Polyamine-modulated c-Myc expression in normal intestinal epithelial cells regulates p21Cip1 transcription through a proximal promoter region

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Maintenance of intestinal mucosal epithelial integrity requires cellular polyamines that regulate expression of various genes involved in cell proliferation, growth arrest and apoptosis. Our previous studies have shown that polyamines are essential for expression of the *c-myc* gene and that polyamine-induced c-Myc plays a critical role in stimulation of normal IEC (intestinal epithelial cell) proliferation, but the exact downstream targets of induced c-Myc are still unclear. The p21Cip1 protein is a major player in cell cycle control, which is primarily regulated at the transcriptional level. The current study was designed to determine whether induced c-Myc stimulates normal IEC proliferation by repressing p21Cip1 transcription following up-regulation of polyamines. Overexpression of the ODC (ornithine decarboxylase) gene increased levels of cellular polyamines, induced c-Myc expression and inhibited p21Cip1 transcription, as indicated by repression of p21Cip1 promoter activity and a decrease in p21Cip1 protein levels. In contrast, depletion of cellular polyamines by inhibiting

ODC enzyme activity with α -difluoromethylornithine decreased c-Myc, but increased p21Cip1 transcription. Ectopic expression of wild-type *c-myc* not only inhibited basal levels of p21Cip1 transcription in control cells, but also prevented increased p21Cip1 in polyamine-deficient cells. Experiments using different p21Cip1 promoter mutants showed that transcriptional repression of p21Cip1 by c-Myc was mediated through Miz-1- and Sp1-binding sites within the proximal region of the p21Cip1 promoter in normal IECs. These findings confirm that p21Cip1 is one of the direct mediators of induced c-Myc following increased polyamines and that p21Cip1 repression by c-Myc is implicated in stimulation of normal IEC proliferation.

Key words: cell proliferation, α -difluoromethylornithine (DFMO), gene expression, intestinal epithelium cell (IEC), ornithine decarboxylase (ODC), transcriptional repression.

INTRODUCTION

The epithelium of the mammalian intestine is a self-renewing tissue serving a number of important physiological functions, including digestion and absorption, secretion and barrier and immune functions [1]. Maintenance of intestinal epithelial integrity is prerequisite to these mucosal functions and depends on a complex interplay between processes involved in cell proliferation, differentiation, migration and apoptosis [2,3]. Proliferating cells are localized to the lower crypt fractions, whereas differentiated cells are localized to the upper half of the colon and the villus fraction of the small intestine. Under physiological conditions, undifferentiated epithelial cells continuously replicate in the proliferating zone within the crypts and differentiate as they migrate up the luminal surface of the colon and villous tips in the small intestine [4,5]. Apoptosis occurs in 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 [5,6]. This rapid dynamic turnover rate of IECs (intestinal epithelial cells) is highly regulated and critically controlled by numerous factors, including the cellular polyamines, spermidine and spermine and their precursor putrescine [7–9].

Polyamines are organic cations found in all eukaryotic cells and have distinct regulatory roles in IECs [7,10]. An increasing body of evidence indicates that the regulation of cellular polyamines is the central convergence point for the multiple signalling pathways driving epithelial cell decisions in control of intestinal mucosal homeostasis [7,10,11]. We [8,12–15] and others [7,16] have demonstrated that normal IEC proliferation in the intestinal mucosa is dependent on the supply of polyamines to the dividing cells in the crypts and that increasing cellular polyamines significantly enhances IEC renewal *in vitro* and stimulates mucosal growth and healing after injury *in vivo*. The exact role of polyamines in regulating cell proliferation at the molecular level is not fully understood. It has been shown that polyamines are necessary for *c-myc* gene expression primarily through transcriptional, but not post-transcriptional, activation [17,18] and that polyamine-induced nuclear c-Myc interacts with Max, binds to specific DNA sequences, and plays an important role in normal IEC growth [19,20]. However, the direct mediators of c-Myc's effect on cell-cycle progression following increases in levels of cellular polyamines are still unknown.

The p21Cip1 protein is a well-characterized CDK (cyclin-dependent kinase) inhibitor that belongs to the Cip/Kip family of CDK inhibitors [21]. p21Cip1 functions by associating with

Abbreviations used: AdMyc, adenoviral vector encoding c-Myc cDNA; CDK, cyclin-dependent kinase; CMV, cytomegalovirus; DFMO, α -difluoromethylornithine; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEC, intestinal epithelial cell; Miz-1, an initiator-binding zinc-finger transcription factor; ODC, ornithine decarboxylase; ODC-IEC, stable ODC-transfected IEC cells; pCMV, cytomegalovirus immediate early gene promoter; pfu, plaque-forming unit(s); Sp1, stimulating protein 1; SPD, spermidine.

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cyclin-CDK complexes to inhibit their kinase activity [22], and increased expression of p21Cip1 results in growth arrest at the G₁-, G₂- or S-phase [23,24,52]. Conversely, p21Cip1-deficient cells fail to undergo cell-cycle arrest in response to p53 activation after exposure to DNA damage [25]. Expression of the p21Cip1 gene is mainly regulated at the transcriptional level by both p53-dependent and -independent mechanisms [21]. Although p21Cip1 transcription is primarily activated by p53 after DNA damage [21,22], other agents and stress also activate p21Cip1 gene transcription by p53-independent pathways through different transcription factors, including Sp1 (stimulating protein-1)/Sp3, Smads (Sma- and Mad-related proteins), AP-2 (activating protein 2), STAT (signal transducer and activator of transcription), BRCA1, E2F-1/E2F-3, and CAAT/enhancer binding protein- α and - β [26]. p21Cip1 has been identified as one of the major targets of the transcription factor c-Myc, but the regulatory effects of induced c-Myc on p21Cip1 gene transcription are paradoxical and depend on cell type and other factors [27–29]. In some cases, c-Myc activates p21Cip1 gene transcription in a p53-dependent manner by inducing p19^{ARF} expression in wild-type mouse embryonic fibroblasts [29], whereas, in other cases, overexpression of *c-myc* represses p21Cip1 gene transcription in Caco-2, HaCaT, and NIH-3T3 cells [27,28,30].

Studies by ourselves and others have shown that *c-myc* expression absolutely requires cellular polyamines in proliferating IECs [7,17,18,19,31] and during healing of damaged mucosa [32], and that polyamine-modulated c-Myc plays a critical role in stimulation of IEC proliferation [20]. In the present study we sought to further determine whether polyamine-modulated c-Myc induces cell proliferation through inhibition of p21Cip1 gene expression. The results presented herein demonstrate that polyamine-induced c-Myc represses p21Cip1 transcription through Miz-1 (an initiator-binding zinc-finger transcription factor) and Sp1 binding sites within the proximal p21Cip1 promoter in normal IECs. 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 (Corning, NY, U.S.A.). Tissue-culture media and dialysed fetal bovine serum were obtained from Invitrogen (Carlsbad, CA, U.S.A.), and biochemicals were from Sigma (St. Louis, MO, U.S.A.). The primary antibody, monoclonal anti-ODC antibody (0-1136), and the secondary antibody, anti-mouse IgG conjugated to horseradish peroxidase (A-8924), were purchased from Sigma. The antibodies against c-Myc and p21Cip1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and DFMO (α -difluoromethylornithine) was from Ilex Oncology Inc (San Antonio, TX, U.S.A.). L-[1-¹⁴C]ornithine (sp. radioactivity 51.6 Ci/mmol) was purchased from NEN (Boston, MA, U.S.A.) and [α -³²P]dCTP (sp. radioactivity 3000 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.).

Recombinant viral construction and infection

Replication-defective retroviral vector for *ODC* gene expression (pLOSN) was a gift from Dr Susan K. Gilmour (Lankenau Medical Research Center, Wynnewood, PA, U.S.A.) [34]. The pLOSN vector encoded the mouse *ODC* cDNA containing an introduced stop codon at position 425, which resulted in a truncated and more stable ODC (ornithine decarboxylase) protein with full enzyme activity [35]. Cells were infected with pLOSN and control retroviral vector lacking *ODC* cDNA (pLXSN) for 4 h with 4 μ g/

ml Polybrene[®]. After 2 days, the cells were selected with medium containing G418 (600 μ g/ml) for 3–7 days. At 3 days after refeeding with serum-containing medium, cells were assayed for levels of ODC protein and its enzyme activity. Adenoviral vectors were constructed by using the Adeno-X Expression System (Clontech) according to the protocol provided by the manufacturer. Briefly, the full-length cDNA of human c-Myc was cloned into the pShuttle by digesting with BamHI/HindIII and ligating the resultant fragment into the XbaI site of the pShuttle vector. pAdeno-c-Myc [AdMyc (adenoviral vector encoding c-Myc cDNA)] was constructed by digesting pShuttle construct with PstI/SceI/I-CeuI and ligating the resultant fragment into the PstI/SceI/I-CeuI sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting HEK-293 cells using Lipofectamine[®] Plus reagent (Gibco–Bethesda Research Laboratory, Gaithersburg, MD, U.S.A.). The adenoviral particles were propagated in HEK-293 cells and purified by CsCl ultracentrifugation. Titres of the adenoviral stock were determined by standard plaque assay. Recombinant adenoviruses were screened for the expression of the introduced gene by Western-blot analysis using anti-c-Myc antibody. pAdeno-X, which was the recombinant replication-incompetent adenovirus carrying no c-Myc cDNA insert (Adnull), was grown and purified as described above and served as a control adenovirus. IEC-6 cells were infected with the AdMyc or Adnull, and expression of c-Myc was assayed at 24 or 48 h after the infection.

Reporter plasmids and luciferase assays

The construct of the p21Cip1 promoter luciferase (Luc) reporter, FL-Luc (2.3 kb regulatory region upstream of the p21Cip1 gene fused to the Luc reporter gene), and its two deletion mutants, 6-Luc and 10-Luc, as depicted in Figure 6(A) (below), were kindly provided by Dr Wafik S. El-Deiry (University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.) [36]. The constructs of CMV/p21Cip1-Luc and CMV/p21Cip1mut-Luc p21Cip1 promoter exchange mutants, which contained a minimal CMV (cytomegalovirus) promoter with multiple binding sites for rtTA transactivator in front of the Luc gene, were a gift from Dr Lars-Gunnar Larsson (Genetic Center, Department of Plant Biology, Swedish University of Agricultural Science, Uppsala, Sweden) [37]. The Sp1 point mutants of the p21Cip1 promoter were generated using QuikChange[®] site-directed mutagenesis kit and performed according to the manufacturer's (Stratagene, La Jolla, CA, U.S.A.) instructions. Briefly, the 6-Luc construct of the p21Cip1 promoter was used as a template. For each mutant, two synthetic oligonucleotide primers were designed, 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 products were used to transform XL-1 competent cells provided by the mutagenesis kit. Mutations of Sp1 binding site within the p21Cip1 promoter were verified by DNA sequencing. Transient transfection was performed by using the Lipofectamine[®] kit as recommended by the manufacturer (Invitrogen). Cells were collected 48 h after the transfection, and a luciferase assay was performed by using Bright-Glo Luciferase Assay System as recommended by the manufacturer (Promega, Madison, WI, U.S.A.). The activity from individual transfection was normalized by the β -galactosidase activity from co-transfected pCMV β -galactosidase plasmid. The experiments were done in triplicate and are reported as the means of relative light units/ β -galactosidase.

Cell culture

The IEC-6 cell line was purchased from the American Type Culture Collection at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. [38]. IEC-6 cells originated from intestinal crypt cells, as judged by morphological and immunological criteria. They are non-tumorigenic and retain undifferentiated characteristics of intestinal crypt cells. Stock cells were maintained in T-150 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 10 µg of insulin/ml and 50 µg/ml gentamicin. Flasks were incubated at 37°C in a humidified atmosphere of air/CO₂ (19:1) and passages 15–20 were used in experiments. IEC-6 cells at passage 15–20 exhibit a stable phenotype [12,39,40].

Assay for ODC enzyme activity

ODC activity was determined by radiometric technique in which the amount of ¹⁴CO₂ liberated from L-[1-¹⁴C]ornithine was estimated [41]. Sample collection and the assay procedure were carried out as described previously [9,19]. Enzymatic activity was expressed as pmol of CO₂/h per mg of protein.

Polyamine analysis

The cellular polyamine content was analysed by HPLC analysis as previously described [12,20]. Briefly, after 0.5 M perchloric acid had been added, the cells were frozen at –80°C until ready for extraction, dansylation and HPLC analysis. The standard curve encompassed 0.31–10 µM. Values that fell >25% below the curve were considered undetectable. The results are expressed as nmol of polyamine/mg of protein.

RNA isolation and Northern-blot analysis

Total RNA was extracted with the RNeasy® Mini Kit from Qiagen by following the instructions provided by the manufacturer. The final RNA sample obtained was dissolved in water and its quantity estimated from its UV absorbance at 260 nm using a conversion factor of 40 units (1 A₂₆₀ = 40 µg of RNA/ml). In most cases, 30 µg of total cellular RNA was denatured and fractionated electrophoretically, using a 1.2%-(w/v)-agarose gel containing 3% formaldehyde, and transferred by blotting to nitrocellulose filters. Blots were prehybridized for 24 h at 42°C with 5 × Denhardt's solution (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA), 5 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 50% formamide, 25 mM potassium phosphate and 100 µg/ml denatured salmon sperm DNA. The cDNA probes for c-Myc, p21Cip1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were labelled with [α-³²P]dCTP using a standard nick-translation procedure. Hybridization was carried out overnight at 42°C in the same solution containing 10% dextran sulfate and ³²P-labelled DNA probes. Blots were washed with two changes of 1 × SSC/0.1% SDS for 10 min at room temperature. After the final wash, the filters were autoradiographed with intensifying screens at –70°C.

Western-blot analysis

Cell samples, placed in SDS sample buffer [250 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, and 5% (v/v) mercaptoethanol], were sonicated, and then centrifuged (10 000 g) at 4°C for 15 min. The supernatant from the cell samples was boiled for 5 min and then subjected to electrophoresis on 7.5% acrylamide gels according to the method of Laemmli [42]. After the transfer of protein on to nitrocellulose filters,

the filters were incubated for 1 h in 5% non-fat dry milk in 1 × PBS/Tween 20 (PBS-T; 15 mM NaH₂PO₄, 80 mM Na₂HPO₄, 1.5 M NaCl, pH 7.5, and 0.5% Tween 20). Immunological evaluation was then performed for 1 h in 1% BSA/PBS-T buffer containing 1 µg/ml of the specific antibody against ODC, c-Myc or p21Cip1 proteins. The filters were subsequently washed with 1 × PBS-T and incubated for 1 h with the second antibody conjugated to peroxidase by protein cross-linking with 0.2% glutaraldehyde. After extensive washing with 1 × PBS-T, the immunocomplexes on the filters were allowed to react for 1 min with a chemiluminescence reagent (NEL-100; NEN, Boston, MA, U.S.A.). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

Statistics

All data are expressed as means ± S.E.M. from six or nine dishes. Autoradiographic and immunoblotting results were repeated three times. Quantitative analysis derived from densitometric scans of Northern and Western blots was performed, and relative levels of mRNAs and proteins were normalized as measured by densitometry of GAPDH and actin respectively. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using the Duncan's multiple-range test [43].

RESULTS

p21Cip1 gene expression in response to c-Myc is regulated by cellular polyamines in IEC cells

To determine the exact role of polyamine-modulated c-Myc in regulation of p21Cip1 gene expression in normal IECs, stable ODC-IEC cells were generated and characterized. The results presented in Figure 1(A) show that two clones of IEC cells resistant to the selection medium containing G418 overexpressed ODC protein. Consistently, these stable ODC-IEC cells were associated with dramatic increases in levels of ODC enzyme activity (Figure 1B). There was a more than 50-fold increase in levels of ODC enzyme activity in stable ODC-IEC cells relative to control transfectants. The increased ODC enzyme activity correlated well with an increase in the levels of the cellular polyamines putrescine, spermidine and spermine (Figure 1C). The level of putrescine in stable ODC-IEC cells was increased by approx. 12 times the value observed in parental IEC-6 cells; the level of spermidine was more than twice the control value, whereas that of spermine was increased by ~25%. On the other hand, cells infected with the retroviral vector lacking ODC cDNA exhibited no significant changes in levels of ODC protein, enzyme activity or cellular polyamine content.

As shown in Figure 2(A), stable ODC-IEC cells exhibited a substantial increase in c-Myc protein expression. The levels of c-Myc protein in stable ODC-IEC cells were much higher than those observed in parental IEC-6 cells and cells infected with the control retroviral vector lacking c-Myc cDNA. Interestingly, induced c-Myc following increased polyamines in stable ODC-IEC cells was associated with a pronounced inhibition of the p21Cip1 transcription, as indicated by repression of p21Cip1 promoter activity and decreased protein levels (Figure 2B). Levels of p21Cip1 promoter activity and its protein in stable ODC-IEC cells were inhibited by ~70% and ~85% respectively as compared with those from parental IEC-6 cells and cells infected with the control vector. Although there was a slight clonal variation, differences in inhibitory effects on p21Cip1 promoter activity and its protein expression between ODC-IEC-C1 cells and

Figure 1 Characterization of stable ODC-IEC cells

(A) Representative immunoblots for ODC protein. IEC-6 cells were infected with either the retroviral vector containing the sequence encoding mouse ODC cDNA or control retroviral vector lacking ODC cDNA. The clones resistant to the selection medium containing 600 μ g/ml G418 were isolated and screened for ODC expression by Western-blot analysis. Whole-cell lysates from each stable ODC-IEC clone, cells infected with the control retroviral vector, and parental IEC-6 cells were harvested, applied to each lane (20 μ g) equally, and subjected to electrophoresis on a 10%-(w/v)-acrylamide gel. Levels of ODC protein were identified by probing nitrocellulose membranes with a specific antibody that recognizes ODC. After the membrane was stripped, it was reprobed with actin, which served as an internal control for equal loading. Three experiments were performed that showed similar results. (B) ODC enzyme activity in cells described in (A). ODC activity was determined by using a radiometric technique, and values are means $\pm$ S.E.M. for six dishes. *** P < 0.001 compared with parent IEC-6 cells and cells infected with control retroviral vector. (C) Cellular polyamine concentrations in cells described in (A). Levels of cellular polyamines were measured by HPLC analysis, and values are means $\pm$ S.E.M. for six dishes. ** P < 0.01 and * P < 0.05 compared with parent IEC-6 cells and cells infected with control retroviral vector.

ODC-IEC-C2 cells were not statistically significant. To exclude the possibility that polyamines promote differentiation of IEC-6 cells, we also examined the effects of increased polyamines on cell differentiation after ectopic expression of the *ODC* gene by measurement of expression of a brush-border enzyme such as sucrase-isomaltase and found that stable ODC-IEC cells retained their undifferentiated characteristics (results not shown).

Changes in p21Cip1 transcription following depletion of cellular polyamines is associated with decreased c-Myc

The role of reduced c-Myc in regulating p21Cip1 transcription following decreased levels of cellular polyamines was examined. Endogenous cellular polyamines were depleted by treatment with DFMO, the specific inhibitor of ODC [7,11]. Exposure of IEC-6 cells to 5 mM DFMO for 6 days completely inhibited ODC enzyme activity and almost totally depleted cellular polyamines. The levels of putrescine and spermidine were undetectable on day 6 after treatment with DFMO, and spermine was decreased by $\sim$ 60% (results not shown). Similar results have been reported in our previous publications [12,19]. Consistent with our previous

findings that polyamines are absolutely required for transcription of the *c-myc* gene [17,19,32], depletion of cellular polyamines significantly decreased the steady-state levels of c-Myc mRNA and protein (Figure 3A). The levels of c-Myc mRNA and protein in DFMO-treated cells were decreased by $\sim$ 80% as compared with those observed in control cells. These decreases in c-Myc mRNA and protein were completely prevented by exogenous SPD (spermidine) (5 μ M) given together with DFMO. Putrescine (10 μ M) had an effect equal to that of SPD on levels of c-Myc mRNA and protein when it was added to cultures that contained DFMO (results not shown).

Decreased levels of c-Myc in the polyamine-deficient cells were associated with a stimulation of p21Cip1 transcription as demonstrated by increases in p21Cip1 promoter activity and its mRNA and protein (Figure 3B). Increased activity of p21Cip1 promoter luciferase reporter in DFMO-treated cells was $\sim$ 4 times the control level on day 6, and levels of p21Cip1 mRNA and protein were $\sim$ 5 times the control values. Furthermore, increased expression of the p21Cip1 gene was associated with an inhibition of cell growth (Figure 3C, panel a), in which number of G₁ phase cells was significantly increased (Figure 3C, panel b). Cell

Figure 2 Changes in expression of c-Myc and p21Cip1 in stable ODC-IEC cells described in Figure 1

(A) Expression of c-Myc protein as measured by Western-blot analysis. Whole-cell lysates were harvested, applied to each lane (20 μ g) equally and subjected to electrophoresis and immunoblotting, and equal loading was monitored by immunoblotting of actin. Three experiments were performed that showed similar results. (B) Expression of p21Cip1 in stable ODC-IEC cells. (B, panel a) p21Cip1 promoter activity as measured using luciferase reporter gene assay. The full-length p21Cip1 promoter luciferase reporter construct (FL-Luc) was used, in which the 2.3 kb regulatory region upstream of the p21Cip1 gene was fused to the luciferase reporter gene. Cells were transfected using the p21Cip1 promoter luciferase reporter construct by the Lipofectamine[®] technique, and transfected cells were harvested and assayed for luciferase activity after 48 h of incubation. Data were normalized relative to β -galactosidase activity from co-transfected plasmid pRSV β -galactosidase and expressed as means $\pm$ S.E.M. for three separate experiments: *** P < 0.001 and ** P < 0.01 compared with parent IEC-6 cells and cells infected with control vector containing no ODC cDNA. (B, panel b) Representative immunoblots for p21Cip1. Levels of p21Cip1 protein were measured by Western-blot analysis and equal loading was monitored by using actin immunoblotting. Three experiments were performed and showed similar results.

numbers were decreased by $\sim 50\%$ at day 4 and by $\sim 70\%$ at days 6 and 8 after polyamine depletion by DFMO. The population of G_1 -phase cells was increased from $\sim 38\%$ in controls to $\sim 70\%$ in cells exposed to DFMO for 6 days, along with a reduced population in the S and G_2/M phases (results not shown). We also examined changes in cellular DNA synthesis as measured by [3H]thymidine incorporation techniques and demonstrated that DNA synthesis was almost completely inhibited in cells treated

Figure 3 Effect of depletion of cellular polyamines by inhibiting ODC on c-Myc and p21Cip1 expression, and cell growth in IEC-6 cells

Cells were grown in control cultures, cultures in which ODC activity was inhibited with 5 mM DFMO and cultures inhibited with DFMO and supplemented with 5 μ M SPD for 6 days. (A) Levels of c-Myc mRNA and protein. Panel (a): representative autoradiograms of Northern blots. Total cellular RNA was isolated and examined by Northern-blotting analysis using a c-Myc cDNA probe. Hybridization to the labelled GAPDH probe served as a marker for equal loading. Panel (b): representative immunoblots for c-Myc protein from Western-blotting analysis. (B) p21Cip1 promoter activity and levels of p21Cip1 mRNA and protein in cells described in (A). Panel (a): p21Cip1 promoter activity as measured using the FL-Luc reporter construct. Cells were grown in medium containing either DFMO alone or DFMO plus SPD for 4 days and then transfected using the p21Cip1 promoter luciferase reporter construct. After 48 h, transfected cells were harvested and assayed for luciferase activity. Values are means $\pm$ S.E.M. for three separate experiments. ** P < 0.01 compared with controls and cells exposed to DFMO plus SPD. Panel (b): representative autoradiograms for p21Cip1 mRNA as measured by Northern-blot analysis. Panel (c): representative immunoblots for p21Cip1 protein from Western-blot analysis using the specific anti-p21Cip1 antibody. Three experiments were performed and showed similar results. (C) Growth of cells described in (A). Panel (a): cell numbers. Values are means $\pm$ S.E.M. for six dishes. Panel (b): percentages of G_1 phase cells. Cells were harvested and stained with propidium iodide and analysed by flow cytometry. Values are means $\pm$ S.E.M. for three separate experiments. * P < 0.05 compared with controls and cells treated with DFMO plus SPD.

with DFMO for 6 and 8 days (results not shown). Similar results have been reported in our previous studies [40]. In the presence of DFMO, exogenous SPD returned c-Myc expression to near normal levels and prevented induced p21Cip1 transcription, which was associated with the restoration of cell growth. The rates of p21Cip1 transcription and cell growth were indistinguishable between cells exposed to DFMO plus SPD and control cells. These results clearly indicate that reducing c-Myc by depletion of cellular polyamines enhances p21Cip1 transcription.

Effect of c-Myc ectopic expression on p21Cip1 transcription

To further define the role of polyamine-modulated c-Myc in the regulation of p21Cip1 transcription in normal IECs, we examined the effect of overexpression of the *c-myc* gene on p21Cip1 expression in the presence or absence of cellular polyamines. The adenoviral vector containing the corresponding human *c-myc* cDNA under the control of the human pCMV (CMV immediate early gene promoter) was constructed as described previously [20]. Adenoviral vectors used in the present study have been shown to infect IECs with near 100 % efficiency [20,39]. In fact, > 95 % of IEC-6 cells were positive when they were infected for 24 h with the adenoviral vector encoding green fluorescent protein (results not shown). As noted in Figure 4(A), the c-Myc protein was expressed in amounts which increased with increasing viral load and reached ~30-fold higher than control levels when adenovirus at a concentration of 100 pfu (plaque-forming units)/cell was used. An adenovirus that lacked exogenous *c-myc* cDNA (Adnull) was used as negative control in this experiment and did not induce c-Myc levels when it was infected at equal concentration (results not shown).

Transient infection with the AdMyc dose-dependently inhibited p21Cip1 transcription in IEC-6 cells (Figure 4B, panel a). The levels of p21Cip1 promoter activity in cells infected with the AdMyc were decreased by ~80 % at a dose of 10 pfu/cell and by ~95 % at doses of 50 and 100 pfu/cells. The inhibition of p21Cip1 promoter activity by induced c-Myc was associated with a decrease in p21Cip1 protein expression (Figure 4B, panel b). Levels of p21Cip1 protein in cells infected with the AdMyc were decreased by ~55 % at a dose of 10 pfu/cells and by ~85 % at doses of 50 and 100 pfu/cell (Figure 4B, panel c). On the other hand, it is interesting and important that the level of adenovirus-mediated c-Myc expression and its inhibitory effect on p21Cip1 expression in polyamine-deficient cells were similar to those observed in control cells. The level of c-Myc protein was increased by more than 10-fold when polyamine-deficient cells were infected with the AdMyc (50 pfu/cell) relative to DFMO-treated cells infected with Adnull at the same concentration (Figure 5A). Consistently, induced c-Myc significantly prevented the increase in p21Cip1 in polyamine-deficient cells (Figure 5B). The level of p21Cip1 protein in DFMO-treated cells infected with the AdMyc was decreased by ~45 % relative to DFMO-treated cells infected with Adnull. These results indicate that induced c-Myc represses p21Cip1 regardless of the presence or absence of cellular polyamines, suggesting that increased polyamines inhibit p21Cip1 transcription through a c-Myc-mediated mechanism in normal IECs.

Involvement of Sp1 in repression of the p21Cip1 promoter by c-Myc

To determine the mechanisms by which polyamine-induced c-Myc represses p21Cip1 transcription, the c-Myc-responsive region of the p21Cip1 promoter was mapped in normal IECs. First, we examined the involvement of Sp1 in p21Cip1 inhibition by c-Myc, because several recent studies have shown that c-Myc

Figure 4 Effect of overexpression of the *c-myc* gene on p21Cip1 expression in IEC-6 cells

(A) Representative immunoblots for c-Myc protein. AdMyc was constructed in which the complete open reading frame of the human *c-myc* cDNA was cloned to pShuttle of the Adeno-X Expression system under the control of the human CMV immediate-early promoter. Cells were infected with the AdMyc or adenoviral vector containing no *c-myc* cDNA (Adnull) at a multiplicity of infection of 10–100 pfu/cell, and expression of c-Myc protein was analysed 48 h after the infection. Three separate experiments were performed that showed similar results. (B) Expression of p21Cip1 in cells described in (A). Panel (a): p21Cip1 promoter activity as measured using the luciferase reporter gene assay. The luciferase activity was measured 48 h after the transfection with the FL-Luc reporter construct, and data were normalized according to β -galactosidase activity from co-transfected plasmid pRSV β -galactosidase. Values are means $\pm$ S.E.M. for three separate experiments. ** P < 0.01 and * P < 0.05 compared with cells infected with the Adnull. Panel (b): representative immunoblots for p21Cip1 protein. Whole-cell lysates were harvested, and levels of p21Cip1 protein were identified by Western-blot analysis. Panel (c): quantitative densitometric Western-blot analysis from cells described in (B), panel (b). Values are means $\pm$ S.E.M. for three separate experiments. ** P < 0.01 and * P < 0.05 compared with cells infected with the Adnull.

represses the p21Cip1 promoter, at least partially, by interactions with Sp1 in certain cell types [27,28,53]. The p21Cip1 promoter deletion mutants were generated as indicated in Figure 6(A), panel a. These deletion mutants of the p21Cip1 promoter exhibited substantial differences in their basal expression after transfection in IEC-6 cells. Basal levels were 54 ± 3 ($n = 6$) at the full-length promoter (FL-Luc), 5.5 ± 0.2 ($n = 6$) at the 6-Luc,

Figure 5 Effect of ectopic expression of c-Myc on p21Cip1 expression in polyamine-deficient IEC-6 cells

(A) Representative immunoblots for c-Myc. Cells were grown in control cultures and cultures containing 5 mM DFMO for 4 days and then infected with the adenoviral expression vector encoding c-Myc cDNA (AdMyc) or adenoviral vector lacking c-Myc cDNA (Adnull) in the concentration of 50 pfu/cell. After 48 h in the presence of DFMO, whole-cell lysates were harvested and levels of c-Myc protein were measured by Western blot analysis. Equal loading was monitored by actin immunoblotting. Three separate experiments were performed and showed similar results. (B) Expression of p21Cip1 in cells described in (A). Panel (a): representative immunoblots for p21Cip1 protein. Panel (b): quantitative densitometric Western-blot analysis from cells described in (B), panel (a). Values are means $\pm$ S.E.M. of data from three separate experiments. * $P < 0.05$ compared with DFMO-treated cells and cells treated with DFMO and then infected with Adnull.

1.2 ± 0.04 ($n = 6$) at the 10-Luc, and 1.06 ± 0.02 at the CD-Luc respectively. Results presented in Figure 6(A), panel b, show that deletion of nucleotides from positions -2326 bp to -291 (6-Luc) relative to the transcriptional start site did not prevent the repression of p21Cip1 promoter activity after overexpression of c-Myc. There were no significant differences in the inhibitory effect of induced c-Myc on p21Cip1 promoter activity when cells were transfected with the 6-Luc or FL-Luc. However, deletions of the sequence containing either two Sp1 binding sites or all six Sp1-binding sites within the proximal region of the p21Cip1 promoter significantly prevented p21Cip1 repression by c-Myc, although they did not completely abolish c-Myc-induced inhibition of p21Cip1 promoter. The inhibitory rates of p21Cip1 promoter by c-Myc were $\sim 80\%$ in the FL-Luc, $\sim 60\%$ in the 10-Luc, and $\sim 45\%$ in the CD-Luc respectively.

To further define the role of Sp1 in p21Cip1 repression by c-Myc, different Sp1 point mutants of the p21Cip1 promoter were generated (Figure 6B, panel a). Consistent with observations from deletion study, the mutation of a single Sp1-binding site or all six Sp1-binding sites within the proximal region of the p21Cip1

Figure 6 Effect of ectopic expression of c-Myc on p21Cip1 promoter activity after mutation of Sp1 binding sites

(A) Changes in p21Cip1 promoter activity after deletion mutation. Panel (a): schematic representation of p21Cip1 promoter luciferase (Luc) reporter deletion constructs. p53, Sp1, and Miz-1 indicate the relative locations of p53-, Sp1- and Miz-1-binding sites within the p21Cip1 promoter respectively. Panel (b): luciferase reporter activity of deletion constructs. After cells were grown in control culture for 2 days, they were infected with either the adenoviral expression vector encoding c-Myc cDNA (AdMyc) or control vector (Adnull) in the concentration of 50 pfu/cell for 24 h and then transfected using different p21Cip1 promoter luciferase reporter deletion constructs by the Lipofectamine[®] technique. After 48 h of incubation, transfected cells were harvested and assayed for luciferase activity. Data were normalized according to β -galactosidase activity from co-transfected plasmid pRSV β -galactosidase and expressed means $\pm$ S.E.M. for data from three separate experiments. ** $P < 0.01$ and * $P < 0.05$ compared with cells infected with Adnull. + $P < 0.05$ compared with cells infected with AdMyc and then transfected with the full-length p21Cip1 promoter (FL-Luc). (B) Changes in p21Cip1 promoter activity after Sp1 point mutation. Panel (a): schematic representation of Sp1 point mutants of the p21Cip1 promoter. The opening shading indicates the relative location of mutated Sp1 binding site within the p21Cip1 promoter. Panel (b): luciferase reporter activity of Sp1 point mutants. * $P < 0.05$ compared with cells infected with Adnull.

promoter also significantly, but not totally, abolished repression of the p21Cip1 promoter by c-Myc in IEC-6 cells (Figure 6B, panel *b*). The inhibitory rates of p21Cip1 promoter by c-Myc were ~60 % when a single Sp1-binding site was mutated (1mut-Luc) and ~48 % when all six Sp1-binding sites were mutated (7mut-Luc) respectively. These results indicate that Sp1-binding sites in the proximal region of p21Cip1 promoter are implicated in the repression of p21Cip1 transcription by c-Myc in IECs. Because deletion mutation and point mutation of all Sp1-binding sites within the p21Cip1 promoter do not completely prevent c-Myc-induced repression of p21Cip1 transcription, it is suggested that other factors are also involved in this process.

Role of Miz-1 in repression of the p21Cip1 promoter by c-Myc

To determine the role of Miz-1 in p21Cip1 repression by c-Myc, we used the p21Cip1 promoter exchange mutant (CMV/p21Cip1-Luc) in which nucleotides -94/-50 of the p21Cip1 promoter were replaced with the corresponding region of the c-Myc-non-responsive CMV immediate-early promoter (Figure 7A). Basal expression levels of the CMV/p21-Luc and CMV/p21mut-Luc were 3.7 ± 0.3 ($n=6$) and 1.1 ± 0.07 ($n=6$) in IEC-6 cells. As shown in Figure 7(B), left, induced c-Myc still significantly repressed p21Cip1 promoter activity as measured by transfection with the CMV/p21Cip1-Luc construct, indicating that the c-Myc-responsive region is situated between -49 and +16 of the p21Cip1 promoter. Because there are three potential Miz-1 binding sites within this region of the p21Cip1 promoter [37,44], we examined the possibility that induced c-Myc inhibited p21Cip1 promoter activity in IEC-6 cells by interacting with Miz-1. A p21Cip1 promoter construct (CMV/p21Cip1mut-Luc) in which the three Miz-1 binding sites were mutated by substituting two nucleotides within the Miz-1 sequence, was used to detect changes in p21Cip1 promoter activity following c-Myc induction. Repression of p21Cip1 promoter by c-Myc was almost completely abolished when the three Miz-1 binding sites were mutated (Figure 7B, right). Under these conditions, there were no significant differences in levels of p21Cip1 promoter activity between cells infected with the AdMyc and cells infected with Adnull.

To determine the role of Miz-1 binding sites in p21Cip1 repression by induced c-Myc following increased polyamines, changes in p21Cip1 promoter activity were measured in stable ODC-IEC cells by using the CMV/p21-Luc and CMV/p21mut-Luc constructs. Consistent with results from the full-length promoter (Figure 2B), the p21Cip1 promoter activity was also substantially inhibited when stable ODC-IEC cells were transfected with the CMV/p21-Luc (Figure 7C, left). However, this inhibitory effect was significantly decreased when ODC-IEC cells were transfected with the CMV/p21mut-Luc construct (Figure 7C, right). The inhibitory rates of p21Cip1 promoter activity in stable ODC-IEC cells were decreased from ~77 % when three Miz-1 binding sites were presented (CMV/p21-Luc) to ~32 % when all three Miz-1 binding sites were mutated (CMV/p21mut-Luc). These results indicate that induced c-Myc following increased polyamines represses p21Cip1 promoter activity, at least in part, by interacting with Miz-1 sites within the proximal region of p21Cip1 promoter in IECs.

DISCUSSION

Increasing evidence indicates that c-Myc is a major biological regulator of intestinal mucosal development and homeostasis [45–47] and plays an important role in maintenance of intestinal

Figure 7 Effect of ectopic expression of c-Myc on p21Cip1 promoter luciferase reporter activity: Miz-1 point mutations within p21Cip1 promoter

(A) Schematic representation of p21Cip1 promoter exchange mutant constructs. Part of p21Cip1 promoter region was replaced by CMV promoter, and the opening shading indicates the relative location of mutated Miz-1 binding sites within the p21Cip1 promoter. (B) Luciferase reporter activity of Miz-1 point mutants of p21Cip1 promoter in IEC-6 cells. Cells were infected with either the adenoviral expression vector encoding c-Myc cDNA (AdMyc) or control vector (Adnull) at a concentration of 50 pfu/cell for 24 h and then transfected with the luciferase reporter constructs of p21Cip1 promoter with or without Miz-1 point mutant by the Lipofectamine® technique. Levels of luciferase activity were assayed 48 h after the transfection. Data were normalized according to β -galactosidase activity from co-transfected plasmid pRSV β -galactosidase and expressed as means $\pm$ S.E.M. for results from three separate experiments. ** $P < 0.01$ compared with cells infected with Adnull. (C) p21Cip1 promoter activity after Miz-1 point mutation in stable ODC-IEC cells. The reporter activity was measured 48 h after transfection and results are expressed means $\pm$ S.E.M. for three separate experiments. ** $P < 0.01$ and * $P < 0.05$ compared with IEC-6 cells infected with control vector containing no ODC cDNA. + $P < 0.05$ compared with ODC-IEC cells transfected with the CMV/p21-Luc.

epithelial integrity [19,31,32]. Increased c-Myc expression is not only essential for the induction of crypt formation during early intestinal development [45], but also stimulates healing of damaged mucosa in adult animals [32]. Our previous studies have demonstrated that expression of the *c-myc* gene requires cellular polyamines in cultured IECs [19,20] and in the regenerating mucosa after injury [32] and that polyamines stimulate transcription without affecting post-transcriptional regulation of the *c-myc* gene [17,20]. Since c-Myc exerts distinct cellular outcomes, depending on activation or repression of specific c-Myc target genes, cell type and tissue location [46], it becomes imperative to define the direct mediators of polyamine-induced c-Myc in the epithelial cells of the gastrointestinal mucosa. We [20] have recently reported that polyamine-induced c-Myc is crucial for stimulation of normal IEC proliferation, because decreased

levels of c-Myc by polyamine depletion or exposure to specific c-myc antisense oligomers inhibit IEC-6 cell proliferation, whereas increased levels of c-Myc after elevation of cellular polyamines or ectopic expression of the c-myc gene enhance cell renewal. The present studies advance our previous observations and provide new evidence for the mechanism by which induced c-Myc stimulates normal IEC proliferation by demonstrating that induced c-Myc inhibits p21Cip1 transcription following polyamine production.

The results reported here clearly show that ectopic expression of the ODC gene increases polyamines and induces c-Myc expression, whereas depletion of cellular polyamines after inhibiting ODC enzyme activity with the specific inhibitor, DFMO, decreases c-Myc in IEC-6 cells. These results are consistent with our previous findings [17,20] that polyamines are required for stimulation of c-myc transcription and that modulating the levels of cellular polyamines, either increased or decreased, alters c-myc gene expression through the regulation of its mRNA synthesis. Most significantly, however, is the observation that polyamine-induced c-Myc inhibits p21Cip1 transcription in normal IECs. Induced c-Myc expression in IECs overexpressing ODC (increased polyamines) was associated with an inhibition of p21Cip1 promoter activity and its protein expression (Figure 2). In contrast, decreasing the levels of c-Myc after depletion of cellular polyamines by treatment with DFMO resulted in a significant induction of p21Cip1 transcription (Figure 3). Because the activation of p21Cip1 promoter and the resultant increases in levels of its mRNA and protein in DFMO-treated cells were completely prevented by the addition of exogenous SPD, these observed changes in p21Cip1 transcription are most likely related to polyamine depletion rather than to the non-specific effect of DFMO.

It has been reported that c-Myc can induce [29,49] or repress [27,28,30] p21Cip1 transcription, depending on the nature of the stress, cell type and other factors. Results from two studies have shown that overexpression of c-Myc induces p21Cip1 transcription through the activation of p53 in normal human and mouse fibroblasts [29,48]. Consistent with our current results, however, several studies have demonstrated that p21Cip1 is one of the targets for repression by c-Myc and that increased c-Myc inhibits p21Cip1 expression at the transcriptional level [27,28,30]. Ectopic expression of c-Myc represses the basal levels of p21Cip1 mRNA in NIH-3T3 cells and also blocks the induction of p21Cip1 transcription by treatment with transforming growth factor- β in human keratinocyte cells [27,28]. Although the exact reasons for these opposing effects of c-Myc on p21Cip1 transcription remain unclear, it may reflect a dual function of p21Cip1. It has been suggested that p21Cip1 is required for cell-cycle progression, although it can induce cell-cycle arrest, depending on threshold levels and cell type [49]. Overexpression of c-myc represses p21Cip1 transcription and results in a stimulation of cell proliferation in cells with mutant p53, whereas the overall effects of repression and activation by c-Myc will be additive in cells with wild-type p53.

Results obtained in the present study also show that repression of p21Cip1 transcription by polyamine-induced c-Myc occurs at the promoter level in normal IECs. As noted in Figures 2 and 3, the p21Cip1 promoter activity was inhibited by induced c-Myc in stable ODC-IEC cells, but it was activated following decreased c-Myc in polyamine-deficient cells. Ectopic expression of the c-myc gene by transient infection with the AdMyc significantly repressed p21Cip1 promoter activity, thus leading to a decrease in its protein expression (Figure 4). In addition, forced expression of the c-myc gene by AdMyc infection in DFMO-treated cells also prevented the induction of p21Cip1 in the absence of cellular

polyamines (Figure 5). Because overexpression of c-myc did not completely prevent the increased p21Cip1 in polyamine-deficient cells (Figure 5B), it is suggested that other factors are also involved in this process. In support of this possibility, we have recently demonstrated that polyamine depletion stabilizes p53 [14,15] and JunD [13], which also activate p21Cip1 transcription in IECs. To localize the region of the p21Cip1 promoter that mediates this repression in IEC-6 cells, results presented in Figures 6 and 7 indicated that induced c-Myc repressed the p21Cip1 promoter through the short GC-rich region immediately upstream of the transcription start site. This proximal region of the p21Cip1 promoter lacks canonical Myc-binding sites and does not overlap with previously identified regions of regulation by factors such as p53 or C/EBP (CCAAT/enhancer-binding protein) proteins [21,26], but it does contain multiple Miz-1- and Sp1-binding sites. These findings are consistent with results obtained by other investigators who have demonstrated that the proximal promoter region (about 100 bp) of p21Cip1 is sufficient for p21Cip1 repression by c-Myc in several cell types [27,28,53].

Although the exact mechanisms of p21Cip1 repression by c-Myc remain to be fully elucidated, it is clear that DNA binding by c-Myc is not essential for this effect [21]. However, it is still not clear which transcription factors interact with Myc on the p21Cip1 promoter. Recent studies suggest that complex formation by c-Myc with Miz-1 plays a critical role in mediating repression by c-Myc [37,44]. Miz-1 is rapidly activated in response to stress, binds to the initiator element, and induces p21Cip1 transcription, whereas c-Myc interferes with Miz-1 to repress p21Cip1 transcription [37,50]. By contrast, a recent study has also shown that p21Cip1 levels are up-regulated after Miz-1 knock-down with small interfering RNA, suggesting that Miz-1 is a repressor of p21Cip1 transcription in certain circumstances [21]. In addition, c-Myc also binds and inhibits Sp1/Sp3 activity through the initiator-independent mechanism [27]. It has been found that c-Myc antagonizes Ras-mediated induction of p21Cip1 by inhibiting Sp1 and that this effect is Miz-1 independent [51]. Results presented in Figures 6 and 7 indicate that mutation of all Miz-1- or Sp1-binding sites in the p21Cip1 promoter significantly but not totally blocks p21Cip1 repression by c-Myc, while selective point mutation of each of the Sp1 binding sites in this region only slightly abolished p21Cip1 inhibition by c-Myc. These findings suggested that c-Myc represses p21Cip1 transcription through a process involving both Miz-1- and Sp1-binding sites in the proximal region of the p21Cip1 promoter in normal IECs.

Repression of p21Cip1 by c-Myc plays an important role in stimulation of normal IEC proliferation following increased cellular polyamines (Figure 3C) and is of biological significance. IECs contain high concentrations of cellular polyamines that are critically regulated under physiological conditions. Our previous studies [17–20] have demonstrated that increased polyamines stimulate c-myc gene transcription, leading to an increase in nuclear c-Myc in IECs. Results obtained in the present study further show that polyamine-induced c-Myc represses p21Cip1 transcription, probably through the proximal region of the p21Cip1 promoter and decreases levels of cellular p21Cip1 protein, thus activating cyclin-CDKs by decreasing the formation of p21Cip1-cyclin-CDK complexes. Activated cyclin/CDKs enhance the G₁-to-S-phase transition during the cell cycle, resulting in stimulation of IEC proliferation during normal mucosal growth and healing after injury. These findings suggest that c-Myc-dependent p21Cip1 repression is involved in the regulation of normal IEC proliferation and plays an important role in maintenance of homeostasis in rapidly self-renewing mucosal tissue.

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