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ORIGINAL PAPER

Cdc37 engages in stable, S14A mutation-reinforced association with the most atypical member of the yeast kinome, Cdk-activating kinase (Cak1)

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Abstract In most eukaryotes, Cdc37 is an essential chaperone, transiently associating with newly synthesised protein kinases in order to promote their stabilisation and activation. To determine whether the yeast Cdc37 participates in any stable protein interactions in vivo, genomic two-hybrid screens were conducted using baits that are functional as they preserve the integrity of the conserved N-terminal region of Cdc37, namely a Cdc37-Gal4 DNA binding domain (BD) fusion in both its wild type and its S14 nonphosphorylatable (Cdc37(S14A)) mutant forms. While this failed to identify the protein kinases previously identified as Cdc37 interactors in pull-down experiments, it did reveal Cdc37 engaging in a stable association with the most atypical member of the yeast kinome, cyclin-dependent kinase (Cdk1)-activating kinase (Cak1). Phosphorylation of the conserved S14 of Cdc37 is normally crucial for the interaction with, and stabilisation of, those protein kinase targets of Cdc37, Cak1 is unusual in that the lack of this Cdc37 S14 phosphorylation both reinforces Cak1:Cdc37 interaction and does not compromise Cak1 expression in vivo. Thus, this is the first Cdc37 client kinase found to be excluded from S14 phosphorylation-dependent interaction. The unusual stability of this Cak1:Cdc37 association may partly reflect unique structural features of the fungal Cak1.

Keywords Yeast two-hybrid · Cdc37 · Molecular chaperone · Cak1

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Introduction

In eukaryotic organisms, the maturation and stabilisation of nascent protein kinase molecules frequently require the action of Cdc37(p50), a molecular chaperone that often subsequently targets these maturing protein kinase molecules on to Hsp90 (Pearl 2005; Mandal et al. 2007). While Cdc37 fulfils an essential function in diverse species from yeast to man, this important molecular chaperone would appear to be lacking in certain eukaryotic phyla (Johnson and Brown 2009). Notably, Cdc37 seems to be absent in the Apicomplexan phylum of protozoan pathogens, possibly a reflection of the protein kinases in these species differing, both structurally and mechanistically, from the protein kinases of their hosts (Talevich et al. 2011).

Since its original discovery of as a component of the v-Src-Hsp90 complex (Brugge et al. 1981), Cdc37(p50) has been found associated with a wide spectrum of protein kinases. A few non-kinase proteins have also been found to bind Cdc37 (Rao et al. 2001; Millson et al. 2004a; Krogan et al. 2006), though the precise function of Cdc37 in the biology of these

non-kinase clients is still poorly understood. While Cdc37 interaction serves to stabilise many newly synthesised protein kinases (Pearl 2005; Mandal et al. 2007), we remain largely ignorant of how such nascent protein kinases interact with Cdc37 at the structural level, especially how the N-terminus of Cdc37—the Cdc37 region highly conserved in evolution—might assist presentation protein kinases to the Hsp90 chaperone machine. A crystal structure has though revealed how the middle region of Cdc37 interacts with the N-terminal domain of Hsp90, thereby transiently arresting the Hsp90 chaperone cycle (Siligardi et al. 2002; Roe et al. 2004).

Important for Cdc37 function is a sequential casein kinase 2 (CK2)-catalysed phosphorylation, then Ppt1(PP5)-catalysed dephosphorylation, of evolutionarily conserved serines at the conserved N-terminal region of Cdc37 (Bandhakavi et al. 2003; Shao et al. 2003; Miyata and Nishida 2004; Vaughan et al. 2008). Yeast Cdc37 becomes phosphorylated on both Ser14 and Ser17, while in the case of the mammalian Cdc37(p50), only Ser13 (equivalent to Ser14 in the yeast Cdc37) is modified in this manner (p50 has a glutamic acid, a possible phosphomimetic residue, at the position equivalent to Ser17 in the yeast Cdc37). A yeast mutant with a nonphosphorylatable S14A mutant Cdc37 (*cdc37(S14A)*) is temperature sensitive, exhibiting poor growth even at moderate temperatures (Bandhakavi et al. 2003). It has ~70 % of its kinome destabilised, revealing that S14 phosphorylation of Cdc37 is important for protecting nascent kinase chains against degradation and maintaining appropriate in vivo levels of protein kinases (Mandal et al. 2007). We and others have shown that this Ser14 phosphorylation is essential for the functionality of several stress-responsive, mitogen-activated protein kinase (MAPK) pathways in yeast, the *cdc37(S14A)* mutation causing loss of Cdc37 interaction with key signalling components of these pathways, notably the kinases Ste11, Kss1, Hog1 and Slt2 (Abbas-Terki et al. 2000; Hawle et al. 2007; Yang et al. 2006). Here, we describe our discovery, through yeast two-hybrid (Y2H) screening, of a Cdc37 client kinase whose stable association with Cdc37 and in vivo expression level appears not to depend upon this Ser14 phosphorylation of Cdc37.

Material and methods

Strains and growth conditions Yeast strains used in this study are listed in Table 1. The *CAK1* coding sequences of DH211-5 were tagged at their C-terminus with the *myc* epitope using the *13myc-TRP1* cassette system (Longtine et al. 1998). Except for the two-hybrid screen (see below), yeast cells were routinely grown at 24 °C in either yeast peptone dextrose (YPD) or synthetic yeast nitrogen base (YNB) medium supplemented with the required amino acids. Analysis of the rescue of osmosensitivity by Cdc37 constructs was as previously described (Yang et al. 2006; Vaughan et al. 2008).

Yeast two-hybrid (Y2H) analysis Y2H bait fusions comprising wild-type and S14A mutant forms of Cdc37 fused at their C-terminus to the Gal4 binding domain (BD) were constructed as previously described (Millson et al. 2003). Briefly, the wild-type *CDC37* gene from plasmid Ycplac111-CDC37 and the mutant *cdc37-S14A* allele present in plasmid Ycplac111-*cdc37-S14A* (Hawle et al. 2007) were used as a template in two-sequential polymerase chain reactions (PCRs). The first PCR used primers GCTTGAAGCAAGCC TCGATGGCCATTGATTACTCTAAGTGGG and CAGTAG CTTCATCTTTCGGTCAACAGTGTCGGCAGTATGT. The second PCR used the product of this initial PCR as a template and previously described universal primers homologous to regions flanking the *NruI* site of plasmid pBDC (Millson et al. 2003). Next, strain PJ694-α was transformed with the product of this second PCR together with *NruI*-digested pBDC. Transformants were selected for growth on YNB agar plates (0.67 % yeast nitrogen base and 2 % glucose) supplemented with all amino acids except tryptophan. Expression of the correct fusion protein was confirmed by western blot analysis using an anti-Gal4 BD antibody (Clontech). Cdc37 baits in PJ694-α were subsequently shown to be not self-activating by plating on selective medium lacking tryptophan and histidine but containing increasing concentrations (0–16 mM) of 3-amino-1,2,4-triazole (3-AT).

Two clones of each bait were mated to a 16-plate, 384-well format array of 6,000 yeast Gal4 activation domain (AD)-protein fusions from an earlier study (Uetz et al. 2000) using the 384-pin replicator of a Biomek 2000 Laboratory Automation Workstation (Beckman) and grown for 2 days at 30 °C. After mating, diploids were selected by plating on selective medium lacking leucine and tryptophan and incubated for 2 days at 30 °C. To screen for protein–protein interactions, the selected diploids were then transferred to selective medium lacking leucine, tryptophan and histidine but containing 4 mM 3-AT. Growth on these plates was monitored after 4, 8 and 16 days at 30 °C. Measurements of the β-galactosidase activity resulting from the interaction-responsive *GAL7* promoter-regulated *LacZ* gene in PJ694a/α (James et al. 1996) were as previously described and are expressed relative to that of the control diploid containing the empty pBDC and the plasmid for the AD fusion of interest (Millson et al. 2003, 2005b).

Cak1 and Cdc37 overexpression in yeast GAL1 promoter-driven overexpression of an N-terminally hexahistidine (His_6) tagged Cak1 used two vector systems: (1) a His_6-*CAK1* gene insert in the *URA3* vector pYES2 (pYES2-His_6-CAK1) (for data in Fig. 2d) and (ii) a His_6-*TEV-CAK1* gene in the *HIS3/CDC28* vector pMH940 (Geymonat et al. 2007) (for data in Fig. 4). For the latter, strain MGY140 (Table 1) was modified so as to give *GAL1* promoter-driven dual overexpressions of both His_6-Cak1 and a Cdc37(S14A)-precision-Streptag fusion protein (the Streptag introduced at level of PCR primer). The latter expression used a version of the

Table 1 Yeast strains

Strain	Genotype	Reference
82a	W303 1A *hsc82::LEU2 hsp82::LEU2 HSP82::HIS3*	Yang et al. (2006)
hsp90-G81S	W303 1A *hsc82::LEU2 hsp82::LEU2 hsp82,G81S::HIS3*	Yang et al. (2006)
TM141	MAT-a *leu2 ura3 trp1 his3*	Hawle et al. (2007)
DH211 CAK1-myc[a]	TM141 *cdc37::HIS3* [Ycplac111 *CDC37-HA*] *CAK1-myc-TRP1*	This study
DH212 CAK1-myc[a]	TM141 *cdc37::HIS3* [Ycplac111 *cdc37-S14A-HA*] *CAK1-myc-TRP1*	This study
DH213 CAK1-myc[a]	TM141 *cdc37::HIS3* [Ycplac111 *cdc37-S14E-HA*] *CAK1-myc-TRP1*	This study
DH214 CAK1-myc[a]	TM141 *cdc37::HIS3* [Ycplac111 *cdc37-S17A-HA*] *CAK1-myc-TRP1*	This study
DH215 CAK1-myc[a]	TM141 *cdc37::HIS3* [Ycplac111 *cdc37-S17E-HA*] *CAK1-myc-TRP1*	This study
XX201	TM141 *cdc37::HIS3* [YCplac33 *CDC37-GFP*]	Yang et al. (2006)
XX300	Hsp82-G81S [YEplac195 *CDC37*]	Yang et al. (2006)
XX301	Hsp82-G81S [YEplac195 N-term *GFP-CDC37*]	This study
XX302	Hsp82-G81S [YEplac195 N-term *GST-CDC37*]	This study
PJ694-a	MAT-A *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::Gal1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al. (1996)
PJ694-α	MATα *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::Gal1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al. (1996)
PH600	PJ694-α [pBDC *CDC37-BD*]	This study
PH601	PJ694-α [pBDC *cdc37(S14A)-BD*]	This study
PH602	TM141 *cdc37::HIS3* [pBDC *CDC37-BD*]	This study
PH603	TM141 *cdc37::HIS3* [pBDC *cdc37(S14A)-BD*]	This study
MGY140	*MATα ura3-1 trp1-289 his3 leu2 lys2Δ0 mob1::kanR cdc28::LEU2 pep4::LYS2* [*pURA3-MOB1 CDC28*].	Geymonat et al. (2007)

[a] Strains DH211-5 from an earlier study (Hawle et al. 2007) here modified to express a myc-epitope tagged Cak1

TRP1/MOB1 vector pMH903 (Geymonat et al. 2007) modified by site-directed mutagenesis so as to introduce an Nde1 site at the start codon of the GST gene (TTCATG➔CATATG). By Nde1 cleavage of this vector (pMH903-Nde1), homologous recombination with PCR-generated genes is able to give vectors for *GAL1* promoter-driven overexpression of protein fusions with a C-terminal tag (in this case, a Cdc37(S14A)-precision-Streptag fusion). Sequences of the PCR primers used here are available on request.

Protein analysis and detection Protein extracts were prepared, and western blots analysed with anti-His, anti-myc, anti-PSTAIRE, anti-actin and anti-GAPDH antisera as previously described (Millson et al. 2005a; Mollapour and Piper 2007; Mandal et al. 2007).

Results

A Cdc37 with a C-terminal BD extension is functional in yeast

Amongst the Cdc37 proteins of distantly related species, it is only the 20–30 amino acids at the extreme N-terminus of Cdc37 that are highly conserved in sequence (MacLean and Picard 2003), indicating that this N-terminal region is important for the functionality of Cdc37. Consistent with this, we have noted that, while Cdc37 overexpression will normally rescue the osmosensitive growth of the *hsp90-G81S* mutant (Yang et al. 2006), overexpressions of N-terminally green fluorescent protein (GFP)- or glutathione transferase (GST)-tagged forms of Cdc37 cannot provide any rescue of this mutant in the presence of 2 M sorbitol (Fig. 1a). Contrasting with this are the experiences of placing sequences at the C-terminus of Cdc37. Cdc37 has been shown to be functional when tagged at the C-terminus with either GFP, the HA epitope (Hawle et al. 2007) or the tandem affinity tag (Puig et al. 2001).

A number of potential interactors of yeast Cdc37 have already been identified in experiments involving immunoprecipitation (Hawle et al. 2007; Abbas-Terki et al. 2000), yeast two-hybrid (Y2H) (Lamphere et al. 1997; Mort-Bontemps-Soret et al. 2002; Millson et al. 2004b) or the mass spectrometry of protein complexes (Gavin et al. 2002; Krogan et al. 2006). We noted that these earlier Y2H screens had invariably used “bait” or “prey” fusions where the BD or AD domain of Gal4 is positioned at the N-terminus of Cdc37. Concerned that these might not have been functional fusions, we constructed (by homologous recombination in strain PJ694-α; see Material and Methods section) vectors

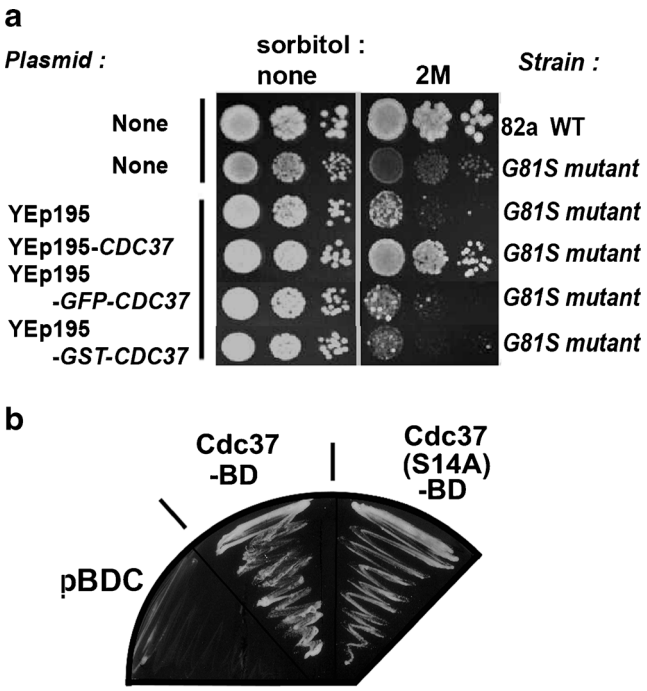

Fig. 1 **a** Overexpression of an N-terminally GFP- or GST-tagged Cdc37 does not rescue the osmosensitivity of the *hsp90-G81S* mutant. Serial dilutions of untransformed wild-type (82a WT) or osmosensitive *hsp90-G81S* mutant cells, as well as *hsp90-G81S* cells transformed with the empty multicopy vector YEplac195 or YEplac195-based plasmids with genes either native Cdc37; N-terminally GFP- or GST-tagged Cdc37 were pinned on YPD, as well as YPD containing 2 M sorbitol. Plates were photographed after 3 days at 24 °C. **b** Growth (3 days at 30 °C) of Trp+ transformants of strain XX201 on FOA, cells containing either empty pBDC vector, or pBDC containing a Cdc37-BD or Cdc37(S14A)-BD gene insert. No growth is apparent with the empty vector pBDC

for the expression of Y2H "bait" fusions where the BD domain is placed at the C-terminus of either the wild-type or a Cdc37(S14A) mutant Cdc37 (Cdc37-BD and Cdc37(S14A)-BD, respectively). After extraction from the original PJ694-α transformants (PH600/1; Table 1), the *TRP1* vectors bearing these fusion genes were retransformed into a strain (XX201; Table 1) in which the essential Cdc37 function is provided by a *CDC37-GFP* gene carried on a centromeric *URA3* plasmid. Transformants of this XX201 growing on minus tryptophan plates were subsequently streaked onto agar containing 5-fluoroorotic acid (FOA), whereupon only the cells able to lose the original *URA3*-based *CDC37* plasmid should grow. As shown in Fig. 1b, the cells containing the Cdc37-BD and the Cdc37(S14A)-BD fusion vectors were capable of growth in presence of FOA, showing that both Cdc37 and Cdc37(S14A) are still functional with a C-terminal BD extension. As is normal for the *cdc37(S14A)* yeast mutant, the cells expressing Cdc37(S14A)-BD as their sole Cdc37 (PH603; Table 1) were temperature sensitive (not shown).

A genomic Y2H screen for interactors of Cdc37-BD and Cdc37(S14A)-BD

PJ694α cells expressing either the Cdc37-BD or the Cdc37(S14A)-BD bait fusion (strains PH600/1; Table 1) were robotically mated with a previously described array of AD fusions to the 6,000 different proteins of yeast, the latter expressed in cells of the opposite mating type (PJ694a) (Uetz et al. 2000). Diploids—now expressing either the Cdc37(Wt)-BD or the Cdc37(S14A)-BD and an AD-protein fusion—were selected on medium lacking tryptophan and leucine and then transferred to medium without tryptophan, leucine and histidine but containing 4 mM 3-AT. Activity of the interaction-responsive *HIS3* reporter gene was monitored as 3-AT resistant growth over 4, 8 and 16 days incubation at 30 ° C. Figure 2a shows a few strong positives appearing on four sample plates, arrayed diploid cells (now expressing both the AD and the Cdc37(S14A)-BD fusions) grown 16 days in the presence of 4 mM 3-AT. Eliminating the known false positives on these arrays, this small number of interactors (15 interactions with the Cdc37-BD fusion and nine with Cdc37(S14A)-BD) had mostly not been found in the earlier Y2H screens for Cdc37 binding targets (Table 2). Repinning (Fig. 2b) and expression measurements of the interaction-responsive *LacZ* reporter gene in PJ694a/α (Fig. 2c) confirmed the interactions with AD-Cak1, AD-Vps71 and AD-Nmd5, and also that the S14A mutation in the bait was moderately reinforcing the interactions with AD-Cak1 and AD-Vps71 and strongly reinforcing the interaction with AD-Nmd5.

To confirm the Cak1:Cdc37 association, an N-terminally His_6-tagged Cak1 (His_6-Cak1) was overexpressed from the *GAL1* promoter of plasmid pYES2-His_6-CAK1 in DH211 and DH212 (Table 1), strains that possess C-terminally HA-epitope tagged forms of either a wild-type or the *cdc37(S14A)* mutant Cdc37, respectively (Cdc37-HA; Cdc37(S14A)-HA). Immobilised metal affinity chromatography (IMAC) retention of this His_6-Cak1 confirmed the enhancement to Cdc37-HA retained by the His_6-Cak1 with S14A mutation of this Cdc37-HA, consistent with the Y2H data (Fig. 2b–d). We subsequently found this Cak1:Cdc37(S14A) complex to be sufficiently stable as to be isolatable by gel filtration (see below).

Analysis of Cak1 and Cdc37 protein levels in Cdc37 phosphorylation mutants

Our Y2H screen did not detect most of the protein kinases thought to be Cdc37 "clients" in yeast, for the probable reasons outlined in the Discussion section below. Remarkably though it did detect Cdc37(S14A)-BD interacting with at least two protein kinases (Ste20, Cak1) which would appear, on the basis of current evidence, might not require

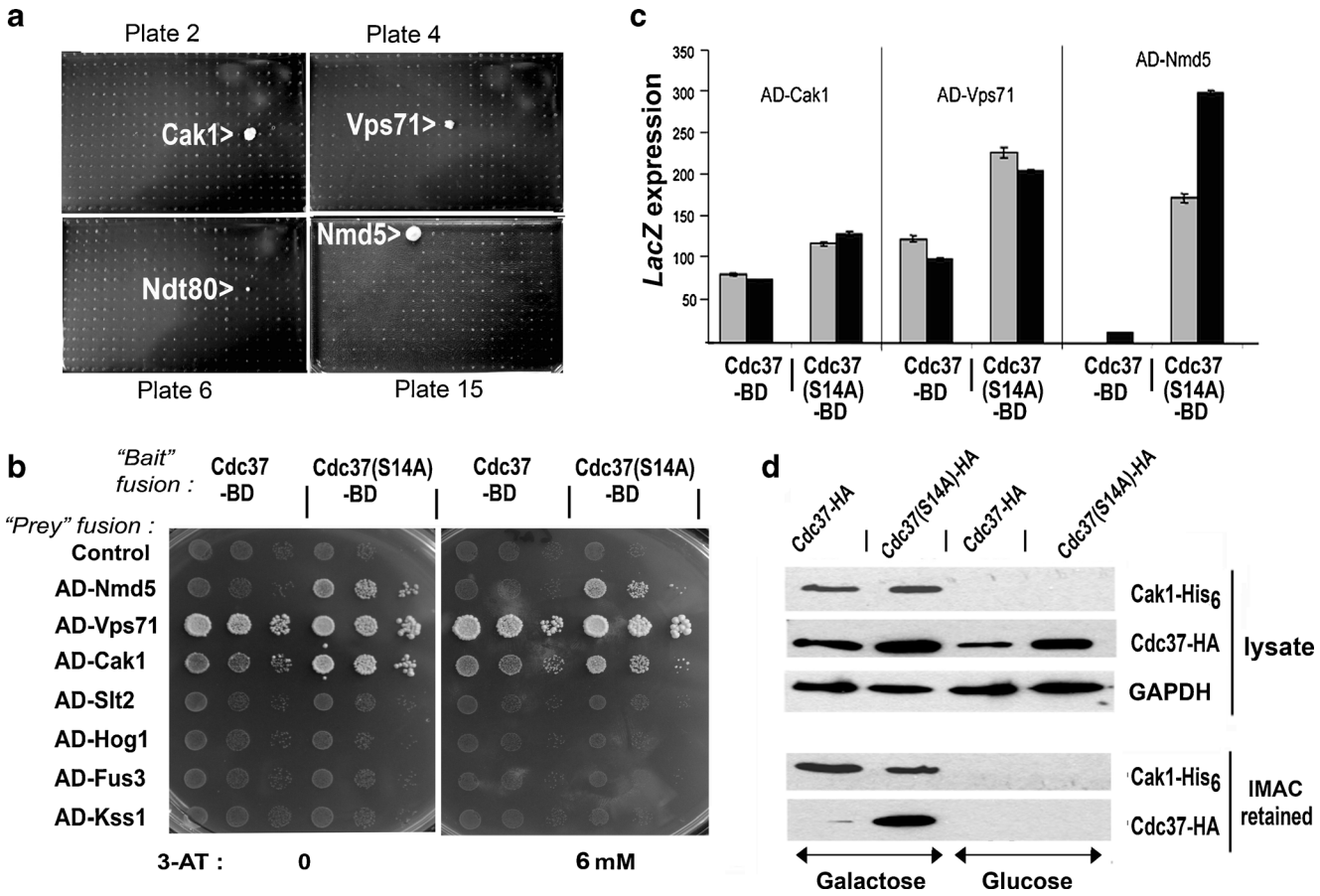

Fig. 2 **a** Four sample plates of the 384-colony format 16-plate library array of PJ694a/α cells containing the Cdc37(S14A)-BD bait. Activation of the interaction-responsive *HIS3* gene is monitored as 3-AT resistant growth (16 days at 30 °C) on SD medium without tryptophan, leucine and histidine containing 4 mM 3-AT; Cdc37(S14A)-BD interactions represented on these plates being: Plate 2: AD-Cak1; Plate 6: AD-Ndt80; Plate 7: AD-Vps71; Plate 15: AD-Nmd5. The colony size is an indicator of interaction strength. **b** Serial dilutions of PJ694a/α cells expressing Cdc37-BD or Cdc37(S14A)-BD baits and the indicated AD fusions after growth (10 days at 30 °C) on this same medium and either zero or 6 mM 3-AT. **c** Measurements of interaction-responsive *LacZ* reporter gene expression in PJ694a/α cells analysed after growth to mid-log phase at 24° (*grey*), or 1 h following a heat shock from 24 to 39 °C (*black*). *LacZ* expression is expressed as percentage increase relative to that of PJ694a/α control cells containing empty pBDC plasmid and the corresponding AD-protein fusion. **d** Total versus IMAC-retained His_6-Cak1, Cdc37-HA and Cdc37(S14A)-HA in extracts from 28 °C glucose and galactose-grown cultures of DH211 and DH212 containing pYES2-His_6-CAK1, a vector for *GAL1* promoter-driven overexpression of His_6-Cak1. Blots were probed with anti-HA, anti-His and (as loading control) anti-GAPDH antisera

Cdc37 phosphorylation for their stability (Table 2). An earlier study indicated that the stability of Ste20 is unaffected by *cdc37(S14A)*, a mutation that destabilises much of the yeast kinome (Mandal et al. 2007). Furthermore, we have found that the *cdc37(S14A)* mutant can efficiently overexpress His_6-Cak1 (Fig. 2d). Though Cak1 may be a Cdc37 "client" in yeast (reduced stability of Cak1 having been reported in the temperature-sensitive *cdc37-1* mutant (Farrell and Morgan 2000)), the eukaryotic Cdc37 chaperone function may not be critical for the proper folding of Cak1 since a GST-Cak1 fusion expressed in *Escherichia coli* is fully active and is able to efficiently phosphorylate Cdk (Song et al. 2001).

Cak1 has not been identified as one of the several protein kinases destabilised in *cdc37(S14A)* yeast cells. So that Cak1 levels could be analysed in this, as well as other Cdc37 phosphorylation mutants, we *myc* epitope tagged the chromosomal *CAK1* coding sequence in strains DH211-5 (Table 1). Analysing protein extracts from these cells (Fig. 3), the levels of Cak1-*myc* in the *cdc37(S14A)* mutant did not appear to be dramatically reduced in contrast to Cdc28 (the latter Cdk, a major phosphorylation target of Cak1, is destabilised by the *cdc37(S14A)* mutation (Mandal et al. 2007)). Levels of Cak1-*myc* were also substantially unaltered in cells expressing the nonphosphorylatable S17A and phosphomimic (S14E and S17E) mutant versions of Cdc37-HA (Fig. 3). This reveals that, while Cdc37 is probably required for the in vivo stability of Cak1 (since Cak1 levels are reduced in the *cdc37-1* mutant (Farrell and Morgan 2000)), such stabilisation is operating independently of the CK2 phosphorylation/Ppt1 dephosphorylation of Cdc37 (Vaughan et al. 2008).

Table 2 Two-hybrid interactors from genomic screen of AD-yeast protein fusions

ORF	Gene	Int[a]
Cdc37-BD bait:		
Heat shock protein		
YJL159W	*HSP150*	2
Protein kinases		
YFL029C	*CAK1*	2[b]
YJL165C	*HAL5*	3[c]
YFL033C	*RIM15*	1
Transcription factor		
YCL055W	*KAR4*	2
YER045C	*ACA1*	3
YGR274C	*TAF1*	2
Vesicle trafficking/vacuolar protein sorting		
YML041C	*VPS71*	3[c]
YBR217W	*ATG12*	1
Nuclear protein import/export		
YGL172W	*NUP49*	1
Chromosome segregation		
YJR089W	*BIR1*	2
Endocytosis		
YOR329C	*SCD5*	2
Enzymes		
YLR405W	*DUS4*	2
YLR134W	*PDC5*	2
Unknown function		
YCL073C		2
YPL107W		1
Cdc27(S14A)-BD bait :		
Protein kinases		
YFL029C	*CAK1*	3
YHL007C	*STE20*	1
Transcription factor		
YGL071W	*RCS1*	1
YHR124W	*NDT80*	1
Vesicle trafficking		
YML041C	*VPS71*	3
Nuclear import		
YJR132W	*NMD5*	3
Flocculation		
YKR102W	*FLO10*	3
Unknown function		
YOL007C	*CSI2*	1
YHL045W		2

[a] Strength of interaction. 1, 2 and 3 indicate 3-AT resistant growth of the cells containing the interacting protein and the Cdc37-BD bait (wild-type or S14A mutant) in the presence of 4 mM 3-AT, determined by colony size

[b, c] Identified as an Cdc37 interactor in earlier Y2H screens ([b] Mort-Bontemps-Soret et al. 2002; [c] Millson et al. 2004a)

While conducting this analysis, we were also intrigued to find that levels of Cdc37-HA are elevated in yeast strains that express, as their sole Cdc37, S14A, S14E and S17A mutant versions of this Cdc37-HA (Figs. 2d and 3). Levels of Cdc37-HA are increased in strains DH212-4 CAK1-myc, relative to the cells expressing either the wild type or S17E mutant Cdc37-HA (strains DH211 CAK1-myc and DH215 CAK1-myc). The underlying mechanisms whereby these mutations in Cdc37 are acting to increase the levels of this chaperone in yeast have yet to be established. It is noteworthy that these three Cdc37 mutations, unlike S17E, also act to render cells highly sensitive to stress and the loss of Hsp90 activity (Bandhakavi et al. 2003; Vaughan et al. 2008).

Purification of Cak1 in complex with Cdc37(S14A)

Cak1 has previously been found to associate with both Cdc37 and Cdc28 in yeast (Lamphere et al. 1997; Mort-Bontemps-Soret et al. 2002; Millson et al. 2004b). In this study, we identified that the presence of the S14A mutation in Cdc37 was enhancing Cak1:Cdc37 association (Fig. 2b–d). To further characterise the protein composition of what appeared to be a relatively stable complex forming in vivo between Cak1 and Cdc37(S14A), we modified a *Saccharomyces cerevisiae* vector autoselection system for recombinant protein expression (Geymonat et al. 2007), so that the cells would simultaneously overexpress both of these yeast proteins (also the native Cdc28 and Mob1) during growth on galactose. Strain MGY140 (Table 1) was engineered (see Material and Methods section) so that it maintained two expression vectors, each with a bidirectional *GAL1* promoter regulating two genes: (1) a pMH940 derivative bearing genes for Cdc28 and His_6-TEV-Cak1 (Cak1 with an N-terminal TEV protease-cleavable His_6 tag) and (2) a pMH903 derivative bearing genes for Mob1 and Cdc37(S14A)-Strep (the latter Cdc37(S14A) with a C-terminal, precision protease-cleavable Strep2 tag).

Following growth on galactose and cell breakage, we isolated the His_6-Cak1 in the cell extract on IMAC resin. This His_6-Cak1 was found to be associated with a substantial amount of Cdc37(S14A)-Strep, but very little Cdc28 and no detectable Hsp90 (Fig. 4). Gel filtration of the IMAC-eluted sample applied to a 16/60HiLoadSuperdex 200 column equilibrated with PBS buffer indicated that it contained a substantial amount of free His_6-Cak1 (yield ~20 mg/l culture), as well as—at lower level—complexes containing His_6-Cak1, Cdc37(S14A) and a little Cdc28 (Fig. 4). Much of it was a complex with an apparent MW of 165 kDa, indicative of a monomer of Cak1 in association with a dimer of Cdc37(S14A).

When this IMAC-eluted protein was subsequently applied to streptavidin agarose resin, there was no selective retention of the His_6-Cak1 (though this resin bound the free Cdc37(S14A)-Strep in cell extracts; not shown). This is an indication that the C-terminal Strep2 tag on the Cdc37(S14A) bound to His_6-Cak1 may be buried in the structure of the His_6-Cak1:Cdc37(S14A)-Strep complex and that for further

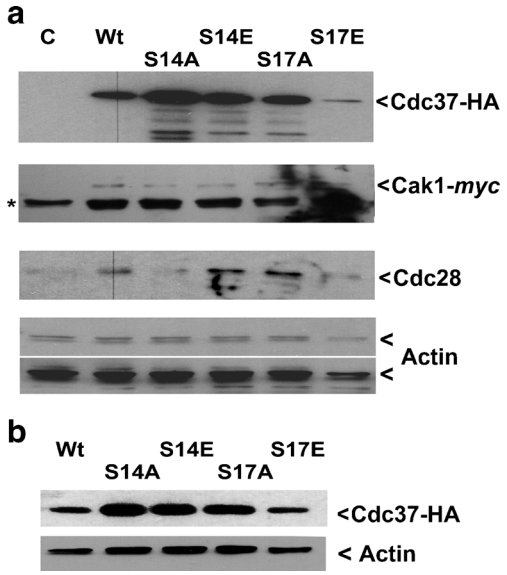

Fig. 3 **a** Analysis of Cak1 and Cdc28 in Cdc37 phosphorylation mutants. Protein extracts from 24 °C cultures of strains DH211-5 CAK1-*myc* were western blotted and probed using anti-HA, anti-myc, PSTAIRE and anti-actin (loading control) antisera. Two exposures of the actin blot are shown; a nonspecific band from the anti-myc probing is indicated with an *asterisk*. The control lane (*C*) is an identically treated extract from strain TM141 in which neither the Cdc37 nor the Cak1 is epitope tagged. **b** An analysis of Cdc37-HA levels in the original DH211-5 (Hawle et al. 2007)

affinity purification of this complex, it would be expeditious to use a longer or larger affinity tag at the C-terminus of Cdc37. Such tagging of Cdc37 with the tandem affinity tag has already been used with success (Puig et al. 2001).

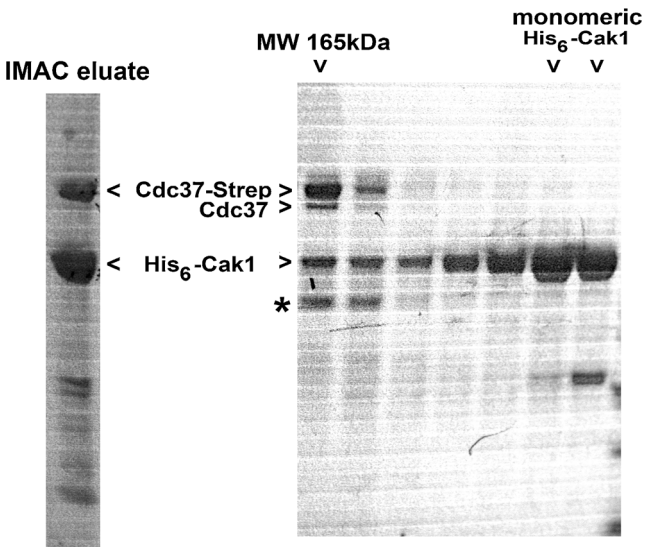

Fig. 4 SDS-PAGE analysis of His_6-TEV-Cak1 (also—at lower level—the His_6-TEV-Cak1/Cdc37-Strep complex) overexpressed in galactose-grown MGY140 cells. A total eluate of the IMAC-retained material (*left*) analysed by gel filtration (*right*), revealing both resin-included material (His_6-TEV-CAK1) and a 165-kDa peak formed substantially of His_6-TEV-Cak1 and Cdc37-Strep (also a little untagged Cdc37 derived from the chromosomal *CDC37* gene of strain MGY140) and Cdc28 (*asterisk*)

Discussion

The novelty of this study is that it has provided the first example of a Cdc37 client kinase whose stable association with Cdc37 and expression level is independent of Cdc37 phosphorylation. Stability of Cak1 is greatly reduced in the temperature-sensitive *cdc37-1* mutant (Farrell and Morgan 2000), yet, despite phosphorylation on the conserved S14 of Cdc37 being crucial for the stability of most protein kinases in yeast (Mandal et al. 2007), the interaction of Cak1 with Cdc37 and Cak1 in vivo expression levels are independent of this phosphorylation (Figs. 2 and 3). This is a protein kinase that appears not to require the CK2 phosphorylation/Ppt1 dephosphorylation of Cdc37 that is so vital for stabilisation of much of the kinome. Not only were we able to overexpress His_6-Cak1 efficiently in the *cdc37(S14A)* mutant (Fig. 2d), but we also found that the levels of Cak1 expression in this mutant were practically normal (Fig. 3). Though not investigated here, novel structural features of fungal Cak1 (see below) may be a factor in these unusual properties of the Cdc37:Cak1 complex.

Earlier Y2H screens for Cdc37 interactors (Lamphere et al. 1997; Mort-Bontemps-Soret et al. 2002; Millson et al. 2004b) all used bait or prey fusions in which the Gal4-BD or AD sequence is positioned at this N-terminus of Cdc37. However, fusion proteins where domains are placed at the N-terminus of Cdc37 appear unable to provide the essential Cdc37 function in yeast (Fig. 1a). Instead, fusions in which the BD is placed at the C-terminus of Cdc37 remain functional (Fig. 1b) and are therefore potentially more suitable for Y2H screening. Using such functional Cdc37-BD and Cdc37(S14A) baits in genomic Y2H screens, this study—as with the earlier Y2H screens—still only identified a few of the 115 protein kinases of yeast as Cdc37 interactors, despite Cdc37 apparently stabilising ~70 % of the yeast kinome (based on the evidence of kinase destabilisation in the *cdc37(S14A)* yeast mutant (Mandal et al. 2007)). Just two protein kinases exhibited a reasonably strong interaction with Cdc37 in our screen (Hal5 and Cak1), with two others displaying weaker interactions (Ste20 and Rim15) (Table 2). Repinning confirmed that Cdc37-BD and Cdc37(S14A)-BD interact with AD-Cak1, but the lack of any detectable interaction with four yeast MAP kinases (Slt2, Hog1, Fus3 and Kss1; Fig. 2b) previously shown to interact with Cdc37 in immunoprecipitation studies (Hawle et al. 2007). The reason that these protein kinases known to require Cdc37 for their stability (Hawle et al. 2007; Mandal et al. 2007) were not detected is probably that Cdc37 interactions with nascent protein kinases are generally highly transient in the environment of the living cell (the Y2H system only detects relatively stable in vivo protein–protein interaction (Bartel and Fields 1997)). Indeed it is conceivable that the isolation of reasonably stable complexes between Cdc37 and protein kinases (Hawle et al. 2007; Vaughan

et al. 2006) may require the dilution of cell contents that occurs upon cell breakage.

The phenotype of *cdc37(S14A)* cells (Bandhakavi et al. 2003) is remarkably similar to that generated with loss of Cak1 activity (*cak1-ts* mutants become elongated at high temperature, most of the budded cells having a short spindle spanning an undivided nucleus at the mother/bud neck, characteristic of G2 arrest (Kaldis et al. 1996)). Indications of limiting Cdc28 activity being a major factor in the *cdc37(S14A)* phenotype come from its rescue by Cdc28 overexpression or with the loss of Swe1 tyrosine kinase (Bandhakavi et al. 2003). Generally, these effects have been attributed to the well-established Cdc37-dependence of Cdc28 itself (Turnbull et al. 2005; Mandal et al. 2007), rather than the loss of Cak1 activity. Consistent with this, we were unable to obtain rescue of either the temperature sensitivity or the elongated cell phenotype of *cdc37(S14A)* cells with either the His_6-Cak1 overexpression from the *GAL1* promoter of plasmid pYES2-His_6-CAK1 or the expression of a Cak1-independent mutant allele of Cdk that overrides the essential requirement for Cak1 (Cdc28-42344; (Cross and Levine 1998))(data not shown). Our analysis did though reveal that the S14A, S14E and S17A mutations in Cdc37 operate to increase the cellular levels of this chaperone (Fig. 3), the first time this effect has been noted. There is currently considerable interest in the factors that might influence the cellular levels of Cdc37, since this is a protein vital for the activity of several of the oncoprotein "drivers" of cancer (Pearl 2005; Smith and Workman 2009).

The unusual strength of the Cdc37(S14A) association with Cak1 (Fig. 2) may reflect the novel structural features of Cak1 protein kinase. In man and in most eukaryotes, Cak is a heterotrimeric complex (Cdk7-cyclin H-Mat1), its catalytic subunit (Cdk7) being activated by either the phosphorylation of its own activating threonine (Thr-170 in human Cdk7) or the binding to MAT1. In contrast *S. cerevisiae* Cak1 has a highly atypical sequence and is active as a monomer (its activity not requiring cyclin binding, phosphorylation or an assembly factor in vivo (Kaldis et al. 1996; Tsakraklides and Solomon 2002)). Active throughout the cell cycle, the *S. cerevisiae* Cak1 has unusual biochemical properties and a substrate specificity that are not shared by the human Cdk7. Thus, while human Cdk7 prefers cyclin-cdk complexes, these yeast CAKs prefer monomeric cyclin-free Cdk substrates. Human Cdks bind cyclin prior to becoming phosphorylated by Cdk7, whereas in the *S. cerevisiae* cell cycle, Cdk is phosphorylated by CAK prior to cyclin binding (Kaldis et al. 1996, 1998; Tsakraklides and Solomon 2002). This difference potentially makes fungal Cak1 a promising target for antifungal drug development.

Structurally, fungal Cak1 lacks features conserved in practically all other protein kinases, notably the canonical "glycine loop" motif (G*X*G*XX*G) that normally stabilises ATP in the nucleotide-binding pocket as well as a number of highly conserved core residues. Furthermore, fungal Cak is also unique in that, while it contains the "invariant lysine" that aligns ATP by interacting with its α- and β-phosphates and is essential for catalysis in practically all other protein kinases, this lysine is completely dispensable for their activity both in vitro and in vivo (fungal Cak is insensitive to the protein kinase inhibitor 5′-fluorosulfonylbenzoyladenosine which, by covalently modifying this lysine, leads to loss of activity in nearly all protein kinases, including Cdk7). Despite this, fungal CAKs still have high affinity for ATP, with a K_m (ATP) ~1.8–5.0 μM (Enke et al. 2000; Tsakraklides and Solomon 2002), indicating that they have compensated for their lack of a glycine loop. It is tempting to speculate that these unusual structural features may contribute to the Cak1:Cdc37 association. Structural analysis of the Cak1:Cdc37 complex in Fig. 4 should reveal this, as well as how the Cdc37 N-terminus—the region of Cdc37 highly conserved in evolution—interacts with protein kinases at the atomic level. It also promises to assist the development of drugs that selectively inhibit the fungal, not human, Cak1.

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