Polyamine depletion is associated with an increase in JunD/AP-1 activity in small intestinal crypt cells

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Patel, Anami R., and Jian-Ying Wang. Polyamine depletion is associated with an increase in JunD/AP-1 activity in small intestinal crypt cells. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G441–G450, 1999.—Activator protein 1 (AP-1) is a group of dimeric transcription factors composed of protooncogene (Jun and Fos) subunits that bind to a common DNA site, the AP-1 binding site. The proteins of c-jun, c-junB, and Fos are essential for initiation of the cell cycle. Conversely, the activation of the JunD gene slows cell growth in some cell types. The current study tests the hypothesis that polyamines influence cell growth by altering the balance of positive and negative Jun/AP-1 activities in intestinal epithelial cells. Studies were conducted in the IEC-6 cell line derived from rat small intestinal crypts. Administration of α-difluoromethylornithine (DFMO), a specific inhibitor for polyamine synthesis, for 4 and 6 days completely depleted cellular polyamine levels, while AP-1 binding activity was significantly increased. Spermidine, when given together with DFMO, restored AP-1 binding activity toward normal. The increased AP-1 complexes in polyamine-deficient cells were dramatically supershifted by the anti-c-jun antibody but not by antibodies against c-jun, c-junB, or Fos proteins. There were significant increases in c-junD mRNA and protein in DFMO-treated cells, although expression of the c-fos, c-jun, and junB genes decreased. The increase in c-junD/AP-1 activity in DFMO-treated cells was associated with a significant decrease in cell division. Exposure of control quiescent cells to 5% dialyzed serum increased c-jun/AP-1 but not JunD/AP-1 activities. DFMO prevented the stimulation of c-jun/AP-1 activity induced by 5% dialyzed serum. These results indicate that 1) polyamine depletion is associated with an increase in AP-1 binding activity and 2) the increase in AP-1 activity in the DFMO-treated cells was primarily contributed by an increase in the JunD/AP-1. These findings suggest that polyamines regulate cell growth at least partially by modulating the balance of positive and negative Jun/AP-1 activities in the intestinal mucosa.

The transcription factor activator protein 1 (AP-1) has been shown to integrate various mitogenic signals in a large number of cell types and plays a critical role in the control of cell proliferation (2). AP-1 is a homo- or heterodimeric DNA-binding protein composed of either two Jun family proteins or one Jun and one Fos family protein via the interaction of the leucine zipper domains (1, 2). The activated AP-1 dimer specifically binds to DNA at a consensus AP-1 site (TGAC/GTCA) in the responsive regions of gene promoter and regulates the transcriptional activity of AP-1-dependent genes (2, 26, 27). Although Jun-Fos heterodimers are more stable and more potent at binding DNA compared with corresponding Jun-Jun dimers, Fos proteins alone do not form stable Fos-Fos homodimers and do not bind DNA without Jun participation. Therefore, Jun proteins are more important than Fos proteins in gene regulation because they are absolutely required for the functioning of the AP-1 transcription factors (2, 30).

Numerous studies have shown that c-jun and junB function as immediate early response genes and are involved in the transition from a quiescent state to a proliferating state after exposure to growth factors (2, 4, 10). Inhibition of expression of the c-jun and junB genes by antisense RNA or oligomers causes reversible growth arrest and withdrawal from the cell cycle (28), including that c-jun and JunB are positive AP-1 factors for cell proliferation. In contrast, the activation of junD expression results in slower cell growth and an increase in the percentage of cells in the G0/G1 phase of the cell cycle (11, 22, 25). It has been shown that the inhibition of proliferation by 1,25-dihydroxyvitamin D3 in chronic myelogenous leukemia cells is accompanied by an increase in AP-1 DNA binding activity and that JunD protein is the major constituent of these AP-1 complexes (14). The activation of junD expression also partially suppresses neoplastic transformation induced by an activated ras oncogene (11). These findings clearly indicate that junD is a negative AP-1 factor that downregulates cell proliferation in some cell types.

A series of observations from our previous studies (32, 33, 35) and others (12, 15, 16) has demonstrated that the polyamines spermidine and spermine and their diamine precursor putrescine are absolutely required for cell proliferation in the crypts of the small intestinal mucosa. The intracellular concentration of polyamines is highly regulated by the cell according to the state of growth (12) and completely depends on the activation or inhibition of ornithine decarboxylase (ODC), the first rate-limiting step in polyamine biosynthesis (17). Decreasing cellular polyamine levels by inhibition of the activity of ODC with α-difluoromethylornithine (DFMO) suppresses cell renewal in intestinal mucosal tissue (16, 35). Although the exact role of polyamines in the regulation of cell proliferation is still unclear, we have recently demonstrated that polyamine deficiency results in a significant decrease in expression of the c-fos, c-myc, and c-jun genes in intestinal crypt cells (36). To further investigate the molecular mechanism of mucosal growth inhibition following polyamine depletion, the current studies were designed to determine whether polyamines alter the
balance of positive and negative Jun/AP-1 binding activities in intestinal epithelial cells and whether depletion of intracellular polyamines by DFMO results in an increase in the negative AP-1 factor JunD. Some of these data have been published in abstract form (18).

MATERIALS AND METHODS

Cell culture and general experiment protocol. The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (23). Originating from intestinal crypt cells as judged by morphological and immunologic criteria, the IEC-6 cells are nontumorigenic and retain the undifferentiated character of epithelial stem cells.

Stock cells were maintained in T-150 flasks in DMEM supplemented with 5% heat-inactivated FBS, 10 µg/ml insulin, and 50 µg gentamicin sulfate/ml. Flasks were incubated at 37°C in a humidified atmosphere of 90% air-10% CO2. Stock cells were subcultured once per week at 1:20, and medium was changed three times weekly. The cells were restarted from original frozen stock every seven passages. Tests for mycoplasma were routinely negative. Passages 15–20 were used in the experiments. There were no significant changes of biological function and characterization from passages 15–20.

The general protocol of the experiments and the methods used were similar to those described previously (20, 36). In brief, IEC-6 cells were plated at 6.25 × 104 cells/cm2 in DMEM + 5% dialyzed FBS + 10 µg insulin and 50 µg gentamicin sulfate/ml (supplemented DMEM). The cells were incubated in a humidified atmosphere at 37°C in 90% by volume of air and 10% by volume of CO2 for 24 h before being subjected to a series of experimental treatments.

In the first series of studies, we examined whether cellular polyamines could alter AP-1 DNA binding activity in IEC-6 cells. Cells were grown in control cultures or cultures containing either DFMO (5 mM) or DFMO plus 5 µM spermidine for 4 and 6 days. The dishes were placed on ice, the monolayers were washed three times with ice-cold Dulbecco’s phosphate-buffered saline (DPBS), and then nuclear proteins were prepared. The AP-1 DNA binding activity was measured by electrophoretic mobility shift assay. To characterize the AP-1 complex in polyamine-deficient cells, the compositional changes of the AP-1 complex were examined by gel supershift assays using various antibodies against either c-Fos, c-jun, J unB, or J unD proteins.

In the second series of studies, we investigated the effect of polyamine depletion by DFMO on the expression of protooncogene junD in IEC-6 cells. After cells were grown in the presence or absence of DFMO for 4 and 6 days after initial plating, J unD mRNA and protein levels were measured by Northern and Western blotting analyses. The cellular distribution of J unD protein in control and polyamine-deficient cells was also examined by immunohistochemical staining.

Preparation of nuclear protein extraction and electrophoretic mobility shift assay. Nuclear extracts were prepared as previously described (38). Briefly, cells were harvested in ice-cold DPBS with a cell scraper and centrifuged at 500 g at 4°C for 5 min. The resulting cell pellets were resuspended in 4 ml of ice-cold STM buffer containing 20 mM Tris-HCl (pH 7.85), 250 mM sucrose, 1.1 mM MgCl2, and 0.2% Triton X-100 and were incubated on ice for 5 min. The cell pellets were then washed once with STM buffer containing 0.2% Triton X-100 and once with STM buffer without Triton X-100. The isolated nuclei were then resuspended in STM buffer (without Triton X-100) that contained 0.4 M KCl and 5 mM β-mercaptoethanol and incubated on ice for 10 min to extract nuclear proteins. After centrifugation at 2,000 g at 4°C for 10 min, the supernatant was collected, aliquoted, and frozen at −80°C before use. The protein content of nuclear extracts was determined by the method described by Bradford (5).

The double-stranded oligonucleotides used in these experiments included 5’-CCCTTGTAGCTACGCGGAA-3’, which contains a consensus AP-1 binding site that is underlined (Santa Cruz Biotechnology, Santa Cruz, CA). These oligonucleotides were radioactively end-labeled with γ-32P]ATP (3,000 Ci/mmol; Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Promega, Madison, WI). For mobility shift assays, 0.035 pmol 32P-labeled oligonucleotides (∼30,000 cpm) and 10 µg nuclear protein (in 2 µl) were incubated in a total volume of 25 µl in the presence of 2 mM Tris-HCl (pH 7.5), 8 mM NaCl, 0.2 mM EDTA, 0.2 mM β-mercaptoethanol, 0.8% glycerol, and 1 µg poly(dI-dC). The binding reactions were allowed to proceed at room temperature for 20 min. Thereafter, 2 µl of bromophenol blue (0.1% in water) were added, and protein-DNA complexes were resolved by electrophoresis on nondenaturing 5% polyacrylamide gels and were visualized by autoradiography.

Gel supershift assays were accomplished by adding 1 µg (in 1 µl) of TransCruz supershift antibody to the reaction mixture and incubating for an additional 30 min at room temperature. The antibodies used in these experiments included rabbit polyclonal IgG raised against either c-jun, J unB, or J unD, and a polyclonal antibody raised against all Fos proteins including c-Fos, FosB, Fra-1, and Fra-2. All gel supershift antibodies were purchased from Santa Cruz Biotechnology.

RNA isolation and Northern blot analysis. Total RNA was extracted with guanidinium isothiocyanate solution and purified by CsCl density gradient ultracentrifugation as described by Chirgwin et al. (8). Briefly, the monolayer of cells was washed with DPBS and lysed in 4 M guanidinium isothiocyanate. The lysates were brought to 2.4 M CsCl concentration and centrifuged through a 5.7-M CsCl cushion at 150,000 g at 20°C for 24 h. After centrifugation, the supernatant was aspirated and the tube was cut about 0.5 cm from the bottom with a flame-scalpel. The resulting RNA pellet was dissolved in Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 µg/ml laurylsarcosine, and 5% phenol (added just before use). The purified RNA was precipitated from the aqueous phase by the addition of 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol in sequence. The final RNA was dissolved in water and estimated from its ultraviolet absorbance at 260 nm using a conversion factor of 40 units. In most cases, 30 µg of total cellular RNA were denatured and fractionated electrophoretically using a 1.2% agarose gel containing 3% formaldehyde and was transferred by blotting to nitrocellulose filters. Blots were prehybridized for 24 h at 42°C with 5× Denhardt’s solution and 5× standard salmon sperm DNA. DNA probes for J unD (Oncogene Science) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ATCC 57090) were labeled with [γ-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-labeled DNA probes. Blots were washed with two changes of 1× saline-sodium citrate-0.1% SDS for 10 min at room temperature. After the final wash, the filters were autoradiographed with intensifying screens at −70°C. The signals were quantitated by densitometry analysis of the autoradiograms.

Western blot analysis of Jun and Fos proteins. Nuclear extracts were prepared as described above. Ten micrograms of nuclear protein were mixed with SDS sample buffer, boiled
for 10 min, and then subjected to electrophoresis on 7.5% acrylamide gels according to Laemmli (13). Briefly, after the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 10x phosphate-buffered saline-Tween 20 (PBS-T: 15 mM NaH₂PO₄, 80 mM Na₂HPO₄, 1.5 M NaCl, pH 7.5, and 0.5% (vol/vol) Tween 20). Immunologic evaluation was then performed for 1 h in 1% BSA-PBS-T buffer containing 1 µg/ml polyclonal antibodies that had been used for gel supershift assays as described above. The filters were subsequently washed with 1x PBS-T and incubated for 1 h with goat anti-rabbit IgG antibody conjugated to peroxidase. After extensive washing with 1x PBS-T, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100, Du Pont NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

Immunohistochemical staining. Immunohistochemical staining for JunD protein was performed in IEC-6 cells by the indirect immunoperoxidase method as described previously (37). Cells were plated at 4.0 x 10⁴ cells/cm² on 22 x 22-mm glass coverslips that were placed in 35-mm dishes in medium consisting of DMEM + 5% dialyzed FBS + 10 µg/ml insulin and 50 µg/ml gentamicin sulfate. DFMO (5 mM) with or without 5 µM spermidine was added as treatment. On day 6 after initial plating, the cells were washed with DPBS and then with DPBS without calcium and magnesium (DPBS-Ca²⁺-Mg²⁺) and were fixed for 5 min at room temperature in 1 part 37% paraformaldehyde + 9 parts PEM buffer (10 mM Pipes, 5 mM EGTA, 2 mM MgCl₂, pH 6.8, containing 0.2% Triton X-100). The cells were postfixed for 5 min with ice-cold methanol. The cells were rehydrated in DPBS-Ca²⁺-Mg²⁺ for 30 min at room temperature and were incubated with 5% goat serum for 10 min at room temperature to reduce nonspecific background staining. The cells were then incubated with rabbit polyclonal anti-junD antibody used for gel supershift assays as described above at a dilution of 1:1,000 in humidified chambers for 20 min at room temperature. The primary antibody recognizes the 40-kDa JunD protein band in immunoblots of IEC-6 cell extracts and does not cross-react with other oncoproteins. Nonspecific slides were incubated without antibody to JunD protein. All the treated slides were incubated for 20 min with second antibody-horseradish peroxidase conjugate at a dilution of 1:500. The final reaction was achieved by incubating with freshly prepared reagent containing 3-amino-9-ethylcarbazole (Sigma) dissolved in dimethylformamide and sodium acetate. The slides were counterstained with hematoxylin, mounted, and viewed with an Olympus microscope.

Measurement of DNA synthesis. DNA synthesis was measured with the use of the [³H]thymidine incorporation technique as previously described (29). Cells in 24-well plates were pulsed with 1 µCi/ml tritiated thymidine for 4 h before harvest. Cells were washed twice with cold DPBS solution and then incubated in cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. After rinsing twice with cold 10% TCA, cells were dissolved in 0.5 ml of 0.5 N NaOH at 37°C in humidified air for 90 min. The incorporation of [³H]thymidine into DNA was determined by counting the aliquot of cell lysate in a Beckman liquid scintillation counter. The protein content of aliquot of cell lysate was determined by the method described by Bradford (5). DNA synthesis was expressed as disintegrations per minute per microgram of protein.

Polyamine analysis. The nuclear polyamine content was analyzed by HPLC as described previously (36). Nuclear pellets were determined according to the method described above, mixed with 0.5 M perchloric acid, and then frozen at −80°C until ready for extraction, dansylation, and HPLC. The standard curve encompassed 0.31–10 µM. Values that fell more than 25% below the curve were considered not detectable. Protein was determined by the Bradford method (5). The results are expressed as nanomoles of polyamines per milligram of protein.

Statistics. All data are expressed as means ± SE from six dishes. Autoradiographic results were repeated three times. 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 (9).

RESULTS

Effect of polyamine depletion on AP-1 DNA binding activity. Exposure of IEC-6 cells to 5 mM DFMO for 4 or 6 days almost completely depleted cellular polyamines. The levels of putrescine and spermidine were undetectable and spermine content was decreased by ~60% on days 4 and 6 in the presence of DFMO (data not shown). Similar results have been published previously (36). Putrescine level was undetectable in nuclei isolated from both control and DFMO-treated cells. Normal basal levels of nuclear spermidine and spermine were slightly lower than those observed in whole cells. Administration of DFMO markedly decreased nuclear spermidine and spermine levels (Fig. 1). In cells exposed to DFMO for 4 and 6 days, nuclear spermidine...
was undetectable and spermine was decreased by 70%.

Depletion of nuclear polyamines by DFMO significantly increased AP-1 binding activity in IEC-6 cells as measured by electrophoretic mobility shift assay (Fig. 2). The AP-1 binding activity was increased by 2.2-fold on day 4 and by 3.1-fold on day 6 in the DFMO-treated cells, although the basal AP-1 binding activity in control cells (without DFMO) increased with time after initial plating. Spermidine (5 μM) given together with DFMO attenuated the increase in AP-1 binding activity at day 4 and prevented the increase at day 6. Putrescine at a dose of 10 μM had an effect equal to spermidine on the AP-1 binding site. The SP1 binding activity in IEC-6 cells was almost completely inhibited when unlabeled SP1 oligonucleotide (×50) was added to the binding reaction mixture (data not shown). Because other transcription factors such as AP-2 showed no significant difference between control cells and polyamine-deficient cells and since SP1 binding activity decreased in DFMO-treated cells, the increase in AP-1 binding activity observed following polyamine depletion was specific.

Effect of polyamine depletion on AP-2 and SP1 binding activities.

The AP-2 DNA binding activity was undetectable in IEC-6 cells regardless of the presence or absence of DFMO for 4 and 6 days (Fig. 4A). As shown in Fig. 4B, basal level of SP1 binding activity was high in normal IEC-6 cells (without DFMO). Treatment with DFMO for 4 and 6 days significantly decreased SP1 binding activity, which was completely prevented by exogenous spermidine. The competition experiments were also performed by using an unlabeled oligonucleotide containing a single SP1 binding site. The SP1 binding activity in IEC-6 cells was almost completely inhibited when unlabeled SP1 oligonucleotide (×50) was added to the binding reaction mixture (data not shown). Because other transcription factors such as AP-2 showed no significant difference between control cells and polyamine-deficient cells and since SP1 binding activity decreased in DFMO-treated cells, the increase in AP-1 binding activity observed following polyamine depletion was specific.

Characterization of AP-1 complexes in polyamine-deficient IEC-6 cells.

Because AP-1 complexes consist of Jun and Fos proteins, we next examined the compositional changes of the induced AP-1 complexes that occur following polyamine depletion. These studies

![Fig. 2. Changes in activator protein (AP)-1 DNA binding activity in IEC-6 cells in control cultures, cultures in which ornithine decarboxylase (ODC) was inhibited with 5 mM DFMO, and cultures inhibited with DFMO and supplemented with 5 μM spermidine (SPD). Nuclear extracts were prepared from cells grown in the presence of DFMO with or without SPD for 4 and 6 days. Electrophoretic mobility shift assays were performed using 10 μg of nuclear protein and 0.035 pmol of [32P]-end-labeled oligonucleotides containing a single AP-1 binding site. Positions of the specifically bound DNA-protein complex and the freely migrating probes are indicated. None, binding reaction mixture without nuclear extracts. Three experiments were performed that showed consistent results.](http://ajpgi.physiology.org/)

![Fig. 3. Effect of unlabeled AP-1 oligonucleotide added to the binding reaction mixture on AP-1 DNA binding activity in control cells (A) and cells treated with DFMO (B). Competitive inhibition experiments were performed by incubating 10 μg of nuclear protein with 0.035 pmol of [32P]-end-labeled AP-1 oligonucleotides in the presence of different concentrations of unlabeled AP-1 oligonucleotides. Positions of the specifically bound DNA-protein complex and the freely migrating probes are indicated. Three experiments were performed that showed consistent results.](http://ajpgi.physiology.org/)
were carried out by performing gel supershift assays using various specific antibodies. As can be seen in Fig. 5, the anti-JunD antibody, when added to the binding reaction mixture, dramatically supershifted the AP-1 complexes present in IEC-6 cells exposed to DFMO for 4 and 6 days. The AP-1 activity attributed to JunD in the DFMO-treated cells was approximately one-third of the total AP-1 binding activity on day 4 and about one-half on day 6. In control cells and cells exposed to DFMO and spermidine, the AP-1 binding activity was slightly supershifted by the anti-JunD antibody. On the other hand, addition of antibodies against c-Jun or JunB to the binding reaction mixture had effect on the AP-1 binding activity in all three treatment groups. There was no stimulation of the AP-1 activity attributed to c-Jun or JunB by DFMO (Fig. 6, A and B). Although the anti-Fos antibody also partially supershifted the AP-1 complexes, there were no significant differences in the AP-1 activity attributed to Fos between control cells and cells exposed to DFMO or DFMO plus spermidine (Fig. 6C). The increased AP-1 binding activities in polyamine-deficient cells were not supershifted by the anti-Myc IgG, which served as a control antibody. These results indicate that the increase in AP-1 activity in polyamine-deficient cells is primarily contributed by an increase in JunD/AP-1 while c-Jun/AP-1 and JunB/AP-1 activity remains essentially unchanged.

Changes in JunD mRNA and protein levels following polyamine depletion. To determine whether the increased JunD/AP-1 activity following polyamine depletion resulted from the activation of JunD gene expression, we measured changes in the JunD mRNA and protein levels in the DFMO-treated cells. Consistent with its effect on Jun/AP-1 binding activity, inhibition of polyamine synthesis by DFMO resulted in a significant increase in steady-state levels of JunD mRNA (Fig. 7). Increased JunD mRNA levels in the DFMO-treated cells were 6.6 times the control level on day 4 and 8.9 times on day 6. Addition of spermidine (5 µM) completely prevented the stimulatory effects of DFMO on expression of the JunD gene. The induced mRNA levels of JunD in cells treated with DFMO returned toward control levels when spermidine was given. There were no changes in GAPDH mRNA levels in IEC-6 cells grown in the presence or absence of DFMO.

Western immunoblotting analysis of IEC-6 cells showed the JunD protein at 40 kDa, the expected molecular mass of JunD in other epithelial cells. Increased levels of JunD mRNA following polyamine depletion were paralleled by an increase in JunD protein (Fig. 8). There were significant increases in JunD protein levels of the nuclear extracts comparable to those used for gel shift assays in IEC-6 cells exposed...
In the presence of DFMO, exogenous spermidine returned the induced levels of JunD protein toward normal values.

We also measured changes in the levels of mRNAs and proteins for c-fos, c-jun, and junB in cells treated with DFMO for 4 and 6 days and demonstrated that polyamine depletion was associated with significant decreases in expression of these genes (data not shown). Similar data have been published previously (20, 36).

Association of increased JunD/AP-1 binding activity with a decrease in cell division. To elucidate the possible role of increased JunD/AP-1 binding activity in cell proliferation, we measured changes in the rate of DNA synthesis and final cell number in IEC-6 cells exposed to DFMO or DFMO plus spermidine. As can be

Results were consistent with our data from Northern and Western blot analyses that revealed a basal level of expression of the junD gene in control cells. In the DFMO-treated cells (Fig. 9B), however, these nuclear immunoreactivities for JunD markedly increased as expected. Identification was facilitated for every experiment by heavily staining nuclei. The appearance of JunD in the cells treated with DFMO plus spermidine was the same as in control cells (Fig. 9C). The distribution and the immunostaining level for JunD in cells grown in the presence of DFMO and spermidine were indistinguishable from those of control cells.

Effects of polyamine deficiency on the distribution of JunD protein in IEC-6 cells. To extend the positive findings of increased JunD following polyamine depletion, immunohistochemical staining was performed to determine the organization of JunD protein in IEC-6 cells. Figure 9 shows the cellular distribution of JunD protein in cells grown with or without spermidine for 6 days. In control cells, the slight immunostaining for JunD protein was visible and present just inside the nuclei (Fig. 9A). These
seen in Fig. 10, increased JunD/AP-1 activity in the DFMO-treated cells was associated with significant decreases in DNA synthesis and cell number. The administration of spermidine completely prevented the effects of DFMO on AP-1 binding activity and cell proliferation. The induced JunD/AP-1 activity and decreased cell proliferation in the DFMO-treated cells returned toward control levels when spermidine was given. In the cells grown in standard culture conditions containing no DFMO, exogenous polyamines had no additional effect on cell division (data not shown). To support the possibility that JunD/AP-1 plays a negative role in intestinal mucosal growth, we measured the changes in Jun/AP-1 binding activities in quiescent cells exposed to a positive treatment of 5% dialyzed FBS. In this study, cells were grown in the presence or absence of 5 mM DFMO for 4 days and were then exposed to 5% dialyzed FBS after 24-h serum deprivation. Figure 11 clearly shows that exposure of normal quiescent cells (without DFMO) to 5% dialyzed FBS markedly increased AP-1 binding activities. However, there was no significant increase in AP-1 binding activity in DFMO-treated cells exposed to 5% dialyzed FBS. The observations from supershift assays showed that increased AP-1 activities in normal quiescent cells exposed to 5% dialyzed serum resulted primarily from the increase in c-Jun/AP-1 and JunB/AP-1, whereas JunD/AP-1 activity in polyamine-deficient cells was not induced by 5% dialyzed FBS. These results suggest that JunD/AP-1 appears to function differently from c-Jun/AP-1 and JunB/AP-1 in regulating cell growth in the intestinal mucosa.

DISCUSSION

The murine junD was cloned in 1989 as a third member of the protooncogene jun family by screening cDNA libraries with c-Jun and/or junB probes (11, 25). Unlike c-Jun and JunB, junD is usually constitutively expressed and is not inducible by growth factors (11, 14). Overexpression of the junD gene promotes neither cell proliferation nor transformation in several cell types (14, 22, 24), although some studies have shown a positive effect of junD on cell proliferation. Pfarr et al. (22) have reported that growth arrest correlates well with high junD and low c-Jun levels while stimulation of cell proliferation is associated with low junD and high c-Jun levels. Similar changes have also been observed in papillomavirus-transformed mouse cell lines in which an increase in the c-Jun- and JunB-to-junD ratio becomes evident as cells progress from fibromatosis to fibrosarcoma (3). Recently, it has been found that...
negative JunD/AP-1 activity increases twofold during differentiation of SV40-transformed 3T3T cells (CSV3–1) and that decreasing JunD levels by antisense oligodeoxyribonucleotides significantly increases cell proliferation (31). These findings indicate that Jun/AP-1 transcription factors can be dissected into two parts: c-Jun/AP-1 and JunB/AP-1 positively regulate cell division, and JunD/AP-1 negatively regulates cell growth.

Our previous studies (16, 35, 36) have demonstrated that cell proliferation in the intestinal mucosa is dependent on the supply of polyamines to the dividing cells. Although the molecular mechanisms by which polyamines are required for the stimulation of cell proliferation are still unclear, changes in gene expression are known to occur (7, 17, 20). In this regard, we have recently reported that polyamine-deficient IEC-6 cells are associated with a significant decrease in expression of the protooncogenes c-fos, c-jun, and junB (36). On the basis of these results, we expected that polyamine depletion by treatment with DFMO would decrease AP-1 DNA binding activity in small intestinal crypt cells. The new finding described in this study, however, shows that inhibition of polyamine biosynthesis markedly increases AP-1 binding activity in the DFMO-treated cells (Fig. 2). The increased AP-1 DNA binding activities following polyamine depletion are specific because there are no significant differences in other transcription factors such as AP-2 and SP1 binding activities between control cells and polyamine-deficient cells (Fig. 4).

To determine whether the increased AP-1 activities following polyamine depletion result from either c-Jun/AP-1 and JunB/AP-1 or JunD/AP-1, we characterized the composition of increased AP-1 complexes in the DFMO-treated cells by performing gel supershift assays using various specific antibodies. As can be seen in Figs. 5 and 6, increased AP-1 complexes in polyamine-deficient cells are dramatically supershifted by the anti-JunD antibody but not by antibodies against c-Jun, JunB, or Fos proteins, suggesting that most of the increased AP-1 activities in the DFMO-treated cells are primarily contributed by an increase in JunD/AP-1. These findings are further supported by the observations that increased JunD/AP-1 binding activity in the DFMO-treated cells is paralleled by increases in the levels of both JunD mRNA and protein (Figs. 7 and 8). Increased levels of JunD protein following polyamine depletion are present just inside the nuclei of intestinal crypt cells (Fig. 9). These results clearly indicate that polyamine depletion significantly increases JunD/AP-1 binding activity and strongly support the hypothesis that intracellular polyamines are required for intestinal mucosal growth in association with their ability to modulate the balance between positive and negative Jun/AP-1 activities.

Because the importance of AP-1 transcription factors in the control of cell proliferation has been documented by numerous studies (2, 10) and because intracellular polyamines are essential for normal intestinal mucosal growth under physiological conditions (16, 19), the results presented in this paper raise a series of interesting questions for future study. The first question that should be addressed is the nature of the molecular mechanisms that activate the expression of the JunD gene following polyamine depletion. Data presented in Fig. 7 show that the increased JunD protein level leading to increased JunD/AP-1 activity is paralleled by a significant increase in JunD mRNA levels in IEC-6 cells exposed to DFMO for 4 and 6 days. It is not clear at present whether increased mRNA level for JunD is due to an increase in gene transcription or results from the alteration of mRNA stabilization.

The second question is whether activated negative JunD/AP-1 plays a role in the growth inhibition process following polyamine depletion in intestinal crypt cells.
Figure 10 clearly shows that increased JunD/AP-1 activity in DFMO-treated cells is accompanied by a significant decrease in cell growth that can be completely restored to normal by concomitant treatment with spermidine. Because JunD has a function opposed to c-jun and JunB and promotes quiescence during the cell cycle in other cell types, the temporal association of increased expression of the JunD gene and decreased cell division after polyamine depletion suggests that activated negative JunD/AP-1 activity and growth inhibition are causally related. In support of this possibility, JunD/AP-1 binding activity is not increased by exposure to a positive treatment of 5% dialyzed serum (Fig. 11). Our previous studies (36) have also demonstrated that inhibition of polyamine synthesis by DFMO significantly decreases steady-state levels of c-fos, c-jun, and JunB mRNAs in IEC-6 cells. The repression of c-fos, c-jun, and JunB expression in the DFMO-treated cells would be expected to facilitate the inhibitory effect of JunD on cell division because decreasing c-jun/AP-1 and JunB/AP-1 activities would allow JunD/AP-1 activity to be dominant. Further studies are needed to determine whether decreasing JunD levels by using antisense RNA or by transfection of dominant negative JunD mutant will promote cell proliferation in polyamine-deficient cells.

The third issue raised by the current study is the interrelationship between c-jun/AP-1, JunB/AP-1, and JunD/AP-1 activities during growth inhibition following polyamine depletion. Although activated JunD/AP-1 could function to prevent cells from entering the cell cycle in polyamine-deficient cells, the exact mechanism concerning how JunD/AP-1 functions in this regard is unclear. Possibilities include that negative JunD/AP-1 may compete with positive c-jun/AP-1 or JunB/AP-1 for DNA binding or that JunD/AP-1 may regulate genes that are primarily involved in negatively controlling cell proliferation, whereas c-jun/AP-1 and JunB/AP-1 may regulate genes that facilitate proliferation. Our previous studies (34, 36) and others (6) have found that expression of the c-fos gene decreases following polyamine depletion, suggesting a limited possibility that Jun proteins may dimerize with Fos. Because JunD is more abundant than c-jun and JunB in the polyamine-deficient cells (Figs. 6 and 7), it may dimerize with c-jun or JunB to suppress their stimulatory effects on cell proliferation.

In summary, these results indicate that polyamines are implicated in the regulation of Jun/AP-1 activities in intestinal epithelial cells. Depletion of intracellular polyamines by DFMO is associated with a significant increase in JunD/AP-1 activity, which is paralleled by increases in the levels of both JunD mRNA and protein in IEC-6 cells. Cell proliferation in the DFMO-treated cells is also significantly decreased. Because JunD/AP-1 has been shown to negatively regulate cell division in a variety of cell types, these findings suggest that polyamine depletion results in growth inhibition in the intestinal mucosa at least partially by altering expression of the JunD gene and Jun/AP-1 binding activity.

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