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.

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-

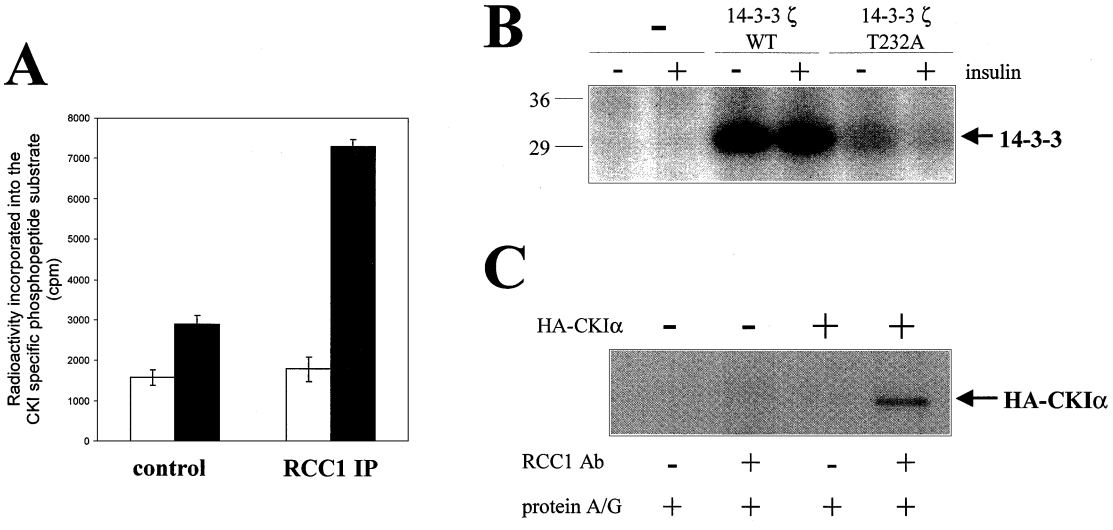

Fig. 1. RCC1 associates in vivo with CKI. A: Brain lysate was incubated with (RCC1 IP) or without (control) RCC1 antibodies and a mixture of protein A and G was then added. A kinase assay was performed on the precipitates without (open bars) or with a CKI specific phosphopeptide as the substrate (closed 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.

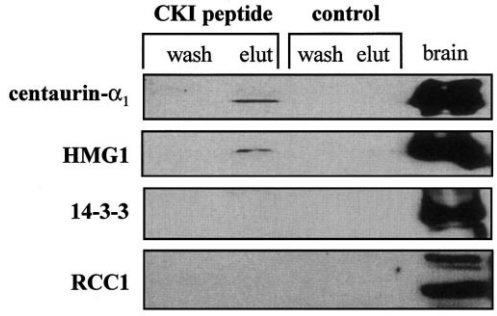

Fig. 2. RCC1 does not associate with residues 217–233 of CKIα. Brain extracts were loaded onto a 1 ml peptide affinity column to which a peptide corresponding to residues 214–233 (C-^{214}FNRTSLPWQGLKAATKKQKY^{233}) of CKIα was coupled (CKI peptide) or onto a control column (control). The columns were 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-

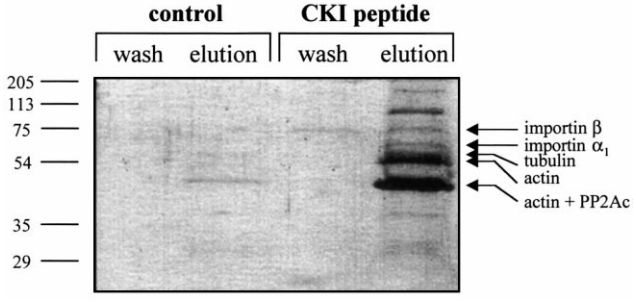

Fig. 3. A number of brain proteins associate specifically with residues 217–233 of CKIα. Brain extracts were loaded onto a peptide column comprising residues 214–233 of CKIα as described in Fig. 2. Eluted proteins were analysed by SDS–PAGE and were visualised by Coomassie blue staining. The bands were 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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