Candida albicans Cdc37 interacts with the Crk1 kinase and is required for Crk1 production

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Received 11 December 2003; revised 11 December 2003; accepted 15 December 2003

First published online 24 February 2004

Edited by Gianni Cesareni

Abstract **Crk1, a Cdc2-related protein kinase from the human pathogenic fungus *Candida albicans*, plays an important role in hyphal development and virulence. To address its regulatory mechanisms, we searched for Crk1 interacting proteins by two-hybrid screening. A *CDC37* ortholog (*CaCDC37*) was cloned from the screening with the Crk1 kinase domain as the bait. The CaCdc37 interacted preferentially with the kinase domain of Crk1 (Crk1N) as shown by two-hybrid and immunoprecipitation experiments. *CaCDC37* could complement a *cdc37* thermosensitive mutant (*cdc37-34*) of *Saccharomyces cerevisiae*. Importantly, Crk1 protein was hardly detectable in the *cdc37-34* mutant at restrictive temperature. However, upon expression of CaCdc37 in the *cdc37* mutant, Crk1 protein was detected even at restrictive temperature. Our data suggested that CaCdc37 was required for the production of Crk1 kinase. Like Cdc37 proteins of *S. cerevisiae* and higher eukaryotes, CaCdc37 might function as a molecular chaperone that stabilized Crk1 and other protein kinases in *C. albicans*. In support of this, *CaSTI1* was identified from a two-hybrid screen with the full-length Crk1 as the bait. CaSti1 showed two-hybrid interactions with both Crk1 and the CaCdc37.**

Key words: *CaCDC37*; Crk1; Chaperone; *Candida albicans*

1. Introduction

CDC37 was initially identified from *Saccharomyces cerevisiae* as an essential gene necessary for cell cycle progression through G1 [1]. Further studies showed that it was required for *CDC28* kinase activity because it was essential for Cdc28 stability during translation [2]. Later, many other kinases, such as Cak1, Ste11 and Mps1, were shown to require the molecular chaperone Cdc37 for their stability and functions in *S. cerevisiae* [2–4]. A vertebrate homolog of Cdc37 was first discovered as a $p50^{Cdc37}$ in a multisubunit complex with $pp60^{v-src}$ [5]. Subsequently, Cdc37 was found in complexes with many protein kinases such as *CDK4*, *CDK9*, *RAF-1* and *MOK1* [6–9], and was shown to be required for stability and function of these kinases.

In mammalian systems, Cdc37 is suggested to act as an adapter between the Hsp90 and kinases [10]. Cdc37 can interact directly with client protein kinase via its N-terminal region [7] and with Hsp90 via its central region [11]. Cdc37 is suggested to act as a molecular chaperone that specifically directs protein kinases to Hsp90 [12–14]. However, in *S. cerevisiae* whether Cdc37 acts as an adapter with Hsp90 or independently as a chaperone is still under debate. The Cdc37 has been co-immunoprecipitated with Hsp90 during maturation of Ste11 and Gcn2 [4,13,15]. But in the cases of Kin28, Cdc28 and Mps1, in vivo interaction between Cdc37 and Hsp90 has not been detected. Moreover, Hsp90 is not essential for Cdc37-dependent maturation of Cdc28 [16].

Sti1/Hop/p60 is a scaffold protein implicated in the recruitment of the steroid hormone receptor–Hsp70–Hsp40 complex to Hsp90 via simultaneous interaction with the C-terminal tails of Hsp70 and Hsp90 in mammalian cells [17]. In *Saccharomyces*, Sti1 interacts with Hsp90 genetically and biochemically [18]. Recently, Sti1 was also found to interact physically and genetically with Cdc37 [19]. Deducing from these phenomena, dynamic assembly of the heterocomplexes may be required for maturation and movement of different client proteins.

A Cdc2-related protein kinase Crk1 plays a significant role in filamentous growth in *Candida albicans* [20]. The *crk1*/*crk1* null mutant is severely impaired in filamentous growth and transcriptional induction of hyphal-specific genes. Crk1 is also important for *C. albicans* virulence. The kinase domain of Crk1 (Crk1N) shares high similarity with a cyclin-dependent kinase Sgv1/Bur1 of *S. cerevisiae* [21] and Cdk9 of human, and has a stronger effect than the full-length Crk1 on filaments growth in *S. cerevisiae* and *C. albicans*. The human Cdk9 is the catalytic subunit of a Rpb1 carboxy-terminal domain (CTD) kinase that associates with cyclin T or cyclin K and is required for regulating transcription elongation by phosphorylating the Rpb1 CTD [22]. Like Cdk9, Bur1 is a *S. cerevisiae* CTD kinase that associates with a cyclin Bur2 and comprises a divergent CDK–cyclin complex that plays a role during transcriptional elongation [23].

To search for Crk1 regulatory proteins, we constructed a *C. albicans* two-hybrid library to identify Crk1-interacting proteins. A Cdc37 ortholog, CaCdc37, was identified in the two-hybrid screening. CaCdc37 is able to physically interact with Crk1, especially with its kinase domain (Crk1N). CaCdc37 is required for production and stabilization of Crk1. Our results imply that Crk1 may be one of the client proteins of CaCdc37 in *C. albicans* and CaCdc37 may be involved in maturation of Crk1.

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0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00172-3

2. Materials and methods

2.1. Strains and growth conditions

Escherichia coli strains used in this study are DH5α (*supE44Δ lacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) [24] and Golden XL10-Gold™ {*Tet*^{R} *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac The*[F′ *proAB lacL*^{q} *ZΔM15 Tn10*] (*Tet*^{R} *Amy Cam*^{R})} (Stratagene). *S. cerevisiae* strains used are EGY48 (*MATα*, *trp1*, *ura3*, *his3*, *leu2*, *lexA*$_{op(\times 6)}$-*leu2*) [25], CJY151-(*MATα*, *his3*, *trp1*,*LexA*$_{op(\times 6)}$-*leu2*, *LexA*$_{op(\times 8)}$-*lacZ*) which was generated by integrating the LacZ reporter and its upstream modulation sequence to the strain EGY48. 8A7 (MATα *cdc37-34 leu2 lys2 trp1 ura3*) [26], kindly provided by Dr. Picard (University of Geneva) [4]. *E. coli* was grown in LB at 37°C. *S. cerevisiae* strains were cultured in YPD at 30°C or SC/Glu or SC/Gal at 30°C.

2.2. Plasmids and constructions

Plasmids pEG202, pJG4-5, pSH17-4 and pRFHM1 [27–29] were used in yeast two-hybrid experiment. pEG202 was used for the expression of LexA-tagged fusion proteins. pJG4-5 was used for expression of HA fusion proteins with the B42 activation domain under the control of the *GAL1* promoter. pSH17-4 was a positive control plasmid for LexA-AD fusion protein. pRFHM1 was a negative control plasmid for expression of a LexA-human lamin C fusion protein.

The DNA fragment encoding the N-terminal part of Crk1 (1–370 aa) [20] was cloned into pEG202, generating the expression vector pEG202CRK1N for expression of the LexA-Crk1N fusion protein. pEG202CRK1N was used as a bait vector in two-hybrid screening. DNA fragments encoding full-length Crk1 (1–746 aa) and the C-terminal part (350–746 aa) were cloned into pEG202, generating LexA fusion protein vectors pEG202CRK1 and pEG202CRK1C respectively. These DNA fragments were also inserted into pJG4-5, generating HA-B42D-fused expression vectors pJG4-5CRK1N, pJG4-5CRK1 and pJG4-5CRK1C for two-hybrid analysis.

The CaCdc37 coding sequence was obtained by polymerase chain reaction (PCR) from *C. albicans* genomic DNA (primer 1: 5′-GTCGGATCCTCATGCCAATAGATTACTCCAAGT-3′, primer 2: 5′-GCTCTCGAGCGCTATTTAATCAACTGTATCTTCA-3′). The sequence of *CaCDC37* was analyzed and deposited in GenBank database under accession number AF397024. The PCR DNA fragment was digested with *Bam*HI and *Xho*I, then inserted into the MCS of pEG202 and the pVT102U [30], generating pEG202CaCDC37 and pVTUCaCDC37. pJG4-5CaCDC37 was constructed by inserting the PCR fragment into pJG4-5. pVTUCaCDC37 was used for complementary analysis. pEG202CaCDC37 and pJG4-5CaCDC37 were used for two-hybrid analysis. The same strategy was used to construct pJG4-5*CaSTI1* for two-hybrid experiment. The *CaSTI1* coding sequence was obtained by PCR from *C. albicans* genomic DNA. Primers for *CaSTI1* were 5′-GTCGAATTCATGACAACAGCTGACGAATACA-3′ and 5′-GCTCTCGAGTTATCTGGTACGAATAACACCAG-3′. The sequence of *CaCDC37* was analyzed and deposited in GenBank database under accession number AF397024.

2.3. Two-hybrid library construction and β-galactosidase assays

To construct a *C. albicans* two-hybrid library, genomic DNA of SC5314 [31] was extracted and partially digested with *Sau*3AI. DNA fragments of 1–2 kb were purified and filled in at ends with dATP and dGTP. The plasmid pJG4-5 was digested with *Xho*I and filled in with dCTP and dTTP. The partially filled ends of the plasmid and *Sau*3AI genomic DNA fragments were ligated and transformed into *E. coli* Golden XL10-Gold™ competent cells (Stratagene). 7.7×10^4 colonies were harvested and pooled. The library was amplified for screening.

Filter assays for β-galactosidase activity were performed as described [32]. X-gal was used as a substrate. Colonies (2 mm diameter) on filters were frozen with liquid nitrogen for 1 min, then incubated with X-gal (334 μg/ml) at 30°C.

2.4. Protein extraction and Western blotting

S. cerevisiae total protein extracts were prepared as described [33]. About 2 mg total protein extracts were used for each co-immunoprecipitation assay. Each sample was incubated with 3 μg antibody (Santa Cruz) for 2 h at 0°C, then pulled down with protein G (Sigma). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli [34] using 8% w/v polyacrylamide gels. The relative molecular mass (M_r) of proteins was estimated using pre-stained protein markers (molecular weight 27–180 kDa, Sigma-Aldrich). Pulled-down proteins that were separated by SDS–PAGE were electroblotted onto nitrocellulose membranes (Hybond-C, Amersham, UK). The transfer conditions were 100 V, 1 h, 4°C. Appropriate antibodies were used to detect the corresponding proteins according to Sambrook et al. [35]. Secondary antibody (Santa Cruz) was visualized by Supersignal West Pio-chemiluminescent Substrate (Pierce).

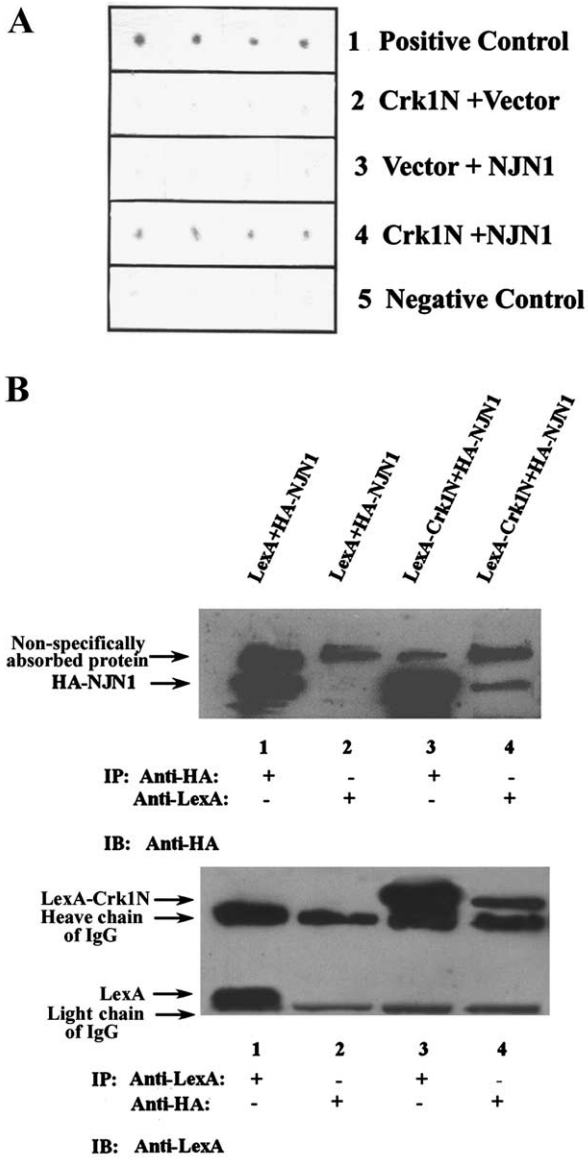

Fig. 1. Interaction between Crk1N and NJN1. A: Two-hybrid assay. CJY151 was co-transformed with plasmids as follows: 1, pSH17-4+pJG4-5; 2, pEG202CRK1N+pJG4-5; 3, pEG202+pJG4-5NJN1; 4, pEG202CRK1N+pJG4-5NJN1; 5, pRFHM1+pJG4-5. β-Galactosidase activities of the transformants were assayed as described in Section 2. B: Co-immunoprecipitation assay. Total proteins were extracted from CJY151 transformants that co-expressed HA-NJN1 and LexA (lanes 1, 2) or HA-NJN1 and LexA-Crk1N (lanes 3, 4). The tagged protein were pulled down with anti-HA antibody or an anti-LexA antibody (as indicated in the figure). Western blots were probed with anti-HA antibody (upper panel) or anti-LexA antibody (lower panel).

3. Results

3.1. Two-hybrid screening for proteins that interact with the Crk1 kinase domain

In order to identify proteins that interact with the Crk1 kinase domain, the plasmid pEG202CRK1N, which expresses the LexA-Crk1N$^{1-370}$ fusion protein, was used as a bait vector to screen the *C. albicans* two-hybrid library in *S. cerevisiae* strain CJY151. Among 1×10^7 colonies screened, 154 colonies could grow on the minimal medium (SC Gal/Raff *ura*$^-$ *his*$^-$ *trp*$^-$ *leu*$^-$), and seven of them showed a positive response in the β-galactosidase assay. Restriction map and DNA sequence analysis demonstrated that these seven inserts represent three different DNA fragments. Four out of the seven clones contained the same gene fragment named *NJN1*.

The interaction between Crk1N and NJN1 was verified by two-hybrid analysis. Co-transforming pJG4-5NJN1 and pEG202CRK1N into the yeast CJY151 showed β-galactosidase activity after 6 h incubation at 30°C (Fig. 1A). pJG4-5NJN1 or pEG202CRK1N, co-transformed with control vectors, did not detect β-galactosidase activity (Fig. 1A). The results indicate that the interaction between Crk1N and NJN1 in two-hybrid is specific.

To verify the two-hybrid interaction between Crk1N and NJN1 we performed co-immunoprecipitation experiments. pJG4-5NJN1 was co-transformed with pEG202CRK1N or pEG202 into CJY151. Total proteins were extracted from these two transformed strains. HA-NJN1 was pulled down with an anti-HA antibody and detected with the anti-HA antibody (Santa Cruz) to confirm that NJN1 was expressed normally in both strains (Fig. 1B, upper panel lane 3). From the same protein extracts, LexA-Crk1N was pulled down with an anti-LexA antibody (Santa Cruz) and the immunoprecipitates were detected with an anti-HA antibody to test whether NJN1 co-immunoprecipitated with Crk1N. An 80 kDa band was detected in the pJG4-5NJN1 and pEG202CRK1N co-transformed clone. The control vector gave a negative result (Fig. 1B, upper panel lane 2). A Western blot was also performed to verify that LexA-Crk1N but not LexA was co-precipitated by HA-NJN1 (Fig. 1B, lower panel). The result confirmed the physical interaction between NJN1 and Crk1N.

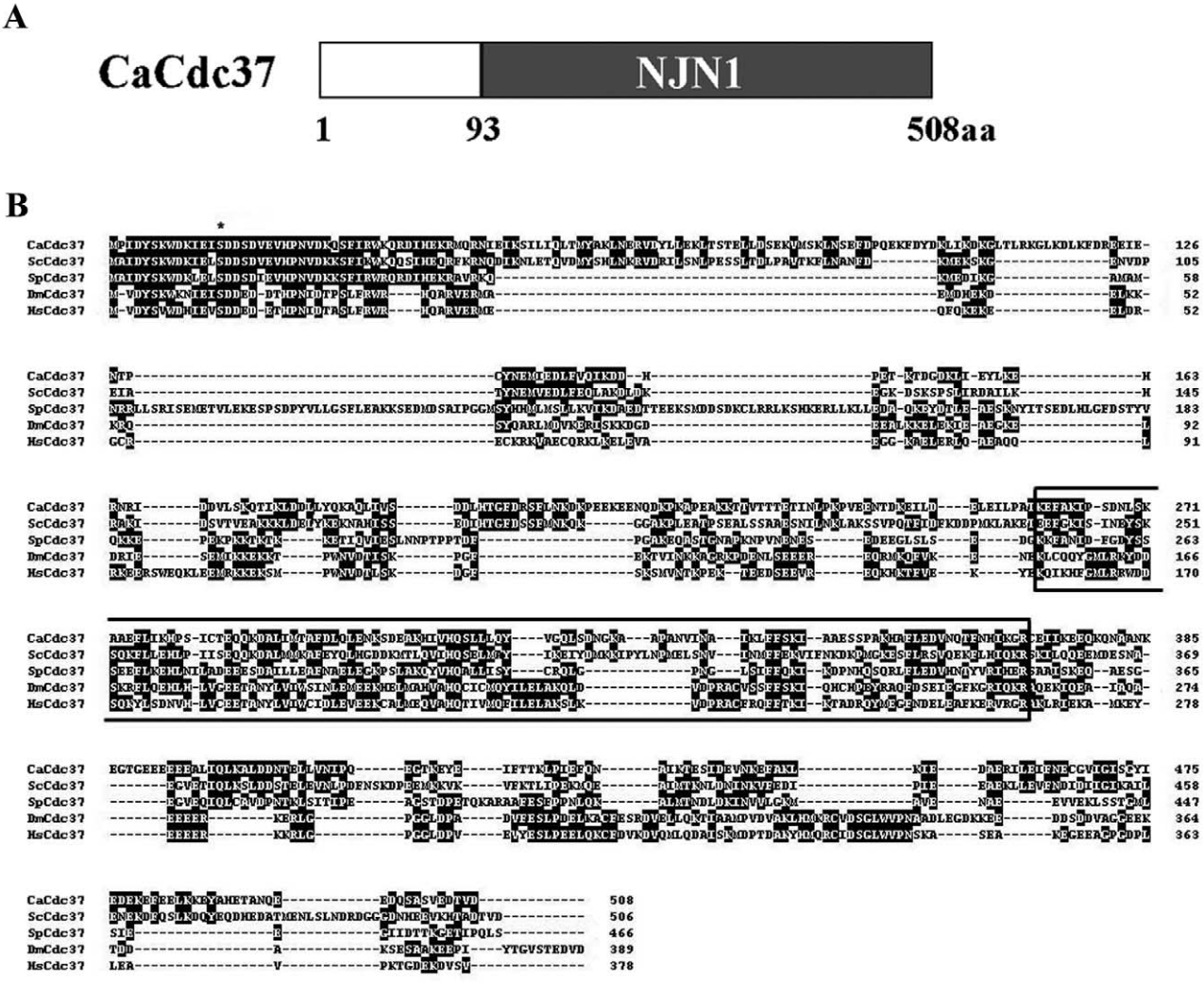

Fig. 2. Sequence alignment of CaCdc37 with other Cdc37 proteins. A: Schematic diagram of CaCdc37. B: Protein sequence alignment of different Cdc37 proteins by the Megalign program. Identical residues among different Cdc37 proteins are shaded, the conserved residue Ser14 is marked by a star, a putative Hsp90 binding region is boxed. Abbreviations: Ca, *C. albicans*; Sc, *S. cerevisiae*; Sp, *S. pombe*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*.

3.2. NJN1 *encodes* CaCDC37

The sequence of *NJN1* was blasted against the Stanford *C. albicans* Genome Database (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html). Through comparing and searching, we obtained a sequence containing a whole open reading frame that matched *NJN1*. The deduced amino acid sequence shares the highest similarity with *S. cerevisiae* Cdc37 (41% identity). The second one from the search is *Schizosaccharomyces pombe* Cdc37 (37% identity). Based on the protein sequence similarity, the gene is designated *CaCDC37* (*C. albicans CDC37*). The *CaCDC37* gene encodes a predicted protein of 508 aa. *NJN1* contains the region between amino acids 93 and 508 of CaCdc37 (Fig. 2A). Like ScCdc37, the similarity between CaCdc37 and human Cdc37 is low (only 21% identity). Both CaCdc37 (508 aa) and ScCdc37 (506 aa) are longer than human Cdc37 (378 aa) and *Drosophila* Cdc37 (389 aa).

The most conserved sequence of Cdc37 proteins is a 60 amino acid region located at the N-terminus (80% conserved) (Fig. 2B), although its function is not very clear yet. The mammalian Cdc37 was proved to be phosphorylated on Ser13 in situ in rabbit reticulocyte lysate. This phosphorylation is required for its kinase binding activity and ability to stimulate the nucleotide-modulated conformational switching of Hsp90 [36]. Conclusive evidence of MALDI-TOF mass spectrometry analysis indicated that the phosphorylation of mammalian Cdc37 at the conserved site is required for its interaction with client Hri molecules [37]. In *Saccharomyces*, two serine residues (Ser14, Ser17) in the N-terminal region are also required for Cdc37 activity, which were phosphorylated in vivo by casein kinase II [38,39]. A yeast thermosensitive mutant *cdc37-34* is caused by a mutation of Ser14. The level of Cdc37 protein in the *cdc37-34* mutant was reduced by approximately 20-fold compared with the level found in the wild-type strain [40]. Ser14 is conserved in CaCdc37 and all other Cdc37 sequences compared (Fig. 2B). The central 136 amino acids of human Cdc37 (127–163) constitute a Hsp90 binding domain (Fig. 2B) [37,41]. This region is also conserved in CaCdc37 and ScCdc37, although interaction between the yeast Cdc37 and Hsp90 has not been detected [42].

3.3. Interaction between Crk1 and full-length CaCdc37

To examine the physical interaction between Crk1 and full-length CaCdc37, We cloned the *CaCDC37* gene by PCR from SC5314 genomic DNA. *CaCDC37* was fused to *LexA* in frame to construct expression vector pEG202CaCDC37. *CRK1N* (1–370 aa), *CRK1C* (350–746 aa) and *CRK1* (1–746 aa, entire coding region) were cloned into pJG4-5 to produce HA-AD fusion proteins. pEG202CaCDC37 was co-transformed into CJY151 with pJG4-5CRK1N, pJG4-5CRK1C and pJG4-5CRK1, respectively, for two-hybrid and co-immunoprecipitation assays.

In yeast two-hybrid system, control strains that expressed LexA-CaCdc37, B42D-Crk1N, B42D-Crk1C, or B42D-Crk1 alone did not detect β-galactosidase activity. Strains carrying pEG202-CaCDC37 and pJG4-5CRK1N activated the *lacZ* reporter gene and turned blue after 6 h of incubation in X-gal plates (Fig. 3A). Interestingly, strains co-expressing LexA-CaCdc37 and B42D-Crk1C did not show any β-galactosidase activity even after an overnight incubation. A weak interaction between CaCdc37 and full-length Crk1 was also observed. The strains co-expressing CaCdc37 and Crk1 turned blue only after an overnight incubation at 30°C. Therefore, the C-terminal part of Crk1 may have an inhibitory effect on the interaction with CaCdc37. The results indicated that the full-length CaCdc37 interacted physically with Crk1 especially through the Crk1 kinase domain. This is similar to the case of Cdc28, where Cdc37 interacts strongly with the N-terminal domain of Cdc28 but not detectably with the full-length Cdc28 protein in a two-hybrid system [12].

Co-immunoprecipitation assay was used to verify the interaction between Crk1 and CaCdc37. pEG202CaCDC37 was

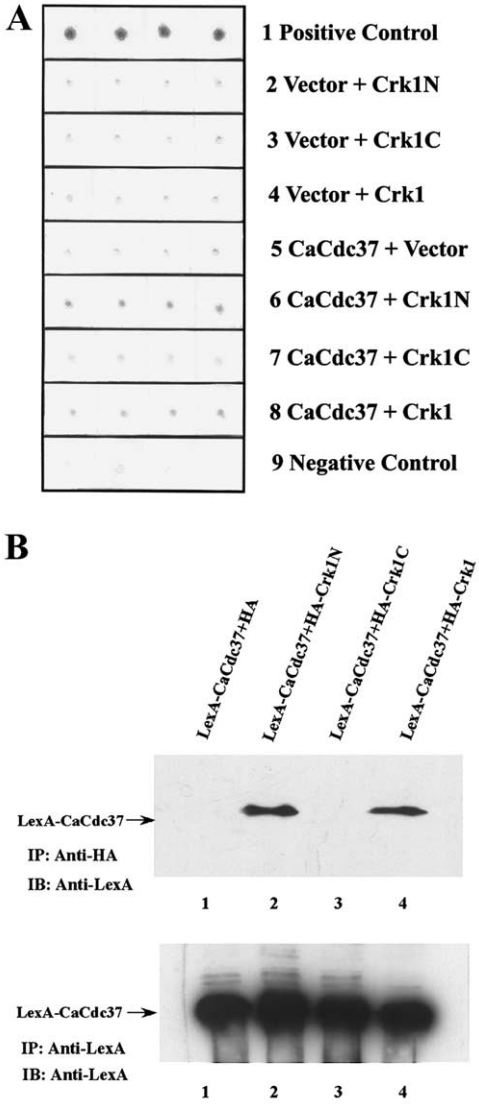

Fig. 3. Interaction between Crk1N and full-length CaCdc37. A: Two-hybrid assay. CJY151 was co-transformed with plasmids as follows: 1, pSH17-4+pJG4-5; 2, pEG202+pJG4-5CRK1N; 3, pEG202+pJG4-5CRK1C; 4, pEG202+pJG4-5CRK1; 5, pEG202CaCDC37+pJG4-5; 6, pEG202CaCDC37+pJG4-5CRK1N; 7, pEG202CaCDC37+pJG4-5CRK1C; 8, pEG202CaCDC37+pJG4-5CRK1; 9, pRFHM1+pJG4-5. β-Galactosidase activity of the CJY151 transformants was assayed as described in Section 2. B: Co-immunoprecipitation assay. Proteins were extracted from the CJY151 transformants that expressed LexA-CaCdc37 and HA (lane 1), LexA-CaCdc37 and HA-Crk1N (lane 2), LexA-CaCdc37 and HA-Crk1C (lane 3) or LexA-CaCdc37 and HA-Crk1 (lane 4). The tagged proteins were pulled down with an anti-HA antibody (upper panel) or an anti-LexA antibody (lower panel). Western blot was performed by using the anti-LexA antibody.

co-transformed respectively with pJG4-5CRK1N, pJG4-5CRK1C, pJG4-5CRK1, or pJG4-5. Total proteins were extracted from the transformed strains. Crk1N, Crk1C, and Crk1 were pulled down with anti-HA antibody. The immunoprecipitated proteins were blotted with an anti-LexA antibody to determine whether CaCdc37 was co-immunoprecipitated with Crk1. The results confirmed that CaCdc37 can physically interact with both Crk1N and Crk1 (Fig. 3B, lanes 2, 4), but not with Crk1C (Fig. 3, lane 3). The control vector (pJG4-5) did not give a signal (Fig. 3B, lane 1).

3.4. CaCDC37 *suppresses the growth defect of a* S. cerevisiae cdc37 *thermosensitive mutant*

cdc37-34 is a temperature-sensitive mutant and *cdc37-34* cells arrest in G1 and G2/M when shifted from the permissive temperature to the restrictive temperature. To determine whether *CaCDC37* is a functional homolog of *S. cerevisiae CDC37*, the *cdc37-34* mutant was used for complementation analysis. *cdc37-34* strain 8A7 transformed with high-copy plasmid pVTUCaCDC37 could grow at both 25°C and 37°C, while both the host strain 8A7 and the strain transformed with the control plasmid pVT102U could only grow at 25°C (Fig. 4). The result showed that *CaCDC37* could suppress the temperature-sensitive growth defect of the *cdc37-34* mutant. The complementation experiment confirmed that the *CaCDC37* gene is a functional homolog of Cdc37. This is not surprising because many Cdc37 functions are conserved between yeast and multicellular organisms despite the fact that the protein sequence of yeast Cdc37 shares only limited similarity with that of mammalian and *Drosophila* $p50^{Cdc37}$/Cdc37. For example, *Drosophila CDC37* can complement a *cdc37* mutant.

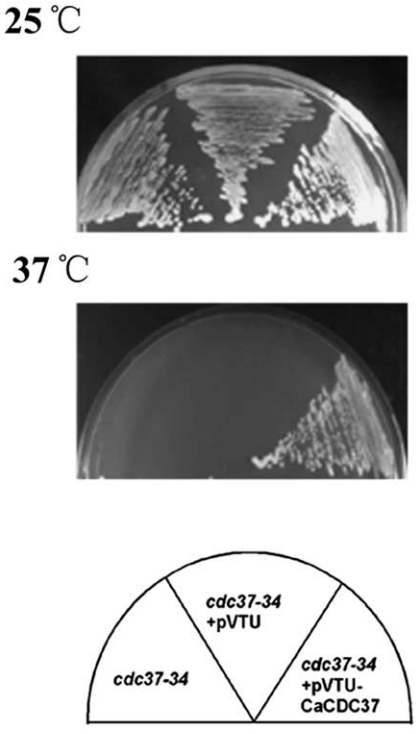

Fig. 4. Complementation of a temperature-sensitive *S. cerevisiae cdc37* mutant by *CaCDC37*. *S. cerevisiae cdc37-34* mutant strain (8A7) was transformed with pVT102U or pVTUCaCDC37. The strains were grown on YPD plates at 37°C or 25°C for 3 days.

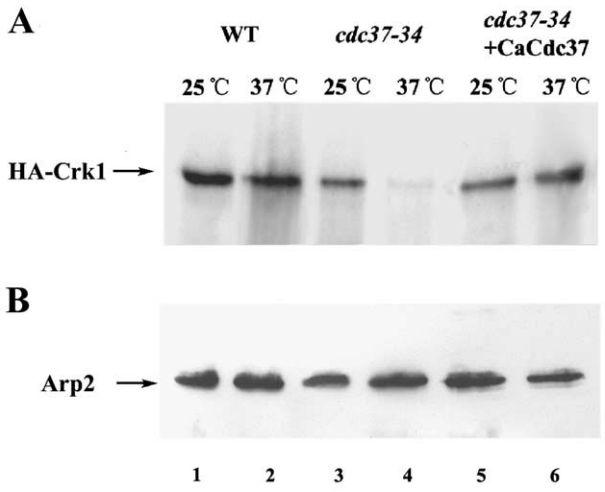

Fig. 5. CaCdc37 can replace the function of Cdc37 in the production of Crk1 in the *cdc37-34* mutant. A: Plasmid pJG4-5CRK1 (*GAL1p-HA-CRK1*) was transformed into a wild-type strain (WT, CJY151), the *cdc37-34* mutant (8A7) and 8A7 carrying pVTU-CaCDC37 (*cdc37-34*+CaCdc37), respectively. The strains were cultured in SC/Glu medium overnight and then transferred to SC/Gal medium for 5 h growth at 25°C or 37°C. Cells were lysed by bead beating, and Crk1 levels were detected by immunoblotting with an anti-HA antibody. B: Equal sample loading was confirmed by probing with an anti-Arp2 antibody.

3.5. *Cdc37 or CaCdc37 is required for the production of Crk1*

Yeast Cdc37 is required for the production and function of many protein kinases. The levels and activity of the protein kinase Cdc28 and Cak1 are significantly reduced in the *cdc37-1* mutant [2]. Cdc37 is also required for activity of the kinase Ste11 [4]. The *cdc37-34* mutant is defective in Ste11-mediated pheromone signaling and in accumulation and functional maturation of the constitutively active Ste11 version Ste11ΔN. Moreover, *CDC37* is required for the activity of vertebrate $pp60^{v-src}$ in yeast [26]. The oncogenic protein kinase $pp60^{v-src}$ is toxic to yeast. When $pp60^{v-src}$ was expressed in the *cdc37-1* mutant, the strain produces wild-type amounts of $pp60^{v-src}$ and is $pp60^{v-src}$-sensitive at the permissive temperature, but becomes $pp60^{v-src}$-resistant at the restrictive temperature. In the *cdc37-34* mutant, $pp60^{v-src}$ is produced at lower levels than in the wild-type. Therefore, the *cdc37-34* mutant is resistant to $pp60^{v-src}$ even at the permissive temperature.

To test whether ScCdc37 is required for production of Crk1 in yeast, *GAL1p-HACRK1* (plasmid pJG4-5CRK1) was introduced into a wild-type strain and the *cdc37-34* mutant strain, respectively. The fusion protein is under the control of the *GAL1* promoter. Overnight-cultured cells were transferred from SC/glucose into SC/galactose medium and grown at 25°C or 37°C for 5 h. The expression level of HA-Crk1 was detected with an anti-HA antibody and an antibody specific for actin-related protein Arp2 (Santa Cruz) was used as a loading control (Fig. 5). Like $pp60^{v-src}$, the expression level of HA-Crk1 in the *cdc37-34* mutant is lower than that in the wild-type at the permissive temperature (compare Fig. 5A, lane 1 with lane 3). But the amount of HA-Crk1 was reduced significantly when it was expressed in the *cdc37-34* mutant at the restrictive temperature (compare Fig. 5A, lane 2 with lane 4) while the amount of Arp2 was not affected.

To investigate whether CaCdc37 has a similar effect on

Crk1 activity in *S. cerevisiae*, the plasmid pVTUCaCDC37 was co-transformed with the plasmid pJG4-5CRK1 into *cdc37-34* mutant cells. The reduction of Crk1 level was suppressed by overexpression of *CaCDC37* (Fig. 5A, lane 6). Co-expression of CaCdc37 in *cdc37-34* mutant cells can restore the production of Crk1 at the restrictive temperature. The results indicated that ScCdc37 is required for the production of Crk1 in yeast cells and CaCdc37 could complement the function of a *cdc37* mutant and promote the production or stability of Crk1 protein in *cdc37* mutant cells at the restrictive temperature. Like the other Cdc37 proteins, CaCdc37 may function as a molecular chaperone involved in folding and maturation of the Crk1 kinase.

3.6. CaSti1 interacts with Crk1 and CaCdc37

Sti1/Hop/p60 has been shown to function together with Cdc37 in the recruitment of client proteins, such as protein kinases and steroid hormone receptors, into the Hsp90 chaperone system [16]. In a two-hybrid screen with the full-length Crk1 as the bait, we isolated a clone that contained the carboxyl part (CaSti1$^{184-544}$) of *C. albicans* Sti1 from the *C. albicans* genome sequence database. The gene designation by the *C. albicans* genome database was based on the 44% identity of the predicted protein sequence to that of *S. cerevisiae* Sti1. Two-hybrid analysis showed that CaSti1$^{184-544}$ had a weak interaction with Crk1 and Crk1N (Fig. 6). The result indicated that CaSti1 might also be involved in assembly of the Crk1 maturation complex. A potential full-length *CaSTI1* gene is identified by the *Candida* sequencing project as Orf6.1022. To test the interaction between CaSti1 and Crk1, a DNA fragment containing full-length *CaSTI1* was cloned by PCR and fused to a HA tag in a two-hybrid vector for two-hybrid assays. Although CaSti1$^{184-544}$ could interact with Crk1 or Crk1N, the full-length CaSti1 failed to interact with Crk1 or Crk1N in our two-hybrid experiments (Fig. 6). An appropriate conformation of CaSti1 may be required for the physical interaction with Crk1.

In yeast, Sti1 interacts genetically with Cdc37 and Hsp90 [18]. Sti1 is also found in the Cdc37 complex in vivo. In vitro, purified recombinant Sti1 can interact with Cdc37 in the absence of Hsp90 [19]. To determine whether CaSti1 functions together with CaCdc37 in kinase maturation, we investigated interactions between CaSti1 and CaCdc37 with two-hybrid system. Yeast strains transformed with both CaSti1 and CaCdc37 turned blue after 12 h incubation in β-galactosidase assays. CaSti1$^{184-544}$ showed a stronger interaction with CaCdc37 than full-length CaSti1. The results indicated that CaCdc37 and CaSti1 could interact, which is consistent with results in *S. cerevisiae*. The fact that Crk1 could interact with CaCdc37 and the C-terminal part of CaSti1 suggested that CaSti1 may also participate in the chaperone function of CaCdc37 during Crk1 maturation.

4. Discussion

In this study, we have identified a novel *CDC37* ortholog in *C. albicans* in a screen for Crk1-interacting proteins through a two-hybrid system. We designated it *CaCDC37* because the amino acid sequence of CaCdc37 has high similarity to that of other Cdc37 proteins from different species. It shares 41% identity with *S. cerevisiae* Cdc37. Its N-terminal sequence shares more than 80% identity with the other Cdc37 proteins and the conserved serine residue at position 14 may be required for phosphorylation. Furthermore, CaCdc37 is a functional homolog of *S. cerevisiae* Cdc37 because expression of CaCdc37 could suppress the temperature-sensitive growth defect of the *S. cerevisiae* mutant *cdc37-34*.

Yeast *CDC37* is required for the maturation and activity of vertebrate p60^{v-src} transcribed in yeast. The p60^{v-src} kinase is produced in wild-type yeast cells and is toxic to cells, while *cdc34* mutants carrying the p60^{v-src} gene can grow because p60^{v-src} is not produced in the mutant. Similar to the p60^{v-src} kinase, maturation of Crk1 kinase also requires the activity of Cdc37 in *S. cerevisiae*. Crk1 kinase is detectable in wild-type *S. cerevisiae* cells, while it is not detectable in a *cdc37* mutant. The dependence of Crk1 production on Cdc37 is rescued by the expression of CaCdc37 in the *cdc37* mutant. Therefore, CaCdc37 is required for the maturation of the Crk1 kinase and perhaps other kinases as well. The proposed function of CaCdc37 as a chaperone in protein kinase maturation is also supported by the interactions that we found between CaCdc37 and Crk1 kinase. CaCdc37 is isolated from a two-hybrid screen with the Crk1 kinase domain, and the interaction is confirmed by in vivo immunoprecipitation. In an independent two-hybrid screen with the full-length Crk1, we have identified CaSti1 and have found that CaSti1 can interact with not only Crk1 but also CaCdc37. The interaction of CaCdc37 with CaSti1 suggests that CaSti1 may be involved in assembly of CaCdc37-kinase heterocomplexes. The requirement of CaCdc37 for Crk1 production in *S. cerevisiae cdc37* mutant, together with the interactions among CaCdc37, Crk1 and CaSti1, suggests that the function and regulation of CaCdc37 in kinase maturation may be similar to that of *S. cerevisiae* Cdc37.

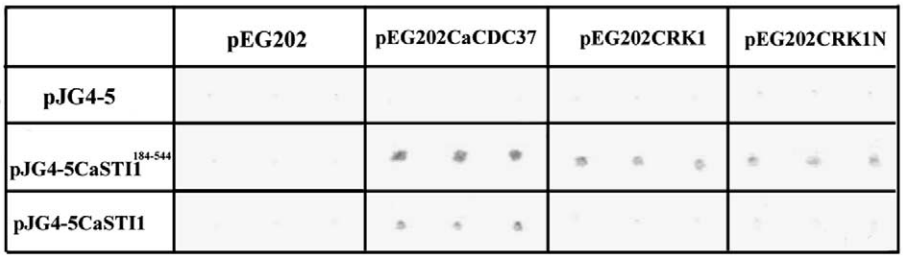

Fig. 6. Two-hybrid interactions between CaSti1 and Crk1 or CaCdc37. CJY151 was co-transformed with plasmids as follows: 1, pEG202+pJG4-5; 2, pEG202+pJG4-5CaSTI1$^{184-544}$; 3, pEG202+pJG4-5CaSTI1; 4, pEG202CaCDC37+pJG4-5; 5, pEG202CaCDC37+pJG4-5CaSTI1$^{184-544}$; 6, pEG202CaCDC37+pJG4-5CaSTI1; 7, pEG202CRK1+pJG4-5; 8, pEG202CRK1+pJG4-5CaSTI1$^{184-544}$; 9, pEG202CRK1+pJG4-5CaSTI1; 10, pEG202CRK1N+pJG4-5; 11, pEG202CRK1N+pJG4-5CaSTI1$^{184-544}$; 12, pEG202CRK1N+pJG4-5CaSTI1.

Cdc37 proteins are known to target protein kinases to Hsp90, which helps kinases fold correctly. How Cdc37 proteins recognize their client protein kinases is not clear yet. The central region of mammalian Cdc37 has been shown to interact directly with Hsp90 and its N-terminal region interacts with the kinase domain of Raf1 and Hri [7,11,36,37]. Different from the human Cdc37, the N-terminal region of CaCdc37 seems not essential for its interaction with kinases because CaCdc37 without the N-terminal 90 amino acids can still interact with Crk1. This is consistent with the findings in *S. cerevisiae* in which both full-length and the CTD of Cdc37 can physically interact with the kinase domain of Cdc28 [12]. A possible reason for the discrepancy is the low identity between Cdc37s from the yeast and mammalian systems. Although the first 30 amino acids are over 80% identical, the rest of the protein shows as little as 20% similarity between human and yeast Cdc37. Some major amino acid insertions are not present in the mammalian Cdc37. Moreover, in the yeast system a physical interaction between Cdc37 and Hsp90 could not be detected but in the mammalian system it is easily detected. So the mechanisms of Cdc37 recognition of the kinases may be different in fungi and mammals. CaCdc37 can interact with both full-length Crk1 and the kinase domain of Crk1 (Crk1N), but not with the C-terminal region of Crk1. In addition, the interaction of CaCdc37 with the kinase domain is stronger than with Crk1. Therefore, CaCdc37 recognizes and interacts with the kinase domain. The C-terminal half of Crk1, on the other hand, may reduce the interaction of the kinase domain with CaCdc37 after Crk1 maturation. This type of differential interaction between Cdc37 and different domains of a kinase has also been reported for other kinases in *S. cerevisiae* [12] and in mammals [43]. The phenomenon also indicates that the interaction of Cdc37 with the kinase can be transient. When the client kinase has been matured into a correct conformation it will dissociate from the Cdc37.

Hsp90 has been shown to form transient heterocomplexes with Sti1 and Cdc37 as intermediate steps in the maturation process of protein kinases in mammalian systems [10]. However, no physical interactions between Cdc37 and Hsp90 in cell extracts are detected in the yeast system [42], although Cdc37 can genetically interact with Hsp90. Furthermore, Hsp90 is not essential for Cdc37 function in Cdc28 maturation [17] and the N-terminal 149 amino acids of Cdc37, without the domain for Hsp90 interaction, are sufficient for yeast viability and Cdc37 function in yeast [42]. We did not isolate any Hsp90 orthologs from our two-hybrid screens with either the Crk1 kinase domain or the full-length Crk1. A potential *CaHSP90* gene is identified by the *Candida* sequencing project as Orf6.7645. We cloned *CaHSP90* by PCR and tested whether CaHsp90 can interact with CaCdc37, CaSti1 or Crk1 in a two-hybrid system. We did not detect any two-hybrid interactions with CaHsp90 (data not shown). However, the interaction between CaCdc37 and CaHsp90 may be too weak or transient to detect, or it is also possible that CaCdc37 acts as a chaperone and functions independently of CaHsp90 during protein kinase maturation.

Acknowledgements: We thank Prof. Picard for his generous offer of strain 8A7. The work was supported by the National Natural Science Foundation of China No. 30028010 and No. 30330010.

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