Negative Regulation of Histone H1 Kinase Expression by Mimosine, a Plant Amino Acid

Sandy T. Feldman and Axel Schöntal

Department of Microbiology, University of Southern California, Los Angeles, California 90033-1054 [A. S.], and Department of Ophthalmology, University of California, San Diego, La Jolla, California 92093-0946 [S. T. F.]

INTRODUCTION

The analysis of cellular growth regulation is the target of intense scrutiny. Understanding the underlying biochemical processes not only will advance our knowledge of cellular proliferation in general but also will have implications for efficient cancer therapies as well. Compounds that arrest cells in culture at specific points in the cell cycle have proven to be extremely useful tools to unravel cell cycle regulatory events. Moreover, these compounds may be useful for therapeutic approaches to cancerous diseases. Mimosine [β-N-(3-hydroxy-4-pyridone)-α-aminopropionic acid], a plant amino acid isolated from the seeds of Leucaena glanca Benth or Mimosa pudica (1, 2), has been shown to reversibly block cells at a point late in G1 of the cell cycle (3–6). Its mechanism of action, however, has remained elusive.

We investigated potential effects of mimosine on two crucial cell cycle regulatory proteins, histone H1 kinase and the Rb3 tumor suppressor gene product. Histone H1 kinase is a heterodimeric complex (at least) consisting of a catalytic subunit and a regulatory subunit (7, 8). The subunits are encoded by two families of related genes. At least two, and likely many more, genes code for the catalytic subunit; cdc2 encodes a protein of Mr 34,000 (p34cdk2) and cdk2 codes for p33cdk2 (7–12). The regulatory subunit is encoded by a set of genes named cyclin A through E. The activity of histone H1 kinase is regulated by physical interactions of regulatory and catalytic subunits, by phosphorylation-dephosphorylation reactions, and by cell cycle-dependent expression and synthesis of its subunits (7–12).

A potential in vivo substrate for H1 kinase is the protein product of the Rb tumor suppressor gene (13–15). The Rb protein is hypophosphorylated during G0 and early G1 phases and becomes highly phosphorylated as cells progress from G1 to S phase. It has been proposed that the hypophosphorylated form of Rb is the active tumor suppressor and that phosphorylation of Rb may act as a key switch in allowing cells to progress through the cell cycle (13–15).

In this report we show that primary cells as well as certain transformed cells treated with mimosine become blocked in the cell cycle. Concomitantly, expression and activity of various forms of histone H1 kinase are blocked. These effects are reversible, because removal of mimosine restores H1 kinase activity and the cells resume growth. Because it has been shown that H1 kinase is essential for cell cycle progression, our experiments suggest that mimosine may block cell growth by inhibiting expression and activity of this enzyme.

ABSTRACT

The plant amino acid mimosine has been shown to reversibly arrest mammalian cells in late G1 phase of the cell cycle. However, the underlying molecular mechanisms of this block are not as yet understood. Here we show that mimosine prevents the serum-stimulated synthesis and activation of histone H1 kinase, a crucial regulator of cell cycle progression. The same effect is observed in logarithmically growing primary cells as well as transformed cells. Concomitantly, hyperphosphorylation of the retinoblastoma tumor suppressor gene product is partially inhibited. These effects are fully reversible, because removal of mimosine restores histone H1 kinase activity and the cells resume growth. Because the activity of histone H1 kinase has been shown to be absolutely required for cell cycle progression, it is conceivable that the cytostatic effect of mimosine is due to its negative effects on synthesis and activity of this enzyme.

MATERIALS AND METHODS

Materials. Mimosine (Sigma Chemical Co., St. Louis, MO) was resuspended in 10 mM HCl to yield a 100 mM stock solution. Immediately before the addition into cell culture medium, the pH was adjusted to 7.4 with 10 N NaOH.

Cell Culture. All cells were grown in DMEM supplemented with 10% FCS, 0.5 mg/ml glucose, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, at 37°C in a 5% CO2 atmosphere. For experiments where starvation of cells was required, the monolayers were rinsed twice with phosphate-buffered saline and incubated further in medium with 0.5% FCS for 48 h.

Histone H1 Kinase Assay. Cells were lysed in RIPA buffer as described (16), and the amount of protein was determined using the bichinchoninic acid protein assay reagent (Pierce, Rockford, IL). Total protein (100–500 µg) was subjected to immunoprecipitation with antibodies to the gene products of either cdc2 (17), cdk2,4 cyclin A or B (18), or cyclin E (19), as described (17). The immunocomplexes were collected with Protein A-agarose and washed four times with RIPA buffer and three times with kinase buffer (50 mM Tris, pH 7.4, 1 mM MgCl2, 1 mM dithiothreitol). Twenty-five µl kinase buffer with 1 µg histone H1, 100 µM ATP, and 10 µCi [γ-32P]-ATP (3000 Ci/mmol) were added, and the reactions were incubated at room temperature for 10–30 min. The reaction was stopped by the addition of 20 µl 5 X Laemmli sample buffer (16) and boiling for 10 min. The reaction products were separated on a 12.5% acrylamide gel, stained with Coomassie blue (to ensure that the same amounts of histone H1 proteins were added), dried, and exposed to Kodak X-AR film. The amount of radioactivity incorporated into histone H1 protein was determined by exposing the dried gel to the AMBIS radiodensitographic imaging system.

Immunoblot Analysis. One mg total cell lysate in RIPA buffer was subjected to immunoprecipitation with p34cdk2 or p60v RCA cyclin A-specific antibodies, as described (17). The immunocomplexes were collected with Protein A-agarose and washed extensively with RIPA buffer. Laemmli sample buffer was added and, after boiling for 5 min, the sample was separated on a 12.5% acrylamide gel and transferred onto a nitrocellulose membrane. To detect p34cdk2 the membrane was incubated with the same anti-cdc2 antibody as before, with a biotinylated secondary antibody, and finally with streptavidin/alkaline phosphatase, essentially as described (16, 17). For the detection of p60v CA on the membrane we used a different antibody (20) than the one used for the immunoprecipitation.

For the analysis of Rb protein, cells from one 15-cm plate were lysed in 1 ml of lysis buffer (25 mM Tris, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 1 mM sodium vanadate, 1 mM sodium fluoride, 0.5 mM EDTA, 2% Nonidet P-40, 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM/ml aprotonin, 1 mg/ml leupeptin, 10 mg/ml pepstatin, 100 mM okadaic acid, 100 mM calyculin A). One mg of the lysate was incubated with purified anti-Rb monoclonal antibody (PharMingen, San Diego, CA) as described in the manufacturer’s protocol. Briefly, immunocomplexes were collected with Protein

1 Parts of this work were performed in the laboratory of James R. Feramisco (University of Southern California, Los Angeles).
2 To whom requests for reprints should be addressed, at Department of Microbiology, University of Southern California, 2011 Zonal Ave., HMR-405, Los Angeles, CA 90033-1054.
3 The abbreviations used are: Rb, retinoblastoma; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; cDNA, complementary DNA.
4 J. Pines, unpublished observations.
A mRNA we used a 1.2-kilobase by an amplification step in which we used streptavidin-biotinylated horseradish cpm/txg. Two gels were run in parallel, blotted onto nylon membranes, and DNA fragments were performed essentially as described (21). To detect cyclin peroxidase complexes. Then developing solution (Luminol in the presence of blasts, we compared various growth parameters of primary human membrane.

were blocked with 5% nonfat milk, the membrane was incubated with the same anti-Rb antibody as before. Then we used the ECL Western blotting detection system (Amerham Corp.), which works on the principle of chemiluminescence. The second antibody was biotinylated sheep anti-mouse IgG, followed by an amplification step in which we used streptavidin-biotinylated horseradish peroxidase complexes. Then developing solution (Luminol in the presence of enhancers) was added and the filter was exposed to Kodak X-AR film.

mRNA Analysis. Isolation of polyadenylated RNA, Northern blot analysis, and hybridization of filter-bound mRNA to the respective radioactively labeled DNA fragments were performed essentially as described (21). To detect cyclin A mRNA we used a 1.2-kilobase PvuII cDNA fragment (22), for cdc2 we used a 2.0-kilobase BamH1 cDNA fragment (kindly provided by Peter Welch, University of California, San Diego), and for choA we used a linearized plasmid containing the cDNA sequence (23). All DNA fragments were labeled with [α-32P]dCTP according to manufacturer’s instructions, using the Amersham Multi Prime labeling kit. The specific activity obtained was 2–10 × 106 cpm/μg. Two gels were run in parallel, blotted onto nylon membranes, and hybridized to probes for either cyclin A or cdc2. After the first hybridization the filters were stripped by boiling in 0.1× standard saline citrate (15 mM NaCl, 1.5 mM sodium citrate, pH 7) and were rehybridized to choA to ensure that equal amounts of RNA were present on the filters. For the same purpose, the gels had been stained with acridine orange before transfer to the nylon membrane.

RESULTS

To analyze whether mimosine would affect the growth of fibroblasts, we compared various growth parameters of primary human foreskin fibroblasts (Hs68) that were treated with different concentrations of mimosine. We found that mimosine at concentrations of 300 μM or higher inhibited cellular proliferation, as well as DNA synthesis (data not shown). These effects were reversible, because upon removal of mimosine the cells underwent DNA synthesis and increased in cell number (data not shown). Thus, our findings with Hs68 cells are consistent with several previous reports that describe cytostatic effects of mimosine in other cell types (3–6).

In an effort to understand the mechanism(s) underlying the growth inhibition by mimosine, we decided to study its effects on different proteins with crucial cell cycle regulatory functions. Therefore, we first analyzed whether mimosine might affect the activity of histone H1 kinase, a key enzyme regulating the progression through various cell cycle stages. Hs68 cells were cultured at reduced serum concentrations to synchronize them in G0, a stage with very low histone H1 kinase activity. Then the cells were stimulated with serum in the presence or absence of mimosine. At different times thereafter, cell lysates were prepared and histone H1 kinase activity was determined. As shown in Fig. 1A, in the absence of mimosine serum stimulation induced increased histone H1 kinase activity associated with cyclin A and cyclin B, two histone H1 kinase subunits that are thought to play a role during the G0/S and G2/M transition, respectively. In contrast, when mimosine was present, no increase in either cyclin A- or cyclin B-associated kinase activity could be detected.

To determine whether mimosine might also affect other subunits of histone H1 kinase, we repeated this experiment and analyzed kinase activity associated with cyclin E, p32cdc2, and p34cdc2. As shown in Fig. 1B, all three subunits exhibited increased kinase activity in response to serum. However, in the presence of mimosine this induction was blocked. These findings demonstrate that inhibition of cellular growth by mimosine correlates with inhibition of a crucial cell cycle regulator, histone H1 kinase.

During the normal cell cycle histone H1 kinase has been shown to be regulated at several levels, e.g., transcriptional and post-translational. We therefore studied the potential level of inhibition by mimosine and analyzed protein synthesis as well as mRNA expression of histone H1 kinase. Because it was established earlier that inhibition of either cyclin A or p34cdc2 alone is sufficient to block cell cycle progression, we focused this part of our analysis on these two subunits. To determine the amount of cyclin A and p34cdc2 protein, Hs68 cells synchronized in G0 were stimulated with serum for 20 h, with or without mimosine, and were subjected to immunoblot analysis. The results are shown in Fig. 2A. While in G0-arrested cells p60p34/cdc2 was undetectable and p34cdc2 was detectable at very low levels, serum-stimulated cells exhibited increased levels of both proteins. In contrast, the accumulation of these two subunits was blocked when mimosine was present (Fig. 2A). To determine whether this inhibition may be due to increased turnover of the protein or inhibition of translation of the respective mRNAs, we performed Northern blot analysis of cyclin A and cdc2 mRNAs. As shown in Fig. 2B, the accumulation of cyclin A and cdc2 mRNA seen in response to serum stimulation was completely blocked in the presence of mimosine. These findings suggest that the major effect of mimosine on histone H1 kinase is the inhibition of its synthesis. In the absence of cdc2 and cyclin A mRNA accumulation, insufficient p60p34/cdc2 and p34cdc2 pro-
tein is synthesized, resulting in a lack of increased kinase activity. Whether mimosine may exert similar effects on the other subunits remains to be determined.

Previous studies reported that mimosine does not inhibit mRNA or protein synthesis in general (24). To exclude a nonspecific effect on protein synthesis in our cells as well, we treated Hs68 cells with various concentrations of mimosine for 16 h. Then we determined the level of ongoing translation by measuring the incorporation of [35S]methionine into newly synthesized protein for 4 h. We found that 300 and 500 mM mimosine slightly reduced the amount of [35S]methionine incorporation (by 5–15% and 10–20%, respectively) (data not shown). Considering the data presented above, it is unlikely that this weak inhibition is sufficient to completely block histone H1 kinase synthesis and activity. Because mimosine-treated cells are growth arrested, the somewhat lower rate of translation likely reflects a general reduction of metabolic activity in these nondividing cells.

Because in the experiments described above we studied mimosine effects on cells synchronized in G0, e.g., at a stage where histone H1 kinase expression and activity are very low, we next analyzed mimosine effects on logarithmically growing cells, where histone H1 kinase expression and activity are already at high levels. For this experiment we analyzed the associated histone H1 kinase activity of two cyclins, p60cycA as an indicator of G1/S transition and p58cycB as an indicator of G2/M. Logarithmically growing Hs68 cells were treated with mimosine for 24 h and analyzed for histone H1 kinase activity. As shown in Fig. 3 (left two lanes), in the presence of mimosine histone H1 kinase activity in these cells was strongly reduced. Thus, mimosine not only prevents increases from low levels but also down-regulates histone H1 kinase activity from already high levels.

Because the cytostatic effects of mimosine are readily reversible upon its removal (data not shown; see also Refs. 3–5), we analyzed whether resumed growth would correlate with increased histone H1 kinase activity. Cells that had been growth arrested in the presence of mimosine were washed and further incubated with fresh growth medium in the absence of mimosine. Fig. 3 shows that upon removal of mimosine histone H1 kinase activity increased and again reached levels similar to those seen before the induced growth arrest. These experiments demonstrate reversibility of mimosine effects not only on the cellular level but also on the molecular level.

Because the Rb tumor suppressor gene product has been shown to be a likely in vivo substrate for histone H1 kinase, we next analyzed whether inhibition of histone H1 kinase activity by mimosine would affect the phosphorylation status of Rb protein. For this purpose, G0-synchronized Hs68 cells were stimulated with serum in the presence or absence of mimosine and Rb protein was analyzed by immunoblotting. Differences in the phosphorylation status of the Rb protein become apparent due to different mobilities of Rb phosphorylated proteins in polyacrylamide gel electrophoresis (25, 26). While Rb protein from G0-synchronized cells ran as a single band indicative of the hypophosphorylated (growth-suppressive) form, serum stimulation caused a shift to at least two more slowly migrating forms, indicative of hyperphosphorylated (inactive) Rb protein (Fig. 4). However, mimosine quantitatively and qualitatively affected this shift. First, in the presence of mimosine less Rb protein was shifted to a hyperphosphorylated form. Second, the hyperphosphorylated form accumulated in only one band, not in two as seen in the absence of mimosine. Thus, the inhibition of histone H1 kinase activity correlates with reduced phosphorylation of the Rb protein. However, because the appearance of some phosphorylated forms of Rb is not blocked, this finding may point to differential inhibition of Rb kinases by mimosine. Therefore, this drug may be useful for future dissection of Rb phosphorylation in vivo.

Fig. 2. Amount of cyclin A and cdc2 protein and mRNA in mimosine-treated cells. Hs68 cells were stimulated with serum in the presence or absence of mimosine as described in the legend to Fig. 1. Twenty h after the onset of stimulation the cells were harvested. Co, cells before stimulation with serum; FCS, cells stimulated with serum; mimo + FCS, cells stimulated with serum in the presence of 300 μM mimosine. In A, cells were lysed in RIPA buffer and the amount of p60cycA and p34cdc2 protein was determined by immunoblot analysis (see "Materials and Methods"). Note that in A, top, only the upper of the two bands represents cyclin A protein (p60cycA). MW, molecular weight markers.

Fig. 3. Reversibility of mimosine effects. Logarithmically growing Hs68 cells in 10% FCS/DMEM were treated for 24 h with either 300 μM mimosine (mimo) or the same amount of solvent without mimosine (Co). Then some of the mimosine-treated cell layers were rinsed three times in warm phosphate-buffered saline and incubated further in fresh 10% FCS/DMEM without mimosine (after mimo). At the times indicated cells were lysed and analyzed for histone H1 kinase activity associated with cyclin A and cyclin B. 32P-H1, phosphorylated histone H1 protein, which was used as the substrate for these in vitro kinase reactions. Numbers below the autoradiographs, cpm of the respective bands, as determined by AMBIS. Coomassie blue staining of the gels was performed to confirm that the same amounts of antibodies (upper band) and histone H1 protein (lower doublet) were used.
Parallel lysates from logarithmically growing Saos-2 cells were subjected to blot analysis (see "Materials and Methods"). As a negative control, we analyzed the first of the two discernible hyperphosphorylated forms. Hs68 cells were synchronized in G0 by starvation in 0.5% FCS for 48 h. Both cell types. In Saos-2 cells, the activity associated with cyclin A is p.M mimosine. After 1 or 20 h cells were lysed, and Rb protein was analyzed by immunoblot analysis (see "Materials and Methods"). As a negative control, we analyzed in parallel lysates from logarithmically growing Saos-2 (Saro) cells, an osteosarcoma cell line with a truncated form of Rb, p60, hypophosphorylated form of Rb protein; p34cdc2, hyperphosphorylated forms. Note that in mimosine-treated cells Rb is shifted only to the first of the two discernible hyperphosphorylated forms.

To determine the importance of Rb phosphorylation during mimosine-induced growth arrest, we analyzed the effects of this amino acid on cells already harboring an inactivated Rb protein. Two different cell lines were studied. The first was Hs68 cells transformed by the large T antigen of simian virus 40 (Hs68-LT cells). Large T antigen is known to bind to the hypophosphorylated form of Rb protein and thereby inactivate it, leading to tumorigenic transformation (27). The second cell line was Saos-2, osteosarcoma cells that harbor a truncated inactivated form of Rb. Introduction of wild-type Rb protein into these cells has been shown to cause them to revert to the untransformed phenotype (28). We treated these two cell lines with mimosine and analyzed cell growth and histone H1 kinase activity. As shown in Fig. 5, mimosine caused down-regulation of histone H1 kinase activity in both cell types. In Saos-2 cells, the activity associated with cyclin A and cyclin B was reduced to 22% and 37%, respectively. In Hs68-LT cells the activity of cyclin A and p34cdc2 dropped to 31% and 4%, respectively. Concomitantly, DNA synthesis in both cell lines was blocked (data not shown). Thus, despite the fact that these cells harbor inactivated forms of Rb, mimosine effectively blocked their progression through the cell cycle. Compared with normal cells this indicates that inhibition of full Rb phosphorylation may be a consequence rather than the decisive event of growth inhibition by mimosine.

**DISCUSSION**

The experiments described above demonstrate potent inhibition of H1 kinase activity by the plant amino acid mimosine. This effect is likely due to a block of synthesis of H1 kinase subunits, as shown for p60 and p34cdc2. This may explain the relatively long lag period after removal of mimosine. Because it takes approximately 14 h for histone H1 kinase activity to reach pretreatment levels again (see Fig. 3), it is conceivable that this may be due to the time required for new subunit synthesis. The importance of H1 kinase in cell cycle progression has been shown by experiments where defined subunits of this kinase have been inhibited by microinjection of antibodies or by antisense oligonucleotides (29-33). In these cases, cell cycle progression is inhibited, indicating that these components are essential for proliferation. Because in the presence of mimosine the synthesis of these subunits is blocked, it is likely that the cytostatic effect of mimosine may, at least in part, be due to inhibition of H1 kinase synthesis. This is further supported by our observation that the resumption of cell growth after removal of mimosine correlates with increased H1 kinase activity.

One of the potential in vivo substrates for histone H1 kinase is the Rb tumor suppressor protein (11, 25, 26). We found (see Fig. 4) that in the presence of mimosine the phosphorylation of Rb in response to stimulation of cells with serum growth factors is partially inhibited. This may reflect a consequence of down-regulated histone H1 kinase activity. Thus, mimosine appears to affect the phosphorylation status of Rb protein indirectly, e.g., via regulation of histone H1 kinase activity. However, even in the presence of mimosine, some Rb kinase activity is stimulated in response to serum treatment (see Fig. 4), although not sufficiently to enable cells to progress through the cell cycle. Because we do not see any apparent increase in associated histone H1 kinase activity of cyclin A, cyclin B, cyclin E, p33cdc2, and p34cdc2, it could be that another kinase, such as one of the other recently discovered subunits of histone H1 kinase, is responsible for this effect. Alternatively, the in vitro phosphorylation of histone H1 protein may not completely reflect the in vivo activity of histone H1 kinase towards Rb protein as a substrate. In any case, the observed partial hyperphosphorylation of Rb protein in the presence of mimosine appears to represent an intermediate step towards full hyperphosphorylation and inactivation of the protein. Thus, because mimosine appears to block some, but not all, Rb kinases in vivo, it will be a valuable tool to further dissect the mechanisms involved in Rb phosphorylation and cell cycle progression.

Because the growth of cells harboring an inactive Rb protein is still effectively inhibited by mimosine, it is unlikely that inhibition of Rb phosphorylation is the ultimate mechanism by which this amino acid blocks cell cycle progression. Clearly, mimosine does not require Rb protein to inhibit cellular proliferation. Although this result is negative, it is still instructive because it suggests that instead, via the inhibition of histone H1 kinase subunits, other components necessary for cell growth may be affected. For example, thymidine kinase, thymidylate synthase, DNA polymerase α, and dihydrofolate reductase belong to a set of enzymes required for DNA synthesis (34). Expression of the respective genes is transcriptionally activated at the G1/S border. The presence of E2F binding sites in the promoter regions of these genes suggests that regulation by the transcription factor E2F may be a characteristic of genes necessary for DNA synthesis (34). Intriguingly, E2F has been found to be present in multi-protein complexes containing Rb, p107 (a Rb-related protein), cyclin A or E, and p34cdc2 or p33cdc2 in a temporally regulated fashion (35-39). It has therefore been suggested that histone H1 kinase may play an active role in the regulation of E2F activity by phosphorylating components of this complex, maybe even E2F itself. Thus, inhibition of histone H1 kinase by mimosine may prevent activation of E2F and consequently may block the expression of genes required for DNA synthesis.

In summary, we present evidence that mimosine may exert its negative effects on cell cycle progression via the inhibition of histone H1 kinase synthesis. Its effects at the cellular level (inhibition of cell growth after removal of mimosine).
proliferation) as well as at the molecular level (inhibition of histone H1 kinase) are readily reversible. For the further evaluation of the potential therapeutic value of this compound it will be important to establish how mimosine is able to affect histone H1 kinase synthesis and whether it can be applied in a way to permanently block this enzyme in selected target cells.

ACKNOWLEDGMENTS

We thank Jonathon Pines, Bryan T-Y. Lin, Vjekoslav Dulic, and Steven L. Reed for generous gifts of antibodies and Andrew M. Thorburn and James R. Feramisco for many useful discussions.

REFERENCES

Negative Regulation of Histone H1 Kinase Expression by Mimosine, a Plant Amino Acid

Sandy T. Feldman and Axel Schönthal


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/54/2/494

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/54/2/494. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.