Alteration of the Protein Kinase Binding Domain Enhances Function of the *Saccharomyces cerevisiae* Molecular Chaperone Cdc37

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Cdc37 is a molecular chaperone that has a general function in the biogenesis of protein kinases. We identified mutations within the putative “protein kinase binding domain” of Cdc37 that alleviate the conditional growth defect of a strain containing a temperature-sensitive allele, tpk2(Ts), of the cyclic AMP-dependent protein kinase (PKA). These dominant mutations alleviate the temperature-sensitive growth defect by elevating PKA activity, as judged by their effects on PKA-regulated processes, localization and phosphorylation of the PKA effector Msn2, as well as in vitro PKA activity. Although the tpk2(Ts) growth defect is also alleviated by Cdc37 overproduction, the CDC37 dominant mutants contain wild-type Cdc37 protein levels. In addition, *Saccharomyces cerevisiae* Ste11 protein kinase has an elevated physical interaction with the altered Cdc37 protein. These results implicate specific amino-terminal residues in the interaction between Cdc37 and client protein kinases and provide further genetic and biochemical support for a model in which Cdc37 functions as a molecular chaperone for protein kinases.

Protein kinases are charged with critical regulatory roles in such diverse cellular processes as cell cycle control, development, stress response, and metabolism. One example is the cyclic AMP (cAMP)-dependent protein kinase (PKA), which is found in all eukaryotes and serves as the biochemical template for all other serine/threonine protein kinases (47). In the yeast *Saccharomyces cerevisiae*, PKA has been implicated in a myriad of processes including growth, carbon store accumulation, and adaptation to stress (6, 48). Like its mammalian counterpart, yeast PKA is a heterotetramer comprised of two negative regulatory subunits and two catalytic subunits. Activation occurs as regulatory subunits, encoded by *BCY1* (Fig. 1), bind the second messenger cAMP and release catalytic subunits redundantly encoded by genes *TPK1*, *TPK2*, and *TPK3* (8, 49). Strains lacking *BCY1* exhibit high cAMP-independent PKA activity, fail to accumulate carbon stores (e.g., glycogen), and are sensitive to stress (7, 8, 50). Strains containing disruptions of any two catalytic genes, *tpk1 TPK2 tpk3*, are largely indistinguishable from their wild-type parent, whereas mutants in which the single intact catalytic subunit gene has been replaced with a temperature-sensitive allele [*tpk1 tpk2(Ts) tpk3*] arrest in *G1*, hyperaccumulate glycogen, and display constitutive resistance to stress (44, 53).

Genetic analyses have identified the general stress response transcription factor Msn2 as well as the protein kinase Yak1 as being potential downstream effectors of PKA function (Fig. 1) (16, 44). Msn2 resides in the cytoplasm of dividing cells and accumulates in the nucleus in response to glucose starvation or exposure to stress (20, 21). The glucose signal is thought to be mediated by the PKA pathway because both glucose starvation and PKA depletion, but not stress, result in the dephosphorylation and nuclear accumulation of a carboxyl-terminal fragment of Msn2 (21). By contrast, nuclear accumulation in response to stress is thought to be mediated by the Tor kinase/PP2A phosphatase signaling pathway, which regulates an amino-terminal export signal (3, 13, 21, 39). As Msn2 accumulates in the nucleus, it stimulates high-level expression of over 150 genes involved in growth arrest (*YAK1*), glycogen accumulation (*GLC3*), and stress survival (*HSP12, CTT1, DDR2*, etc.) (5, 9, 18, 31). Thus, Msn2 regulates several PKA-dependent processes, whereas Yak1 functions exclusively to inhibit growth.

Given the general importance of protein kinases to cell growth, it is not surprising that cells expend significant energy and genetic capacity to maintain protein kinase function. This is achieved, in part, through the action of the multisubunit Hsp90-chaperone complex. Hsp90 is an abundant and highly conserved protein that acts in concert with several cochaperone subunits to maintain a select group of signaling proteins in their functional form by preventing aggregation and facilitating folding (33–35, 37). The importance of Hsp90 is underscored by the observation that yeast viability relies on the presence of at least one of the two highly related Hsp90-encoding genes, *HSC82* and *HSP82* (4). Although the mechanism of Hsp90 chaperone function is incompletely understood, recent studies have identified a set of cochaperones that are thought to be dedicated to substrate recognition and binding. For example, the cochaperone Sti1 (and its mammalian counterpart Hop) interacts with the carboxyl-terminal tails of Hsp70 and Hsp90, allowing the efficient transfer of select client proteins from the former chaperone to the more exclusive Hsp90 machinery (11, 40). Sti1 binding also inhibits ATP hydrolysis by Hsp90 (29, 36, 37), thereby preventing Hsp90 from completing its ATP-de-
pathway have focused on genes whose loss of function or over-expression alleviates the conditional growth of a *tpk2(Ts)* strain (16, 44, 53). To identify novel effectors of this pathway by eliminating recessive suppressors, we isolated temperature-resistant revertants of the conditional growth defect of a homozygous *tpk2(Ts)/tpk2(Ts)* diploid strain. One class of dominant suppressors falls within the putative protein kinase binding domain of the Hsp90 cochaperone Cdc37. Growth suppression corresponds to the restoration of PKA catalytic function as judged by physiological effects and monitoring of Msn2 localization and function. This conclusion is buttressed by the observation that the dominant *CDC37* mutations enhance both wild-type and mutant Tpk2 activity and is consistent with the synthetic lethality of a *tpk2(Ts) cdc37(Ts)* double mutant.

**MATERIALS AND METHODS**

**Media and growth conditions.** Cells were grown in rich (yeast extract-peptone-dextrose [YPED]) medium or synthetic complete medium lacking the appropriate amino acids (24). Temperature shifts were achieved by suspending harvested cells in prewarmed media.

**Yeast strains.** The yeast strains used in the study are listed in Table 1.

**Dominant suppressors of the *tpk2-63(Ts)* growth defect.** Independent colonies of homozygous *tpk2-63(Ts)/tpk2-63(Ts)* diploid strain NOY16 were patched onto rich medium (YPEPD) agar at 23°C for 36 h and then replicated to 34°C for 2 days. A single, temperature-resistant colony from each patch was purified at 23°C and retested for growth at the nonpermissive temperature. Ten temperature-resistant revertants were subjected to tetrad analysis and backcrosses to determine if suppression was due to a single mutation and if any of the suppressors were linked.

**Plasmids used and constructed in this study.** The C-terminal Msn2-green fluorescent protein (GFP) plasmid [pDH-PKI-Msn2(576-704)-GFPp1] was described previously (21). The low-copy-number *CDC37-101* plasmid pCB4a2 was isolated from a low-copy-number library constructed with genomic DNA from haploid revertant strain SGY530. Briefly, Sau3A fragments of approximately 10 kb were purified by agarose gel electrophoresis separation after partial digestion and cloned into the BamHI site of low-copy-number *URA3* vector pRS316. DNA from over 10,000 bacterial transformants was used to transform yeast strain SGY446 to temperature resistance on minimal yeast medium lacking uracil. Plasmid pCB42a contains the full-length *CDC37* gene on a 7.4-kbp chromosomal fragment. Subcloning and sequence analysis showed that the *CDC37-101* allele was the result of a single amino acid change (L62F). Plasmid pGS224 was constructed by inserting a 6.5-kbp KpnI-SpeI fragment from pCB42a into the KpnI-XbaI sites of pRS316. Plasmid pGS226 was digested with XbaI, diluted, and then ligated to generate cdc37A rescue plasmid pGS226. Wild-type and mutant alleles of *CDC37* were rescued by transforming SGA46, SGA47, SGA53, SGY355 (CDC37-106), and SGY355 (CDC37-109) to Ura+ with XbaI-cut pGS226. Corresponding plasmids were shown to contain wild-type (pYZ1 *CDC37*) and mutant (pYZ2 CDC37-106; pYZ5 CDC37-109) genes by sequence (Fig. 2C), complementation (see Fig. 5A), and suppression (Fig. 2A) studies. The high-copy-number *CDC37* plasmid pYZ4 was constructed by inserting a 4.5-kb KpnI-Smal *CDC37* fragment from pYZ1 into the KpnI-XbaI sites of URA3 vector pRS202 (19a). Integrating Tpk2-Myc12 (pMR1) and Tpk2-Myc12 (pMR3) plasmids were constructed by inserting NotI- and SalI-digested PCR fragments from SSOY59 (TPK2) and SGY446 (tpk2-63) into the corresponding sites of integrating Myc12 vector pRS306-GAL*SWE1*-myc12 (30). Those manipulations replaced the *GAL*SWE1* fragment with the promoter and coding regions of TPK2 and *tpk2-63* and fused the 3′ ends of these genes to the 12 repeats of the Myc epitope. Plasmids were integrated into the chromosome at the *ura3*-52 locus by digestion with HindIII and selection of Ura+ transformants. The galactose-inducible, His6-tagged Ste11 fusion plasmid His6*-V5-Stc11AN* was described previously (27).

**Plasmid mutagenesis.** The low-copy-number *CDC37* plasmid pYZ1 was transformed into midD strain LE30 (F′ *midD5 rpsL azi galU95*) (14), and five mutagenized DNA pools were prepared from independent colonies grown overnight in rich bacterial medium containing ampicillin. Each pool was used to transform yeast strain SGY562 to Ura+, and a single temperature-resistant revertant was selected from each pool for further analysis. Plasmids recovered from two of the temperature-resistant yeast transformants were retransformed into SGY446 and SGY562 to confirm that the temperature resistance was plas-
mid dependent. Mutations identified by sequencing of the CDC37 coding region in both directions are shown in Fig. 2C.

**Table 1. Strains of S. cerevisiae used in this study**

| Strain         | Genotype                                                                 | Reference or source |
|----------------|--------------------------------------------------------------------------|---------------------|
| SGY398         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-63(Ts) ptk3::TRP1 bcy1Δ::LEU2 | 53                  |
| SGY446         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-63(Ts) ptk3::TRP1 | 44                  |
| SGY448         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::URA3 ptk2-63(Ts) ptk3::TRP1 | 44                  |
| SGY562         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-62(Ts) ptk3::TRP1 | This study          |
| SGY559         | MATa Cdc37-101              | This study          |
| NOY16          | MATa Cdc37-101              | This study          |
| SGY398         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-63(Ts) ptk3::TRP1 | This study          |
| SGY530         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-63(Ts) ptk3::TRP1 CDC37-101 | This study          |
| SGY533         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-63(Ts) ptk3::TRP1 CDC37-106 | This study          |
| SGY535         | MATa ura3-52 his3 leu2-3,112 trp1 ade6 ptk1::ADE8 ptk2-63(Ts) ptk3::TRP1 CDC37-109 | This study          |
| MRY140         | MATa Cdc37A::KanMX lys2 (pRS202-CDC37) | This study          |
| MRY141         | MATa Cdc37A::KanMX lys2 (pRS316-CDC37-101) | This study          |
| FY70           | MATa ura3-52 his3 leu2-3,112 trp1 ade1 TP2K | This study          |
| FY70.1         | FY70 ura3::pRS306-tpk2-63-myc12 | This study          |
| FY70.2         | FY70 ura3::pRS306-TPK2-myc12 | This study          |
| MRY31          | MATa Cdc37A::KanMX lys2 (pRS316)(pADH-PIK-MSN2-(576-704)-GFP3) | This study          |
| MRY43          | MATa Cdc37A::KanMX lys2 (pRS316-CDC37-101) | This study          |
| MRY45          | MATa Cdc37A::KanMX lys2 (pRS316-CDC37-101) | This study          |
| MRY47          | MATa cdc37A::KanMX lys2 (pRS316-CDC37-101) | This study          |
| MRY5           | MATa cdc37A::KanMX met15 (pRS316-CDC37) | This study          |
| MRY8           | MATa cdc37A::KanMX met15 (pRS316-CDC37) | This study          |
| MRY11          | MATa cdc37A::KanMX met15 (pRS316-CDC37-101) | This study          |
| MRY10          | BRY742(pRS316+cde37A) | This study          |
| MRY75          | MATa cdc37A::KanMX his3Δ1 leu2Δ1 met15Δ0 ura3Δ1 (pRS315-CDC37-101) | This study          |
| MRY79          | MATa cdc37A::KanMX his3Δ1 leu2Δ1 met15Δ0 ura3Δ1 (pRS315-CDC37-106) | This study          |
| MRY81          | MATa cdc37A::KanMX his3Δ1 leu2Δ1 met15Δ0 ura3Δ1 (pRS315-CDC37-106) | This study          |
| ACRY77-3       | MATa ade2 leu2 his3 trp1 ura3 sti1-1::HIS3 | This study          |
| Y703           | MATa cdc25-1 ura3-52 leu2-3,112 trp1 ade1 | Jim Broach          |
| WX241-12b      | MATa mpr1-1 ura3-52 his3Δ200 | 42                  |

**RESULTS**

Dominant suppressors of the **tpk2(Ts)** growth defect. Previous suppressor analyses of the **tpk2(Ts)** growth defect identified loss-of-function mutations in the **YAK1** and **MSN2** genes (16, 44). To identify rare dominant suppressors, we isolated temperature-resistant revertants of a homozygous **tpk2-63(Ts)/tpk2-63(Ts)** diploid strain. The presence of a second, wild-type allele of the suppressor gene would also alleviate potential
growth defects that resulted from an altered function of an essential, PKA-dependent gene. Separate colonies of strain NOY16 were patched onto YEPD agar, incubated for 36 h at 23°C, and then replicated in YEPD agar for 2 days at 34°C. Tetrads analyses of 10 independent revertants gave rise to four-spored asci that formed colonies at 23°C. In each tetrad, two of the four colonies grew at 34°C, indicating that suppression was due to a single mutation. Tetrads from 7 of the 10 revertants gave rise to two colonies that grew slowly, ranging from very slowly to moderately slowly, at 23°C. Interestingly, these slow-growing colonies corresponded (100% linkage in >20 complete tetrads for each revertant) to the colonies that grew (slowly) at 34°C.

The dominance of each of the suppressors was confirmed by mating temperature-resistant haploid segregants from each revertant with a compatible haploid tpk2-63(Ts) parent (SGY446 or SGY448) and showing that the resulting diploids grew at 34°C. Moreover, diploids generated by mating the slow-growing haploid strains with the tpk2-63(Ts) parent of the opposite mating type grew as well at 25°C as the homozygous tpk2-63(Ts)/tpk2-63(Ts) parent, indicating that, in contrast with suppression, the growth defect conferred by the seven “slow-growth” suppressors was recessive. Subsequent crosses showed that at least four of the dominant suppressors that conferred a slow-growth defect were tightly linked (100% linkage in >10 tetrads) with one another and unlinked with the other three suppressors. The slow-growth suppressors have been tentatively named SOK5 (suppressor of kinase 5). Finally, all three “normal-growth” suppressors were tightly linked (100% linkage in >10 tetrads) with one another and are described below.

**tpk2(Ts) suppression by alteration of the kinase-specific chaperone CDC37.** The gene corresponding to one of the “normal-growth” suppressors was identified by transforming tpk2-63(Ts) haploid strain SGY446 to temperature resistance (growth at 34°C) with a low-copy-number plasmid library containing DNA isolated from the corresponding revertant (Fig. 2A) (see Materials and Methods). Suppression was not due to an alteration of the PKA negative regulatory subunit Bcy1 because suppression also occurred in a bcy1Δ derivative (Fig. 2B). Subcloning and sequence analysis showed that the smallest suppressing fragment contained a single open reading frame that, with the exception of a single base transversion (G to T) at the third position of codon 62, was identical with CDC37. A gapped version of the suppressor plasmid was then used to isolate the other two mutations as well as the wild-type CDC37* gene from the corresponding strains by homologous repair (see Materials and Methods). Sequence analysis showed that each of the three suppressor mutations resulted from a single, and different, base change within a small region toward the 5′ end of the CDC37 coding region (Fig. 2C). The suppressors have been designated CDC37-101, CDC37-106, and CDC37-109. Two additional mutations isolated by random mutagenesis of a CDC37* plasmid (see Materials and Methods) also fell within the same region of CDC37 and were designated CDC37-111 (Y59C) and CDC37-112 (R65Q). Interestingly, the CDC37-109 suppressor converts a residue (W33R) that is found in most eukaryotic Cdc37 proteins (43).

The CDC37 gene product is thought to function as a protein kinase-specific molecular chaperone that functions in association with Hsp90. Accordingly, the CDC37 dominant mutations may suppress the tpk2-63(Ts) growth defect by restoring function to the temperature-sensitive PKA catalytic subunit. This interpretation is consistent with previously reported observations, which have noted that CDC37 overexpression can enhance the functions of several conditional yeast protein kinases, including the debilitated spindle pole body duplication protein kinase Mps1 that is found in the mps1-1(Ts) mutant (42). As expected, the conditional growth defects of tpk2-63(Ts) and tpk2-62(Ts) mutants were alleviated, albeit to different degrees, by a high-copy-number plasmid bearing wild-type CDC37* as well as by low-copy-number plasmids bearing CDC37-101, CDC37-106, and CDC37-109 (Fig. 2A and Table 2). However, a truncated version of CDC37-106 that contained only the protein kinase binding domain and that was deleted for the Hsp90-binding domain was unable to suppress the growth defect of the tpk2(Ts) mutants (data not shown). This finding suggests that the dominant mutants function in association with Hsp90. The dominant CDC37 alleles also alleviated the conditional growth defect of the mps1-1(Ts) mutant (Table 2), suggesting that suppression was neither allele nor gene

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**FIG. 2.** The tpk2(Ts) growth defect is suppressed by alterations of the amino-terminal sequence of Cdc37. (A) Suppression of the tpk2(Ts) growth defect by the CDC37-101 mutation. Strains were streaked onto minimal medium and incubated for several days. Wild-type (WT) [SGY559(pRS316)], tpk2-63(Ts) CDC37-101 [SGY446(pYZ1)], tpk2-63(Ts) CDC37-101 [SGY446(pGS224)], tpk2-62(Ts) CDC37-101 [SGY562(pGS224)] strains were used. (B) Suppression of the tpk2(Ts) growth defect in the absence of Bcy1. Strain SGY398 (tpk2-63 bcy1Δ) was transformed with a low-copy-number vector or the same vector containing wild-type (CDC37) or mutant (CDC37-101) alleles of CDC37 and incubated on minimal medium for several days at 23°C and 34°C. (C) The dominant CDC37 mutations fall within the amino terminus of Cdc37. The individual mutations are indicated by lines within the putative protein kinase interaction domain (filled box) of Cdc37.
specific. By contrast, neither high-copy-number CDC37+ nor any of the mutant CDC37 alleles were capable of suppressing the conditional growth defect of the cdc25-1(Ts) mutant (Table 2), which contains a lesion in an upstream component of the PKA pathway.

To further examine the physiological interaction between Cdc37 and Tpk2, we monitored the growth of strains containing the tpk2-63(Ts) allele and the temperature-sensitive cdc37-1(Ts) mutation, which expresses a truncated version of the Cdc37 protein. Interestingly, none of the 24 predicted tpk2-63(Ts) cdc37-1(Ts) double mutant spores formed colonies at 23°C when a heterozygous TPK2/cdc37-1(Ts) double mutant strain was grown at 23°C; none of these 15 double mutant colonies grew on minimal medium containing 5-fluoroorotic acid, which is toxic to uracil. None of these 15 double mutant colonies grew on minimal medium containing 5-fluoroorotic acid, which is toxic to uracil. Thus, the suppression of the tpk2-63(Ts) strain by CDC37+ is specific. The dominant CDC37 suppressors alter Msn2 localization and phosphorylation. The simplest explanation for tpk2-63(Ts) suppression by the dominant CDC37 mutations is reactivation of the mutant PKA catalytic subunit by the altered chaperone (Fig. 1). Alternative possibilities include interdiction downstream of Msn2 function (e.g., relieving Yak1-specific growth inhibition), direct inactivation of the Msn2 transcriptional activator, and alteration of Msn2 localization by a PKA-independent mechanism (such as through the hyperactivation of the stress/Tor kinase signaling pathway). To differentiate among these four possibilities, we examined the effect of the CDC37 dominant mutations on glycogen accumulation (a growth-independent process) as well as Msn2 localization and phosphorylation (a PKA-dependent process). Whereas the tpk2-63(Ts) CDC37+ strain accumulates much more glycogen than its TPK2 CDC37+ parent, the tpk2-62(Ts) strain containing CDC37-101 (Fig. 3A) or the other suppressors (data not shown) accumulates near-wild-type levels of glycogen. However, the suppression of the tpk2-62(Ts) growth defect is accompanied by the suppression of the glycogen hyperaccumulation defect, implying that the dominant Cdc37 mutations do not act downstream of Msn2. Consistent with this conclusion, Msn2 localization was altered by the CDC37 dominant suppressors, as shown by comparing the cytoplasmic localization of the carboxyl-terminal, PKA-responsive Msn2-GFP fusion (21) in the tpk2-62(Ts) strain containing CDC37-101 (Fig. 3B) or CDC37-106 and CDC37-109 (data not shown) with its nuclear localization in the isogenic tpk2-62(Ts) CDC37+ strain (Fig. 3B). Because the localization of the carboxyl-terminal Msn2-GFP fusion responds to glucose and PKA depletion, but not stress or changes in the Tor kinase pathway (21, 39), these results also rule out the possibility that suppression results from an effect of Cdc37 on the stress-specific Tor kinase pathway (Fig. 1). Finally, we probed extracts of several Msn2-GFP strains with anti-phospho-CREB antibody, which specifically recog-

### Table 2. Suppression by CDC37

| Strain                  | Genotype                  | Growth |
|-------------------------|---------------------------|--------|
|                         |                           | 23°C   | 30°C | 34°C |
| SGY46(pYZ1)             | tpk2-63 cdc37+ (LC CDC37+) | +     | –    | –    |
| SGY46(pGS24)            | tpk2-63 cdc37+ (LC CDC37+) | +     | +    | +    |
| SGY46(pYZ2)             | tpk2-63 cdc37+ (LC CDC37+) | +     | +    | +    |
| SGY46(pYZ5)             | tpk2-63 cdc37+ (LC CDC37+) | +     | +    | +    |
| SGY46(pRS202)           | tpk2-63 cdc37+ (HC CDC37+) | +     | +    | +    |
| SGY46(pYZ4)             | tpk2-63 cdc37+ (HC CDC37+) | +     | +    | +    |
| SGY56(pYZ1)             | tpk2-62 cdc37+ (LC CDC37+) | +     | +    | +    |
| SGY56(pGS24)            | tpk2-62 cdc37+ (LC CDC37+) | +     | +    | +    |
| SGY56(pYZ2)             | tpk2-62 cdc37+ (LC CDC37+) | +     | +    | +    |
| SGY56(pYZ5)             | tpk2-62 cdc37+ (LC CDC37+) | +     | +    | +    |
| Y703(tpk2-62)           | cdc25-1 cdc37+ (LC CDC37+) | +     | +    | +    |
| Y703(pYZ1)              | cdc25-1 cdc37+ (LC CDC37+) | +     | +    | +    |
| Y703(pGS224)            | cdc25-1 cdc37+ (HC CDC37+) | +     | +    | +    |
| WX241-128(pYZ1)         | map1-1 cdc37+ (LC CDC37+) | +     | +    | +    |
| WX241-128(pGS224)       | map1-1 cdc37+ (LC CDC37+) | +     | +    | +    |
| WX241-128(pYZ2)         | map1-1 cdc37+ (LC CDC37+) | +     | +    | +    |
| WX241-128(pRS202)       | map1-1 cdc37+ (HC CDC37+) | +     | +    | +    |
| WX241-128(pYZ4)         | map1-1 cdc37+ (HC CDC37+) | +     | +    | +    |

a +, growth; –, no growth; +/–, slow growth.
b LC, low copy number; HC, high copy number

tPK2 CDC37+ parent, the tpk2-62(Ts) strain containing CDC37-101 (Fig. 3A) or the other suppressors (data not shown) accumulates near-wild-type levels of glycogen. Thus, the suppression of the tpk2-62(Ts) growth defect is accompanied by the suppression of the glycogen hyperaccumulation defect, implying that the dominant Cdc37 mutations do not act downstream of Msn2. Consistent with this conclusion, Msn2 localization was altered by the CDC37 dominant suppressors, as shown by comparing the cytoplasmic localization of the carboxyl-terminal, PKA-responsive Msn2-GFP fusion (21) in the tpk2-62(Ts) strain containing CDC37-101 (Fig. 3B) or CDC37-106 and CDC37-109 (data not shown) with its nuclear localization in the isogenic tpk2-62(Ts) CDC37+ strain (Fig. 3B). Because the localization of the carboxyl-terminal Msn2-GFP fusion responds to glucose and PKA depletion, but not stress or changes in the Tor kinase pathway (21, 39), these results also rule out the possibility that suppression results from an effect of Cdc37 on the stress-specific Tor kinase pathway (Fig. 1). Finally, we probed extracts of several Msn2-GFP strains with anti-phospho-CREB antibody, which specifically recog-
nizes PKA-dependent phosphorylation of Msn2 (21, 39), to examine the effect of the dominant CDC37 mutations on PKA activity. As shown in Fig. 3C, Msn2 phosphorylation is reduced in the tpk2-62(Ts) CDC37" strain relative to the wild-type TPK2 CDC37" strain, and this phosphorylation is partially restored by the presence of the CDC37-101 mutation. Msn2 protein levels remained the same, as shown by reprobing stripped blots with anti-GFP antibody (Fig. 3C). Together, these results suggest that tpk2(Ts) suppression results from an altered form of Cdc37 impinging upon PKA.

The CDC37 mutations restore PKA-specific activity but not stability. The stability of most yeast protein kinases is affected by Cdc37 function (28). To compare PKA stability in the presence of wild-type and dominant Cdc37 proteins, we examined levels of Myc-tagged Tpk2 and Tpk2-63 proteins in extracts of CDC37" and CDC37-106 strains after treatment with cycloheximide. In the wild-type CDC37" strain, both Tpk2-Myc and Tpk2-63–Myc fusions were stable for at least 4 h at 34°C (Fig. 4A). Moreover, levels of both tagged proteins were identical in the CDC37" and CDC37-106 strains (Fig. 4B), indicating that dominant suppression does not result from Tpk2-63 accumulation.

To measure PKA activity directly, we purified Myc-tagged wild-type (Tpk2) and mutant (Tpk2-63) proteins from strains grown at 23°C. Purified PKA was then used in an in vitro peptide phosphorylation assay. As shown in Fig. 4C, wild-type Tpk2-Myc exhibited greater activity when it was purified from the CDC37-106 strain than when it was purified from the CDC37" wild-type strain. Unfortunately, the Tpk2-63–Myc fusion protein was devoid of measurable activity regardless of whether it was purified from the wild-type CDC37" or CDC37-106 strain (Fig. 4C) or if it was immunoprecipitated from 10 times as much extract (data not shown). This defect is consistent with our previous observation that the tpk2-63(Ts) allele confers a partial PKA defect even at the permissive temperature (23, 44, 53). Using a fluorescent peptide assay, we also monitored PKA activities of whole-cell extracts made from strains containing either a wild-type or mutant allele of CDC37. Once again, PKA activity of the TPK2 CDC37-106 strain was greater than that of the TPK2 strain containing the wild-type CDC37" allele (Fig. 4D). Moreover, extracts of the tpk2-63(Ts) cdc37-106 strain exhibited greater PKA activity than extracts of the isogenic tpk2-63(Ts) CDC37" strain (Fig. 4D). Thus, at least one dominant CDC37 mutation enhances the specific activity of both wild-type and mutant forms of the PKA catalytic subunit Tpk2.

Dominant mutations alter kinase affinity, but not levels, of Cdc37. One trivial explanation for the suppression of the tpk2-63(Ts) growth defect is that each of the CDC37 mutations increases the amount of Cdc37 available for chaperone function. Cdc37 levels of strains containing wild-type and mutant CDC37 alleles, as well as a strain containing CDC37" on a high-copy-number plasmid, were monitored by probing extracts with anti-Cdc37 antibody. Interestingly, none of the three dominant mutants examined (CDC37-101, CDC37-106, and CDC37-109) contained elevated levels of Cdc37 protein compared with those of the wild-type strain (Fig. 5A and data not shown). This was not due to an inherent insensitivity of the assay because the strain containing the high-copy-number CDC37" plasmid exhibited significantly higher levels of Cdc37 protein than strains bearing either the wild-type or mutant CDC37 allele on the low-copy-number plasmid. Moreover, suppression did not correlate with Cdc37 levels because the tpk2-63(Ts) growth defect was more efficiently alleviated by the
dominant mutants than the \( CDC37^+ \) high-copy-number plasmid (Table 2).

All of the dominant \( CDC37 \) mutations fall within the conserved protein kinase interaction domain within the amino terminus of Cdc37 (Fig. 2C). Thus, the mutations might alter the physical interaction between Cdc37 and its client protein kinase subunit. We were unable to observe a physical interaction between Tpk2-Myc and either Cdc37 or \( CDC37-106 \) (data not shown), consistent with previous conclusions that yeast Cdc37 binds protein kinases much more weakly than does its mammalian counterpart. Instead, we exploited a previously documented interaction between yeast Cdc37 and the catalytic domain of the pheromone-sensing pathway kinase Ste11 (1, 27). Although we failed to observe wild-type Cdc37 binding to the Ste11 catalytic domain (Ste11\(^{11AN}\)) in our strain background, we did observe the interaction of Ste11\(^{11AN}\) with Cdc37-106 in immunoprecipitation experiments (Fig. 5B), suggesting that the dominant mutant form of Cdc37 has increased affinity for its kinase clients.

**Synthetic interaction between \( \text{tpk2-63(Ts)} \) and mutations in \( \text{Hsp90 complex genes} \).** Although Cdc37 is generally thought to serve as a co-chaperone of the Hsp90 complex, several observations are consistent with a model in which Cdc37 is capable of functioning alone (25–27). To examine if Hsp90 or its co-chaperones contribute to PKA function, we combined the conditional \( \text{tpk2-62(Ts)} \) mutation with an \( \text{sti1A} \) deletion. The \( \text{STT1} \) gene encodes an Hsp90 regulatory factor that is dispensable for viability at 30°C but that becomes essential for normal growth in cells shifted to elevated temperatures (10, 32). Interestingly, \( \text{STT1} \) could not be deleted from the \( \text{tpk2-62(Ts)} \) strain unless the strain also contained \( \text{CDC37-101} \) (Fig. 6A) or one of the other dominant alleles (data not shown). Thus, the \( \text{tpk2-62(Ts)} \) allele is synthetically lethal with \( \text{CDC37-101} \) strain unless the strain also contained \( \text{CDC37-101} \) (Fig. 6A) or one of the other dominant alleles (data not shown). Thus, the \( \text{tpk2-62(Ts)} \) allele is synthetically lethal with \( \text{ CDC37-101} \) strain unless the strain also contained \( \text{CDC37-101} \) (Fig. 6A) or one of the other dominant alleles (data not shown).

**DISCUSSION**

We report here the isolation of mutations in the Hsp90 cochaperone Cdc37 that alleviate the growth defect of a conditional PKA mutant. These mutations identify residues of the N-terminal domain that play critical roles in the physical and functional interaction between Cdc37 and its protein kinase clients. Our results also suggest that the suppression of the conditional growth defect results from the restoration of PKA activity.

General consensus holds that Cdc37 functions as the kinase-
specific targeting subunit of the Hsp90-chaperone complex. Biochemical support for this role comes from binding studies, which have assigned the protein kinase interaction domain of mammalian Cdc37 to the N-terminal 126 residues and the Hsp90-binding domain to a region between residues 127 and 283 (22, 43). Alanine-scanning mutagenesis has shown that several N-terminal residues are necessary for protein kinase binding (2, 43); however, it has been difficult to determine if those residues are directly involved in protein-protein interactions or if their effect on protein binding is a result of global changes within the protein. For example, although residues 2, 3, 4, and 7 are critical for the interaction between mammalian Cdc37 and heme-regulated HRI kinase, the W7A alteration also affected the interaction between Cdc37 and Hsp90, implying that relatively minor changes can have global effects (43). By contrast, residues identified in our suppressor screen fall within a small region (residues 33, 58, 59, 62, and 65) of the N-terminal domain and enhance, rather than abrogate, Cdc37 client binding and function. Of the five residues altered, W33 is the most evolutionarily conserved. It is found in Cdc37 proteins from such diverse organisms as humans, mice, fruit flies, and the yeasts Schizosaccharomyces pombe and Candida albicans, with only a limited number of fungi such as Neurospora crassa and Magnaporthe grisea containing an A at the same position. Neither of the other residues is as conserved, although L62 is found within all fungi with sequenced genomes, including S. pombe, C. albicans, N. crassa, and M. grisea. Because the screen was not saturating, it will be interesting to see if other conserved residues can be affected and if each residue has only a limited spectrum of acceptable changes.

The yeast CDC37 gene has been isolated in several screens for high-copy-number suppressors of growth defects conferred by conditional protein kinases. This is consistent with the conclusion that Cdc37, not Hsp90, is the limiting component of the Hsp90-chaperone complex (2, 25, 41, 46). Although Cdc37 overproduction can suppress the tpk2(Ts) growth defect, at least three of the five dominant mutations suppressed without elevating Cdc37 levels. Thus, the limiting nature of Cdc37 can be overcome by either increased levels or enhanced function. Because at least one of the mutant Cdc37 alleles enhanced protein kinase binding, we speculate that the interaction between Cdc37 and its client protein is a limiting factor in Cdc37 function.

This result is also intriguing because all five mutations were dominant and in different, although clustered, residues. The fact that all five mutations are different would imply that there are many ways in which the amino-terminal region might be altered to increase the binding affinity between Cdc37 and the kinase client. Although the interaction between Cdc37 and its large number of kinase clients must be sufficiently flexible to accommodate sequence and structural variation found in both Ser/Thr and Tyr kinases, it is still intriguing that all five mutations were in different amino acids. One way to reconcile this apparent paradox would be to imagine that the interaction between Cdc37 and the kinase is actually mediated by a domain of the amino-terminal region that is linked to, but separate from, the domain identified by the dominant mutations that fall between residues 33 and 65. In this scenario, the domain identified by the suppressor mutations would function as an intramolecular kinase-binding inhibitory domain, much like the autoinhibitory domain found in many protein kinases. Alteration of this domain would decrease its affinity for the kinase interaction domain within Cdc37, thereby increasing the relative affinity between Cdc37 and its client kinase. One prediction of the model is that Cdc37-kinase binding could be enhanced by relatively frequent alterations in the autoinhibitory domain (as exemplified by the mutations shown in Fig. 1) or relatively rare alterations of the (unknown) kinase interaction domain. Of course, both mutations would be dominant. Unfortunately, while the amino terminus is the most structurally conserved region of Cdc37, the absence of a three-dimensional structure for this portion of Cdc37 makes it hard to predict how these changes would affect this interaction. Nevertheless, these mutations should prove useful in future studies of Cdc37 binding and function.

Although Cdc37 is often described as a client-specific targeting subunit of the Hsp90-chaperone complex, several observations are consistent with Cdc37 possessing Hsp90-independent chaperone function. For example, purified yeast Cdc37 is capable of maintaining unfolded proteins in a reaction-competent state (25), and yeast viability is supported by elevated levels of a Cdc37 truncation fragment that lacks the carboxyl-terminal Hsp90 interaction domain (26, 51). These results imply that Hsp90 interaction and function are not absolutely essential for Cdc37 function. Interestingly, while our genetic results suggest that Sti1 (and hence Hsp90) contributes to PKA function, they also show that Cdc37 function is not completely dependent upon Sti1 for function. These results are compatible with a model in which Cdc37 and Sti1/Hsp90 play interdependent roles in PKA maintenance.

The dominant CDC37 mutations ameliorate the tpk2(Ts) growth defect by enhancing the function of the mutant PKA catalytic subunit as judged by their effects on Msn2 localization and function as well as an increase in both in vivo and in vitro PKA activities. These results add PKA to a growing list of protein kinases whose functions can be influenced by Cdc37. Interestingly, this influence is not limited to PKA subunits that have been inactivated by extreme conditions or mutation, because the activity of the wild-type PKA subunit is also enhanced. Thus, Cdc37 may optimize proper folding during PKA synthesis as well as ensure that PKA catalytic activity remains elevated during periods of stress. The notion that Cdc37 plays a physiological role in PKA function is further supported by the synthetic growth defect exhibited by strains containing the cdc37-2(Ts) and tpk2-63(Ts) mutations.

In addition to the rare, dominant suppressors in Cdc37, our selection allowed us to identify a second, intriguing class of suppressors that combined dominant suppression with a recessive growth defect. We have not characterized this second class of suppressors; however, most, if not all, fall within a single linkage group. The simplest explanation for the combined phenotype is that the same modification that alleviates the loss of PKA activity compromises an essential function. In the diploid strain, this growth defect is masked by the complementing wild-type allele. An alternative explanation is that both dominant suppression and the recessive growth defect result from a loss of function. According to this alternative model, diploid strains lacking one copy of the suppressor gene would have insufficient gene product to inhibit PKA-dependent growth but enough product to support viability, whereas haploid strains
containing only the inactive allele would exhibit slow or no growth. This issue will be resolved by molecular characterization of the suppressors; however, in the meantime, we favor the former explanation because both dominant suppressor classes were isolated much less frequently (at least 100-fold) than loss-of-function mutations in YAK1 and MSN2 (16, 44).

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REFERENCES

1. Abbas-Terki, T., O. Donze, and D. Picard. 2000. The molecular chaperone Cdc37 is required for Ste11 function and pheromone-induced cell cycle arrest. FEMS Microbiol. Lett. 187:111–116.
2. Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Abbas-Terki, T., O. Donze, and D. Picard. 2000. The molecular chaperone Cdc37 is required for Ste11 function and pheromone-induced cell cycle arrest. FEMS Microbiol. Lett. 187:111–116.
3. Chang, H. C., D. F. Nathan, and S. Lindquist. 1988. cAMP-independent growth. This issue will be resolved by molecular characterization of the suppressors; however, in the meantime, we favor the former explanation because both dominant suppressor classes were isolated much less frequently (at least 100-fold) than loss-of-function mutations in YAK1 and MSN2 (16, 44).

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6.

Broach, J. R., and R. J. Deschenes. 1990. Control of sporulation, glycogen metabolism, and heat shock resistance in S. cerevisiae. Adv. Cancer Res. 54:111–116.
7. Broach, J. R., and R. J. Deschenes. 1990. Control of sporulation, glycogen metabolism, and heat shock resistance in S. cerevisiae. Adv. Cancer Res. 54:111–116.
8. Chang, H. C., D. F. Nathan, and S. Lindquist. 1997. In vivo analysis of the Hsp90 co-chaperone Sti1 (p60). Mol. Cell. Biol. 17:318–325.
9. Chen, S., V. Prapapanich, R. A. Rimerman, B. Honore, and D. F. Smith. 1996. Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. Mol. Endocrinol. 10:682–693.
10. Chen, S., V. Prapapanich, R. A. Rimerman, B. Honore, and D. F. Smith. 1996. Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. Mol. Endocrinol. 10:682–693.
11. Dey, B., B. J. Lighthbody, and F. Boscelli. 1996. CDC37 is required for p56lck activity in yeast. Mol. Biol. Cell 7:1405–1417.
12. Duan, K., A. F. S. Garrett, J. Schneper, and J. R. Broach. 2003. Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast. Mol. Cell 11:1467–1478.
13. Enquist, L., and R. A. Weisberg. 1977. A genetic analysis of the att-int-xis region of coliphage lambda. J. Mol. Biol. 111:97–120.
14. Freeman, Y. Miyata, I. Yahara, B. C. Freeman, L. Yue, R. L. Morimoto, and S. Lindquist. 1997. Cdc37 is a molecular chaperone with specific functions in signal transduction. Genes Dev. 11:1775–1785.
15. Frey, W. B., and T. O. A. T. J. Fox, L. Piper, and L. H. Pearl. 1999. Regulation of Hsp90 ATPase activity by tetraoctopeptide repeat (TPR)-domain co-chaperones. EMBO J. 18:754–762.
16. Frey, W. B., and T. O. A. T. J. Fox, L. Piper, and L. H. Pearl. 1999. Regulation of Hsp90 ATPase activity by tetraoctopeptide repeat (TPR)-domain co-chaperones. EMBO J. 18:754–762.
17. Gardiner, A., S. Gillard, R. O’Brien, D. N. Woolfson, L. Regan, B. Panaretou, J. E. Ladbury, P. W. Piper, and L. H. Pearl. 2001. Regulation of Hsp90 ATPase activity by tetraoctopeptide repeat (TPR)-domain co-chaperones. EMBO J. 18:754–762.
18. Gardiner, A., S. Gillard, R. O’Brien, D. N. Woolfson, L. Regan, B. Panaretou, J. E. Ladbury, P. W. Piper, and L. H. Pearl. 2001. Regulation of Hsp90 ATPase activity by tetraoctopeptide repeat (TPR)-domain co-chaperones. EMBO J. 18:754–762.
19. Gardiner, A., S. Gillard, R. O’Brien, D. N. Woolfson, L. Regan, B. Panaretou, J. E. Ladbury, P. W. Piper, and L. H. Pearl. 2001. Regulation of Hsp90 ATPase activity by tetraoctopeptide repeat (TPR)-domain co-chaperones. EMBO J. 18:754–762.
20. Gardiner, A., S. Gillard, R. O’Brien, D. N. Woolfson, L. Regan, B. Panaretou, J. E. Ladbury, P. W. Piper, and L. H. Pearl. 2001. Regulation of Hsp90 ATPase activity by tetraoctopeptide repeat (TPR)-domain co-chaperones. EMBO J. 18:754–762.
21. Garber, J., D. A. Garrett, R. J. Deshaies, I. Herskowitz, and D. O. Morgan. 1995. Cdc37 is required for association of the protein kinase Cdc28 with G1 cyclins. J. Cell Biol. 131:4651–4655.
22. Gimeno, C. J., and G. R. Finke. 1994. Induction of pseudohyphal growth by overexpression of PHD1, a Saccharomyces cerevisiae gene related to transcriptional regulators of fungal development. Mol. Cell. Biol. 14:2100–2112.
23. Gorner, W., E. Duschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamam, K. E. Rats, and C. Schüller. 1998. Nuclear localization of the C2H2 zinc finger protein Msnp2 is regulated by stress and protein kinase A activity. Genes Dev. 12:586–597.
24. Gorner, W., E. Duschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamam, K. E. Rats, and C. Schüller. 1998. Nuclear localization of the C2H2 zinc finger protein Msnp2 is regulated by stress and protein kinase A activity. Genes Dev. 12:586–597.
25. Gorner, W., E. Duschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamam, K. E. Rats, and C. Schüller. 1998. Nuclear localization of the C2H2 zinc finger protein Msnp2 is regulated by stress and protein kinase A activity. Genes Dev. 12:586–597.
26. Gorner, W., E. Duschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamam, K. E. Rats, and C. Schüller. 1998. Nuclear localization of the C2H2 zinc finger protein Msnp2 is regulated by stress and protein kinase A activity. Genes Dev. 12:586–597.
targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. 33:904–918.

49. Toda, T., S. Cameron, P. Sass, M. Zoller, and M. Wigler. 1987. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell 50:277–287.

50. Toda, T., S. Cameron, P. Sass, M. Zoller, J. D. Scott, B. McMullen, M. Hurwitz, E. G. Krebs, and M. Wigler. 1987. Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7:1371–1377.

51. Turnbull, E. L., I. V. Martin, and P. A. Fantes. 2005. Cdc37 maintains cellular viability in *Schizosaccharomyces pombe* independently of interactions with heat-shock protein 90. FEBS J. 272:4129–4140.

52. Valay, J. G., M. Simon, M. F. Dubois, O. Bensaude, C. Facca, and G. Faye. 1995. The KIN28 gene is required both for RNA polymerase II mediated transcription and phosphorylation of the RpB1p CTD. J. Mol. Biol. 249:535–545.

53. Ward, M. P., and S. Garrett. 1994. Suppression of a yeast cyclic AMP-dependent protein kinase defect by overexpression of SOK1, a yeast gene exhibiting sequence similarity to a developmentally regulated mouse gene. Mol. Cell. Biol. 14:5619–5627.