamber with 5% CO_2.

2.3. Kinase assays on the immunoprecipitates

Brain tissue and cells were lysed as described [19]. Lysates were precleared with Pansorbin cells (Roche Molecular Biochemicals) and incubated with an affinity-purified goat polyclonal RCC1 antibody (Santa Cruz Biotechnology, Inc.). A mixture of protein A and G (Pharmacia) was then added to precipitate the antibody. A kinase assay was performed on the bead precipitates as described [19] using 14-3-3 ζ wild-type or T232A (purified as in [30]) or the phosphopeptide KRRRALS^{P}VASLPGL (where S^P is a phosphoserine; New England Biolabs) as CKI specific substrates. 14-3-3 ζ T232A was used as a control. Kinase assays using 14-3-3 proteins were analysed by SDS–PAGE followed by autoradiography. In contrast, the phosphopeptide substrate was spotted on P81 paper squares (Whatman) and washed four times with 1% phosphoric acid. Radioactivity retained on the papers was quantified by liquid scintillation counting [19].

2.4. Transfection and co-immunoprecipitation

COS-7 cells were transfected using Fugene (Roche Molecular Biochemicals) for 24–36 h in 60-mm diameter petri dishes with 4 μg of DNA (HA-tagged CKIα wild-type or with the empty vector, pCDNA3.1). Cells were serum-starved for 16 h and were either stimulated for 10 min with 100 nM insulin (Sigma) or left untreated. The cells were lysed and RCC1 was immunoprecipitated as described above. Proteins were transferred onto nitrocellulose (Bio-Rad) and the presence of HA-CKI was detected by Western blotting with a rat anti-HA (Roche Molecular Biochemicals) antibody and ECL detection (Amersham Pharmacia Biotech).

2.5. Identification of proteins which interact with residues 214–233 of CKIα by affinity chromatography

This experiment was performed as described [19]. Briefly, rat brain lysate was loaded onto a 1 ml Sulfo-Link (Pierce) column to which 1 mg of a peptide corresponding to residues 214–233 (C-^{214}FNRTSLPWQGLKAATKKQKY^{233}) of CKI was coupled. Brain extract was also loaded onto a control column. Both columns were washed extensively and bound proteins were eluted with 1 M NaCl. Eluted proteins were revealed by mass spectrometry after in-gel trypsin digestion [19] or by Western blotting using high mobility group protein 1 (HMG1; Pharmingen), RCC1 (Santa Cruz Biotechnology, Inc.), centaurin-α_1 [19] and pan 14-3-3 [19] antibodies.

3. Results and discussion

3.1. Identification of CKIα co-purifying proteins in brain

During the purification of CKIα from brain as a protein kinase which phosphorylates 14-3-3 proteins [30], several proteins co-purified with the kinase after four conventional chromatography steps (SP-Sepharose, Blue Affi-gel Blue, Mono S and gel filtration) [19]. These co-purifying proteins may represent novel CKI-partners and we therefore attempted to identify them by mass spectrometry. These proteins were identified as Erf, RCC1, synaptotagmin IX, HMG1/2, centaurin-α_1 and CPI-17 (Table 1). It should be noted that the in vitro and in vivo association of CKIα with centaurin-α_1 has been shown by our laboratory previously [19].

Some of the proteins that we have identified here have previously been implicated in CKI functions. For instance, HMG1, an architectural DNA binding protein [31,32], has recently been shown to be phosphorylated by CKI [33]. Erf is a transcription factor of the Ets family [34] and Ets1 has been used as an in vitro CKI substrate [3]. Moreover, we have found that Erf is also phosphorylated in vitro by CKIα and preliminary data indicate that it occurs within the serine/threonine-rich domain of Erf (T. Dubois, G. Mavrothalassitis, and A. Aitken, unpublished data). CPI-17, a protein phospha-

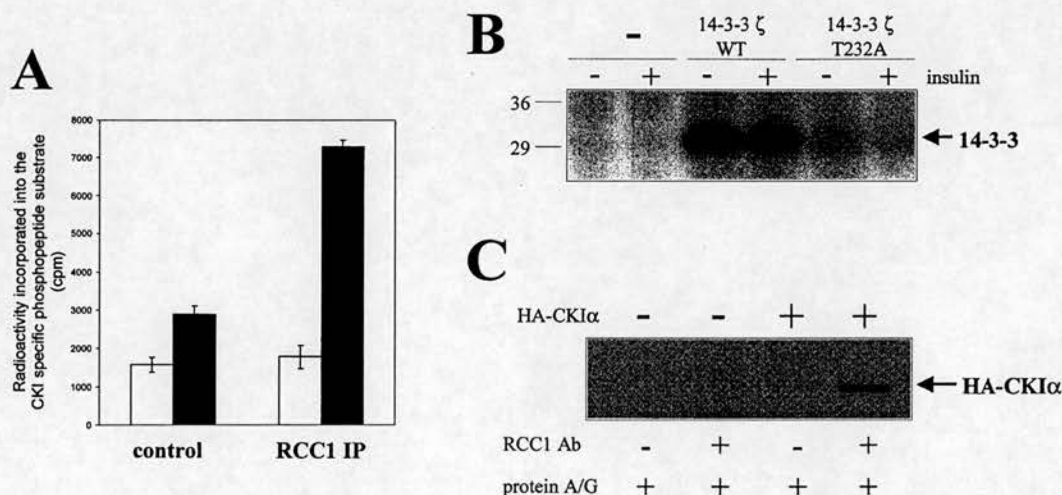

Fig. 1. RCC1 associates in vivo with CKI. A: Brain lysate was incubated with (RCC1 IP) or without (control) RCC1 antibodies and a mixture of protein A and G was then added. A kinase assay was performed on the precipitates without (open bars) or with a CKI specific phosphopeptide as the substrate (closed bars). The presence of radioactivity (^{32}P) incorporated into the peptide was quantified by liquid scintillation counting and represents CKI activity associated with the beads. B: COS-7 cells lysate from insulin-stimulated cells or unstimulated cells was immunoprecipitated with RCC1 antibodies. A kinase assay was performed using no CKI substrate (−), 14-3-3 ζ wild-type (14-3-3 ζ WT) or a mutant of 14-3-3 ζ whereby the CKI phosphorylation residue was mutated to alanine (14-3-3 ζ T232A). The kinase assay was analysed on SDS–PAGE and the gel was autoradiographed. The position of 14-3-3 is indicated as well as the molecular weight markers (kDa). C: COS-7 cells were transfected with a plasmid expressing HA-tagged CKIα (+, top of the figure) or with the empty vector (−, top of the figure). Cells were lysed and incubated with (+) or without (−) RCC1 antibodies followed by the addition of a mixture of protein A and G. The presence of CKIα in the immunoprecipitates was detected by Western blotting using a rat anti-HA monoclonal antibody. The position of HA-CKIα is indicated.

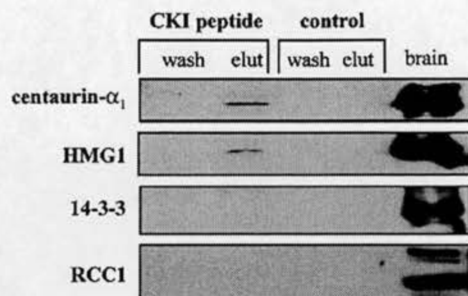

Fig. 2. RCC1 does not associate with residues 217–233 of CKIα. Brain extracts were loaded onto a 1 ml peptide affinity column to which a peptide corresponding to residues 214–233 (C-[214]FNRTSLPWQGLKAATKKQKY[233]) of CKIα was coupled (CKI peptide) or onto a control column (control). The columns were washed, and bound proteins were eluted with 1 M NaCl. Eluted fractions (elut) and the last washes (wash) were subjected to SDS–PAGE and analysed by immunoblotting using RCC1 or HMG1 antibodies. Antibodies against centaurin-α_1 or 14-3-3 were used as positive and negative controls, respectively [19]. A brain extract was also analysed as a positive control for the antibodies (brain lysate).

tase 1 inhibitor [35], associates with and regulates the activity of members of the CKI family (E. Zemlickova, F.J. Johannes, A. Aitken, and T. Dubois, manuscript in preparation). Synaptotagmin IX has been shown to associate with a mRNA binding protein [36]. The association of synaptotagmin IX with CKIα is interesting in light of evidence which suggests that CKIα may regulate certain steps of mRNA metabolism [20].

Therefore, it appears that all the co-purifying proteins we have identified are related in some way to the function(s) of CKI. It also means that the affinity of these proteins is strong enough to withstand four different chromatography steps. It is noteworthy that the interactions between CKI and the co-purifying proteins may represent in vivo complex(es) as they were identified from brain. Whether these proteins interact simultaneously or in a mutually exclusive manner with CKI is not known. It is interesting to note that some of the proteins are mainly localised in the nucleus (RCC1, HMG1/2, centaurin-α_1 and Erf). This may explain the observed association of CKIα with mitotic spindles and speckles within the nucleus [1].

3.2. *CKIα and RCC1 co-purify in brain*

We also identified RCC1 as a protein which co-purified with CKIα (Table 1). RCC1 is a guanosine exchange factor for Ran, a small G protein of the Ras superfamily. RCC1 has been involved in nuclear transport and mitotic spindle formation (reviewed in [27–29]). Importantly, CKIα has been found to be associated with mitotic spindles [23]. Other members of the CKI family (CKIδ and CKIε) have been proposed to play a role in the regulation of centrosome or spindle function during cell division. Both have been found to be recruited to the centrosomes and to the spindle apparatus [11–13]. In addition, yeast CKIs have been linked to chromosome segregation and cytokinesis [1]. Moreover, members of the CKI family contain a kinesin homology domain [37]. Kinesin, which possesses this domain that has been proposed to be involved in microtubule interactions, plays a role in the assembly of mitotic spindle and the segregation of chromosomes. Therefore, experiments were carried out to characterise the interaction of RCC1 with CKIα.

Table 1
Identification of CKIα co-purifying proteins in brain

CKI co-purifying proteins	Function
HMG1/HMG2	DNA binding proteins
RCC1	mitotic spindle formation, nucleo-cytoplasmic shuttle
Synaptotagmin IX	associates with a mRNA binding protein
Erf	transcription factor of the Ets family (repressor)
CPI-17	protein phosphatase 1 inhibitor
Centaurin-α_1	membrane trafficking

We have identified six proteins by mass spectrometry that we found to co-purify with CKIα in brain. These proteins are listed in the table and their functions are indicated.

3.3. *RCC1 associates with a CKI activity in brain*

We tested whether RCC1 associates with CKI in brain. For that purpose, endogenous RCC1 was immunoprecipitated from brain lysate using RCC1 antibodies. A kinase assay using a specific CKI phosphopeptide substrate was performed on the RCC1 immunoprecipitates. Results showed that RCC1 associates with a kinase which is capable of phosphorylating the specific CKI phosphopeptide substrate (Fig. 1A, 'RCC1 IP'). The CKI activity is detected only in the RCC1 immunoprecipitate and not in the control, thus validating the experiment (Fig. 1A, 'control'). Therefore, results from Fig. 1A indicate that a CKI activity specifically associates with RCC1 in brain.

3.4. *RCC1 associates with a CKI activity in cultured cells*

In order to further confirm the endogenous association between CKI and RCC1, we immunoprecipitated RCC1 from COS-7 cells and performed a kinase assay using another CKI substrate. We have previously shown that CKI phosphorylates 14-3-3 ζ on Thr-232 [30]. We have used 14-3-3 ζ as a substrate because the CKI phosphopeptide substrate may be more prone to non-specific phosphorylation. Endogenous RCC1 associates with a kinase which is able to phosphorylate 14-3-3 ζ WT but not 14-3-3 ζ in which the CKI phosphory-

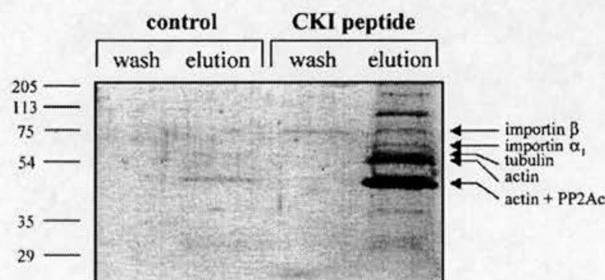

Fig. 3. A number of brain proteins associate specifically with residues 217–233 of CKIα. Brain extracts were loaded onto a peptide column comprising residues 214–233 of CKIα as described in Fig. 2. Eluted proteins were analysed by SDS–PAGE and were visualised by Coomassie blue staining. The bands were excised and subjected to trypsin digestion and electrospray mass spectrometry. The peptide map analysis identified some of the proteins as importin-α_1, importin-β, actin, tubulin and PP2Ac. The positions of these proteins are indicated as well as the molecular weight markers (kDa).

lation site has been mutated to an alanine (14-3-3 ζ T232A) (Fig. 1B). In addition, the kinase activity associated with RCC1 was not sensitive to insulin stimulation. This is consistent with CKI activity which is proposed to be second messenger-independent. In conclusion, Fig. 1B indicates that endogenous RCC1 associates with an endogenous CKI activity in COS-7 cells.

3.5. RCC1 associates with ectopically expressed CKIα in cultured cells

To further demonstrate the association between RCC1 and CKIα, we have transfected COS-7 cells with HA-CKIα. We have used COS-7 cells as CKIα was found to be expressed poorly or not at all (not detectable) in other cell lines tested (discussed in [19]). Endogenous RCC1 was immunoprecipitated and the presence of CKIα in the immunoprecipitate was detected by Western blot analysis using an anti-HA antibody. The results showed that endogenous RCC1 forms a protein complex with ectopically expressed CKIα in COS-7 cells (Fig. 1C).

3.6. RCC1 does not associate with residues 217–233 from CKIα

We have previously mapped the centaurin-α_1 binding site within residues 217–233 of CKIα using several approaches [19]. These residues belong to a loop between helices E and F of CKIα that have been proposed to be the target region for protein–protein interactions [38]. A brain extract was passed through a column to which a peptide comprising residues 217–233 from CKIα was coupled [19]. We tested whether RCC1 eluted from the peptide column by Western blotting using RCC1 antibodies. However, we were unable to identify RCC1 in any of the fractions eluted from the CKI peptide column (Fig. 2), thus indicating that residues 217–233 of CKIα do not represent the RCC1 binding site. Centaurin-α_1 and 14-3-3 antibodies were used as positive and negative controls, respectively [19].

3.7. Identification of HMG1, importin-α_1/β, catalytic subunit of protein phosphatase 2A (PP2Ac), tubulin and actin as putative CKI partners interacting with residues 217–233 of the kinase

Several proteins from brain specifically eluted from the CKI peptide column as judged by Coomassie blue staining (Fig. 3). We attempted to identify these putative CKI protein partners by mass spectrometry. Proteins were digested with trypsin and peptide mass map analysis identified them as HMG1, importin-α_1, importin-β, PP2Ac and actin (Fig. 3). Therefore, these proteins represent novel putative CKI partners interacting with residues 217–233 of CKIα.

Intriguingly, the roles of importins are closely related to those of RCC1. Indeed, RCC1 activates Ran which uses importin-α/β as effectors in both nuclear transport and spindle assembly (reviewed in [28]). The finding that importins bound to the CKI peptide column emphasised a function for CKI in these biological events and further supports our present results regarding the CKI/RCC1 association. In addition, importin-α recognises NLS-bearing proteins, and importin-β associates with the importin-α–NLS complex to facilitate the transport to the nucleus through the nuclear pore. Interestingly, residues 217–233 of CKIα contain a putative NLS (amino acids KKQK) [1] and we show that these residues represent the CKI importin binding site. As CKIα is also expressed in the nucleus, our results suggest that the transportation of CKI to the nucleus is via its interaction with importin-α_1/β.

We also identified PP2Ac as a protein which specifically associates with residues 217–233 of CKIα (Fig. 3). In yeast, a genetic link has been found between CKI and PP2A [39]. In addition, phosphorylation/dephosphorylation events by the CKI/PP2A complex are important in endocytosis and actin cytoskeleton organisation in yeast [40]. PP2Ac and CKI may form a complex in which the two activities may regulate each other [40]. Supporting the existence of such a complex, PP2A has been shown to associate with several protein kinases including casein kinase II [41], calcium-calmodulin-dependent kinase IV [42], p21-activated kinase [43], p70 S6 kinase [43], Raf-1 [44] and cyclic adenosine monophosphate-dependent protein kinase [45]. Our data suggest that PP2A also associates with CKI within residues 217–233 but further experiments are required to characterise the interaction.

Actin was also found to interact with residues 217–233 of CKIα (Fig. 3). This result is consistent with a report showing that a protein having CKI activity and a molecular weight of 37 kDa associates with and phosphorylates actin [46]. The M_r of the kinase suggested that it could be CKIα and we have found that CKIα phosphorylates actin in vitro (T. Dubois, S.K. Maciver, and A. Aitken, unpublished data). Taken together, this suggests that CKIα associates with and phosphorylates actin, and we have mapped the site of interaction to residues 217–233. Tubulin associates also specifically to the peptide column (Fig. 3) and it is interesting to find this protein in the context of the association of CKI with mitotic spindles.

Finally, we have identified HMG1 as a protein which associates specifically with residues 217–233 of CKIα by Western blot analysis (Fig. 2). This result strongly supports the idea that they form a protein complex after our finding that they co-purified from brain (Table 1). In support of this finding, HMG1 has recently been shown to be phosphorylated by CKI [33].

4. Conclusions

We have identified numerous potential CKI protein binding partners including RCC1. Our findings are consistent with a role of RCC1 in mitotic spindle formation and the association of several CKI mammalian isoforms with the mitotic spindle. However, we do not know whether the association is direct or via another molecule, and the possibility for RCC1 to be a substrate for CKI has not been tested yet. In conclusion, the association of CKI with RCC1 supports a role for this kinase in mitotic spindle formation. The identification of RCC1, HMG1, importin-α_1/β, PP2Ac, tubulin, actin, Erf, synaptotagmin IX, centaurin-α_1 and CPI-17 as CKI protein partners gives a further insight into the biological roles of CKI. Indeed, these interactions need to be fully characterised in order to better understand the role of CKI in mammalian cells.

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