Coevolution of Cyclin Pcl5 and Its Substrate Gcn4

Tsvia Gildor, Revital Shemer, Avigail Atir-Lande, and Daniel Kornitzer*

Department of Molecular Microbiology, B. Rappaport Faculty of Medicine, Technion–IIT, and Rappaport Institute for Research in the Medical Sciences, Haifa, Israel

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Gcn4, a transcription factor that plays a key role in the response of *Saccharomyces cerevisiae* to amino acid starvation, is regulated at both the levels of translation and of protein stability. Regulated degradation of Gcn4 depends on its phosphorylation by the cyclin-dependent kinase Pho85, in conjunction with the cyclin Pcl5. The pathogenic yeast *Candida albicans* contains a functional homolog of Gcn4, which is involved in amino acid metabolism, as well as in the regulation of filamentous growth in response to starvation. Here, we show that *C. albicans* Gcn4 (CaGcn4) is rapidly degraded and that this degradation depends on a Pho85 cyclin homolog, CaPcl5. The regulatory loop that includes Gcn4 and Pcl5 is conserved in *C. albicans*: like in *S. cerevisiae*, CaPcl5 is transcriptionally induced by CaGcn4 and is required for CaGcn4 degradation. However, the proteins have coevolved so that there is no cross-recognition between the proteins from the two species: phosphorylation-dependent degradation of CaGcn4 occurs only in the presence of CaPcl5, and *S. cerevisiae* Gcn4 (ScGcn4) requires ScPcl5 for its degradation. Phenotypic analysis of the Capcl5 mutant indicates that CaPcl5 also modulates the filamentous response of *C. albicans* in amino acid-rich media.

Cyclin-dependent kinases (CDKs) are the principal regulators of cell proliferation (46). CDKs absolutely require binding of an ancillary subunit, the cyclin, for their activity (43). The function of the cyclin subunit in activation of the kinase is well established (26), but it is thought that specific cyclins also participate in targeting the kinase to specific substrates (23, 55, 63; reviewed in reference 40). Not all CDKs are required for cell cycle progression. In *Saccharomyces cerevisiae*, only one, Cdc28, is essential for progression through the cell division cycle. Cdc28 can bind nine different cyclins, and the various Cdc28-cyclin complexes are active at different phases of the cell cycle. Pho85, another *S. cerevisiae* CDK, can bind up to 10 different cyclins (38). Pho85, although not essential for cell cycle progression, does display cell cycle-related phenotypes (11, 29, 37). Pho85 is structurally and functionally related to the mammalian kinase CDK5 (24, 48). The target sites on the CDK substrates consist of threonine or serine residues followed by a proline. In Cdc28/CDK1-like kinases, there is in addition a strong preference for a basic amino acid at position +3, yielding the consensus S/TPXK/R (22, 58). In contrast, Pho85 has a predilection for a hydrophobic residue at +3 (50). The recognition site of this motif is probably on the CDK itself rather than on the cyclin (3), which explains the dependence of this consensus on the identity of the kinase. Cyclins are assumed to play a role in substrate recognition as well, although few sequence determinants were identified on substrates that are recognized by the cyclin. One exception is the RXL or Cy motif, found in some inhibitors and substrates of CDK2-cyclin A and CDK2-cyclin E, which is likely recognized by the cyclin (1, 5, 35, 64). Based on its effect on the *Km* of the kinase reaction, this second motif appears to play a role in the affinity of the substrate for its CDK-cyclin complex (59).

Gcn4 is an *S. cerevisiae* transcriptional activator involved in biosynthesis of amino acids and purines (19), which regulates a significant proportion of the yeast genes (47). Starvation for amino acids leads to an increase in Gcn4 translation by a mechanism that involves phosphorylation of the general translation initiation factor eIF-2α by the kinase Gcn2 (20). In addition, Gcn4 is normally rapidly degraded, but it is stabilized under conditions of amino acid limitation or of partial inhibition of protein synthesis (30, 39; reviewed in reference 25). Degradation of Gcn4 depends on its phosphorylation at a specific residue, Thr165 (39), and on its ubiquitination by the ubiquitin-conjugating enzyme Cdc34 (39) in conjunction with the ubiquitin ligase SCF<sup>CDC48</sup> (6, 39). Two CDKs are involved in Gcn4 degradation: Pho85 (39) and Srb10 (6). On the basis of the phenotype of the respective deletion mutants, Pho85 is solely involved in regulation of Gcn4 degradation by starvation (6, 39). The specific Pho85 cyclin required for Gcn4 phosphorylation and degradation is Pcl5 (56). Pcl5 is itself under the transcriptional regulation of Gcn4; thus, a negative feedback loop is generated, which ensures that Gcn4 activity is kept in check under normal growth conditions (56).

*Candida albicans*, a commensal yeast able to cause disseminated systemic infection in immunocompromised individuals, is dimorphic: it has the ability to grow in either yeast or pseudohyphal or hyphal forms. The ability to switch between various morphologies is thought to contribute to the virulence of this organism (34). A Gcn4 homolog was identified in *C. albicans* which, in addition to its expected role in promoting amino acid synthesis, was suggested to play a role in the induction of filamentous growth in response to amino acid starvation (61). The presence of a subset of amino acids in the medium and/or their uptake into the cell is also known to...
induce filamentous growth (2, 31, 36). Here, we show that the regulatory loop that includes Gcn4 and Pcl5 is conserved in C. albicans and that C. albicans Pcl5 (CaPcl5) plays a role in the modulation of filamentation in response to amino acids.

MATERIALS AND METHODS

Plasmids and strains. Plasmids GAL1-ScGcn4 (KB843) and GAL1-ScPCL5 (KB1093) were described before (56). CaGcn4 was cloned by PCR amplification from the C. albicans CAI4 strain. Our clone is identical in sequence to the sequence in the GenBank locus AF18140. pGAL1-CaGcn4-Myc6 (KB1345) was built by substituting a CaGcn4-EcoRI-NdeI PCR fragment for the EcoRI-NdeI insert of plasmid KB895 (39) and then introducing the resulting CaGcn4-Myc6-EcoRI-Xhol fragment into the p414GAL1 vector (44). pGAL1-CaGcn4-Myc6 T222A (KB1346) was obtained by site-directed mutagenesis of KB1345. Plasmid CaMAL2-CaGcn4-Myc6 (KB1192) was constructed by cloning the CaGcn4-Myc6 SmaI-KpnI fragment from KB1345 into BESt19 (12) digested with EcoRV and KpnI. Plasmid 2µm CaPCL5 (KB1209) was isolated as a CaGcn4 toxicity-suppressing plasmid from a C. albicans genomic library (33). It carries open reading frame 19.4012 (CaPCL5), as well as 2,087 nucleotides (nt) 5’ and 1,359 nt 3’ to the open reading frame. The adjacent sequences contain no additional complete open reading frames. Two additional overlapping but different library isolates, 4M2 and 6M1, contained, respectively, an additional 2,623 nt and 1,274 nt 3’ of the open reading frame. The site of disruption is at nucleotide position 324 of the open reading frame, within the predicted cyclin box domain. BglII, into KB1209 digested with BglII. The site of disruption is at nucleotide position 324 of the open reading frame, within the predicted cyclin box domain. KB1325-1 and KB1325-6 carry the his-Gal-Ura3-hisG fragment in opposite orientations.

The S. cerevisiae strains W303 and KY827 (pe5Δ) were described previously (56), as was the S. cerevisiae strain KY546 (39). The wild-type C. albicans strain used was CAI4 (13). The Capcl5/Capcl5 mutant strain (GTC44) has been described (61). For the growth tests, we consistently used the CAI4 and GTC44 strains transformed to uridine prototrophy with plasmid BES116 (12). The Capcl5/Capcl5 mutant strain was obtained by disrupting the first allele of CaPCL5 with plasmid KB1325-1, generating two independent heterozygotes, KC117 and KC118. After elimination of the CaURA3 sequence by selection on 5-fluoroorotic acid, the second allele was disrupted with KB1325-6, yielding strains KC121 and KC122 from KC117 and KC123 and KC124 from KC118. The disruptions were initially screened by PCR and confirmed by Southern blotting. Ura+ versions of the double disruptants were obtained by 5-fluoroorotic acid selection, to yield KC125 and KC128 from KC121 and KC124, respectively.

Media. Yeast media (YPD and synthetic complete [SC]) are described in reference 57. Lee’s liquid medium (33) contains, per liter, 5 g of (NH4)2SO4, 0.2 g of MgSO4·7H2O, 25.5 g of KH2PO4, 12.5 g of glucose, 5 g of NaCl, 0.5 g each of alanine, phenylalanine, proline, and threonine, 1.3 g of leucine, 1 g of lysine, 0.1 g of methionine, 0.07 g of ornithine, and 1 mg of biotin. Lee’s solid medium contains, in addition, 20 g of agar (Difco) per liter.

Methods. Pulse-chase analysis was performed as described previously (39), using the SE10 anti-Myc antibody throughout. Cells were grown overnight in 2% raffinose, diluted into 2% maltose (for the CaMAL2 promoter) or 2% galactose (for the GAL1 promoter), and the cells were grown for another 3 h in the inducing carbon source before being labeled. Dephosphorylation of CaGcn4 was performed by immunoprecipitating the Myc-tagged protein from cells similarly induced with galactose, performing a last wash with phosphate buffer (50 mM Tris [pH 7.9], 10 mM MgCl2, 100 mM NaCl, 1 mM diethiothreitol), and then incubating the protein A bead immunoprecipitate for 2 h at 37°C in 20 µl of phosphate buffer plus antiproteases (1 mM phenylmethylsulfonyl fluoride and 20 µg each of chymostatin, pepstatin A, leupeptin, and antipain/ml) in the presence or absence of 20 U of calf intestinal alkaline phosphatase (New England Biolabs). Hyphal induction in liquid Lee’s medium was achieved by growing the strains overnight in this medium at 25°C and then diluting the cells 1:10 into Lee’s medium and growing in a shaker at 37°C. Sequence data for C. albicans were obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida.

RESULTS

Rapid degradation of C. albicans Gcn4. The Gcn4 homolog of C. albicans, CaGcn4, displays high similarity to its S. cerevisiae counterpart, ScGcn4, only at the C terminus of the protein, i.e., in the bZIP domain (61). In other parts of the protein, very little homology is detected. However, a short stretch of high homology between the two proteins stands out, in the vicinity of the threonine residue (Thr165 in the S. cerevisiae Gcn4 sequence) that was previously shown to be functionally important (39). The corresponding position in the CaGcn4 sequence is Thr222 (Fig. 1). The conserved sequence (residues 160 to 169 in ScGcn4) overlaps almost completely with a sequence shown to be sufficient for binding in vitro to Cde4, the F-box protein involved in Gcn4 ubiquitination (residues 160 to 170) (45, 51). The conservation of this degradation signal suggested that like ScGcn4, CaGcn4 would also be short-lived. To test this, the CaGcn4 protein was epitope tagged at the C terminus with the Myc epitope and cloned under the inducible MAL2 promoter of C. albicans and the degradation of the protein in C. albicans was followed by pulse-chase analysis. As shown in Fig. 2A, in SC medium, i.e., under conditions where in S. cerevisiae ScGcn4 is rapidly degraded (39), the CaGcn4 protein is also unstable, with a half-life of less than 5 min.

Given the conservation of the sequence shown in ScGcn4 to be necessary for degradation in vivo and sufficient for ScCde4 binding in vitro, we expected that CaGcn4 would be efficiently degraded in S. cerevisiae as well. However, this is not the case: under the same growth conditions, the same CaGcn4 epitope-tagged construct that is rapidly degraded in C. albicans was stable in S. cerevisiae (Fig. 2B). We also found that expression in S. cerevisiae of CaGcn4-Myc from the strong, inducible GAL1 promoter strongly inhibited growth (Fig. 3). This is probably due to the inherent toxicity of Gcn4 when overexpressed (56, 60) coupled with the high stability, and therefore high steady-state concentration, of CaGcn4 when it is expressed in S. cerevisiae.

Isolation of a suppressor of CaGcn4 toxicity. We reasoned that if S. cerevisiae is lacking a specificity factor (e.g., a specific kinase or ubiquitin ligase) required for CaGcn4 degradation, then such a factor might be isolated from a C. albicans genomic library based on its expected suppression of CaGcn4 toxicity in S. cerevisiae. We performed such a suppressor screen using a C. albicans genomic library cloned into a high-copy-number (2µm) plasmid vector (33) and isolated four independent library plasmids carrying overlapping fragments of the same region of the C. albicans genome. Figure 3 shows the CaGcn4 toxicity phenotype and its suppression by one of the clones isolated in our screen, KB1209.

A single complete open reading frame, encoding a protein of a predicted length of 304 amino acids (C. albicansorf19.4012; http://www-sequence.stanford.edu/group/candida), was found in all the suppressing plasmids. When compared with the predicted S. cerevisiae proteome, this open reading frame displays the highest homology to Pho85 cyclins, and specifically to Pcl5 (Fig. 4). However, the overall homology between this protein and Pcl5, over a region of 140 residues encompassing the
predicted cyclin box domains, did not exceed 28%. Nonetheless, based on its ability to suppress CaGcn4 toxicity, we assumed that this sequence encodes the functional Pcl5 homolog of *C. albicans*, and we therefore called it CaPcl5. This assignment is supported by the mutant phenotype of the CaPcl5<sup>−/−</sup> disruption (see below).

**CaPcl5 induces hyperphosphorylation and degradation of CaGcn4 in *S. cerevisiae***. The presence of CaPcl5 in the *S. cerevisiae* cells expressing CaGcn4 led to a suppression of the toxicity of CaGcn4. We tested whether this reduced toxicity correlated with phosphorylation and enhanced degradation of CaGcn4. As shown in Fig. 5A, in the presence of CaPcl5, a slower-migrating form of CaGcn4 became apparent, strongly suggesting that CaPcl5 induces the phosphorylation of CaGcn4. Furthermore, the stability of CaGcn4 was somewhat reduced in the cells expressing CaPcl5, with a resulting half-life of about 30 min. This rate of degradation, although higher than in the absence of CaPcl5, was still significantly lower than that observed for either CaGcn4 or ScGcn4 in their native cellular environments. The weaker labeling seen in the control strain compared to the CaPCL5-expressing strain, despite the use of equivalent number of cells, was consistently observed in several separate experiments. It may reflect the toxicity of CaGcn4 in *S. cerevisiae*. To show that the slower-migrating bands do indeed represent phosphorylation of CaGcn4, the protein was immunoprecipitated from cells coexpressing CaPcl5 and subjected to dephosphorylation by alkaline phosphatase. Figure 5B shows that after phosphatase treatment, the four (at least) detectable protein bands collapse to the single, highest-mobility band.

The obvious candidate CDK to function with CaPcl5 to phosphorylate CaGcn4 in *S. cerevisiae* was Pho85. To test whether Pho85 is indeed required for CaPcl5 activity, we tested whether CaPcl5 could suppress CaGcn4 toxicity in a pho85<sup>−/−</sup> mutant. Figure 6A shows that in the pho85<sup>−/−</sup> mutant, suppression of CaGcn4 toxicity by CaPcl5 is disabled. In addition, the homology of CaGcn4 Thr222 to ScGcn4 Thr165, a residue that is phosphorylated by ScPcl5/Pho85 and required for the degradation of ScGcn4 (39), suggested that Thr222 might be the relevant target of CaPcl5. Indeed, we found that, whereas CaPcl5 can suppress the toxicity of overexpression of wild-type CaGcn4, it is unable to suppress the toxicity of CaGcn4 Thr222Ala (Fig. 6B).

Pulse-chase analysis of the CaGcn4 Thr222Ala mutant indicated that this mutant, even in the presence of CaPcl5, is stable (Fig. 6C): quantitation of the band intensities indicated that 40 min into the chase, the CaGcn4 Thr222Ala band had still 103% of the signal left compared to that at the start of the chase.

**FIG. 2.** CaGcn4 is rapidly degraded in *C. albicans* but not in *S. cerevisiae*. (A) Six tandem repeats of the Myc epitope were fused C terminally to CaGcn4, and the fusion protein was expressed from the CaMAL2 promoter in *C. albicans* (plasmid KB1192). The W303 cells were grown in SC medium containing 2% maltose, an inducer of the CaMAL2 promoter. The cells were labeled with [35S]methionine for 5 min and then chased with cold methionine. At the indicated times after the chase, an aliquot was removed and subjected to immunoprecipitation with the anti-Myc monoclonal antibody 9E10 followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The position of the CaGcn4-6xMyc band is indicated with an arrowhead. The no-tag control lane is indicated by “C.” (B) The same epitope-tagged CaGcn4 protein was expressed from the GAL1 promoter in *S. cerevisiae* (plasmid KB1345). The cells were induced with 2% galactose and treated as described for panel A.

**FIG. 3.** Suppression of toxicity of CaGcn4 overexpression by coexpression of CaPCL5. Shown are *S. cerevisiae* W303 cells carrying a control vector plasmid, the GAL1-CaGCN4 KB1345 plasmid by itself, or the KB1345 plasmid together with one of the suppressing plasmids from the *C. albicans* genomic library (KB1209). The cells were grown for 3 days on SC-Trp + 2% galactose plates.

**FIG. 4.** Phylogenetic tree of the 10 *S. cerevisiae* Pho85 cyclins (38) together with CaPcl5 (obtained with CLUSTALW using the PAM250 weight matrix).
versus 33% after 40 min for the wild-type protein (Fig. 5). Surprisingly, the band migration pattern of this mutant in the presence of CaPcl5 was at first sight indistinguishable from that of the wild-type protein (Fig. 5), suggesting that it is similarly phosphorylated. However, closer comparison, achieved by loading a smaller amount of the wild-type protein, revealed that the broad upper band of the wild-type protein can be resolved into a doublet of two closely migrating bands; the slower-migrating band of the doublet is absent from the Thr222Ala mutant, suggesting that this species represents phosphorylation of the threonine at position 222. Taken together, the results indicate that CaPcl5 directs phosphorylation of several residues on CaGcn4, including Thr222. Furthermore, Thr222, the homolog of the critical Thr165 residue of ScGcn4, is essential for the suppression of toxicity and the degradation of CaGcn4.

Coevolution of Pcl5 and Gcn4. The presence of the genomic copy of PCL5 in wild-type S. cerevisiae is not sufficient to suppress CaGcn4 toxicity, suggesting that ScPcl5 has little affinity for CaGcn4. However, it was previously shown that overexpression of ScPcl5 is required in order to suppress the overexpression toxicity of ScGcn4 (56). Thus, it was possible that suppression of CaGcn4 overexpression toxicity could be achieved if ScPcl5 was overexpressed. To test this, cells were transformed with the GAL1-CaGCN4 plasmid together with either the GAL1-ScPCL5 plasmid or the 2μH9262mCαPCL5 plasmid. As shown in Fig. 7, the GAL1-ScPCL5 plasmid efficiently suppressed GAL1-ScGCN4 toxicity but was unable to suppress GAL1-CaGCN4 toxicity. Conversely, the 2μm CaPCL5 plasmid efficiently suppressed GAL1-CaGCN4 toxicity but was able to only very weakly suppress GAL1-ScGCN4 toxicity.
Thus, Pcl5 specifically recognizes the Gcn4 substrate with which it coevolved.

**CaGen4 induces CaPCL5 transcription.** In *S. cerevisiae*, PCL5 is transcriptionally induced by Gcn4, thereby generating a negative feedback loop, since an increase in Gcn4 activity leads to an increase in PCL5 and Pcl5 represses Gcn4 activity posttranslationally (56). In order to test whether the transcriptional regulation of PCL5 by Gcn4 is conserved in *C. albicans*, we cloned the CaGcn4 open reading frame under the control of the maltose-inducible CaMAL2 promoter. As shown in Fig. 8, in rich medium, i.e., even in the absence of amino acid starvation, ectopic induction of CaGcn4 by the addition of maltose lead to an almost 10-fold increase in the CaPCL5 mRNA. A predicted Gcn4 binding sequence (TGAGCTCA) is found in the CaPCL5 promoter at position −385 relative to the translation initiation codon, supporting the possibility that CaGen4 binds directly to the CaPCL5 promoter.

**Phenotypes of the CaPCL5 disruption in *C. albicans*.** In *S. cerevisiae*, Pcl5 is the single Gcn4-specific Pho85 cyclin, as it is the only Pcl found to be necessary for Gcn4 degradation (56). To test whether the same is true in *C. albicans*, we disrupted both alleles of CaPCL5 from the *C. albicans* genome sequentially (see Materials and Methods). Gcn4 was isolated as a gene required for resistance to 3-amino-nitroazole (3-AT), an analog of the histidine biosynthesis precursor histidinol and a competitive inhibitor of imidazolglycerol phosphate dehydratase (His3): Gcn4 counteracts the histidine starvation imposed by 3-AT by increasing *HIS3* expression (19). Similarly, the *C. albicans* Cagen4/Cagen4 mutant was shown to be hypersensitive to 3-AT by increasing HIS3 expression (19). Similarly, the C. albicans Cagen4/Cagen4 mutant was shown to be hypersensitive to 3-AT by increasing HIS3 expression (19). 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the phenotype of the Capcl5−/Capcl5− mutant was intermediate: although some hyphal induction occurred, the efficiency was lower and the hyphae were shorter (Fig. 9D). The microscopic phenotypes were reflected macroscopically in the rapid sedimentation of the wild-type cells, but not of the mutant cells, from Lee’s medium after 6 h of incubation at 37°C.

**DISCUSSION**

The role of the cyclin in conferring substrate specificity has been hard to ascertain in cell-cycle CDKs due to the small number of known substrates and the apparent redundancy of the cyclins. Although it should be noted that this redundancy...
may often be at the level of cell cycle regulatory pathways, rather than at the level of the cyclin-substrate interaction (e.g., see reference 16; reviewed in reference 54), some initial observations of redundancy may have been due to artifacts of overexpression (e.g., see reference 8). The role of the cyclin in conferring substrate specificity is probably best established in the case of Pho85. The different functions of Pho85 in metabolic regulation can be explained by targeting of the kinase by specific Pho85 cyclins (Pcls) to specific substrates. The role of Pho85 in phosphate assimilation depends on its targeting to the transcription factor Pho4 by the Pcl Pho80 (21, 28), whereas its role in glycogen synthesis depends on its targeting to the glycogen synthase Gsy2 by Pcl8 and Pcl10 (23, 63). The role of Pho85 in cell morphogenesis, which depends chiefly on Pcl1 and Pcl2 (32, 42), may be due to phosphorylation of Ste24 by Pho85/Pcl1,Pcl2 (42). The ancillary role of Pho85 in cell cycle progression (11, 29, 37) may be due in part to targeting by Pcl1 of Pho85 to the CDK inhibitor Sic1 (49) and to the proteins Epa1, Hms1, and Ncp1 (29). Finally, degradation of the bZIP transcription factor Gen4 depends on the phosphorylation of a specific residue, Thr165, by Pho85 in conjunction with Pcl5 (39, 56).

The data presented here provide the clearest evidence yet for a role for the cyclin in substrate selectivity: the Pho85-Pcl5 substrate Gen4 and the cyclin Pcl5 have coevolved in the S. cerevisiae and C. albicans lineages such that interspecies recognition has been lost. In other words, the substrate specificity seen among Pcl paralogs (the Pho85 cyclins within a single species) also can extend to Pcl orthologs. Whereas differential expression, or differential cellular localization, could be invoked to explain the substrate specificity of the paralogs, the substrate specificity of the orthologs can best be explained by invoking a direct role of the cyclin in substrate selection. The implication is that species-specific sequences exist on the substrates that are recognized by their cognate cyclins; analysis of hybrid substrates should enable us to identify the location of these cyclin-binding sequences on the substrate. Additionally, coevolutionary analysis of interacting protein families has been suggested as a way to gain insight, e.g., into receptor-ligand interactions, based on the idea that phylogenies of the interacting partners will overlap (17, 52, 53). To the extent that the cyclin can be viewed as a receptor for the substrate on the cyclin-CDK complex, similar analysis may prove useful for identifying interactions between substrates and cyclin families.

In contrast to the cyclin Pcl5, the CDK Pho85 of S. cerevisiae was still able both to interact with the C. albicans cyclin and to phosphorylate the C. albicans substrate. The C. albicans Pho85 homolog has been described (41); its similarity to the ScPho85 protein is markedly higher (62% identity) than the ScPcl5-CaPcl5 similarity (28% identity within the cyclin box domain). The relative conservation of Pho85 supports the correlation that was found between the number of interacting partners of a protein and its evolutionary conservation (14, 15). Pho85, with 10 cyclins in S. cerevisiae, and an unknown number of substrates, clearly falls within the category of the more prolific interactors (or “hubs”) (27) and would therefore be predicted to evolve more slowly. Our findings show that a conservation of function underlies the observed sequence conservation. A corollary of this analysis is that the high evolutionary rate of the cyclin is predictive of a small number of interactors. To date, Gen4 is the single known substrate of Pcl5; although the existence of additional substrates cannot be excluded, the high evolutionary rate for Pcl5 would predict that their number would be small.

In spite of the evolutionary divergence of the Gen4-Pcl5 protein interaction between S. cerevisiae and C. albicans, it is notable that the regulatory feedback loop that ensures Gen4 homeostasis, i.e., the induction of PCL5 by Gen4 and the degradation of Gen4 induced by Pcl5-mediated phosphorylation, is conserved in C. albicans. Conservation of regulatory networks even in the absence of protein homology is a recurring motif. This was recently underscored with the identification of a common G1 regulatory pathway between yeast and mammalian cells, despite a lack of homology between some of the key regulators (7, 10).

Phylogenetic footprinting (18), i.e., the conservation across species of sequences that are functionally important, is increasingly used for determination of protein binding sites on DNA. The strong conservation between ScGen4 and CaGen4 of the short fragment (residues 160 to 169 in ScGen4) implicated in its ubiquitination and degradation (39, 45) suggests that short protein sequences might be analyzed similarly. The interacting partner in this case has already been identified as Cdc4, one of the F-box proteins that confer substrate specificity to the SCF ubiquitination complex (45). The sequence conservation would thus predict that ScCdc4 will efficiently interact with CaGen4, once it is phosphorylated at Thr222. Surprisingly however, although CaGen4 was hyperphosphorylated in the presence of CaPcl5 in S. cerevisiae, degradation was only slightly increased. One possibility is that the hyperphosphorylation detected in the presence of CaPcl5 (Fig. 5) does not represent phosphorylation at Thr222. However, this was ruled out by showing that the suppression of toxicity of CaGen4 by CaPcl5 depends on Thr222 and that while several CaGen4 residues are phosphorylated by CaPcl5, Thr222 is among them (Fig. 6). Another possibility is that in spite of the sequence conservation in the Cdc4 binding site, ScCdc4 recognizes CaGcn4 only inefficiently. Our preliminary data tend to support the latter possibility (T. Gildor and D. Kornitzer, unpublished observations).

CaGcn4 was shown to play a role in C. albicans filamentous growth in response to starvation for amino acids: amino acid starvation induced pseudohyphal growth, dependent on the presence of a functional CaGNC4 copy; furthermore, ectopic expression of CaGCN4 under the control of the ACT1 promoter in normal medium also induced filamentous growth (61). Here we show that the hyphal growth induced by Lee’s medium depends on CaGNC4 as well. In Lee’s medium, it is the presence of specific amino acids, rather than starvation for amino acids, that is thought to induce hyphal growth. Recently, the C. albicans Csy1 sensor of extracellular amino acids was shown to be necessary for the filamentation response in this medium (2). It should be noted, however, that growth of the Cagcn4 mutant was reduced on Lee’s medium (Fig. 9A, right panel), suggesting that the cells experience some form of starvation on this medium, which is normally counteracted by Gen4. Unexpectedly, the Capcl5− mutant strain, which displays increased CaGNC4 activity and stability, also displayed a reduction in filamentous growth on Lee’s medium. Thus, the relation between CaGcn4 activity and filamentous growth on Lee’s medium appears to be more complex than the Cagcn4−
phenotype by itself would suggest. One possibility to explain the contribution of CaPcl5 to the filamentation response on Lee’s medium (Fig. 9C) is that CaGcn4 activity plays a negative role in this signaling pathway. The lack of filamentation of the Cagcn4 mutant itself could be a secondary effect of the slow growth of this mutant on Lee’s medium. This is, however, unlikely: in liquid medium, the growth of the Cagcn4 strain was robust, and filamentation was nonetheless greatly impaired (Fig. 9D). Alternatively, CaPcl5 could play a positive role in the filamentation response on Lee’s medium independently of its effect on CaGcn4 activity. According to this possibility, the lack of filamentation of the Cagcn4 mutant on Lee’s medium may simply reflect the positive role of CaGcn4 activity per se, but rather adequate modulation of CaGcn4 activity, that is required to achieve the filamentation response.

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