Cdc37 has distinct roles in protein kinase quality control that protect nascent chains from degradation and promote posttranslational maturation

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Cdc37 is a molecular chaperone that functions with Hsp90 to promote protein kinase folding. Analysis of 65 *Saccharomyces cerevisiae* protein kinases (∼50% of the kinome) in a *cdc37* mutant strain showed that 51 had decreased abundance compared with levels in the wild-type strain. Several lipid kinases also accumulated in reduced amounts in the *cdc37* mutant strain. Results from our pulse-labeling studies showed that Cdc37 protects nascent kinase chains from rapid degra-dation shortly after synthesis. This degradation phenotype was suppressed when *cdc37* mutant cells were grown at reduced temperatures, although this did not lead to a full restoration of kinase activity. We propose that Cdc37 functions at distinct steps in kinase biogenesis that involves protecting nascent chains from rapid degradation fol-lowed by its folding function in association with Hsp90. Our studies demonstrate that Cdc37 has a general role in kinome biogenesis.

Introduction

Quality control mechanisms in eukaryotic cells involve molecular chaperone-dependent protein folding and ubiquitin/proteasome-dependent degradation. In addition, environmental stress such as heat shock or endoplasmic reticulum stress leads to a general down-regulation of translation (Wickner et al., 1999; Schneider, 2000; Frydman, 2001; Harding et al., 2002).

Hsp90 is a molecular chaperone that functions in association with many cochaperones to fold transcription factors, protein kinases, and many other clients (Millson et al., 2005; Zhao et al., 2005). Protein kinase folding is collaborative between Hsp90 and the molecular chaperone Cdc37. Cdc37 functions by stabilizing protein kinases or maintaining them in a folding-competent conformation (Kimura et al., 1997). In addition, Cdc37 promotes the assembly of Hsp90–protein kinase complexes (Stepanova et al., 1997), and expression of a dominant-negative form that lacks the Hsp90-binding domain inhibits kinase activation in animal cells (Grammatikakis et al., 1999). Interestingly, similar truncations promote yeast cell viability, suggesting that complex formation between Hsp90 and Cdc37

is not essential for their function in protein kinase maturation in all cases (Lee et al., 2002; Turnbull et al., 2005). Both chape-rones interact directly with kinases during folding, and inhibi-tion of Hsp90 with geldanamycin or other ansamycin antibiotics results in the rapid proteasome-dependent degradation of client kinases and transcription factors (MacLean and Picard, 2003; Pearl, 2005; Whitesell and Lindquist, 2005).

Protein kinase folding requires the action of several cha-perones and cochaperones in addition to Cdc37 and Hsp90. Like nuclear receptors, initial chaperone interactions with un-folded kinases requires Hsp70/40 chaperones that appear to pre-pare the kinase for interaction with Cdc37 (Arlander et al., 2006). Cdc37 needs to be phosphorylated at Ser13 (mammals) or Ser14/17 (yeast) by casein kinase II to interact with its kinase clients (Bandhakavi et al., 2003; Shao et al., 2003; Miyata and Nishida, 2004). Hsp90 is subsequently recruited into this com-plex in a manner that is stimulated by the action of Sti1/Hop (Lee et al., 2004; Arlander et al., 2006). A stable ternary com-plex between Hsp90, Cdc37, and the client kinase then forms. Interestingly, Cdc37 exists in dimeric form in solution but in monomeric form in this ternary complex (Vaughan et al., 2006). How the folding reaction proceeds from this point is unknown.

Although it is clear that Cdc37 is vital for the biogenesis of protein kinases, it remains unknown what proportion of the kinome

requires this and other chaperones for maturation. The binding site for Cdc37 on client protein kinases has been localized to the N lobe of the catalytic domain (Prince and Matts, 2004; Zhao et al., 2004) via highly conserved sequence motifs that stabilize nucleotide binding. These findings suggest a very general role for Cdc37 in kinome biogenesis because the sequences that mediate chaperone interactions are present in virtually all kinases.

Previous studies have noted that Cdc37 is up-regulated in cancer cells and can act as an oncogene (Stepanova et al., 2000a,b). As such, it may play an important role in regulating the signaling capacity of the cell, especially if it has a broad function across the kinome. To clarify the extent to which Cdc37 controls protein kinase biogenesis, we first adopted a proteomic approach to understand how many different kinases are affected by the loss of Cdc37 function. By analyzing a large proportion of the yeast kinome, we observed that Cdc37 has a general function in maintaining the levels of most but not all kinases. In addition, we characterize a novel function for Cdc37 in protecting nascent kinase chains against rapid degradation by the proteasome.

Results

Cdc37 has a general role in kinome biogenesis

To establish what proportion of the yeast kinome is dependent on Cdc37 function, we performed a large-scale analysis of
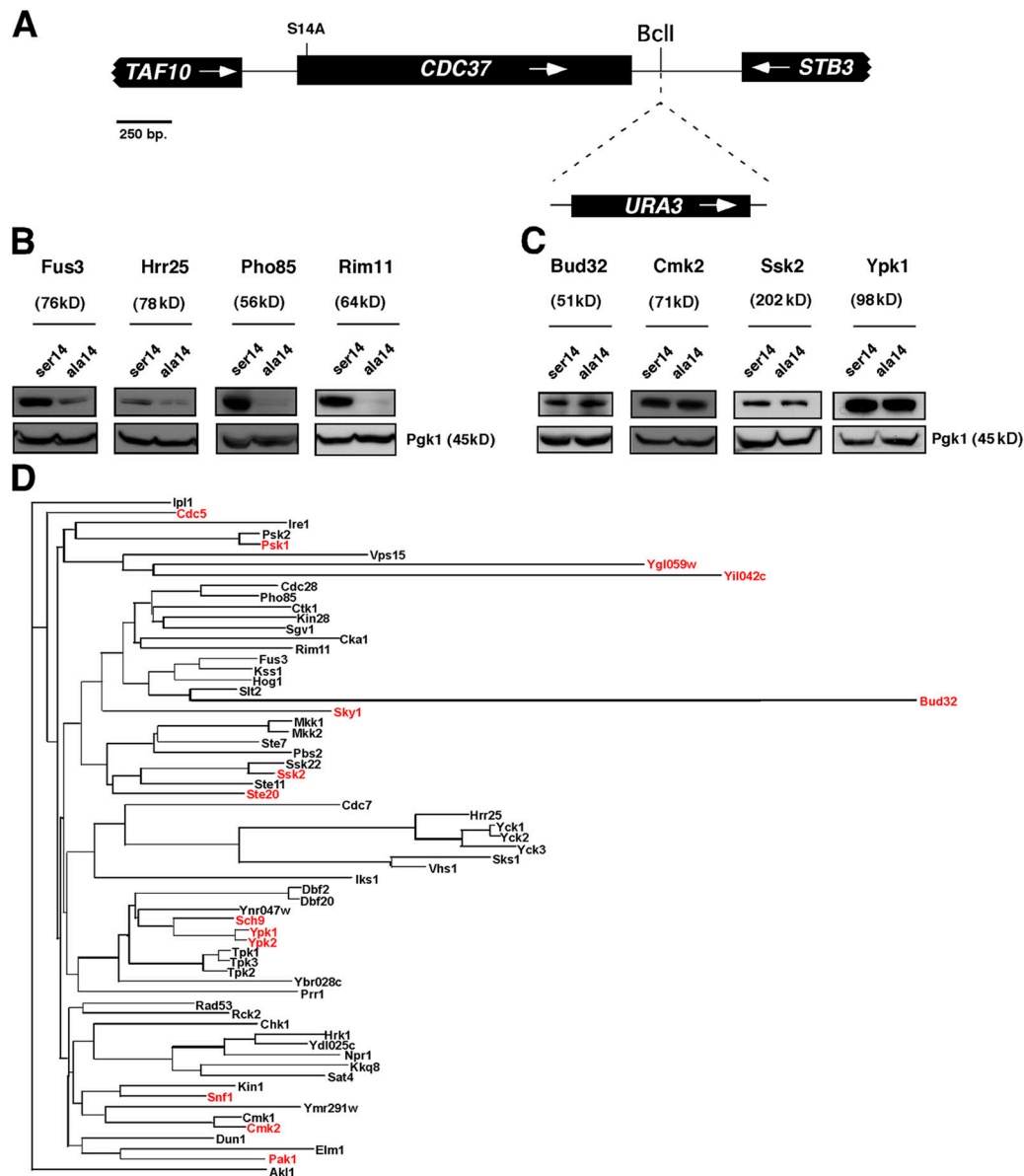

Figure 1. **Effect of *CDC37* mutation on protein kinase levels.** (A) Schematic of the *CDC37* locus showing the position where *URA3* was inserted as well as the serine 14 to alanine mutation (S14A) in *CDC37*. Arrows denote the direction of transcription. (B) Four examples of unstable kinases identified with anti-TAP (cells grown at 30°C). Western blots of the same samples with anti-Pgk1 are shown as loading controls. (C) Four examples of kinases that were unchanged after culturing wild type and *cdc37*^*S14A* at 30°C. (D) Phylogenetic analysis of protein kinases used in this study. Kinases that accumulated to wild-type levels in the *cdc37*^*S14A* mutant are shown in red. Kinases in black accumulated at less than twofold compared with wild-type levels.

kinase steady-state levels in wild-type and *cdc37* mutant yeast. This analysis used the tandem affinity-purified (TAP)–tagged yeast library, in which each strain contains a different TAP-tagged gene (Ghaemmaghami et al., 2003). The TAP tag was used as a means of identifying individual kinases after the replacement of wild-type *CDC37* with a mutant version known to affect kinase maturation (Bandhakavi et al., 2003). This mutation, serine 14 to alanine (*cdc37^S14A*), precludes phosphorylation at amino acid 14 by casein kinase II. A second phosphorylation site at serine 17 was left intact. Selection of the mutant was made possible by insertion of an auxotrophic marker (*URA3*) in the intergenic region between *cdc37^S14A* and the downstream gene *STB3* (Fig. 1 A). *Ura^+* transformants were rescreened for a temperature-sensitive lethal growth phenotype at 37°C, which was diagnostic for replacement of the wild-type gene with the *cdc37^S14A* mutant (Dey et al., 1996; Bandhakavi et al., 2003).

A large-scale analysis was performed using TAP-tagged kinases isolated from the library. 65 strains containing different TAP-tagged kinases (∼50% of the kinome) were converted to *cdc37^S14A*, and the steady-state levels of each were determined by Western blotting in comparison with the levels found in wild-type strains. Kinase identity was verified by size (predicted size of the protein plus the 21-kD tag) and by PCR analysis of the kinase-encoding gene from individual strains. Steady-state levels of each kinase were assessed by Western blotting in at least two independent transformants. Phosphoglycerate kinase was used as a loading control. Some kinases were not readily observed by direct Western blot analysis of crude extracts as a result of low abundance, and these were instead assayed after a single round of pull down with IgG–Sepharose beads (see Materials and methods).

Of the 65 kinases assayed, 51 displayed reduced steady-state levels by at least twofold in the *cdc37^S14A* mutant strain compared with the wild type. 14 kinases accumulated to the same levels in both wild-type and *cdc37^S14A* strains (less than a twofold difference; $n > 4$) when the cells were grown at 30°C. Examples of the primary data are shown in Fig. 1 (B and C), and the combined dataset is presented in Fig. 1 D as a phylogenetic tree. Multiple sequence alignment revealed no strong evolutionary relationships that distinguish those kinases affected by mutation in CDC37 from those that were not. However, three kinases that were unaffected by the *cdc37* mutant, Bud32, Ygl059W, and Yil042C, represent outliers based on their phylogenetic distance from others in the group. All three have atypical structure: Bud32 utilizes Mn^{2+} instead of Mg^{2+} (Facchin et al., 2002), and Ygl059W and Yil042C are similar to mitochondrial branched-chain α-ketoacid dehydrogenase kinases. The overwhelming majority of the other kinases analyzed have broad distributions in cytosolic or nuclear compartments (55 kinases). Three are localized to vesicle intermediates in the secretory pathway (Akl1, Ire1, and Vps15), and four have ambiguous distributions (Mkk1, Ydl025c, Sat4, and Iks1; Huh et al., 2003).

The binding of Cdc37 is restricted to the N domain of protein kinases, which comprises five β sheets that pack against a single conserved α helix (the αC helix). This structure is also found in lipid kinases (Hanks and Hunter, 1995; Walker et al., 1999).

Therefore, we tested whether any lipid kinases required Cdc37 for stability. Strains expressing TAP-tagged Pik1 (PI4 kinase) and Vps34 (a PI3 kinase) strains were converted to *cdc37^S14A*, and their steady-state levels were assayed by Western blotting (Fig. 2). Both had reduced accumulation in the *cdc37^S14A* mutant compared with the wild-type strain. Two other kinases that phosphorylate metabolic intermediates, Guk1 (a guanylate kinase) and Prs5 (a subunit of ribose phosphate diphosphokinase), accumulated to wild-type levels in the *cdc37^S14A* mutant strain. These combined data indicate that most protein kinases have access to and require Cdc37 for biogenesis at normal growth temperatures, whereas a smaller proportion of kinases accumulate to wild-type levels independent of Cdc37.

Protein kinases can function in pathways that have a self-regulatory capacity to control their own cellular abundance. In the pheromone-responsive pathway, kinase components such as Ste11, Ste7, and Fus3 are under transcriptional control that depends on a basal level of pathway activation (Fields et al., 1988; Hagen et al., 1991). Deletion of any of these kinases leads to reduced basal signal transmission, resulting in decreased levels of other proteins within the pathway. Therefore, it is possible that the reduced kinase levels we observed in the *cdc37^S14A* mutant could result from the inhibition of just a single kinase within the pathway, and this leads to further indirect decreases in the levels of other kinases. This is illustrated in Fig. 3, in which deletion of the known chaperone client *STE11* (Abbas-Terki et al., 2000) resulted in decreased levels of Fus3, a mitogen-activated protein kinase that is downstream in the pheromone-responsive pathway. However, the mutation of *CDC37* results in a further reduction in Fus3 levels. Similar results were observed when *BCK1* was deleted and the levels of Slt2 protein kinase (which is downstream in the PKC signaling pathway) were measured. The deletion of *BCK1* resulted in reduced levels of Slt2, and these were further reduced in the *bck1Δ/cdc37^S14A* double mutant (Fig. 3 B). These results show that kinase expression can be affected indirectly by the activity of other kinases but that Cdc37 has additional functions to control kinase abundance.

The results shown in Fig. 3 demonstrate that protein kinases affect the levels of other kinases within a pathway.
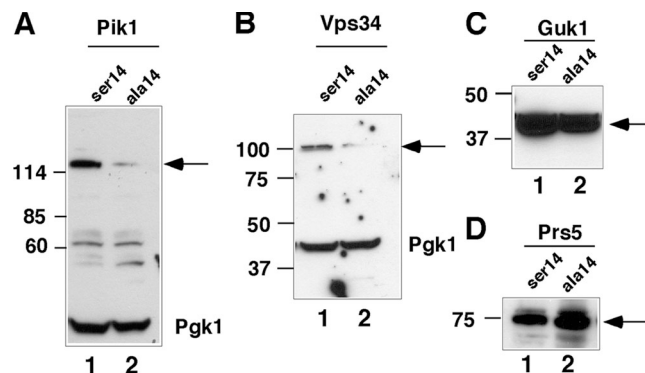

Figure 2. **Analysis of lipid kinases in a *cdc37^S14A* strain.** (A) Western blot analysis of TAP-tagged Pik1 (arrow) and Pgk1 in wild-type (ser14) and *cdc37^S14A* (ala14) strains. (B) Analysis of Vps34; details are as described in A. (C and D) Analysis of Guk1 and Prs5 steady-state levels by Western blotting. In each case, the arrow indicates the position of the TAP-tagged protein.

Figure 3. **Regulation of kinase levels by other kinases and Cdc37.** (A) Schematic of the pheromone-responsive mitogen-activated protein kinase pathway is shown at the left. Levels of Fus3 in wild-type (WT) cells (lane 1), *ste11Δ* cells (lane 2), and *ste11Δ/cdc37^{S14A}* cells (lane 3) are shown at the right. (B) Schematic at left shows the PKC signaling pathway. Levels of the Slt2 mitogen-activated protein kinase in wild-type cells (lane 1), *bck1Δ* cells (lane 2), and *bck1Δ/cdc37^{S14A}* cells (lane 3) are shown at the right. (A and B) Pgk1 levels are shown as a loading control. Vertical lines denote nonconsecutive lanes from the same gel and Western blots.
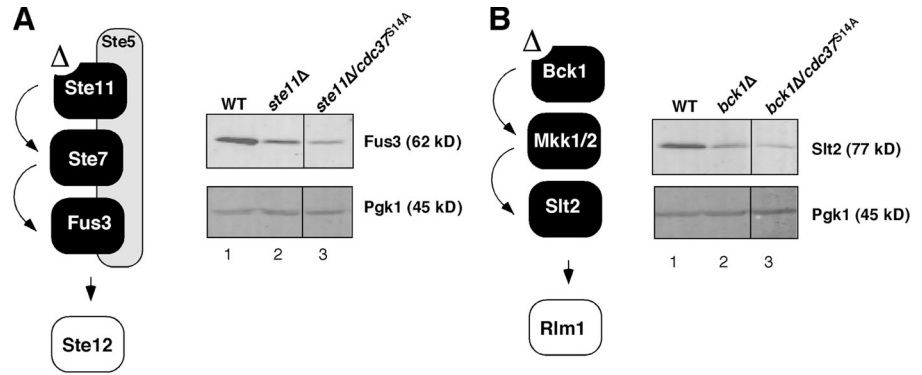
In the case of pheromone-responsive and PKC-dependent pathways, this regulation is at a transcriptional level (Fields et al., 1988; Hagen et al., 1991; Jung and Levin, 1999). Accordingly, if Cdc37 affects protein kinase abundance via the indirect method, this should result in changes in kinase gene mRNA levels. To test this hypothesis, we performed a microarray analysis to determine whether the *cdc37^{S14A}* mutation affects kinase gene expression on a general level. However, a comparison of transcriptomes between wild-type and *cdc37^{S14A}* mutant strains showed no statistically significant changes in kinase gene expression when the cells were grown at 30°C. This was confirmed by quantitative PCR analysis of several kinases whose protein levels were reduced in the *cdc37^{S14A}* mutant (unpublished data). In contrast, statistically significant changes ($P < 0.05$) in expression were observed for a small number of genes, with the maximal fold change being less than twofold. The top three genes with higher expression in the mutant strain (*YLR168C*, *RSM22*, and *ODC2*) all function in mitochondria.

Cdc37 protects nascent chains from rapid degradation

We next investigated the fate of kinases that accumulated at reduced levels in the *cdc37^{S14A}* mutant, focusing first on Tpk2. Pulse-chase analysis showed that Tpk2 levels were reduced by at least twofold in the *cdc37^{S14A}* mutant immediately after a 10-min pulse compared with the wild type (Fig. 4 A). Once synthesized, the levels of Tpk2 altered little in either strain over a 2-h period. Pulse labeling of Pho85 and Rim11 resulted in similarly decreased kinase levels in the *cdc37^{S14A}* mutant (Fig. 4, E and F). Experiments with Cmk2, whose accumulation at steady state was normal in the *cdc37^{S14A}* mutant, showed that it also accumulated normally under pulse-labeling conditions (Fig. 4 B).

The decrease in Tpk2, Pho85, and Rim11 levels under pulse-labeling conditions could represent the rapid degradation of each kinase or a defect in translation. Degradation by the vacuolar pathway was ruled out by experiments in a *pep4Δ* mutant and incubation of cells with PMSF. Incubation of cells with PMSF or deletion of the gene encoding the vacuolar peptidase Pep4 did not lead to substantial increases in the levels of Tpk2 or Rim11 in *cdc37* mutant cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200604106/DC1). Translation was assessed by examination of polysome profiles and by quantitation of the levels of kinase mRNA on the polysomes from

wild-type and *cdc37* mutant cells. However, polysome profiles from wild-type and *cdc37^{S14A}* strains were identical, suggesting that general defects in translation initiation and elongation do not occur in the *cdc37^{S14A}* mutant (Fig. S2). Furthermore, the *cdc37^{S14A}* strain did not display growth hypersensitivity on media containing paromomycin (Fig. S2), which is common for mutants that affect translation, such as *ssb1Δ/ssb2Δ* (Nelson et al., 1992). Quantitative analysis of kinase mRNA levels in polysomes and 80S ribosomes also failed to reveal any differences between the wild-type and *cdc37^{S14A}* mutant strains.

Further studies addressed whether kinase levels were reduced in the *cdc37^{S14A}* mutant as a result of rapid degradation by the proteasome. MG132 was incubated with cells for 30 min before the pulse labeling. Under these conditions, we observed that Tpk2 levels were increased in the *cdc37^{S14A}* mutant strain compared with DMSO alone and that the kinase appeared as two closely migrating bands (Fig. 4 C). Levels of Tpk2 in wild-type cells were unaffected by the presence of MG132, suggesting that the kinase is not subject to degradation during translation under normal conditions. However, these results strongly support the notion that Cdc37 protects nascent chains from degradation either during or immediately after translation. Importantly, this type of quality control is mechanistically distinct from that which occurs upon Hsp90 inhibition with geldanamycin. Under conditions in which geldanamycin is incubated with cells for 30 min before pulse labeling, there is no difference in the amount of Tpk2 synthesized after the pulse, yet there is a profound subsequent reduction in kinase levels after 2 h of chase (Fig. 4 D). Similar results were observed for Slt2 and Kss1 kinases (unpublished data).

The effect of MG132 on the levels of several other kinases during pulse labeling was also examined. Pho85, a cyclin-dependent kinase, behaved in a similar manner to Tpk2. The kinase was present in reduced amounts in the *cdc37^{S14A}* mutant compared with the wild type after a 5-min pulse, and MG132 partially suppressed this defect (Fig. 4 E). Rim11 levels were also reduced in the *cdc37^{S14A}* mutant, and, in this case, MG132 led to the accumulation of a slower migrating band that is present in all samples but is typically present as a minor component of the immunoprecipitation (Fig. 4 F). This band may represent a nonphosphorylated form of Rim11. Similar pulse-labeling studies were performed with Cdc28, the cyclin-dependent kinase that promotes cell cycle progression. Surprisingly, levels
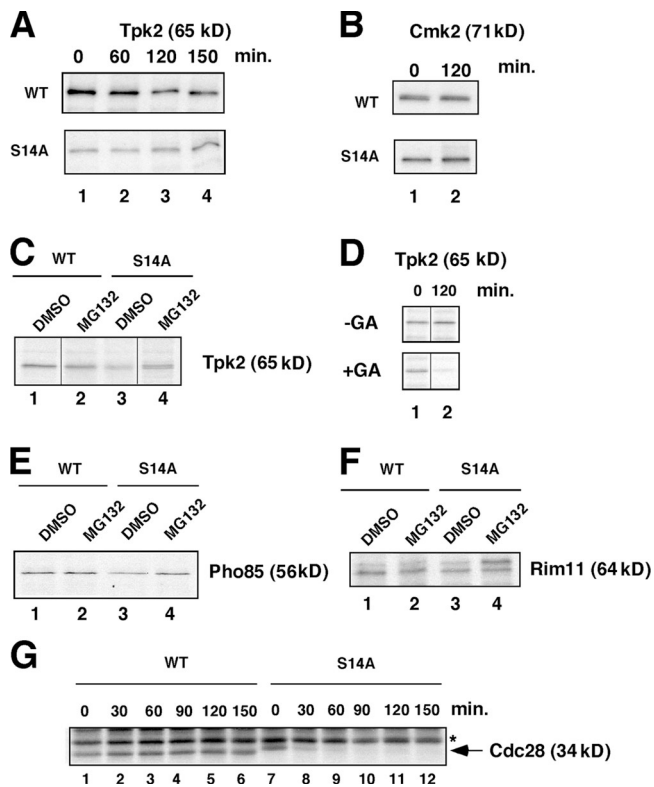
A Tpk2 (65 kD)

0   60   120   150   min.

WT

S14A

1   2   3   4

B Cmk2 (71kD)

0   120   min.

WT

S14A

1   2

C   WT   S14A

DMSO MG132 DMSO MG132

Tpk2 (65 kD)

1   2   3   4

D Tpk2 (65 kD)

0   120   min.

-GA

+GA

1   2

E   WT   S14A

DMSO MG132 DMSO MG132

Pho85 (56kD)

1   2   3   4

F   WT   S14A

DMSO MG132 DMSO MG132

Rim11 (64 kD)

1   2   3   4

G   WT   S14A

0 30 60 90 120 150   0 30 60 90 120 150   min.

*←Cdc28 (34 kD)

1   2   3   4   5   6   7   8   9   10 11 12

Figure 4. **Pulse-chase analysis of kinases in the *cdc37^{S14A}* mutant.** (A) Pulse-chase analysis of TAP-tagged Tpk2 in wild-type (WT) and *cdc37^{S14A}* mutant (S14A) cells. Chase times are indicated in minutes. (B) Pulse-chase analysis of TAP-tagged Cmk2 in wild-type and *cdc37^{S14A}* mutant (S14A) cells. (C) Pulse labeling of Tpk2 in wild-type cells and *cdc37^{S14A}* mutant (S14A) cells in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 μM MG132 added 30 min before pulse labeling (5-min pulse). (D) Pulse-chase experiment of Tpk2 in the presence of 50 μM geldanamycin (+GA) or DMSO (−GA) added 1 h before labeling. (C and D) Vertical lines denote nonconsecutive lanes from the same gel. (E) Pulse labeling and immunoprecipitation of Pho85 in wild-type (lanes 1 and 2) and *cdc37^{S14A}* mutant (S14A) cells in the absence (lanes 1 and 3) or presence of 100 μM MG132 (lanes 2 and 4) added 30 min before labeling. (F) As in E except that Rim11 kinase was immunoprecipitated. (G) Pulse-chase analysis of untagged Cdc28 immunoprecipitated with anti-PSTAIRE in wild-type (lanes 1–7) and *cdc37^{S14A}* mutant (S14A; lanes 7–12) cells. The band labeled with an asterisk is nonspecific. Chase times are given in minutes. The strain background for the experiments shown in C–G is *erg6Δ*, which improves MG132 permeability (Lee and Goldberg, 1996; Lee et al., 1996).

of Cdc28 were similar in both wild-type and *cdc37^{S14A}* strains after pulse labeling (Fig. 4 G) even though we observed greatly reduced levels of the kinase in the *cdc37^{S14A}* strain at steady state (Fig. 1 and not depicted). This paradox was resolved by pulse-chase analysis (Fig. 4 G), which shows that Cdc28 is synthesized in the same amount in both wild-type and mutant strains, yet its levels decrease sharply in the *cdc37^{S14A}* strain within a 30-min period. As with Rim11, nascent Ccd28 expressed in the mutant cells migrates more slowly in the gel compared with Cdc28 from wild-type cells, as noted previously (Gerber et al., 1995). These findings are consistent with previous studies on Cdc28 biogenesis in a different *cdc37* mutant strain (Gerber et al., 1995; Farrell and Morgan, 2000) but contrast with the more rapid degradation observed for Tpk2, Pho85, and Rim11 (Fig. 4, C–E). This could reflect differences in chaperone binding between kinases that will be discussed later.

Cdc37 promotes kinase maturation

Protein folding is a temperature-dependent process, and chaperone dependence for folding is more stringent at higher growth temperatures. We investigated the temperature dependence of kinase accumulation in the *cdc37^{S14A}* mutant. Three protein kinases that accumulated at low levels in the *cdc37^{S14A}* mutant were chosen for analysis (Rim11, Tpk2, and Yck2) based on their representation of different subgroups of the kinome (Hunter and Plowman, 1997). Notably, the kinases displayed similar stability profiles that were inversely correlated with increasing temperature (Fig. 5 A). Furthermore, each of the kinases accumulated to levels that were similar to those found in the wild-type strain after growth at 26°C (Fig. 5 B). Next, we measured the activity of Tpk2 and Rim11 after growth of the cells at 26°C. The TAP-tagged kinases were assayed for activity after isolation on IgG–Sepharose beads from wild-type and mutant strain lysates. A synthetic peptide substrate was used for Tpk2, and myelin basic protein (MBP) was used for Rim11 (Zhu et al., 2000). The results of these assays (Fig. 5 C) showed that Rim11 and Tpk2 both had reduced activity when isolated from the *cdc37^{S14A}* mutant strain compared with wild-type yeast even though similar amounts of kinase were present in the assays.

A further study addressed whether Tpk2, a cAMP-dependent protein kinase, could interact with its negative regulatory subunit Bcy1 in the *cdc37^{S14A}* mutant strain (Toda et al., 1987). As with mammalian PKA, inhibitory binding of the regulatory subunit is relieved at high cAMP levels, leading to increased kinase activity (Johnson et al., 1987). An HA-tagged version of Bcy1 was expressed in wild-type and *cdc37^{S14A}* mutant strains grown at 26 and 30°C. Similar amounts of HA-Bcy1 were expressed in both wild-type and *cdc37^{S14A}* mutant strains grown at both temperatures. TAP-tagged Tpk2 was isolated using IgG–Sepharose bead resin, and the amount of coprecipitating Bcy1 was detected by Western blotting. When the cells were grown at 30°C, there was a decreased yield of TAP-Tpk2 by threefold (Fig. 5 D) as expected because of the decreased kinase levels (Fig. 4). The amount of coprecipitating Bcy1 was decreased by fivefold relative to those amounts isolated from the wild-type strain extracts. When the cells were grown at 26°C, however, similar amounts of TAP-Tpk2 were isolated, but twofold reduced levels of Bcy1 were still bound. This suggests that some population of Tpk2 failed to bind to Bcy1 in the *cdc37^{S14A}* mutant, yet this correlated with the decreased activity of the kinase.

To determine whether Cdc37 was required for the activity of kinases that were synthesized normally at 30°C, we investigated Sky1, a constitutively active kinase (Nolen et al., 2001). Sky1 has an extra helix between the αC helix and β4 sheet that helps stabilize the kinase. Because the αC/β4 loop is implicated in Cdc37 binding and for mediating kinase sensitivity to geldanamycin (Tikhomirov and Carpenter, 2003; Xu et al., 2005), we hypothesized that Sky1 folding might be chaperone independent. However, Sky1 isolated from the *cdc37^{S14A}* mutant grown at 30°C was ~50% less active than Sky1 isolated from the wild-type strain (Fig. 6), indicating that this kinase also requires Cdc37 for efficient activation. When assayed after growth at 26°C, however, Sky1 had a similar activity when isolated
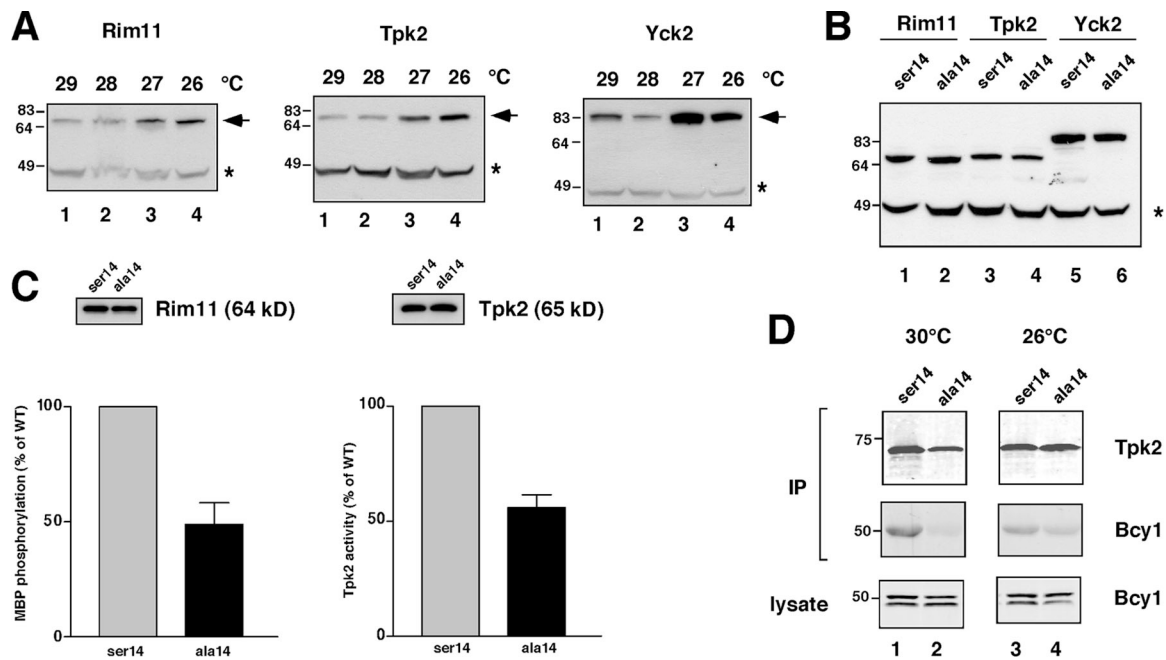
Figure 5. **Effect of temperature on kinase accumulation and activity.** (A) Western blot analysis of Rim11, Tpk2, and Yck2 (arrows) after growth of the relevant TAP-tagged $cdc37^{S14A}$ strains at the indicated temperatures. Asterisks denote Pgk1, which was used as a loading control. (B) Comparison of Rim11 (lanes 1 and 2), Tpk2 (lanes 3 and 4), and Yck2 (lanes 5 and 6) levels by Western blotting in whole cell extracts from wild-type (ser14) and $cdc37^{S14A}$ (ala14) cells grown at 26°C. The asterisk denotes Pgk1. (C) Activity of protein kinases in the $cdc37$ mutant strain. Activity of Rim11 and Tpk2 after growth of the relevant TAP-tagged wild-type (WT) and $cdc37^{S14A}$ cells at 26°C. The blots above the graph show Western blot analysis of protein levels of the kinases used in the assays. Error bars represent SEM. (D) Binding of HA-Bcy1 to Tpk2 in wild-type (ser14; lanes 1 and 3) and $cdc37^{S14A}$ (ala14; lanes 2 and 4) cells grown at 30 and 26°C as indicated. Top panels show Western blots of Tpk2; middle panels show HA-Bcy1 (Bcy1) after immunoprecipitation (IP) of TAP-Tpk2 using IgG–Sepharose beads; bottom panel shows levels of HA-Bcy1 in cell lysates. Note that two bands appear in the anti-HA Western blots, but only the slower migrating band coimmunoprecipitated with TAP-Tpk2 (not depicted).

from either strain, suggesting that it became independent of Cdc37 at the lower growth temperature. Overall, kinases that are stable at 26 or 30°C in the $cdc37^{S14A}$ mutant are less active than those in wild-type strains. Similar findings were made with the kinase Mps1, which is stably expressed in a $cdc37$ mutant but has decreased activity (Schutz et al., 1997). However, the chaperone dependence for kinase activity is inversely proportional to growth temperature.

Discussion

There is accumulating evidence that Cdc37 has a general role in kinase biogenesis, but there has been no systematic analysis at a kinomic level. In our studies, we addressed this issue by analysis of ∼50% of the yeast kinome for dependence on Cdc37. Our results are consistent with Cdc37 having a general role in kinome biogenesis and functioning either during or immediately after translation to protect nascent chains from degradation. In addition, Cdc37 promotes kinase maturation to the folded state.

The finding that Cdc37 plays a general role in kinase biogenesis is consistent with the growing number of kinases that have been characterized to interact with this chaperone (MacLean and Picard, 2003). Two other large-scale screens for Hsp90-interacting proteins also uncovered protein kinases. In a two-hybrid screen with a mutant form of Hsp90 as bait (Millson et al., 2005), six kinases were identified as interacting with

Hsp90, and, in a multiapproach study (Zhao et al., 2005), 27 kinases interacted with Hsp90 either physically or genetically. Interestingly, the kinases found in these studies did not overlap, indicating that neither screen was saturating.

The results of our studies show that Cdc37 functions immediately after translation to protect nascent kinase chains from degradation. Even after a subminute pulse labeling, we noted that kinase levels were reduced in the $cdc37^{S14A}$ mutant strain (unpublished data). However, proteasome inhibition followed by pulse labeling largely restored Tpk2, Pho85, and Rim11 levels, providing strong support for Cdc37's role in nascent chain quality control. This phenotype was only observed when MG132 was incubated for brief periods (30 min) in the cells before pulse labeling. Prolonged treatments (2 h) failed to restore Tpk2 levels, although this may result from reduced transcription occurring from long-term proteasome inhibition (unpublished data).

The finding that some kinases require Cdc37 to protect them against rapid degradation shortly after synthesis suggests that the chaperone functions as a conformational sensor at or near to the polysome. The degradation of several kinases during pulse labeling is so fast that we initially considered that translation itself was impaired. However, polysome profiles and measurement of the rate of protein synthesis suggested that Cdc37 was not involved in regulating translation, at least in a general manner (Fig. S2). Also, there is a precedent for the very rapid degradation of ribosomal proteins shortly after synthesis
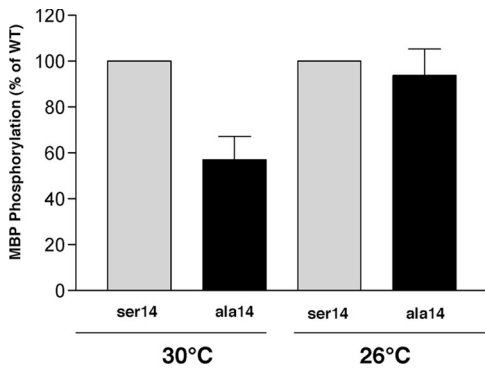
Figure 6. **Sky1 dependence on Cdc37 is temperature dependent.** Activity of Sky1 after the isolation of wild-type (ser14) and *cdc37*[S14A] mutant (ala14) cells grown at 30 and 26°C as indicated. Bars represent SEM (*n* = 3).

(Warner et al., 1985; Maicas et al., 1988). Although it is unclear whether this quality control pathway is the same as the one observed here, a recent study has discovered a role for Hsp90 in the biogenesis of some ribosomal proteins (Maicas et al., 1988; Kim et al., 2006).

Therefore, our results are consistent with the hypothesis that Cdc37 protects some nascent kinase chains from rapid degradation immediately after translation. Whether Cdc37 binds directly to the nascent chain as it is being synthesized or binds immediately afterward may depend on where in the polypeptide chain the kinase domain is located. Scroggins et al. (2003) have observed Cdc37 binding to polysomes programmed with heme-regulated inhibitor kinase, for example, but not with lymphocyte-specific protein tyrosine kinase. In our own studies, we failed to observe Cdc37 binding to polysomes isolated from yeast even when such polysomes were enriched for Ste11 kinase mRNA (unpublished data). What remains unclear is how such a function for Cdc37 is integrated with those of Hsp70/40 chaperones that interact with misfolded kinases before Cdc37 binding, at least in vitro (Arlander et al., 2006). The function of Cdc37 in stabilizing kinase nascent chains suggests that it belongs to the newly defined group of chaperones linked to protein synthesis (Albanese et al., 2006). This group includes others that are known to promote protein kinase biogenesis such as Ydj1 and Sse1. However, Cdc37 appears to be distinct from this group in other ways because the *cdc37* mutant did not display hypersensitivity to a translation inhibitor nor is *CDC37* transcriptionally coregulated with other components of the translational machinery. As pointed out by Albanese et al. (2006), however, some chaperones appear to function as both chaperones linked to protein synthesis and as stress-regulated chaperones that function in protein refolding, and this appears to apply to Cdc37.

Rapid degradation of nascent kinase chains in the *cdc37* mutant during pulse labeling was not observed in all cases. Cdc28 levels were similar in wild-type and *cdc37* mutant strains after pulse labeling, but the kinase was degraded rapidly within a 30-min period. These findings are consistent with previous studies showing that Cdc37 promotes Cdc28 stability (Gerber et al., 1995; Farrell and Morgan, 2000). Whether Cdc28 represents

a distinct class of kinase that is degraded by a different pathway is unclear. In our experiments, Cdc28 was untagged, contrasting with the C-terminal TAP-tagged kinases used in all other experiments and providing a possible source for the phenotypic distinction. However, it is also possible that Cdc28 is protected in its prefolded form by other chaperones and that loss of Cdc37 function manifests at a slightly later stage in its maturation. This explanation for the delayed degradation of Cdc28 compared with Tpk2 or Rim11 may also account for why geldanamycin promotes degradation only after kinase synthesis. As shown in Fig. 4 D, Tpk2 is completely synthesized and stable for the duration of a 5-min pulse even in the presence of geldanamycin, although it is rapidly degraded thereafter. These results are consistent with the posttranslational role attributed to Hsp90 and with nascent kinases being stabilized by other chaperones, including Cdc37 and Ydj1/Hsp70, before interacting with Hsp90 for the final stages in maturation.

The temperature-sensitive phenotype for kinase stability in the *cdc37*[S14A] mutant allowed us to further dissect the different roles for this chaperone in kinase biogenesis. For Rim11 and Tpk2, the increase in kinase stability in *cdc37*[S14A] when grown at 26°C did not lead to full activity; rather, a twofold decrease in activity was observed. For Tpk2, the decrease in activity of the isolated kinase correlated with decreased binding of its inhibitory subunit Bcy1 in cell lysates. This could be caused by Tpk2 misfolding and/or by increased cAMP levels in the *cdc37*[S14A] cells caused by a combined decrease in Tpk1/2/3 activity (Nikawa et al., 1987). These combined results are consistent with decreased kinase activity in *cdc37*[S14A] mutant cells even when the triage system for degrading misfolded kinases is suppressed by growth at low temperature.

A previous study supports the hypothesis that kinases can accumulate as misfolded conformers that are not degraded. In this case, the deletion of *STI1* or *HSC82* resulted in very low v-Src levels, an effect that was suppressed by the overexpression of full-length or truncated forms of Cdc37 (Lee et al., 2002). Although the full-length Cdc37 also restored v-Src activity, the truncated forms promoted stabilization without large increases in activity. Because v-Src is constitutively active in the absence of other kinases, these data indicate that v-Src accumulates in an improperly folded form that is not degraded. The same is likely true for Sky1, which is also constitutively active (Nolen et al., 2001). Furthermore, the accumulation of misfolded proteins upon the deletion of several different chaperone proteins was recently demonstrated (McClellan et al., 2005). In this case, components of the Hsp90 chaperone machinery were implicated in promoting degradation but not in the folding of a heterologously expressed protein in yeast. For kinases, the triage system may operate to clear the cell of misfolded kinases close to their site of synthesis before they have a chance to aggregate. This can be bypassed by growth at low temperature even in the absence of chaperone function (Fig. 5 A). In this case, the effect of low temperature may compensate for decreased chaperone function and allow the kinases to proceed to a later stage of maturation with a greater probability of achieving the native state. We suspect this has physiological relevance because some kinases interact persistently with chaperones even after initial folding.

For these kinases, chaperones are constantly needed to promote the folded state rather than target the polypeptide for degradation.

In conclusion, our findings show that Cdc37 has a general role in protecting nascent kinase chains from degradation in addition to its function in posttranslational maturation. These findings suggest that Cdc37 is a gatekeeper to cellular kinase abundance. Importantly, yeast Cdc37 synthesis is fairly constant compared with other chaperones whose expression is stress regulated, and this may be a means of limiting kinase abundance in cells.

Materials and methods

Strains and plasmids

The TAP-tagged yeast library based on strain S288C was purchased from Open Biosystems. Strain genotype was verified by sizing individual gene products by Western blotting and PCR analysis using one primer specific to the tag and one primer specific to the gene of interest.

The $URA3$-marked $cdc37^{S14A}$ mutant (pS14AU) was constructed by inserting a 1.2-kb DNA fragment containing the $URA3$ gene into Bcl1-digested pRS314.$cdc37S14A$ (a gift from C. Glover III, The University of Georgia, Athens, GA). A similar plasmid was constructed using wild-type $CDC37$ for microarray analysis. A plasmid encoding HA-tagged Bcy1 was a gift from J. Hirsch (Mount Sinai School of Medicine, New York, NY). A strain deleted for SSB1 and SSB2 was a gift from E. Craig (University of Wisconsin-Madison, Madison, WI).

The $cdc37^{S14A}$ mutant strains were constructed as follows. Wild-type cells containing TAP-tagged kinases were grown overnight. A DNA fragment containing $cdc37^{S14A}$::$URA3$ was prepared for transformation by digesting pS14AU with BamH1, HindIII, and Kpn1. Transformation by the $cdc37^{S14A}$::$URA3$ fragment into the TAP-tagged strains was performed using the standard lithium acetate method as previously described (Gietz et al., 1995) and transformants selected for growth in media lacking uracil. Ura$^+$ colonies were selected for further study based on having a temperature-sensitive lethal growth phenotype at 37°C.

The yeast $erg6\Delta$ knockout strains were constructed as follows. The deletion allele (replaced by the KanMX4 module) was amplified by PCR using primers that were 200 bp upstream of the start codon and 200 bp downstream of the stop codon. The resulting 2-kb products were used to replace the wild-type genes in the TAP-tagged strains under study by a modified version of the transformation protocol of Gietz et al. (1995), which included a heat-shock step at 42°C for 20 min followed by washing the cells with water, resuspension in YPD (yeast extract/peptone/glucose), and incubation at 30°C for 2 h before plating. Successful homologous recombination was determined by selection on YPD media containing 400 μg/ml G418 followed by reselection on complete synthetic dextrose media containing 200 μg/ml G418 minus histidine. Gene replacement was verified by PCR analysis of the KanMX4 module in the proper location using one primer specific to the module (KanB; 5'-CTGCAGCGAGGAGC-CGTAAT-3') and one 300 bp upstream from the start codon where the wild-type gene resided.

Western blot analysis

Western blot analysis was performed on whole cell extracts prepared in IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% NP-40) plus Complete protease inhibitor (Boehringer) by glass bead lysis. An HRP-labeled rabbit Fc fragment followed by chemiluminescent detection was used in most experiments. In some experiments, anticalmodulin-binding peptide antibody was used (Open Biosystems). Some kinases were not detected in whole cell extracts but were readily detected after a single round of isolation on IgG–Sepharose bead resin. Kinases in this class (a total of 16) were Rad53, Sgv1, Cdc7, Prr1, Ydl025c, Sat4, Ste11, Ynr047c, Hrk1, Npr1, Kin1, Ctk1, Ssk22, KKq8, Ipl1, and Ire1.

Pulse labeling and polysome profile analysis

Pulse labeling, immunoprecipitations, and sucrose gradient analysis of polysome profiles were performed as described previously (Brodsky et al., 1998).

Kinase assays

Wild-type and $cdc37^{S14A}$ cells expressing TAP-tagged forms of Rim11, Tpk2, and Sky1 were grown overnight (at 26°C for Rim11 and Tpk2 and at 30°C for Sky1) in 100 ml synthetic complete media (minus His and Ura) to log phase. Cells were harvested, and extracts were prepared using IPP150 (Seraphin et al., 2002). The cell extract concentrations were adjusted to 0.45 ml at 3 mg/ml in IPP150 and were incubated with 50 μl of 50% vol/vol IgG–Sepharose bead resin (GE Healthcare) for 1 h at 4°C. The resin was washed four times using the same buffer and divided into two aliquots. One aliquot was used for Western blot analysis (see above), whereas the other was used for the kinase assay. Kinase assay for Tpk2 was performed using the PepTag Nonradioactive Protein Kinase Assay kit (Promega) according to instructions provided by the manufacturer. Kinase activity was determined by absorbance at A$_{570}$. Rim11's and Sky1's kinase activities were assayed by measuring their ability to phosphorylate MBP (Upstate Biotechnology). In addition to the aforementioned four washes, the resin was subsequently washed once with kinase buffer (10 mM Tris-HCl, pH 7.5, and 10 mM MgCl$_2$). The washed resin was incubated in 20 μl kinase buffer containing 1 μg MBP, 100 μM ATP, and 10 μCi [γ^{32}]ATP (PerkinElmer) for 20 min at room temperature. 20 μl of 2× SDS sample buffer (4°C) was added to stop the kinase reaction. The samples were resolved by denaturing gel electrophoresis. The gel was fixed and dried before autoradiography. Quantitation of MBP phosphorylation was determined using a phosphoimager (Storm 860; Molecular Dynamics) and ImageQuant software (GE Healthcare).

Microarray analysis

Wild-type S288C yeast were converted to $CDC37$::$URA3$ or $cdc37^{S14A}$::$URA3$ as described above. Cultures of each strain were grown to log phase, and total RNA was prepared as described previously (Schmitt et al., 1990). A clean-up procedure was used with an RNeasy kit (QIAGEN) and on-column DNA digestion. Hybridization to S98 chips (Affymetrix, Inc.) and data analysis were performed by the Mount Sinai microarray facility.

Phylogenetic analysis

The analysis shown in Fig. 2 was performed on isolated kinase domains downloaded from http://kinase2.salk.edu. A multiple alignment was performed with ClustalW in the MacVector software package (version 7; Accelrys) followed by phylogenetic analysis using Protdist and Neighbor present in the Phylip 3.6 package (http://evolution.genetics.washington.edu/phylip.html). The tree was drawn using Hypertree (Bingham and Sudarsanam, 2000).

Online supplemental material

Fig. S1 shows that deletion of the vacuolar peptidase $PEP4$ or incubation of yeast cells with PMSF results in the accumulation of kinases Rim11 and Tpk2 but in a manner that is similar in wild-type and $cdc37^{S14A}$ cells. These findings rule out a role for the vacuole as a means of preferential kinase depletion in the $cdc37^{S14A}$ mutant cells. Fig. S2 shows polysome profiles that are similar in wild-type and $cdc37^{S14A}$ mutant cells. The sensitivity of yeast growth on paromomycin is also shown and demonstrates that $cdc37^{S14A}$ mutant cells have a similar sensitivity to wild-type cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200604106/DC1.

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