Research Article

Protein kinase CK1 interacts with and phosphorylates RanBPM *in vitro*

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Abstract

Members of the casein kinase 1 (CK1) family of serine/threonine kinases are highly conserved from yeast to mammals and are involved in the regulation of various cellular processes. Specifically, the CK1 isoforms δ and ε have been shown to be involved in the regulation of proliferative processes, differentiation, circadian rhythm, as well as in the regulation of nuclear transport. In this report we show that CK1δ and ε interact with murine RanBPM in the yeast two-hybrid system (YTH) and that the putative CK1δ-interacting domains of RanBPM are located between aa 155-386 and aa 515-653. Furthermore, in mammalian cells CK1δ partially co-localizes with RanBPM and can be co-immunoprecipitated with RanBPM. In addition, CK1δ strongly phosphorylates RanBPM within aa 436-514 *in vitro*. The identification of the interacting and scaffolding protein RanBPM as a new substrate of CK1δ points towards a possible function for CK1δ in modulating RanBPM specific functions.

Introduction

Members of the casein kinase 1 (CK1) family form a group of highly related, ubiquitously expressed serine/threonine-specific kinases which can be found in all eukaryotic organisms (Knippschild *et al.* 2014, Venerando *et al.* 2014). CK1 isoforms can be detected in the nucleus and cytoplasm, and are associated with cellular structures like the cytoskeleton, the centrosomes, transport vesicles and the plasma membrane. Members of the CK1 family are able to phosphorylate a wide variety of substrates, suggesting an involvement of CK1 in the regulation of various cellular processes, including chromosome segregation, circadian rhythm, centrosome-specific functions, differentiation and apoptotic processes, and nuclear import processes (Cruciat 2014, Knippschild *et al.* 2014, Venerando *et al.* 2014). Furthermore, deregulation of CK1 expression and activity has been associated with several diseases, among them neurodegenerative diseases (Flajolet *et al.* 2007, Hanger *et al.* 2007, Perez *et al.* 2011, Rubio de la Torre *et al.* 2009) and cancer (Knippschild *et al.* 2014, Schittek & Sinnberg 2014). On protein level the activity of CK1 is modulated by several mechanisms, among them subcellular localization, (auto-) phosphorylation, dephosphorylation, site-specific cleavage by endoproteases, and interaction with cellular structures and proteins (Cruciat 2014, Knippschild *et al.* 2014).

The Ran Binding Protein in the Microtubule-organizing center (MTOC), RanBPM, has first been described as a centrosomal protein with a molecular mass of 55 kDa (RanBPM55) (Nakamura *et al.* 1998). Later it was determined that RanBPM55 was a truncated variant of a 90 kDa protein (RanBP9) with both nuclear and cytoplasmic distribution (Nishitani *et al.* 2001). The majority of Ran binding proteins (RanBPs) are related to the importin-β family of receptors, which regulate nuclear trafficking, and are regulated by the Ras-like nuclear small GTPase Ran (Murrin & Talbot 2007). However, RanBPM lacks the consensus Ran-binding domain (Beddow *et al.* 1995) and rather acts as an interaction partner for multiple receptors and as scaffolding protein for signal transduction components (Suresh *et al.* 2012). RanBPM contains several functional domains, including a SPRY domain as well as LisH and CTLH motifs. The SPRY domain is involved in mediating several of the known protein–protein interactions of RanBPM (Cheng *et al.* 2005, Hafizi *et al.* 2005, Mikolajczyk *et al.* 2003, Rao *et al.* 2002, Wang *et al.* 2002a, Yuan *et al.* 2006), whereas the LisH and CTLH motifs are important for the regulation of homo-dimerization and binding to microtubules (Emes & Ponting 2001, Gerlitz *et al.* 2005, Mateja *et al.*

Table 1. Sequences of RanBPM55-specific primers (RanBPM, a 55 kDa protein, the nucleotide position refers to the sequence of the murine RanBP9 NM_019930.2) (nt: nucleotide).

Primer	Sequence	RanBP9 Sequence
5'RanBPM-1	5′-TCTCGAGACCCGGGTATGGGAATTGGTCTTT-3′ (5′ RanBP9, *Xho*I/*Xma*I)	nt 463-478
5'RanBPM-2	5′-TCTCGAGACCCGGGTATGTTTGTGTTTGATAT-3′ (5′ RanBP9, *Xho*I/*Xma*I)	nt 775-788
5'RanBPM-3	5′-TCTCGAGACCCGGGTATGCGGTGTTTGGGA-3′ (5′ RanBP9, *Xho*I/*Xma*I)	nt 1156-1167
5'RanBPM-4	5′-TCTCGAGACCCGGGTATGGAAAGTTGTAGC-3′ (5′ RanBP9, *Xho*I/*Xma*I)	nt 1306-1317
5'RanBPM-5	5′-TCTCGAGACCCGGGTATGGAAGATTGTGAC-3′ (5′ RanBP9, *Xho*I/*Xma*I)	nt 1543-1557
3'RanBPM-6	5′-TCTCGAGGGTACCTAAGGATGTTGCCCAAA-3′ (3′ RanBP9, *Xho*I/*Kpn*I)	nt 760-774
3'RanBPM-7	5′-TCTCGAGGGTACCTACACTTCACTGTCTGT-3′ (3′ RanBP9, *Xho*I/*Kpn*I)	nt 1141-1155
3'RanBPM-8	5′-TCTCGAGGGTACCTATACACCATTGCTACAAC-3′ (3′ RanBP9, *Xho*I/*Kpn*I)	nt 1310-1326
3'RanBPM-9	5′-TCTCGAGGGTACCTATTCGTGGTCATGTTTAG-3′ (3′ RanBP9, *Xho*I/*Kpn*I)	nt 1526-1542
3'RanBPM-10	5′-TCTCGAGGGTACCTAATGTAGGTAGTCTTCC-3′ (3′ RanBP9, *Xho*I/*Kpn*I)	nt 1944-1959

2006).

RanBPM interacts with a variety of receptors and signal transduction components, such as MET (Wang *et al.* 2002b), CDK11p46 (Mikolajczyk *et al.* 2003), Mirk/Dyrk1B (Zou *et al.* 2003), Axl (Hafizi *et al.* 2002, 2005), and TrkA/B (Yin *et al.* 2010; Yuan *et al.* 2006). Furthermore, RanBPM acts as a scaffolding protein modulating downstream signaling of neural cell adhesion molecule L1 (Cheng *et al.* 2005), β2-integrin LFA-1 (Denti *et al.* 2004), semaphorins (Togashi *et al.* 2006), and BM88/Cend1 (Tsioras *et al.* 2013). By interacting with p73α, RanBPM seems to enhance the pro-apoptotic activity of p73α, by preventing its degradation via the ubiquitin proteasome pathway (Kramer *et al.* 2005). In addition, RanBPM has been shown to exhibit enhancing effects on amyloid β peptide generation in Alzheimer's disease by scaffolding APP (amyloid precursor protein), BACE1 and LRP (lipoprotein receptor-related protein) (Lakshmana *et al.* 2008, 2009, 2010, 2012). Moreover, RanBPM interacts with HDAC6, a component of the aggresome complex. There is increasing evidence that RanBPM promotes aggresome formation since silencing of RanBPM impairs aggresome formation (Salemi *et al.* 2014).

In the present study we identified RanBPM as an interaction partner of CK1 family members in the yeast two-hybrid (YTH) system, especially of CK1δ and ε. The putative CK1δ-interacting domains of RanBPM are located between aa 155-386 and aa 515-653. Furthermore, RanBPM co-immunoprecipitates with CK1δ, and is phosphorylated by CK1δ at several amino acids within aa 436-514 *in vitro*. These findings point towards a possible function for CK1δ in regulating RanBPM specific functions.

Materials and methods

Expression vectors

Construction of the bait plasmids pGBKT7-wt-CK1δ and pGBKT7-mt-CK1δ has been described previously (Wolff *et al.* 2005). pGBKT7-CK1α, pGBKT7-CK1γ3 and pGBKT7-CK1ε were kindly provided by Dr. David Meek, Dundee, Scotland (Sillibourne *et al.* 2002). Primers listed in Table 1 were used to amplify cDNAs of several fragments of murine RanBPM55 from a GAL4 Matchmaker mouse testis library in pACT2 (Clontech, Takara Bio Inc., USA). PCR products were ligated into pcDNA3.1/V5-His©-TOPO® (Invitrogen, USA) and subcloned into

Table 2. Generation of murine RanBPM55 expression vectors (bp: base pair; aa: amino acid).

Primer pair	bp RanBP9	aa RanBP9	Vector names	Fusion Protein (FP)
5'RanBPM-1 / 3'RanBPM-10	1497	155-653	pcDNA3.1-RanBPM pGADT7-RanBPM pGEX-RanBPM	FP 974 FP 980 FP 986
5'RanBPM-1 / 3'RanBPM-6	312	155-258	pGADT7-RanBPM pGEX-RanBPM	FP 969 FP 975
5'RanBPM-2 / 3'RanBPM-7	381	259-385	pGADT7-RanBPM pGEX-RanBPM	FP 970 FP 976
5'RanBPM-3 / 3'RanBPM-8	171	386-442	pGADT7-RanBPM pGEX-RanBPM	FP 971 FP 977
5'RanBPM-4 / 3'RanBPM-9	237	436-514	pGADT7-RanBPM pGEX-RanBPM	FP 972 FP 978
5'RanBPM-5 / 3'RanBPM-10	417	515-653	pGADT7-RanBPM pGEX-RanBPM	FP 973 FP 979

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pVII16          ------------LKRLYPAVDEQETPLPRSWSPKDKFSYIGLSQNNLRVHYKGHGKTPKD 48
NP_064314.2     ALNEQEKELQRRLKRLYPAVDEQETPLPRSWSPKDKFSYIGLSQNNLRVHYKGHGKTPKD 180
                            ************************************************

pVII16          AASVRATHPIPAACGIYYFEVKIVSKGRDGYMGIGLSAQGVNMNRLPGWDKHSYGYHGDD 108
NP_064314.2     AASVRATHPIPAACGIYYFEVKIVSKGRDGYMGIGLSAQGVNMNRLPGWDKHSYGYHGDD 240
                ************************************************************

pVII16          GHSFCSSGTGQPYGPTFTTGDVIGCCVNLINNTCFYTKNGHSLGIAFTDLPPNLYPTVGL 168
NP_064314.2     GHSFCSSGTGQPYGPTFTTGDVIGCCVNLINNTCFYTKNGHSLGIAFTDLPPNLYPTVGL 300
                ************************************************************

pVII16          QTPGEVVDANFGQHPFVFDIEDYMREWRTKIQAQIDRFPIGDREGEWQTMIQKMVSSYLV 228
NP_064314.2     QTPGEVVDANFGQHPFVFDIEDYMREWRTKIQAQIDRFPIGDREGEWQTMIQKMVSSYLV 360
                ************************************************************

pVII16          HHGYCATAEAFARSTDQTVLEELASIKNRQRIQKLVLAGRMGEAIETTQQLYPSLLERNP 288
NP_064314.2     HHGYCATAEAFARSTDQTVLEELASIKNRQRIQKLVLAGRMGEAIETTQQLYPSLLERNP 420
                ************************************************************

pVII16          NLLFTLKVRQFIEMVNGTDSEVRCLGGRSPKSQDSYPVSPRPFSSPSMSPSHGMSIHSLA 348
NP_064314.2     NLLFTLKVRQFIEMVNGTDSEVRCLGGRSPKSQDSYPVSPRPFSSPSMSPSHGMSIHSLA 480
                ************************************************************

pVII16          PGKSSTAHFSGFESCSNGVISNKAHQSYCHSKHQLSSLTVPELNSLNVSRSQQVNNFTSN 408
NP_064314.2     PGKSSTAHFSGFESCSNGVISNKAHQSYCHSKHQLSSLTVPELNSLNVSRSQQVNNFTSN 540
                ************************************************************

pVII16          DVDMETDHYSNGVGETSSNGFLNGSSKHDHEMEDCDTEMEVDCSQLRRQLCGGSQAAIER 468
NP_064314.2     DVDMETDHYSNGVGETSSNGFLNGSSKHDHEMEDCDTEMEVDCSQLRRQLCGGSQAAIER 600
                ************************************************************

pVII16          MIHFGRELQAMSEQLRRECGKNTANKKMLKDAFSLLAYSDPWNSPVGNQLDPIQREPVCS 528
NP_064314.2     MIHFGRELQAMSEQLRRECGKNTANKKMLKDAFSLLAYSDPWNSPVGNQLDPIQREPVCS 660
                ************************************************************

pVII16          ALNSAILETHNLPKQPPLALAMGQATQCLGLMARSGVGSCAFATVEDYLH 578
NP_064314.2     ALNSAILETHNLPKQPPLALAMGQATQCLGLMARSGVGSCAFATVEDYLH 710
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Figure 1. RanBPM sequence aligments on protein level. Comparison of the amino acid sequence of the cDNA sequence cloned into pVII16 with the amino acid sequence of murine RanBPM (gi: 161353515, accession number NP_064314.2).

pGADT7, pEYFP-C1 (both Clontech, Takara Bio Inc., USA) and pGEX-4T-3 (GE Healthcare, USA) (Table 2).

Yeast two-hybrid assay

To screen for novel CK1δ interacting proteins, yeast two-hybrid analyses were carried out as described previously (Wolff *et al.* 2005). The yeast strain AH109 was co-transformed with pGBKT7-CK1δ and the mouse testis library in pACT2 (Clontech, Takara Bio Inc, USA). To eliminate auto-activating library plasmids or plasmids containing proteins, which interacted with the GAL4 DNA binding domain, library plasmids isolated from positive yeast clones were co-transformed with pGBKT7 into AH109. False positive library plasmids were identified after co-transformation with pAS2-LaminC. The cDNA inserts of true positive plasmids were sequenced.

Cell culture and transfection of cells

MiaPaCa2 (Yunis *et al.* 1977), HeLa (Gey, 1952), and CV1 cells (Manteuil *et al.* 1973) were maintained in Dulbecco's modified Eagle's medium (DMEM) whereas BxPC3 cells (Loor *et al.* 1982) were maintained in a 1:1 mixture of DMEM and Roswell Park Memorial Institute medium (RPMI). All media contained 10% heat-inactivated fetal calf serum (FCS) (Gibco, USA), 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco, Germany), and 2 mM glutamine. Cell cultures were incubated at 37°C in a humidified 5% (v/v) CO_2 atmosphere. Cell lines HeLa and CV1 were transfected with the plasmid pEYFP-C1-RanBPM55. Transient transfections were performed using subconfluent cultures in 10-cm dishes or six-well tissue culture plates using Effectene Transfection Reagent (Qiagen, Germany) according to the manufacturer's instructions.

Antibodies and immunocytochemistry

The peptide GFLNCSSKHDHEMED (aa 579 - 593 of human or aa 503 - 517 of murine RanBP9, respectively) was coupled to KLH, emulsified in TiterMax Gold (Sigma-Aldrich, Germany) and used for subsequent subcutaneous injections of rabbits. After three to four boosts the animals were bled out. Depletion experiments using the RanBPM-specific peptide for preincubation with the RanBPM-specific serum clearly demonstrated the specificity of the anti-RanBPM serum.

To determine the subcellular localization of CK1δ in HeLa and CV1 cells, the monoclonal mouse

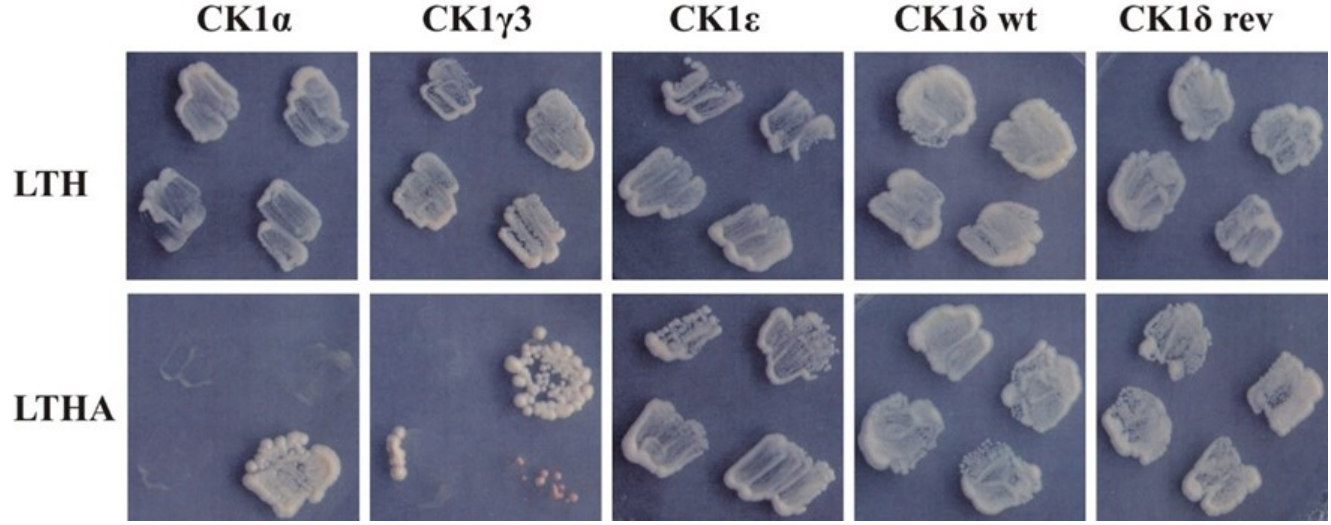

Figure 2. Interaction of CK1 isoforms and RanBPM. Vector pVII16 expressing RanBPM55 was cotransformed with pGBKT7-CK1α, pGBKT7-CK1γ3, pGBKT7-CK1ε pGBKT7-CK1δrev or pGBKT7-CK1δwt into the yeast strain AH109. Positive clones were selected by growing the transformed yeast first on SD/-Trp/-Leu/-His plates (LTH) followed by platting onto more stringent SD/-Trp/-Leu/-His/-Ade plates (LTHA) The ability of the transformants to grow was analyzed.

anti-CK1δ antibody 128A (ICOS Corporation, now Lilly, USA) was used. An Alexa 568-conjugated anti-mouse antibody (MoBiTec GmbH, Germany) was used as fluorophore-labeled secondary antibody. Cells were fixed and permeabilized with ice-cold methanol. Stained cells were analyzed using an epifluorescence microscope (IX70 Olympus, Germany).

Cell lysis, immunoprecipitation, SDS-PAGE, Western blotting, and IP-Western analysis

Cellular lysates were obtained as described previously (Wolff *et al.* 2005). Cleared extracts were subjected to immunoprecipitation using RanBPM-specific serum (CGV1) and protein A sepharose beads (PAS) (GE Healthcare, USA). Negative controls were done using mouse IgG (Jackson ImmunoResearch Europe Ltd., UK). Immunoprecipitates were analyzed as described before (Wolff *et al.* 2005). For IP-Western experiments, the endogenous RanBPM was detected in BxPC3 and MiaPaCa2 cells by using the RanBPM specific rabbit serum (CGV1). After immunoprecipitation of RanBPM, the precipitates were separated by SDS-PAGE and transfered to nitrocellulose membranes. The detection of CK1δ in the immune complex was performed using the antibody 128A (ICOS Corporation, now Lilly, USA).

Expression and purification of recombinant proteins

Expression and purification of GST-RanBPM55 fusion proteins was done as described previously (Knippschild *et al.* 1997). *In vitro* transcription/translation of His-RanBPM55 was done using TNT reticulocyte extract (Promega, USA) according to the manufacturer's instructions.

***In vitro* kinase assays**

In vitro kinase assays were carried out as described previously (Knippschild *et al.* 1996, Wolff *et al.* 2005) using His-RanBPM55 or GST-RanBPM55 fusion proteins (expressed from pGEX-4T-3) encoded by the pGEX-vectors shown in Table 2. Reactions were started by adding CK1δ kinase domain (CK1δKD; NEB, USA) as enzyme and incubated at 30°C for 30 min or as indicated. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography.

Results

CK1 isoforms interact with RanBPM55

In order to identify new protein interaction partners for CK1δ, a cDNA library from mouse testis was screened for interactions with the mutant CK1δ variant CK1δrev (Hirner *et al.* 2012) as bait in a yeast-two-hybrid (YTH) screen. Variant CK1δrev was chosen for the

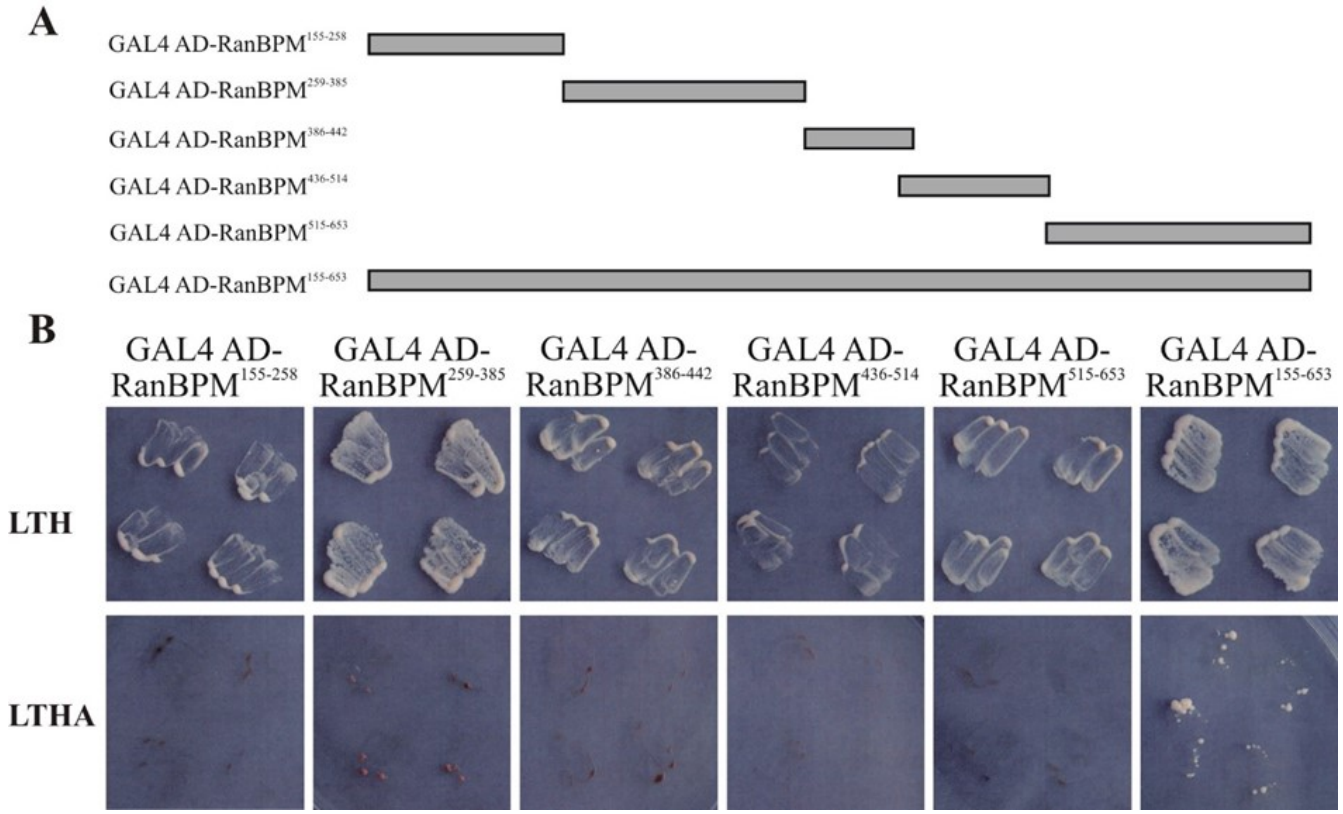

Figure 3. Interaction of CK1δ and RanBPM. (A) Schematic representation of the RanBPM55 fragments. (B) The vectors with RanBPM fragments were cotransformed with pGBKT7-CK1δ rev into the yeast strain AH109. Positive clones were selected by growing the transformed yeast first on SD/-Trp/-Leu/-His plates (LTH) followed by platting onto more stringent SD/-Trp/-Leu/-His/-Ade plates (LTHA). The ability of the transformants to grow was analyzed.

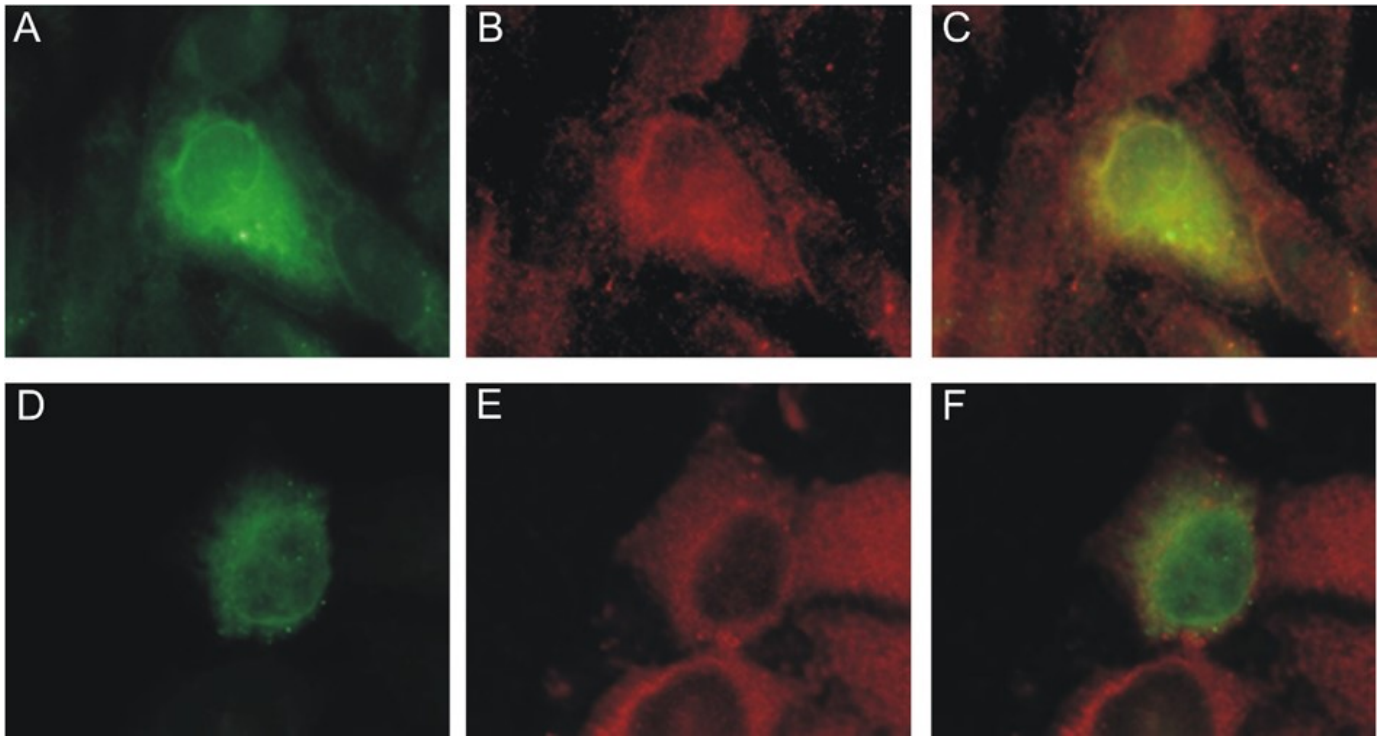

Figure 4. Subcellular localization of CK1δ and EYFP-RanBPM55 in CV1 and HeLa cell lines. Two days after transfection CV1 cells and HeLa cells were fixed with ice-cold methanol and permeabilized. (A) and (D) show EYFP-RanBPM55. In panels (B) and (E), CK1δ was labeled with the antibody 128A and an Alexa 568-conjugated secondary antibody. In the superposition of the fluorescence channels shown in panels (C) and (F), co-localization of RanBPM55 and CK1δ appears as a yellow color.

initial experiments since this mutant only exhibits one third of the kinase activity of wt CK1δ and therefore its overexpression does not cause the same cytotoxic effects which can be observed for overexpression of wt CK1δ.

Transformed yeast cells were selected on semi-stringent SD/-Trp/-Leu/-His (LTH) plates, followed by re-platting positive colonies onto SD/-Trp/-Leu/-His/-Ade (LTHA) plates for a more stringent selection. From one positive clone the plasmid termed pVII16 was isolated containing a N-terminally truncated sequence of murine RanBPM (Figure 1). pVII16 together with either pGBKT7-CK1α, pGBKT7-CK1γ3, pGBKT7-CK1ε, pGBKT7-rev-CK1δ, or pGBKT7-CK1δwt, were cotransformed into AH109 to analyze its interaction with the different CK1 isoforms in the YTH system. Cells cotransformed with RanBPM/CK1δrev, RanBPM/CK1δwt, and RanBPM/CK1ε exhibited growth on SC/-Leu/-Trp/-His/-Ade medium. RanBPM/CK1α, RanBPM/CK1γ3 showed only partial growth on more stringent medium (Figure 2). The specificity of the interaction of RanBPM with the indicated CK1 isoforms was confirmed by the inability of RanBPM to interact with Gal4 DBD or GAL4 DBD-lamin C (data not shown). For further studies the full-length sequence of murine RanBPM55, a N-terminally truncated form of RanBP9 (accession ID AAD01272.1), was amplified from the mouse testis cDNA library.

Identification of CK1δ and RanBPM55 interaction domains

Fragments of RanBPM55 were generated (Figure 3a) and used in the YTH system in order to identify the CK1δ domain(s) necessary for the interaction with RanBPM55. On LTH plates (Figure 3b), fragment GAL4 AD-RanBPM$^{259\text{-}385}$ could mediate an interaction with CK1δ as strongly as full length RanBPM55

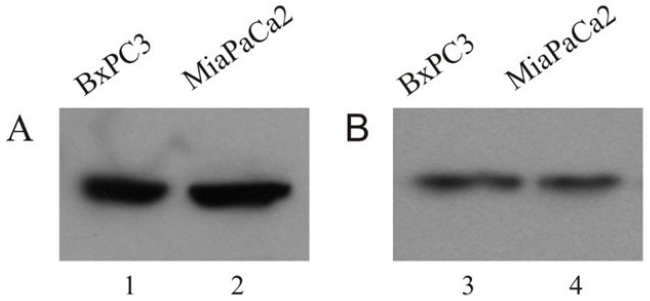

Figure 5. CK1δ co-immunoprecipitates with RanBPM. (A) Detection of RanBPM in cellular lysates of BxPC3 and MiaPaCa2 cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The detection of RanBPM in Western blot analysis was performed using the polyclonal rabbit serum CGV1 (lane A1: BxPC3, anti-RanBPM; lane A2: MiaPaCa2, anti-RanBPM). (B) RanBPM was immunoprecipitated from BxPC3 cells and MiaPaCa-2 cells using an anti-RanBPM antibody. The detection of CK1δ in the immune complex was performed using the antibody 128A. (lane B1: BxPC3, IP against RanBPM; lane B2: MiaPaCa2, IP against RanBPM).

(GAL4 AD-RanBPM$^{155-653}$). Fragments including aa 155-258, aa 386-442 and aa 515-653 of RanBPM55 also showed interaction whereas GAL4 AD-RanBPM$^{436-514}$ only showed a weak interaction with CK1δ. All described interactions were not observed using more stringent SD/-Trp/-Leu/-His/-Ade medium (LTHA).

Characterization of the interaction between RanBPM and CK1δ in mammalian cells

Additional cell biological and biochemical analyses were performed to confirm the physiological relevance of the interaction between RanBPM and CK1δ detected in the YTH system. First, analysis of the subcellular localization of CK1δ and RanBPM55 in HeLa and CV1 cells transfected with the plasmid pEYFP-C1-RanBPM55 by immunofluorescence microscopy, using the CK1δ-specific antibody 128A, revealed a partial co-localization of both proteins in the perinuclear area (Figure 4). Secondly, IP-Western-analysis revealed that CK1δ co-immunoprecipitates with RanBPM in cellular lysates of BxPC3 and MiaPaCa2 cells (Figure 5).

RanBPM55 is phosphorylated by CK1δ *in vitro*

Since sequence based analysis of RanBPM55 identified several serine and threonine residues as putative targets for CK1 mediated phosphorylation, *in vitro* kinase assays were performed to verify RanBPM55 phosphorylation by CK1. His-tagged RanBPM55, *in vitro* translated from pcDNA3.1-RanBPM (His-RanBPM$^{155-653}$), was phosphorylated using C-terminally truncated CK1δ (CK1δKD). The appearance of a phosphorylated band at around 55 kDa indicated that CK1δ can phosphorylate RanBPM55 *in vitro* (Figure 6a). No phosphate incorporation into the substrate was detected for control reactions performed without kinase (data not shown).

In order to localize the phosphorylation sites targeted by CK1δ, different GST-RanBPM55 fragments were purified and same protein amounts of these were used as substrates for *in vitro* kinase assays. As shown in Figure 6b CK1δKD differently phosphorylated the different GST-RanBPM fusion proteins. Highest phosphate incorporation was detected for the GST-RanBPM$^{436-514}$ protein pointing to the existence of one or more major phosphorylation sites in the respective region. GST-RanBPM$^{515-653}$ as well as GST-RanBPM259-385 showed significantly weaker phosphorylation intensity. Nearly all GST-RanBPM fusion proteins showed different degrees of degradation after the performed purification steps, especially GST-RanBPM$^{259-385}$ and GST-RanBPM$^{515-653}$ were highly degraded, and most phosphate incorporation could be detected in degradation products.

Discussion

CK1δ, a member of the CK1 family, plays an important role in the regulation of various cellular processes including circadian rhythm, vesicle transport, chromosome segregation, and centrosome specific functions. Therefore, CK1δ has to be tightly regulated by several mechanisms, including interaction with cellular structures and proteins (reviewed by Knippschild *et al.* 2014). However, little is known about protein-protein interactions which may influence activity or localization of CK1 isoforms. In the present study we identified RanBPM as a new interaction partner for CK1 isoforms α, γ3, δ, and ε in the yeast two-hybrid system. However, interaction of RanBPM with CK1δ and ε was significantly stronger compared to those with CK1α and γ3. Using different RanBPM fragments the domain responsible for mediating the interaction with CK1δ could be located between aa 259-385. This region is located directly N-terminal to the SPRY domain (aa 212–333) of RanBPM, which has been shown to mediate most of the so far reported protein-protein interactions (Ponting *et al.* 1997). In subsequent experimental approaches, we were able to confirm the interaction of RanBPM and CK1δ using mammalian cell lines. Whereas our immunofluorescence analyses only revealed a partial co-localization in the perinuclear region, our IP-Western analyses clearly showed that CK1δ co-immunoprecipitates with RanBPM. The partial co-localization of both proteins could be explained by the fact that RanBPM is predominantly localized in chromatin-free areas, the so-called nuclear speckles

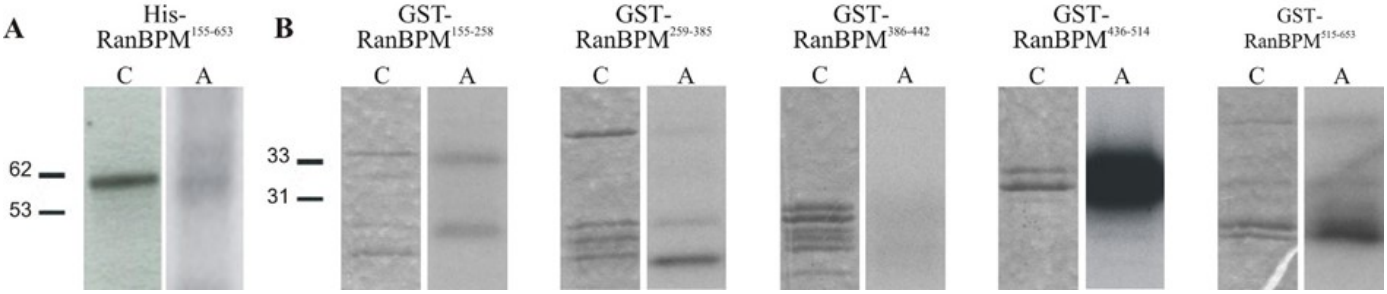

Figure 6. *In vitro* phosphorylation of RanBPM by CK1δ. (A) *In vitro* kinase assays were performed using His-RanBPM55 as substrate and C-terminally truncated CK1δ. Proteins were separated in SDS-PAGE, stained with Coomassie staining solution (C), and the phosphorylation was detected by autoradiography (A). (B) *In vitro* kinase assays were performed using GST-RanBPM55 fragments (described in Figure 1) as substrate and C-terminally truncated CK1δ. The proteins were separated in SDS-PAGE, stained with Coomassie staining solution (C), and the phosphorylation was detected by autoradiography (A).

(Lamond & Spector 2003) in association with various proteins (Ideguchi *et al.* 2002, Mikolajczyk *et al.* 2003, Rao *et al.* 2002, Wang *et al.* 2002b, Zou *et al.* 2003), whereas CK1δ can be mainly detected in the perinuclear region in association with membrane structures, transport vesicles, microtubules, and centrosomes (reviewed by Knippschild *et al.* 2014). However, in some cases it has been reported that RanBPM is located in the cytoplasm thereby interacting with various cellular structures and the plasma membrane (Lamond & Spector 2003). RanBPM often acts as scaffold protein containing a LisH/CTLH motif, which is present in proteins involved in microtubule dynamics, cell migration, nucleokinesis, and chromosome segregation (Kobayashi *et al.* 2007). By phosphorylation CK1δ might regulate these functions of RanBPM. CK1δ actually is able to phosphorylate RanBPM *in vitro* especially between aa 436-514. Interestingly, this strongly phosphorylated RanBPM fragment exhibits the weakest interaction with CK1δ as determined by YTH assays. This fact indicates, that binding of RanBPM55 to CK1δ is mediated by a certain domain (i.e. aa 259-385) which is not identical to the domain which appears to be the main target for CK1δ-mediated phosphorylation (aa 436-514). RanBPM has been reported to be phosphorylated by several kinases, including CDK11p46, Plk (Mikolajczyk *et al.* 2003), Dyrk1B (Tsioras *et al.* 2013), PKC gamma/delta (Rex *et al.* 2010), and p38 (Denti *et al.* 2004) pointing to an important role of site-specific phosphorylation in regulating RanBPM-specific functions as well as its subcellular localization (Suresh *et al.* 2012). However, additional experiments have to be set up to identify the amino acids of RanBPM targeted by CK1δ and to identify the physiological consequences of CK1δ-mediated site-specific phosphorylation on RanBPM cellular functions.

In summary, RanBPM has been identified as a new interaction partner of CK1δ. The specificity of the biological relevance is underlined by the following facts: (i) RanBPM specifically interacts with various CK1 isoforms, namely with CK1α, γ3, δ, and ε, (ii) RanBPM is phosphorylated by CK1δ *in vitro*, and (iii) RanBPM interacts with CK1δ in pancreatic tumor cell lines, as confirmed by IP-Western analysis. However, additional analysis is required to evaluate the physiological relevance of the interaction between CK1δ and RanBPM in detail.

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Conflicts of interest

The authors declare no conflicts of interest.

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