The Cdc37 protein kinase–binding domain is sufficient for protein kinase activity and cell viability

Paul Lee,1 Jie Rao,1 Albert Fliss,1 Emy Yang,1 Stephen Garrett,2 and Avrom J. Caplan1

1 Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, NY 10029
2 Department of Microbiology and Molecular Genetics, University of Dentistry and Medicine of New Jersey, New Jersey Medical School, Newark, NJ 07103

Cdc37 is a molecular chaperone required for folding of protein kinases. It functions in association with Hsp90, although little is known of its mechanism of action or where it fits into a folding pathway involving other Hsp90 cochaperones. Using a genetic approach with Saccharomyces cerevisiae, we show that CDC37 overexpression suppressed a defect in v-Src folding in yeast deleted for STI1, which recruits Hsp90 to misfolded clients. Expression of CDC37 truncation mutants that were deleted for the Hsp90-binding site stabilized v-Src and led to some folding in both sti1Δ and hsc82Δ strains. The protein kinase–binding domain of Cdc37 was sufficient for yeast cell viability and permitted efficient signaling through the yeast MAP kinase–signaling pathway. We propose a model in which Cdc37 can function independently of Hsp90, although its ability to do so is restricted by its normally low expression levels. This may be a form of regulation by which cells restrict access to Cdc37 until it has passed through a triage involving other chaperones such as Hsp70 and Hsp90.

Introduction

The role of Hsp90 in protein folding appears to be restricted to signal-transducing proteins, represented largely by steroid hormone receptors and protein kinases (Caplan, 1999). Although protein kinases represent a large and abundant protein family, much more is known about Hsp90 mechanism of action in folding of steroid hormone receptors (Pratt and Toft, 1997). The overall scheme involves sequential action by Hsp70/Hsp40 chaperones followed by Hsp90 and several of its cochaperones. It is thought that Hsp70 acts first by interacting with nascent polypeptide chains in association with Hsp40s. Should a client polypeptide require Hsp90, then it must be recruited. This function is likely to be performed by Hop, a cochaperone that interacts with Hsp90 and Hsp70 via distinct tetratricopeptide repeat sequences (Chen et al., 1996; Scheufler et al., 2000). Hop also regulates Hsp90’s ATPase by stabilizing the ADP-bound form of yeast Hsp90 and by inhibiting client-stimulated ATP hydrolysis by the human homologue (Prodromou et al., 1999; McLaughlin et al., 2002). Recruitment of Hsp90 via Hop leads to the establishment of an “intermediate” complex containing the client polypeptide in association with Hsp70/Hop–Hsp90. Hsp70 and Hop subsequently dissociate from Hsp90 and are replaced by p23 and TPR-containing immunophilins (Smith, 1993). The change from intermediate to “mature” complexes is associated with a change in nucleotide bound to Hsp90, since p23 binds only to Hsp90-ATP (Johnson and Toft, 1995; Fang et al., 1998; Grenert et al., 1999). These interactions ultimately result in establishment of a hormone-binding competent conformation by steroid receptors. Investigators that reconstituted this pathway with purified proteins have proposed that Hsp70 and Hsp90 are the only essential chaperone components for folding of glucocorticoid receptor (GR)* and that other cochaperones such as Hsp40 or Hop play supporting roles that stimulate the reaction (Morishima et al., 2000; Rajapandi et al., 2000). Similar studies with the closely related progesterone receptor, however, showed that Hsp40, Hop, and p23 are also necessary for folding and hormone binding (Kosano et al., 1998). Similarly, studies with a viral reverse transcriptase indicated that Hop and Hsp40, in addition to Hsp90 and Hsp70, are required for activity, whereas p23 is not essential but stimulates the reaction (Hu et al., 2002). The requirement of cochaperones for folding has also been demonstrated in yeast, since deletion of YDJ1 (a yeast Hsp40) or STI1 (yeast Hop) strongly affects the activity of exogenous Hsp90 clients such as the androgen receptor and v-Src (Caplan et al.,

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Address correspondence to Avrom J. Caplan, Department of Pharmacology and Biological Chemistry, Box 1603, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Tel.: (212) 241-6563. Fax: (212) 860-1174. E-mail: avrom.caplan@mssm.edu

*Abbreviation used in this paper: GR, glucocorticoid receptor.
Figure 1. **Cdc37 overexpression suppresses v-Src activity defect in sti1Δ yeast.** (A) Western blot analysis using antiphosphotyrosine (top), anti-v-Src (middle), and antiphosphoglycerate kinase (bottom, Pak). Extracts were prepared after induction of v-Src for 6 h from wild type (lane 1) and sti1Δ strains minus and plus Cdc37 overexpression (lanes 2 and 3). Cells were grown at 25°C. (B) Extracts were prepared as described for A, except that cells were grown at 30°C. (C) Pull-down assay with GST-Hsp90. V-Src was induced by growth of wild-type (WT) and sti1Δ cells in galactose-containing media. Lane 1, wild-type cells not expressing GST-hsp90; lane 2, wild-type cells expressing GST-Hsp90; lane 3, wild-type cells expressing GST-Hsp90 plus overexpressed Cdc37; lane 4, sti1Δ cells expressing GST-Hsp90; lane 5, sti1Δ cells expressing GST-Hsp90 plus overexpressed Cdc37. The bottom two panels show Western blots of proteins isolated on GST-agarose resin. The top two panels show Western blots of proteins in cell extracts.

**Results**

**STI1** encodes an *S. cerevisiae* homologue of Hop, the hsp-organizing protein that coordinates the combined action of Hsp70 and Hsp90 on unfolded or misfolded proteins (Fryman and Hohfeld, 1997). Deletion of **STI1** has little effect on cell growth under normal conditions, but results in decreased growth rate at high temperatures (Nicolet and Craig, 1989). Even under normal growth conditions, however, **STI1** is important for folding of the oncogenic tyrosine kinase, v-Src, when heterologously expressed in yeast (Chang et al., 1997). To further investigate chaperone function, we searched for suppressors of v-Src loss of activity in yeast cells deleted for **STI1** (sti1Δ) using a reverse genetic approach. Three genes were chosen for initial studies: **CDC37**, **SBA1**, and **CNS1**. **CDC37** was chosen since it is essential for v-Src folding yet its action within the pathway of chaperone action is unknown. **CNS1** was chosen since it has some similarity with **STI1** (Dolinski et al., 1998; Marsh et al., 1998). **SBA1** is the yeast homologue of mammalian p23, which is thought to function downstream of Hop in steroid receptor activation and displayed genetic interactions with **STI1** (Smith, 1993; Bohen, 1998; Fang et al., 1998). Each of these genes was overexpressed in sti1Δ cells and v-Src induced by growth in galactose-containing medium for 6 h. The short induction period was necessary since prolonged expression of v-Src is lethal to wild-type cells (Florio et al., 1994). Since yeast has very little endogenous tyrosine kinase activity, it is possible to measure v-Src activity with a monoclonal antiphosphotyrosine on Western blots using whole cell extracts. Induction of v-Src in wild-type cells led to the appearance of multiple bands that reacted with antiphosphotyrosine, as shown in Fig. 1. The intensity of these bands was reduced in sti1Δ cells at 25°C, and almost disappeared when the cells were grown at 30°C. The difference in v-Src activity when cells were grown at the two temperatures partly correlates with the lev-
els of v-Src, which were markedly lower at 30°C. The decreased levels of v-Src in the sti1Δ strain did not reflect the levels of all protein kinases in yeast, since we observed no decrease in the levels of phosphoglycerate kinase (Fig. 1, A and B). Overexpression of SBA1 or CNS1 had no effect on v-Src levels or activity in sti1Δ cells (not shown). However, CDC37 overexpression resulted in increased v-Src activity at close to wild-type levels. The suppression phenotype was more pronounced when the cells were grown at 30°C compared with those grown at 25°C (Fig. 1). CDC37 overexpression also lead to increased levels of v-Src, especially at 30°C, perhaps by stabilizing the protein.

Our hypothesis regarding Cdc37 action in suppressing v-Src loss of function in sti1Δ yeast was based on the known or proposed functions of both Hop and Cdc37 as recruitment factors for Hsp90 (Stepanova et al., 1996; Frydman and Hohfeld, 1997). Both proteins bind to Hsp90 (at distinct sites; Silverstein et al., 1998), although only Cdc37 has been characterized to have chaperone activity. Thus, sti1Δ deletion may affect Hsp90 recruitment to v-Src, and CDC37 overexpression may compensate for this loss by acting to bypass the Hop requirement for Hsp90 recruitment. We therefore analyzed whether increasing Cdc37 dosage in sti1Δ cells stimulated v-Src binding to Hsp90 as a method of testing this hypothesis. The experiment was performed by inducing v-Src expression in yeast cells expressing GST-Hsp90 in the absence or presence of overexpressed Cdc37. Extracts from these cells were used for pull-down assays to determine whether or not v-Src was binding to Hsp90 (Fig. 1 C). In wild-type cells, v-Src binding to Hsp90 was observed using the pull-down assay and GST-tagged Hsp90 with or without Cdc37 overexpression. By contrast, there was no observable binding of v-Src to GST-Hsp90 in extracts from sti1Δ cells (Fig. 1 C), and Cdc37 overexpression failed to stimulate this association. These data support the hypothesis that Sti1 functions to recruit Hsp90 to client proteins, since this interaction was lost in the sti1Δ strain. On the other hand, it seems clear that Cdc37 does not substitute for Sti1 by promoting stable recruitment of Hsp90 to v-Src in the sti1Δ strain.

The data shown in Fig. 1 C may be explained by one of two possibilities: either Cdc37 functions in association with Hsp90 but fails to promote stable association of Hsp90–v-Src complexes, or it functions independently of Hsp90. To differentiate between these two possibilities, we devised an experimental strategy that uncoupled the ability of Cdc37 to interact with v-Src from its ability to interact with Hsp90. This strategy was based on prior studies showing that the NH2-terminal region of human Cdc37 interacts with protein kinases, whereas the Hsp90-binding site resides toward the middle portion of the protein (Grammatikakis et al., 1999; Scholz et al., 2001). We confirmed this for interaction between human Cdc37 and v-Src. In the experiment shown in Fig. 2 B, we used purified full-length human Cdc37, or a COOH-terminal truncation (Cdc371–173) that was deleted for the putative Hsp90-binding site. Incubation of these recombinant proteins in rabbit reticulocyte lysates resulted in binding of Hsp90 to the full-length Cdc37 only, whereas in vitro synthesized 35S-labeled v-Src bound to both proteins. Based on these results, we generated a series of truncation mutants of yeast Cdc37 that spanned the Hsp90-binding region. Each contained an NH2-terminal His6 tag for subsequent detection by Western blot analysis. Of the eight mutants that were generated, three failed to express in yeast, whereas the rest were soluble (Fig. 2 D). We performed several experiments to assay for interaction between His-tagged Cdc37 or the truncation mutants with Hsp90 in cell extracts, although we failed to detect any physical interactions.
Figure 3.  **Effect of different Cdc37 truncation mutants on v-Src activity in a sti1Δ strain.**  (A) Western blot analysis of tyrosine kinase activity (P-Tyr) by v-Src in sti1Δ yeast overexpressing wild-type (lane 3) or truncated forms of CDC37 (lanes 4–7) as indicated. V-Src–dependent tyrosine kinase activity in wild-type cells and sti1Δ cells with empty vector is shown in lanes 1 and 2. Middle and bottom panels show levels of v-Src and phosphoglycerate kinase (PGK) proteins in the same extracts. Cells were grown at 25°C. (B) Extracts were prepared as described for A except that the cells were grown at 30°C.

This is consistent with studies showing that interaction between yeast Cdc37 and Hsp90 is very weak (Kimura et al., 1997; Siligardi et al., 2002).

We expressed each of the cdc37 truncation mutants in sti1Δ yeast and tested whether any of them could suppress the v-Src activity defect. These studies were performed at both 25°C and 30°C. Remarkably, the Cdc371–388 mutant was as proficient as full-length Cdc37 for suppressing the sti1Δ phenotype, and its expression restored v-Src activity at both temperatures. None of the other truncations was as effective in this regard, although even the Cdc371–148 mutant was capable of stabilizing v-Src protein when induced at 30°C in the sti1Δ background and restoring a small amount of v-Src activity (Fig. 3 B). None of the truncation mutants smaller than Cdc371–388 was very effective at suppressing the v-Src activity defect in sti1Δ at 25°C. This may reflect the relative stability of v-Src at this lower temperature even in the absence of Sti1 (Fig. 3, compare lane 2 of A with that of B). The modest suppression phenotype observed at 30°C by the two smaller truncations (Cdc371–235 and Cdc371–148) may therefore relate to their ability to stabilize v-Src levels. Since these smaller truncations were deleted for the putative Hsp90-binding site, their function in activating v-Src may be Hsp90 independent. However, there is a sharp drop in v-Src activity in cells expressing truncated forms of Cdc37 smaller than Cdc371–388, which correlates with loss of the Hsp90-binding site (Fig. 3 B, compare lanes 3–7). These combined data therefore suggest that Cdc37 cooperates with Hsp90 when it is able to do so, although it can function independently in a less efficient manner when it has to.

We performed further studies to address the relationship between Cdc37 and Hsp90 in v-Src activity. Our approach was to test whether full-length or truncated versions of Cdc37 could suppress v-Src activity defects arising from decreased levels of Hsp90. The rationale was to differentiate between suppression due to Hsp90 interaction with Cdc37 versus Cdc37 being able to function by itself. Previous studies showed that deletion of the yeast HSC82 gene caused a substantial reduction in the cellular concentration of Hsp90 (by >90%) (Borkovich et al., 1989; Xu and Lindquist, 1993), with just enough expression from a second gene (HSP82) to maintain cell viability. Previous studies also demonstrated that v-Src was inactive in an hsc82Δ strain (Xu and Lindquist, 1993), and that Cdc37 overexpression partially suppressed v-Src loss of activity in a conditional hsp82 mutant (Kimura et al., 1997). We first confirmed that v-Src activity was decreased in the hsc82 mutant (Fig. 4), and then assayed for the ability of each Cdc37 truncation mutant to suppress the v-Src activity defect. As shown in Fig. 4 A, the results of this experiment were very similar to those observed for the sti1Δ mutant, although overall levels of suppression by full-length Cdc37 were reduced compared with the sti1Δ strain (Fig. 3). The similarity between the results using the hsc82Δ and sti1Δ strains extended the findings that Cdc371–388 was as effective as full-length Cdc37, whereas the smaller Cdc371–335, Cdc371–259, and Cdc371–148 proteins were effective, but to a lesser degree. Also, each truncation mutant stabilized v-Src to a similar extent independently of its size. Since the hsc82Δ strain was limiting for Hsp90 to begin with, and the smaller truncations were deleted for the putative Hsp90-binding site, we conclude that Hsp90 is not essential for Cdc37 function in protein kinase folding, but that both chaperones cooperate to optimize the reaction. One further possibility that was addressed is whether Cdc37 stimulated intrinsic Hsp90 activity. This was tested by analyzing the effect of Cdc37 on the activity of a protein that depends on Hsp90 but is not a client of Cdc37. The GR represents such a client, and its activity was analyzed in wild-type and sti1Δ cells in which its activity was known to be reduced (Chang et al., 1997). GR activity was measured as deoxycorticosterone induced β-galactosidase activity (from a reporter plasmid under control of hormone responsive elements). In sti1Δ cells, the basal levels of β-galactosidase was increased relative to that found in wild-type cells, but the inducible levels were reduced (Fig. 4 B). The hormone-dependent induction of β-galactosidase was therefore greatly reduced in sti1Δ cells compared with the wild-type from 30-fold induction to approximately sixfold induction. Im-
importantly, Cdc37 overexpression had very little effect on GR activity in either strain, suggesting that Cdc37 overexpression does not affect intrinsic Hsp90 activity.

These findings led us to test whether the protein kinase–binding domain of Cdc37 was sufficient for yeast cell viability. Yeast strains expressing truncated forms of Cdc37 constructed in a cdc37Δ deletion background isolated from sporulated diploids. The growth phenotype of each haploid was scored after tetrad dissection. The results from this study demonstrated that all the truncated forms of Cdc37 could support yeast cell growth with the exception of Cdc371–335. Further studies addressed whether the viability of cells expressing the truncations was dose dependent. We prepared plasmid constructs that expressed full length, Cdc371–335, Cdc371–148, and Cdc371–148, were slow growing and displayed a temperature sensitive lethal phenotype at 37°C (Fig. 5 A). Further studies (not shown). Using a plasmid swap procedure with 5-fluoro-oortic acid, we then determined whether these truncations in low copy number could support growth of cdc37Δ yeast cells. In low copy, only full-length Cdc37 and Cdc371–388 suppressed the lethality of cdc37Δ. In high copy, further suppression was observed by plasmids expressing Cdc371–239 and Cdc371–148, as expected based on the tetrad dissection analysis. The only truncation that failed to support growth in either high or low copy was Cdc371–335 (not shown). These data demonstrate that the protein kinase–binding domain of Cdc37 is sufficient for cell viability if present in a high enough concentration. We also tested whether these mutants could suppress the temperature-sensitive lethal phenotype of cdc37–34, which has a point mutation (serine 14 to leucine; Fliss et al., 1997). As expected, the small truncations failed to do so since they failed to promote growth of cdc37Δ at 37°C, although expression of Cdc371–388 led to viable cells (Fig. 5 D). Interestingly, Cdc371–335 expression had a negative effect on cdc37–34 growth at 25°C. These combined data indicate that the COOH-terminal 118 amino acids of Cdc37 are dispensable for normal function and that Cdc371–335 has abnormal function that is partially dominant–negative in cdc37–34.

The ability of Cdc371–148 to promote v-Src folding in sti1Δ and hsp82Δ strains supports the hypothesis that Cdc37 is sufficient for v-Src folding when Hsp90 is limiting or cannot be recruited to the client. We further tested how well Cdc371–148 could substitute for full-length Cdc37 in cells that were wild type for Hsp90 and Sti1. To this end, we induced v-Src in cdc37Δ yeast cells that expressed Cdc371–148 from a multi-copy plasmid. As shown in Fig. 6 A, the activity of v-Src was much lower in the presence of Cdc371–148 compared with full length Cdc37, similar to the results observed in hsp82Δ and sti1Δ strains. However, a major difference was that the levels of v-Src protein were also markedly lower in the cdc37Δ background than in hsp82Δ and sti1Δ cells. We extended this analysis to an endogenous protein kinase pathway. The system used was signaling from the α-factor mating pheromone via a G-protein–coupled receptor and the MAP kinase pathway. Previous studies showed that one of the kinases in this pathway, Ste11, interacts with Cdc37 and Hsp90, and that signaling was impaired in cdc37Δ and hsp82Δ mutant strains (Abbas-Terki et al., 2000). Using β-galactosidase as a reporter for this pathway, we tested how well Cdc371–148 could substitute for full-length Cdc37. As shown in Fig. 6 B, there was very little signaling in the cdc37Δ–34 mutant, consistent with a previous report (Abbas-Terki et al., 2000). However, the Cdc371–148 truncation facilitated signal transmission at a level that was ~70% of the wild-type value. These data suggest that folding is optimal when Cdc37 and Hsp90 interact with each other, but is also quite strong in the presence of the isolated protein kinase–binding domain of Cdc37.

### Discussion

The role of Cdc37 in protein kinase folding has become firmly established over the past few years. Cdc37 interacts

Figure 4. Effect of Cdc37 overexpression on v-Src activity in an hsp82Δ strain and GR in the sti1Δ strain. (A) Western blot analysis of tyrosine kinase activity (P-Tyr) by v-Src in hsp82Δ yeast overexpressing wild-type (lane 3) or truncated forms of Cdc37 (lanes 4–7) as indicated. V-Src–dependent tyrosine kinase activity in wild-type cells and hsp82Δ cells with empty vector is shown in lanes 1 and 2. Middle and bottom panels show levels of v-Src and phosphoglycerate kinase (PGK) proteins in the same extracts. Cells were grown at 30°C. (B) Cdc37 overexpression does not suppress loss of GR activity in sti1Δ cells. Wild-type (WT) and sti1Δ cells expressing GR were grown to mid-log phase and GR-dependent β-galactosidase activity was induced by addition of 1 μM deoxycorticosterone. Gray bars represent basal β-galactosidase activity in wild-type or sti1Δ cells; black bars represent GR-dependent β-galactosidase activity in WT cells; hatched bars represent β-galactosidase activity in sti1Δ cells. The absence (−) or presence (+) of overexpressed CDC37 is noted. Fold induction (ratio of induced β-galactosidase levels divided by basal levels) is shown above bars. Data shown are the mean of three independent experiments ± standard error.
with many different protein kinases and facilitates their folding in association with Hsp90. Although the exact relationship between these two chaperones is unknown, it has been suggested that Cdc37 targets Hsp90 to misfolded protein kinases (Stepanova et al., 1996). In this capacity, Cdc37 would bear some homology with Hop, which also recruits Hsp90 to misfolded polypeptides bound to Hsp70 (Frydman, 2001). In this report we show that CDC37 overexpression suppressed the defect in v-Src activity resulting from deletion of STI1, the yeast Hop homologue.

The defect in v-Src activity in sti1 Δ was temperature dependent, with the defect being greater when the cells were

Figure 5. The protein kinase–binding domain of Cdc37 is sufficient for viability. (A) Spot test analysis of cdc37Δ cells expressing wild-type (Cdc37) or truncated versions of Cdc37 as indicated in the figure. 10-fold dilutions of saturated cultures were plated (3 μl each) and incubated at 30°C and 37°C. (B) Western blot analysis of proteins eluted from Ni-NTA resin after incubation with extracts expressing vector alone (lane 1) or full-length histidine-tagged Cdc37 on a multicopy plasmid (lanes 2–5). Cdc371–239 was coexpressed with full-length Cdc37 on low copy number (lane 2) or high copy number plasmids (lane 4). Cdc371–148 was similarly expressed from low copy number or high copy number plasmids (lanes 3 and 5, respectively). Proteins that reacted with anti-His6 that bound nonspecifically to the resin are labeled with an asterisk. (C) Growth of cells expressing different forms of CDC37 from low copy or high copy number plasmids as indicated. Cells are shown spotted onto plates or presence of 5-fluoroorotic acid (5-FOA) to counterselect for full-length Cdc37. (D) Spot test analysis of growth of cdc37–34 mutant yeast expressing truncated versions of Cdc37 as indicated. The cells were grown at 25°C or 37°C.

Figure 6. Protein kinase activity in cdc37Δ cells expressing Cdc371–148 protein. (A) Phosphotyrosine activity in cells expressing v-Src. Lane 1, wild-type cells; lane 2, cdc37Δ cells expressing full-length Cdc37; lane 3, cdc37Δ cells expressing Cdc371–148. Western blot with anti-phosphotyrosine (top), Western blot with anti-v-Src (middle), and Western blot with anti-phosphoglycerate kinase (PGK) (bottom). (B) β-galactosidase activity as a measure of MAP kinase signaling. Cells treated with or without α-fator were assayed for Ste12-dependent lacZ gene expression. All strains were cdc37Δ expressing either cdc37–34, full-length Cdc37, or Cdc371–148 proteins as indicated.
grown at 30°C compared with 25°C. This is correlated with decreased levels of v-Src when the cells were grown at 30°C. We interpret these findings in terms of v-Src being less stable at the higher temperature combined with its inability to undergo refolding in the sti1Δ mutant. This defect probably arises due to lack of Hsp90 recruitment to v-Src (Fig. 1 C). Although overexpression of CDC37 suppressed the loss of v-Src activity, it does so in a manner that is independent of any stable recruitment of v-Src to Hsp90; although this finding alone does not rule out the possibility that full-length Cdc37 acts via Hsp90 in the suppression process. Since Cdc37 and Hsp90 are only weakly interacting in yeast, it is possible that Cdc37 interacts with both v-Src and Hsp90 in sti1Δ, but in a complex that rapidly dissociates or is sensitive to our extraction procedure. Our model is that Cdc37 acts downstream of Sti1 in the folding pathway, and that Sti1 normally functions to recruit Hsp90 (and perhaps Cdc37) (Abbas-Terki et al., 2002) to misfolded clients already bound to Hsp70 (Fig. 7). The bypass function of overexpressed CDC37 probably reflects the ability of Cdc37 protein to interact with both Hsp90 and v-Src. Since it is normally expressed at low levels, Cdc37 probably represents a limiting factor for protein kinase folding that must be recruited to the client via interaction with Hsp90 and Sti1. However, our results are also consistent with Cdc37 being able to function in an Hsp90-independent manner. This is because Cdc37 truncations lacking the Hsp90-binding site were still functional for v-Src folding in sti1Δ and hsc82Δ cells, albeit at reduced efficiency. The isolated protein kinase–binding domain of Cdc37 was also sufficient for cell viability and signaling via the MAP kinase–signaling pathway (Figs. 5 and 6). However, whether Cdc37 can function in a completely Hsp90-independent manner remains unclear, since it is still possible that both chaperones could function together even though they fail to interact with each other. Supporting this notion is the intriguing finding that a truncated version of Cdc37 that failed to interact directly with Hsp90 could still stimulate Hsp90 recruitment to a protein kinase in animal cells, although this was far less than the effect of full-length Cdc37 (Scholz et al., 2000). Thus, it is still possible that truncated versions of Cdc37 could permit association of a protein kinase with Hsp90, perhaps via allosteric effects on the client itself. However, since Cdc371–148 facilitated some v-Src activity under conditions where Hsp90 could not be recruited to the client (Fig. 3), or was already present in reduced amounts (Fig. 4), we propose that the protein kinase–binding domain of Cdc37 is sufficient for a limited amount of v-Src folding independently of Hsp90 function. We also conclude that the COOH-terminal 118 amino acids of Cdc37 are dispensable for function since Cdc371–388 behaved just like full-length Cdc37 for v-Src folding and cell viability (Figs. 3, 4, and 5). This is consistent with a previous finding showing that cells expressing Cdc37 truncated to amino acid 360 were viable but temperature-sensitive for growth (Gerber et al., 1995).

Although Cdc37 overexpression suppressed loss of function by Hop and Hsp90, we have thus far been unable to suppress defects in v-Src folding in YDJ1 mutant strains (Dey et al., 1996) in the same manner. YDJ1 encodes an Hsp40 protein that functions in association with Hsp70 probably at the earliest stages of protein folding (Cheetham and Caplan, 1998; Frydman, 2001). We have recently shown, however, that SSE1 overexpression suppressed loss of v-Src activity in a ydj1 mutant strain (Goeckeler et al., 2002). SSE1, a member of the Hsp110 family, is similar in structure to Hsp70 and also interacts with Hsp90 (Liu et al., 1999). It is therefore possible that SSE1 suppressed defects in the ydj1 mutant because it has homologous action to Hsp70. This suggests that Hsp70 function is an essential step in v-Src folding that cannot be bypassed by Cdc37, unlike the actions of Sti1 or Hsp90. The role of other cochaperones such as p23 (encoded by SBA1) is more speculative, since its deletion does not strongly affect v-Src folding in yeast (Fang et al., 1998), although it is possible that Cdc37 and p23 form a complex with Hsp90 in animal cells (Hartson et al., 2000).

The ability of Cdc37 to bypass Hsp90 chaperone components may occur only under certain conditions such as its overexpression, which occurs in cancer cells (Stepanova et al., 2000b). Previous studies have shown that Cdc37 is onco-
genic when overexpressed in mice, and its levels are greatly increased in human prostate cancer tissues (Stepanova et al., 2000a,b). These data suggest that Cdc37 levels must be tightly regulated. We propose that this reflects a form of regulation whereby Cdc37 is recruited only to those protein kinases that have been through a trisome system involving other chaperones, such as Hsp70/Hsp40 and Hsp90. In this manner, the cell maintains tight control over exposure of client kinases to Cdc37, and prevents inappropriate stabilization or activation. Indeed, recent studies demonstrated that Cdk4 activation destabilized by C/EBPα correlates with dissocation of Cdc37/Hsp90 from Cdk4, suggesting that chaperone-dependent stabilization plays an important role in cell cycle progression (Wang et al., 2002a). It is possible that Cdc37 overexpression could override such controls and contribute to the transformation process.

Materials and methods

Yeast strains and methods

The W3031b (MATα) strain background was used for the sti1Δ and hisc2Δ studies. The cdc37 Δ mutants were derived from strain BY4743 as part of S. cerevisiae gene deletion project (Winston et al., 1999). Haploids containing the cdc37 Δ gene were isolated from tetrads. Yeast cells were grown in selective media (0.67% yeast nitrogen base, 2% glucose supplemented with amino acids to complement auxotrophic markers). Yeast transformation was performed by the lithium acetate method as described previously (Gietz et al., 1995). Plasmid swap experiments were performed by plating cells onto media containing 5-Fluoroorotic acid to counterselect for plasmids encoding URA3 as described previously (Sikorski and Boeke, 1991).

v-Src induction, extract preparation, and Western blot analysis

v-Src was induced from the Gal1–10 promoter by growth in media containing 2% galactose for 6 h. Cells were grown in 2% rafinose containing media before induction. Extracts were prepared by glass bead lysis in 20 mM HEPES, pH 7.5, 100 mM KCl, 0.1 mM EDTA plus protease inhibitors contained in the complete protease inhibitor cocktail by Boehringer. Cells were lysed with 0.4-mm glass beads using 3× 1-min pulses in a bead beater with 1-min rests in between at 4°C. Extracts were cleared at 14,000 g for 10 min. Lysates were resolved by denaturing gel electrophoresis and transferred to Immobilon-P membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20 overnight). Levels of tyrosine kinase activity were determined by the lithium acetate method as described previously (Gietz et al., 1995). Plasmid swap experiments were performed by plating cells onto media containing 5-Fluoroorotic acid to counterselect for plasmids encoding URA3 as described previously (Sikorski and Boeke, 1991).

GST-Hsp90 pull-down assays

Wild-type and sti1Δ yeast cells expressing GST-Hsp90 (from p2U/4SP52-GST; a gift from D. Picard, Université de Geneve, Geneva, Switzerland) were grown in 200 ml 0.67% yeast nitrogen base plus 2% rafinose to an OD600 of 0.2. Galactose was added to 2% to induce v-Src expression for an additional 6 h. The cells were harvested, and cell extracts were prepared as described above, but with 20 mM Tris-HCl, pH 8, 200 mM NaCl, 1 mM EDTA, pH 8, 0.5% NP-40, 25 μM PMSF plus protease inhibitors contained in the complete protease inhibitor cocktail by Boehringer. The extracts were adjusted to 3 mg/ml in the same buffer and incubated with 50 μl glutathione-agarose resin (ICN Biomedicals) for 2 h at 4°C. The resin was collected by low speed centrifugation for 2 min, followed by four washes in the same buffer. Proteins retained by the resin were dissolved in sample buffer, resolved by gel electrophoresis, and analyzed by Western blot.

Plasmid assay

The plasmid-encoding poly-histidine (His6) tagged yeast Cdc37 under control of the yeast ADH1 promoter, pPL2 (TRP1, 2 μm), was described previously (Rao et al., 2001). To construct a URA3 version of this plasmid, the insert and promoter region were excised with Kpn1 and Sac1, and ligated into pRS426 (Christianson et al., 1992) to generate pPL3. Truncated forms of His6-Cdc37 were generated by one of two different methods. Cdc371–173, Cdc371–239, and Cdc371–148 were generated by digesting pPL3 with XbaI/Kpn1, StuI/Kpn1, EcoRI/Kpn1, PstI/Kpn1, and ClaI/Kpn1, respectively. The digested plasmids were gel-purified and converted to blunt ends with DNA polymerase I klenow fragment. They were subsequently ligated with T4 DNA ligase and transformed into E. coli XLI-Blue. Cdc371–335 and Cdc371–282 were generated by PCR amplification of Cdc37 from pRS52 (Fliss et al., 1997). The upstream primer 5′-AAGCTT-GCATACCCACACACGACGCTATGATCTGAAG-3′ was used for both constructs. The downstream primer for Cdc371–173 was 5′-GGATCC-TATGCACCACCACACCCACGCCATGATTACTCTAAG-3′ and for Cdc371–282 5′-GGTACCATCACGTTCCCTTGAAGAT-3′. PCR products were generated with Pfu1 polymerase in 20 cycles using 50°C annealing temperature. The products were gel-purified and sub-cloned first into pCR-Script and then using HindIII and Kpn1 into pRS426. Plasmids encoding Cdc371–335, Cdc371–235, and Cdc371–135 were subsequently subcloned into pRS423 (His3, 2 μm) and pRS313 (His3, CEN/AARS) after Pfu1 digestion. The plasmid encoding v-Src used in these studies was constructed by digestion of prs316-vsrc (a gift from Dr. Frank Boschelli, Wyeth-Ayerst, Pearl River, NY) with NotI/XhoI, and ligation into prs425 (Leu2, 2 μm). The plasmid encoding full-length His6 human Cdc37 for expression in E. coli (pET15b,Cdc31) was described previously (Rao et al., 2001). The truncated His6 human Cdc371–173 was prepared by PCR amplification from pET15b.Cdc37 using the primers 5′-GATATGCTGGAACATCGGCTG-3′ and 5′-GATACCTCCTATTTGCTGATCCGAGGC-3′ using Pfu1 in a 20-cycle reaction at 55°C annealing temperature. The product was gel-purified and sub-cloned into pCR-Script. The resulting plasmid was digested with Nde1 and BamH1 and the fragment ligated into pET15b.

Cdc37 binding reactions

The expression and purification of His6,Cdc37 and His6,Cdc371–173 and the binding reactions with v-Src translated in rabbit reticulocyte lysates were performed exactly as described previously (Rao et al., 2001).

MAP kinase and GR signaling assays

Wild-type and cdc37Δ/cdc37Δ cells were transformed by pPRE-LacZ (a gift from K. Morano, University of Texas Medical School, Houston, TX) that expresses β-galactosidase under control of Ste12 response elements. These cells were grown to mid-log growth phase at 25°C and incubated with 5 μM α-factor (Sigma-Aldrich) for 3 h. Extracts were prepared as above in 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.1 mM EDTA plus protease inhibitors contained in the complete protease inhibitor cocktail by Boehringer, and β-galactosidase activity was measured exactly as described previously (Caplan et al., 1995). GR activity was assayed by a similar assay in the absence or presence of 1 μM deoxycorticosterone (Fliss et al., 1997) using the Tropix kit.

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