

# Physical Interaction of Mammalian CDC37 with CDK4\*

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**CDC37 was originally identified as a Start gene in budding yeast and has been shown to be required for association of CDC28 with cyclins. The exact functional mechanism by which CDC37 promotes this association, however, remains unknown. CDK4 is a cyclin D-dependent kinase that controls progression through G<sub>1</sub> of the mammalian cell cycle. We have detected a specific association of CDK4 with the molecular chaperon HSP90 and a 44-kDa protein that we identify as mammalian CDC37. A physical interaction between CDC37 and CDK4 suggests that CDC37 may regulate the mammalian cell cycle through a direct effect on CDK4. Association of CDK4 with both CDC37 and HSP90 may also imply a mechanistic link between the functions of CDC37 and HSP90.**

In mammalian cells, ordered activation of cyclin-dependent kinases (CDKs)<sup>1</sup> governs the progression through different phases of the cell cycle (1–4). CDK4 is one of the CDKs that control the G<sub>1</sub>-to-S phase transition. Activation of CDK4 requires binding to one of the D-type cyclins (D1, D2, or D3) (5) and phosphorylation by the CDK-activating kinase (CAK) (6). As cells enter the cycle from quiescence (G<sub>0</sub>) in response to growth signals, CDK4 and cyclin D are synthesized and assemble into CDK4-cyclin D complexes. The assembly of CDK4-cyclin D complexes may also require an as yet unidentified upstream regulatory factor(s), since complex assembly in cells with ectopically expressed CDK4 and cyclin D is still dependent on mitogenic signals (7). Although required for the activation of CDK4, phosphorylation by CAK appears not needed for the assembly of CDK4-cyclin D complexes (6). Therefore, although much has been known about the functional mechanism and regulation of CDKs and their cyclin partners, new regulatory proteins and regulation pathways remain to be uncovered.

In order to identify other proteins involved in CDK4 regulation, we analyzed CDK4 complexes in various mammalian cells by an approach employed in our previous studies (8–11). We report the discovery of a direct association of CDK4 with mammalian CDC37, a protein potentially essential for activation of CDK4.

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<sup>1</sup> The abbreviations used are: CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

## EXPERIMENTAL PROCEDURES

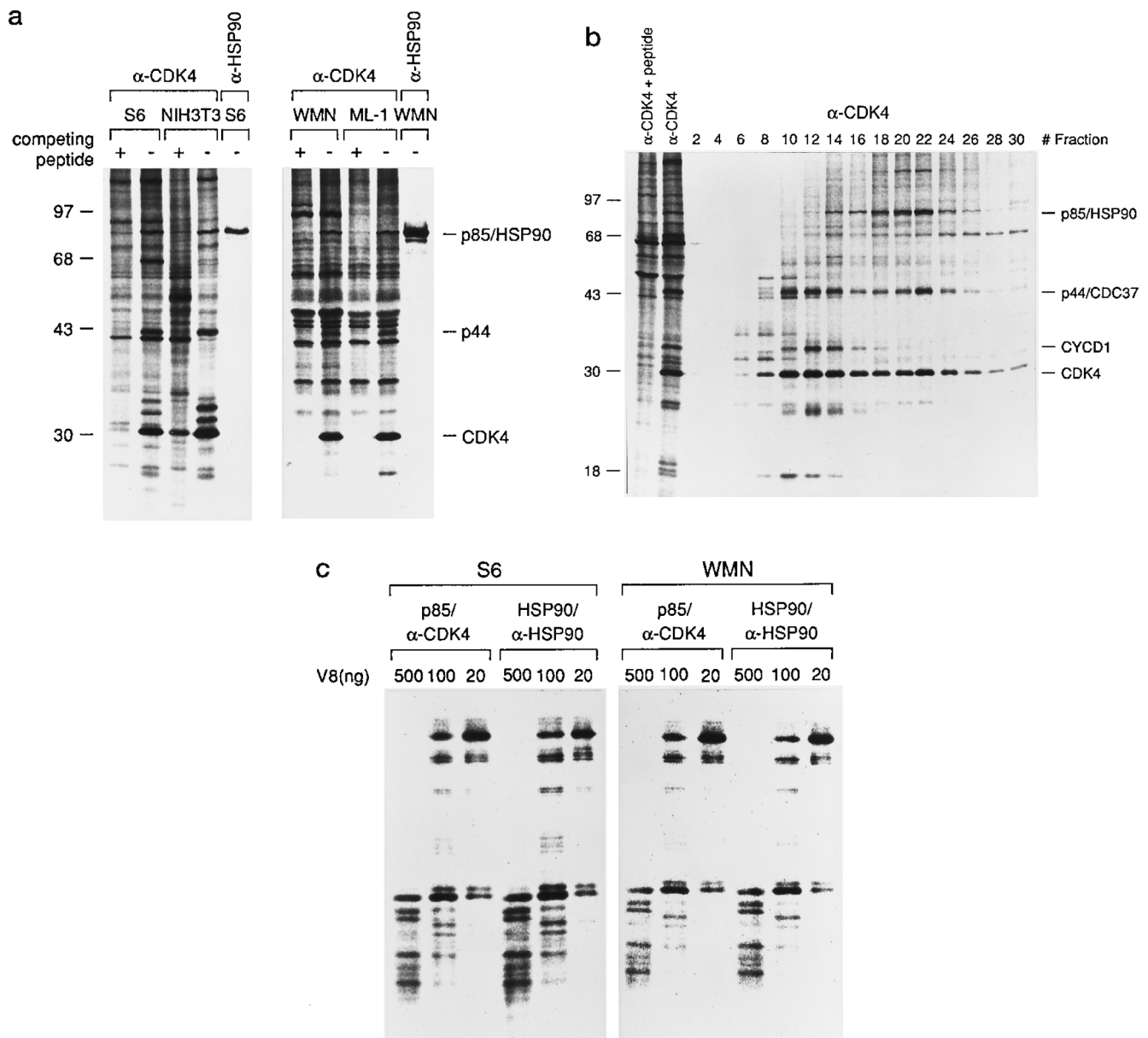
**Cell Lines and Antibodies**—S6, WMN, and ML-1 cells were grown in RPMI 1640 containing 10% fetal bovine serum. NIH3T3 (ATCC CRL 1658) cells were grown in DMEM supplemented with 10% calf serum. The S6 is a subclone of mouse myeloid cell line M1 (13). WMN, a human Burkitt's lymphoma cell line, was kindly provided by Dr. Patrick M. O'Connor (National Cancer Institute, Bethesda, MD). ML-1, a human myeloid cell line, was obtained from cell culture facilities at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY). The antibody used to immunoprecipitate CDK4 from mouse cells was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA: catalog number sc-260). It was raised against a peptide at the carboxyl terminus of CDK4 of mouse origin. The antibody used to immunoprecipitate CDK4 from human cells has been described (8). The anti-HSP90 monoclonal antibody was purchased from StressGen Biotechnologies Corp. (catalog number SPA-830, Victoria, British Columbia, Canada). It exhibits cross-reactivity with HSP90 from human and mouse. In immunoprecipitation, it strongly favors free HSP90 over complexed HSP90. The anti-CDC37 antibody was raised against the GST-CDC37 (human) fusion, and it cross-reacts with mouse CDC37 in immunoprecipitation assay.

**Immunoprecipitation and Glycerol Gradient Sedimentation**—Metabolic labeling of cells with [<sup>35</sup>S]methionine and immunoprecipitation of labeled proteins were performed as described before (9). For glycerol gradient sedimentation analysis, log-phase growing S6 cells were labeled with [<sup>35</sup>S]methionine. The cells were lysed in 300  $\mu$ l of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM sodium vanadate, 50 mM NaF, 5  $\mu$ g/ml each of the following protease inhibitors: aprotinin, leupeptin, soybean trypsin inhibitor, and 1 mM benzamidin). The lysates were cleared of nuclear fractions and loaded onto a 15–35% glycerol gradient prepared in the lysis buffer. The gradient was centrifuged at 40,000 rpm in a SW41.Ti rotor (Beckman) at 4 °C for 36 h. After centrifugation, 300- $\mu$ l fractions were collected from the top of the gradient, diluted in 700  $\mu$ l of lysis buffer, and immunoprecipitated with anti-CDK4 antibody.

**In Vitro Translation, Protein Binding Assay, and V8 Protease Mapping**—Human CDC37, CDKs, CAK, and cyclin D1 were *in vitro* translated using the TNT-lysate *in vitro* translation kit (Promega) following manufacturer's instructions. *In vitro* protein binding assay was carried out essentially as described previously (12). Briefly, GST-CDC37 or GST (negative control) (1  $\mu$ g) was incubated with the *in vitro* translated CDK or cyclin D1 for 30 min at 30 °C in 50  $\mu$ l containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM EGTA. After incubation, 200  $\mu$ l of lysis buffer and 15  $\mu$ l of glutathione-Sepharose were added to the mixture, and the incubation was continued for 1 h. Beads were washed four times with 1 ml of IP buffer before heated for SDS-PAGE analysis. Partial *Staphylococcus aureus* V8 protease mapping of CDC37 and HSP90 was performed as described before (8, 14).

## RESULTS

**Association of CDK4 with p44 and HSP90**—To uncover novel CDK4 regulatory protein(s), we analyzed CDK4 complexes in various cells by immunoprecipitation using anti-CDK4 antibodies. In S6 cells, a mouse myeloid cell line (13), two proteins, one with a molecular mass of 44 kDa, the other 85 kDa, were found to co-immunoprecipitate with CDK4 (Fig. 1a). Similar proteins were also found in CDK4 immunoprecipitates from several other mouse and human cells (Fig. 1a and not shown). No proteins with similar molecular masses have been reported previously to associate with CDK4, and the two proteins were therefore further characterized. To confirm the association of the two proteins with CDK4, extracts of S6 cells were separated by glycerol gradient centrifugation, and the



**FIG. 1. Association of CDK4 with HSP90 and p44.** *a*, analysis of CDK4-associated proteins by immunoprecipitation. [ $^{35}$ S]Methionine-labeled lysates of mouse (*S6*, *NIH3T3*) and human (*WMN*, *ML-1*) cells were immunoprecipitated with anti-CDK4 antibody with (+) or without (–) preincubation with the antigen peptide. The immunoprecipitates were analyzed by 15% SDS-PAGE. The HSP90 proteins in mouse and human cells were immunoprecipitated with an anti-HSP90 antibody and run in the same gel for comparison with CDK4-associated p85. Positions of the molecular mass markers (kDa), CDK4, p85/HSP90, and p44, are marked. *b*, analysis of CDK4 complexes by glycerol gradient sedimentation. [ $^{35}$ S]Methionine-labeled *S6* cell lysate was sedimented in 15–35% glycerol gradient. Proteins in every second fraction were immunoprecipitated with anti-CDK4 antibody and the immunoprecipitates were separated by 15% SDS-PAGE. Immunoprecipitates from the starting total lysate were shown on the left. Positions of CDK4-cyclin D1 (CYCD1), p85/HSP90, and p44/CDC37 are marked. *c*, comparison of p85 with HSP90 by V8 protease mapping. Immunoprecipitates by antibodies to CDK4 or HSP90 were first separated by 15% SDS-PAGE. Protein bands corresponding to p85 and HSP90 (Fig. 1*a*) were excised and digested with V8 protease, and the digests were analyzed by 17.5% SDS-PAGE.

fractions were immunoprecipitated with anti-CDK4 antibodies. Both p44 and p85 were found to co-sediment with CDK4 (Fig. 1*b*). The levels of CDK4 and p44 peaked both in fraction 12 and the heavier fraction 22. Fraction 12 also displays a peak of cyclin D1, whereas the cyclin is absent from the heavier fraction (22), which instead contains p85. Co-sedimentation of CDK4, p44, and p85, however, may suggest that they form a ternary complex.

Previously, we purified an 85-kDa protein as a CDK6-associated protein and identified it as HSP90 by peptide sequencing.<sup>2</sup> To determine whether the CDK4-associated p85 is the

same protein, an anti-HSP90 monoclonal antibody was used to immunoprecipitate HSP90 from mouse or human cells. The immunoprecipitated HSP90 co-migrated with p85 (Fig. 1*a*). V8 proteolytic digest patterns of HSP90 and p85 from both mouse and human cells were essentially identical (Fig. 1*c*). Based on these results, we conclude that the CDK4-associated p85 is HSP90.

**Identification of p44 as Mammalian CDC37**—In order to identify p44, the protein was purified from *S6* cells using an anti-CDK4 affinity column essentially as described previously (11). *S6* was chosen mainly because of the abundance of p44 in the CDK4 immunoprecipitates from these cells. Peptide sequence analysis was carried out on four p44 fragments gener-

<sup>2</sup> K. Dai, R. Kobayashi, and D. Beach, unpublished results.

a

CGCCGTCGCCGCCACCCGAGCCGAGCGGGCTGGGCCGCCAAGCAAGATGGTGGACTAC 60  
M V D Y

AGCGTGTGGGACCACATTGAGGTGTCTGATGATGAAGACGAGACGCCACCCCAACATCGAC 120  
S V W D H I E V S D D E D E T H P N I D

ACGCCAGTCTCTTCGCTGGCGGCATCAGGCCGGGTGGAACGCATGGACAGTTCCAG 180  
T A S L F R W R H Q A R V E R M E Q F Q

AAGGAGAAGGAGAACTGGACAGGGCTGCGCGAGTGCAAGCGCAAGGTGGCCGAGTGC 240  
K E K E E L D R G C R E C K R K V A E C

CAGAGGAACTGAAGGAGCTGGAGGTGGCGGAGGGCGCAAGGCAGAGCTGGAGCGCCTG 300  
Q R K L K E L E V A E G G K A E L E R L

CAGGCCGAGGCACAGCAGCTGCGCAAGGAGGAGCGAGCTGGGAGCAGAAGCTGGAGGAG 360  
Q A E A Q Q L R K E E R S W E Q K L E E

ATGCGCAAGAGGAGAGAGCATGCCCTGGAACGTGGACACGCTCAGCAAGAGCGGCTTC 420  
M R K K E K S M P W N V D T L S K D G F

AGCAAGAGCATGGTAAATACCAAGCCCGAGAAGACGAGGAGGAGTCTCAGAGGAGTGAGG 480  
S K S M V N T K P E K T E E D S E E V R

GAGCAGAAACACAAGACCTTCGTGAAAAATACGAGAAACAGATCAAGCACTTTGGCATG 540  
E Q K H K T F V E K Y E K Q I K H F G M

CTTCGCGCTGGAGTACAGCCAAAGTACCTGTGACAGCAACGTCACCTGGTGTGCGAG 600  
L R R W D D S Q K Y L S D N V H L V C E

GAGACAGCAATTACCTGTGCTATTGGTGCATTGACCTAGAGGTGGAGGAGAAATGTGCA 660  
E T A N Y L V I W C I D L E V E E K C A

CTCATGGAGCAGGTGGCCACACGACAACTGTCATGCAATTTATCTGGAGCTGGCCAAAG 720  
L M E Q V A H Q T I V M Q F I L E L A K

AGCCTAAAGGTGAGCCCGCGGCTGCTTCGCGAGTCTCTCACTAAGATTAAGACAGCC 780  
S L K V D P R A C F R Q F F T K I K T A

GATCGCAGTACATGGAGGGCTTCAACGACGAGCTGGAAGCCTTCAAGAGCGTGTGCGG 840  
D R Q Y M E G F N D E L E A F K E R V R

GGCGTGGCAAGCTGCGCATCGAGAAGGCCATGAAGAGTACGAGGAGGAGGAGCGCAAG 900  
G R A K L R I E K A M K E Y E E E R K

AAGCGCTCGGCCCGCGCGCTGGACCCGCTCGAGGTCTACGAGTCCCTCCCTGAGGAA 960  
K R L G P G G L D P V E V Y E S L P E E

CTCCAGAAGTGCTTCGATGTGAAGACGTGCAGATGCTGCAGGACGCCATCAGCAAGATG 1020  
L Q K C F D V K D V Q M L Q D A I S K M

GACCCACCGACGCAAGATACCATGACAGCGCTGCATGTGACTTGGCTCTGGGTCCCC 1080  
D P T D A K Y H M Q R C I D S G L W V P

AACTCTAAGGCCACGAGGCCAAGAGGAGGAGGAGGAGGAGTCTGGGAGCCATTACTG 1140  
N S K A S E A K E G E E A G P G D P L L

GAAGCTGTCCCAAGACGGCGATGAGAAGGATGTGAGTGTGAGTCTGCCACGCTAC 1200  
E A V P K T G D E K D V S V

ACCGCCACCTGCTTCCAGGCCCTATGTGCCCCCTTTTCAGAAAACAGATAGATGCCATCT 1260  
CGCCGCTCTGACTTCTCTAC 1283

b

1 M - V D Y S V W D H I E V S D D E D - E T H P N I D T A S L Human  
1 M - V D Y S V W D H I E V S D D E D - E T H P N I D T P S L Drosophila  
1 M A I D Y S K W D K I E L S D D S D V E V H P N V D K K S F Yeast

29 F R W R H Q A - - - - - Human  
29 F R W R H Q A - - - - - Drosophila  
31 I K M K Q Q S I H E Q R F K R N Q D I K N L E T Q V D M Y S Yeast

36 - - - R V E R M - - - - - Human  
36 - - - R V E R M - - - - - Drosophila  
61 H L N K R V D R I L S N L P E S S L T D L P A V T K F L N A Yeast

41 - - E Q F Q K E K - E E L D R G C R E C K R K V A E C Q R K Human  
41 - - A E M D H E K - D E L K K K R Q S V Q A R L M D V K E R Drosophila  
91 N F D K M E K S I G E N V D P E I A T Y N E M V E D L F E Q Yeast

68 L K E - - - L E V A E G G - K A E L - - E R L Q A E A Q Q L Human  
68 I S K - - - K D G D E A L K K E L - - E K I E A E G K E L Drosophila  
121 L A K D L D K E G K D S K - S P S L I R D A I L K H R A K I Yeast

92 R K E E R S W E Q K L E E M R K K - - - - - E K S M P W N Human  
93 D R I S - - - - - S E M I R K - - - - - E K K T P W N Drosophila  
150 D S V T - - - - - V E A K K K L D E L Y K E K N D H I S Yeast

V D T L S K

116 V D T L S K D G F S K E M V N T K P E - - - - - K T E E D Human  
110 V D T I S K P O F E K T V I N K K A G - - - - - R K F D E Drosophila  
173 S E D I H - T G F D S E F M N R Q K G A K P L E A T P S E Yeast

140 - - S E E V R E Q K H K T F V E K Y - - - - - Human  
134 N L S E E R E R M K Q F V K E N - - - - - Drosophila  
202 A L S S A A E S N I L N K L A K S S V P Q T F I D F K D D P Yeast

H F G M L

156 - - E K Q I K H F G M L R - - R W D D S O K Y L S D N V H Human  
152 - - E K L C Q Q Y G M L R - - K Y D D S K R K L Q E H L H Drosophila  
232 M K L A N E T E E F G K I S I N E Y S K S Q K F L L E H L P Yeast

181 L V C E E T A N Y L V I W C I D L E V E E K C A Human  
177 L V G E E T A N Y L V I W C I D L E V E E K C A Drosophila  
262 I I S E Q Q K D A L M M K A F E Y Q L H G D D K M T L Q V I Yeast

211 H Q T I V M C E I L E L A K S L K V - - - - - D P R A C F Human  
207 H Q C I C M Q V I L E L A K Q L D V - - - - - D P R A C V Drosophila  
292 H Q S E L M A V I K E I Y D M K K I P Y L N P M E L S N V I Yeast

Y E L E A F K E

235 R Q F F T K I - - K T A D R Q V M E G F N D E L E A F K E R Human  
231 S S P F F S K I - - Q H C H P E Y R A E F D S E I E G F K G R Drosophila  
322 N M F F E K V I F N K D K P M G K E S F L R S V Q E K F L H Yeast

263 V R G R A K - - - - - L R I E K A M K E Y E E E E R K R L G Human  
259 I Q K R A Q - - - - - E K I Q E A I A Q A E E E E R K E R L G Drosophila  
352 I O K R S K I L Q Q E E M D E S N A E G V E T I Q L K S I L D Yeast

289 - - - - - P G G L D P V E V Y E S L - Human  
285 - - - - - P G G L D P A D V F E S L - Drosophila  
382 D S T E L E V N L P D F N S K D P E E M K K V K V F K T L I Yeast

302 P E E L O K C P D V K D V Q M L Q D A I S K M D P T D A K Y Human  
298 P D E L K A C P E S R D V E L L K O K T I A A M P V D V A K L Drosophila  
412 P E K M Q E A I M T K N L D N I N K E F D E D I P I E A E K Yeast

332 H M K R C I D S G L W V P N S K A - - - - - S - - - - Human  
328 H M K R Q V D S G L W V P N S K A - - - - - S - - - - Drosophila  
442 L L E V F N I D I - - I G I K A I L E N E K D F Q S L K D Q Yeast

E A G P G D P L L E A V P K

350 - E A K E G E - - - - - E A G P G D P L L E A V P K Human  
351 - E E D D S D - - - - - D V A G G E E K T D D A K S Drosophila  
471 Y E Q D H E A T M E N L S L N D R D G G G D N H E E V R H Yeast

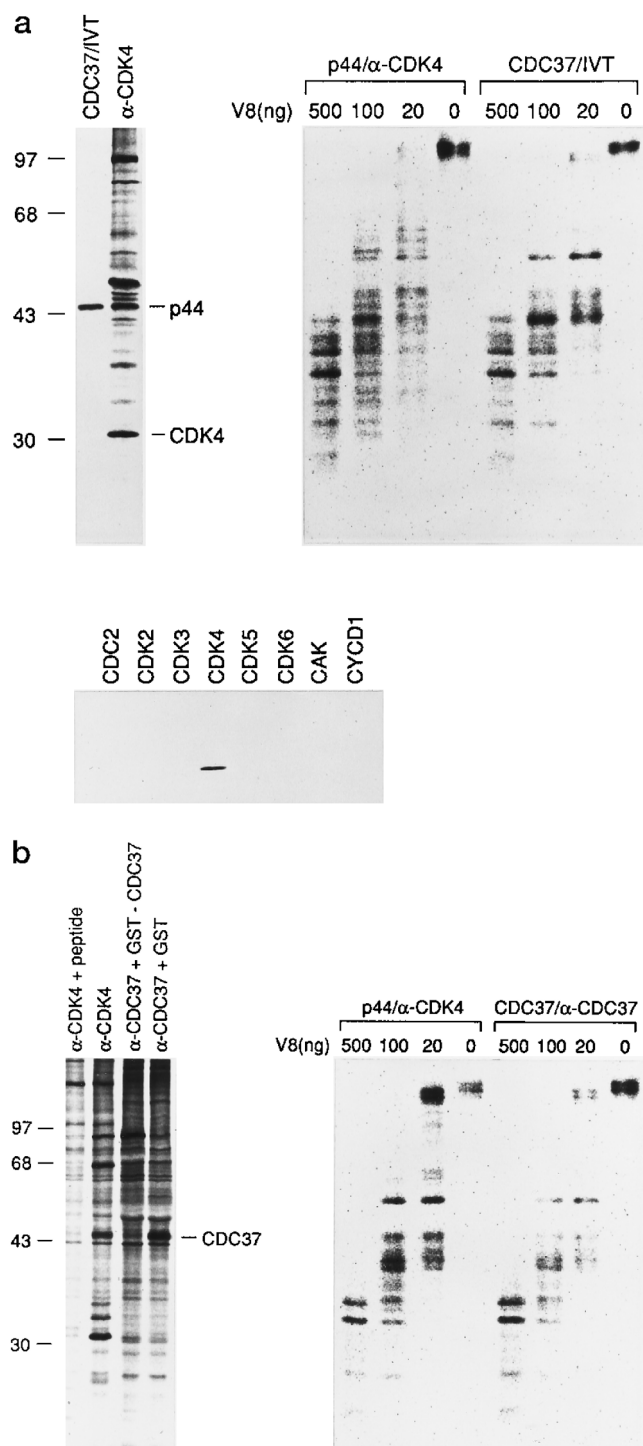
370 T G D E K D V S V Human  
371 E S A A K E E P I Y T G V S T E D V D Drosophila  
501 T A D T V D Yeast

FIG. 2. Sequences of CDC37. *a*, the nucleotide sequence of the human CDC37 cDNA along with the deduced amino acid sequence of the human CDC37 protein. *b*, the deduced amino acid sequence of the human CDC37 protein (Human) is compared with those of the *D. melanogaster* (Drosophila) and *S. cerevisiae* (Yeast) CDC37 proteins. Four peptide sequences obtained from the purified mouse CDC37 is also shown at the top (boxed). Areas of homology was identified using DNA Star MegAlign program. The shaded regions indicate identity.

ated by *Achromobacter lyticus* protease I digestion. Sequences for three of these were either identical or highly related to sequences contained in the chicken and *Drosophila* homologues of CDC37 (15, 16) (Fig. 2*b*). Four human cDNA clones in the dbEST data base that encode a peptide with homology to known CDC37 proteins were found. Clone R87892 (GenBank™ ID) that contains the longest cDNA insert (1.7 kilobase pairs) was obtained from Genome System Inc., and its nucleotide sequence was determined. An open reading frame of 378 amino acids was found (Fig. 2*a*). The putative polypeptide has a predicted molecular mass of 44.5 kDa, and the amino acid sequence has a nearly perfect match with the four mouse p44-derived peptide sequences (Fig. 2*b*). A search of the existing data bases did not reveal significant homology to known polypeptides other than CDC37. The protein shares 45% amino acid identity with *D. melanogaster* CDC37 (16) and 25% amino

acid identity with *S. cerevisiae* CDC37 (17, 18), while the latter two share amino acid identity of 27%. The most conserved region of the CDC37 proteins of different species is at the NH<sub>2</sub> terminus, indicating a possible critical role of this region in the function of CDC37 proteins. Based on the sequence similarity, we conclude that this cDNA encodes the human CDC37 protein.

Several experiments confirmed that CDC37 and the CDK4-associated p44 are the same proteins. The cDNA for human CDC37 was translated *in vitro* and the translation product co-migrated with p44 from human cells in SDS-PAGE gels (Fig. 3*a*, left panel). In addition, the V8 protease digestion patterns of the two proteins were almost indistinguishable (Fig. 3*a*, right panel, note particularly the 500 ng lanes). Furthermore, an anti-CDC37 serum was used to immunoprecipitate mouse CDC37 from S6 cells (Fig. 3*b*). Comparison of the mouse



**FIG. 3. Identification of p44 as mammalian CDC37.** *a*, human CDC37 was *in vitro* translated and compared with the CDK4-associated p44 from WMN cells by electrophoresis in 15% SDS-PAGE (left panel) and by V8 protease mapping (right panel). Lower panel, human CDC37 specifically binds to CDK4. A fusion protein consisting of glutathione *S*-transferase and human CDC37 (GST-hCDC37) was expressed and purified from *E. coli* and mixed with equal amounts of *in vitro* translated, <sup>35</sup>S-labeled CDC2, CDK2, CDK3, CDK4, CDK5, CDK6, CAK, or cyclin D1. Bound proteins were recovered on glutathione-Sepharose and analyzed by 12.5% SDS-PAGE. GST was used as negative control, and it had no binding to any of the above proteins (data not shown). *b*, mouse CDC37 was immunoprecipitated from S6 cells using an anti-CDC37 antibody (left panel) and compared with mouse p44 from the same cells by V8 protease mapping (right panel).

CDC37 with mouse p44 following V8 protease digestion and electrophoresis also demonstrates that they are the same proteins. Finally, the association of CDC37 with CDK4 was tested *in vitro* using a GST-CDC37 fusion expressed and purified from *Escherichia coli*. The human CDC37 fusion protein specifically bound to CDK4 translated *in vitro* (Fig. 3a, lower panel).

#### DISCUSSION

We have demonstrated a physical interaction between mammalian CDC37 and CDK4 in both mouse and human cells. We have also shown evidence for an association of CDK4 with HSP90. The *CDC37* gene was first identified in *S. cerevisiae* by mutations that cause cell cycle arrest in G1 (17). Recently, it has been shown that the yeast CDC37 protein is required for the association of CDC28 with cyclins and that a defect in CDC37 prevents CDC28 activation (19). A similar role might be expected for mammalian CDC37 in the activation of CDK4. Previously, a CAK-associated protein (MAT1) was cloned and shown to facilitate the assembly of the CAK-cyclin H complex (20, 21). If mammalian CDC37 would similarly stimulate CDK4-cyclin D complex assembly remains to be determined. It seems likely, however, that "facilitator" proteins might be a general requirement for the generation of active CDK-cyclin complexes in mammalian cells. Playing a possibly essential role in the activation of their associating CDKs, these proteins could be potential regulation points.

HSP90 functions as a molecular chaperone (22, 23). It is associated with and required for the activity of a number of kinases and steroid receptors (22, 24–27). Increasing evidence suggests that CDC37 may functionally interact with HSP90. In *S. cerevisiae*, either mutations in *CDC37* or a reduction in the level of the yeast HSP90 protein were found to suppress the lethality of overexpression of v-Src (28, 29). In *D. melanogaster*, mutations in *CDC37* and *hsp90* have been isolated in a screen for mutations that exacerbate a defect in the sevenless receptor tyrosine kinase (16). Our finding, in mammalian cells, that CDC37 and HSP90 are both associated with CDK4 and that the three proteins may possibly form a ternary complex could suggest a direct mechanistic link between CDC37 and HSP90. It is possible that CDK37 and HSP90 act in concert to facilitate the proper folding of CDK4 for complex assembly with D-type cyclins. Partner proteins that presumably function in concert with HSP90 have been found in a number of HSP90 complexes with its target proteins, including steroid hormone receptors, Raf and v-Src kinases (22, 27, 30, 31). The exact role and functional mechanism of these proteins are yet to be elucidated. Some studies suggest that association with HSP90 and the partner proteins is important for the stabilization and intracellular trafficking of the target protein (30, 31). Whether CDC37 and HSP90 have a similar effect on CDK4 stability and transport is currently under investigation.

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