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SHORT REPORT

Interaction between Cdc37 and Cdk4 in human cells

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Using the yeast two-hybrid system we have identified novel potential Cdk4 interacting proteins. Here we described the interaction of Cdk4 with a human homologue of the yeast *Drosophila CDC37* gene products. Cdc37 protein specifically interacts with Cdk4 and Cdk6, but not with Cdc2, Cdk2, Cdk3, Cdk5 and any of a number of cyclins tested. Cdc37 is not an inhibitor nor an activator of the Cdk4/cyclin D1 kinase, while it appears to facilitate complex assembly between Cdk4 and cyclin D1 *in vitro*. Cdc37 competes with p16 for binding to Cdk4, suggesting that p16 might exert part of its inhibitory function by affecting the formation of Cdk4/cyclin D1 complexes via Cdc37.

Keywords: Cdk; cyclin; cdc37; hsp90

Progression of eukaryotic cells through the division cycle is controlled by a family of serine/threonine protein kinases, the cyclin dependent kinases (Cdks). Each member of this protein family regulates a specific transition in the cell cycle (Morgan, 1995). The regulated activation of every Cdk at the required cell cycle stage is crucial for maintaining the accuracy of the division process, to the extent that checkpoint mechanisms will intervene to block Cdk activity and cell cycle progression upon perturbation of the system. Cdk activation is controlled by multiple biochemical mechanisms, which include the regulated synthesis of the cyclin and Cdk subunit, the phosphorylation of the Cdk subunit on a conserved threonine residue in the large lobe, the phosphorylation on a tyrosine and a threonine residue within the glycine loop of the small lobe which is counteracted by Cdc25 phosphatases, and the association with inhibitory proteins of the p16 and p21 family (Morgan, 1995). It has also been proposed that additional factor(s) intervene to regulate Cdk complex formation (Matsushime *et al*., 1994). Indeed, the assembly of the Cdk4/cyclin D kinase is thought to be mediated by molecule(s) whose activity is regulated by growth factors (Matsushime *et al*., 1994).

The Cdk4/cyclin D kinase plays an essential role during progression through the G_1 phase of the cell cycle and links mitogen stimulation to the activation of other Cdk kinases (Draetta, 1994; Sherr, 1994; Sherr and Roberts, 1995). In an effort to discover novel, biologically relevant, targets of G_1 cyclin-dependent kinases we set-up a genetic screen using the yeast two hybrid system with the Cdk4 protein as a bait. Through this screen we have identified 74 positive clones, which have presently been characterized. Upon sequencing, one cDNA showed homology to the *CDC37 S. cerevisiae* gene product. *CDC37* was first identified in a screen aimed at the isolation of genes whose function is essential for the proper execution of START, a point in G_1 beyond which yeast cells become committed to a new round of cell division (Reed, 1980). Analysis of genetic interactions between *CDC37* and *CDC28* in *S. cerevisiae* and recently in *D. melanogaster* suggested that CDC37 and CDC28/CDC2 act on a common regulatory pathway to control cell division (Cutforth and Rubin, 1994; Reed *et al*., 1985). Biochemical analysis of the Cdc28 kinase activity in *cdc37*^{ts} cells at the non-permissive temperature showed that G_1 arrest is accompanied by a decrease of the Cdc28 kinase activity. This was shown to be the consequence of decreased complex formation between Cdc28 and both G_1 and mitotic cyclins, suggesting that Cdc37 regulates their association (Gerber *et al*., 1995). A genetic screen in *D. melanogaster* also identified *CDC37* as a component of the *sevenless* tyrosine kinase signaling pathway (Cutforth and Rubin, 1994). During the course of our work, reports have appeared which describe the interaction of the mammalian Cdk4 kinase with Cdc37 (Dai *et al*., 1996; Stepanova *et al*., 1996). Here we describe a genetic screen that led to the identification of human Cdc37 as a subunit of Cdk4, and the potential role of Cdc37 in favoring the interaction of Cdk4 with cyclin D1. The specific interaction of Cdc37 with Cdk4 and Cdk6 suggests that this protein plays a role in controlling cell cycle progression during the G_1 phase of the cell cycle.

We devised a screen to isolate human cDNAs that specifically interact with a LexA-Cdk4 bait in the yeast two-hybrid system using a cDNA library from quiescent, adherent human WI38 (Fields and Song, 1989; Gyuris *et al*., 1993). 5×10^6 independent yeast transformants were screened for interaction with LexA-Cdk4 and 297 clones were isolated. The recovered plasmids were subjected to restriction analysis and from this screen 74 clones were identified that correspond to unique cDNAs. One of the cDNA fragments encoded for a predicted protein having a region of similarity to the *S. cerevisiae* and *D. melanogaster CDC37* gene products (Figure 1) (Cutforth and Rubin, 1994; Ferguson *et al*., 1986) and was also found to be identical to the sequence of

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the human Cdc37 protein recently identified (Stepanova *et al*., 1996; Dai *et al*., 1996). The homology with the yeast and insect proteins started at the first ATG, suggesting that the isolated cDNA spanned the entire open reading frame. Human Cdc37 is 51% identical to the *D. melanogaster* and 25% identical to the *S. cerevisiae* Cdc37 proteins with the highest degree of homology at the N-terminal 40 amino acids (data not shown) (Stepanova *et al*., 1996; Dai *et al*., 1996).

a

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1     CGCCGCCACCCGAGCCGGAGCGGGTTGGGCCGCCAAGGCAAG ATG GTG GAC
3                                                 M   V   D

52    TAC AGC GTG TGG GAC CAC ATT GAG GTG TCT GAT GAT GAA GAC
4      Y   S   V   W   D   H   I   E   V   S   D   D   E   D

94    GAG ACG CAC CCC AAC ATC GAC ACG GCC AGT CTC TTC CGC TGG
18     E   T   H   P   N   I   D   T   A   S   L   F   R   W

136   CGG CAT CAG GCC CGG GTG GAA CGC ATG GAG CAG TTC CAG AAG
32     R   H   Q   A   R   V   E   R   M   E   Q   F   Q   K

178   GAG AAG GAG GAA CTG GAC AGG GGC TGC CGC GAG TGC AAG CGC
46     E   K   E   E   L   D   R   G   C   R   E   C   K   R

220   AAG GTG GCC GAG TGC CAG AGG AAA CTG AAG GAG CTG GAG GTG
60     K   V   A   E   C   Q   R   K   L   K   E   L   E   V

262   GCC GAG GGC GGC AAG GCA GAG CTG GAG CGC CTG CAG GCC GAG
74     A   E   G   G   K   A   E   L   E   R   L   Q   A   E

304   GCA CAG CAG CTG CGC AAG GAG GAG CGG AGC TGG GAG CAG AAG
88     A   Q   Q   L   R   K   E   E   R   S   W   E   Q   K

346   CTG GAG GAG ATG CGC AAG AAG GAG AAG AGC ATG CCC TGG AAC
102    L   E   E   M   R   K   K   E   K   S   M   P   W   N

388   GTG GAC ACG CTC AGC AAA GAC GGC TTC AGC AAG AGC ATG GTA
116    V   D   T   L   S   K   D   G   F   S   K   S   M   V

430   AAT ACC AAG CCC GAG AAG ACG GAG GAG GAC TCA GAG GAG GTG
130    N   T   K   P   E   K   T   E   E   D   S   E   E   V

472   AGG GAG CAG AAA CAC AAG ACC TTC GTG GAA AAA TAC GAG AAA
144    R   E   Q   K   H   K   T   F   V   E   K   Y   E   K

514   CAG ATC AAG CAC TTT GGC ATG CTT CGC CGC TGG GAT GAC AGC
158    Q   I   K   H   F   G   M   L   R   R   W   D   D   S

556   CAA AAG TAC CTG TCA GAC AAC GTC CAC CTG GTG TGC GAG GAG
172    Q   K   Y   L   S   D   N   V   H   L   V   C   E   E

598   ACA GCC AAT TAC CTG GTC ATT TGG TGC ATT GAC CTA GAG GTG
186    T   A   N   Y   L   V   I   W   C   I   D   L   E   V

640   GAG GAG AAA TGT GCA CTC ATG GAG CAG GTG GCC CAC CAG ACA
200    E   E   K   C   A   L   M   E   Q   V   A   H   Q   T

682   ATC GTC ATG CAA TTT ATC CTG GAG CTG GCC AAG AGC CTA AAG
214    I   V   M   Q   F   I   L   E   L   A   K   S   L   K

724   GTG GAC CCC CGG GCC TGC TTC CGG CAG TTC TTC ACT AAG ATT
228    V   D   P   R   A   C   F   R   Q   F   F   T   K   I

766   AAG ACA GCC GAT CGC CAG TAC ATG GAG GGC TTC AAC GAC GAG
242    K   T   A   D   R   Q   Y   M   E   G   F   N   D   E

808   CTG GAA GCC TTC AAG GAG CGT GTG CGG GGC CGT GCC AAG CTG
256    L   E   A   F   K   E   R   V   R   G   R   A   K   L

850   CGC ATC GAG AAG GCC ATG AAG GAG TAC GAG GAG GAG GAG CGC
270    R   I   E   K   A   M   K   E   Y   E   E   E   E   R

892   AAG AAG CGG CTC GGC CCC GGC GGC CTG GAC CCC GTC GAG GTC
284    K   K   R   L   G   P   G   G   L   D   P   V   E   V

934   TAC GAG TCC CTC CCT GAG GAA CTC CAG AAG TGC TTC GAT GTG
298    Y   E   S   L   P   E   E   L   Q   K   C   F   D   V

976   AAG GAC GTG CAG ATG CTG CAG GAC GCC ATC AGC AAG ATG GAC
312    K   D   V   Q   M   L   Q   D   A   I   S   K   M   D

1018  CCC ACC GAC GCA AAG TAC CAC ATG CAG CGC TGC ATT GAC TCT
326    P   T   D   A   K   Y   H   M   Q   R   C   I   D   S

1060  GGC CTC TGG GTC CCC AAC TCT AAG GCC AGC GAG GCC AAG GAG
340    G   L   W   V   P   N   S   K   A   S   E   A   K   E

1102  GGA GAG GAG GCA GGT CCT GGG GAC CCA TTA CTG GAA GCT GTT
354    G   E   E   A   G   P   G   D   P   L   L   E   A   V

1144  CCC AAG ACG GGC GAT GAG AAG GAT GTC AGT GTG TGA CCTGCCC
368    P   K   T   G   D   E   K   D   V   S   V   *

1191  CAGCTACCACCGCCACCTGCTTCCAGGCCCCTATGTGCCCCCTTTTCAAGAACAA
1246  GATAGATGCCATCTCGCCCGCTCCTGACTTCCTCTACTTGCGCTGCTCGGCCCAG
1301  CCTGGGGGGCCCGCCCAGCCCTCCCTGGCCTCTCCACTGTCTCCACTCTCCAGCG
1356  CCCAATCAAGTCTCTGCTTTGAGTCAAGGGGCTTCACTGCCTGCAGCCCCCCATC
1411  AGCATTATGCCAAAGGCCCGGGGGTCCGGGGAAGGGCAGAGGTCACCAGGCTGGT
1466  CTACCAGGTAGTTGGGGAGGGTCCCCAACCAAGGGGCCGGCTCTCGTCACTGGGC
1521  TCTGTTTTCACTGTTCGTCTGCTGTCTGTGTCTTCTAATTGGCAAACAACAATGA
1576  TCTTCCAATAAAAGATTTCAGATGCC - 1601
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We tested the ability of Cdc37 to bind to a panel of known cell cycle regulators using yeast interaction assays. We found that the activator domain-tagged Cdc37 strongly interacted with Cdk4 and Cdk6 but not with Cdc2, Cdk2, Cdk3, Cdk5 (Figure 2a) or cyclins C, D1, D2, D3 and E (data not shown). To validate the results of the yeast interaction assays in a cell free system, we performed a series of *in vitro* binding experiments using glutathione-Sepharose bound glutathione-S-transferase (GST)-Cdc37 fusion protein and various, *in vitro* translated, ^{35}S-methionine labeled proteins. Consistent with the results of the yeast experiments, we detected strong specific binding of Cdk4 and Cdk6 to GST-Cdc37 (Figure 2b). No binding of GST-Cdc37 to Cdc2, Cdk2, Cdk3, Cdk5 or cyclin A, C, D1, D2, D3, E was observed (data not shown). The exclusive binding of Cdc37 to Cdk4 and Cdk6 suggests a possible role for the Cdc37 in regulating progression through the G_1 phase of the cell division cycle.

To explore the possibility that Cdc37 and Cdk4 might associate *in vivo*, we performed immunoprecipitations with Cdc37, Cdk4, and cyclin D1 specific antisera in extracts from human SK-UT-1B leiomyosarcoma cells. We then detected the precipitated proteins by immunoblotting using the same antibodies. As expected, the Cdc37, Cdk4, and cyclin D1 specific antisera precipitated their respective antigens. The Cdk4 antiserum co-precipitated the Cdk4-bound cyclin D1 and conversely, the anti-cyclin D1 antibody brought down the cyclin D1-associated Cdk4. The Cdk4 antiserum co-precipitated Cdc37 as well, and we also detected small amounts of Cdc37 in the cyclin D1 immunoprecipitation. In the converse experiment using the Cdc37 antiserum, we were able to detect a small amount of co-precipitating Cdk4 and cyclin D1 (Figure 2c). Under similar experimental conditions, we detected stable association of Cdc37 with Cdk4 in Rko colon

b

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1    M-VDYSVWDHIEVSDDED-ETHPNIDTASLFRWRHQARVERMEQFQKEKEELDRGCRECKRKVAECQRKLK  Hs Cdc37
1    M-VDYSKWKNIEISDDED-DTHPNIDTPSLFRWRHQARVERMAEMDHEKDELKKKRQSYQARLMDVKERIS  Dm Cdc37
1    MAIDYSRWDKIELSDDSDVEVHPNVDKKSPIKWRQQGIIHEQRFKRNQDIKNLETQVDMYGILNKRVDRILS  Sc Cdc37

70   ELEVAEGG---------KAELERLQ-AEAQQLRKEERSWEQKLEEMRK----------KEKSMPW------  Hs Cdc37
70   KKDGDEEA---------LKKELEKIE-AEGKELDRIES-------EMIK----------KEKKTPW------  Dm Cdc37
72   NLPESSLTDLPAVTKFLNANFDKMEKSKGENVDPEIATYNEMVEDLFEQLAKDLDKEGKDSKSPSLIRDAI  Sc Cdc37

115  ----------------NVDTLSKD------------GFSKSMVNTKPE------KTEED--SEEVREQKHK  Hs Cdc37
109  ----------------NVDTISKP------------GFEKTVINKKAG------RKPDENLSEEEREQRMK  Dm Cdc37
143  LKHRAKIDSVTVEAKKKLDELYKENKDHISSEDIHTGFDSSFMNKQKGGAKPLEATPSEALSSAAESNILN  Sc Cdc37

150  TFVE---------------KVEKQIKHFGMLR--RWDDSQKYLSDNVELVCEETANYLVIWCTDLEVEEKC  Hs Cdc37
146  QFVK---------------ENEKLCQQYGMLR--KYDDSKRFLQEHLSLVGEETANYLVIWSINLEKEEKH  Dm Cdc37
214  KLAKSSVPQTFIDFKDDPMKLAKETEEFGKISINEYSKSQKFLLELPIISEQQKDALMMKAFEYQLHSDD  Sc Cdc37

204  ALMEQVAHQTIVMQFILELAKSLKV------DPRACFRQFFTKI--KTADKQYMEGFNDELEAFKERVRGR  Hs Cdc37
200  ELMAHVAHQCICMQYILELAKQLDV------DPRACVSSFFSKI--QHCHEYRAQFDSEIEGFKGRIQKR  Dm Cdc37
285  KMTLQVIHQSELMAYIKEIYDMKKIPYLNPMELSNVINMIFEKVIFNKDKPMGKESFLRSVQEKFLHIQKR  Sc Cdc37

267  AKLRIEKAMKEYEEEERK----KRLG----------------PGGLDPVEVYESL-PEELQKCFDVKDVQM  Hs Cdc37
263  AQEKIQEAIAQAEEEEKK----ERLG----------------PGGLDPADVFESL-PDELKACFESRDVEL  Dm Cdc37
356  SKILQQEEMDESNAEGVETIQLKSLDDSTSLEVNLPDFNSKDPEEMKKVKVFKTLIPEKMQEAIMTKNLDN  Sc Cdc37

317  LQDAISKMDPTDAK-----YHMQRCIDSGLWVPMSKASEAKESE-EAGPGDPLLEAVPKTGDEKDVS----  Hs Cdc37
313  LQKTIAAMPVDVAK-----LHMKRCVDSGLWVPMAADLEGDKKE-EDDSDD-VAGGEEKTDDAKSESAAKE  Dm Cdc37
427  INKVFEDIPIEEAEKLLEVFNDIDIIGIKAILEWEKDFQSLKDQYEQDHEDATMENLSL--NDRDGGDNH  Sc Cdc37

378  ----------Y-*          Hs Cdc37
377  EPIYTGVSTEDVD*         Dm Cdc37
496  EEV--KHTADTVD*         Sc Cdc37
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Figure 1 (**a**) The nucleotide and predicted amino acid sequence of human Cdc37 and (**b**) its comparison to other Cdc37 homologues. The cDNA contains a predicted open reading frame of 378 amino acids, encoding a protein with a molecular weight of 44.4 kDa and having an isoelectric point of 5.11. The screening based on the interaction trap system was performed with a cDNA library constructed (Gyuris *et al*., 1993) with mRNA isolated from serum starved, contact inhibited WI38 cells and containing 5.7×10^6 independent members. The yeast strain EGY048 that contained the *LEXAop-LEU2* and *LexAop-LacZ* reporter genes as well as the LexA-Cdk4 bait was used to perform the screening as described (Fiore *et al*., 1995)

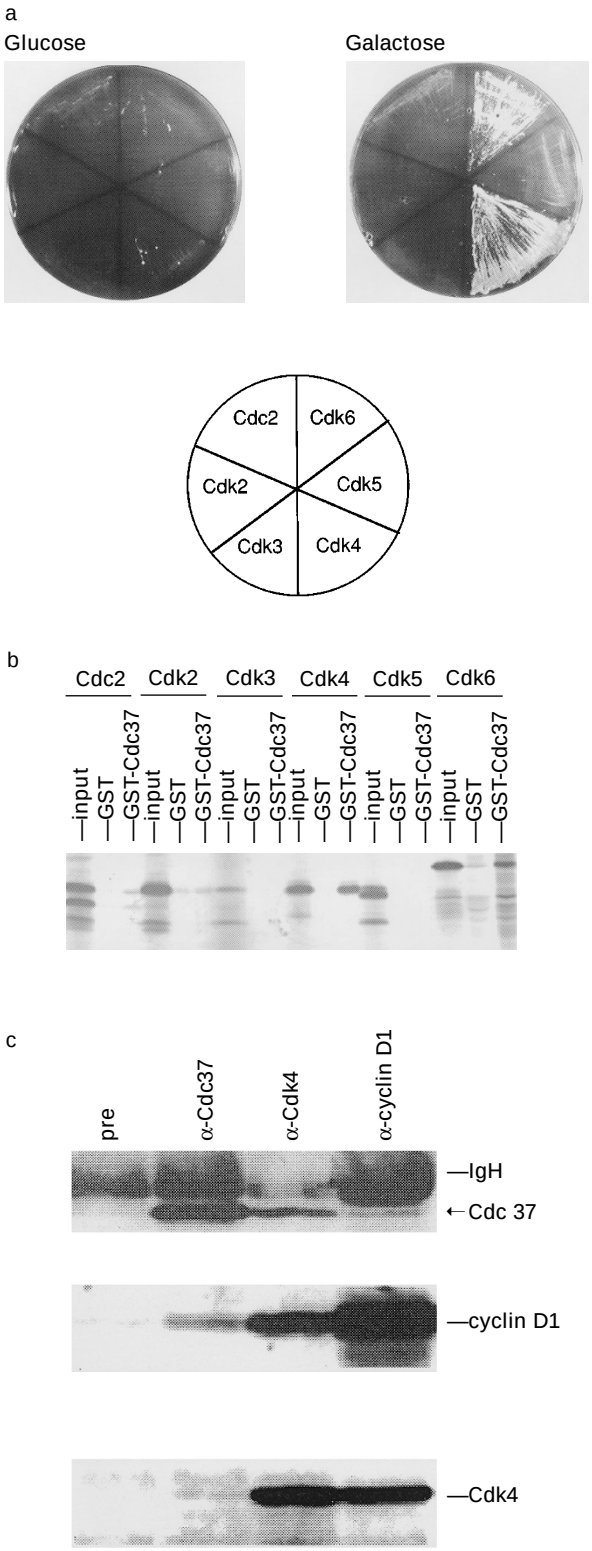

Figure 2 Interaction of Cdc37 with Cdk4 in yeast, *in vitro* and in human cells. (**a**) Interaction of Cdc37 with Cdk4 and Cdk6 in yeast. The interaction between the transcriptional activator domain tagged Cdc37 and the various baits we detected through the activation of the *LexAop-LEU2* reporter gene as growth on media lacking leucine in the presence of galactose. (**b**) The binding of Cdc37 to *in vitro* translated Cdks. The *in vitro* binding studies were done as described (Toyoshima and Hunter, 1994) except that approx 25 μg of glutathione-Sepharose bound GST or GST-Cdc37 proteins were used. A fraction of the input lysates and the GST as well as the GST-Cdc37 bound proteins were visualized by autoradiography after separation on SDS/PAGE. (**c**) The association of Cdc37 and Cdk4 in human cells. To detect the *in vivo* association of Cdc37 and Cdk4, SK-UT-1B (ATCC HTB 115) cells were lysed in lysis buffer (50 M Tris/Cl pH 7.5, 250 mM NaCl, 0.5% NP40, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4) in the presence of protease inhibitors. 15 mg of the soluble fraction were precipitated for 2 h at 4°C with antibodies prebound to 50 μl Protein A-Sepharose and washed extensively with lysis buffer. The precipitated proteins were immunoblotted and detected using the indicated antibodies. Rabbit polyclonal antibodies against human cyclin D1 and Cdk4 have been described previously. The Cdc37 specific antibody was generated against recombinant human GST-Cdc37

carcinoma cells, but not in extracts made from WI38 human primary fibroblasts or Saos-2 osteosarcoma cells (data not shown) suggesting either that the association of Cdc37 with Cdk4 is cell type dependent or that other factors might influence their stable association in different cellular settings.

To investigate the capacity of Cdc37 to bind to Cdk4 in human cell extracts, we incubated GST-Cdc37 and control GST with ^{35}S-methionine/^{35}S-cysteine labeled cell lysates made from Rko cells, and detected the proteins bound to GSH-Sepharose by SDS–PAGE electrophoresis and autoradiography. In these experiments the predominant partner of GST-Cdc37 was a protein of apparent M_r 90 000 (Figure 3a). In *D. melanogaster* both a CDC37 homologue and a homologue of the mammalian Hsp90 protein had been identified, as the encoded products of two genes that affected the regulation of the *sevenless* receptor tyrosine kinase (Cutforth and Rubin, 1994). Although no biochemical interaction had been suggested between these two proteins, this led us to test whether the 90k protein bound to GST-Cdc37 was human Hsp90. The GST and GST-Cdc37 associated proteins were immunoblotted using a monoclonal antibody that specifically recognized Hsp90. This antibody indeed recognized a M_r 90 000 protein in the Gst-Cdc37 bound complexes, but not in the control ones, demonstrating the ability of Cdc37 and Hsp90 to form stable complexes *in vitro* (Figure 3b). An interaction between Cdc37 and HSP90 has also been recently demonstrated by Stepanova *et al*. (1996) and Dai *et al*. (1996).

To test the effect of Cdc37 on the activity of the Cdk4/cyclin D1 kinase *in vitro*, we added increasing amounts of GST-Cdc37 to Sf9 extracts that contained active Cdk4/cyclin D1 (Figure 4, top panel) or to isolated Cdk4/cyclin D1 complexes immunoprecipitated from Cdk4/cyclin D1 overexpressing insect cells (Figure 4, lower panel). We then measured the kinase activity of the Cdk4/cyclin D1 complex using GST-Rb as a substrate. We did not observe any effect of the added GST-Cdc37 on the Cdk4/cyclin D1 kinase activity in either experiment, whereas the addition of the known Cdk4 inhibitor $p16^{Ink4-A}$ effectively blocked this activity. These results show that Cdc37 is neither an inhibitor, nor an activator of the Cdk4/cyclin D1 kinase.

To further investigate the effect of Cdc37 on Cdk4/cyclin D1 complex formation, we measured the amounts of cyclin D1 bound to GST-Cdk4 in the absence or the presence of increasing amounts of Cdc37. Since it has been shown that phosphorylation of the Cdk subunit by CAK stabilizes certain Cdk/cyclin complexes, we also included purified CAK in one set of binding reactions. As shown in Figure 5, Cdc37 enhanced the binding of cyclin D1 to Cdk4,

while the addition of CAK to the reactions did not have any significant effect. The increase was dependent on the concentration of added Cdc37, reaching a maximum at Cdc37 concentrations comparable to the amounts of total GST-Cdk4 and cyclin D1 (100 nM) added to the reaction.

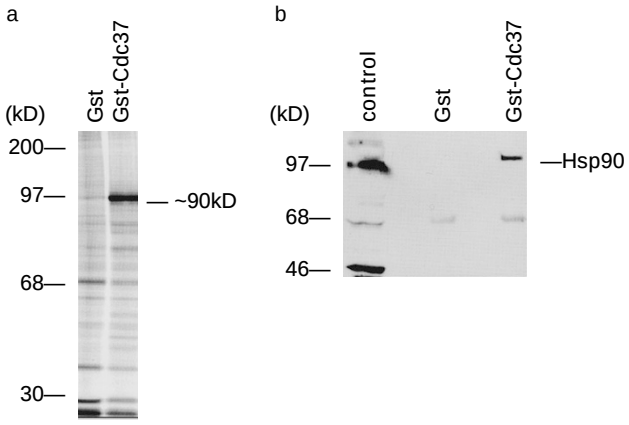

Figure 3 The association of Cdc37 with Hsp90 in cell extract. (**a**) GST-Cdc37 preferentially binds to a M_r 90 000 protein in cell extracts. For the co-precipitation assay 20 μg of GST or GST-Cdc37 fusion protein prebound to the glutathione-Sepharose beads were incubated in lysis buffer, with 0.5 mg of ^{35}S-methionine/^{35}S-cysteine labeled cell lysate from RKO, for 2 h at 4°C. Unbound proteins were removed by three washes with lysis buffer. The labeled bound proteins were visualized by autoradiography after separation on SDS/PAGE. (**b**) HSP90 binding to GST-Cdc37 in cell extracts. The same experimental procedure was performed with cold extracts and bound proteins were immunoblotted with a specific monoclonal antibody against Hsp90

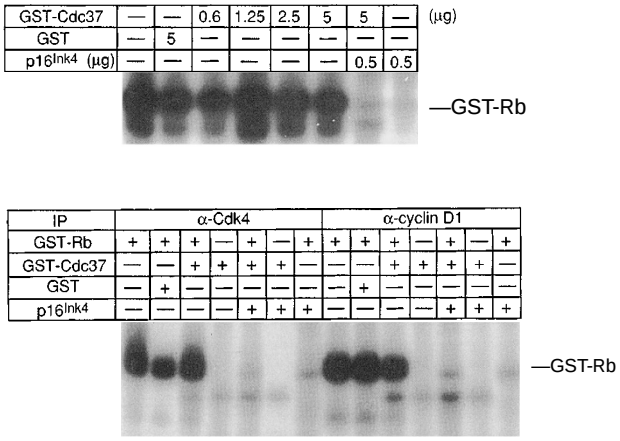

Figure 4 Lack of inhibition of the Cdk4/cyclin D1 kinase by Cdc37. Top panel: Insect cells derived from Cdk4 and cyclin D1 co-infected Sf9 cells were lysed in 50 mM Tris/Cl pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 2 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% Tween 20. 10 mg were incubated in 25 μl for 30 min at room temperature with either buffer or the indicated amounts of GST, GST-Cdc37, p16^{Ink4-A}, and with the combination of GST-Cdc37 and p16^{Ink4-A}. After incubation, the kinase reaction was done as described (Matsushime *et al*., 1994) using 2.5 μg of GST-Rb as substrate at 30°C for 30 min. Reactions were terminated by the addition of 5 μl 5 × SDS sample buffer, heated for 1 min at 100°C, and the phosphorylated GST-Rb were resolved on 10% SDS/PAGE. Gel was first stained with Coomassie blue to confirm comparable loading per each lane, then dried and the labeled proteins visualized by autoradiography. The positions of the phosphorylated GST-Rb in indicated. Lower panel: Cdk4/cyclin D1 complex was immunoprecipitated by Cdk4 or Cyclin D1 specific antibodies from 50 mg of Sf9 lysates and incubated in 25 μl with the indicated amounts of GST, GST-Cdc37, and p16^{Ink4-A} at room temperature for 30 min. After the incubation the kinase reaction was done as before

It has been demonstrated that p16^{Ink4-a} directly inhibits the activity of the Cdk4/cyclin D1 complex. It also prevents the binding of cyclin D1 to Cdk4 in *in vitro* mixing experiments (Parry *et al*., 1995). Since Cdc37 facilitates the formation of the Cdk4/cyclin D1 complex we investigated the effect of p16 on the binding of Cdc37 to Cdk4 using a microtiter plate based assay (Wick *et al*., 1995). In this experiment immobilized p16 was incubated with a constant amount of Cdk4 containing insect lysate in the presence of increasing amounts of Cdc37. The amount of p16 bound to Cdk4 was determined by ELISA utilizing a Cdk4-specific antiserum. As shown in Figure 6, Cdc37 inhibited the interaction between Cdk4 and p16 in a concentration dependent manner. The inhibitory concentration (IC_{50}) needed to achieve 50% inhibition of the Cdk4-p16 binding was 1.27 μM. This suggests that p16 and Cdc37 bind to at least partially overlapping regions of Cdk4 and may play opposing roles in regulating Cdk4/cyclin D function.

The above experiments demonstrate that Cdc37 is a subunit of Cdk4, one that does not alter directly the activity of the Cdk4/cyclin D1 kinase while it may control its activity by regulating its assembly. Cdc37 is also able to form stable complexes with the molecular chaperone Hsp90, suggesting that it is either a target or a subunit of Hsp90. *In vivo*, Hsp90 is part of a larger protein complex required for the activity of a number of different kinases and other proteins (for a review, Jakob and Buchner, 1994). Hsp90 was found to form trimeric complexes with the Src and Raf kinases and an unknown protein called p50 (Whitelaw *et al*., 1991;

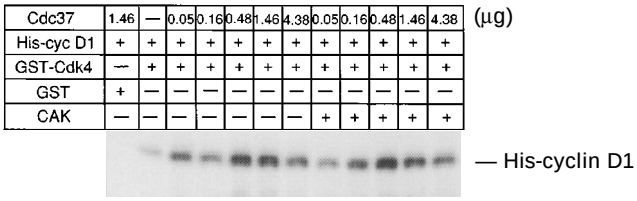

Figure 5 Cdc37 facilitates the binding of cyclin D1 to Cdk4 *in vitro*. The *in vitro* binding assay was performed with 1 μg of GST-Cdk4 or GST. The proteins were incubated with 1 μg of His-cyclin D1 in 100 μl of buffer (50 mM Tris/Cl pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% NP40, 0.1 mM ATP) for 1 h at room temperature in the presence of 0.5 μg of CAK where indicated. The reactions were diluted to 500 μl in buffer and GST-Cdk4 was captured on 30 μl of glutathione-Sepharose beads. The beads were washed four times with 500 μl of buffer and the GST-Cdk4 associated His-cyclin D1 was detected by immunoblotting. GST-Cdk4 and GST-Cdc37 were produced in BL21 cells and purified on glutathione-Sepharose beads (Pharmacia) according to the manufacturer's instruction. The glutathione-Sepharose bound GST-Cdc37 was digested with thrombin to remove the GST tag and the released Cdc37 was used further. The largely insoluble His-cyclin D1 (gift of X Xu) was prepared under denaturing condition from BL21 (DE3) cells on Ni-NTA-agarose (Invitrogen) and renatured by sequential dialysis against decreasing concentrations of urea (XX and LB, unpublished). To generate CAK, glutathione-Sepharose bound GST-cyclin H was incubated with Sf9 extracts made from cells that were coinfected with a baculovirus construct that directed the expression of Cdk7/MO15. The unbound proteins cyclin H/Cdk7 complexes were eluted with glutathione as described above. Approximately 20% of the eluted proteins corresponded to GST-cyclin H/Cdk7 complexes

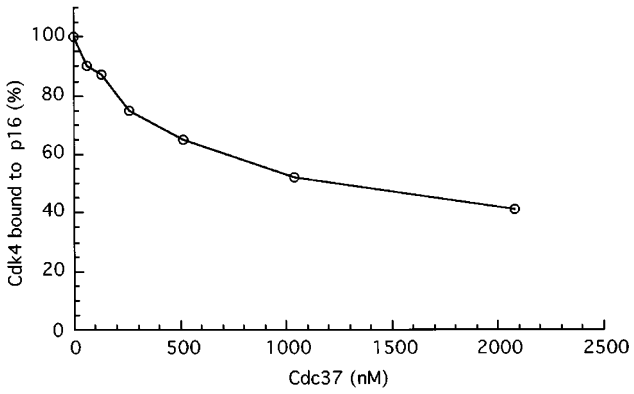

Figure 6 p16 competes with Cdc37 for the binding to Cdk4. The effect of Cdc37 on the binding of p16 to Cdk4 was tested using a microtiter plate based assay, as described (Wick *et al*., 1995). Immobilized p16 was incubated with constant amount of Cdk4 containing insect lysate in the presence of increasing amounts of Cdc37

Wartmann and Davis, 1994). The binding of Hsp90 to these kinases is essential for their biological activity (Whitesell *et al*., 1994; Schulte *et al*., 1995). It has now been demonstrated that Cdc37 is identical to Hsp90 associated protein, p50 (Dai *et al*., 1996; Stepanova *et al*., 1996).

The assembly of the Cdk4/cyclin D kinase is thought to be mediated by an assembly factor whose activity is regulated by growth factors (Matsushime *et al*., 1994). The function of this hypothetical assembly factor is reminiscent of that of Cdc37 in yeast. We have demonstrated that *in vitro* Cdc37 facilitates the binding of cyclin D1 to Cdk4. In this regard Cdc37 may have a similar function to $p36^{MAT1}$ whose binding to Cdk7 facilitates the assembly of the Cdk7/cyclin H complex (Fisher *et al*., 1995). Alternatively, Cdc37 or the Cdc37/Hsp90 protein complex could act as a molecular chaperone whose binding to Cdk4 is required for the proper folding of Cdk4 prior to its association with the cyclin D subunit.

A positive role of Cdc37 in the regulation of the Cdk4/cyclin D kinase is supported by the observation that the binding of Cdc37 to Cdk4 is prevented by p16. p16 exclusively inhibits the activity of the Cdk4/cyclin D kinase, either by preventing the interaction of Cdk4 with cyclin D1 (Parry *et al*., 1995) or by directly blocking catalysis (Hirai *et al*., 1995). The inhibition of Cdc37 binding to Cdk4 by p16 suggests another inhibitory mechanism for p16. In this scenario, p16 interferes with a protein that either directly facilitates the assembly of the Cdk4/cyclin D complex or whose function is to catalyze the folding of Cdk4 into a conformation that is accessible for the binding of cyclin D1.

In mammalian cells, Cdk4/cyclin D complexes function as receivers of various growth factor-induced signals that link the effect of different signal transduction pathways to the activation of other cyclin-dependent kinases. The first step in the activation of the Cdk4/cyclin D kinase is the assembly of the Cdk4 and cyclin D subunit that could be mediated by a mitogen dependent assembly factor. The possible involvement of Cdc37 in the assembly of the Cdk4/cyclin D1 kinase prompted us to follow the expression of Cdc37 in human fibroblasts after serum stimulation of cells synchronized by serum deprivation. In these cells, Cdc37 was expressed at high levels in serum starved, quiescent cells and its levels remained constant as cells moved from G_0 into G_1 and did not change dramatically as cells progressed through the cell cycle (data not shown). Other mechanisms therefore could be involved in regulating Cdc37 activity. They could involve post-translational modifications as well as changes in the subcellular localization of the protein. The elucidation of the mechanisms responsible for the association of Cdk4 with Cdc37 and the nature of the specific interaction of Cdc37 with CDK4/6 are likely to be important for our understanding of how growth factors stimulate cell cycle progression of normal cells and how oncogenic activation of the components of various signal transduction pathways leads to uncontrolled cell proliferation in cancer.

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