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

Sandy T. Feldman and Axel Schöntthal

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. E.]

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 it has been shown that histone 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.

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)-o-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 (p34cdc2) and cdk2 codes for p33cdc2 (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 phosphotyrosyl-phosphatophorylation 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.

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 glutamine, 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 bicinchoninic 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, 10 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% 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 AMBIOS radioanalytic imaging system.

Immunoblot Analysis. One mg total cell lysate in RIPA buffer was subjected to immunoprecipitation with p34cdc2 or p60v-src 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 p34cdc2 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-src on the membrane we used a different antibody (20) than the one we 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 dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 10 mg/ml pepstatin, 100 μM okadaic acid, 100 μM 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 A-agarose, washed extensively with RIPA buffer, and subjected to SDS-PAGE. The membrane was incubated with the same anti-Rb antibody as before, with a biotinylated secondary antibody, and finally with streptavidin/alkaline phosphatase, essentially as described (16, 17). For the detection of p60v-src on the membrane we used a different antibody (20) than the one we used for the immunoprecipitation.

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1 Parts of this work were performed in the laboratory of James R. Feramisco (University of Southern California, San Diego).

2 To whom requests for reprints should be addressed, at Department of Microbiology, University of Southern California, 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 cpm/txg. Two gels were run in parallel, blotted onto nylon membranes, and DNA fragments were performed essentially as described (21). To detect cyclin enhancers) was added and the filter was exposed to Kodak X-AR film.

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, p33cdc2, 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 p60cyclA 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 p60cyclA and p34cdc2 pro-mRNAs were present in the cells.

RESULTS

To determine 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).

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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 Go, 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, p60\(^{\text{cycl}A}\) as an indicator of G3/S transition and p58\(^{\text{cycl}B}\) 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 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.
parallel lysates from logarithmically growing Saos-2 cells received 20% FCS/DMEM in the absence or presence (mim + FCS) of 300 μM 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 the first of the two discernible hyperphosphorylated forms.

Hs68 cells were synchronized in Go by starvation in 0.5% FCS for 48 h followed by treatment with mimosine. After 1 or 20 h cells were lysed and analyzed for histone H1 kinase activity associated with cyclin A, cyclin B, and pp34<sup>cdc2</sup>. This may explain the relatively long lag period before growth inhibition by mimosine. 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.

The experiments described above demonstrate potent inhibition of H1 kinase activity by the amino acid mimosine. This effect is likely due to a block of synthesis of H1 kinase subunits, as shown for p60<sup>cycA</sup> and p34<sup>cdc2</sup>. 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, p33<sup>cdc2</sup>, and p34<sup>cdc2</sup>, 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.

**DISCUSSION**

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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.

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