The molecular chaperone Cdc37 is required for Ste11 function and pheromone-induced cell cycle arrest

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Abstract The molecular chaperone Cdc37 is thought to act in part as a targeting subunit of the heat-shock protein 90 (Hsp90) chaperone complex. We demonstrate here that Cdc37 is required for activity of the kinase Ste11 in budding yeast. A cdc37 mutant strain is defective in Ste11-mediated pheromone signaling and in accumulation and functional maturation of the constitutively active Ste11ΔN. Moreover, Cdc37, Ste11ΔN and Hsp90 coprecipitate pairwise. Thus, Hsp90 and Cdc37 may transiently associate with Ste11 to promote proper folding and/or association with additional regulatory factors. Our results establish Ste11 as the first endogenous Cdc37 client protein in yeast.

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1. Introduction

cdc37 was initially discovered in the budding yeast Saccharomyces cerevisiae as a temperature-sensitive cell division cycle (Cdc) mutation that arrests cells at Start in G1 at the non-permissive temperature [1]. Why it does that and why the null mutation is lethal [1,2] remains poorly understood. Genetic interactions have been found between CDC37 and the genes encoding the p34cdc2-type cell cycle kinase homologs Cdc2 in Drosophila [3] and Cdc28 in yeast [2,4], the kinases Mps1 [5] and Kin28 [6], and the isoforms Hsc82 and Hsp82 of the molecular chaperone heat-shock protein 90 (Hsp90) [7] in yeast. Whereas biochemical interactions have not been reported for these four proteins, the reduced levels and/or activities of Mps1 and Cdc28 in yeast strains with cdc37 mutations might account for the observed defects in spindle pole body duplication [5] and cell cycle progression [2].

In contrast to Drosophila and yeast, the vertebrate homolog p50Cdc37 has been discovered by virtue of it being a 50 kDa component of multisubunit complexes with pp60src and other members of the Src kinase family [8–14], the kinases Cdk4 [15,16], Cdk6 [17] and Raf-1 [18,19]. These complexes also contain Hsp90, which binds Cdc37 directly [20]. In vitro, yeast Cdc37 has been demonstrated to be a molecular chaperone [7]. Thus, work with a variety of organisms over the last decade has led to the view that Cdc37 is a molecular chaperone that may have some specificity for protein kinases and perhaps as a co-chaperone also direct Hsp90 to these substrates (reviewed in [21–23]).

Yeast Cdc37 diverges from mammalian and Drosophila p50Cdc37/Cdc37 quite considerably. Overall, it shares only about 20% sequence identity at the amino acid level with human Cdc37 with the highest conservation in the N-terminal 50 amino acids [15]. Several observations suggest that at least some Cdc37 functions may be conserved between yeast and multicellular organisms: (i) Drosophila CDC37 can complement a cdc37 yeast strain [3]; (ii) yeast CDC37 is required for activity of vertebrate pp60src expressed in yeast [24], and (iii) its overexpression can rescue pp60src activity in a hsp90 mutant strain [7].

p50Cdc37/Cdc37 is required for sevenless signaling in Drosophila [3], and in mammalian cells for Raf-1 function and thus for growth factor signaling to mitogen-activated protein kinase (MAPK) [25]. We therefore wondered whether the Cdc37 function in MAPK pathways is conserved in the budding yeast despite the sequence divergence. We set out to examine the pheromone signaling pathway since it is one of those MAPK pathways that are dependent on the Raf equivalent Ste11 (reviewed in [26,27]), which we have previously shown to require the Cdc37 co-chaperone Hsp90 [28]. Binding of mating pheromone to transmembrane receptors elicits a series of events including the sequential activation of the kinases Ste11, Ste7 and Fus3, leading to morphological changes, a cell cycle arrest in G1 and the expression of specific genes in preparation for mating. For the first time, we provide combined genetic and biochemical evidence for an endogenous Cdc37 function in yeast.

2. Materials and methods

2.1. Plasmids

The wild-type CDC37 open reading frame was obtained by PCR amplification of yeast genomic DNA using the following oligos: 5’-CTGGATCCTCAAAGTCAAATAATGCCCATTTGATTCTT-3’ and 5’-TGGAGCTCTTTAGTCGCGCATGTTAATAGCTA-3’. The BamHI-SacI cut PCR product and a SacI-BglII fragment from plasmid pGHer [29] were introduced into the BamHI site of the yeast expression vector pG-1 [30] to yield the plasmid pG1/Cdc37. The PCR product was also introduced into the BamHI-SacI of plasmid pSP73 (Promega). To obtain construct pGEX2/CDC37 for expression of a glutathione-S-transferase (GST)/Cdc37 fusion protein, BamHI-EcoRI and EcoRI-EcoRI fragments from pSP73/CDC37 were introduced into BamHI-EcoRI sites of the bacterial expression vector pGEX2T (Pharmacia). The recombinant pYesGST/Cdc37 for galactose-inducible expression of the same fusion protein in yeast contains the GST coding sequences as a SacI-BamHI fragment linked to the above-mentioned CDC37 fragments in pYES2.0 (Invitrogen). Recombinant pYesADE2/HA-Ste11 is identical to plasmid pYes/HA-

Abbreviations: Cdc, cell cycle division; GST, glutathione-S-transferase; Hsp90, heat-shock protein 90; MAPK, mitogen-activated protein kinase

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2.2. Yeast strains

Strain DP007 was made by mating of strain 8A7 (MATa cdc37-34 leu2 lys2 trp1 ura3-52) with p360 (VPI1, leu2-3,112 trp1-1 ura3-52). After sporulation and tetrad dissection, spots were tested for the cdc37-34 phenotype (temperature sensitivity). A MATa cdc37-34 spore was selected as DP007.

2.3. Growth arrest induced by α-factor or by Ste11α expression

To monitor the cell cycle arrest in response to α-factor, cells were diluted to a density of 1.2×10^5 cells/ml and spotted or streaked onto YEPD plates containing 10 mM Na-citrate pH 4.3 and, where indicated, 0.2 μM α-factor (Bachem, Switzerland), and incubated at 32°C. For the Ste11α-induced growth arrest, the two strains, RMY326 and DP007, were transformed with plasmids pYES2.0, pYes/Ste11α or pYes/Ste11α-GST, and single colonies were streaked on plates containing glucose or galactose as carbon sources. The plates were incubated at 30°C for several days.

2.4. GST pull-down experiments

GST pull-down experiments were performed as follows: yeast cells (strain 8A7) were grown to 1×10^7 cells/ml with 1 ml of DTT and 1 mM PMSF, and once with TEG (25 mM Tris-HCl pH 7.5, 15 mM EGTA, 10 mM DTT, 1 mM PMSF, 3 μg/ml chymostatin, 1.5 μg/ml pepstatin A, 0.75 μg/ml leupeptin, 3.8 μg/ml antipain) containing 150 mM NaCl. Cell pellets were then resuspended in a small volume of TEG but with glass beads by 30 s pulses at maximum speed in a Mini-BeadBeater-8 (Biospec, Bartlesville) at 4°C. After centrifugation at 15'000 rpm in a table top centrifuge, the supernatant was quantitated and adjusted to 0.1% Triton X-100. Glutathione-Sepharose beads (Pharmacia) were added to the extracts, tumbled for 30 min at 4°C, washed three times with TEG containing 150 mM NaCl, 0.1% Triton X-100 and twice with TEG and 10% glycerol. Membranes were washed three times for 10 min with PBS and once with TEG. 3 mg of yeast protein extract from wild-type strain strain RMY326 was added to each sample and incubated with PBS and once with TEG. After the GST pull-down, the beads with Ste11α were adjusted to 0.1% Triton X-100 and separated by 10% SDS-PAGE. The recombinant proteins GST and GST-Cdc37 were expressed in the Escherichia coli strain XLI-Blue (Stratagene) and extracted in a phosphate-buffered saline buffer (PBS) with 1 mM DTT, 1 mM PMSF, 3 μg/ml chymostatin, 1.5 μg/ml pepstatin A, 0.75 μg/ml leupeptin and 3.8 μg/ml antipain by sonication after induction of protein expression of the amino-terminal regulatory domain of Ste11 (Ste11α) greatly facilitates the biochemical analysis (see below), and it is therefore used in most subsequent experiments. The strain DP007 with the cdc37-34 allele fails to be growth-arrested by Ste11α-GST unless it is complemented by the wild-type CDC37 gene (Fig. 1C). The same pattern was observed with the original cdc37-34 strain 8A7, which has the opposite mating type (MATα) (data not shown). Thus, these experiments showed that the requirement for Cdc37 is independent of mating type and possibly at the level of Ste11 or downstream of it.

3.2. Accumulation and maturation of Ste11 are defective in a cdc37-34 background

We have previously reported that the accumulation of Ste11
is reduced in strains in which the molecular chaperone Hsp90 is altered genetically [28]; this effect can be mimicked pharmacologically by blocking Hsp90 function with the benzoxquinone ansamycin Macbecin I (data not shown). Moreover, the vertebrate tyrosine kinase pp60c-src is produced at lower levels and is less active upon expression in a cdc37-34 yeast strain at permissive temperature [24]. We therefore decided to examine the levels and the activity of Ste11N.GST expressed in strain 8A7 with and without wild-type CDC37. Fig. 2A shows that the steady-state level of Ste11N.GST expressed from a galactose-inducible promoter is reduced in the mutant strain. The levels of other proteins, for example of endogenous Hsp82 or of β-galactosidase also expressed from a galactose-inducible promoter, were not affected by the mutation (data not shown). Since Ste11N.GST is expressed from a heterologous promoter, this suggests that it might be the degradation of Ste11N.GST that is increased in the cdc37-34 strain.

We next performed an in vitro kinase assay with Ste11N.GST pulled down from yeast extracts with glutathione beads (Fig. 2B). Phosphorylation of Ste11N.GST is reduced 8-fold in the absence of wild-type Cdc37. Thus, both accumulation of Ste11N.GST and relative phosphorylation of what does accumulate are reduced in the mutant strain. These findings are consistent with the idea that Cdc37 is required for the accumulation and maturation not only of the exogenously expressed Ste11N.GST, but also of the endogenous wild-type Ste11 protein, and that these defects are responsible for the signaling defect described above.

3.3. Pairwise complexes of Cdc37, Ste11 and Hsp90

Several lines of evidence indicated that Ste11 might associate with both Cdc37 and Hsp90. We had previously shown that Ste11 forms complexes with Hsp90 in yeast [28], and the vertebrate Ste11 equivalent Raf-1 had been demonstrated to form complexes with both Cdc37 and Hsp90 [25]. We therefore examined the presence of pairwise complexes in wild-type cells with GST pull-down experiments. Fig. 3A shows that Ste11N.GST expressed in yeast is associated with endogenous Cdc37 whereas GST only pulls down background levels. In the converse experiment, a GST-Cdc37 fusion protein, but not GST alone, pulls down exogenously expressed epitope-tagged full-length Ste11 (Fig. 3B). This demonstrates that the association of Cdc37 and Ste11 is not restricted to the N-terminally truncated Ste11N. Moreover, the yeast Hsp90 isoform Hsp82, expressed as a GST fusion protein in yeast, but not GST alone, pulls down endogenous Cdc37 (Fig. 3C). To characterize the latter interaction further, we examined whether yeast Hsp90 could be retained by a recombinant GST-Cdc37 fusion protein from a total yeast extract. Note that such an N-terminally tagged Cdc37 can be fully functional as the same fusion protein is able to complement a cdc37-34 strain at non-permissive temperature (data not shown).
shown). As can be seen in Fig. 3D, GST-Cdc37 specifically pulls out the two Hsp90 isoforms Hsp82 and Hsc82. Since no other band of similar intensity is visible in the Coomassie-stained gel, we tentatively conclude that the interaction between Cdc37 and yeast Hsp90 is direct. Due to the severely reduced levels in a cdc37-34 strain of both the mutant Cdc37 itself [35] and its client Ste11p [9,10], we have not been able to assess the effects of the mutation on all relevant pairwise complexes although Ste11-Hsp90 complexes still seem to form (data not shown).

4. Discussion

We have shown that CDC37 is required for pheromone signaling, growth arrest induced by a constitutive Ste11 kinase, and accumulation and activity of Ste11. Moreover, we have demonstrated that Cdc37 and Ste11 form complexes. Taken together, these results argue very strongly that Ste11 function depends on Cdc37. Whether all signaling pathways that require Ste11 [26,27] are defective remains to be determined. However, this seems highly likely in view of the low level and activity of Ste11 in a cdc37 mutant strain. Hence, Cdc37 might be required for pheromone signaling as well as the osmoregulatory response and the response to low nitrogen levels.

Several observations support the conclusion that Ste11 is a direct target of Cdc37: (i) two different epitope-tagged versions of Ste11 associate with Cdc37; (ii) accumulation and phosphorylation of a Ste11-GST fusion protein are reduced; (iii) Hsp90, another molecular chaperone that is required for...
Stell1 function [28], associates with Cdc37. These results parallel those obtained in the vertebrate system. The vertebrate Ste11 equivalent Raf-1, with its catalytic domain, binds directly to human p50Cdc37 [20]. Interaction of Raf-1 with Hsp90 appears to be both direct and indirect through the formation of a ternary complex with p50Cdc37 [25]. Raf-1 function and signaling are blocked by a truncated version of p50Cdc37 that is unable to recruit Raf-1 into a ternary complex with Hsp90 [25]. Although we have been able to demonstrate pairwise associations of Cdc37, Ste11 and Hsp90 in the yeast system (Fig. 4), novel approaches need to be developed to determine the stoichiometry and dynamics of these complexes, in general, and whether the three proteins form a ternary complex, in particular.

Our findings are consistent with the idea developed for the metazoan systems [25] that the two molecular chaperones Hsp90 and Cdc37 coordinate, and perhaps only transiently, intervene in the folding of newly synthesized Ste11 and/or in directing additional cofactors to Ste11. When Cdc37 or Hsp90 functions are defective, Ste11 may not properly fold or associate with other factors. As a consequence, it does not become functional and is eventually degraded more rapidly (see also [28]). Allele-specific effects, such as those observed with Ste11 (see above and [28]), have been reported for pp60SRC expressed in cdc37 [24] and hsp90 [7,36–38] mutant yeast strains. Indeed, the cdc37-34 allele was selected for as a mutation that relieves the toxicity of overexpressed pp60SRC in yeast. Decreases in accumulation and specific activity of pp60SRC both contribute to reduce the toxicity in cdc37-34 cells even at permissive temperature [24]. In contrast, cdc37-1 is a milder allele that is associated with resistance to both pp60SRC [24] and Ste11ΔN at permissive temperature. We have recently discovered that the eIF2α kinase Gen2 is an Hsp90 substrate and that the general amino acid control, a Gen2-dependent response to limiting levels of amino acids, is defective in both cdc37-1 and cdc37-34 strains [39]. Whereas reduced accumulation and functional ‘maturation’ was also reported for Cdc28 in a cdc37-1 mutant strain [2], Cdc28 defects may not lead to increased degradation of all Cdc37 client proteins. Mps1, another putative Cdc37 client protein in yeast, accumulates to the same levels but its kinase activity is markedly reduced [5].

It is interesting to compare Cdc37 with Hsp90. They are both essential for viability, biochemically and genetically associated with a whole panel of kinases, and they have very similar chaperone activities in vitro (reviewed in [21–23]). However, they cannot replace each other in yeast or in Drosophila except perhaps for a subset of substrates such as pp60SRC expressed in yeast [7]. Whereas Hsp90 is very highly conserved with 60% sequence identity between human and yeast, Cdc37 is not (only 20%). It is conceivable that higher order structural elements of the Cdc37-Hsp90 interface are conserved. Although Cdc37 appears to bind to Hsp90 directly both in vertebrates [20] and in yeast (see above), Cdc37 and Hsp90 certainly do not always come together. Hsp90, the more abundant partner, associates with several other cofactors whose binding to Hsp90 sterically prevents the binding of p50Cdc37 [20]. As a result, Hsp90 in association with these other cofactors is likely to have a distinct set of client proteins. It remains to be seen whether the converse is true as well, whether there are Cdc37 clients that do not need Hsp90. Unlike Ste11 [28] and Gen2 [39], Cdc28, Kin28 and Mps1, although genetically linked to Cdc37, have not been shown to form complexes with either Cdc37 or Hsp90 in yeast [2,5,6]. To explore further the similarities and differences between Cdc37 and Hsp90, it will be interesting to determine whether SSF1, HCH1 and CNS1, which have been discovered as allele-specific suppressors of temperature-sensitive hsp90 mutations [40], are able to suppress cdc37 mutations. It is noteworthy, though, that CDC37 itself is the only gene that we have been able to recover as a high-copy suppressor of cdc37 (data not shown). More proteins that interact with Cdc37 need to be identified to assess whether it acts ‘solely’ as one of several Hsp90 co-chaperones.

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