Induced JunD in intestinal epithelial cells represses CDK4 transcription through its proximal promoter region following polyamine depletion

Lan XIAO*‡, Jaladanki N. RAO*‡, Tongtong ZOU*‡, Lan LIU*‡, Bernard S. MARASA*†‡, Jie CHEN*‡, Douglas J. TURNER*‡, Antonino PASSANITI†‡ and Jian-Ying WANG*†‡¹

*Cell Biology Group, Department of Surgery, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A., †Department of Pathology, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A., and ‡Baltimore Veterans Affairs Medical Center, Baltimore, MD 21201, U.S.A.

Maintenance of intestinal epithelial integrity requires cellular polyamines that regulate expression of various genes involved in cell proliferation, growth arrest and apoptosis. In prior studies, depletion of cellular polyamines has been shown to stabilize JunD, a member of the AP-1 (activator protein-1) family of transcription factors, leading to inhibition of intestinal epithelial cell proliferation, but the exact downstream targets of induced JunD remain elusive. CDK4 (cyclin-dependent kinase 4) is essential for the G₁- to S-phase transition during the cell cycle and its expression is primarily controlled at the transcriptional level. In the present study, we show that induced JunD in IECs (intestinal epithelial cells) is a transcriptional repressor of the *CDK4* gene following polyamine depletion. Increased JunD in polyamine-deficient cells was associated with a significant inhibition of CDK4 transcription, as indicated by repression of CDK4-promoter activity and decreased levels of CDK4 mRNA and protein, all of which were prevented by using specific antisense

JunD oligomers. Ectopic expression of the wild-type *junD* also repressed CDK4-promoter activity and decreased levels of CDK4 mRNA and protein without any effect on CDK2 expression. Gel shift and chromatin immunoprecipitation assays revealed that JunD bound to the proximal region of the CDK4-promoter *in vitro* as well as *in vivo*, while experiments using different CDK4-promoter mutants showed that transcriptional repression of CDK4 by JunD was mediated through an AP-1 binding site within this proximal sequence of the CDK4-promoter. These results indicate that induced JunD in IECs represses CDK4 transcription through its proximal promoter region following polyamine depletion.

Key words: activator protein-1 (AP-1), α -difluoromethylornithine, growth arrest, intestinal epithelium, ornithine decarboxylase, transcriptional regulation.

INTRODUCTION

The epithelium of the intestinal mucosa is a rapidly self-renewing tissue in the body, and maintenance of its integrity depends on a complex interplay between cell proliferation, growth arrest and apoptosis [1,2]. Undifferentiated epithelial cells continuously replicate in the proliferative zone within crypts and differentiate as they migrate up the luminal surface of the colon and the villous tips in the small intestine. Apoptosis occurs in both the crypt area, where this process maintains the balance in cell number between newly divided and surviving cells, and at the luminal surface of the intestine, where differentiated cells are lost [3–5]. This rapid dynamic turnover rate of IECs (intestinal epithelial cells) is highly regulated and critically controlled by numerous factors, including cellular polyamines [6–9]. The natural polyamines spermidine and spermine and their precursor putrescine are organic cations found in all eukaryotic cells [10–12]. The regulation of cellular polyamines has been recognized for many years as a central convergence point for the multiple signalling pathways driving IEC functions. It has been shown that IEC proliferation in the intestinal mucosa is dependent on the supply of polyamines to the dividing cells in the crypts and that decreasing cellular polyamines inhibits cell renewal *in vivo* as well as *in vitro* [7,13–15]. Although few specific functions of polyamines at the molecular level have been identified to date, an increasing body of evidence indicates that polyamines regulate IEC proliferation by virtue of their ability to modulate the expression of various growth-related genes [6,14,16].

JunD belongs to the family of Jun proteins, which are primary components of the AP-1 (activator protein-1) transcription factors. Via their basic-leucine zipper domains, Jun proteins can form AP-1 homodimers or heterodimers among themselves or with members of the related Fos family or the ATF family [17,18]. These Jun/AP-1 dimers bind to promoters at specific DNA elements such as TGAGTCA and TGACGTCA and regulate transcription of their target genes, either positively or negatively [19–21]. Although all three Jun proteins (c-Jun, JunB and JunD) are similar in their DNA-binding affinity, their expression exhibits different patterns in response to stress and plays distinct roles in regulation of cell proliferation and transformation [16,22,23]. *c-jun* and *junB* function as immediate early response genes upon mitogenic stimulation, and activation of these two genes enhances the G₁- to S-phase transition during cell cycle progression [19,22]. In contrast, JunD has been shown to inhibit cell proliferation in a variety of cell types [14,23–25]. In the absence of JunD (*junD*^{−/−}), immortalized fibroblasts and T-cells display higher proliferation rates than their wild-type counterparts [23,26]. Our previous studies have demonstrated that JunD is implicated in the negative control of IEC proliferation and that polyamines regulate expression of the *junD* gene at the post-transcriptional level [14,27]. Depletion of cellular polyamines by treatment with DFMO (α -difluoromethylornithine), a specific inhibitor for polyamine biosynthesis, stabilizes JunD mRNA and increases JunD-dependent transcriptional activity, resulting in an inhibition of IEC proliferation. However, the exact downstream targets of induced JunD following polyamine depletion remain to be elucidated.

Abbreviations used: AP-1, activator protein-1; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; DFMO, α -difluoromethylornithine; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEC, intestinal epithelial cell; Luc, luciferase; PBS-T, PBS containing Tween 20; Q-PCR, quantitative PCR; Rb, retinoblastoma tumour suppressor protein; RT, reverse transcriptase.

¹ To whom correspondence should be addressed (email jwang@smail.umaryland.edu).

CDK (cyclin-dependent kinase) 4 controls how cells enter into and are committed to progress through the cell cycle, and its activity is critically regulated at different levels including modulation of *CDK4* gene expression. It has been reported that *CDK4* is a target of the c-Myc transcription factor and that increased c-Myc activates *CDK4* expression [28]. On the other hand, the calcineurin-regulated transcription factor, NFAT (nuclear factor of activated T-cells), is shown to repress *CDK4* gene transcription and inhibit *CDK4* activity [29]. The main target of activated *CDK4* is the phosphorylation of Rb (the retinoblastoma tumour suppressor protein) that represses transcription of E2F family members [30]. In quiescent cells, Rb associates with and inactivates E2F activity, but this Rb-dependent repression of E2F is relieved upon phosphorylation of Rb by CDKs, allowing E2F to target S-phase-specific genes and commits the cell to enter the cell cycle [31]. Specific inhibition of *CDK4* activity delays cell-cycle progression and induces the accumulation in the G₁-phase growth arrest [30], similar to the phenotype of polyamine-deficient IECs [14]. Consistently, embryonic fibroblasts derived from *CDK4*^{-/-} mice exhibit a prolonged transition from G₁-to S-phase after serum stimulation [32]. In the present study, we sought to determine whether increased JunD inhibits IEC proliferation through suppression of *CDK4* gene expression following polyamine depletion. The results shown in the present study demonstrate that *CDK4* is a target of JunD in IECs and that induced JunD in polyamine-deficient cells represses *CDK4* transcription through an AP-1 binding site within a proximal region of the *CDK4*-promoter. Some of these results have been published previously in abstract form [33].

MATERIALS AND METHODS

Chemicals and supplies

Disposable cultureware was purchased from Corning Glass Works. Tissue culture medium and dialysed FBS (fetal bovine serum) were purchased from Invitrogen, and biochemicals were obtained from Sigma. Antibodies against JunD, *CDK4* and *CDK2* were purchased from Santa Cruz Biotechnology. DFMO was purchased from Ilex Oncology. JunD antisense or sense oligodeoxyribonucleotides were from Biognostik. [γ -³²P]ATP (3000 Ci/mmol) was purchased from PerkinElmer.

Cell culture

The IEC-6 cell line, derived from normal rat intestinal crypt cells [34], was purchased from the ATCC (American Type Culture Collection) at passage 13 and used at passage 15–20 in the current experiments. Cells were maintained in DMEM (Dulbecco's modified Eagle medium) supplemented with 5 % heat-inactivated FBS, 10 μ g/ml insulin and 50 μ g/ml gentamicin. Flasks were incubated at 37 °C in a humidified atmosphere of 90 % air and 10 % CO₂, and medium was changed three times weekly. The Caco-2 cell line (a human colon carcinoma cell line) was also obtained from ATCC at passage 16. It was maintained similarly to the IEC-6 cell line except that it was maintained in an atmosphere of 95 % air and 5 % CO₂. The medium used was Eagle's minimum essential medium with 10 % heat-inactivated FBS, and passages 18–23 were used for the experiments.

Plasmid construction and transfection

The plasmid clone (pRSV-hjD) containing the human *junD* gene was obtained from ATCC. Two PCR primers (sense: 5'-TACCGC-

TAG-CGGAGGATGGAAACACCCTTC-3'; antisense: 5'-GTC-AGGTACCCTCAGTACGCCGGGACCTG-3') were used to amplify the complete open reading frame of *junD* from pRSV-hjD. The resulting PCR product was sequenced to confirm that there were no mutations introduced by PCR and then cloned into an expression vector pcDNA3.1(+) (Invitrogen) with the CMV promoter. The constructs of the *CDK4* promoter luciferase (Luc) reporter, 8-Luc (0.8 kb regulatory region upstream of the *CDK4* gene fused to the Luc reporter gene), and its deletion mutant, 4-Luc, were provided by Dr Burakoff (Harvard Institutes of Medicine, Boston, MA, U.S.A.). The 3-Luc construct (–319/+43) of the 5'-deletion *CDK4* promoter was generated using the QuikChange[®] site-directed mutagenesis kit from Stratagene. Briefly, the 4-Luc construct of the *CDK4* promoter was used as a template. Two mutagenic oligonucleotide primers were designed and synthesized, each of which was complementary to the opposite strand of template DNA and contained the desired mutation. The oligonucleotide primers were extended during temperature cycling, and incorporation of the primers generated the mutated plasmid. After digestion with DpnI, 4 μ l of product was used to transform XL1-Blue competent cells provided in the mutagenesis kit, and the positive clones were selected for DNA extraction. The deletion mutant of the *CDK4*-promoter was confirmed by DNA sequencing. The AP-1 point mutant of the *CDK4* promoter was also generated using the QuikChange[®] site-directed mutagenesis kit, and the 4-Luc construct of the *CDK4* promoter was used as a template. Experiments were performed according to the manufacturer's instructions as described above. The final PCR product was sequenced to verify that it contained the desired AP-1 point mutation. Transient transfection was performed with Lipofectamine[™] reagent from Invitrogen. All *CDK4*-promoter constructs were from firefly luciferase reporters. The promoter constructs were transfected into cells along with pRL-null, a *Renilla* luciferase control reporter vector from Promega, to monitor transfection efficiencies. The transfected cells were lysed for assays of promoter activity using the Dual Luciferase Reporter Assay System (Promega). The luciferase activity from individual constructs was normalized by *Renilla*-driven luciferase activity in every experiment.

RT (reverse transcriptase)-PCR and real-time Q-PCR (quantitative PCR) analysis

Total RNA was isolated using the RNeasy Mini Kit from Qiagen and used in the RT reaction as described previously [16]. Real-time Q-PCR was performed using an Applied Biosystems instrument using specific primers, probes and software (Applied Biosystems). The levels of *CDK4* mRNA were quantified by Q-PCR analysis and normalized by mRNA levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Western blot analysis

Cell samples, placed in SDS sample buffer [250 mM Tris/HCl, (pH 6.8), 2 % SDS, 20 % glycerol and 5 % 2-mercaptoethanol], were sonicated, and then centrifuged at 10 000 g for 15 min at 4 °C. The supernatant from cell samples was boiled for 5 min and then subjected to electrophoresis on 7.5 % acrylamide gels according to the method of Laemmli [35]. After the transfer of protein on to nitrocellulose filters, the filters were incubated for 1 h in 5 % non-fat dried skimmed milk in 1 × PBS/Tween 20 [PBS-T; 15 mM NaH₂PO₄, 80 mM Na₂HPO₄, 1.5 M NaCl (pH 7.5) and 0.5 % (v/v) Tween 20]. Immunological evaluation was then performed for 1 h

in 1 % BSA/PBS-T buffer containing 1 $\mu\text{g/ml}$ of the specific antibody against JunD, CDK4 or CDK2 proteins. The filters were subsequently washed with $1 \times$ PBS-T and incubated for 1 h with the secondary antibody conjugated to peroxidase by protein cross-linking with 0.2 % glutaraldehyde. After extensive washing with $1 \times$ PBS-T, the immunocomplexes on the filters were developed using the ECL[®] method according to the manufacturer's instruction (Amersham Pharmacia Biotech).

Preparation of nuclear protein and EMSAs (electrophoretic mobility-shift assays)

Nuclear proteins were prepared via the procedure described previously [36,37], and the protein contents in nuclear preparations were measured using the Bradford method [38]. The double-stranded oligonucleotides used in these experiments included 5'-CGCTTGATGAGTCAGCCGAA-3', which contains a consensus AP-1 binding site (underlined); and 5'-CCGGTAGTGAG-ACAATCCTTC-3', which is from a proximal region of the CDK4-promoter at -335/-315 and contains an AP1-like sequence (underlined). These oligonucleotides were end-labelled with [γ -³²P]ATP using the end-labelling system from Promega. For EMSAs, 0.035 pmol of ³²P-labelled oligonucleotides (approx. 30000 c.p.m.) and 10 μg of nuclear protein were incubated in a total volume of 20 μl in the presence of 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT (dithiothreitol), 50 % glycerol and 1 μl of poly(dI-dC). The binding reactions were allowed to proceed at room temperature (25 °C) for 20 min. Thereafter, 2 μl of Bromophenol Blue (0.1 % in water) was added, and protein-DNA complexes were resolved by electrophoresis on non-denaturing 4 % polyacrylamide gels and visualized by autoradiography.

ChIP (chromatin immunoprecipitation)

ChIP assays were performed using the Upstate Biotechnology ChIP assay kit according to the provided protocol with minor modification. Cells were grown to 80 % confluence and fixed with 1 % formaldehyde to cross-link chromatin. Cells were washed with ice-cold PBS, suspended in SDS lysis buffer and then sonicated. After centrifugation (11 400 *g* for 10 min at 4 °C), the supernatant was transferred and diluted in 10-fold ChIP dilution buffer, and then pre-cleared with a 50 % slurry of salmon sperm DNA/Protein A agarose with agitation. Supernatant was incubated with the anti-JunD antibody or control IgG overnight with constant rotation. The immunocomplexes were captured and eluted with fresh elution buffer (1 % SDS and 0.1 M NaHCO₃). The DNA-protein cross-links were reversed and deproteinized, and DNA was recovered and amplified by PCR. A pair of primers to amplify the proximal region of the CDK4-promoter containing an AP-1 binding site were 5'-ATGCAGACAGGCTGAAAGAC-3' and 5'-GATGGCAGCCACGTGATCTG-3'. The DNA isolated through IgG ChIP was used as a negative control. Input DNA, obtained from chromatin that was cross-link reversed similarly to the sample, served as a positive control for PCR effectiveness.

Statistics

Values are means $\pm$ S.E.M. from 3 to 6 samples. Autoradiographic and immunoblotting results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined by using Duncan's multiple range test [39].

RESULTS

Increased levels of endogenous JunD repress CDK4 transcription following polyamine depletion

Since overexpression of JunD in IECs inhibited cell proliferation and induced G₁-phase growth arrest [13,14], the focus of the present study was to further determine whether increased JunD inhibited cell proliferation by repressing CDK4 transcription following polyamine depletion. Exposure of IEC-6 cells to 5 mM DFMO for 6 days completely inhibited ODC (ornithine decarboxylase) enzyme activity (the first rate-limiting step for polyamine biosynthesis) and almost totally depleted cellular polyamines. Levels of putrescine and spermidine were undetectable on day 6 after treatment with DFMO, and spermine had decreased by approx. 60 % (results not shown). Similar results have been reported in our previous publications [40,41]. Consistent with our previous findings [14,27], polyamine depletion by DFMO significantly increased the steady-state levels of JunD protein, which was completely prevented by exogenous putrescine (10 μM) given together with DFMO (Figure 1A). Spermidine (5 μM) had an effect equal to that of putrescine on levels of JunD protein when it was added to cultures that contained DFMO (results not shown).

Increased levels of endogenous JunD in the polyamine-deficient cells were associated with inhibition of CDK4 transcription as demonstrated by decreases in CDK4-promoter activity and CDK4 mRNA (Figure 1B and 1C). Levels of CDK4-promoter activity and CDK4 mRNA were decreased by approx. 65 % in cells exposed to DFMO for 6 days, which was associated with a significant decrease in CDK4 protein (Figure 1D). Expression of CDK4 protein was inhibited by approx. 60 % in DFMO-treated cells. To determine the effect of increased JunD on CDK4 expression in other cell lines, we examined changes in levels of JunD and CDK4 proteins following polyamine depletion in Caco-2 cells. As shown in Figure 2, depletion of cellular polyamines also increased expression of JunD, which was associated with a decrease in CDK4 expression in Caco-2 cells. The level of JunD protein in cells exposed to DFMO for 6 days was approx. 3.4 times the normal values, while CDK4 protein was decreased by approx. 70 %. Furthermore, this repressed CDK4 expression following increased JunD in polyamine-deficient cells was accompanied by an inhibition of cell proliferation, in which most cells were arrested in G₁-phase as shown in our previous study [14]. In the presence of DFMO, exogenous putrescine not only prevented induced JunD but also returned CDK4 transcription to near normal levels. Consistently, cell growth was also returned to normal when putrescine was administered together with DFMO (results not shown).

To determine the causal relationship between increased JunD and CDK4 repression, the effect of inhibition of JunD by using specific JunD antisense oligomers on CDK4 expression was examined in polyamine-deficient cells. Cells were grown in the presence of 5 mM DFMO for 4 days and then exposed to JunD antisense or sense oligomers at the concentration of 4 μM . Levels of JunD and CDK4 proteins were measured 48 h after the incubation. Results presented in Figure 3 clearly show that inhibition of JunD expression by treatment with JunD antisense oligomers promoted CDK4 protein expression. When the DFMO-treated cells were exposed to JunD antisense oligomers for 48 h, CDK4 protein expression returned to near normal levels. To verify the specificity of JunD antisense oligomers used in the present study, the membranes were reprobed with an anti-JunB antibody and showed that levels of JunB protein were not affected by the antisense to JunD (results not shown). In addition, treatment with JunD antisense oligomers also significantly promoted cell proliferation in

Figure 1 Changes in JunD protein levels and CDK4 expression in control IEC-6 cells and in cells treated with either DFMO (5 mM) alone or DFMO plus PUT (putrescine; 10 μ M) for 6 days

(A) Representative immunoblots of JunD protein. Whole cell lysates were harvested, applied to each lane (20 μ g) equally, and subjected to electrophoresis on a 7.5% acrylamide gel. Levels of JunD (~40 kDa) were identified by probing nitrocellulose with a specific anti-JunD antibody. After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. (B and C) Changes in CDK4 expression in cells described in (A). (B) CDK4 promoter activity as measured using luciferase reporter gene assays. After cells were grown in cultures containing DFMO or DFMO plus PUT for 4 days, they were transfected using the CDK4-promoter luciferase reporter construct using LipofectamineTM. Transfected cells were harvested and assayed for luciferase activity after 48 h of incubation in the presence of DFMO or DFMO plus PUT. Data were normalized by *Renilla*-driven luciferase activity and expressed as means $\pm$ S.E.M. of data from 3 separate experiments. * P < 0.05 compared with control cells and cells treated with DFMO plus PUT. (C) Levels of CDK4 mRNA as measured by real-time quantitative PCR analysis. Values are means $\pm$ S.E.M. of data from 3 separate experiments. (D) Representative immunoblots of CDK4 protein in cells described in (A). Levels of CDK4 protein (~34 kDa) were detected by Western blot analysis, and equal loading was monitored by actin immunoblotting. Data are representative from three independent experiments showing similar results.

polyamine-deficient cells. Cell numbers were increased by approx. 30% in the DFMO-treated cells exposed to 4 μ M JunD anti-

Figure 2 Effect of polyamine depletion on expression of JunD and CDK4 in Caco-2 cells

Cells were grown in control cultures and cultures containing either 5 mM DFMO alone or DFMO plus PUT (putrescine; 10 μ M) for 6 days. (A) Representative immunoblots of JunD and CDK4 proteins. Whole cell lysates were harvested, and levels of JunD and CDK4 proteins were measured by Western blot analysis with the specific antibody against JunD or CDK4. Loading control was monitored by actin immunoblotting. (B and C) Quantitative analysis of Western blots by densitometry from Caco-2 cells described in (A). Values are means $\pm$ S.E.M. of data from 3 separate experiments. * P < 0.05 compared with control cells and cells treated with DFMO plus PUT.

sense oligomers for 48 h (results not shown). Similar results have been reported in our previous publication [14]. On the other hand, treatment with JunD sense oligomers at the same concentration had no effect on expression of JunD and CDK4 proteins and cell growth. These results indicate that CDK4 is a down-stream target of JunD in IECs and that increased levels of endogenous JunD following polyamine depletion repress CDK4 expression, thus leading to inhibition of cell proliferation.

Effect of JunD ectopic expression on CDK4 transcription

To further define the exact role of JunD in regulation of CDK4 expression, we examined the effect of overexpression of the wild-type *jund* gene on CDK4 transcription in Caco-2 cells. This line of cells was used in the present study based on the following reasons: (i) polyamine depletion also increased JunD expression (Figure 2) resulting in inhibition of Caco-2 cell growth [14]; and (ii) this cell line is an excellent model for transient transfection. A JunD expression vector containing the corresponding human *jund* cDNA under the control of pCMV was constructed as indicated in Figure 4(A). Transient transfection with the JunD vector dramatically increased JunD protein expression (Figure 4B). The levels of JunD protein were increased by approx. 8-fold at 24 h and by approx. 15-fold at 48 h after the transfection respectively. The vector that lacked exogenous *jund* cDNA (Null) was used as a negative control and did not induce JunD protein levels (Figure 4B). Importantly, overexpression of the *jund* inhibited CDK4-promoter activity (Figure 4C, left-hand panel) and decreased levels of CDK4 mRNA (Figure 4C, right-hand panel) and protein (Figure 4D). The activity of CDK4-promoter luciferase reporter was inhibited by approx. 60% 48 h after the transfection, while levels of CDK4 mRNA and protein were decreased by approx. 55%. This inhibitory effect of increased JunD on CDK4 transcription is specific because ectopic expression of the *jund* did not alter levels of CDK2 expression (Figure 4D). On the other hand, increased JunD by transient transfection also inhibited Caco-2

Figure 3 Effect of inhibition of JunD expression by antisense JunD oligodeoxyribonucleotides on CDK4 expression in polyamine-deficient cells

IEC-6 cells were initially treated with 5 mM DFMO for 4 days and then exposed to 4 μ M antisense JunD oligomers in the presence of DFMO for an additional 48 h. JunD sense oligomers served as a control. **(A)** Representative immunoblots of JunD and CDK4 proteins. Whole cell lysates were harvested, and JunD and CDK4 proteins were detected by Western blot analysis. Loading control was monitored by actin immunoblotting. **(B)** Quantitative analysis derived from densitometric scans of Western blots of CDK4 protein from cells described in **(A)**. Values are means $\pm$ S.E.M. of data from 3 separate experiments. * $P < 0.05$ compared with control cells and cells treated with DFMO plus JunD sense oligomers.

cell proliferation as indicated by a decrease in levels of DNA synthesis and cell numbers (results not shown). Similar results have been reported in our previous studies [14]. Neither CDK4-promoter activity nor levels of CDK4 mRNA and protein were affected by the transfection with the vector lacking *junD* cDNA. This result indicates that overexpression of JunD represses CDK4 transcription and inhibits cell proliferation, suggesting that repression of CDK4 by JunD plays a critical role in the negative control of IEC proliferation.

Involvement of a proximal region of the CDK4-promoter in its repression by JunD

To define the mechanism by which induced JunD represses CDK4 transcription, the JunD-responsive region of the CDK4-promoter was mapped, and several deletions in the CDK4-promoter luciferase reporter genes were constructed as illustrated in Figures 5(A) and 5(B). Consistent with previous findings [29], the elements that contained an 824 bp region of the CDK4 promoter and 43 bp of the first untranslated exon are required for basal and regulatory CDK4 expression in Caco-2 cells. Results presented in Figure 5(C) show that induced JunD repressed CDK4-promoter activity when cells were transfected with either the 8-Luc construct (full-length promoter) or 4-Luc construct (–447 bp promoter fragment). However, deletion of nucleotides from the positions –824 bp to –404 (4-Luc) relative to the transcriptional start site enhanced the inhibitory effect of induced JunD on CDK4-promoter activity. The inhibitory rates of CDK4-promoter by JunD were approx.

Figure 4 Effect of ectopic expression of the *junD* gene on CDK4 transcription

(A and B) Characterization of transient JunD-transfected cells. **(A)** Structure of JunD expression vector. The complete open reading frame of human *junD* cDNA was amplified by PCR, sequenced, and then cloned to an expression vector pcDNA3.1(+). PCMV, human cytomegalovirus immediate-early promoter. **(B)** Representative immunoblots of JunD protein. Caco-2 cells were grown in minimum essential medium containing 5% dialysed FBS and transfected using the expression vector containing human *junD* cDNA (JunD) or control vector lacking *junD* cDNA (Null) using LipofectamineTM. JunD protein levels were measured by Western blot analysis 24 and 48 h after the transfection. **(C and D)** Changes in CDK4 transcription in cells described in **(A)**. **(C)** Left-hand panel: CDK4-promoter activity as measured using luciferase reporter gene assays. Cells were co-transfected with the CDK4-promoter luciferase reporter and the JunD expression vector or Null, and the activity of CDK4-promoter activity was measured 48 h after transfection. Values are means $\pm$ S.E.M. of data from 3 separate experiments. * $P < 0.05$ compared with control cells and cells transfected with the Null for 48 h. Right-hand panel: levels of CDK4 mRNA as measured by real-time Q-PCR analysis. Values are means $\pm$ S.E.M. of data from 3 separate experiments. * $P < 0.05$ compared with controls and cells transfected with the Null. **(D)** Representative immunoblots for CDK4 and CDK2 (~34 kDa) proteins as measured by Western blot analysis. Three experiments were performed that showed similar results.

Figure 5 Effect of ectopic expression of the *junD* gene on CDK4-promoter activity after deletion mutation of AP-1 binding site

(A and B) Basal levels of activity of various CDK4-promoter deletion mutants. (A) Schematic representation of CDK4-promoter luciferase (Luc) reporter constructs. AP-1 indicates the relative location of the AP-1 binding site within the CDK4-promoter. (B) Basal activity of various deletion mutants of the CDK4-promoter in Caco-2 cells without JunD overexpression. Cells were transfected with different deletion mutants of CDK4-promoter, and levels of the luciferase reporter activity were detected 48 h after the transfection. Results were normalized relative to *Renilla*-driven luciferase activity and expressed as means $\pm$ S.E.M. of data from three separate experiments. (C) Changes in luciferase reporter activity of deletion constructs after JunD overexpression. Cells were co-transfected with different CDK4-promoter luciferase reporter deletion constructs and the JunD expression vector or Null. After 48 h of incubation, transfected cells were harvested and assayed for luciferase activity. Values are means $\pm$ S.E.M. of data from three separate experiments. * $P < 0.05$ compared with cells transfected with the Null. + $P < 0.05$ compared with cells co-transfected with the JunD expression vector and the 8-Luc construct.

55 % in the 8-Luc and approx. 77 % in the 4-Luc respectively. In contrast, further deletion of the nucleotides from positions from -404 bp to -319 bp (3-Luc) of the CDK4-promoter completely abolished repression of the CDK4-promoter by JunD, indicating that this proximal region of the CDK4-promoter is crucial for JunD-mediated CDK4 repression. Interestingly, this region of the CDK4-promoter contains an AP-1-like binding sequence (TGAGACA) at -322, in which there was only one nucleotide difference compared with the canonical AP-1 core binding sequence (TGAGTCA). Because JunD is a member of the AP-1 family of proteins, these results suggest the possibility that increased JunD represses CDK4 transcription through its interaction with this AP-1 binding site within the proximal region of the CDK4-promoter.

A functional AP-1 binding site is located at a proximal region of the CDK4 promoter

To characterize the exact role of this AP-1-like sequence of the CDK4-promoter in repression of CDK4 transcription by JunD, the following three studies were performed. First, we used EMSAs to determine the binding characteristics of the AP-1-like site within the CDK4-promoter. The sequence from positions -335 to -315 of the CDK4-promoter, as illustrated in Figure 6(A), was

Figure 6 AP-1 binding activity *in vitro* and *in vivo*

(A) Sequences of a synthetic (commercial) AP-1 probe and the oligonucleotides corresponding to the sequence -335/-315 (AP-1-like) of the human CDK4-promoter. (B) AP-1 binding activity as measured by electrophoretic mobility-shift assay (EMSA). Left-hand panel: representative autoradiogram of AP-1 binding activity by using commercial AP-1 oligonucleotides. Middle and right-hand panels: changes in AP-1 binding activity by using AP-1-like sequences of the CDK4-promoter. EMSAs were performed using 10 μ g of nuclear protein and 0.035 pmol of 32 P-end-labelled AP-1 or AP-1-like oligonucleotides, and positions of the specifically bound DNA-protein complex and the freely migrating probes are indicated. Competitive inhibition experiments (middle panel) were performed in the presence of unlabelled (cold) AP-1 or Sp1 oligonucleotides at the concentration of $\times 50$. In studies determining the effect of addition of the antibody against JunD on the AP-1 binding activity (right-hand panel), nuclear proteins were initially incubated with the 32 P-end-labelled AP-1-like oligonucleotides for 20 min. The antibody against JunD was then added to the binding reaction mixture, and the reaction was allowed to proceed for an additional 30 min. No-NE, lane in which no nuclear extract was added. None, lane without cold AP-1 or Sp1 oligos. No-AB, lane with nuclear extract and no antibody. (C) Binding activity as measured by ChIP assays. Association of JunD with a proximal region of CDK4-promoter (between -412 and 43) was determined using anti-JunD antibody, while IgG was used as a negative control. Cross-linked chromatin isolated from Caco-2 cells was immunoprecipitated by the anti-JunD antibody, and the associated chromosomal DNA fragments were amplified by PCR using CDK4-promoter specific primers as described in the Materials and methods section. The expected size of the PCR product was 270 bp. Chromosomal DNA input was subjected to the same PCR amplification. Three experiments were performed that showed similar results.

used in an EMSA analysis, while an oligonucleotide containing a single consensus AP-1 binding site (available commercially) served as a positive control. Nuclear proteins were isolated from Caco-2 cells that highly expressed JunD. As shown in Figure 6(B), increased JunD was observed to bind to the 32 P-labelled

nucleotides from the positions -328 to -322 of the CDK4-promoter, which contained the AP-1-like sequence. The binding complexes were identical with those using canonical AP-1 oligonucleotides and were almost completely inhibited when the unlabelled AP-1 oligonucleotide, but not Sp1 (stimulating protein-1) oligonucleotide, was added to the binding reaction mixture at a concentration of $\times 50$. Furthermore, the specific antibody against JunD, when added to the binding reaction mixture, dramatically supershifted the AP-1 complexes present in cells overexpressing JunD (Figure 6B, middle panel).

Secondly, ChIP was used to further confirm the binding of JunD to the CDK4-promoter by examining an *in vivo* association of JunD with a proximal region of the CDK4-promoter. In the present study, the nuclear fractions were immunoprecipitated using a specific anti-JunD antibody, and the associated DNA was purified. Using specific primers to the CDK4 promoter, a 270 bp PCR product was obtained, which completely matched the sequence of a proximal region of CDK4 promoter from -384 bp to -115 bp relative to the transcriptional start site. This association was specific for JunD, since no PCR product was detectable when using a non-specific antibody IgG or when using primers to an unrelated promoter such as GAPDH. Thirdly, we determined the effect of an AP-1 point mutation on CDK4 repression by JunD. The AP-1 point mutant of the CDK4-promoter was generated, in which the AP-1-like binding site at $-328/-322$ was eliminated by making one base change as indicated in Figure 7(A). As shown in Figure 7(B), left-hand panel, induced JunD repressed CDK4-promoter activity as measured by transfection with the 4-Luc construct. However, CDK4 repression by JunD was almost completely prevented when the AP-1 binding site within the proximal promoter region was mutated (Figure 7B, right-hand panel). Under these conditions, there were no significant differences in levels of CDK4-promoter activity between cells transfected with the JunD expression vector and cells transfected with the Null. These results indicate that there is a functional AP-1 binding site within a proximal region of the CDK4-promoter and that transcriptional repression of CDK4 by JunD is mediated through this functional AP-1.

Finally, we determined the role of the AP-1 binding site within a proximal promoter region in CDK4 repression by increased endogenous JunD following polyamine depletion. As shown in Figure 7(C), left-hand panel, the CDK4-promoter activity was substantially inhibited in polyamine-deficient cells when they were transfected with the 4-Luc construct that contained the AP-1 binding site. However, this inhibition completely disappeared when polyamine-deficient cells were transfected with the 4Mut-Luc construct, in which the AP-1 binding site was mutated (Figure 7C, right-hand panel). There were no significant differences in CDK4 promoter activity between control cells and cells treated with DFMO alone or DFMO plus putrescine for 6 days. These results indicate that induced JunD in IECs represses CDK4-promoter activity by interacting with the AP-1 binding site within its proximal promoter region following polyamine depletion.

DISCUSSION

JunD is the most abundantly expressed Jun family member and functions as a negative regulator of cell proliferation in a variety of cell types [14,23,24]. Overexpression of JunD inhibits cell proliferation, whereas cells lacking JunD derived from JunD^{-/-} mice exhibit increased rates of cell division [26]. Our previous studies [14,27] have shown that IECs highly express JunD when quiescent and that increasing polyamines stimulate intestinal epithelial cell proliferation, at least in part, by decreasing JunD. Our

Figure 7 Effect of ectopic expression of the *junD* gene on CDK4-promoter luciferase reporter activity: AP-1 point mutation within CDK4-promoter

(A) Schematic representation of AP-1 point mutants of the CDK4 promoter. Mutagenic oligonucleotides were designed to hybridize to the CDK4-promoter construct to create the mutant where the AP-1 consensus site at $-328/-322$ was eliminated by making a base change. (B) Luciferase reporter activity of AP-1 point mutant of CDK4-promoter. Caco-2 cells were co-transfected with the JunD expression vector and the luciferase reporter constructs of CDK4-promoter with or without AP-1 point mutation using LipofectamineTM. Levels of luciferase activity were measured 48 h after the transfection. Values are means $\pm$ S.E.M. of data from three separate experiments. * $P < 0.05$ compared with cells transfected with the Null. (C) Changes in levels of CDK4-promoter activity after AP-1 point mutation in the presence or absence of cellular polyamines. Cells were grown in the medium containing either DFMO alone or DFMO plus PUT (putrescine) for 4 days and then transfected using the CDK4-promoter luciferase reporter constructs with (left-hand panel) or without (right-hand panel) AP-1 point mutation. Luciferase activity was measured 48 h after the transfection. Values are means $\pm$ S.E.M. of data from three separate experiments. * $P < 0.05$ compared with control cells and cells treated with DFMO plus PUT.

studies further indicate that polyamines negatively regulate JunD expression at the post-transcriptional level and that depletion of cellular polyamines stabilizes JunD mRNA without affecting its gene transcription [14]. Induced levels of nuclear JunD in polyamine-deficient cells form JunD/AP-1 homodimers and increase JunD-dependent transcriptional activity, resulting in the inhibition of IEC proliferation. In the present study, we provide new evidence that CDK4 is a downstream target of induced JunD following polyamine depletion and that repression of CDK4 transcription by JunD occurs at the promoter level, thereby advancing our understanding of cellular functions of JunD in IECs.

The results reported in the present study clearly show that increased levels of endogenous JunD in DFMO-treated cells were associated with a significant inhibition of CDK4 expression as indicated by repression of CDK4-promoter activity and decreases in CDK4 mRNA and protein (Figure 1). Because both increased JunD and inactivation of CDK4 expression in DFMO-treated cells were completely prevented by the addition of exogenous

putrescine, these observed changes in levels of JunD and CDK4 expression are most likely related to polyamine depletion rather than to the non-specific effect of DFMO. These results are consistent with our previous studies [6,15,42] and others [43,44] that show that polyamine depletion alters expression of various cell-cycle-related proteins such as CDKs, cyclins, p53, p27 and p21 in IECs, and caused cell growth arrest at the G₁-phase. Most significantly, however, is the observation that polyamine-depletion-induced JunD inhibits CDK4 transcription in IECs. In polyamine-deficient cells, treatment with specific JunD antisense oligomers prevented increased levels of endogenous JunD and thus promoted CDK4 expression (Figure 3). In contrast, ectopic expression of the wild-type *junD* repressed CDK4-promoter activity and decreased levels of CDK4 mRNA and protein, but had no effect on CDK2 expression (Figure 4). The present study provides new evidence, for the first time, that JunD is a transcriptional repressor of the *CDK4* gene, results that are consistent with studies demonstrating that expression of the *CDK4* gene is primarily regulated at the transcriptional level [24,28,29,45,46].

The results reported in the present study also indicate that there is a functional AP-1 binding site within the proximal region of the CDK4-promoter and that increased JunD represses CDK4 transcription through direct interaction with this AP-1 site in IECs. In comparison with the canonical AP-1 motif (TGAGTCA) [17–19], this AP-1-like sequence (TGAGACA) within the proximal region of the CDK4-promoter has only one nucleotide difference (underlined). Several pieces of evidence from the current studies demonstrate that this AP-1-like sequence is crucial for CDK4 repression by JunD. First, deletion of this AP-1-like sequence from the CDK4-promoter (3-Luc) prevented repression of CDK4 transcription after overexpression of JunD (Figure 5). In contrast, deletion nucleotides from positions –824 to –404 relative to the transcription start site but with this AP-1-like sequence (4-Luc) did not block the repression of CDK4 promoter by JunD. In fact, the inhibitory effect of increased JunD on the CDK4-promoter is much stronger as measured by using the 4-Luc construct than that observed in full-length promoter (8-Luc), although the reason causing this difference remains to be elucidated. It is possible that there may be transcriptional enhancers in the distal region of the CDK4-promoter. Secondly, gel shift assays and ChIP analysis revealed that JunD bound to the CDK4-promoter *in vitro* (Figure 6B) as well as *in vivo* (Figure 6C). Thirdly, repression of CDK4 by either ectopic expression of *junD* gene or increased endogenous JunD following polyamine depletion was also completely prevented when this AP-1-like binding site was mutated (Figure 7). Together, these experiments establish that the sequence located at –328/–322 within the CDK4-promoter is a functional AP-1 binding site and that JunD represses CDK4 through this AP-1. These findings are consistent with results from others [24,26] who have demonstrated that the transcriptional activity of JunD is mediated through AP-1 in the promoters of its target genes. It has been reported that both JunD-induced inhibition of the *atrial natriuretic peptide* gene transcription [24] and activation of the *ferritin H* gene promoter by JunD [46] require AP-1 motifs within their promoters.

Repression of CDK4 transcription by increased JunD plays an important role in inhibition of IEC proliferation following polyamine depletion and is of biological significance. JunD is a transcription factor with a C-terminal DNA-binding domain and N-terminal transcriptional activation domain and can act as a repressor or an activator of transcription of its target genes, depending on the composition of the heterodimeric complexes, the promoter elements, interactions with other factors and the cell type [17,26,47]. For example, JunD is converted from a growth suppressor into a growth promoter when its binding to menin is

prevented [26]. Because expression of other AP-1 members such as c-Fos, c-Jun and JunB decrease dramatically following polyamine depletion in IECs [42,48], it is likely that the inhibition of CDK4-promoter activity in DFMO-treated cells is primarily due to the JunD/JunD homodimers. In support of this possibility, disruption of JunD expression in polyamine-deficient cells by using JunD antisense oligomers promoted CDK4 expression (Figure 3). Our previous studies [14,27] have demonstrated that decreased levels of cellular polyamines stabilize JunD mRNA, leading to an accumulation of nuclear JunD in IECs. Results obtained in the present study further show that polyamine-depletion-induced JunD represses CDK4 transcription through the proximal region of the CDK4 promoter and decreases levels of cellular CDK4 protein. Since CDK4 activation is essential for entry into the cell cycle after mitogenic stimuli [30] and cells derived from CDK4^{–/–} mice exhibit a prolonged transition from G₁- to S-phase through cell cycle progression [32], the ability of induced JunD to inhibit IEC cell proliferation is in part due to its ability to directly repress the transcription of CDK4 following polyamine depletion. Given the fact that IECs highly express JunD under physiological conditions, these findings suggest that JunD-dependent CDK4 repression is implicated in the negative control of IEC proliferation and plays a critical role in maintenance of intestinal mucosal homeostasis.

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