Over-expression of cyclin D1 regulates Cdk4 protein synthesis

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Abstract. Increased Cdk4 expression occurs coincident with over-expression of cyclin D1 in many human tumours and tumourigenic mouse models. Here, we investigate both *in vivo* and *in vitro* the mechanism by which Cdk4 expression is regulated in the context of cyclin D1 over-expression. Cdk4 mRNA levels in cyclin D1-over-expressing tissue and cultured cells were unchanged compared with controls. In contrast, Cdk4 protein levels were increased in cyclin D1-over-expressing tissue and cells versus their respective controls. This increase was not due to altered protein stability, but appeared to be due to an increase in Cdk4 protein synthesis. We also performed immunoprecipitation and *in vitro* kinase assays to demonstrate an increase in cyclin D1-Cdk4 complex formation and associated kinase activity. Blocking cyclin D1 expression resulted in diminished Cdk4 protein but not mRNA levels. These findings suggest a mechanism by which Cdk4 expression is increased in the context of cyclin D1 over-expression during tumourigenesis.

INTRODUCTION

Normal cellular proliferation requires progression through four distinct phases of the cell cycle: G₁, S, G₂, and M. Passage through these phases is regulated by protein complexes consisting of cyclins and cyclin-dependent kinases (Cdks). Cyclin D1 and cyclin-dependent kinase 4 (Cdk4) are key proteins involved in the G₁ to S phase transition. Cyclin D1 forms a complex with Cdk4 and regulates Cdk4 activity. Activation of the cyclin D1-Cdk4 complex enables Cdk4 to phosphorylate the tumour suppressor protein, retinoblastoma (pRb). Hyperphosphorylation of pRb results in dissociation from E2F family members, enabling S-phase entry (Connell-Crowley *et al.* 1997; Santoni-Rugiu *et al.* 1998).

Over-expression of cyclin D1 characterizes several tumour types (Jiang *et al.* 1993; Nishida *et al.* 1994; Shapiro *et al.* 1995). During carcinogenesis, increased cyclin D1 expression leads

to increased pRb phosphorylation (Ramljak *et al.* 1998; Santoni-Rugiu *et al.* 1998), suggesting a role for Cdk4 in carcinogenesis, as cyclin D1 functions through the catalytic activity associated with Cdk4 to phosphorylate pRb. In addition, many human tumours and tumourigenic mouse models display a coincident increase in cyclin D1 and Cdk4 expression. Among these examples are human ovarian carcinoma (Masciullo *et al.* 1997), cervical carcinoma (Cheung 2001), and hepatoblastoma (Kim *et al.* 1998), and mouse models of intestinal (Zhang *et al.* 1997; Wang *et al.* 1998) and hepatocellular carcinoma (Ramljak *et al.* 1998). Also, cyclin D1 transgenic mice engineered to target expression to the epithelium of the upper aerodigestive tract (Nakagawa *et al.* 1997) demonstrate increased Cdk4 levels in cyclin D1-targeted tissues. These mice develop severe dysplasia and exhibit increased Cdk4 levels during early stages of disease progression, suggesting that increased Cdk4 expression may play a role in initiating these pre-neoplastic events. Although the mechanisms accounting for increased cyclin D1 expression are well characterized, the mechanisms promoting the coincident increase in Cdk4 expression have yet to be determined.

Here, using a transgenic mouse model and cultured cells over-expressing cyclin D1, we provide evidence that over-expression of cyclin D1 plays an important role in increasing Cdk4 protein levels. We also observe an increase in cyclin D1-Cdk4 complex formation and associated kinase activity. Interestingly, over-expression of cyclin D1 does not enhance Cdk4 protein stability, but the rate of Cdk4 protein synthesis is increased. Finally, transducing hepatocyte cells with antisense cyclin D1 inhibits cyclin D1 expression, which leads to decreased Cdk4 protein but not mRNA levels.

MATERIALS AND METHODS

Cell culture

Conditionally immortalized mouse hepatocyte (ImHep) cells were isolated from a 6-week-old male C57BL/6 transgenic mouse carrying a thermolabile SV40 T gene (Allen *et al.* 2000), using a two-step collagenase perfusion method (Seglen 1976). Immortalized hepatocytes were maintained in RPMI medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 5 units/ml mouse γ -interferon (Gibco-BRL, Grand Island, NY, USA). The medium for hepatocytes infected with retroviral constructs containing a puromycin resistance gene was supplemented with 2 μ g/ml puromycin. Cells were maintained at 33 °C in the presence of 5% CO₂ and the medium was changed three times per week. DNA synthesis was assessed by bromo-deoxy-uridine (BrdU) and [³H]thymidine uptake. Proliferation was determined by construction of growth curves. For BrdU experiments 1 $\times$ 10⁴ cells were plated in 4-well chamber plates and incubated for 2 days at 39 °C. One hour prior to staining, cells were incubated with 5 μ M BrdU (Sigma; St Louis, MO, USA). Staining procedures were performed according to the manufacturer's protocol (Zymed; San Francisco, CA, USA). For [³H]thymidine assays, 1 $\times$ 10⁴ cells were plated in triplicate in 24-well plates and incubated for 2 days at 39 °C. Cells were incubated with 1 μ Ci/ml of [³H]thymidine and harvested 1 h later. DNA synthesis was estimated by [³H]thymidine incorporation into trichloroacetic acid-insoluble material as previously described (Ko *et al.* 1994). Data were expressed as cpm/10 000 cells. For growth curves, 1 $\times$ 10⁴ cells were plated in triplicate in 6-well plates. The next day, cells were trypsinized and counted using a haematocytometer. Cells were then counted by the same method every other day for the next 8 days.

Western blot analysis

Polyclonal rabbit anti-human cyclin D1 antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Polyclonal rabbit anti-mouse Cdk4 antibody was obtained from Santa

Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal mouse anti-human β -actin antibody (Sigma Chemical, St. Louis MO, USA). Cells were washed in phosphate-buffered saline (PBS) and lysed directly on the plate using RIPA buffer [PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] plus a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). Lysates were clarified by centrifugation and protein concentrations quantified by the Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA). For immunoblotting, protein samples (25 μ g) were resolved by 10% (acrylamide) sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and probed with the appropriate antibodies. Proteins were visualized using the enhanced chemiluminescence (ECL) system (Amersham Life Sciences Inc., Arlington Heights, IL, USA) and exposed to XAR5 film (Eastern Kodak Company; Rochester, NY, USA).

Immunohistochemistry

Sections (5 μ m) were deparaffinized with xylene, rehydrated in a graded series of ethanols and finally with PBS. Endogenous peroxidase activity was quenched in 3% H₂O₂ followed by an alkaline antigen retrieval step (pH 10.0; Biogenix; San Ramon, CA, USA). Additional non-specific background staining was blocked using the MOM peroxidase-based kit (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was a polyclonal rabbit anti-mouse Cdk4 antibody obtained from Santa Cruz Biotechnology at a dilution of 1 : 100 in a 1% goat serum PBS solution. Amplification of the reaction was achieved by incubating sections with the Elite Avidin-Biotin kit supplied by Vector laboratories. Sites of immunoreactivity were visualized using 3,3'-diaminobenzidine as the chromogen (Dako, Carpinteria, CA, USA). Specimens were viewed under a Zeiss Axioplan 2 microscope, and images were captured using a model HRP042-CMT digital camera from Diagnostic Instruments and Zeiss Image 3.0 software. Images were processed using Adobe Photoshop software (Adobe Systems Inc.; San Jose, CA, USA).

Northern blot analysis

Total RNA was extracted according to manufacturer's protocol using Trizol reagent (Gibco-BRL) from transgenic and age-matched control mouse liver tissue. Tissues from a total of 10 mice ranging in age from 3 to 12 months were analysed. From control and cyclin D1 transduced hepatocytes, a total of eight clones were analysed in three independent experiments. RNA samples (10 μ g/lane) were separated by electrophoresis on 1.1% denaturing agarose gels and blotted onto nylon + membranes (Schleicher & Schuell, Rockford, IL, USA). Blots were probed using antisense riboprobes for cyclophilin and human Cdk4 [full-length Cdk4 cDNA was a gift from Dr Steven Hanks (Hanks 1987)]. Riboprobes were labelled using digoxigenin-11-UTP and signals visualized by Digoxigenin Luminescent Detection following manufacturer's recommendations (Roche, Indianapolis, IN, USA).

Retroviral transduction

The mouse cyclin D1 pBabe/puro and pBabe/puro control retroviral vectors were used to transfect Phoenix A cells (a gift from Gary Nolan, Stanford University). The 1.3-kb mouse cyclin D1 cDNA was subcloned in the opposite orientation into the pBabe/puro vector for antisense procedures. All transfections were conducted using lipofectamine 2000 following manufacturer's instructions (Gibco-BRL). In brief, 2.4×10^5 Phoenix A cells were seeded in 6-cm tissue-culture plates and transfected using 8 μ g of DNA and 10 μ l of lipofectamine for 6–7 h, at which time the media were changed. Media were also changed the following day and 24 h later viral supernatant was collected and filtered through a 0.45- μ m filter (Sarstedt, Newton, NC, USA). To start infection, conditionally immortalized hepatocytes were seeded at 2.4×10^5 cells

in 6-cm plates at 33 °C. Hepatocyte culture medium was replaced with viral supernatant containing 5 µg/ml polybrene (Sigma). After 24 h, the cell media were changed. The following day, cells were split 1 : 10 and selected in 2 µg/ml puromycin. Stable pools were incubated at 39 °C and gene expression assessed 48–72 h later.

Kinase assay

Cells were lysed in immunoprecipitation buffer (1% Triton-X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 0.5% NP40). To 500 µg of total protein, 1 µg of cyclin D1 antibody was added and incubated for 1 h with shaking at 4 °C. Following incubation, 30 µl of protein G + sepharose beads (Santa Cruz Biotechnology) were added and incubated overnight. The beads were washed four times, twice in immunoprecipitation buffer and twice in kinase buffer. Following washes, the immunoprecipitated proteins were collected and incubated in kinase buffer (10 mM Tris pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT) plus 100 ng of Rb C-terminal substrate (Santa Cruz Biotechnology) and 20 µM ATP for 20 min at 37 °C. Proteins were resolved by 10% SDS-PAGE, transferred to a PVDF membrane and probed using control and phospho-specific pRb antibodies (New England Biolabs, Beverly, MA, USA). Proteins were visualized using the ECL system (Amersham). The latter antibody recognizes phosphorylated serine 790, which is specifically phosphorylated by Cdk4 containing complexes (Kitagawa *et al.* 1996; Connell-Crowley *et al.* 1997). Experiments were performed using four cyclin D1 transgenic mouse liver lysates obtained from animals ranging in age from 3 to 6 months. Comparisons were made using age-matched controls. Four control and cyclin D1 transduced hepatocyte cell clones were utilized for cell culture experiments. Relative changes in kinase results were quantified by densitometry using a Paperport flatbed scanner and the ImageJ software, provided by Wayne Rasband, National Institutes of Health.

Pulse-chase and metabolic labelling

Pulse-chase experiments were performed by seeding 2.4×10^5 cells in 6-cm plates at 33 °C. The next day, cells were brought up to 39 °C. Following a 24-h period, cells were labelled for 16 h with [³⁵S]methionine (50 µCi/ml) in methionine minus media. The labelling period was followed by PBS and plating media washes and a 0, 2, 4 and 6 h chase in plating media plus 2 mM cold methionine. Cells were lysed and Cdk4 immunoprecipitations performed. Immunoprecipitations were resolved by 10% SDS-PAGE. Gels were fixed, treated with Amplify (Amersham, Arlington Heights, IL, USA), dried, and visualized by exposure to XAR5 film (Eastern Kodak Company, Rochester, NY, USA). For metabolic labelling experiments, 2.4×10^5 cells were seeded in 6-cm plates at 33 °C. The next day, cells were placed at 39 °C. Following a 24-h period, cells were labelled for 1 h with [³⁵S]methionine (50 µCi/ml) in methionine minus media. To prepare lysates, cells were lysed directly on the plate and Cdk4 immunoprecipitations performed from 250 µg of total protein. Immunoprecipitations were resolved by SDS-PAGE (10%). Gels were fixed, treated with Amplify (Amersham), dried, and visualized by exposure to XAR5 film (Eastern Kodak Company). Densitometry was used to quantify Cdk4 protein levels using a Paperport flatbed scanner and the ImageJ program, provided by Wayne Rasband, National Institutes of Health. Pulse-chase and metabolic labelling were repeated three times.

Statistical analysis

Statistical comparisons between groups were performed using a Student's unpaired *t*-test and a two-tailed *P* < 0.05 was considered statistically significant.

RESULTS

Over-expression of cyclin D1 is associated with increased Cdk4 protein expression both *in vivo* and *in vitro*. In an effort to ascertain the role of cyclin D1 in tumour initiation and progression in intestinal and liver tissues, we generated liver fatty acid binding protein (LFABP) promoter driven cyclin D1 transgenic mice. The LFABP promoter directs expression primarily to the liver, small bowel, and large bowel (Simon *et al.* 1993). These mice develop hepatocellular adenoma (HCA) and carcinoma (HCC) with high penetrance (Deane *et al.* 2001). Using this mouse model, we determined the relative levels of Cdk4 protein expression in control and cyclin D1 transgenic mouse livers. We observed an increase in Cdk4 protein expression. Western blot analysis showed that cyclin D1 transgenic mice displayed higher levels of Cdk4 protein expression compared with control littermates (Fig. 1a). Immunohistochemical analysis confirmed that Cdk4 protein levels were increased in hepatocyte nuclei of pre-neoplastic liver tissues compared to control (Fig. 1b).

Because the LFABP promoter also directs cyclin D1 expression to the small bowel and large bowel, we examined whether Cdk4 levels were increased in those tissues. By western blot analysis we found that Cdk4 levels were increased in all tissues where cyclin D1 was over-expressed. As a control, we examined stomach tissue where cyclin D1 expression was not detectable and found low to undetectable levels of Cdk4 (Fig. 2a). We also examined Cdk6 expression as cyclin D1 can form a complex with either Cdk4 or Cdk6 to regulate the G₁/S transition. We found no change in Cdk6 expression comparing lysates obtained from control and transgenic mouse livers (Fig. 2b). These data demonstrate that over-expression of cyclin D1 is associated with increased expression of Cdk4, suggesting Cdk4 expression is dependent upon up-regulation of cyclin D1.

Figure 1. Increased Cdk4 expression in pre-cancerous cyclin D1 transgenic liver tissue. (a) Western blot analysis of liver lysates obtained from cyclin D1 transgenic (Tr) and control (Ctl) mice illustrating increased expression of endogenous Cdk4 in association with over-expression of cyclin D1. Blots were also probed with actin as an internal loading control. (b) Paraffin-embedded sections from control (Ctl) and pre-cancerous LFABP-cyclin D1 transgenic (Tr) livers were stained using a Cdk4 antibody showing specific nuclear hepatocyte staining in transgenic liver tissue sections ($\times 400$).

Figure 2. Cdk4 expression is increased specifically in tissues over-expressing cyclin D1. (a) Western blot analysis of various tissues from control (Ctl) and cyclin transgenic (Tr) mice showing increased Cdk4 expression in tissues over-expressing cyclin D1: L. Bow., large bowel; S. Bow., small bowel; Liv., liver; Stom., Stomach. (b) Western blot analysis of liver lysates obtained from control (Ctl) and cyclin D1 transgenic (Tr) mice showing no change in Cdk6 expression. Blots were probed with cyclin D1, Cdk4, and Cdk6 antibodies. Blots were also probed with actin as an internal loading control.

To explore the mechanism of Cdk4 regulation, conditionally immortalized hepatocyte (ImHep) cells were used. We determined the effect of cyclin D1 over-expression on cellular proliferation and Cdk4 regulation. Cyclin D1 transduced ImHep cells were placed at the non-permissive temperature when endogenous levels of cyclin D1 and Cdk4 proteins are low (Fig. 3a) and in an effort to simulate the *in vivo* growth characteristics of hepatocytes in the adult liver. Proliferation was determined by assessing DNA synthesis based on the incorporation of tritiated thymidine and bromo-deoxy-uridine. DNA synthesis was significantly increased in hepatocyte cultures infected with pBabe-cyclin D1 versus pBabe control retroviral vectors (Fig. 3b and c). Cdk4 protein expression was greatly increased in hepatocyte cells over-expressing cyclin D1 (Fig. 3d). From these experiments, we were able to conclude that like hepatocytes *in vivo* (Deane *et al.* 2001), over-expression of cyclin D1 in cultured hepatocyte cells is associated with increased Cdk4 expression levels and increased proliferation.

Over-expression of Cyclin D1 induces increased Cdk4 complex formation and Cdk4 activity

During normal proliferation in a variety of cell types, increased cyclin D1 expression is accompanied by an increase in cyclin D1-Cdk4 associated kinase activity, which in turn is dependent upon complex formation (Sherr 1996; Albrecht *et al.* 1998). Therefore, we wanted to determine whether the increase in proliferation with Cdk4 expression was associated with an increase in cyclin D1-Cdk4 complex formation and kinase activity in cyclin D1 over-expressing mouse tissue and ImHep cells versus control. To conduct these experiments, total protein lysates were immunoprecipitated with a cyclin D1 antibody and blots probed with an antibody to Cdk4. Relative levels of complexed Cdk4 were compared among the various lysates. These experiments showed that the level of complexed Cdk4 was increased in cyclin D1 over-expressing cells and

Figure 3. Infection of conditionally immortalized hepatocytes with cyclin D1 retrovirus results in increased DNA synthesis and sustained Cdk4 expression. (a) Western blot showing coincident loss of endogenous cyclin D1 and Cdk4 expression in ImHep cells cultured at the non-permissive temperature (39 °C). DNA synthesis was measured in control and cyclin D1 retrovirally transduced cells placed at the non-permissive temperature (39 °C) for 72 h. (b) [^3H]-thymidine and (c) BrdU incorporation were measured and presented as the mean $\pm$ SD (* $P < 0.05$). (d) Western blot analysis of control (Ctl) and cyclin D1 (D1) transduced ImHep cells cultured at the non-permissive temperature for 72 h.

tissue compared with controls (Fig. 4a and b). To evaluate changes in Cdk4 activity, we performed kinase assays *in vitro*. Again, cyclin D1 immunoprecipitations were performed from total protein lysates. Complexes were incubated with Rb-GST substrate. The level of phosphorylated Rb-GST was determined by western blot analysis using an Rb phospho-specific antibody as described in MATERIALS and METHODS. Rb phosphorylation was increased 2–3-fold in cyclin D1 transgenic mouse livers (Fig. 4a) and cyclin D1-transduced cells (Fig. 4b) relative to their respective controls. These results suggest that over-expression of cyclin D1 leads to an increase in the formation of active Cdk4-cyclin D1 complexes.

Cdk4 mRNA expression is unaffected by alterations in cyclin D1 expression

Northern blot analysis was performed to determine if the increase in Cdk4 reflected an increase in Cdk4 mRNA expression. Total RNA was isolated from cyclin D1 transgenic and non-transgenic

Figure 4. Over-expression of cyclin D1 induces increased cyclin D1-Cdk4 complex formation and associated kinase activity. (a) Cyclin D1 immunoprecipitations from control (Ctl) and transgenic (Tr) mouse liver tissue lysates and (b) control (Ctl) and cyclin D1 (D1)-transduced ImHep cell lysates were blotted with Cdk4 and cyclin D1 to show relative levels of complex formation. Cyclin D1 immunoprecipitations were also used in kinase assays. A GST-C-terminal pRb fusion protein was used as a substrate. Phosphorylated pRb (GST-Rb-P) was detected in (a) control (Ctl) and transgenic (Tr) mouse liver tissue lysates and (b) control (Ctl) and cyclin D1 (D1) transduced ImHep cell lysates using a phospho specific pRb antibody. As a control hypophosphorylated pRb (GST-Rb) is also shown.

littermates and from ImHep cell cultures retrovirally infected with cyclin D1-pBabe and pBabe control vectors. In three independent experiments, no change in Cdk4 mRNA expression was observed. Endogenous levels of cyclin D1 were undetectable in either control or transgenic mouse tissue lysates (indicated by arrow). Endogenous mouse cyclin D1 mRNA migrates at 3.8 kb and is expressed at low levels in the liver (Santoni-Rugiu *et al.* 1998) (Fig. 5a). Similarly, comparisons of Cdk4 mRNA expression in cyclin D1 and pBabe infected ImHep cells revealed no difference (Fig. 5b). Based on these results, we were able to conclude that the observed increase in Cdk4 protein expression was not due to an increase in Cdk4 mRNA levels, suggesting the increase reflects alterations in protein stability or protein synthesis.

Over-expression of cyclin D1 induces an increase in Cdk4 protein synthesis

We determined whether cyclin D1 over-expression influences Cdk4 protein stability by performing [³⁵S]methionine pulse chase experiments. Following a 16-h pulse, ImHep cells were chased for 0, 2, 4 and 6 h. Cells were harvested and Cdk4 immunoprecipitations performed from equal amounts of total protein lysate. Analysis of three independent experiments revealed that the Cdk4 protein half-life was $2.7 \text{ h} \pm 0.38$ in cyclin D1 and $2.9 \text{ h} \pm 1.02$ in control transduced hepatocyte cells (Fig. 6). We conducted ³⁵S-pulse-labelling experiments to determine if the observed increase in Cdk4 protein expression was due to enhanced protein synthesis. Cells were pulsed

Figure 5. Cdk4 mRNA expression is not regulated by over-expression of cyclin D1. (a) Representative northern blot showing mRNA expression of cyclin D1 and Cdk4 in control (Ctl) and cyclin D1 transgenic (Tr) mouse tissue lysates. Arrow indicates approximate position of endogenous cyclin D1, which was not detectable. (b) Representative northern blot showing mRNA expression of cyclin D1 and Cdk4 in control (Ctl) and cyclin D1 (D1) transduced ImHep cells. Cyclophilin served as an internal control.

Figure 6. Cdk4 half-life is not regulated by cyclin D1 over-expression. Pulse-chase experiments were performed in ImHep cells transduced with control or cyclin D1 retroviral vectors. Cells were harvested at 0, 2, 4 and 6 h post chase. The upper panel shows a representative experiment and the lower panel shows the half-life quantified by densitometry and plotted as a percentage of the 0-time point. In three independent experiments the Cdk4 half-life was 2.9 h $\pm$ 1.02 in control cells and 2.7 h $\pm$ 0.38 in cyclin D1 transduced cells.

Figure 7. Over-expression of Cyclin D1 regulates Cdk4 protein synthesis. The upper panel shows control (Ctl) and cyclin D1 (D1) transduced ImHep cells. Cells were pulsed with [35 S]methionine label for 1 h. Following the pulse, lysates were immunoprecipitated with anti Cdk4 antibodies, resolved by SDS-PAGE, enhanced by flouorography, and exposed to film overnight. Prior to immunoprecipitation, lysates were immunoblotted with cyclin D1 and actin antibodies. The lower panel shows densitometry comparing the relative amounts of Cdk4 protein synthesized in 1 h between control (Ctl) and cyclin D1 transduced ImHep cells. Data is presented as the mean $\pm$ SD (* $P < 0.05$).

for 1 h and harvested. Cdk4 was immunoprecipitated from equal amounts of total protein lysates. These studies showed that Cdk4 protein synthesis was accelerated threefold in cyclin D1 versus control-transduced hepatocyte cells (Fig. 7).

Blocking cyclin D1 expression leads to diminished Cdk4 protein expression

We used control and antisense cyclin D1 retroviral constructs to assess whether over-expression of cyclin D1 is necessary to sustain increased Cdk4 expression. ImHep cells were transduced at 33 °C when endogenous levels of cyclin D1 and Cdk4 are elevated (Fig. 3a). Northern blot analysis revealed no change in Cdk4 mRNA expression between control and cyclin D1 antisense transduced cells. Cyclophilin was used to normalize expression between control and cyclin D1 antisense cells. In comparison with controls, cyclin D1 mRNA was reduced by 40% in antisense expressing cells (Fig. 8a). This 40% reduction in cyclin D1 mRNA levels was adequate to confer dramatic decreases in cyclin D1 protein expression. Transduction of antisense cyclin D1 resulted in decreased levels of Cdk4 protein expression (Fig. 8b). From these experiments, we concluded that blocking cyclin D1 expression resulted in diminished Cdk4 protein levels. Furthermore, proliferation and DNA synthesis were significantly diminished based on tritiated thymidine incorporation and growth curve experiments (Fig. 8c and d).

DISCUSSION

The results presented in this study demonstrate that over-expression of cyclin D1 regulates Cdk4 levels by increasing Cdk4 protein synthesis. In addition, an increase in cyclin D1-Cdk4 complex

Figure 8. Antisense cyclin D1 diminishes Cdk4 protein expression and diminishes cell proliferation. (a) Northern blot comparing changes in mRNA expression of cyclin D1 and Cdk4 in control (Ctl) and antisense cyclin D1 (D1⁺) transduced cells. Blots were also probed with cyclophilin as an internal control. (b) Western blot showing cyclin D1 and Cdk4 protein expression in control (Ctl) and antisense cyclin D1 (D1⁺) transduced cells. Blots were also probed with actin as an internal control. (c) Cell proliferation was examined by measuring DNA synthesis based on tritiated thymidine incorporation and cell growth. For tritiated thymidine assays, cells were plated in triplicate, at a density of 1×10^4 cells per well in a 24 well plate. DNA synthesis was compared in control (Ctl) and antisense cyclin D1 (D1⁺) transduced Imhep cells. (d) For growth curves, cells were plated in triplicate at a density of 1×10^4 cells per well in a 6-well plate. Proliferation was compared in control (Ctl) and cyclin D1 antisense (AS) ImHep cells. Data is presented as the mean $\pm$ SD (* $P < 0.05$).

formation and Cdk4-associated kinase activity was observed in cyclin D1 over-expressing cells and tissue compared with normal controls. This was associated with an increase in cellular proliferation and the development of hepatocellular carcinoma in our cyclin D1 transgenic mouse model (Deane *et al.* 2001). We also demonstrated that blocking cyclin D1 expression with a cyclin D1 antisense construct diminishes Cdk4 protein expression and decreases cell proliferation. Together these results show that modulation of cyclin D1 expression has profound regulatory effects on Cdk4 protein expression and activity, which impact cell proliferation.

As the regulatory subunit in cyclin D1-Cdk4 complexes, cyclin D1 plays a pivotal role in cell growth such that de-regulated cyclin D1 expression leads to abnormal cell cycle progression. Our results further underscore the ability of cyclin D1 to de-regulate the cell cycle. The preponderance of BrdU-positive cells due to cyclin D1 overexpression (Fig. 3c) suggest that these cells are experiencing a shortened G₁ phase and early S-phase entry. This alteration in cell cycle progression sets the stage for the development of a number of genetic alterations leading to carcinogenic consequences. This is further emphasized by the fact that cyclin D1 over-expression characterizes a variety of tumour types including parathyroid adenomas, certain B-cell lymphomas, breast, bladder, head and neck, colon and hepatocellular carcinomas (Zhang *et al.* 1993; Bartkova *et al.* 1994; Nishida *et al.* 1994; Donnellan & Chetty 1998). Cyclin D1 exerts its

proliferative effects by regulating Cdk4 activity. Cdk4 is the catalytic subunit in cyclin D1-Cdk4 complexes (Bartek *et al.* 1996). Mounting evidence suggests that co-ordinate up-regulation of this catalytic subunit is also important in cyclin D1-associated carcinogenesis. The present study clearly demonstrates that increased expression of cyclin D1 is sufficient to cause the increase in Cdk4 expression. Coincident over-expression of cyclin D1 and Cdk4 characterizes a variety of human carcinomas (Kitahara *et al.* 1996; Masciullo *et al.* 1997; Kim *et al.* 1998; Dong *et al.* 2001). In addition, several carcinogenic mouse models display increased levels of both cyclin D1 and Cdk4. Among these are included intestinal adenomas of MIN mice and azoxymethane-induced mouse colon tumours (Zhang *et al.* 1997; Wang *et al.* 1998). Moreover, characterization of *Helicobacter hepaticus*-induced liver tumours showed that cyclin D1 and Cdk4 levels are increased in early pre-neoplastic and neoplastic liver lesions (Ramljak *et al.* 1998). Furthermore, EBV promoter-driven cyclin D1 transgenic mice display increased Cdk4 expression coincident with transgene expression in pre-neoplastic lesions of the oesophagus (Mueller *et al.* 1997). Taken together, these studies provide compelling evidence that co-ordinate regulation of cyclin D1 and Cdk4 has an important role in carcinogenesis.

Cyclin D1 and Cdk4 are critical modulators of cell cycle progression and play an important role in neoplastic transformation. Previous studies have shown that cyclin D1 antisense expression reduces cyclin D1 protein levels and associated kinase activity in human cell lines derived from oesophageal (Zhou *et al.* 1995), colon (Arber *et al.* 1997), pancreatic (Kornmann *et al.* 1998a) and lung (Driscoll *et al.* 1997) cancer. Associated with the decrease in cyclin D1 and Cdk4 kinase activity is inhibition of cell proliferation (Zhou *et al.* 1995; Arber *et al.* 1997; Driscoll *et al.* 1997; Kornmann *et al.* 1998b). This underscores the likely importance of cyclin D1-Cdk4 complexes in sustaining elevated levels of proliferation found in transformed cells. In the present study, we demonstrated that blocking cyclin D1 expression with a cyclin D1 antisense construct diminished Cdk4 protein expression and decreased cell proliferation. This further supports the role of cyclin D1 in modulating Cdk4 protein expression and suggests that this may be a key step in regulating proliferation.

This study was intended to address the mechanism by which over-expression of cyclin D1 regulates increased Cdk4 expression. Although coincident over-expression of cyclin D1 and Cdk4 has been reported in a variety of tumours, the mechanism surrounding the increased expression of Cdk4 has never been fully explored. Previous studies addressing the regulation of Cdk4 translation have shown that p53 binds the 5' UTR of the Cdk4 mRNA to mediate translational inhibition of the Cdk4 protein (Ewen *et al.* 1993; Ewen *et al.* 1995; Miller *et al.* 2000). The studies presented here demonstrate that over-expression of cyclin D1 leads to increased translation of the Cdk4 protein, but does not increase Cdk4 protein stability. The increased expression of Cdk4 likely perturbs the co-ordinated response of various Cdk inhibitors and growth inhibitory cytokines, resulting in incomplete suppression of the kinase and escape of cells from normal growth constraints. Therefore, increased Cdk4 expression may represent an important step in the carcinogenic process and an important target for therapeutic purposes.

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