Cdc37 is a molecular chaperone that is important for the stability and activity of several protein kinases, including Cdk4 and Raf1. We first determined, using in vitro assays, that Cdc37 binds to the amino-terminal lobe of Cdk4. Subsequent mutagenesis revealed that Gly-15 (G15A) and Gly-18 (G18A) were critical for Cdc37-Cdk4 complex formation. Gly-15 and Gly-18 of Cdk4 are within the conserved Gly-X-Gly-X-Gly motif that is required for ATP binding to the kinase. Mutation of either glycine at the equivalent positions of Raf1 (G358A and G361A) also inhibited Cdc37 binding to Raf1. Replacing another conserved residue critical for ATP binding and kinase activity, Lys-35 (K35A), reduced Cdc37-Cdk4 complex formation but to a lesser extent. The interaction of Cdk4 with Cdc37 in vitro was not sensitive to changes in ATP levels. Cell-based assays indicated that Cdk4G15A and Cdk4G18A were present at the same level as wild type Cdk4. Equivalent amounts of p16 bound to Cdk4G15A and Cdk4G18A relative to wild type Cdk4, suggesting that Cdc37G15A and Cdc37G18A adopt significant tertiary structure. However, in contrast to wild type Cdk4, Cdk4G15A and Cdk4G18A had greatly reduced binding of cyclin D1, Cdc37, and Hsp90. Importantly, overexpression of Cdc37 not only stimulated cyclin D1 binding to wild type Cdk4 but also restored its binding to Cdk4G15A. Under the same conditions, p16 binding to wild type Cdk4 was suppressed. Our findings show that the interaction of Cdc37 with its client protein kinases requires amino acid residues within a motif that is present in many protein kinases.

Molecular chaperones assist protein folding reactions in cells. They function by repeated binding and release cycles that facilitate the folding reaction and prevent polypeptide aggregation. Molecular chaperones of the Hsp90 class function in the folding and activation of client proteins involved in signal transduction, including many transcription factors and protein kinases. Hsp90 does not act alone in this capacity but in association with several different co-chaperones. Hsp90 also functions downstream of Hsp70, which interacts with many nascent polypeptide chains (1).

Co-chaperones are non-client chaperone-binding proteins that can also have chaperone activity. Among the many co-chaperones that interact with Hsp90, Cdc37 is distinguished by having a preferential interaction with protein kinases. Cdc37 was originally identified as a gene required for cell cycle progression in the budding yeast *Saccharomyces cerevisiae* (2). Yeast strains with temperature-sensitive alleles of CDC37 (cdc37-1 or cdc37-34) arrest in late G1 or in G2/M (2, 3). Subsequent studies indicated that cdc37-1 confers a defect in the binding and activation of the yeast cyclin-dependent kinase Cdc28 by the G1 cyclin Cln2 or the G2/M cyclin Clb2 (4). Genetic and biochemical evidence has suggested Cdc37 interacts with other protein kinases in yeast, including casein kinase II (5), Ste11 (6), Kin28 (7), and Mps1 (8), and is important for the stability and activity of these protein kinase mutants. Mutations in the *Drosophila* CDC37 gene impair the signaling activity of the sevenless receptor tyrosine kinase (9) and also lead to defects in chromosome segregation and cytokinesis via its action on the aurora B kinase (10).

Vertebrate Cdc37 was originally identified as a 50-kDa component of a complex that includes v-Src and Hsp90 (11). Mammalian Cdc37 shares 20 and 45% sequence identity with yeast and *Drosophila* Cdc37, respectively. It can bind to or affect the activity of a broad range of protein kinases, including cyclin-dependent kinases (12–14), v-Src (15, 16), Raf1 (17–19), and Akt (20). Clients of mammalian Cdc37 also include other proteins such as viral reverse transcriptase (21), androgen receptor (22), and atrial natriuretic peptide receptor (23). The fact that Cdc37 usually acts in concert with Hsp90 leads to the hypothesis that Cdc37 is a kinase-targeting subunit of Hsp90 (24). However, it is far from clear whether all the protein kinases that interact with Cdc37 are Hsp90-dependent. Several lines of evidence indicate that Cdc37 itself has chaperone activity. Overexpression of Cdc37 can compensate for Hsp90 in terms of maintaining v-Src activity, although these two proteins are not completely interchangeable (25, 26). Cdc37 regulates the ATPase activity of Hsp90 by suppressing ATP turnover, which is hypothesized to facilitate client loading onto Hsp90 (27). A truncated form of Cdc37 that no longer binds Hsp90 is still capable of partially rescuing the catalytic activity of a temperature-sensitive mutant of Hck and promoting its association with Hsp90 (28). A similar Cdc37 mutant defective in Hsp90 binding maintains the ability to interact specifically with the reverse transcriptase of the duck hepatitis B virus both in vitro and in vivo (21).
Whether Cdc37 facilitates cyclin D1 binding independently of its role in Cdk4 folding is unclear. Increasing the levels of Cdc37 in insect cells stimulated the activity of Cdk4 without any apparent change in the amount of cyclin D1 binding to the kinase. On the other hand, studies performed with recombinant proteins showed that Cdc37 could stimulate cyclin D1 binding to Cdk4 (12, 14). In the present study, we define critical amino acids in Cdk4 that form part of the Cdc37-binding site and are important for subsequent interaction between Cdk4 and cyclin D1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HCT116 cells (ATCC CCL-247) were maintained in Invitrogen. RPMI 1640 medium was supplemented with 10% fetal bovine serum, 1% minimum Eagle’s medium non-essential amino acid solution, and 50 μg/ml gentamicin (Invitrogen). Cells were plated the day before transfection and transfected at 90–95% confluence using LipofectAMINE 2000 (Invitrogen) and standard procedures.

The total amount of DNA was kept at 24 μg for the transfection of a 10-cm dish: 24 μg (wild type or mutant) of FLAG-Cdk4 plasmid for Fig. 4A; 12 μg (wild type or mutant) of FLAG-Cdk4 plasmid and 12 μg of cyclin D1 plasmid for Fig. 4B; 6 μg (wild type or mutant) of FLAG-Cdk4 plasmid, 6 μg of cyclin D1 plasmid; and 12 μg of Cdc37 plasmid (or the control plasmid) for Fig. 5.

DNA Cloning and Site-directed Mutagenesis—For in vitro transcription and translation, the Kozak sequence and GST open reading frame from pGEX-5 (Amersham Biosciences), followed by a spacer containing the Not1 restriction site, were subcloned between HindIII and BamHI sites of pSP64 poly(A) vector (Promega). BamHI and Not1 restriction sites were introduced at the 5′ and 3′ ends of full-length Cdk4 and all deletion constructs. PCR products were gel-purified, enzyme-digested, and inserted between BamHI and NotI sites of pSP64 poly(A) in-frame with GST. For in vivo expression, DNA oligonucleotides that encode the Kozak sequence and FLAG tag were inserted between the Nhel and BamHI sites of pIRE5neo2 (Invitrogen), followed by Cdk4 sequence as a BamHI-NotI fragment. The open reading frame of cyclin D1 was cloned into the Nhel-NotI sites of pIRE5neo2. The construction of the Cdc37 expression plasmid was described previously (22). Mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Stratagene). All constructs were confirmed by DNA sequencing.

Protein Purification, in Vitro Transcription/Translation, Binding Experiment, and Kinase Assay—Cdc37 was directly cloned by PCR from a human placenta cDNA library (Invitrogen). Subcloning, expression, and purification of His6-Cdc37 were described previously (22). For in vitro transcription/translation, 1 μg of DNA template was added to 40 μl of Tnt Quick Master Mix (Promega), supplemented with 20 μM methionine, and incubated at 30 °C for 90 min. Once the reaction was complete, the mixture was left on ice for 5 min before protease inhibitors (Calbiochem) and 1 μg of His6-Cdc37 were added. Proteins were allowed to bind at 4 °C for 2 h with gentle agitation. Nickel-nitrilotriacetic acid-agarose (Qiagen) was washed three times using wash buffer. For immunoprecipitation, 80 μl of 1× sample buffer was added, and the sample was heated at 95 °C.

Western Blot—GST, Cdk4, and phospho-MEK-1 antibodies were obtained from Upstate Biotechnology, Inc. Cdc37 antibody was from BD Biosciences. Hsp70, Hsp90, cyclin D1, and p16 antibodies were obtained from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies and chemiluminescence reagents were obtained from Amersham Biosciences.

RESULTS

Cdc37 Binds to the Amino-terminal Lobe of Cdk4—Several groups have reported interaction between Cdk4 and Cdc37 both in vitro and in vivo (12–14). We developed an in vitro binding assay based on this relatively tight association to define the minimal sequence that Cdc37 recognizes. GST-Cdk4, produced by reticulocyte lysate-based coupled transcription/translation, was able to bind His6-Cdc37 purified from bacteria. Under these conditions, GST-Cdk4 did not bind a control His6-tagged protein (47-2, a gift from Dr. Kiran Khandoke) nor did GST associate with His6-Cdc37, indicating this interaction was specific (data not shown). We then generated a series of deletions extending inward from the carboxyl terminus of Cdk4 (Fig. 4A), and we examined the ability of these polypeptides to associate with Cdc37 in the binding assay. Two of them exhibited even higher affinity to Cdc37 than the full-length protein (lanes 7 and 8, Fig. 1B).
CDks are structurally similar to one another, consisting of an NH₂-terminal lobe and COOH-terminal lobe, with the ATP-binding pocket and catalytic site located between the two lobes (29). The shorter one of the two CDk4 polypeptides that bind tighter to Cdc37, consisting of amino acids 1–103, turned out to be the NH₂-terminal lobe of Cdk4 (Cdk4-N). Mort-Bontemps-Soret et al. (30) made a similar observation that yeast Cdc37 associates strongly with the NH₂-terminal lobe of Cdc28 but not with full-length Cdc28, in a two-hybrid assay. The conservation of this interaction, despite the fact that yeast and human Cdc37 proteins share only ~20% sequence identity, suggests that the association of the NH₂-terminal lobe with Cdc37 is of fundamental importance. We therefore examined this association in greater detail.

Gly-X-Gly-X-Gly Motif Is Required for Cdc37-Kinase Interaction—The amino termini of CDks consist primarily of β-sheets, with the P3(L)STIRE helix located between β3 and β4 (Fig. 2A) (31–36). We found that upon deleting from the COOH-terminal end of Cdk4-N, polypeptides lacking β5 and β4 maintained most of the binding capacity, but further removal of the loop between β4 and the PLSTIRE helix caused complete loss of interaction (Fig. 2B). Reducing the length of the loop sequence by two amino acids at a time revealed that His-68 and Pro-69 were critical for binding to Cdc37 (Fig. 2C). However, substitution of His-68 and Pro-69 by alanine either individually or together did not reduce the interaction (data not shown).

Examination of deletions extending in from the NH₂ terminus of Cdk4-N revealed that removal of the first 15 amino acids greatly reduced the association of Cdk4-N with Cdc37 (Fig. 2D). However, deletion of up to 10 amino acids from the NH₂ terminus caused no effect, indicating that amino acids 11–15 are involved in Cdc37 binding (Fig. 2E). This region partially overlaps with the Gly-X-Gly-X-Gly motif found in many protein kinases and nucleotide-binding proteins, which helps to anchor the non-transferable phosphates of ATP (37, 38). To explore the role of the Gly-X-Gly-X-Gly motif for the in vitro Cdk4-Cdc37 interaction, the three conserved glycine residues were individually mutated. While changing the first glycine to alanine had no effect, replacing the second or the third glycine almost abolished binding of Cdk4-N to Cdc37 (Fig. 3A). Interestingly, when the same mutations were introduced into full-length Cdk4, similar results were observed for the in vitro binding reaction (Fig. 3B). The Gly-X-Gly-X-Gly motif, however, is not sufficient for interaction with Cdc37. A 30-amino acid peptide containing the Gly-X-Gly-X-Gly motif and adjacent sequence failed to bind Cdc37 (data not shown).

In an effort to determine the generality of the Gly-X-Gly-X-Gly motif requirement, these equivalent mutations were introduced into Raf1, which is another Cdc37 client protein kinase. As observed with Cdk4, replacing the second or third glycine with alanine also prevented the association of Raf1 with Cdc37 (Fig. 3C). Therefore, involvement of the Gly-X-Gly-X-Gly motif in binding to Cdc37 is not unique to Cdk4. The Gly-X-Gly-X-Gly motif is believed to participate in anchoring the non-transferable phosphates of ATP (37, 38). We determined the effect of individually mutating these glycines on the kinase activity of Raf1. Changing the first glycine to alanine slightly reduced the kinase activity of Raf1, as measured by MEK-1 phosphorylation (Fig. 3C). By contrast, mutating either the second or third glycine to alanine almost completely abolished the kinase activity of Raf1. This reduced kinase activity could be due to reduced binding of Raf1 to ATP, to Cdc37, or to both.

The crystal structure of cyclin A-bound Cdk2 indicates that the hydrophobic base of ATP fits into the cleft between the two lobes, and three conserved residues, Lys-33, Glu-51, and Asp-
Gly-X-Gly-X-Gly Motif Required for Cdc37-Kinase Interaction

The data shown above demonstrate the importance of Gly-15 and Gly-18 residues within the amino-terminal lobe of Cdk4 for Cdc37 binding. These glycines are located between the β1- and β2-sheets that form part of the p16-binding site on Cdk6 (36). However, neither glycine is predicted to interact directly with p16 based on the Cdk6 crystal structure. Our identification of Gly-15 and Gly-18 as being important for Cdc37 binding is consistent with previous observations that Cdc37 and p16 bind competitively with each other (14), indicating that the sites of interaction of Cdc37 and p16 on Cdk4 are adjacent or overlapping.

The Gly-X-Gly-X-Gly motif is found in many protein kinases and nucleotide-binding proteins and helps to anchor the non-transferable phosphates of ATP (37, 38). We envision three possible models for the interaction of Cdc37 with Cdk4. One model is that Cdc37 recognizes the ATP-Cdk4 complex and that a decrease in ATP binding to Cdk4G15A or Cdk4G18A accounts for the observed reduction in Cdc37 binding. Against this possibility is the finding that the same amount of Cdk4 (either wild type or G15A mutant) is bound to Cdc37 regardless of whether no ATP or 5 mM ATP (or 5 mM ATP-s) is included in cyclin D1, it did lead to an increase in the amount of cyclin D1 associated with wild type Cdk4 and, more importantly, restored cyclin D1 binding to Cdk4G15A (Fig. 5). By contrast, overexpression of Cdc37 inhibited p16 binding to Cdk4 by more than 50%. These changes were not due to enhanced Hsp90 binding, since the immunoprecipitation complex contained approximately the same amount of Hsp90, with or without Cdc37 overexpression. Immunoprecipitation of cyclin D1 revealed similar results, where the amount of Cdc37 recovered in the cyclin D1 complex correlates well with that of FLAG-Cdk4 (Fig. 5). The presence of Cdc37 in the cyclin D1 immunoprecipitates is dependent on Cdk4 because it requires co-overexpression of FLAG-Cdk4 (data not shown) and because mutation of Gly-15 to alanine in FLAG-Cdk4 reduces the amount of Cdc37 in the cyclin D1 immunoprecipitates (Fig. 5). Therefore, a stable ternary complex can be formed where cyclin D1 binds to Cdk4, which in turn is bound to Cdc37.

**DISCUSSION**

Cdc37 Promotes Cyclin D1 Binding and Inhibits p16 Binding—The role of Gly-X-Gly-X-Gly motif in the Cdc37-Cdk4 interaction was further studied in cells. Wild type and mutant forms of full-length Cdk4 were transiently expressed as FLAG-tagged proteins and immunoprecipitated from cell lysates using anti-FLAG M2-agarose. Both Cdc37 and Hsp90 were detected in immunoprecipitates of wild type Cdk4 and Cdk4G15A. By contrast, binding of both Cdc37 and Hsp90 to Cdk4G18A was substantially reduced (Fig. 4A), consistent with the in vitro data. We also co-expressed cyclin D1 with Cdk4 in order to monitor cyclin D1 interaction with Cdk4. Interestingly, when cyclin D1 was co-expressed with Cdk4, the G15A and G18A mutations greatly reduced the amount of cyclin D1 associated with Cdk4 (Fig. 4B). Not all protein interactions, such as Hsp70 and p16, with Cdk4 are affected by mutations in the Gly-X-Gly-X-Gly motif. The binding of Hsp70, an early and critical step in proper folding of newly synthesized protein, is not affected (24). In addition, the binding of the CDK inhibitor p16 to Cdk4 was not reduced by the G15A or G18A mutation (Fig. 4B). These findings lead to two conclusions: one, that Cdk4G15A and Cdk4G18A adopt a tertiary structure; and two, that the Gly-X-Gly-X-Gly motif is required specifically for interaction of Cdk4 with Cdc37 but not with p16. Although overexpression of Cdc37 did not change the level of Cdk4 and binding in cells.

**Fig. 4.** Cdc37 promotes cyclin D1 binding and inhibits p16 binding in cells. A, G15A and G18A mutation reduces binding of Cdk4 to Cdc37 and Hsp90. Wild type (WT) and mutant FLAG-Cdk4 were transiently expressed in HCT116 cells, and immunoprecipitation was performed using anti-FLAG M2 resin. Samples were analyzed by SDS-PAGE and probed with the indicated antibodies. B, loss of Cdc37 and Hsp90 binding leads to reduced association of Cdk4 with cyclin D1 but not Hsp70 and p16. Wild type and mutant FLAG-Cdk4 were co-expressed with cyclin D1. Immunoprecipitation was performed using anti-FLAG M2 resin, and samples were blotted with appropriate antibodies. Cont. 1, wild type FLAG-Cdk4 was expressed without cyclin D1; Cont. 2, cyclin D1 was expressed without FLAG-Cdk4.

The data shown above demonstrate the importance of Gly-15 and Gly-18 residues within the amino-terminal lobe of Cdk4 for Cdc37 binding. These glycines are located between the β1- and β2-sheets that form part of the p16-binding site on Cdk6 (36). However, neither glycine is predicted to interact directly with p16 based on the Cdk6 crystal structure. Our identification of Gly-15 and Gly-18 as being important for Cdc37 binding is consistent with previous observations that Cdc37 and p16 bind competitively with each other (14), indicating that the sites of interaction of Cdc37 and p16 on Cdk4 are adjacent or overlapping.

The Gly-X-Gly-X-Gly motif is found in many protein kinases and nucleotide-binding proteins and helps to anchor the non-transferable phosphates of ATP (37, 38). We envision three possible models for the interaction of Cdc37 with Cdk4. One model is that Cdc37 recognizes the ATP-Cdk4 complex and that a decrease in ATP binding to Cdk4G15A or Cdk4G18A accounts for the observed reduction in Cdc37 binding. Against this possibility is the finding that the same amount of Cdk4 (either wild type or G15A mutant) is bound to Cdc37 regardless of whether no ATP or 5 mM ATP (or 5 mM ATP-s) is included in
the binding assay. A second model is that the reduced interaction of Cdc37 with Cdk4\(^{G15A}\) or Cdk4\(^{G18A}\) is indirect and due to the reduced kinase activity of these altered Cdk4. In this model, Cdk4 may phosphorylate itself or some other unknown factor(s), and this phosphorylation is required for the interaction of Cdc37 with Cdk4. Against this model is that alterations in the Gly-X-Gly-X-Gly motif reduce the kinase activity of Cdk4.

Most important, while the binding of p16 to Cdk4\(^{G15A}\) and Cdk4\(^{G18A}\) mutants was unimpaired, Cdc37 binding was almost abolished, and cyclin D1 binding was greatly reduced. These findings suggest that Cdc37 assists in the formation of complexes between Cdk4 and cyclin D1 but not between Cdk4 and p16. It would place Cdc37 function in a more regulatory role rather than as a chaperone that functions in general protein kinase folding. On the other hand, there is fairly strong evidence to support the hypothesis that Cdc37 functions to promote protein folding and that its absence renders polypeptide clients susceptible to degradation. This notion has been demonstrated with Cdc28 in yeast (41) and with Cdk4 in terminally differentiating liver cells (42). In our studies, however, we note that Cdk4 mutants that fail to interact with Cdc37 had similar steady state levels compared with the wild type Cdk4 protein, suggesting no great change in polypeptide stability.

Previous studies (14) have already demonstrated the competitive nature of p16 and Cdc37 binding to Cdk4, an observation that is consistent with the tumor suppressor function of the former and the oncogenic character of the latter. Overexpression of Cdc37 not only led to an increase in the amount of cyclin D1 associated with wild type Cdk4 but also restored cyclin D1 binding to Cdk4\(^{G15A}\). At the same time, p16 interaction with wild type Cdk4 was suppressed, consistent with previous findings. The amount of Cdc37 recovered from cyclin D1 immunoprecipitation complex correlates well with that of Cdk4. Overall, the identification of the binding site for Cdc37 on Cdk4 should facilitate development of drug-based therapies that would be effective under conditions where mutant forms of p16 are incapable of inhibiting cell cycle progression.

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