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Cells in tissues have only three serious options in life; they can grow and divide, remain static, or die by apoptosis. Upon growth factor stimulation a cell enters the so called cell cycle which will eventually lead to the division of the cell. Cell cycle can be divided into four phases; G1, S, G2 and M. The current model of the cell cycle control holds that the transitions between different cell cycle states are regulated by cyclin dependent kinases (CDK) with their activator subunits, the cyclins.

CDK regulation can be separated into four distinct mechanisms, one of which being phosphorylation on the so called T-loop leading to complete activation. This phosphorylating activity is mediated by apparently a single enzymatic activity termed the CDK activating kinase, CAK. CAK activity was originally isolated as a biochemical purification extract and the enzyme was surprisingly noticed to be structurally related to CDKs. Since a novel cyclin was identified to be associated to it, the enzyme exercising CAK activity was named CDK7 and the cyclin was designated cyclin H. An entirely new perspective on CDK7 function was opened when CDK7 was identified as a subunit of transcription factor IIH (TFIIH) and shown to phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII). CDK7 has also been suggested to be involved in irradiation sensitivity pathways and nucleotide excision repair functions.

To elucidate the intriguing *in vivo* role of CDK7, proteins interacting with CDK7 were screened for using the yeast two-hybrid method as part of previous studies of the laboratory. The results showed that 15 out of 144 (10,4%) positive clones were identified to encode a peptide sequence of a protein previously known as the inhibitor/interactor of protein kinase C (PKCI). These yeast colonies had an unexpected phenotype; contradictory to a dark blue color of the colonies, indicating strong interaction, the size of the PKCI colonies was small compared to others, indicating a possible growth inhibition effect.

Several DNA open reading frames (ORF) coding for proteins related to human PKCI have been identified in a broad range of species representing mammalian, plant, fungal and bacterial kingdoms, all these forming a HIT (conserved triad of histidines) protein family. Another human member, part of this now super family, named FHIT (fragile triad of histidines) was identified with a dinucleoside 5',5'''-P^1,P^3-triphosphate hydrolase activity. These molecules; substrates of FHIT and related enzymes have been proposed to have various intracellular functions, including signalling stress responses.

The aim of this study was to extend the investigation of the interaction between CDK7 and PKCI observed in yeast two-hybrid by means of several genetic and biochemical approaches to determine if this observed interaction and growth phenotype has any physiological significance. Investigations included performing yeast two-hybrid screening for PKCI, developing yeast three-hybrid system and carrying out growth rate assays for yeast liquid cultures. These studies also included performing biochemical purifications of over-expressed proteins, immunoprecipitations, western blot analysis and kinase activity assays. Protein extracts originated from transformed yeast cells, transfected mammalian cells or from *in vitro* transcription and translation reactions.

On basis of growth rate assays it can be concluded that PKCI has an inhibitory growth effect in yeast. The preliminary finding of a specific PKCI-CDK7 interaction in yeast two-hybrid, however could not be conclusively verified by the other methods that were used in this study.

Studies of PKCI characterisation also included examination of the subcellular localisation of PKCI in mammalian cells by immunofluorescence labelling of HA-PKCI. Results showed PKCI to localize both in the nucleus and in the cytoplasm. Also, studies to elucidate the function of PKCI were performed; whether it possesses enzymatic activity related to that of FHIT. By NMR spectroscopy using bacterially produced GST-PKCI, hydrolase activity towards ADP was indeed observed.

Future studies will include elucidation of possible links between growth inhibition and hydrolase activity, in the form of stress signalling functions. The main focus of our future studies will be the generation of mice with targeted PKCI alleles offering powerful means to reveal the function of PKCI through observing phenotypes and through detailed analysis of these mice harbouring wild type, hypomorphic or null alleles.

Avainsanat - Nyckelord
cell cycle, CDK7, yeast two-hybrid, inhibition, stress signalling

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Studies on PKCI, a putative CDK7 interacting growth inhibitor

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ABBREVIATIONS

5-FOA	5-fluoro-oratic-acid
A595	absorbance at 595 nm
ADP	adenosine 5'-diphosphate
AMP	adenosine monophosphate
Ap_2A	diadenosine 5',5'''-P^1,P^2-diphosphate
Ap_3A	diadenosine 5',5'''-P^1,P^3-triphosphate
Ap_3C	adenosine 5'-cytosine 5'-P^1,P^3-triphosphate
Ap_3G	adenosine 5'-guanosine 5'-P^1,P^3-triphosphate
Ap_4A	diadenosine 5',5'''-P^1,P^4-tetraphosphate
Ap_4G	adenosine 5'-guanosine 5'-P^1,P^4-tetraphosphate
Ap_5A	diadenosine 5',5'''-P^1,P^5-pentaphosphate
Ap_6A	diadenosine 5',5'''-P^1,P^6-hexaphosphate
AT	ataxia telangiectasia
ATP	adenosine 5'-triphosphate
β-Gal	β-galactosidase
βGP	β-glycerol phosphate
BSA	bovine serum albumin
Bun	Boy1 URA3 negative
CAK	CDK activating kinase
CDK	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CKI	CDK inhibitor
CMV	immediate early human cytomegalovirus promoter
CTD	carboxy-terminal domain
C-terminal	carboxy terminal
D	one letter symbol for amino acid aspartic acid
D-MEM	Dulbecco's modified Eagle medium
dATP	deoxy adenosine 5'-triphosphate
dGTP	deoxy guanine 5'-triphosphate
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
dTTP	deoxy thymidine 5'-triphosphate
E	one letter symbol for amino acid glutamic acid
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
FHIT	fragile triad of histidines
FITC	fluorescein isothiocyanate
Gp_3G	diguanosine 5',5'''- P^1,P^3-triphosphate
Gp_4G	diguanosine 5',5'''- P^1,P^4-tetraphosphate
GST	glutathione S-transferase
h, his, His	abbreviations for amino acid histidine
HA	hemagglutinin
HIT	histidine triad
HSP90	heat shock protein 90
I	one letter symbol for amino acid isoleucine
Ig	immunoglobulin

IPTG	isopropyl-β−D-thiogalactopyranoside
IVT	in vitro translation
K	one letter symbol for amino acid lysine
kD	kilodalton(s)
L, l	one letter symbols for amino acid leucine
LB	Luria-Bertani
LSB	Laemmli sample buffer
M	one letter symbol for amino acid methionine
m^7Gp_3G	m^7 diguanosine 5',5'''- P^1,P^3-triphosphate
$m^7Gp_3Gm^7$	m^7 diguanosine 5',5'''- P^1,P^3-triphosphate m^7
MAD	multiwavelength anomalous diffraction
MATα	mating type α
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
N-terminal	amino terminal
OD	optical density
ORF	open reading frame
PAS	protein A sepharose
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
P_i	inorganic phosphate
PKC	protein kinase C
PKCI	protein kinase C inhibitor/interactor
PMSF	phenylmethylsulfonyl fluoride
pRb	retinoblastoma
Q	one letter symbol for amino acid glutamine
RNAPII	RNA polymerase II
S	one letter symbol for amino acid serine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	single strand DNA
T	one letter symbol for amino acid threonine
t, trp	abbreviations used in this study for amino acid tryptophane
TFIIH	transcription factor IIH
u	uracil
UV	ultraviolet
w/v	weight per volume
X-Gal	bromo-4-chloro-3-indolyl β-D-galactoside

INTRODUCTION

THE CELL CYCLE

Cells in tissues have only three serious options in life; they can grow and divide, remain static, or die by apoptosis. Upon growth factor stimulation a cell enters the so called cell cycle which will eventually lead to the division of the cell. The two most important events of the eukaryotic cell cycle are S phase (for synthesis) and M phase (for mitosis) where chromosomes are replicated (S) and segregated into the dividing cells (M). The phases proceeding S and M are called G1 and G2, respectively. The current model of the cell cycle control holds that the transitions between different cell cycle states are regulated at checkpoints by enzymes called cyclin dependent kinases (CDK) with their activator subunits, the cyclins.

Cyclins and cyclin dependent kinases (CDK)

The point of the cell cycle that a given cell is in can be determined on the basis of the different CDK-cyclin pairs' temporal activities. The G1 phase is governed by CDK4-cyclin D and CDK6-cyclin D. The substrate of CDK4-cyclin D is the retinoblastoma protein pRb (Ewen et al., 1993; Kato et al., 1993). Loss of pRb function contributes to the development not only of retinoblastoma but also other tumors (reviewed in Weinberg, 1995) The time point of pRb phosphorylation is called the restriction point, a point of no return after which the cell cannot do nothing else but to proceed through the cell cycle to division (Pardee, 1989). Unphosphorylated, active pRb is bound to a transcription factor E2F, a protein thought to be central in activating genes that promote cellular proliferation. Upon pRb phosphorylation pRb-E2F complex dissociates and E2F is freed to activate the transcription of its target genes (Chellappan et al., 1991). Cyclin E being one of the activated gene products then binds to its CDK partner CDK2 forming the next CDK-cyclin complex; CDK2-cyclin E. During S phase CDK2 binds to another cyclin, cyclin A, CDK2-cyclin A being the predominant S phase complex. G2 and M phases involve cdc2-cyclin A and later in mitosis cdc2-cyclin B activity. When cell division is completed, rapid degradation of cyclin B by ubiquitin targeted pathway signals the exit

from the cell cycle (Glotzer et al., 1991; Hershko et al., 1991). See Figure 1 (modified from Sherr, 1993) for the schematic illustration of the cell cycle and the subsequent CDK-cyclin pairs.

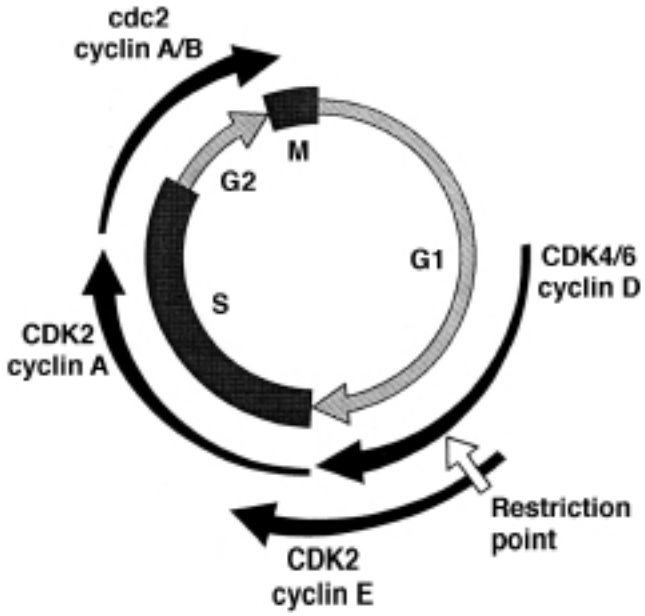

Figure 1. Schematic illustration of the mammalian cell cycle. Cell cycle phases G1, S, G2 and M and the subsequent CDK-cyclin pairs governing them are marked. Restriction point is marked with a white arrow.

The substrates of CDK-cyclin pairs and thus the downstream events are less clear. In addition to pRb, substrates like transcription factors and histone H1 have been proposed. Recently it has been shown that the replication initiator protein $p65^{cdc18}$ is regulated by CDK phosphorylation (Jallepalli et al., 1997) in fission yeast. This verifies the contribution of CDK activity controlling one of the key events in cell proliferation, the initiation of DNA replication. A lot depends on the timing of the CDK activation which is indeed regulated by multiple mechanisms.

Regulation of CDKs

A cyclin dependent kinase itself is completely inactive. The principles of CDK regulation have been elucidated in part by structural studies. Four mechanisms can be separated, all contributing to the regulation of CDKs. Firstly, partial activation of a CDK occurs upon cyclin binding, the positive regulatory subunit (Jeffrey et al., 1995; Kobayashi et al., 1992; Lees and Harlow, 1993). This interaction leads to the correct positioning of ATP

(adenosine 5'-triphosphate) phosphates bound by the CDK subunit for phosphotransfer reaction and the partial opening of the substrate binding site.

The activity of the CDK-cyclin complex can be reduced by at least two major mechanisms: the binding of protein inhibitors known as CKIs (CDK inhibitors) (Russo et al., 1996), and the phosphorylation of the CDK subunit on inhibitory sites (De Bondt et al., 1993).

Another level of regulation by phosphorylation is required for complete activation, phosphorylation of the CDK subunit at a conserved threonine residue on the so called T-loop of most CDKs. This phosphorylating activity is mediated by apparently a single enzymatic activity termed the CDK activating kinase, CAK (Russo et al., 1996). The T-loop guarding the substrate binding site is flattened enabling the subsequent binding.

This fourth mechanism of CDK regulation, the activating phosphorylation by CAK, will be discussed more thoroughly in the following paragraphs.

CAK AND CDK7

Purification of CAK activity

CAK activity was originally isolated as a biochemical purification extract capable of phosphorylating the conserved threonine residue within the T-loops of various CDKs (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). The enzyme exercising this CAK activity was surprisingly noticed to be structurally related to CDKs and it had previously been isolated from *Xenopus* under the name MO15 (Shuttleworth et al., 1990).

A novel cyclin was identified by biochemical purification methods and was found to be associated with MO15 by yeast two-hybrid method (Fisher and Morgan, 1994; Mäkelä et al., 1994). The cyclin was designated cyclin H and MO15 was renamed CDK7. A third subunit, MAT1 (for ménage-à-trois) has also been identified to be associated with the CDK7-cyclin H complex, which is a 36 kD RING-finger protein. MAT1 is thought to function as an assembly factor (Devault et al., 1995; Tassan et al., 1995), promoting a stable interaction between CDK7 and cyclin H and increasing the activity of CDK7-cyclin H complex (Fisher et al., 1995).

CDK7 as a part of transcription factor TFIIH

An entirely new perspective on CDK7 function was opened when CDK7 was identified as a subunit of transcription factor IIH (TFIIH) and shown to phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII) (Roy et al., 1994). Cyclin H and MAT1 are also present in TFIIH (Serizawa et al., 1995; Shiekhattar et al., 1995). Indeed, CDK7 activity in TFIIH has been shown to be required for transcription in vitro (Akoulitchev et al., 1995). A role of CDK7 in nucleotide-excision repair has been suggested based on the observation that TFIIH-associated CDK7 activity drops in response to UV irradiation (Adamczewski et al., 1996). The activity of non-TFIIH associated CDK7 was not altered.

Evidence seems to be accumulating supporting a function of the TFIIH-associated CDK7-cyclin H-MAT1 in class II transcription while the role of the *in vivo* cell cycle function of the complex still remains unverified in mammals. Recently CDK7 was shown to be the physiological CAK in *Xenopus* egg extracts (Fesquet et al., 1997). Clearly more detailed studies are needed to elucidate the intriguing *in vivo* role of CDK7, whether it lies in regulation of transcription, cell cycle progression, or both. To find answers to some of the questions, proteins interacting with CDK7 were screened for using the yeast two-hybrid method (Mäkelä et al., 1994 and Mäkelä, T.P., unpublished data). As the yeast two-hybrid method is relevant to the rationale for beginning this study as well as it being a tool used to generate further data I will expound more upon this powerful tool in the following paragraphs.

YEAST TWO HYBRID SYSTEM

Molecular events are often coordinated by protein-protein interactions. Detailed analysis of protein-protein interactions are thus necessary for the fundamental understanding of the biology of living organisms. To detect protein-protein interactions, several techniques such as co-immunoprecipitation, co-purification and affinity chromatography are currently used. While some interactions are of high affinity and readily detectable by physical separation techniques, others are weaker and more transient. These kinds of interactions require more sensitive detection

strategies. Following the work of Ptashne's group (Sadowski et al., 1988), a sensitive technique to detect protein-protein interactions called the two-hybrid system was established by Stanley Fields and coworkers (Fields and Song, 1989).

The two-hybrid system is a yeast-based genetic assay designed to detect interactions *in vivo*. It has three major applications: testing known proteins for interaction, defining the domains critical for interaction and screening libraries for unknown proteins that bind the target protein. The yeast two-hybrid system is based on the ability of the interacting proteins to bring a transcription activator domain into close proximity with a DNA-binding domain on a DNA-binding site that regulates the expression of an adjacent reporter gene (Fields and Song, 1989).

The most commonly used DNA-binding domains are Gal4 and LexA. Both of these proteins bind DNA as dimers and therefore the reporter genes contain several copies of the binding site. The target gene construct is a fusion with a DNA-binding domain. The most widely used activation domains are derived from the *Saccharomyces cerevisiae (S. cerevisiae)* Gal4 and *Herpes simplex* VP16 proteins. Activation domains of bacterial origin can also be used. Accordingly, a gene construct is built to form a fusion protein with an activator domain. When screening is done to detect unknown proteins interacting with the target gene, a cDNA library is cloned to produce the fusion product with the activator domain. A common marker gene is *Eschericia coli (E. coli) lacZ,* which produces blue yeast colonies on plates containing 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). In addition, a reporter gene for growth selection is also commonly used, which are usually genes involved in amino acid biosynthesis like *HIS3* and *LEU2.*

Robert Brent's yeast two-hybrid system, first published in 1993 (Gyuris et al., 1993) contains a LexA DNA-binding domain expressed from the pEG202 plasmid and an *E. coli* produced B42 (Ma and Ptashne, 1987) as the transcription activation domain expressed from pJG4-5. The resulting fusion proteins are called the bait and the prey, respectively. Marker genes *lacZ* and *LEU2* are used, in the EGY48 *S. cerevisiae* yeast strain *(MATα leu2 ura3 trp1 his3 lexAop::LEU2)* with pSH18-34 *(LexAop-lacZ)* plasmid.

Figure 2 shows a schematic drawing of this yeast two-hybrid system. Brent's yeast two-hybrid system has been used in this study to screen unknown proteins that bind PKCI target bait. It has also been used in the CDK7 two-hybrid screening, the results of which prompted us to initiate this present study.

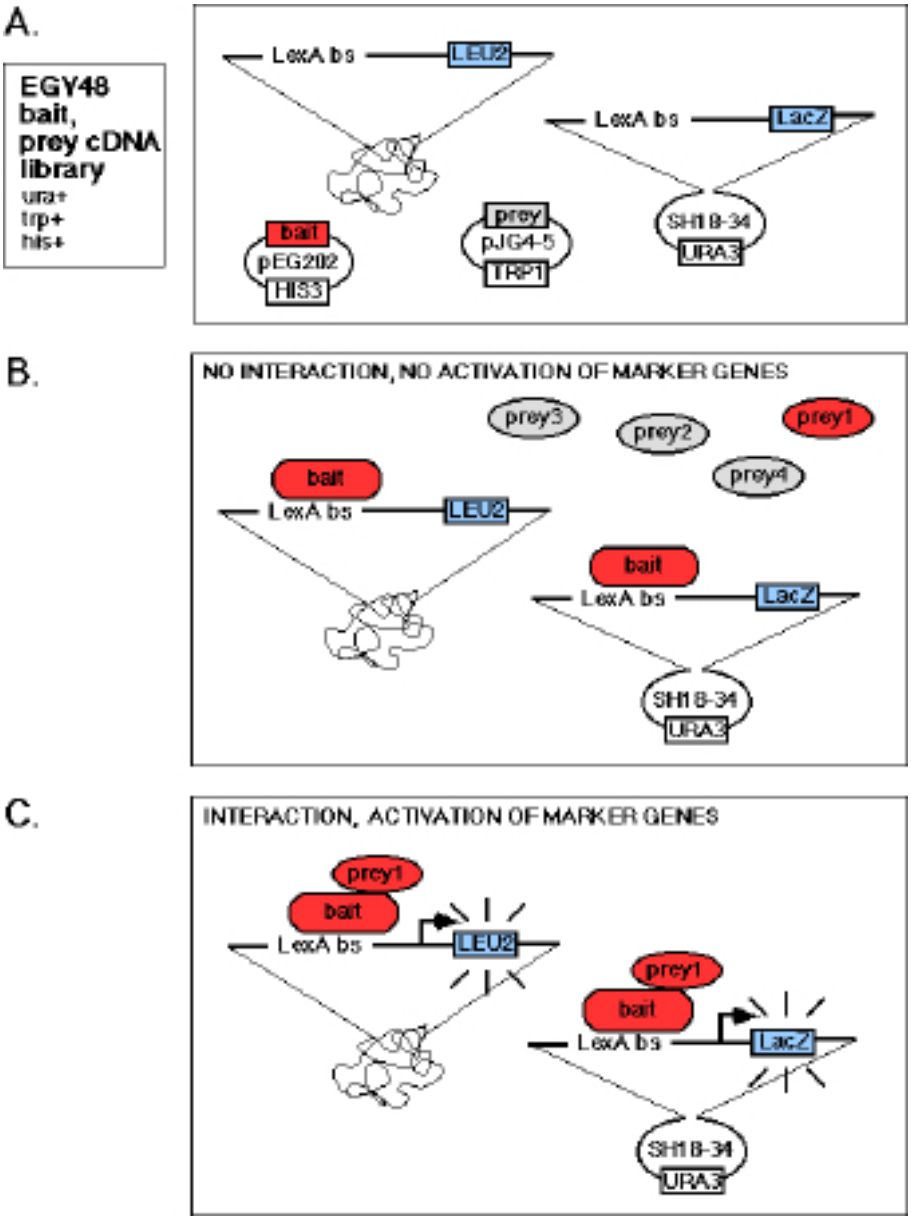

Figure 2. Schematic drawing of the Brent's yeast two-hybrid system. (A) EGY48 strain with SH18-34 (*URA3* selection) transformed with bait / pEG202 vector (*HIS3* selection) and prey cDNA library / pJG4-5 vector (*TRP1* selection). Genomic integration of LexA binding site (LexA bs) and *LEU2* marker gene and SH18-34 integrated LexA bs and *LacZ* marker gene. (B) No activation of the marker genes in the absence of interaction between bait and prey. Bait protein expressed and bound to LexA bs. Prey cDNA library expressed different proteins; prey1, prey2, prey3 and prey4. (C) Activation of *LEU2* and *LacZ* in response to the reconstitution of transcriptional activator complex through interaction between bait and prey1.

Screening with CDK7

EGY48 pSH18-34 using CDK7 as the bait was transformed with a HeLa cDNA library. See Table 1 for the summary of this screening (Mäkelä et al., 1994 and Mäkelä, T.P., unpublished data). The results showed that 15 out of 144 (10,4%) positive clones were identified to encode a peptide sequence of a protein previously known as the inhibitor of protein kinase C (PKCI). This represented the most numerous group of clones after β2 tubulin which was considered unspecific and thus uninteresting.

Table 1. Summary of CDK7 yeast two-hybrid screening.

CLONE NAME	NUMBERS FOUND	IDENTIFIED AS
G11	15	PKCI[1]
Goc2.1	2	PP5[2]
C2	4	
F11	3	cyclin H
F8	4	
A3	6	PCNA[3]
E8	48	β2-tubulin
A9	2	HSP90[4]

[1] protein kinase C inhibitor / interactor
[2] protein phosphatase 5
[3] proliferating cell nuclear antigen
[4] heat shock protein 90

At the time, only the peptide sequence of PKCI was published (Pearson et al., 1990) and thus the full-length cDNA isolated multiple times in the screen represented a cDNA of an uncharacterized gene.

Further characterization of this strikingly strong interaction observed between CDK7 and PKCI has been the focus of this study. The following is an introduction to what was known about PKCI at the time.

PKCI

Early studies on PKCI

PKCI was initially purified from bovine brain as a potent inhibitor of protein kinase C (PKC) with a mass of 17 kD (McDonald and Walsh, 1985),

hence the name. Protein kinase C is one of the key enzymes in cellular signal transduction. Subsequent work has cast some doubt on the physiological relevance of PKCI's inhibitory effect on PKC (Fraser and Walsh, 1991). In addition to this, others have isolated PKCI and have not been able to reproduce protein kinase C inhibition (Brenner et al., 1997; Lima et al., 1996; Mozier et al., 1991; Robinson et al., 1995). The acronym PKCI has since been changed to protein kinase C *interacting* protein although this still poorly describes the *in vivo* nature of it. The human gene was sequenced and it was found out to be 94,4% identical to the bovine sequence (our unpublished results and Brzoska et al., 1995). The human PKCI gene encodes a protein of 126 amino acids with a predicted molecular mass of 14 kD.

Genomic mapping and subcellular localization

The genomic mapping and *in vivo* localization of human PKCI has been studied. The PKCI gene maps to human chromosome 5q31.2 by fluorescence *in situ* hybridization (Brzoska et al., 1996). According to Brozoska et al., PKCI localizes to cytoskeletal structures in the cytoplasm of a human fibroblast cell line. The experiment was done by indirect immunofluorescence using transfected constructs expressing hemagglutinin (HA)-tagged PKCI. (See also our results in Subcellular localization of CDK7 and PKCI.)

Members of HIT protein family

Conserved in evolution but of unknown function

Several DNA open reading frames (ORF) coding for proteins related to human PKCI have been identified in a broad range of species representing mammalian, plant, fungal and bacterial kingdoms (Brenner et al., 1997; Bustos et al., 1990; Frohlich et al., 1991; Robinson et al., 1995; reviewed in Robinson and Aitken, 1994). The proteins have been grouped into the HIT protein family, in reference to a triad of histidines which is highly conserved among the family members, (His51 / His110, His112 and His114 in human PKCI). Figure 3A shows an alignment of the amino acid

sequences of a representative group of the HIT family members. The amino acid sequence identities for the entire coding region between, for example, human and bovine PKCI (94,4%), between human and maize (53,0%) and between human and mycoplasma PKCI (30,6%), indicate that the structure of this protein is well conserved and this then implies a function of importance.

Despite of the high degree of conservation in evolution, so far no function has been reported for the proteins belonging to this family. The function may soon be resolved as another human member of this now super family has recently been reported to have an enzymatic activity (Barnes et al., 1996).

Figure 3. Amino acid sequence alignments of HIT protein family members (A) and human PKCI and FHIT sequences (B). Numbers designate the GeneBank accession numbers.

FHIT is an interesting human relative

A human chromosomal fragile site at 3p14.2, the hereditary renal cancer translocation breakpoint and site of homozygous deletions and aberrant transcripts leading to malignancies has been shown to be encompassed by the *FHIT* locus (Ohta et al., 1996). *FHIT* (fragile triad of histidines) was identified by exon amplification. The encoded 147 amino acid sequence of FHIT shows 20,7% identity to the human PKCI over a 110 amino acid core region overlap.

The highest degree of amino acid conservation between PKCI and FHIT is observed in the so called ligand-binding pocket and notably includes the three histidines; PKCI residues His51, His112 and His114, FHIT residues His35, His96 and His98. The amino acid sequence alignment of human PKCI and FHIT is seen in Figure 3B.

FHIT has been shown to have the highest sequence similarity (52% identity over 110 amino acids) to a *Schizosaccharomyces pombe* HIT family member, which has previously been shown to have diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) asymmetrical hydrolase activity (Huang et al., 1995; Robinson et al., 1993). A third member belonging to this FHIT subfamily of HIT proteins is *S. cerevisiae* dinucleoside triphosphatase hydrolase (Brevet et al., 1991) for which the preferred substrates are Ap_3A (diadenosine 5',5'''-P^1,P^3-triphosphate), Ap_3G (adenosine 5'-guanosine 5'-P^1,P^3-triphosphate) and Ap_3C (adenosine 5'-cytosine 5'-P^1,P^3-triphosphate).

The enzymatic activity of the human FHIT has recently been identified (Barnes et al., 1996). It has dinucleoside 5',5'''-P^1,P^3-triphosphate hydrolase activity having Ap_3A as its preferred substrate (relative hydrolysis 100%). Other reported substrates are Ap_4A (11%), Ap_5A (diadenosine 5',5'''-P^1,P^5-pentaphosphate) (7,9%), Ap_6A (diadenosine 5',5'''-P^1,P^6-hexaphosphate) (2,3%), Ap_3G (44%), Ap_4G (adenosine 5'-guanosine 5'-P^1,P^4-tetraphosphate) (15%), Gp_3G (diguanosine 5',5'''- P^1,P^3-triphosphate) (36%), Gp_4G (diguanosine 5',5'''- P^1,P^4-tetraphosphate) (8,5%), m^7Gp_3G (m^7 diguanosine 5',5'''- P^1,P^3-triphosphate) (35%) and $m^7Gp_3Gm^7$ (m^7 diguanosine 5',5'''-P^1,P^3-triphosphate m^7) (17%). The hydrolysis always results in a nucleotide monophosphate being one of the reaction products. Dinucleoside

polyphosphates (n=3-6) are generally present in prokaryotic and eukaryotic organisms at basal concentrations of 10mM to 1μM (Garrison and Barnes, 1992). Ap_4A and Ap_3A have been proposed to have various intracellular functions, including regulation of DNA replication and signaling stress responses (reviewed in Kitzler et al., 1992; Ogilvie, 1992; Remy, 1992). On basis of site-directed mutagenesis substitution of histidines 35, 94, 96 and 98 one at a time to asparagine residues clearly reduced the catalytic activity of FHIT, to 64%, 31%, zero and to 2,5%, respectively (Barnes et al., 1996). These results as well as structural data on ligand binding (Lima et al., 1997) strongly suggest that the three conserved histidines combine to comprise a major portion of the active site involved in catalysis. It appears likely that the non-FHIT-type HIT proteins, including PKCI, also interact with nucleotide phosphates and that the central histidine (PKCI His112) will be critical to function. The amino acid residues surrounding the catalytically active triad of histidines are less well conserved between PKCI and FHIT suggesting that alternative ligands / bases could interact with PKCI binding site.

Deletions of both alleles within the FHIT locus have been observed in cell lines derived from cancers of the esophagus, stomach, colon, breast, kidney and lung, and aberrant transcripts were observed in several types of primary tumors (Gartenhaus, 1997; Gemma et al., 1997; Man et al., 1996; Mao et al., 1996; Negrini et al., 1996; Ohta et al., 1996; Sozzi et al., 1996; Sozzi et al., 1996; Virgilio et al., 1996; Yanagisawa et al., 1996). The molecular mechanisms of tumorigenicity of this putative tumor suppressor remain to be elucidated. Whether PKCI possesses an effect of this nature is an extremely intriguing question and yet to be seen.

Structural studies

X-ray crystallography was used to solve the three dimensional structure of human PKCI (Lima et al., 1996). This study shows it to be expressed as a dimer of two 13,7 kD polypeptides both consisting of two α-helical regions and five β strands. These two polypeptides come together to form extensive contacts between a helix and carboxy terminal amino acids.

Three dimensional structure of rabbit PKCI (Brenner et al., 1997) reveals striking similarity to the structure of the human counterpart.

The report on the three dimensional structure from multiwavelength anomalous diffraction (MAD) data of FHIT (Lima et al., 1997) shows the tertiary structures of the core domains of PKCI and FHIT to be quite similar. *FHIT* encodes two helices and seven β strands. PKCI and FHIT diverge notably at both ends with FHIT having amino and carboxy terminal extensions (Figure 4).

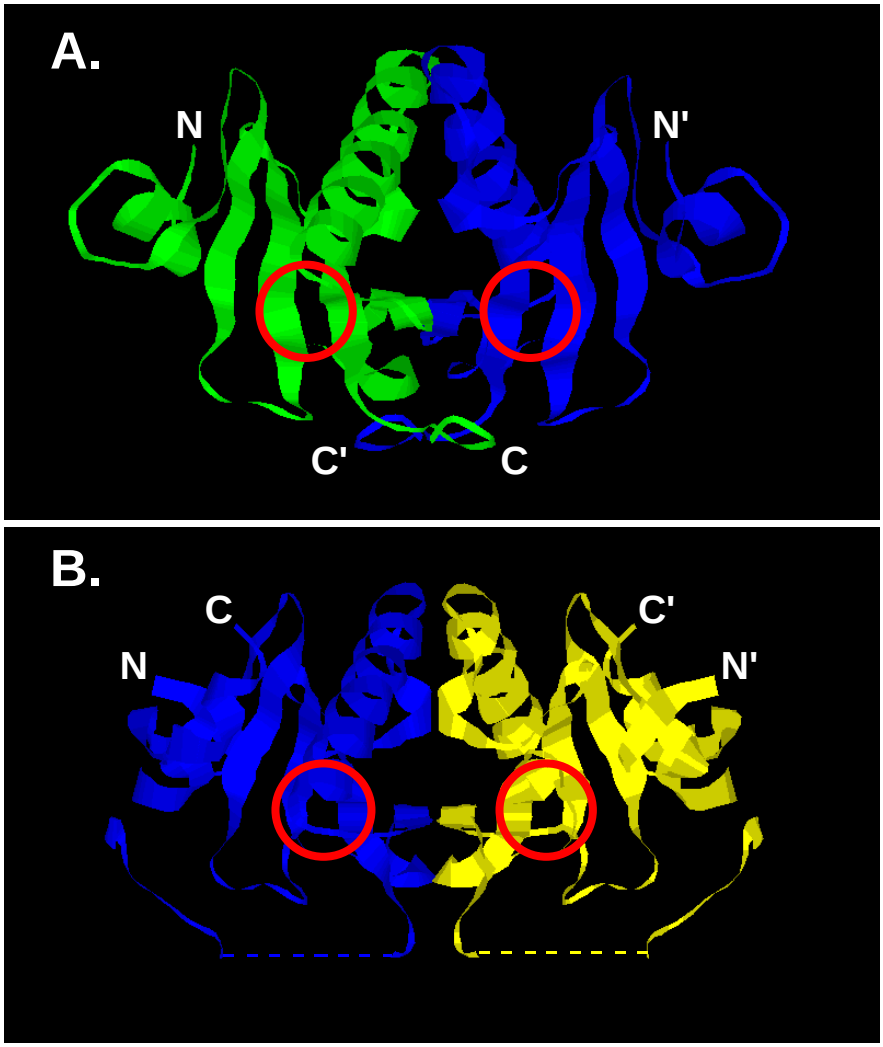

Figure 4. Ribbon diagrams of PKCI (A) and FHIT (B) dimer structures. The amino and carboxy termini of each monomer marked with N / N' and C / C', respectively. The histidine triad regions are circled. Dashed lines in FHIT represent disordered residues between amino acids 108 and 125. PKCI PDB accession code 1kpb, FHIT PDB accession code 1fit.

A novel type of zinc binding motif was reported in PKCI by radioactive zinc overlays. There is no similarity between this motif and the cysteine rich 'zinc-finger' motifs which also bind zinc (Mozier et al., 1991). The amino acid sequence with zinc binding ability was narrowed down to the histidine triad. No support on zinc binding has since been presented; in structural studies of human PKCI, rabbit PKCI and human FHIT it was not observed (Brenner et al., 1997; Lima et al., 1997; Lima et al., 1996). Moreover, evidence has been presented showing zinc to be inhibitory to the enzymatic activity of FHIT (Barnes et al., 1996).

AIM OF THIS STUDY

The aim of this study was to extend the investigation of the interaction between CDK7 and PKCI observed in yeast two-hybrid by means of several genetic and biochemical approaches to determine if this observed interaction has any physiological significance.

MATERIALS AND METHODS

BACTERIAL STRAINS, GROWTH MEDIA AND TRANSFORMATIONS

Eschericia coli (E. coli) strain DH5α *(supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1)* was routinely used in transformations and bacterial plasmid DNA cultures. *E. coli* strain KC8 *(pyrF::Tn5 hsdR leuB600 trpC9830 lacΔ74 strA galK hisB436)* was used in transformation of yeast plasmid purification to provide positive selection of the pJG4-5 plasmid. *E. coli* protease deficient AD202 strain was used as the host in GST (glutathione S-transferase)-PKCI and GST protein cultivations from PKCI/GEX-4T and GEX-4T plasmids, respectively. GST-FHIT production was performed in DH5α.

Bacterial cultures were routinely cultivated at $+37^0$C in Luria-Bertani (LB) medium, 1% (w / v, weight per volume) Bacto-tryptone, 0,5% Bacto-yeast extra, 0,5% NaCl, (plates adding 1.5% Bacto-agar) supplemented with 100μg / ml ampicillin for plasmid selection.

Transformations of cloning products were performed following the one-step preparation of competent *E. coli* cells (Chung et al., 1989).

Clones number 3, 4, 10, 13, 25, 27, 28 and 40 from the PKCI yeast two-hybrid screening were transformed by electroporation as advised by the manufacturer as extracted yeast plasmid DNA. Approximately 80ng DNA was used for each transformation. This worked as positive selection for pJG4-5 prey plasmid.

E. coli KC8 cells were plated on M9 minimal medium (Sambrook et al., 1989) after transformation by electroporation. All necessary amino acids were added except for tryptophane.

Transformed plasmid DNA was purified following Promega's Wizard™ Minipreps DNA purification system protocol using solutions described by Sambrook, J. et al. (Sambrook et al., 1989). Two bacterial plasmid DNA purifications were made from each electroporation transformation plate, named as follows; 3a, 3b, 4a, 4b, 10a, 10b, 13a, 13b, 25a, 25b, 27a, 27b, 28a, 28b, 40a and 40b.

PLASMIDS, CLONING AND OTHER DNA MANIPULATIONS

Plasmid constructs used in this study are listed in Table 2.

Table 2. Plasmid constructs used in this study.

PLASMID NAME	TYPE OF WORK	SELECTION	FUSION/TAG	REFERENCE
pJG4-5	yeast	trp, amp	N-terminal B42-HA	Gyuris et al., 1993
PKCI/pJG4-5	yeast	trp, amp	N-terminal B42-HA	Mäkelä, T.P.
cyclin H/pJG4-5	yeast	trp, amp	N-terminal B42-HA	Mäkelä, T.P.
C5/pJG4-5	yeast	trp, amp	N-terminal B42-HA	Mäkelä, T.P.
CDK7/pJG4-5	yeast	trp, amp	N-terminal B42-HA	This work
pEG202	yeast	his, amp	N-terminal LexA	Gyuris et al., 1993
PKCI/pEG202	yeast	his, amp	N-terminal LexA	This work
CDK7/pEG202	yeast	his, amp	N-terminal LexA	Mäkelä, T.P.
bicoid/pEG202	yeast	his, amp	N-terminal LexA	Gyuris et al., 1993
MAT1/pEG202	yeast	his, amp	N-terminal LexA	Ylikorkala, A.
pYES	yeast	ura, amp	none	Promega
pYF	yeast	ura, amp	N-terminal FLAG	This work
PKCI/pYF	yeast	ura, amp	N-terminal FLAG	This work
cyclin H/pYF	yeast	ura, amp	N-terminal FLAG	This work
pSH18-34	yeast	ura, amp	none	Gyuris et al., 1993
pAHP	transfection	amp	N-terminal HA	Mäkelä, T.P.
PKCI/pAHP	transfection	amp	N-terminal HA	Mäkelä, T.P.
cyclin H/pAHP	transfection	amp	N-terminal HA	Mäkelä, T.P.
PP5/pAHP	transfection	amp	N-terminal HA	Mäkelä, T.P.
PKCI/pAHC	IVT#	amp	N-terminal HA	This work
CDK7-3M/pCIneo	IVT#/transfection	amp	C-terminal 3xmyc	Mäkelä, T.P.
cyclin H/pCIneo	IVT#	amp	none	Mäkelä, T.P.
MAT1/pCIneo	IVT#	amp	none	Mäkelä, T.P.
GEX-4T	hydrolase activity	amp	N-terminal GST	Pharmacia Biotech
PKCI/GEX-4T	hydrolase activity	amp	N-terminal GST	Mäkelä, T.P.
FHIT/GEX-4T	hydrolase activity	amp	N-terminal GST	This work
CMV-β-Gal	transfection	amp	none	MacGregor et al., 1989

[#] in vitro translation

All clonings were performed according to standard protocols (Sambrook et al., 1989) unless otherwise mentioned. New England Biolabs (NEB) restriction enzymes and Boehringer-Mannheim T4 DNA ligase were used. Removal of 5' phosphates was done using Shrimp Alkaline Phosphatase (USB).

All digestions were performed according to basic protocols (Sambrook et al., 1989) using New England Biolabs (NEB) restriction enzymes to confirm cloning products and to analyze cDNA clones from the PKCI yeast two-hybrid screening.

PKCI was cloned into the pAHC mammalian expression vector into *Eco*RI-(*Xho*I-*Sal*I) sites and into pEG202 bait vector using *Eco*RI-*Xho*I sites.

CDK7 was cloned into pJG4-5 yeast expression vector to *Eco*RI-*Xho*I sites, the reading frame was then corrected with Klenow enzyme (2U, Boehringer-Mannheim), T4 DNA ligase and *Eco*RI restriction enzyme incubation. 0,1mM dATP (deoxy adenosine 5'-triphosphate) and dTTP

(deoxy thymidine 5'-triphosphate) (Boehringer-Mannheim) were used as a nucleotide source in the 1 hour incubation at $+37^0$C.

For preparation of the bacterial expression vector polymerase chain reaction (PCR) amplified FHIT cDNA was purified with Qiagen PCR QiaQuick purification kit, cleaved with *Eco*RI-*Xho*I and ligated into GEX-4T to produce FHIT / GEX-4T.

Into the pYES yeast expression vector (Promega) a FLAG-tag (underlined, Eastman Kodak) was cloned. Oligos pYF1 (5' A GCT GTC GAC AGA TCT ACC ACC ATG GAC TAC AAA GAC GAT GAC GAC AAG GGA TCC GGT ACC GAG CTC G 3') and pYF2 (5' AAT TCG AGC TCG GTA CCG GAT CCC TTG TCG TCA TCG TCT TTG TAG TCC ATG GTG GTA GAT CTG TCG AC 3') were annealed by heating at 95^0C for 5 minutes, and cooled at room temperature for 30 minutes. T4 Polynucleotide Kinase (NEB) was used to add 5' phosphates to the annealed oligo. Ligation was directed to the *Hin*dIII-*Eco*RI sites of the pYES vector, resulting in destruction of the *Hin*dIII site. The obtained FLAG-tagged pYES expression vector was designated pYF.

To obtain PKCI / pYF and cyclin H / pYF the following directional cloning strategies were used. PKCI was cloned into *Eco*RI-*Xho*I sites and cyclin H into *Bam*HI-*Xho*I of pYF multiple cloning site.

PCR was performed as the first selection method for PKCI yeast two-hybrid cDNA clones 1-40 and 51-94. The primer pair used was pJG4-5 vector primer (5' CCT CTT GCT GAG TGG AGA TG 3') as forward and PKCI internal sequence primer (5' GAC TGT CCA CCA TCT GAA CC 3'), nucleotides 320-301 as reverse. Amplification was performed in 50µl final volume of 10mM Tris-HCl (pH 8.3), 50mM KCl, 1,5mM $MgCl_4$, 0.001% Gelatin, 0,2mM deoxynucleotide triphosphates (dNTP), 30pmol primers, 2,5U of AmpliTaq (Perkin-Elmer). The PCR program consisted of 30 cycles; 30 seconds of denaturation at $+95^0$C, 30 seconds of annealing at $+54^0$C and an extension step at $+72^0$C of 1 minute. PCR was performed either on a whole cell preparate or for yeast plasmid DNA extractions and it was used as the negative selection method for PKCI cDNA clones.

PCR was also used to obtain FHIT cDNA for hydrolase studies. The primer pair FF3r 5' G GAA TTC ACC ACC ATG TCG TTC AGA TTT GGC 3' (nucleotides -1 to 18 of the FHIT sequence) and FR3r2 5' CCG CTC GAG TCA CTG AAA GTA GAC CCG 3' (nucleotides 445 to 427) (Barnes et al., 1996) with *Eco*RI - *Xho*I restriction sites (underlined) was used in a PCR amplification from Wi38 G0 library. Amplification was performed in 100µl final volume of 20mM Tris-HCl (pH 8.8), 10mM KCl, 10mM $(NH_4)_2SO4$, 2mM $MgSO_4$, 0,1% Triton X-100, 100µg / ml bovine serum albumin (BSA), 0,2mM dNTP, 500ng primers, 2,5U Pfu DNA Polymerase (Stratagene) and 2µg template DNA. The PCR program consisted of 30 cycles; 30 seconds of denaturation at $+95^0$C, 30 seconds of annealing at $+50^0$C and an extension step at $+72^0$C for 1 minute.

From the selective cDNA library transformation plates of PKCI yeast two-hybrid screening five positive cDNA clones 95, 96, 97, 98 and 99 were immediately analyzed by an automated sequencing service (Perkin Elmer ABI 373). Prior to sequencing, a PCR reaction with the forward pJG4-5 vector primer (5' CCT CTT GCT GAG TGG AGA TG 3') was performed using yeast plasmid DNA extractions as template DNA. QIAquick PCR Purification Kit (Qiagen) was used for the PCR reactions.

Sequencing was also performed for seven other clones, 3b, 4b, 10b, 13a, 40a and 40b as the final analysis step of positive clones. The sequences were then studied by comparing them to the sequences in the GenBank+EMBL+DDBJ+PDB database.

YEAST STRAINS, GROWTH MEDIA AND TRANSFORMATIONS

S. cerevisiae yeast strains used in this study are listed in Table 3 and strains with plasmids in Table 4.

Table 3. *S. cerevisiae* strains used in this study.

S. cerevisiae NAME	MATING TYPE	SELECTION MARKERS	INTEGRATED	REFERENCE
EGY48	MAT α	leu2-, ura3-, trp1-, his3-	6LexAop-LEU2	Gyuris et al., 1993
Boy1	MAT α	leu2-, ura3-, trp1-, his3-	6LexAop-LEU2, LexA-lacZ-URA3	Gyuris et al., 1993
Bun	MAT α	leu2-, ura3-, trp1-, his3-	6LexAop-LEU2, LexA-lacZ	This work

Table 4. Yeast strains with plasmids used in this study.

PLASMID 1	sel.#	PLASMID 2	sel.#	PLASMID 3	sel.#	STRAIN	sel.#	DROP OUT MEDIA	TYPE OF WORK IN THIS STUDY
PKCI/pEG202	his					Boy1	ura	hu	PKCI yeast two-hybrid
PKCI/pEG202	his	PKCI/pJG4-5	trp			Boy1	ura	hut	Yeast three-hybrid
PKCI/pEG202	his	PKCI/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Growth experiment
CDK7/pEG202	his	PKCI/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Growth experiment/interaction studi
pEG202	his	PKCI/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Growth experiment
bicoid/pEG202	his	PKCI/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Growth experiment
CDK7/pEG202	his	cyclin H/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Interaction study
CDK7/pEG202	his	PP5/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Interaction study
CDK7/pEG202	his	C5/pJG4-5	trp	pSH18-34	ura	EGY48		hut	Interaction study
CDK7/pEG202	his	pJG4-5	trp	pSH18-34	ura	EGY48		hut	Interaction study
				pYF	ura	Bun		u	Growth experiment
				PKCI/pYF	ura	Bun		u	Growth experiment
CDK7/pEG202	his	PKCI/pJG4-5	trp			Bun		ht	Growth experiment
CDK7/pEG202	his	cyclin H/pJG4-5	trp			Bun		ht	Growth experiment
PKCI/pEG202	his	PKCI/pJG4-5	trp			Bun		ht	Growth experiment
CDK7/pEG202	his	PKCI/pJG4-5	trp	cyclin H/pYF	ura	Bun		hut	Growth experiment
CDK7/pEG202	his	cyclin H/pJG4-5	trp	PKCI/pYF	ura	Bun		hut	Growth experiment
PKCI/pEG202	his	PKCI/pJG4-5	trp	PKCI/pYF	ura	Bun		hut	Growth experiment
MAT1/pEG202	his	CDK7/pJG4-5	trp	cyclin H/pYF	ura	Bun		hut	Yeast three-hybrid

selection marker

As non-selective growth medium YPD was used; 1% Bacto-yeast extract, 2% Bacto-peptone, 2% Dextrose, (plates adding 2,5% Bacto-agar) and for selection purposes Synthetic complete with drop-out mix; 0,17% Bacto yeast nitrogen base without amino acids, 0.5% Ammonium sulfate, 2% Dextrose (here SD), 2% Galactose (SG) or 2% Galactose with 1% Raffinose (SGR), (2,5% Bacto-agar), 1x drop-out mix lacking histidine (-h), tryptophane (-t), uracil (-u), or combinations of them (-hut, -ht).

For selection of colonies expressing *lacZ* gene and leucine (l) X-Gal indicator plates were used, medium made of Solution I, (8% Galactose, 4% Raffinose, 4x drop-out mix (-hutl or -htl), pH 7,0 adjusted with KOH), Solution II, (0,4 M KH_2PO_4, pH 7,0 adjusted with KOH) and X-agar, (0,34% Bacto yeast nitrogen base without amino acids, 1% Ammonium sulfate, 5% Bacto-agar) mixed 1:1:2. X-Gal was added at 0,08 mg / ml concentration. Plates were labeled XGR-hutl or XGR-htl.

For the positive selection of spontaneous *URA3* mutations yeast colonies were plated on SD-ht plates containing 0,1% 5-fluoro-oratic-acid (5-FOA, SIGMA) (Boeke et al., 1984).

Yeast transformation was performed as follows: an over night culture was diluted to optical density (OD) 0,200 at A595 (absorbance at 595 nm). For ten transformations 50 ml was cultured to OD 1,000, cells were then washed two times with phosphate-buffered saline (PBS) and resuspended in 1ml of

100mM LiAc, centrifuged and resuspended again, in 400µl of 100mM LiAc. For one transformation 50µl of that resuspension was used, the cells were centrifuged and 240µl 50% Polyethylene glycol (PEG 3350 SIGMA), 36µl 1,0M LiAc and 25µl ssDNA (single strand DNA; salmon sperm 2,0 mg/ml) were added on top. Plasmid DNA was added in 50µl volume dilution. When more than one plasmid was transformed at the same time 1µg of each plasmid DNA was used. After mixing, incubation of 30 minutes at $+30^0$C and heat shock of 15 minutes at $+42^0$C took place. Cells were plated at two different densities, at 1:20 ratio on suitable selection plates.

HeLa cDNA library transformation in PKCI yeast two-hybrid screening was performed as fifteen individual transformations to Boy1 PKCI/pEG202 strain using 1,4µg DNA each. The cDNA library was generated from HeLa human cervical carcinoma cell line (Gyuris et al., 1993). The number of primary recombinants in the original library was $9,6 \times 10^6$, the library used here had been amplified twice, and thus the number of primary recombinants is unknown. Library transformations were plated on fifteen XGR-hutl selection plates, 145mm in diameter. Total of 3,7 million cDNA clones were screened. Testing of the PKCI bait, library transformation and the following plating were performed by the Yeast Two-hybrid Core Facility technician Kirsi Mänttäri.

YEAST TWO HYBRID SCREENING

A yeast two-hybrid screening for PKCI was performed using Brent's system and the Boy1 strain. PKCI was cloned into the pEG202 bait vector, see Plasmids, Cloning and Other DNA manipulations for this method. The bait vector and the cDNA library were transformed in Boy1 as described above.

From the cDNA library transformation plates, 89 colonies were picked into 100µl 10% glycerol; numbered 1-40 and 51-99. Seventy-nine of them expressed the *lacZ* positive selection marker, and ten white colonies were picked as controls.

Analysis of positive clones was done by PCR, sequencing and diagnostic restriction enzyme digestions, see Plasmids, Cloning and Other DNA manipulations for these methods.

DEVELOPING A YEAST THREE-HYBRID SYSTEM

To further investigate the observed interaction between PKCI and CDK7 a yeast three-hybrid system was developed. The principle of this system is to express a third, nonfusion protein in addition to the DNA binding domain- fused and activator domain- fused proteins of two-hybrid system. This enables screening of protein-protein interactions which occur through a mediator protein and further characterization of the nature of a known interaction in the form of possible effect of a third protein. The three-hybrid system also has the obvious advantage of simultaneous expression of three proteins instead of two. In developing such a system the need of an extra selection marker became evident.

The *URA3* gene was chosen to be deleted to enable the transformation of the third expression vector pYES (Promega) with *URA3* selection marker. A FLAG-tag (Eastman Kodak) was cloned into pYES to obtain pYF for detection and immunoprecipitation purposes of the third protein (see Plasmids, Cloning and Other DNA manipulations for this method). Boy1 with integrated *6LexAop-LEU2* and *LexA-lacZ-URA3* and expression vectors PKCI / pEG202 and PKCI / pJG4-5 was used. PKCI bait and prey fusion proteins interact with each other leading to the expression of the marker genes *LEU2* and *lacZ*. (our unpublished results).

Mutating the *URA3* gene was performed as follows, schematized in Figure 5. Yeast was inoculated from XGR-hutl $+4^0$C plate into 5ml SD-ht liquid (day 1). The purpose of this was to release the uracil selection and thus make it possible for spontaneous mutations to occur on the *URA3* gene. The liquid culture was diluted every second or third day 1:10. On the 9th day 1 million cells were plated on SD-ht plate containing 0,1% 5-FOA, Figure 5A. After two days of incubation at $+30\ ^0$C three hundred resistant colonies were observed. Out of the 300 colonies 50 were streaked onto SD-ht plate. Boy1 PKCI / pEG202, PKCI / pJG4-5 was streaked as a control.

The quality of the mutations in the fifty 5-FOA resistant colonies was determined by phenotype. The aim was to allow spontaneous *URA3* mutation but to maintain the LacZ function. Having the positive interactors PKCI / pEG202 and PKCI / pJG4-5 present, marker genes'

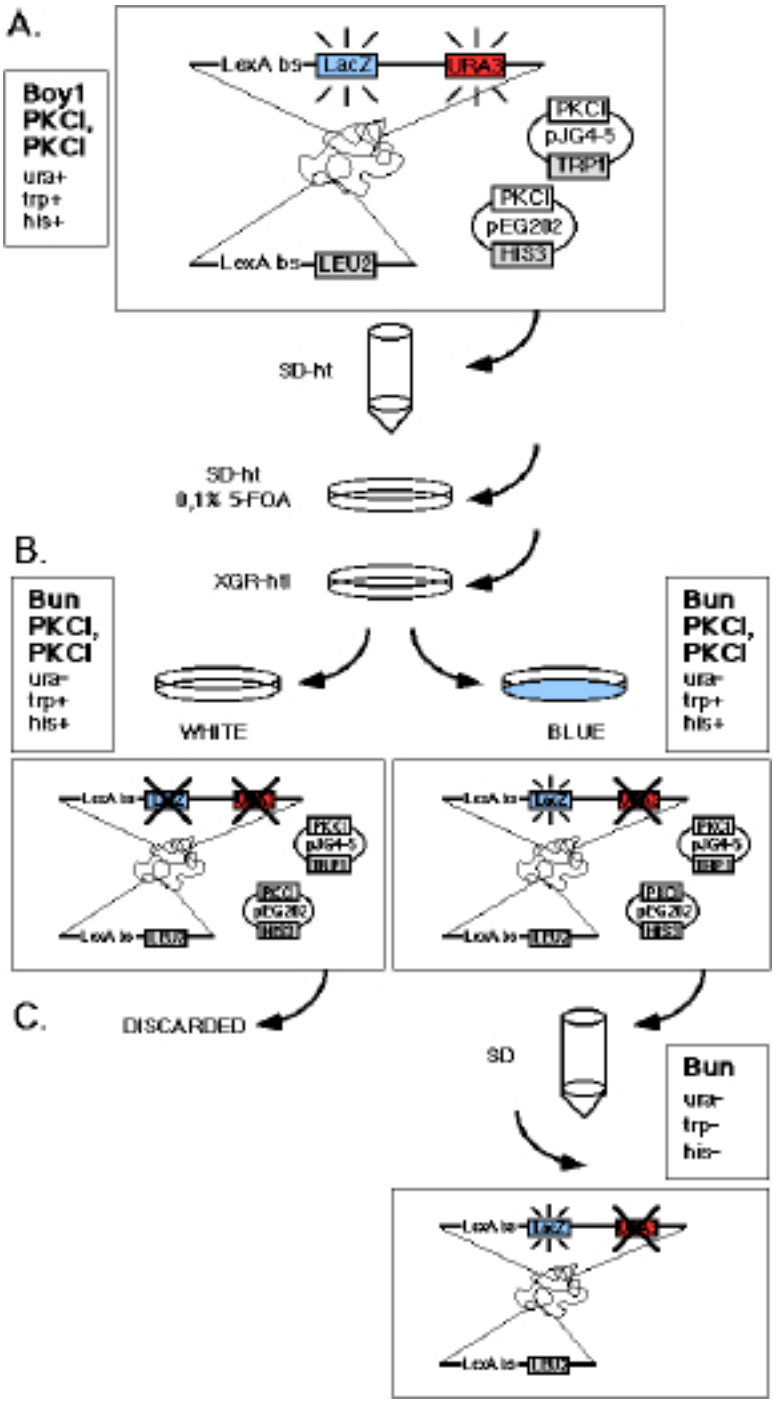

Figure 5. Schematic representation of creating the Bun (Boy1 *URA3* negative) strain by *URA3* mutation and selection strategies. Genomic integrations in Boy1 of LexA binding sites (LexA bs) and their adjacent *LEU2* and *LacZ* marker genes, integrated *URA3* selection gene, PKCI bait (pEG202, *HIS3* selection) and PKCI prey vectors (pJG4-5, *TRP1* selection) indicated. (A) Release of uracil selection of Boy1 PKCI / pEG202 PKCI / pJG4-5 by SD-ht liquid culture and plating of cells on ura3 negative selection plate SD-ht 0,1% 5-FOA. (B) Determination of the quality of *URA3* mutations by plating cells on XGR-htl selection plate and the detected different phenotypes; expression of *LacZ* (blue, no mutation) or absence of expression (white, mutated in some way, discarded). (C) Release of histidine and tryptophane selections by SD liquid culture to obtain Bun. Mutations are marked with a cross over a gene.

expression *(lacZ* and *LEU2)* functioned as an indicator of the desired quality of mutation. After two days four replica plates were made; SD-ht, SD-hut, XGR-htl and SD-ht, in this order. The third replication plate XGR-htl revealed the state of the LacZ gene, (Figure 5B). On this plate clones no. 35 and 40 plus control (cont) grew blue (Figure 6). Clones 35 and 40 were picked as *URA3* defective but *lacZ* functional. They were named Bun35 PKCI/pEG202, PKCI/pJG4-5 and Bun40 PKCI/pEG202, PKCI/pJG4-5. Bun for Boy1 *URA3* negative.

The selective pressure (his and trp) for the plasmids PKCI/pEG202 and PKCI/pJG4-5 was released in the following way (Figure 5C). Clones were inoculated into 5ml SD liquid (day 1). Cultures were diluted 1:40 every day. On the 4th day a small amount of the Bun35 PKCI/pEG202, PKCI/pJG4-5 and Bun40 PKCI/pEG202, PKCI/pJG4-5 cultures was plated on SD. After two days of growth, five replicas were made; SD, SD-h, SD-t, SD-ht and SD in this order. Three colonies of both 35 and 40 were picked, having lost both expression vectors (colonies not growing on SD-h, SD-t and SD-ht). Clones were named Bun35.1 Bun35.2 Bun35.3 and Bun40.1 Bun40.2 Bun40.3. Strain Bun35.1 is being currently used, (name has been shortened to Bun35 or Bun).

To illustrate this three-hybrid system, the property of three polypeptides, CDK7, cyclin H, and MAT1 to form a biologically active CDK-activating kinase (CAK) complex was used. It was observed that the addition of cyclin H, seemed to build the interaction between the DNA binding domain of MAT1 (LexA) and the activator domain of CDK7 (VP16) thereby turning the yeast colonies blue due to the expression of the marker gene (data not shown). Bun MAT1/pEG202, CDK7/pJG4-5 otherwise grows white. Cyclin H was expressed from the pYF yeast three-hybrid expression vector.

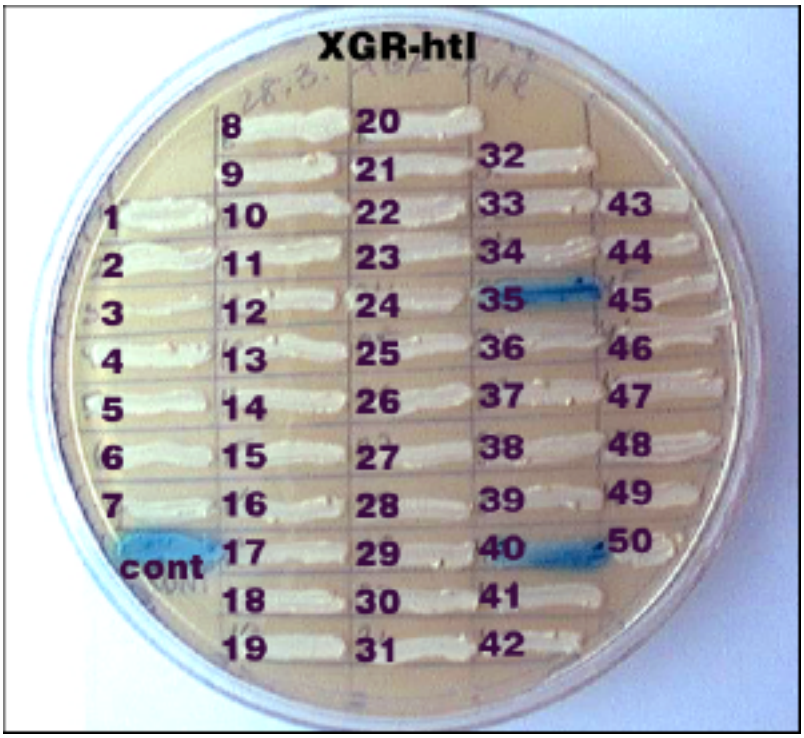

Figure 6. Determination of the quality of *URA3* mutations on XRG-htl selection plate. Tested clones are numbered from 1 to 50. Clones number 35 and 40 express the marker genes *LEU2* and *LacZ*, concluded from the survival and blue color of the clones, respectively. Also the control strain (cont) expresses the marker genes.

YEAST GROWTH RATE ASSAYS

Two separate experiments, I and II were made to measure the growth rates of yeast strains expressing different proteins of interest by measuring the optical densities of the liquid cultures at 595 nm.

The four strains used in growth rate assay I were 1. PKCI/pEG202 PKCI/pJG4-5 pSH18-34 EGY48, 2. -/pEG202 PKCI/pJG4-5 pSH18-34 EGY48, 3. CDK7/pEG202 PKCI/pJG4-5 pSH18-34 EGY48 and 4. bicoid/pEG202 PKCI/pJG4-5 pSH18-34 EGY48. Cultures were started from fresh SD-hut and SG-hut plates setting over night inoculations into 5 ml corresponding liquid media. On day 1 cultures were diluted to OD 0,100, three parallel 7 ml cultures of each were made. The optical density of the cultures was measured at different time points; 4.50, 9.25, 15.50, 22.33, 29.50, 48.50, 64.50 and 107.25 hours after starting the cultures. Growth curves were drawn based on this primary data. Optical densities of three parallel cultures were pooled, only the averages are shown in the results.

Experiment II was done with strains PKCI / pYF Bun35 and - / pYF Bun35, to elucidate the growth inhibitory effect of PKCI itself in the most simple way. Cultures were started from fresh SD-u and SGR-u plates setting over night inoculations into 5 ml corresponding liquid media. On day 1 cultures were diluted to OD 0,100, and again three parallel 7 ml cultures of each were made. The optical density of the cultures was measured at different time points; 7.50, 12.75, 21.50, 27.00 and 32.50 hours of cultivation in growth rate assay II. Growth curves were drawn based on this primary data. Optical densities of three parallel cultures were pooled, and only the averages are shown in the results.

PRODUCTION OF BACTERIAL GST FUSION PROTEINS

Production of GST-PKCI and GST-FHIT was done in bacterial AD202 and DH5α cultures, respectively, of 1 liter volume. The isopropyl-β−D-thiogalactopyranoside (IPTG, Fluka) (0,126mM) induction begun at approximately OD 1,000 and the cultures were incubated at +25^{0}C for 15 hours. Lysis of bacteria was achieved by resuspending the pellets in 100 ml PBS, 1% Triton X-100, 10mM β-glycerol phosphate (βGP), 1μg / ml leupeptin l2,5 μg / ml aprotinin, 0,5mM phenylmethylsulfonyl fluoride (PMSF), 1mM dithiothreitol (DTT), 0,01% lysozyme, incubating on ice for 25 minutes and sonicating twice for 1 minute. After centrifugation the supernatants were filtered through 0,45μm filter. Filtered supernatants were run twice through columns containing 1,2 ml of prewashed 75% slurry Glutathione sepharose (Pharmacy). Elution of the GST-PKCI and GST-FHIT fusion proteins was done with 50mM Tris, 200mM NaCl, 10mM Glutathione, pH 7,7 into eight 300μl fractions. Protein concentrations and purity of the fractions were measured with Bio-Rad Protein Assay and with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Part of the fractions were pooled, 10% glycerol was added and they were stored at -70^{0}C.

IN VITRO TRANSCRIPTION AND TRANSLATION

For the interaction studies PKCI and CDK7 proteins were produced by *in vitro* transcription and translation methods. Three reactions were made

using the following expression vectors; 1. CDK7-3M/pCIneo cyclin H/pCIneo MAT1/pCIneo PKCI/pAHC 2. CDK7-3M/pCIneo PKCI/pAHC and 3. PKCI/pAHC thus translating one, two or four proteins at the same time in the same reaction. In these vector constructs CDK7 has a myc tag (Myc protein one letter amino acid sequence EQKLISEEDL repeated three times, hence 3M), cyclin H and PKCI have HA tags.

In vitro translation was performed with Promega's TnT T7 Coupled Reticulocyte Lysate System following their General Protocol (http://www.promega.com/tbs/tb126/tb126.html) using 1,0 to 2,0 μg DNA per reaction and 40 μCi ^{35}S-labeled methionine (New England Nuclear). Detection was done from fixed (65% water, 25% 2-propanol, 10% acetic acid, 30 minutes), amplified (30 minutes, Amplify™ Amersham) and dried SDS-PAGE by ^{35}S autoradiography. Four percent (2 μl) of the reaction volume was used for the total gel samples.

TRANSFECTION TO MAMMALIAN CELLS

All transfections to U2-OS human osteosarcoma cells and COS African green monkey fibroblasts were performed by the standard calcium-phosphate precipitate protocol (Sambrook et al., 1989). Total DNA amount used in transfections prior to immunoprecipitation was 20 μg per tissue culture dish 9 cm in diameter. Two micrograms of total DNA was transfection reporter CMV-β-Gal (immediate early human cytomegalovirus promoter-β-galactosidase) (MacGregor and Caskey, 1989). To examine the interaction between PKCI and CDK7, co-transfections with CDK7-3M/pCIneo of PKCI/pAHP, cyclin H/pAHP, PP5/pAHP and pAHP to U2-OS cells were made. Cyclin H was used as positive control for interaction, PP5 (protein phosphatase 5, Mäkelä, T.P., unpublished results) and the empty vector were used as negative controls. Nine micrograms of each DNA preparation was used. The transfection precipitate was left to sit on the cells for 10 hours at +37^{0}C in 3% CO_2. The cells were then cultivated for 24 more hours before lysis. Transfections were performed by Arno Pihlak in our laboratory.

Transfections prior to localization determination by immunofluorescence were done to U2-OS and COS cells in a six well dish on coverslips using 7µg PKCI/pAHC DNA preparation, 7µg pCIneo carrier DNA and 1µg transfection reporter CMV-β-Gal per well. Cells were incubated with the transfection precipitate for 24 hours, then washed twice with PBS and cultivated for 47 more hours before labeling.

Cells were routinely cultured in Dulbecco's modified Eagle medium (D-MEM) containing 10% fetal calf serum, supplemented with penicillin/streptomycin and glutamin.

IMMUNOFLUORESCENCE LABELING

Cells were fixed with 3,5% (w/v) paraformaldehyde for 20 minutes followed by quenching the remaining fixative using 50 mM NH_4Cl for 10 minutes and then permeabilized with 0,1% Triton X-100 for 5 minutes. All incubations were carried out in PBS, pH 7,4 at room temperature.

Prior to immunolabeling at room temperature the cells were washed again in PBS, transferred to 10% fetal bovine serum (FBS) for 30 minutes, and then labeled with the primary antibodies αHA mouse at 1:50 and αβ-Gal rabbit at 1:100 in 10% FBS for 30 minutes. The coverslips were rinsed three times for 5 minutes and incubated with fluorescently labeled secondary antibodies fluorescein isothiocyanate (FITC)-α-mouse at 1:200 (Boehringer Mannheim) and Texas Red-α-rabbit at 1:50 (Jackson Laboratories), respectively in 10% FBS for 30 minutes. The two primary and the two secondary antibodies were applied simultaneously. After extensive washing and one short wash in water, the coverslips were mounted in Mowiol containing 2,5% (w/v) 1,4-Diazabicyclo-[2.2.2]octane (DABCO) on glass slides and examined with an Axiophot fluorescence microscope. Transfections, fixation and immunofluorescence labeling were performed by Dr. Päivi Ojala in our laboratory.

IMMUNOPRECIPITATION

Yeast cell extracts

Yeast two hybrid system fusion proteins were used in immunoprecipitation experiments to further study the observed interaction between CDK7 and PKCI. The following yeast strains were used: 1. CDK7/pEG202 PKCI/pJG4-5 pSH18-34 EGY48, 2. CDK7/pEG202 cyclin H/pJG4-5 pSH18-34 EGY48, 3. CDK7/pEG202 PP5/pJG4-5 pSH18-34 EGY48, 4. CDK7/pEG202 C5/pJG4-5 pSH18-34 EGY48, 5. CDK7/pEG202 pJG4-5 pSH18-34 EGY48. Strain number 2 was used as positive control for interaction and strains number 3, 4 and 5 as negative controls. αHA antibody was used to immunoprecipitate the HA-tagged proteins expressed from the pJG4-5 prey vector. Detection of co-immunoprecipitated LexA-CDK7 was made with αLexA antibody from a western blot.

Liquid cultures of 15 ml in volume were grown over night in SGR-hut medium. After centrifuging and washing twice with PBS the cells were lysed in 300 µl volume of ELB (150 mM NaCl, 50 mM HEPES, pH 7,4, 5 mM EDTA, 0,1% NP-40) with 1% NP-40, 10 mM βGP, 1 µg/ml leupeptin l2,5 µg/ml aprotinin, 0,5 mM PMSF, 1 mM DTT added. 300 µl of Glass Beads (SIGMA) were added prior to mechanical lysis of 9 times 45 seconds on vortex shaker. The volume was then increased to 500 µl. Protein concentrations were measured, 20 µg protein lysate was loaded to the total samples and 750 µg of total protein was used for the immunoprecipitations in the volume of 500 µl. Protein A sepharose (PAS) beads (SIGMA), 100 µl of 25% slurry per reaction were pre-coated with αHA antibody (at 1:10) for 1 hour at $+4^0$C and washed two times. For the immunoprecipitation, the antibody was diluted 1:60. The reactions were incubated for 4 hours at $+4^0$C. PAS beads were washed three times and divided into two, one half was mixed with 20 µl of water and 7 µl of 5 x Laemmli sample buffer (LSB) (Sambrook et al., 1989) and incubated at $+95^0$C for 5 minutes. Half of each was run on SDS-PAGE for western blot analysis with αHA and αLexA antibodies.

The other halves of PAS beads were used for kinase activity studies.

Mammalian cell extracts

Transfected U2-OS cells were lysed in 1000 µl ELB (150 mM NaCl, 50 mM HEPES, pH 7,4, 5 mM EDTA, 0,1% NP-40) with 10 mM βGP, 1 µg/ml leupeptin l2,5 µg/ml aprotinin, 0,5 mM PMSF, 1 mM DTT added, plates were kept on ice for 30 minutes. Supernatants were tested for total protein concentrations and transfection efficiency using the CMV-β-Gal reporter gene, 450 µg of total protein was used for both αHA and αmyc immunoprecipitations. Ascites antibodies were used at 1:600 with bridging antibody rabbit-α-mouse IgG at 1:600. 100 µl of 25% PAS beads was added for the 2 hours of incubation at $+4^0$C. PAS beads were washed four times with lysis buffer. These immunoprecipitation samples were next used for a kinase activity assay and after that for western blot analysis with αHA and αmyc antibodies.

Endogenous CDK7 was immunoprecipitated from U2-OS cell lysate with αCDK7 8543.4 polyclonal antibody, used at 1:500. Cells on plate were washed three times with PBS, lysed in 500 µl ELB (150 mM NaCl, 50 mM HEPES, pH 7,4, 5 mM EDTA, 0,1% NP-40) with 10 mM βGP, 1 µg/ml leupeptin l2,5 µg/ml aprotinin, 0,5 mM PMSF, 1 mM DTT added, and incubated on ice for 45 minutes. Another 500 µl of lysis buffer was added on to a plate, and the supernatant was collected and centrifuged. Total protein concentration was then measured. 320 µl 25% PAS bead slurry was used for the 2 hours of incubation at $+4^0$C. PAS beads were washed three times and divided to four for kinase assays with bacterial GST fusion proteins GST-PKCI, GST-PP5 and GST.

***In vitro* translated proteins**

Immunoprecipitations were performed using half of the *in vitro* translation reaction mixture increasing the volume to 170 µl with ELB (150 mM NaCl, 50 mM HEPES, pH 7,4, 5 mM EDTA, 0,1% NP-40) with 10 mM βGP, 1 µg/ml leupeptin l2,5 µg/ml aprotinin, 0,5 mM PMSF, 1 mM DTT. Monoclonal antibodies αHA and αmyc were used at 1:270 with rabbit-α-mouse IgG at 1:270 and 100 µl 25% PAS slurry. Incubation was done at $+4^0$C for 4 hours after which the beads were washed three times, 30 µl water was

added and the samples were then incubated at +95^{0}C for 5 minutes, half of the amounts were run on SDS-PAGE.

WESTERN BLOTTING

For SDS-PAGE gels, semi-dry western blotting to nitrocellulose membrane (0,45 µm pore size, Protran) was performed. Antibody incubations were done at room temperature, αHA at 1:1000 (1h-15h) αLexA at 1:5000 (1h-16h) and αmyc at 1:2000 (over night) in blocking solution; 5% (w/v) milk powder, 0,05% Tween in PBS. Secondary antibodies horseradish peroxidase rabbit-α-mouse or goat-α-rabbit were used at 1:2000 and incubated for 30 minutes. Prior to detection by enhanced chemiluminescence (ECL) method, membranes were stained with Ponceau reagent (SIGMA).

KINASE ACTIVITY ASSAY

The kinase activity assay for transfected or yeast expressed CDK7 after immunoprecipitation was performed using 4 µg GST-CDK2 and 4 µg CTD peptide (RNA polymerase II C terminal domain heptapeptide YSPTSPS repeated three times and labeled with biotin, Research Genetics, Inc.) as substrates. Incubation was carried out in 20 mM Tris pH 7,5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT buffer using 20 µCi γ-^{32}P-ATP (ICN) per reaction. Prior to kinase incubation the PAS beads from immunoprecipitation were washed once with kinase buffer. Incubation was done at +30^{0}C for 30 minutes, 7 µl of 5 x LSB was then added and samples were incubated at +95^{0}C for 5 minutes and half of the samples were loaded on SDS-PAGE gels. Exposures were taken either directly from the gel (yeast extracts) or from nitrocellulose filter after western blot (mammalian cell extracts) at -20^{0}C.

When testing the inhibitory effect of PKCI on endogenous CDK7 kinase activity 1 µg of GST-PKCI, GST-PP5 or GST was added into the kinase reaction incubation mixture. GST-PP5 and GST functioned as negative controls and results were compared to the basal level of activity where no GST protein was added. 10 µl of 5 x LSB was added and samples were

incubated at +95^{0}C for 5 minutes and then half of the samples were loaded on SDS-PAGE gels. Exposures were taken directly from the gel at -20^{0}C.

HYDROLASE ACTIVITY ASSAY BY NMR SPECTROSCOPY

Hydrolase activity studies were performed for bacterially produced GST-PKCI and GST-FHIT fusion proteins, used at 8,75 µM concentrations. GST was used as a control with the substrate Ap_4A. Reactions were performed in 50 mM HEPES, 0,5 mM $CaCl_2$ pH 6,8 buffer in a volume of 800 µl. Substrates ADP (adenosine 5'-diphosphate), ATP, dATP, dGTP (deoxy guanine 5'-triphosphate), Ap_2A (diadenosine 5',5'''-P^1,P^2-diphosphate), Ap_3A, Ap_4A and Ap_5A were used at 5mM concentrations. The reactions were incubated at +37 ^{0}C over night, then put into -20^{0}C and thawed prior to NMR (nuclear magnetic resonance) spectroscopy which was used as the detection method of the phosphorous in the substrates. Spectra of the substrates alone were used as references.

The NMR measurements were carried out using a Varian Unity 600 MHz NMR spectrophotometer. The phosphorous (242 MHz) spectra were measured with broad band probe at +37 ^{0}C. The preacquisition delay was 4,9 seconds and the length of the pulse was 12,8 micro seconds. The FID signal was collected for 682 ms, at that time protons were decoupled. The width of the spectrum was 12 kHz. The measurement time varied from 10 to 60 minutes depending on the concentration of the sample. The NMR spectroscopic part of this study was done by Arto Annila in the Technical Research Center of Finland, VTT Chemical technology.

RESULTS

DEVELOPING A YEAST THREE HYBRID SYSTEM

A yeast three-hybrid system was developed as a part of these studies, see Materials and Methods for procedures.

INTERACTION BETWEEN CDK7 AND PKCI

A protein-protein interaction was observed between CDK7 and PKCI in the yeast two-hybrid screening prior to the beginning of this study. In addition to the encouraging primary finding of multiple independent PKCI cDNA clones, the yeast colonies had an unexpected phenotype. Contradictory to a dark blue color of the colonies, indicating strong interaction, the size of them was small compared to others (unpublished results, data not shown). Thus the over-expression of these two proteins, CDK7 and PKCI interacting with each other seemed to cause a physiological change of some kind leading to small colony phenotype in yeast.

Specificity of interaction between CDK7 and PKCI in yeast two-hybrid system

The specificity of any interaction observed in yeast two-hybrid screening needs to be investigated. PKCI/pJG4-5 was retransformed into several strains expressing different bait fusion proteins; bicoid, cdc2 and CDK7 mutants KM, TE and N terminal deletion of 100 amino acids. In CDK7 KM mutant the ATP binding lysine (K) 41 in the catalytic domain has been changed to methionine (M) thus resulting in kinase inactive CDK7, and in the TA mutant the activation phosphorylation threonine (T) 170 has been changed to glutamic acid (E) resulting in incapability to be activated by phosphorylation. Transcription of the marker genes was observed in both cases of CDK7 single amino acid mutants KM and TE (data not shown). No interaction was observed between PKCI and bicoid or cdc2, thus verifying the specificity of the interaction between CDK7 and PKCI in the yeast two-hybrid system. The interaction was abolished by the N terminal deletion of CDK7. This retransformation study was performed by Tomi Mäkelä (unpublished results).

Growth inhibition by PKCI

Growth rate assays in liquid culture were done to verify the phenotype observed on plates thus using a method, the results of which are easier to compare with each other, and to address the question whether the growth inhibitory phenotype of the CDK7 / pEG202 PKCI / pJG4-5 pSH18-34 EGY48 strain was dependent on the interaction between the two over-expressed proteins or a function of PKCI alone.

In the growth rate assay I with four EGY48 strains a growth inhibitory effect of PKCI was observed. By culturing these strains in galactose (SG-hut), the expression of the PKCI prey protein resulted in a growth inhibition. This observed inhibition was most pronounced in the PKCI / pEG202 PKCI / pJG4-5 pSH18-34 EGY48 strain i.e. when PKCI was expressed both from the bait and the prey expression vector. This suggests the effect to be PKCI dependent. The second strongest growth inhibition was observed in the strain expressing CDK7 and PKCI as noticed already on the plates from the primary CDK7 yeast two-hybrid screening. Growth curves of strains cultured in SD-hut and SG-hut media are shown in Figure 7A and B, respectively. In SD-hut media the growth rate of the strains was the same which suggests that any growth effect is dependent on the subsequent expression of the prey protein after the switch to galactose. The lower optical density of PKCI cultures was not a function of smaller cell size but rather the amount of cells (data not shown). The relative numbers for the optical densities of these strains with the following baits PKCI / pEG202, CDK7 / pEG202, - / pEG202 and bicoid / pEG202 were 0,46 : 0,64 : 0,86 : 1,00, respectively, at 15,5 hours time point in SG-hut media, Figure 7C four bottom columns. The four upper columns represent cultures in SD-hut media at the same time point. Primary data is shown in Table 5.

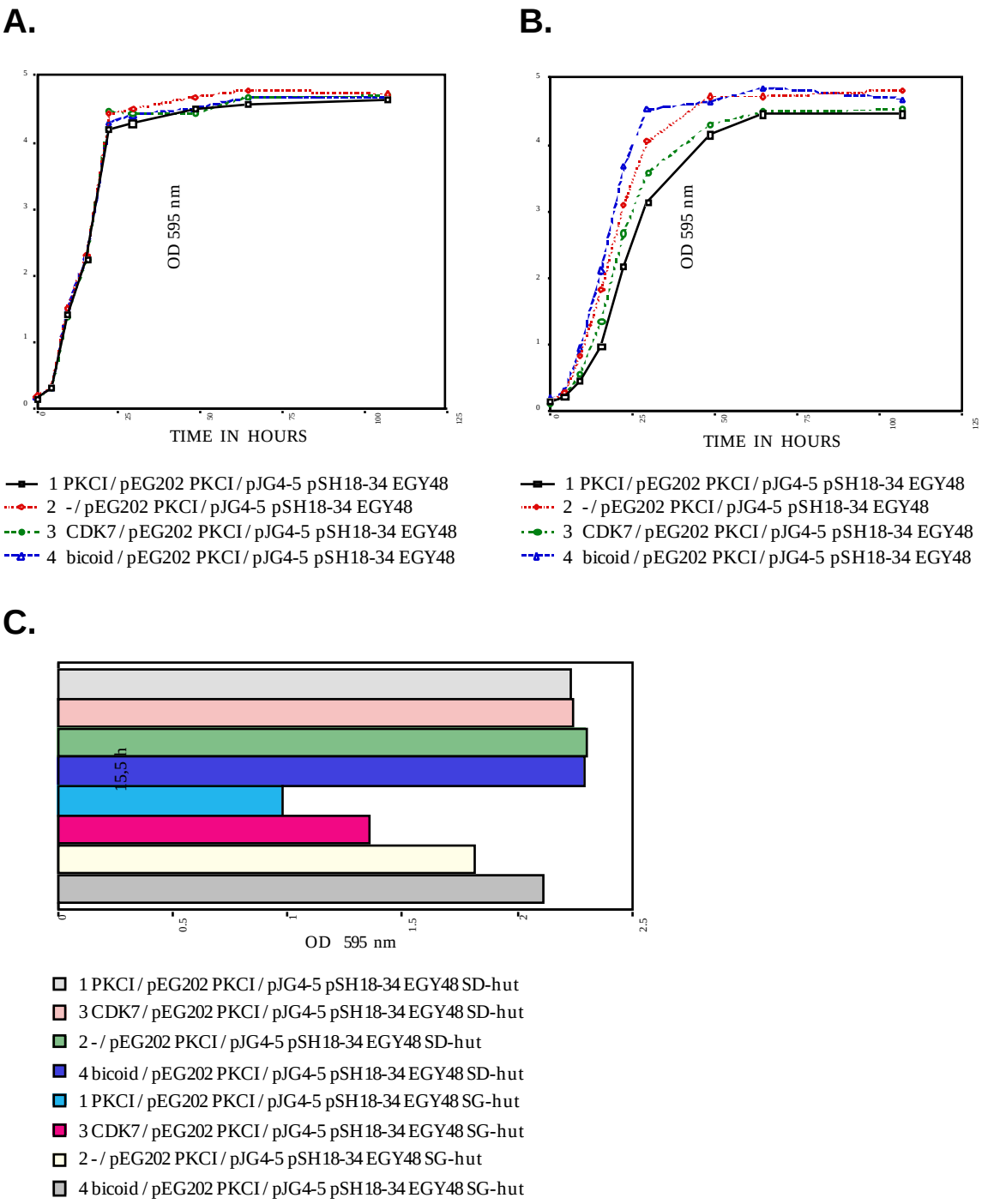

Figure 7. Expression of PKCI causes a growth inhibitory effect in yeast. Growth curves of growth rate assay I based on optical densities (595 nm) measured at different time points of liquid cultures in SD-hut media (A) and in SG-hut media (B). Primary data in Table 5. (C) Column representation of the optical densities measured at 15,5 hours time point in SD-hut and SG-hut media. Cultures were started from fresh SD-hut and SG-hut plates setting over night inoculations into 5 ml corresponding liquid media. On day 1 cultures were diluted to OD 0,100 and three parallel 7 ml cultures of each was made.

Table 5. Primary data of yeast growth rate assay I.

TIME IN HOURS	STRAIN 1 SD-hut	STRAIN 2 SD-hut	STRAIN 3 SD-hut	STRAIN 4 SD-hut	STRAIN 1 SG-hut	STRAIN 2 SG-hut	STRAIN 3 SG-hut	STRAIN 4 SG-hut
0.00	0.153	0.180	0.162	0.178	0.153	0.160	0.129	0.193
4.50	0.310	0.327	0.301	0.305	0.211	0.269	0.223	0.295
9.25	1.398	1.495	1.376	1.521	0.453	0.828	0.566	0.955
15.50	2.233	2.299	2.244	2.289	0.977	1.820	1.358	2.113
22.33	4.195	4.420	4.463	4.300	2.158	3.095	2.668	3.675
29.50	4.273	4.493	4.420	4.403	3.113	4.030	3.573	4.520
48.50	4.505	4.683	4.440	4.510	4.140	4.716	4.293	4.640
64.50	4.570	4.780	4.680	4.663	4.443	4.716	4.483	4.840
107.25	4.630	4.720	4.690	4.673	4.440	4.813	4.516	4.680

STRAINS: 1. PKCI / pEG202 PKCI / pJG4-5 pSH18-34 EGY48, 2. - / pEG202 PKCI / pJG4-5 pSH18-34 EGY48, 3. CDK7 / pEG202 PKCI / pJG4-5 pSH18-34 EGY48 4. bicoid / pEG202 PKCI / pJG4-5 pSH18-34 EGY48

In the growth rate assay II, the inhibitory effect of PKCI alone was verified. Growth curves of SD-u and SGR-u cultures (Figure 8), based on the primary data (Table 6) show expression of PKCI (SGR-u medium) to reduce the growth rate from 100% to 76% [(0.650 : 0.849) x 100% = 76%] observed at 12,75 hours time point. In the same figure it can be seen that the growth rates of the two strains are exactly the same in SD-u media, when expression of prey proteins is shut down.

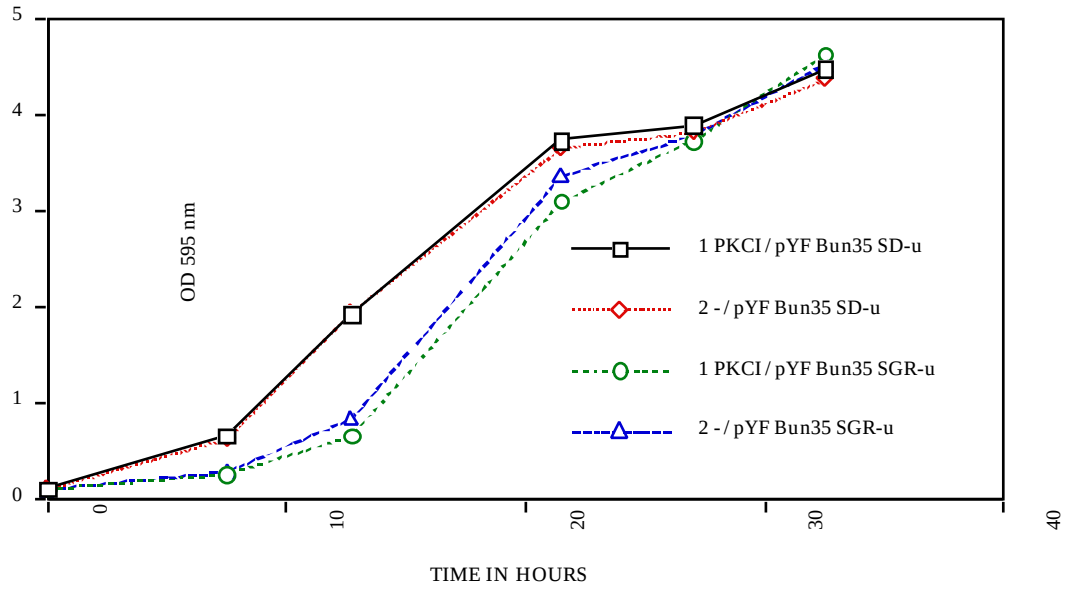

Figure 8. PKCI expression alone inhibits the growth of the Bun strain. Growth curves of growth rate assay II based on optical densities (595 nm) measured at different time points of liquid cultures in SD-u and SGR-u media. Primary data in Table 6. Liquid cultures were started from fresh SD-u and SGR-u plates setting over night inoculations into 5 ml corresponding media. On day 1 cultures were diluted to approximately OD 0,100 and three parallel 7 ml cultures of each were made.

Table 6. Primary data of yeast growth rate assay II.

TIME IN HOURS	STRAIN 1 SD-u	STRAIN 2 SD-u	STRAIN 1 SGR-u	STRAIN 2 SGR-u
0.00	0.122	0.118	0.120	0.121
7.50	0.656	0.644	0.258	0.296
12.75	1.916	1.954	0.650	0.849
21.50	3.744	3.658	3.093	3.360
27.00	3.884	3.808	3.732	3.773
32.50	4.460	4.356	4.623	4.530

STRAINS: 1. PKCI / pYF Bun35, 2. - / pYF Bun35

On basis of these experiments addressing the question of PKCI's growth effect it may be concluded that PKCI does indeed have an inhibitory growth effect in yeast, and in the two-hybrid system, it thus becomes very interesting to find out more about the CDK7-PKCI complex.

Detection of the CDK7-PKCI complex

To further characterize the interaction between PKCI and CDK7 immunoprecipitations of these proteins over-expressed in the yeast two-hybrid system, mammalian cells and in vitro system were performed.

Yeast cell extracts

After the αHA immunoprecipitation of HA-PKCI, HA-cyclin H, HA-PP5, HA-C5 and HA-tag no co-immunoprecipitated LexA-CDK7 could be observed from the αLexA immunoblot in any of these samples. αHA immunoblot part shows the immunoprecipitated HA-tagged proteins (Figure 9A). The total lysate samples in Figure 9B shows the expression of LexA-CDK7 (αLexA immunoblot) and HA-tagged proteins (αHA immunoblot). The HA-tag does not have sufficient molecular mass to be detected in this gel.

The kinase activity assay of these αHA immunoprecipitates however showed slightly elevated levels of GST-CDK2 and CTD peptide phosphorylation in the HA-PKCI immunoprecipitate sample in addition to the HA-cyclin H sample, suggesting a presence of co-immunoprecipitated LexA-CDK7. This is shown in Figure 10.

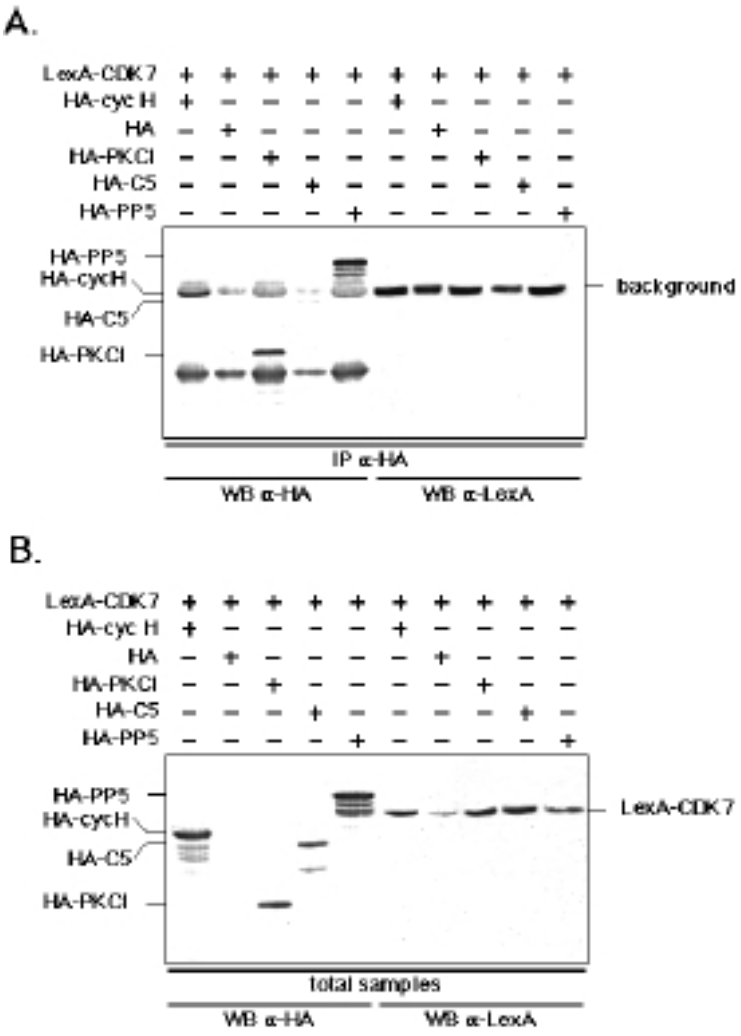

Figure 9. CDK7-PKCI complex is barely detectable with immunoprecipitation method from yeast extracts. Western blot analysis of αHA immunoprecipitated proteins (A) and total protein lysate samples (B) from yeast extracts using αHA and αLexA antibodies. Yeast from liquid cultures of 15 ml were lysed as described in Materials and Methods, 20 µg protein lysate was loaded to the total samples (B), protein amounts in αHA immunoprecipitation samples (A) represents yields from 190 µg of total protein lysate. Immunoprecipitations were carried out by using 100 µl of 25% slurry PAS beads per reaction which were pre-coated with αHA antibody (used at 1:10) for 1 hour at $+4^0$C and washed two times. For the immunoprecipitation the antibody was diluted 1:60. The reactions were incubated for 4 hours at $+4^0$C. PAS beads were washed three times, one half of them was mixed with 20 µl of water and 7 µl of 5 x LSB and incubated at $+95^0$C for 5 minutes. 50% of each sample was loaded on SDS-PAGE for Western blot analysis. SDS-PAGE gels were blotted to nitrocellulose membrane, blocking solution (5% (w / v) milk powder, 0,05% Tween in PBS) incubation was performed over night, primary antibody incubations were carried out in blocking solution for 1h; αHA was used at 1:1000 and αLexA at 1:5000. Filters were washed three times for 10 min. Secondary antibodies horseradish peroxidase rabbit-α-mouse and goat-α-rabbit were used at 1:2000 for αHA and αLexA, respectively, incubated for 30 minutes. After two 5 min. washes in PBS-0,05% Tween filters were analyzed by ECL method. Exposure times were 2 seconds for αHA and 35 seconds for αLexA western blots. Background designates an unspecific protein band, IP for immunoprecipitation and WB for western blot

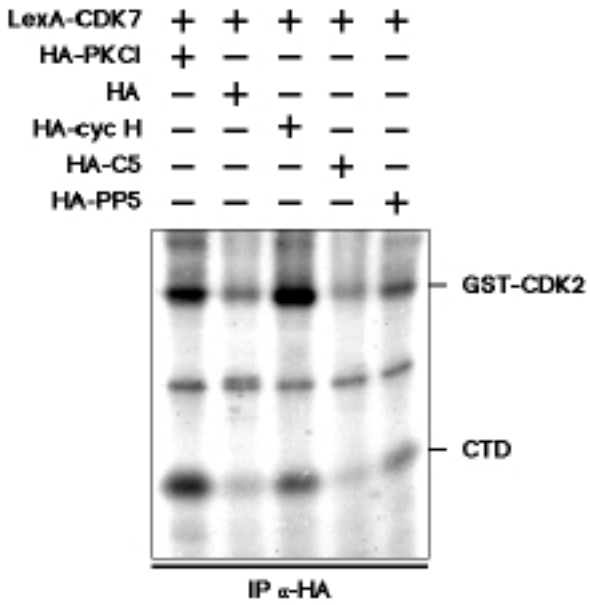

Figure 10. Elevated levels of kinase activity suggesting a presence of LexA-CDK7 co-immunoprecipitated with HA-PKCI. Autoradiography of kinase activity assay of αHA co-immunoprecipitated LexA-CDK7 from yeast cell extracts using GST-CDK2 and RNApolII CTD peptide substrates for phosphate incorporation from γ-^{32}P-ATP. Immunoprecipitations were carried out as described in Materials and Methods, kinase assay incubations were performed in 20 mM Tris pH 7,5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT buffer using 20 µCi γ-^{32}P-ATP per reaction, incubated at +30^{0}C for 30 minutes. 7 µl of 5 x LSB was added to the samples, they were incubated at +95^{0}C for 5 minutes, and 50% of them was loaded on SDS-PAGE gels. Samples represent kinase activities of αHA immunoprecipitations from 190 µg of total protein lysate. Exposure was carried out at -20^{0}C for 48 hours from the SDS-PAGE gel. IP for immunoprecipitation.

Detection of the interaction through co-precipitation from yeast cell extracts proved to be difficult. Repetition of positive co-immunoprecipitation result observed in an experiment lacking proper negative controls (data not shown) has so far been unsuccessful. With the slightly encouraging result from kinase activity assay the CDK7-PKCI complex was next studied in mammalian cells.

Mammalian cell extracts

The interaction of myc-CDK7 with HA-PKCI was studied from the co-transfection samples by αHA and αmyc immunoprecipitations. From αmyc immunoblot, myc-CDK7 was not detected to co-immunoprecipitate with HA-PKCI (Figure 11A, last lane). Myc-CDK7 can be detected from immunoprecipitation sample of HA-cyclin H which was used as a positive control (Figure 11A, next to last lane). Accordingly, in αHA western blot, HA-PKCI was not detected to co-immunoprecipitate with myc-CDK7 PKCI (Figure 11A, fourth lane). Positive control HA-cyclin H was detected from the αmyc immunoprecipitation (Figure 11A, third lane). In the total samples, PKCI cannot be detected (Figure 11B). The insufficient expression level of PKCI may explain this result.

The CDK7 kinase activity of these immunoprecipitated samples was examined using a kinase assay. All αmyc IP samples showed kinase activity towards GST-CDK2, and co-transfection with cyclin H increased the activity of CDK7 (Figure 12). No kinase activity was detected in the αHA immunoprecipitation of HA-PKCI, (Figure 12, last lane). With HA-cyclin H, kinase activity towards GST-CDK2 was immunoprecipitated.

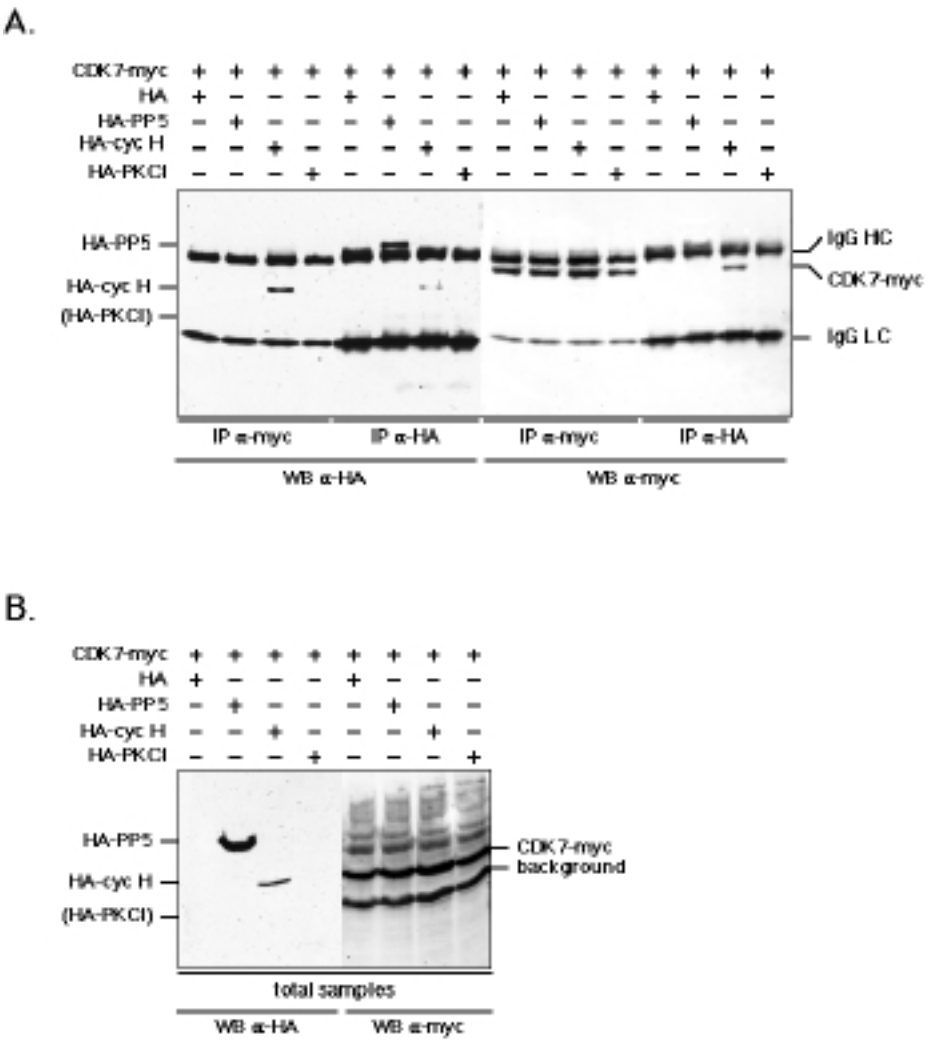

Figure 11. Insufficient expression level of PKCI demolishes the detection of CDK7-PKCI complex of transfected proteins by immunoprecipitation method. Western blot analysis of αHA and αmyc immunoprecipitated proteins (A) and total lysate samples (B) from transfected mammalian cell extracts. Transfections and lysis were carried out as described in Materials and Methods. 30 µg protein lysate was loaded to the total samples (B), protein amounts in αHA and αmyc immunoprecipitation samples (A) represents yields from 225 µg of total protein lysate. Immunoprecipitations with αHA and αmyc antibodies were performed by using them at 1:600 with bridging antibody rabbit-α-mouse IgG at 1:600. 100 µl of 25% PAS beads was added for the 2 hours of incubation at $+4^0$C. PAS beads were washed four times. Kinase activity assay was performed for the samples as described in Materials and Methods, 7 µl of 5 x LSB was added and samples were incubated at $+95^0$C for 5 minutes, half of the samples were loaded on SDS-PAGE gels. Gels were blotted to nitrocellulose membrane, exposures for kinase activity were taken prior to western blot analysis with αHA and αmyc antibodies. Blocking solution (5% (w / v) milk powder, 0,05% Tween in PBS) incubations were performed for 8 hours, primary antibody incubations were carried out in blocking solution; at αHA 1:1000 for 15h and αmyc at 1:2000 over night. Filters were washed three times for 10 min., secondary antibody horseradish peroxidase rabbit-α-mouse was used in incubations of 30 minutes. After two 5 min. washes in PBS-0,05% Tween filters were analyzed by ECL method. Exposure times were 15 seconds for αHA and αmyc western blots. In the absence of HA-PKCI protein, the predicted size of HA-PKCI is shown in brackets. IgG HC for Immunoglobulin G heavy chain and LC for light chain. Background designates an unspecific protein band, IP for immunoprecipitation and WB for western blot.

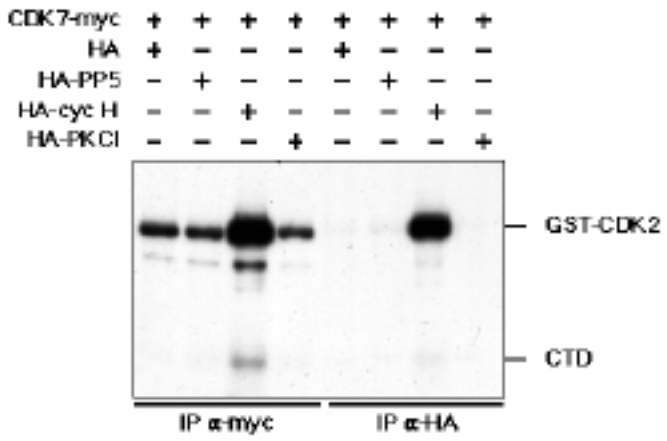

Figure 12. Kinase activity does not co-immunoprecipitate with HA-PKCI. Autoradiography of CDK7 kinase activity assay of αHA and αmyc immunoprecipitated extracts from transfected U2-OS cells using GST-CDK2 and RNApolII CTD peptide substrates for phosphate incorporation from γ-^{32}P-ATP. Immunoprecipitations were carried out as described in Materials and Methods, kinase assay incubations were performed in 20 mM Tris pH 7,5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT buffer using 20 µCi γ-^{32}P -ATP per reaction, incubated at +30^{0}C for 30 minutes. 7 µl of 5 x LSB was added to the samples, incubated at +95^{0}C for 5 minutes, and 50% of them was loaded on SDS-PAGE gels. Western blot to nitrocellulose filter was performed as described in Materials and Methods, exposure from that was carried out at -20^{0}C for 48 hours. Samples represent kinase activities of immunoprecipitations from 225 µg of total protein lysate. IP for immunoprecipitation.

In vitro translated proteins

Expression level and thereby detection is usually not a matter of concern in experiments done with *in vitro* translated proteins because of the high sensitivity granted by this particular method, thus the CDK7-PKCI complex was also studied using *in vitro* translated proteins. Even with this technique, co-immunoprecipitation was not observed between myc-CDK7 and HA-PKCI. HA-PKCI could not be detected from αmyc immunoprecipitation from CDK7-3M/pCIneo cyclin H/pCIneo MAT1/pCIneo and PKCI/pAHC co-expression where as cyclin H and MAT1 were present (Figure 13A, next to last lane). αmyc immunoprecipitation from reaction where HA-PKCI and myc-CDK7 were co-translated did not show HA-PKCI (Figure 13A, last lane). Immunoprecipitation with αHA from reaction of PKCI/pAHC alone (Figure 13A, third lane), reveals the false origin of myc-CDK7 sized band observed in αHA immunoprecipitation samples (Figure 13A, first two lanes). The MAT1 and the faint cyclin H sized bands cannot be interpreted to be MAT1 and cyclin H immunoprecipitated with PKCI on basis of this

and other evidence (Figure 13A, last lane and unpublished results). A clearly detectable protein band (indicated by an arrow in Figure 13A) is most probably a degradation product of one of the expressed proteins. In Figure 13B are the total samples of each reaction.

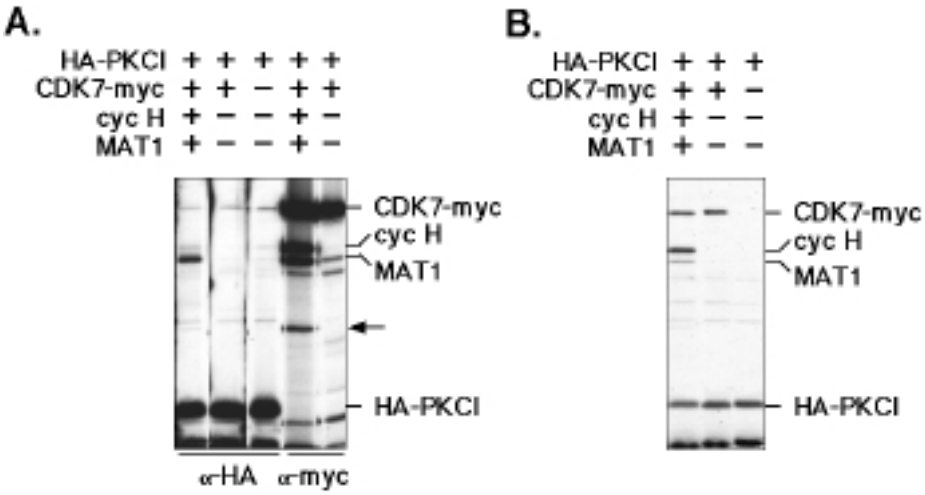

Figure 13. Detection of CDK7-PKCI complex was unsuccessful using *in vitro* translated proteins. Autoradiographies of *in vitro* transcribed and translated αHA and αmyc immunoprecipitated proteins (A) and total reaction samples (B). In vitro translation was performed as described in Materials and Methods and references therein. Half of each reaction was used for αHA (at 1:270) and αmyc (at 1:270) immunoprecipitations with bridging rabbit-α-mouse IgG (at 1:270) and 100 µl 25% PAS bead slurry. Incubation was done at $+4^0$C for 4 hours after which the beads were washed three times, 30 µl water was added, samples incubated at $+95^0$C for 5 minutes and 50% of the volume was run on SDS-PAGE. Total samples (B) contain 4% (2 µl) of the original reaction volume, immunoprecipitation samples (A) represent the yields from 20% (10 µl) of the reaction volume. Detection was done from fixed, amplified and dried SDS-PAGE by ^{35}S autoradiography. Exposure times were 7 days for (A) and 19 hours for (B), at -70^0C. Black arrow points to the putative degradation product of one of the expressed proteins.

Influence of PKCI on CDK7's kinase activity

Regardless of the inability to show the interaction using an immunoprecipitation methodology, we further tested whether the observed growth inhibition could in part be due to an effect of PKCI on CDK7 function as measured by its kinase activity.

The kinase activity of endogenous CDK7 immunoprecipitated from U2-OS cells was studied in the presence of GST-PKCI. From the Figure 14 it can be seen that the activity of CDK7 did not change from the basal level in the presence of PKCI. (Compare the three first lanes to the basal level (-)). We did however observe an interesting, unidentified protein band (indicated

by an arrow in Figure 14) when GST-PP5 was added to the kinase activity incubation. Also note a faint band of similar size on the fourth lane.

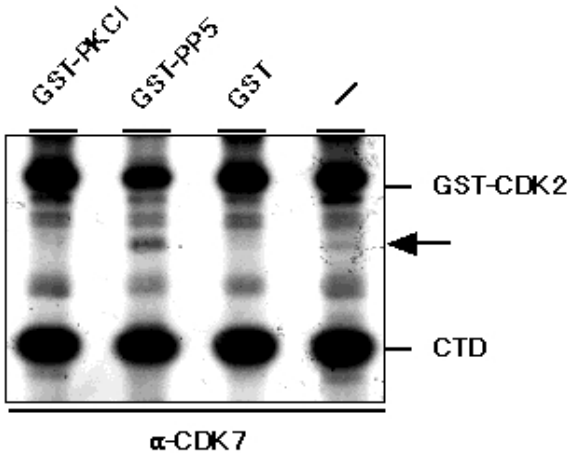

Figure 14. Kinase activity of CDK7 remained unchanged in the presence of GST-PKCI. Autoradiograph from kinase activity assay of immunoprecipitated endogenous CDK7 in the presence of GST-PKCI, GST-PP5, GST compared to the basal level (-). Lysis of the cells was performed as described in Materials and Methods. 1700 µg total protein was used for immunoprecipitation with αCDK7 antibody (at 1:500) and 320 µl 25% PAS bead slurry. Incubation was carried out for 2 hours at $+4^0$C. Beads were washed three times with lysis buffer, once with kinase incubation buffer and divided into four. Kinase activity assays were performed in 20 mM Tris pH 7,5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT buffer using 20 µCi γ-^{32}P-ATP per reaction using GST-CDK2 and RNApolII CTD peptide substrates for phosphate incorporation from γ-^{32}P-ATP. Bacterially produced GST proteins (1 µg) were added and the incubations were carried out at $+30^0$C for 30 minutes. 10 µl of 5 x LSB was added, samples were incubated at $+95^0$C for 5 minutes and half of the samples were loaded on SDS-PAGE gel. Each sample represent immunoprecipitations from 215 µg total protein. Exposures were taken directly from the gel, here for 1 hour 10 minutes at -20^0C. Black arrow points to the interesting, unidentified protein band observed on lanes 2 and 4.

Subcellular localization of CDK7 and PKCI

A requisite for the physiological interaction between any two proteins is that they be localized in the same cellular compartment at the same time. CDK7 is known to localize in the nucleus (Bartkova et al., 1996) and therefore it was examined whether PKCI co-localizes with CDK7 in the nucleus.

After immunofluorescence labeling of transfected HA-PKCI, its subcellular localization was visualized (Figure 15). On basis of our studies PKCI was observed to localize both in the nucleus and in the cytoplasm. This can be seen both in U2-OS (Figure 15A) and COS (Figure 15C) cells. Figures 15B and D, respectively, are β-Gal labelings of the same cells. Thus the

localization of PKCI makes the interaction with CDK7 possible according to our studies.

Figure 15. PKCI localizes both to the nucleus and cytoplasm. Immunofluorescence labeling of transfected HA-PKCI in U2-OS (A) and COS cells (C) and the labeling of β-Gal transfection control in U2-OS (B) and COS cells (D). Cells were transfected and fixed as described in Materials and Methods. Immunolabeling with the primary antibodies αHA mouse (at 1:50) and αβ-Gal rabbit (at 1:100) was carried out in 10% FBS for 30 min. After rinsing the coverslips three times, cells were incubated with fluorescently labeled secondary antibodies FITC-α-mouse (at 1:200) and Texas Red-α-rabbit (at 1:50), respectively, in 10% FBS for 30 min. After careful washing with water the coverslips were mounted in Mowiol. Analysis was done with an Axiophot fluorescence microscope.

STUDYING THE INTERACTION FROM THE SIDE OF PKCI

Proteins interacting with PKCI

To further study the potential role of PKCI in the cell, the yeast two-hybrid was used to identify proteins interacting with PKCI.

As a result from the PKCI two-hybrid screening a total of approximately 76% (60/79) of positive clones were PKCI itself. Most of the positive clones were identified to be PKCI by PCR analysis. Sequenced clones, 95, 96, 97, 98 and 99 were found out to be PKCI cDNA clones. Numbers 95, 96 and 97 were the same cDNA clone starting from the first amino acid of PKCI peptide. Number 98 starts 72 nucleotides upstream of the first amino acid and clone number 99 starts from the 26th amino acid of PKCI. Clone 25

(25a and 25b) was identified to be PKCI by it's recognizable digestion pattern (data not shown). As PKCI occurs in nature as a dimer, the result was expected and will be discussed later.

Clone 3b was identified to be human calmodulin from the nineteenth amino acid on to the carboxy terminus and beyond. In a retransformation experiment to PKCI/EG202 Boy1 human calmodulin cDNA failed to activate the marker genes *LEU2* and *lacZ* indicating that it was a false positive. The other sequenced clones (4b, 10b, 13a, 40a and 40b) encoded short, few amino acid peptides or vector sequence.

Function of PKCI

Continuing on the path of investigations from the side of PKCI, we determined to look for an enzymatic activity of PKCI which might offer further possibilities to elucidate the influence that PKCI and CDK7 have on each other. Knowing that the human relative FHIT possesses hydrolytic activity towards several dinucleotide phosphates a similar assay was performed for PKCI with multiple substrates.

In these experiments hydrolase activity of bacterially produced GST-PKCI towards ADP was detected. During the experimental reaction time, PKCI was shown to cleave 33% of the ADP available into adenosine monophosphate (AMP) and inorganic phosphate (P_i). GST-PKCI did not show hydrolase activity towards any of the other substrates used.

GST-FHIT was used as a control hydrolase and it was shown to cleave Ap_3A to completion, 50% of Ap_5A, 14% of Ap_2A and 11% of ADP in the given reaction time. Table 7 summarizes the results. The NMR spectra of the reactions follows in Appendixes (Appendixes 1-23). The area of each peak is indicated in the spectrum (Appendixes 1-23). For example, the percentage of ADP cleavage for GST-PKCI (Appendix 2) as follows: [0,17 (AMP) + 0,16 (P_i)] : 2 = 0,165 representing the cleaving products. Similarly [0.33 + 0,34 (ADP)] : 2 = 0,335 representing uncleaved ADP. As 0,165 + 0,335 = 0,500, we get (0,165 : 0,500) x 100% = 33% for the amount of cleaved ADP. Other percentages have been calculated accordingly.

Table 7. Hydrolase activities of GST-PKCI and GST-FHIT.

SUBSTRATE	GST-PKCI	appendix	GST-FHIT	appendix
ADP	33%#	2	11%	3
ATP	0%	5	0%	6
dATP	0%	8	0%	9
dGTP	0%	11	0%	12
Ap_2A	0%	13	14%	14
Ap_3A	0%	16	100%	17
Ap_4A	0%	19		
Ap_5A	0%	22	50%	23

[#] [0,17 (AMP) + 0,16 (P_i)] : 2 = 0,165. [0.33 + 0,34 (ADP)] : 2 = 0,335.
0,165 + 0,335 = 0,500, (0,165 : 0,500) x 100% = 33%. Other percentages accordingly.

DISCUSSION

PKCI INTERACTING PROTEINS

The preliminary finding of a specific PKCI-CDK7 interaction in yeast two-hybrid (Mäkelä et al., 1994 and Mäkelä, T.P., unpublished data) could not be conclusively verified in this study by co-immunoprecipitation techniques using yeast and mammalian cell extracts and *in vitro* translated protein extracts. This suggests that some additional component required for mediation of the interaction could be needed and present in the yeast cells. Another reason may lie in the sensitivity difference of these two methods. In addition, the variability of the results and the variations in expression levels of transfected proteins could have been factors impairing the detection. This leaves us with questions about the nature of the interaction and the physiological strength of it. Whether these results shake the foundations of the specificity of PKCI-CDK7 interaction or not will be discussed next in the light of other reports.

Two other reports have been published detailing PKCI interactions in the yeast two-hybrid system. Ataxia telangiectasia (AT) is a human genetic disorder characterized by immunodeficiency, neurological abnormalities, extreme radiosensitivity, cell cycle anomalities and predisposition to lymphoid and other malignancies (Meyn, 1995; Shiloh, 1995). A true single AT gene, *ATM* has been identified (Savitsky et al., 1995). In 1995 human PKCI (C-terminal 80 amino acids) was observed to interact with a bait of ataxia-telangiectasia (AT) group D complementing gene, ATDC (Brzoska et al., 1995). ATDC has been shown to rescue the radiosensitivity phenotype of AT group D, indicating that it functions in a pathway that is defective in AT (Brzoska et al., 1995). This result could implicate a role for PKCI in stress signaling pathways which may involve the hydrolysis of adenosine phosphate compounds which have been suggested to be involved in stress signaling pathways.

Another piece of evidence supporting this hypothesis is the fact that CDK7 is a part of TFIIH which performs nucleotide excision repair function. This kind of DNA damage is caused by UV irradiation and other mechanisms. Also, the kinase activity of TFIIH, which is constant during the cell cycle, is

reduced after UV light irradiation (Adamczewski et al., 1996). Thus CDK7 may also be involved in irradiation sensitivity pathways.

PKCI has also been reported to interact in yeast two-hybrid with the regulatory domain (N-terminal 317 amino acids) of protein kinase C-β isoform (Lima et al., 1996). A single clone was identified to be a PKCI cDNA lacking the nucleotides encoding the amino terminal 31 amino acids. There is no detailed information available about the experiment, and its physiological relevance remains to be seen.

Both of these above mentioned screenings reported only a single PKCI clone as oppose to the CDK7 screening which yielded 15 PKCI clones. This may reflect the strength of the interaction, although it is very difficult to compare individual screenings with each other.

One question that must be asked is whether PKCI could be one of those proteins that tend to interact unspecifically in yeast two-hybrid screening with numerous baits. This is not supported by the fact that PKCI has not occurred in any other screening performed by the Haartman Institute Yeast Two-hybrid Core Facility, nor has it been reported on Erica Golemis's collection of typical false positives (http://www.fccc.edu:80/research/labs/golemis/InteractionTrapInWork.html). Is there a common feature then that can be found between CDK7, ATDC and PKC-β? The fact that two of these proteins are kinases (CDK7 and PKC-β) does point to the direction of nucleotide phosphate compounds. ATDC possesses sequence similarity to a RING finger domain.

The results of the PKCI two-hybrid screening with vast number of PKCI clones reflects an extremely strong preference of interaction with itself to all other proteins. The fact that PKCI forms homodimers in nature is an obvious reason for this result but one could imagine that preformation of PKCI bait dimers in yeast cells could lead to its ability to interact with other proteins besides itself. It is also expected that PKCI prey dimers form in the yeast cells. The abundance of PKCI mRNA in the HeLa cDNA library has not been investigated. Sixty clones out of 3.7×10^6 screened makes 0,0016% which is not an unreasonably high frequency.

FUNCTION OF PKCI

The growth inhibitory effect of over-expressed PKCI in yeast observed in this study has not been reported elsewhere. Under these careful experimental conditions the results were clear and statistically significant. Surprisingly, it has been reported that the *inactivation* of cyanobacterial PKCI open reading frame results in slow growth phenotype for the organism (Bustos et al., 1990), the effect thus being counter intuitive to our results. No further reports have been published of that observation.

The ADP hydrolase activity shown here and also reported by Lima et al. during this study (Lima et al., 1997) is an important hall mark in the search for PKCI's *in vivo* function. It is very likely that nucleotide phosphates are the substrates for the non-FHIT -like HIT proteins; it was recently reported that a third family member, the rabbit HIT homologue also shows an affinity for interacting with purine monophosphate nucleotides (Brenner et al., 1997). Stress signaling pathways are a good candidate for PKCI and family members to function in, possibly as phosphotransferases of some kind.

CONCLUSIONS AND FUTURE DIRECTIONS

As a conclusion, the interaction between PKCI and CDK7 could not be confirmed by other methods besides yeast two-hybrid. The growth inhibitory effect of over-expressed PKCI in yeast is an interesting novel finding, the mechanisms of which remain to be part of future investigations which could be aimed at studying the role of PKCI in stress signaling. The enzymatic activity of human PKCI; whether it is the *in vivo* physiological ADP hydrolase or not, will be resolved in the near future by studies of ours and others. *S. cerevisiae* PKCI knock-out cells will offer a tool for further elucidation. The main focus of our future studies will be the generation of mice with targeted PKCI alleles offering powerful means to reveal the function of PKCI through observing phenotypes and through detailed analysis of these mice harboring wild type, hypomorphic or null alleles.

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