Modulation of Differentiation-related Gene 1 Expression by Cell Cycle Blocker Mimosine, Revealed by Proteomic Analysis*

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L-Mimosine, a plant amino acid, can reversibly block mammalian cells at late G₁ phase and has been found to affect translation of mRNAs of the cyclin-dependent kinase inhibitor p27, eIF3a (eIF3 p170), and ribonucleotide reductase M2. The effect of mimosine on the expression of these genes may be essential for the G₁ phase arrest. To determine additional genes that may be early responders to the mimosine treatment, we performed two-dimensional gel electrophoretic analysis of [³⁵S]methionine-labeled cell lysates followed by identification of the altered protein spots by LC-tandem mass spectrometry. In this study, the synthesis of two protein spots (MIP42 and MIP17) was found to be enhanced by mimosine, whereas the formation of another protein spot (MSP17) was severely blocked following mimosine treatment. These protein spots, MIP42, MIP17, and MSP17, were identified to be differentiation-related gene 1 (Drg-1; also called RTP, cap43, rit42, Ndrg-1, and PROXY-1), deoxyhypusine-containing eIF5A intermediate, and mature hypusine-containing eIF5A, respectively. The effect of mimosine on eIF5A maturation was due to inhibition of deoxyhypusine hydroxylase, the enzyme catalyzing the final step of hypusine biosynthesis in eIF5A. The mimosine-induced expression of Drg-1 was mainly attributable to increased transcription likely by the c-Jun/AP-1 transcription factor. Because induction of Drg-1 is an early event after mimosine treatment and is observed before a notable reduction in the steady-state level of mature eIF5A, eIF5A does not appear to be involved in the regulation of Drg-1 expression.

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L-Mimosine, a plant amino acid derived from seeds of Leucaena leucocephala or Mimosa pudica, can specifically and reversibly block mammalian cells at late G₁ phase (1, 2). It is commonly used as a synchronizing agent for investigating cell cycle progression in mammalian cells (3, 4) and may be developed as an anticancer agent (5). However, the molecular mechanism of mimosine action on the cell cycle progression has not been elucidated. Several independent studies suggest that mimosine may modulate the synthesis of some proteins, which in turn may cause inhibition of DNA replication and cell cycle arrest (6–9).

Recently we reported that a short term mimosine treatment reduced global protein synthesis by 20% at 4 h and up to 50% by 8 h (10). We have identified several proteins that showed altered expression after mimosine treatment. Although eukaryotic translation initiation factor 3α (eIF3α<sup>†</sup> or eIF3 p170), tyrosinated α-tubulin, and ribonucleotide reductase M2 were decreased, the cyclin-dependent protein kinase inhibitor p27 was elevated by mimosine treatment (10, 11). The change in the expression level of these proteins by mimosine may be an important prelude to mimosine-mediated G₁ cell cycle arrest. These data suggest that eIF3 p170 mediates the effect of mimosine on the synthesis of α-tubulin, M2, and p27. Thus, the effect of mimosine on gene expression regulation appears to be complex and may involve a subset of specific proteins.

To identify additional genes affected by mimosine treatment, we performed two-dimensional (2D) gel electrophoresis of metabolically labeled HeLa cell lysates and identified the differentially expressed proteins by tandem MS analysis. We found that the differentiation-related gene 1 (Drg-1) was significantly up-regulated at 2 h following mimosine treatment. The induction of Drg-1 expression by mimosine was determined to occur mainly at transcriptional levels activated likely by the transcription factor c-Jun/AP-1. We also observed that the hypusine modification of the translation initiation factor eIF5A was severely impaired by mimosine due to inhibition of deoxyhypusine hydroxylase.

<sup>†</sup> The abbreviations used are: eIF, eukaryotic initiation factor; Drg-1, differentiation-related gene 1; 2D, two-dimensional; HIF, hypoxia-inducible factor; DFO, deferoxamine mesylate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC7, N<sup>2</sup>-guanyl-1,7-diaminoheptane; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase; AP1, activator protein 1.

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**EXPERIMENTAL PROCEDURES**

Materials—The rabbit polyclonal anti-Drg-1 antibody was a gift from Fred Bosman (Institut Universitaire de Pathologie, Lausanne, Switzerland) (12). Monoclonal antibodies against HIF-1α, c-Myc, and p53; polyclonal antibody against c-Jun; and oligonucleotides with consensus binding sequences for AP-1, HIF-1α, p53, and c-Myc/Max and their corresponding mutant oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL system for Western blot analysis, AmplifyTM, and [γ-32P]ATP were purchased from Amersham Biosciences. Mimosine, deoxyferoxamine mesylate (DFO), curcumin, and sequencing grade trypsin were from Sigma. [35S]Methionine and [1,8-3H]spermidine were purchased from PerkinElmer Life Sciences. SequiBlotTM PVDF membranes and protein assay reagents were from Bio-Rad. Cell culture media and reagents were obtained from Invitrogen. All other reagents were of molecular biology grade and were purchased from Sigma or Fisher Scientific.

Cell Culture, Treatment, and Metabolic Labeling—HeLa cells were cultured in modified Eagle’s medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO2 at 37 °C. HeLa cells were seeded at 6 × 105 cells in 100-mm dishes and cultured for 3 days before treatment with mimosine or other reagents for various times as indicated in the figure legends. For double treatment, curcumin was added to cells to a final concentration of 70 μM and incubated for 1 h before mimosine was added for further treatment for 6 h. For metabolic labeling with [35S]methionine, cells were washed twice with PBS and once with Dulbecco’s modified Eagle’s medium lacking methionine followed by incubation for 2 h in the same medium supplemented with 75 μCi/ml [35S]methionine. The pulse-labeled cells were then washed three times with PBS and harvested for cell lysate preparation and 2D gel electrophoresis as described below. For [3H]spermidine labeling, HeLa cells were incubated with [1,8-3H]spermidine (10 μCi/ml) with or without mimosine (600 μM) for 8 h. Cells were washed once with PBS and harvested, and cell lysates were prepared for 2D gel electrophoresis as described below. The Coomassie Blue-stained MIP17 or MSP17 spots were excised, and proteins in each gel piece were hydrolyzed in 6 N HCl at 108 °C for 18 h. The radioactive hypusine or deoxyhypusine in the hydrolysates was identified by ion exchange chromatographic separation as described previously (13).

Two-dimensional Gel Electrophoresis and Western Blot—Cells were lysed in TNE-SDS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Lysates were cleared by centrifugation (10,000 × g for 10 min at 4 °C), and protein concentrations were determined by the Bradford method (14).

About 170 μg of cell lysate proteins were diluted to 0.9 μg/μl in a total volume of 185 μl with Bio-Rad premade sample loading buffer (8 M urea, 50 mM DTT, 4% CHAPS, 0.2% carrier ampholytes, 0.0001% bromphenol blue) and loaded onto the IPG strips (pH 3–10 and 5–8) using the Bradford method (14).

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Western blot analyses were performed as described previously (15). Briefly cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane. The blot was then probed with primary antibody followed by reaction with horseradish peroxidase-conjugated secondary antibody. The signal was detected using enhanced chemiluminescence.

**Tryptic Digestion**—Protein spots of interest were cut manually from 2D gels. Each excised gel spot was placed in an Eppendorf tube, cut into smaller (less than 1 mm in each dimension) pieces, covered with 200 μl of 200 mM ammonium bicarbonate in 40% acetonitrile, and incubated at 37 °C for 30 min for removal of dye, salts, and SDS. This step was repeated once, and the solution was discarded. The gel pieces were completely dehydrated by drying in a SpeedVac (Eppendorf, Westburg, NY). To the dried gel pieces 20 μl of 20 μg/ml trypsin solution in 36 mM ammonium bicarbonate, 8% acetonitrile was added followed by 50 μl of 40 mM ammonium bicarbonate in 9% acetonitrile. The samples were incubated at 37 °C for 18 h. Following incubation, the liquid (70 μl) was removed from gel pieces, dried in a SpeedVac, and reconstituted in 10 μl of 1% formic acid. These solutions were kept frozen for further analyses.

**Nano-LC-MS/MS**—The nano-LC separations were performed using a CapLC system (Waters Corp., Milford, MA) or an LC Packings system (Dionex, Sunnydale, CA). The latter consists of a FamosTM autosampler, SwitchosTM switching valve and pump (used for sample trapping and washing), and UltiMate gradient pump. Using either of the two systems, 3 or 6 μl of the tryptic digests of the protein spots were first loaded onto a trapping column (15 mm × 100-μm inner diameter) in-house-packed with 5-μm, 200-Å Magic C18AQ packing material (Microm BioResources, Inc., Auburn, CA). The trapping column was then washed to remove any salts and unretained materials prior to elution, and the retained peptides were separated on a pulled-tip capillary column (150 mm × 75-μm inner diameter) packed with the same materials used for the trapping column but with 100-Å pore size. The separation was accomplished using a 15-min gradient from 0 to 35% solvent B at a 250 nl/min flow rate. Solvent B consisted of acetonitrile with 0.1% formic acid, whereas solvent A consisted of 3% acetonitrile and 97% water with 0.1% formic acid. At the end of the column, ions were electrosprayed directly into either a Thermal-Finnigan (San Jose, CA) LQD Deca XP ion trap mass spectrometer or a Micromass QTOF Global mass spectrometer (Waters Corp.), which recorded mass spectra and data-dependent tandem mass spectra of the separated peptide ions. Finally the acquired MS/MS spectra were searched against human protein sequences in the Swiss-Prot data base using Mascot (56) for peptide recognition and consequently protein identification.

**RNA Extraction and Ribonuclease Protection Assay**—Total RNA was extracted using the RNeasy minikit (Qiagen) as described previously (10). Nuclear RNAs were prepared by first isolating nuclei as described previously (16, 17). Briefly HeLa cells were harvested and treated with lysis buffer (PBS containing 0.5% Nonidet P-40) for 1–2 min at room temperature. The nuclei were then collected by centrifugation and washed three times with the lysis buffer followed by purification on a sucrose gradient. RNase protection assay was performed, as described previously, with 20 μg of total RNA (18) using the RPA-III kit (Ambion, Austin, TX) according to the instructions of the supplier. The constitutively expressed human GAPDH gene obtained from Ambion was used as a control for RNase protection assay.

**Nuclei Extract Preparation and Gel Shift Assay**—The nuclei extract preparation and the gel shift assay were performed as described previously (19–21). Briefly nuclei extracts were prepared from HeLa cells following the treatment with or without 600 μM mimosine, 400 μM kojic acid, or 70 μM curcumin. Oligonucleotides containing the consensus AP-1, c-Myc/Max, or HIF-1α binding sequences were labeled with [γ-32P]ATP using T4 polynucleotide kinase. The reaction mixtures for the gel shift assay contained 50,000 cpm (−4 fmol) 32P-labeled oligonucleotides and 10–20 μg of nuclear protein in a final volume of 20 μl of mixture containing 12.5 mM HEPES (pH 7.9), 100 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg BSA, and 3 μg poly(dI-dC) as nonspecific competitors. The reaction was incubated for 20 min at room temperature. Competition binding experiments were performed by first incubating cold competitor oligonucleotides, in 150-fold molar excess, with the nuclear protein and binding buffer for 10 min on ice. The labeled probe was then added and incubated.
for an additional 20 min at room temperature. The reaction mixtures were then separated by electrophoresis on 5% non-denaturing polyacrylamide gels. The gels were subsequently dried for autoradiography analysis.

RESULTS

Effect of Mimosine on the Profile of Global Protein Synthesis—To identify genes up-regulated by mimosine treatment, we performed a two-dimensional gel proteomic analysis of lysates from the cells that had been pulse-labeled with $[^{35}\text{S}]$methionine following 8-h mimosine treatment. As shown in Fig. 1, the synthesis of most proteins was decreased (Fig. 1, compare B with A), although the steady-state levels of these proteins stained by Coomassie Blue did not change significantly (Fig. 1, compare C with D). However, one $^{35}\text{S}$-labeled spot (indicated as MSP17) was completely lost following mimosine treatment, although its steady-state level detected by Coomassie Blue staining remained similar. Interestingly the labeling of two protein spots (indicated as MIP42 and MIP17 in Fig. 1, A and B, inset) was drastically increased following mimosine treatment. The increase in intensity of the spot MIP17 was also clearly visible by Coomassie Blue staining, whereas that of the spot MIP42 was not due to the presence of other adjacent protein spots (Fig. 1, compare C and D, insets). To detect the spot MIP42 by Coomassie Blue staining for further analysis, we performed a two-dimensional gel analysis using a narrower range of ampholytes (pI 5–8) in the first dimension combined with 10% SDS-PAGE in the second dimension. As shown in Fig. 2, the synthesis ($[^{35}\text{S}]$methionine labeling) of the spot MIP42 as detected by autoradiography as well as the steady-state level of the protein as detected by Coomassie Blue staining was increased in mimosine-treated cells.

Identification of the Protein Spots MIP42, MIP17, and MSP17 by LC-MS/MS—For identification, the protein spots MIP42, MIP17, and MSP17 were excised from the Coomassie Blue-stained gel for LC-MS/MS analysis. As shown in Table I, MIP42 was identified as Drg-1 based on the seven peptides identified individually by their tandem mass spectra. The sequences of these peptides account for 29% of the 394-residue protein sequence. MSP17 was identified as elf5A based on four peptides identified by tandem mass spectrometry.
etry (Table II). These four peptides account for 33% of 154 residues in the protein. The gel spot MIP17 was also identified as eIF5A by the same method (data not shown). The identification of both MIP17 and MSP17 as eIF5A suggests that mimosine treatment caused a shift in the pI of the newly synthesized eIF5A (MIP17) to more acidic than that (MSP17) of the untreated sample.

Verification of the Spots of MIP-17 and MSP-17—It is known that Lys50 of the human eIF5A is modified to hypusine co-translationally or shortly after synthesis by deoxyhypusine synthase and deoxyhypusine hydroxylase, and this modification converts the lysine-containing eIF5A precursor to a slightly less acidic hypusine-containing mature protein, eIF5A (22–25). Mimosine inhibits deoxyhypusine hydroxylase (26, 27) and thereby would lead to accumulation of a deoxyhypusine-containing eIF5A intermediate. To confirm that MSP17 is indeed the mature hypusine-containing eIF5A and the MIP17 is the deoxyhypusine-containing eIF5A intermediate, we cultured HeLa cells in the presence of [1,8-3H]spermidine with or without mimosine and analyzed the labeled protein by two-dimensional gel electrophoresis. In control cells without mimosine treatment, a predominant radiolabeling of MSP17 was observed, whereas, in mimosine-treated cells, MIP17 was labeled instead (Fig. 3 A). Upon acid hydrolysis and ion exchange chromatographic separation (13), the 3H-labeled component in MIP17 from cells treated with mimosine was identified as [3H]deoxyhypusine and that of MSP17 from cells treated without mimosine was identified as [3H]hypusine (Fig. 3 B). Thus, the spots MSP17 and MIP17 are the mature hypusine-containing eIF5A and the deoxyhypusine-containing eIF5A intermediate, respectively.

Verification of Mimosine Effect on Drg-1 Expression—To verify that MIP42 is indeed Drg-1 as indicated by LC-MS/MS, we performed a Western blot analysis of cell lysates at different...
ent times following mimosine treatment. As shown in Fig. 4A, Drg-1 was indeed increased following mimosine treatment. An approximately 3-fold increase of Drg-1 was observed at 2 h following mimosine treatment. In a dose-response study, the induction of Drg-1 expression was observed at ~300 \(\mu M\) or higher concentrations of mimosine (Fig. 4B).

**Mimosine Treatment Increases the Transcriptional Activity of Drg-1 Gene**—To determine whether the mimosine effect on the increased expression of Drg-1 is due to the increased transcriptional activity of Drg-1 gene, we first performed an RNase protection analysis of the level of Drg-1 mRNA following mimosine treatment. As shown in Fig. 5A, the level of the Drg-1 mRNA increased about 2-fold at 2 h followed by a drastic increase at 4 h following mimosine treatment. These increases are similar to the increase at the protein level shown by Western blot analysis (Fig. 4A). We next performed an RNase protection assay of the newly synthesized RNAs isolated from nuclei to determine whether the transcription of Drg-1 gene is increased by mimosine treatment. As shown in Fig. 5B, the amount of newly synthesized Drg-1 mRNA was drastically increased following mimosine treatment. Thus, the mimosine-induced increase in Drg-1 protein is likely mainly due to the increased transcriptional activity of the Drg-1 gene.

**Comparison of the Effects of Mimosine on Drg-1 Expression with Other Compounds**—To investigate whether the effect of mimosine on the increase in Drg-1 mRNA is due to its effect on formation of hypusinated eIF5A, we tested the effect on Drg-1 expression of another compound, kojic acid, a mimosine analogue that has no effect on the synthesis of hypusine in eIF5A (26). As shown in Fig. 6, treatment with GC7 increased the Drg-1 protein level (Fig. 6A). However, no increase in Drg-1 mRNA was observed (Fig. 6B), indicating that the effect of GC7 is distinct from that of mimosine. Furthermore DFO, a more effective inhibitor of DOHH than mimosine, was less effective than mimosine in the induction of Drg-1 expression following the treatment of mimosine is probably unrelated to eIF5A or its hypusine modification.

We also tested the effect on Drg-1 expression of another compound, kojic acid, a mimosine analogue that has no effect on the synthesis of hypusine in eIF5A (26). As shown in Fig. 6D, kojic acid effectively induced the expression of Drg-1 gene at a concentration as low as 100 \(\mu M\), similar to that of mimosine (compare Fig. 6D with Fig. 4B). This result further
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As controls, these compounds were also tested for their effect on the hypusine formation using 2D gel analysis following the labeling of cells with [35S]methionine. As expected, both DFO (an inhibitor of DOHH) and GC7 (an inhibitor of DHS) effectively inhibited the formation of the hypusinated eIF5A, whereas kojic acid did not (Fig. 6E, circled spots). It is noteworthy that GC7 inhibits the first step enzyme DHS and thus inhibits the conversion of eIF5A precursor into deoxyhypusine-containing intermediate. Indeed the deoxyhypusine-containing eIF5A intermediate was not observed in the GC7-treated cells (Fig. 6E, spot indicated by rectangle symbol in the third panel). Instead a spot with a smaller pi accumulated after GC7 treatment (Fig. 6E, spot indicated by the arrow), likely representing the eIF5A precursor lack modifications.

The Effect of Mimosine on the Expression and Activity of Transcription Factors—As shown above in Fig. 5, the mimosine-induced up-regulation of Drg-1 expression is mainly due to the increased transcriptional activity of the Drg-1 gene. Previously it has been reported that four transcription factors, c-Jun/AP-1, HIF-1α, p53, and N-Myc/c-Myc were involved in metal ion-, hypoxia-, and DNA damage-induced up-regulation of Drg-1 expression (29–32). To identify the possible mechanism of mimosine-induced up-regulation of Drg-1 transcription, we used Western blot analysis and gel shift assay to measure the expression levels of these transcription factors and their activities following treatment with mimosine and its analogue kojic acid, two effective Drg-1 inducers. As shown in Fig. 7, mimosine treatment increased the level of both expression and binding activities of c-Jun/AP-1 and HIF-1α, whereas treatment with kojic acid increased the level of expression and binding activity of only c-Jun/AP-1. The expression and activity level of c-Myc were not altered significantly by either mimosine or kojic acid. We also found that the expression and activity level of p53 were not changed by either mimosine or kojic acid (data not shown). These results suggest that c-Jun/AP-1 is likely the direct common mediator involved in mimosine- and kojic acid-induced up-regulation of Drg-1 transcription.

To further confirm that the transcription factor AP-1 mediates the mimosine-induced transcription of Drg-1, we performed another experiment to determine whether the mimosine-induced Drg-1 expression can be blocked by inhibiting AP-1 activity using curcumin, a yellow pigment of turmeric that inhibits formation of AP-1-DNA complex (33, 34). As shown in Fig. 8A, treatment with mimosine induced the AP-1 activity, similar to that shown in Fig. 7. However, pretreatment of cells with curcumin abolished the mimosine-induced AP-1 binding activity. This observation is consistent with the previous findings (33, 34). We next determined the expression level of Drg-1 following these treatments. As shown in Fig. 8B, the mimosine-induced Drg-1 expression was also abolished by curcumin. Thus, it is likely that the transcription factor AP-1 mediates the mimosine-induced Drg-1 expression.

DISCUSSION

We have shown here that the expression level of Drg-1 can be modulated by mimosine mainly at the transcriptional level. Our results also suggest that mimosine inhibits the hypusine modification of eIF5A but not the translation of its mRNA. Both the increase in Drg-1 expression and inhibition of eIF5A modification induced by mimosine treatment occurred prior to the G1/S cell cycle arrest. Mimosine has been reported to act as an iron chelator and as an inhibitor of DOHH (26, 27, 35, 36), the enzyme responsible for the second step in the biosynthesis of the unique amino acid hypusine in eIF5A. Our results further suggest that the mimosine-induced early increase in Drg-1 expression is not due to a decrease in mature eIF5A formation or to the inhibition of DOHH.

The human Drg-1 (also called RTP, cap43, rit42, Ndrg-1, and PROXY-1) gene was originally found to be a homocysteine-responder gene in vascular endothelial cells and was named RTP (37). It is a homologue of the mouse Ndr-1/TDD5 gene (38). The human Drg-1 gene encodes a protein of 43 kDa with three unique 10-amino acid tandem repeats at the carboxyl-terminal end. Drg-1 was thought to be a differentiation-related gene and found to be up-regulated upon differentiation of colon epithelial cells (39). It has since been found to be induced by multiple factors such as hypoxia (40), Ni2+ compound (41), Ca2+ ionophore (42), DNA-damaging agents such as mitomycin C (32), ligands of nuclear receptors such as androgen (43), and retinoic acid (44). The stimulant-induced increase in Drg-1 expression in these previous studies was attributed to the increase in transcription (41, 43). However, it has also been found that some human tissues express high levels of Drg-1 mRNA but with little or no expression of Drg-1...
protein, suggesting that the expression of the Drg-1 gene may also be under post-transcriptional control (12).

In normal mammary epithelial cells, the expression of Drg-1 was found to oscillate with cell cycle, and its level was depressed in the S phase and elevated in the G1 and G2/M phases (32). The elevation of Drg-1 expression in G1 and G2/M phases was thought to be due to p53-mediated transcription activation, which is known to cause cell cycle arrest upon DNA damage. Consistent with this concept is the induced expression of Drg-1 by DNA-damaging agents and also by enforced expression of wild-type p53 (32). This observation suggests that Drg-1 may be an important factor in G1 arrest. Thus, the finding that Drg-1 is an early responding gene to mimosine treatment suggests that Drg-1 may be one of the important factors that mediate mimosine-induced G1 cell cycle arrest. This argument is also supported by the finding that the enforced expression of Drg-1 in the human breast cancer cell line MCF-7 and the bladder cancer cell line EJ inhibits their growth (32).

In addition to p53, other transcription factors such as HIF-1, c-Jun/AP-1, E2a-Pbx1 fusion protein, N-Myc, and c-Myc were also implicated to play roles in Drg-1 expression. Whereas HIF-1 is required for Ni2+/H11001 compound-induced Drg-1 expression, c-Jun/AP-1 plays an important role in Ca2+/H11001 ionophore and hypoxia-induced Drg-1 expression (30). E2a-Pbx1 is a fusion protein of transcription factors E2a and Pbx1 generated by chromosomal translocation that possibly causes pediatric acute lymphoblastic leukemia (45). Enforced expression of E2a-Pbx1 induced the expression of endogenous Drg-1 and activated the Drg-1 promoter-mediated reporter activity (46). In contrast to the transcription factors p53, HIF-1, c-Jun/AP-1, and E2a-Pbx1, the transcription factors N-Myc and c-Myc were found to suppress the expression of Drg-1 by corroboration with Max (29).

Previously it has been shown that mimosine functions as an iron chelator (10, 35, 36). Although chelating iron would increase the expression and activity of HIF-1 and c-Jun/AP-1 transcription factors (30), it also decreases the expression and activity of N-Myc and c-Myc (47, 48). In the present study, we found that mimosine up-regulated the expression and activity level of AP-1 and HIF-1 but had no effect on the c-Myc and p53 expression and activity. Thus, it is possible that both HIF-1 and AP-1 are involved in the mimosine-induced increase in Drg-1 transcription. However, kojic acid, another iron chelator, increased only the expression and binding activity of c-Jun/AP-1 but not HIF-1, c-Myc, and p53 at the
concentration used. Furthermore use of an AP-1 inhibitor, curcumin, completely abolished the mimosine-induced Drg-1 expression. Thus, we believe that c-Jun/AP-1 plays a major role in the mimosine-induced expression of Drg-1, and it is likely the common mediator involved in the expression regulation of Drg-1 by mimosine and kojic acid in HeLa cells.

It is unknown why the iron chelator kojic acid did not increase the expression level and activity of HIF-1. Because kojic acid is a weak iron chelator compared with mimosine (49) and the concentration used in this study is lower (400 versus 600 µM, Fig. 7), it is possible that HIF-1 cannot be affected by kojic acid under the conditions used. Furthermore it has been shown that not all iron chelators induce HIF-1 expression and activity (50), and no study on kojic acid induction of HIF-1 has been reported. Thus, whether HIF-1 also plays some role in mediating mimosine-induced Drg-1 expression is unknown.

Reduced expression of Drg-1 has been implicated in cancer cell proliferation and metastasis (32, 51, 52). It has also been reported that Drg-1 expression was reduced in a number of tumor cell lines and tissues compared with their normal counterparts and that the enforced expression of Drg-1 inhibits growth of these cancer cells (32). In a differential display study (51), Drg-1 expression was detected only in normal and primary colon cancer cells but not in the metastatic tumors. An enforced expression of Drg-1 in metastatic colon cancer cell line SW620 decreased its invasive potential both in vitro and in vivo. Reduced Drg-1 expression was associated with advanced prostate cancers, and overexpression of Drg-1 suppressed prostate cancer metastasis (52). These findings suggest that Drg-1 expression inducers such as mimosine and kojic acid may be considered and developed as therapeutic agents to treat and prevent metastatic tumors.

It has been suggested previously that mimosine modulates the expression of specific genes at the translational level (6, 8) through the inhibition of the formation of hypusinated eIF5A (53). The hypusine residue of eIF5A is critical to its function (24, 54, 55). The hypusine-containing eIF5A is stable without the significant decrease following 8 h of mimosine treatment (see Fig. 1 and its Coomassie-stained spot MSP17). Thus, the mimosine inhibition of hypusine formation may not be responsible for the increased level of Drg-1 protein or its mRNA. This is further supported by the observation that GC7, another inhibitor for hypusine formation, did not cause a marked induction of Drg-1 mRNA, whereas its protein appeared to be somewhat increased, and that kojic acid, which does not inhibit hypusine formation, induced Drg-1 expression with effectiveness similar to mimosine. We previously reported that the translation of eIF3 p170 was decreased by the iron chelating activity of mimosine and that the decrease in eIF3 p170 level was responsible for the altered expression level of tyrosinated a-tubulin, p27, and ribonucleotide reductase M2 (10, 11). It is not known whether eIF3 p170 is a mediator of mimosine effect on the expression of Drg-1 indirectly by altering the expression of c-Jun/AP-1 transcription factors. We are currently investigating the role of eIF3 p170 in the modulation of Drg-1 expression.

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REFERENCES

1. Lalande, M. (1990) A reversible arrest point in the late G1 phase of the mammalian cell cycle. Exp. Cell Res. 186, 332–339
2. Mosca, P. J., Dijkwel, P. A. & Hamlin, J. L. (1993) The plant amino acid mimosine may inhibit initiation at origins of replication in Chinese hamster cells. Mol. Cell. Biol. 12, 4375–4383
3. Wang, Y., Zhao, J., Clapper, J., Martin, L. D., Du, C., DeVore, E. R., Harkins, K., Dobbs, D. L. & Benbow, R. M. (1995) Mimosine differentially inhibits DNA replication and cell cycle progression in somatic cells compared to embryonic cells of Xenopus laevis. Exp. Cell Res. 217, 84–91
4. Hughes, T. A. & Cook, P. R. (1996) Mimosine arrests the cell cycle after cells enter S-phase. Exp. Cell Res. 222, 275–280
5. Chang, H. C., Weng, C. F., Yen, M. H., Chuang, L. Y. & Hung, W. C. (2000) Modulation of cell cycle regulatory protein expression and suppression of tumor growth by mimosine in nude mice. Int. J. Oncol. 17, 659–665
6. Kajetra, R. F. & Hamlin, J. L. (1997) The dual effect of mimosine on DNA replication. Exp. Cell Res. 231, 173–183
7. Tsai, W. C. & Ling, K. H. (1975) Toxic action of mimosine. I. Inhibition of mitosis and DNA synthesis of H-Ep cell by mimosine and 3,4-dihydroxyprydine. Toxicol 9, 241–247
8. Hanauke-Abel, H. M., Slowinska, B., Zagulaska, S., Wilson, R. C., Staiano-Coico, L., Hanauske, A. R., McCaffrey, T. & Szabo, P. (1995) Detection of a sub-set of polysomal mRNAs associated with modulation of hypusine formation at the G1-S boundary. Proposal of a role for eIF-5A in onset of DNA replication. FEBS Lett. 366, 92–98
9. Wang, G., Miskimins, R. & Miskimins, W. K. (2000) Mimosine arrests cells in G1 by enhancing the levels of p27Kip1. Exp. Cell Res. 254, 64–71
10. Dong, Z. & Zhang, J. T. (2003) EIF3 p170, a mediator of mimosine effect on protein synthesis and cell cycle progression. Mol. Biol. Cell 14, 3942–3951
11. Dong, Z., Liu, L. H., Han, B., Pincheira, R. & Zhang, J. T. (2004) Role of eIF3 p170 in controlling synthesis of ribonucleotide reductase M2 and cell growth. Oncogene 23, 3790–3801
12. Lachat, P., Shaw, P., Gebhard, S., van Belzen, N., Chaubert, P. & Bosman, F. T. (2002) Expression of NDRG1, a differentiation-related gene, in human tissues. Histocom. Cell Biol. 118, 399–408
13. Park, M. H., Cooper, H. L. & Folk, J. E. (1982) The biosynthesis of protein-bound hypusine (Nε-(4-aminobutyrylo)lysine). Lysine as the amino acid precursor and the intermediate role of deoxyhypusine (Nε-(4-aminobutyrylo)lysine). J. Biol. Chem. 257, 7217–7222
14. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254
15. Pincheira, R., Chen, Q., Huang, Z. & Zhang, J. T. (2001) Two subcellular
localizations of elf3 p170 and its interaction with membrane-bound microfilaments: implications for alternative functions of p170. Eur J Cell Biol. 80, 410–418

16. Elfink, C. J. & Reiners, J. J., Jr. (1996) Quantitative RT-PCR on CYP1A1 heterogeneous nuclear RNA: a surrogate for the in vitro transcription run-on assay. BioTechniques 25, 470–477

17. Matsuda, K., Tomozawa, S., Fukushima, S., Yoshino, T., Murakami, T. & Mitsuhashi, M. (2002) Gene expression analysis from nuclear Poly(A) RNA. BioTechniques 32, 1014–1020

18. Dong, Z., Wang, X., Zhao, Q., Townsend, C. M., Jr., & Evers, B. M. (1998) DNA methylation contributes to expression of the human neurotensin/ neuromedin N gene. Am J. Physiol. 274, G353–G354

19. Digiam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11, 1475–1489

20. Schreiber, E., Matthais, P., Muller, M. M. & Schaffner, W. (1989) Rapid detection of octamer binding proteins with “mini-extracts,” prepared from a small number of cells. Nucleic Acids Res. 17, 6419

21. Dong, Z., Wang, X. & Evers, B. M. (2000) Site-specific DNA methylation contributes to neurotensin/neuromedin N expression in colon cancers. Am. J. Physiol. 279, G1139–G1147

22. Park, M. H. (1988) The identification of an eukaryotic initiation factor 4D precursor in spermdepleted Chinese hamster ovary cells. J. Biol. Chem. 263, 7447–7449

23. Park, M. H., Lee, Y. B. & Joe, Y. A. (1997) Hypusine is essential for eukaryotic cell proliferation. Biol. Signals 6, 115–123

24. Smit-McBride, Z., Schnier, J., Kaufman, R. J. & Hershey, J. W. (1989) Protein synthesis initiation factor elf-4D. Functional comparison of native and unhyposynized forms of the protein. J. Biol. Chem. 264, 18527–18530

25. Duncan, R. F. & Hershey, J. W. (1986) Changes in eIF-4D hypusine modification or abundance are not correlated with translational repression in HeLa cells. J. Biol. Chem. 261, 12903–12906

26. Hanauke, Abel, H. M., Park, M. H., Hanauke, A. R., Popowicz, A. M., Andrus, L., Szabo, P., Grady, R. W., Hanauske, A. R., Huima-Byron, T., Slowinska, B., Zagulinska, A. & Hanauke-Abel, H. M. (1998) Antitretroviral effects of deoxyhypusyl hydroxylase inhibitors: a hypusine-dependent host cell mechanism for replication of human immunodeficiency virus type 1 (HIV-1). Biochem. Pharmacol. 55, 1807–1818

27. Jakus, W., Wolf, E. C., Park, M. H. & Folk, J. E. (1993) Features of the spermidine-binding site of deoxyhypusine synthase as derived from inhibition studies. Effective inhibition by bis- and mono-guanylated diamines and polyamines. J. Biol. Chem. 268, 13151–13159

28. Shimono, A., Okuda, T., & Kondoh, H. (1999) N-myc-dependent repression in vascular endothelial cells identified by differential display analysis. GRP78/BiP and novel genes. J. Biol. Chem. 274, 29659–29665

29. Lin, T. M. & Chang, C. (1997) Cloning and characterization of TDDS, an androgen target gene that is differentially repressed by testosterone and dihydrotestosterone. Proc. Natl. Acad. Sci. U. S. A. 94, 4986–4993

30. van Beilen, N., Dinjens, W. N., Diesfeld, M. P. F. Groen, W. E., van der Maade, A. C., Nozawa, Y., Viletstra, R., Trapman, J. & Bosman, F. T. (1997) A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. Lab. Invest. 77, 85–92

31. Park, H., Adams, M. A., Lachat, P., Bosman, F., Pang, S. C. & Graham, C. H. (2000) Hypoxia induces the expression of a 43-kDa protein (PROXY-1) in normal and malignant cells. Biochem. Biophys. Res. Commun. 276, 321–328

32. Zhou, D., Salinikow, K. & Costa, M. (1998) Cap43, a novel gene specifically induced by N(2)-compounds. Cancer Res. 58, 2182–2189

33. Salnikow, K., Kluz, T. & Costa, M. (1999) Role of Ca(2+) in the regulation of nickel-inducible Cap43 gene expression. Toxicol. Appl. Pharmacol. 160, 127–132

34. Ulrich, W., Swinnen, J. D., Heyns, W. & Verhoeven, G. (1999) The differentiation-related gene 1 (Drg1), is markedly upregulated by androgens in LNCAp prostatic adenocarcinoma cells. FEBS Lett. 455, 23–26

35. Piquemal, D., Jouila, D., Balaguer, P., Bassett, A., Marti, J. & Commes, T. (1999) Differential expression of the RTP/Drg1/Ndr1 gene product in proliferating and growth arrested cells. Biochim. Biophys. Acta 1450, 364–373

36. Nourse, J., Mellemkin, J. D., Galili, N., Wilkinson, J., Starbidge, E., Smith, S. H., Clessey, M. L. (1999) Chromosomal translocation t(1;19)(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 60, 535–545

37. Rutherford, M. N., Bayly, G. R., Matthews, B. P., Okuda, T., Dinjens, W. M., Kondoh, H. & LeBrun, D. P. (2001) The leukemogenic transcription factor E2A-Pbx1 induces expression of the putative N-myc and p53 target gene NDRG1 in Ba/F3 cells. Leukemia 15, 362–370

38. Fan, L., Iyer, J., Zhu, S., Frick, K. K., Wada, R. K., Eskanazi, A. E., Berg, P. E., Ikegaki, N., Kennett, R. H. & Frantz, C. N. (2001) Inhibition of N-myc expression and induction of apoptosis by iron chelation in human neuroblastoma cells. Cancer Res. 61, 1073–1079

39. Kyriakoulis, D., Eliopoulos, A. G., Papadakis, A., Alexandrakis, M. & Eliopoulos, G. D. (1998) Decreased expression of c-myc oncprotein by peripheral blood mononuclear cells in thalassaemia patients receiving desferrioxamine. Eur. J. Haematol. 60, 21–27

40. Katoh, S., Toyama, J., Kaminaka, K., Akita, T. & Abe, T. (1992) Protective action of iron-chelating agents (catechol, mimosine, deferoxamine, and kojic acid) against ischemia-reperfusion injury of isolated neonatal rabbit hearts. Eur. Surg. Res. 24, 349–355

41. Creighton-Gudderie, M. & Tyrell, R. M. (2002) A novel iron chelator that does not induce HIF-1 activity. Free Radic. Biol. Med. 33, 356–363

42. Guan, R. J., Ford, H. L., Fu, Y., Li, Y., Shaw, L. M. & Pardee, A. B. (2000) Drg-3 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. Cancer Res. 60, 749–755

43. Bandypadhyay, S., Pai, S. K., Gross, S. C., Hirota, S., Hossobe, S., Miura, K., Saito, K., Combes, T., Hayashi, S., Watabe, M. & Watabe, K. (2003) The Drg-1 gene suppresses tumor metastasis in prostate cancer. Cancer Res. 63, 1731–1736

44. McCaffrey, T. A., Pomerantz, K. B., Sanborn, T. A., Spokojny, A. M., Du, B., Park, M. H., Folk, J. E., Lamberg, A., Kivirikko, K. I., Falcone, D. J., Mehta, S. B. & Hanauke-Abeill, H. M. (1995) Specific inhibition of eIF-5A and collagen hydroxylation by a single agent. Antiproliferative and fibroblastic effects on smooth muscle cells from human coronary arteriomegaly compounds. Toxicol. Appl. Pharmacol. 134, 83–96

45. Perkins, D. N., Pappin, D. J., Creasy, D. M. & Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567