Mimosine Targets Serine Hydroxymethyltransferase*

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The plant amino acid, mimosine, is an extremely effective inhibitor of DNA replication in mammalian cells (Mosca, P. J., Dijkwel, P. A., and Hamlin, J. L. (1992) Mol. Cell. Biol. 12, 4375–4383). Mimosine appears to prevent the formation of replication forks at early-firing origins when delivered to mammalian cells approaching the G1/S boundary, and blocks DNA replication when added to S phase cells after a lag of ~2.5 h. We have shown previously that [3H]mimosine can be specifically photocross-linked both in vivo and in vitro to a 50-kDa polypeptide (p50) in Chinese hamster ovary (CHO) cells. In the present study, six tryptic peptides (58 residues total) from p50 were sequenced by tandem mass spectrometry and their sequences were found to be at least 77.5% identical and 96.5% similar to sequences in rabbit mitochondrial serine hydroxymethyltransferase (mSHMT). This assignment was verified by precipitating the [3H]mimosine-p50 complex with a polyclonal antibody to rabbit cSHMT. The 50-kDa cross-linked product was almost undetectable in a mimosine-resistant CHO cell line and in a CHO gly− cell line that lacks mitochondrial, but not cytosolic, SHMT activity. The gly− cell line is still sensitive to mimosine, suggesting that the drug may inhibit both the mitochondrial and the cytosolic forms. SHMT is involved in the penultimate step of thymidylate biosynthesis in mammalian cells and, as such, is a potential target for chemotherapy in the treatment of cancer.

Our laboratory's interest is the regulation of DNA synthesis in mammalian cells and, in particular, the nature of origins of replication. Although it is known that mammalian DNA is replicated from bidirectional origins spaced ~100 kilobase pairs apart (1), the molecular mechanisms of this process remain elusive (see Ref. 2, for review).

In the absence of a viable assay for identifying the genetic elements (replicators) that control initiation in mammalian cells, attention has been focused on determining the positions at which replication initiates, which should lie close to replicators. This approach requires methods for obtaining cell populations in which initiation at a given origin is occurring at the same time. In a commonly used synchronization protocol, cells are first arrested in the G0 (non-proliferating) compartment by nutritional or serum starvation, followed by release into an inhibitor of DNA synthesis (e.g. Refs. 3–5). The drug treatment is enforced for a time long enough to allow all cells in the population to arrive at the beginning of the S period (a time when at least some origins are sure to be firing); the drug is then removed, allowing cells to enter S in a semi-synchronous wave. Unfortunately, this protocol is not entirely satisfactory for examining initiation events at the beginning of S, because even the most efficacious replication inhibitors do not inhibit initiation per se, rather, they slow the rate of replication fork movement by affecting DNA polymerases (e.g. aphidicolin) or by lowering deoxyribonucleotides pools (e.g. hydroxyurea (7) and 5'-fluorodeoxyuridine (8)).

About five years ago it was reported that the plant amino acid, mimosine, arrests mammalian cells at a specific point in the late G1 phase of the cell cycle (9, 10). Therefore, mimosine could be a superior agent for synchronizing cells prior to initiation at early-firing origins. However, when we examined the effects of mimosine on cell cycle progression, specific G1 arrest was not observed (11). Instead, we showed that the drug inhibits replication per se, but in a manner different from any known chain elongation inhibitor. For example, mimosine completely prevents the uptake of [3H]thymidine into DNA when added to CHO1 cells that have already entered the S period, but only after ~2.5 h, whereas aphidicolin and hydroxyurea inhibit replication almost immediately (e.g. Ref. 12). After the 2.5-h lag, mimosine effectively prevents S phase cells from progressing any further in the cell cycle for at least 48 h, as assessed by fluorescence-activated cell sorter analysis; this contrasts with aphidicolin and hydroxyurea, which are relatively leaky even at high concentrations and allow cells to slowly traverse the S period (11, 13). When added to cells as they attempt to cross the G1/S boundary, mimosine completely prevents the formation of replication forks in the dihydrofolate reductase origin in CHO cells, while aphidicolin and hydroxyurea do not (12, 14). Finally, the initial rate of DNA synthesis is zero regardless of how long cells are maintained in mimosine after release from a G0 block, suggesting again that mimosine prevents the formation of replication forks; in contrast, with aphidicolin or hydroxyurea, the initial rate of [3H]thymidine incorporation increases with the duration of the block, arguing that both initiation and a significant amount of chain elongation occur in their presence (14).

Thus, the possibility arose that mimosine could inhibit initiation itself, either by interfering with an initiator protein or by somehow preventing the formation of replication forks. However, it is also known that mimosine chelates iron, which is required by ribonucleotide reductase (15). Indeed, at relatively high concentrations, mimosine has been reported to lower deoxyribonucleotide pools in mammalian cells (16, 17). In addition, it

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¶ The abbreviations used are: CHO, Chinese hamster ovary; p50, 50-kDa polypeptide; mSHMT, mitochondrial serine hydroxymethyltransferase; cSHMT, cytosolic serine hydroxymethyltransferase; PAGE, polyacrylamide gel electrophoresis.
has no apparent effect on DNA synthesis either in frog embryos (18) or in vitro replication extracts prepared from mammalian cells (16, 17), both of which contain large deoxynucleotide pools. Furthermore, the inhibitory effects of mimosine on DNA replication in CHO cells can be overcome by adding iron to the culture medium (19). It has therefore been suggested that mimosine functions solely by inhibiting ribonucleotide reductase (16, 17).

It is difficult to prove or disprove the latter assertion (see arguments in Ref. 19). However, a simple lowering of deoxynucleotide pool levels by inhibiting ribonucleotide reductase does not explain why mimosine is such an efficacious inhibitor and why it appears to prevent initiation of nascent DNA chains. We recently obtained evidence for a different (or additional) intracellular target. We showed that [4H]mimosine can be specifically cross-linked to a 50-kDa polypeptide (p50) both in vivo and in vitro at concentrations equal to the minimum effective dose in vivo (19, 20). Furthermore, we demonstrated that the p50 mimosine binding activity is almost absent in a CHO cell line selected for resistance to 1 mM mimosine (−10 times the lethal dose; 19, 20). p50 partitions largely with the soluble cytoplasmic and nuclear fractions, and the ability to bind mimosine does not fluctuate demonstrably during the cell cycle (19).

In the present study, we have partially purified the major 50-kDa mimosine binding activity and have sequenced six of its tryptic peptides by tandem mass spectrometry (21). The sequences of all six peptides (58 residues total) are consistent with 96.5% similarity and at least 77.5% identity to sequences in rabbit mitochondrial serine hydroxymethyltransferases (mSHMT), and all are closely related to rabbit cytosolic SHMT (cSHMT). Both of these enzymes are involved in the biogenesis of thymidine (among other activities; Refs. 22 and 23). Evidence is presented that mSHMT and cSHMT are, indeed, the major mimosine-binding species in vivo, as well as bona fide targets for the drug.

Given the role of SHMT in deoxynucleotide metabolism, mimosine would be expected to have an effect only on chain elongation, even though its overall effect on replication differs greatly from other drugs that inhibit replication by lowering nucleotide pools (e.g. hydroxurea, methotrexate, and 5-fluorodeoxyuridine). Possible reasons for this dichotomy are discussed.

MATERIALS AND METHODS

Cell Culture—A donal derivative of CHO-K1 cells with a doubling time of ≈13 h was the source of purified p50 in this study. CHO-K1 and CHOC 400 (a methotrexate-resistant CHO variant; Ref. 24) were maintained in minimal essential medium supplemented with non-essential amino acids (Life Technologies, Inc./BRL), 10% Fetal Clone II serum; 1 g/ml trypsin inhibitor, 40 μg/ml Gentamicin (Life Technologies, Inc./BRL), and 50 μg/ml G-418 (Invitrogen Corp., San Diego, CA).

The cells were maintained in the same manner.

Purification of the 50-kDa Mimosine-Binding Protein—CHO-K1 cells were plated into 20–40 15-cm tissue culture dishes ( ≈3 × 10⁷ cells/plate) 36 h before use and were harvested and photo-cross-linked to [4H]mimosine (Amersham, custom labeling) while still in exponential growth. All operations were carried out at room temperature except where noted. Plates were washed once with PBS (135 mM NaCl, 5 mM KCl, 1.1 mM KH₂PO₄, 1.1 mM Na₂HPO₄, pH 7.2) and were drained almost to dryness. To each plate were added 100 μl of lysis buffer (20 mM Hapes, pH 7.4, 3 mM MgCl₂, 1 mM EDTA, 2% Triton X-100, 1 μg/ml dithiothreitol, 40 μg/ml trypsin inhibitor, 40 μg/ml leupeptin, 1 μM 1-chloro-3-tosylamido-7- amino-2-heptanone). Cells were scraped with a plastic policeman, triturated to a uniform suspension, pooled into 1.5-ml microfuge tubes, and centrifuged for 3 min at 5,000 rpm in an Eppendorf microcentrifuge (final volume of the cell pellet was 180–200 μl/plate). The supernatant was removed, 225-μl aliquots were distributed to the wells of a microtiter dish, and 25 μl of [4H]mimosine (100 μCi; 7.4 Ci/mmol; Amersham custom labeling) was added. Samples were then incubated at 37 °C for 30 min and were cross-linked with a Xenon lamp (ILC model LX3000 UV) for 15 s at a distance of 10 cm while maintaining the plates on ice.

The sample was then transferred to a 1.5-ml tube and centrifuged at 8,000 rpm for 5 min in a microcentrifuge. The supernatants were brought to 40% (NH₄)₂SO₄, incubated on ice for 10 min, and centrifuged at 10,000 rpm for 20 min at 4 °C in an HB-4 rotor (DuPont/Sorval). Pellets were resuspended in lysis buffer without Triton X-100 and were subjected to gel filtration on a 1.2×20-cm Sepharose CL-6B column (Sigma). Fractions (1.2 ml fractions; 100 μl aliquots) were collected and 100 μl aliquots were analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel (PAGE) (25). The gel was impregnated with ENTENSYFY (DuPont Fluoroform kit) following the manufacturer’s instructions, and radioactive spots were identified fluorographically, using Kodak X-Omat AR film.

The [3H]-labeled p50 protein fractions thus identified were subjected to DE52 anion exchange chromatography (1.5×10 cm, Whatman); after washing the column with 40 mM KCl in lysis buffer lacking Triton X-100, fractions were eluted sequentially with 100, 200, 500, and 1,000 mM KCl in lysis buffer without Triton X-100. The p50-containing fraction (the 100 mM KCl wash) was then separated by PAGE and blotted onto a nitrocellulose membrane. The membrane was stained with Ponceau S, and the p50 spot or band, as well as several surrounding bands, were individually excised. Approximately 5% of each was used for scintillation counting to confirm the identity of [3H]-labeled p50, and the remainder was subjected to tryptic digestion (see below).

Enrichment and recovery values at each purification step were determined by assessing protein content with the Bradford assay (26) and radioactivity with trichloroacetic acid precipitation. Although a large percentage of the [3H]mimosine label in the initial cell lysate is cross-linked to proteins other than p50 (19, 20), the majority is associated exclusively with p50 after the 8,000 rpm centrifugation step (see Fig. 2).

Peptide Sequencing—The stained p50 band (1–2 μg of protein) was excised from the nitrocellulose sheet and digested in situ with trypsin, using the procedure of Aebersold et al. (27). The trypsin was removed from the proteins by washing in 200 μl NaOH, followed by several washes with water. The nitrocellulose fragment was then incubated in 0.5% polyvinylpyrrolidone (Sigma) in 0.6% acetic acid for 30 min at 37 °C; excess polyvinylpyrrolidone was removed by extensive rinsing with water, and the fragment was finally submerged in 100 mM ammonium acetate, pH 8.0, containing 0.1 μg of sequencing grade modified trypsin (Promega Biotech; just enough liquid to cover the sample). Digestion was carried out overnight at 37 °C. The digestion solution was removed, the nitrocellulose fragment was rinsed twice with 100 μl of digestion buffer, and all solutions were pooled. The digest was then acidified with acetic acid (1% final concentration), and the volume was reduced to ≈40 μl in a Speed Vac. Aliquots of the digest were analyzed on an Applied Biosystems 140B Solvent Delivery System (Foster City, CA) (21). Protein data base searches were submitted to the National Center for Biotechnology Information of the National Institutes of Health (Bethesda, MD), using the BlastP program (28).

Immunoprecipitation of p50 from Whole Cell Lysates—Ten microliter of polyclonal antiserum raised against rabbit [4H]mimosine (Amersham, custom labeling) while still in exponential growth was added to the beads and incubated at 37 °C for 1 h with periodic agitation. After washing the beads extensively with phosphate-buffed saline, 7 μl of [4H]mimosine-labeled cell lysate (≈20 μg of protein) were added to the beads and incubated at 37 °C for 3 h. The beads were collected three times with phosphate-buffered saline, boiled in PAGE sample buffer, and pelleted in a microcentrifuge. The supernatants were subjected to one-dimensional SDS-PAGE.

Schiff Base Reduction—Aliquots of cell lysate (80 μl; ≈100 μg of protein) were incubated with different concentrations of sodium borohydride for 10 min at 37 °C (see figure legend). As a control, [4H]mimosine was pretreated with 1 mM sodium borohydride at 37 °C for 30 min and was added to the lysate and cross-linked as described above. Separate aliquots of lysate were treated identically, substituting NaOH, dithiothreitol, or β-mercaptoethanol for sodium borohydride, to
control for any possible effects of pH and reducing equivalents on the NaBH₄-treated extract.

SHMT Enzyme Assays—CHO cell lysates were prepared exactly as described for cross-linking studies, except that 60 mM potassium phosphate buffer (pH 7.5) was substituted for Hepes buffer in the cell washing and lysis steps. Triton X-100 was removed by extraction with isoamyl alcohol and protein concentrations were measured with the Bradford assay (26). The assay for SHMT enzyme activity measures the transfer rate of a [³⁵S]methylenegroup from serine to tetrahydrofolate, and was performed according to the method of Snell (29), with the following modifications: 1) solid L-tetrahydrofolic acid (Sigma) was added just prior to assay to minimize oxidation; 2) [³⁵S]serine was present at 20 mM and 20 μCi/ml final concentration; and 3) mercaptoethanol was replaced with dithiothreitol. Data were plotted using SigmaPlot (version 4.1), and the means of duplicate determinations is shown. Error bars indicate the range of values of two determinations in each case.

RESULTS

Conservation of p50 in Mammalian Cells—In previous studies on CHO-K1 cells, we showed that [³H]mimosine can be photo-cross-linked to a 50-kDa polypeptide (p50) (19, 20). However, the 50-kDa mimosine binding activity was virtually undetectable in CHO-K1 cells that had been selected for resistance to 1 mM mimosine (10 times the lethal dose; Refs. 19 and 20). This result strongly suggested that p50 is a biologically relevant target for mimosine. Since mimosine also inhibits DNA synthesis in cell lines derived from human, monkey, and murine sources (9, 10) it would therefore be predicted that each of these cell lines should also exhibit [³H]mimosine binding activity.

To address this question, extracts from eight different cell lines of human, monkey, mouse, and hamster origin were incubated with [³H]mimosine and illuminated with a xenon lamp. The extracts were separated on a polyacrylamide gel, and the corresponding radioactive products were detected by fluorography. The Coomassie Brilliant Blue-stained gel is shown in Fig. 1A and the fluorogram in Fig. 1B. Seven of the eight cell lines contain a prominent [³H]mimosine-binding species that migrates at ~50 kDa. Although the CV-1 extract displays only a faint band in this experiment, p50 mimosine binding activity has been observed in other preparations. Also note that monkey COS cells, which were derived from CV-1 cells, display a prominent radioactive band at ~50 kDa. Thus, a mimosine binding activity appears to be conserved among four different mammalian species. Furthermore, although some minor differences in molecular weight are detected (e.g. compare human 293 and monkey COS cell extracts, Fig. 1B), the binding proteins all migrate in the size range 50–53 kDa.

Additional, less prominent ³H-labeled bands are also detected, as well as significant amounts of labeled high molecular weight material at the top of the gel. Since the spectrum and intensity of the faint bands are not reproducible from experiment to experiment, and since additional experiments indicated that they are probably not relevant (see below), we have not studied any of these bands further. The high molecular weight material is not detected in non-irradiated extracts (19), and we therefore assume that it represents aggregates of p50, either with itself or with other polypeptides. We have also examined extracts prepared from Saccharomyces cerevisiae and Escherichia coli, but did not reproducibly observe specific [³H]mimosine binding activity at any position in the gels. This is consistent with the observation that mimosine does not inhibit DNA replication in either of these species at concentrations as high as 1 mM.

Purification of p50 from CHO-K1 Cells—The observation that p50 is present in all of the cell lines that are sensitive to mimosine inhibition further argues that p50 represents a biologically important target for drug action. We therefore developed a fractionation scheme for obtaining p50 of sufficient purity to sequence by tandem mass spectrometry (21).

Exponentially growing CHO-K1 cells in tissue culture dishes were harvested, and a soluble extract was prepared by lysing with Triton X-100 and removing nuclei by centrifugation. The resulting extract was cross-linked with [³H]mimosine and aggregated material was removed by low speed centrifugation. The bulk of the p50 was then precipitated in a 40% saturated ammonium sulfate solution, and the resulting pellet was redissolved and fractionated by size on Sepharose CL-6B. The p50-enriched fractions were pooled and subjected to chromatography on DE52, and those fractions containing the [³H]mimosine binding activity were finally subjected to preparative polyacrylamide gel electrophoresis.

The approximate degrees of purification and recovery at each step in a typical experiment are outlined in Table I, and the progress of the purification is shown in Fig. 2. Note that equal amounts of protein were loaded into each well of the polyacrylamide gel in this experiment. As can be seen in the stained gel in Fig. 2A and the corresponding fluorogram in Fig. 2B, DE52 chromatography and polyacrylamide gel electrophoresis represented the most effective purification steps. The arrow in Fig. 2A indicates the stained band that co-migrates with the [³H]mimosine-binding band in Fig. 2B. This stained band must represent several 50-kDa polypeptide species in the early stages of the purification, since its intensity relative to total protein does not markedly increase in the first two steps, while the [³H]mimosine binding activity at the corresponding posi-
TABLE I

| Purification scheme | Purification steps | Fold of purification | Recovery |
|---------------------|-------------------|----------------------|----------|
| Starting            | 1 x               | 100                  |
| 40% (NH₄)₂SO₄/PPT   | 2 x               | 80                   |
| Sepharose CL-6B     | 4 x               | 64                   |
| DE52                | 15 x              | 57                   |
| SDS-PAGE            | 50 x              | 57                   |
| Total               | 6000 x            | 57                   |

Fig. 2. Progress of the purification of p50. Extract from ~6 × 10⁸ CHO-K1 cells was mixed with [³H]mimosine, irradiated, centrifuged to remove aggregated material, and adjusted to 40% saturated ammonium sulfate. The resulting pellet was resuspended and fractionated on Sepharose CL-6B, followed by DE52. Approximately equal amounts of protein (~20 μg) from the pooled fractions resulting from each step were separated on a 10% denaturing polyacrylamide gel and stained with Coomassie Brilliant Blue. The polypeptides were then subjected to fluorography as described in the legend to Fig. 1. Panel A, stained gel. Panel B, fluorogram.

Fig. 3. Six tryptic peptides from CHO p50 are highly homologous to rabbit mSHMT. A tryptic digest of p50 was separated by high performance liquid chromatography and subjected to analysis by the tandem mass spectrometer. The sequence of six different peptides (54 residues total) were compared to sequences of cSHMT and mSHMT from humans and rabbits (GenBank). Note that the quadrupole instrument cannot discriminate between leucine and isoleucine, resulting in ambiguity at these residues; in the CHO peptides shown, we have used the residues that are common to four other mammalian SHMT sequences. The period denotes probable identity with the lysine or arginine in the amino-terminal positions of the other comparison peptides, although these were not actually sequenced in the hamster peptide.

Antibody against Rabbit CSMT Precipitates p50—Therefore, the predominant polypeptide migrating at the position of the major [³H]mimosine-binding band appears to be mSHMT. However, the progress of the purification regimen suggested that there could be additional polypeptides migrating at this position, since the [³H]mimosine binding activity increased at each step (Fig. 2B), while the intensity of the protein staining at the p50 position relative to other bands did not dramatically change (Fig. 2A). Because we have not been able to sequence the actual peptide that is cross-linked to [³H]mimosine, it was important to determine whether mSHMT (which constitutes the majority of the protein at the 50 kDa position in the acrylamide gel based on sequence analysis) actually represents the mimosine-binding species.

The primary sequences of both cSHMT and mSHMT are highly conserved among mammals (30). We therefore asked whether a polyclonal antibody against rabbit cSHMT would cross-react with the mimosine-binding p50 polypeptide. An aliquot of the starting cell lysate was cross-linked to radiolabeled mimosine and was then incubated with Protein A beads, either alone or coupled to the polyclonal antibody preparation. As shown in Fig. 4B, the majority of [³H]-labeled p50 partitioned with the antibody-coated Protein A beads (compare +Ab pellet to +Ab supe), while no detectable [³H]-labeled p50 partitioned with control beads (−Ab pellet).

A gly¢ CHO-K1 Cell Line Has Lost the Majority of the p50 Mimosine Binding Activity—By the criteria of primary sequence and cross-reactivity with an antibody to rabbit cSHMT, the major mimosine binding activity in CHO cells appears to correspond to mSHMT. In normal mammalian cells, SHMT
transfers a methyl group from serine to tetrahydrofolate to form $N_5, N_{10}$-methylenetetrahydrofolate and glycine (Fig. 5). The methyl group is then donated to dUMP to form thymidine in a reaction catalyzed by thymidylate synthase. Thus, SHMT is involved in both glycine and thymidine biosynthesis. In CHO cells, the predominant form of SHMT is the mitochondrial species (31, 32). In rabbits, rats, monkeys, and humans, the cytosolic form predominates (31, 33).

In an independent test of whether mSHMT represents the major mimosine binding activity, we analyzed the effects of mimosine on a variant CHO cell line (CHO/51-11) that lacks mSHMT activity, probably as the result of a point mutation (32, 34). This cell line was isolated as a glycine-requiring mutant, and was subsequently shown to have greatly reduced levels of mSHMT enzyme activity but near-normal levels of cSHMT activity (32).

As shown in Fig. 6, extracts prepared from the gly$^-$ CHO/51-11 mutant lack the predominant 50-kDa $[^3]$H]mimosine binding activity that characterizes wild-type CHO-K1 cells. Furthermore, p50 could not be detected in extracts of CHO/51-11 cells regardless of whether they were propagated in minimal essential medium supplemented with non-essential amino acids (our standard culture medium) or in F12 (the medium in which this cell line is usually maintained; Ref. 32). Thus, a single mutation has affected both catalysis and mimosine binding to mSHMT in the CHO/51-11 cell line, suggesting that mimosine may bind directly to the active site.

The Binding of Mimosine to p50 May Involve Schiff Base Formation at or Near the Active Site—To further investigate the possibility that mimosine may inhibit DNA replication by binding to the active site of mSHMT, we studied the competition between mimosine and pyridoxal phosphate for binding to the wild-type enzyme. Pyridoxal phosphate is required for cSHMT and mSHMT activity both in vivo and in vitro (33), and has been shown to interact with a lysine residue in the active site through the formation of a Schiff base (35). In initial studies, we were able to show that, in fact, 100 μM pyridoxal phosphate does prevent mimosine binding to wild-type mSHMT. However, mimosine itself also binds to pyridoxal phosphate through a Schiff base (36, 37). Therefore, this exper-

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8 V. Schirch, personal communication.
Mimosine Binds to SHMT

Fig. 7. Reduction of Schiff bases in CHO-K1 extracts abolishes the cross-linking of mimosine (Mimo) to p50. Mimosine was pre-treated with sodium borohydride as described under "Materials and Methods" and was then incubated with extracts that had been treated with the indicated concentrations of sodium borohydride, dithiothreitol (DTT), or β-mercaptoethanol (BME) as described under "Materials and Methods." After irradiation, the extracts were separated on a 10% denaturing polyacrylamide gel. Panel A, stained gel. Panel B, fluorograph.

The reduction of Schiff bases in CHO-K1 extracts abolishes the cross-linking of mimosine (Mimo) to p50. Mimosine was pre-treated with sodium borohydride as described under "Materials and Methods" and was then incubated with extracts that had been treated with the indicated concentrations of sodium borohydride, dithiothreitol (DTT), or β-mercaptoethanol (BME) as described under "Materials and Methods." After irradiation, the extracts were separated on a 10% denaturing polyacrylamide gel. Panel A, stained gel. Panel B, fluorograph.

The basis for mimosine action has been studied for years, ever since it was shown to be the agent responsible for hair loss...
in the cattle feed, *Leucaena pudica*. We became interested in this compound as a potential synchronizing agent when it was reported to arrest cells in the late $G_2$ period (9, 10). However, we have shown that mimosine is actually an extremely effective inhibitor of DNA replication (11, 12, 14). In fact, several of its properties suggested that mimosine might act during initiation at origins, either at the strand separation stage or when the replication machinery is loaded into the melted region to form the replication forks (11, 12, 14).

Mimosine is also known to chelate iron. Thus, it has been suggested that mimosine inhibits DNA replication by depriving ribonucleotide reductase of required iron (16, 17). Three lines of evidence were advanced to support this proposal: 1) mimosine reduces deoxynucleotide pool levels and inhibits DNA replication, both of which effects can be reversed with added iron (17, 19); 2) mimosine has no effect on DNA replication in frog embryos or in vitro, in which deoxyribonucleotide precursors are present in excess (18); and 3) mimosine inhibits ribonucleotide reductase activity in vitro, and inhibition can be overcome by the addition of excess iron (17).

However, we have argued that these experiments are not conclusive, since the inhibition of many different metal-requiring enzymes could ultimately result in imbalanced deoxyribonucleotide pools through secondary effects (19); furthermore, excess iron could inactivate mimosine itself instead of resupplying iron to an iron-deprived ribonucleotide reductase (19). We have also been able to demonstrate that cell lysates prepared from mimosine-treated cells have a reduced capacity for supporting SV40 replication in vitro. In addition, CHO cell lines selected for resistance to 300 $\mu$M mimosine are still sensitive to the iron chelator, deferoxamine, at a concentration that chelates the same amount of iron (100 $\mu$M; 38, 40). Finally, mimosine is much more efficacious and has markedly different properties than does hydroxyurea, which inhibits DNA replication by inactivating ribonucleotide reductase directly (7, 15); in particular, dATP and dGTP pool levels are maximally reduced only after a 4-h exposure to mimosine (16), whereas hydroxyurea requires only a few minutes to maximally affect deoxynucleotide pool levels (7).

It has also been suggested that mimosine may affect histone kinase activity (41), although, again, it is difficult to separate primary from downstream effects on this activity.

We have taken a direct approach to identifying possible intracellular targets for mimosine. A photochemical cross-linking strategy allowed us to detect a 50-kDa $[^3H]$mimosine-binding species in CHO cell extracts (19, 20). Importantly, this 50-kDa mimosine-binding activity appears to be absent in CHO cells made resistant to 1 mM mimosine, strongly arguing that p50 is a biologically relevant target of mimosine (19, 20). We also show in this report that the 50-kDa mimosine-binding activity is present in all of the wild-type mammalian cell lines examined (Fig. 1); therefore, p50 and its ability to bind to mimosine have been conserved in mammals.

In the present report, we also describe a simple fractionation scheme in which extracts from a modest amount of cells ($\sim 6 \times 10^8$) yields a band on a polyacrylamide gel of sufficient purity and quantity ($\sim 20$ pmol) to analyze by tandem mass spectrometric analysis. Six tryptic peptides from this fraction were sequenced, all of which show a high degree of similarity with rabbit mitochondrial SHMT: four of the six are identical to rabbit mSHMT and are nearly identical to human mSHMT (Fig. 3). Although we have not yet been able to purify the mimosine-binding peptide itself for sequence analysis, $[^3H]$mimosine-labeled p50 was quantitatively precipitated by an antibody to rabbit cSHMT (Fig. 4). Furthermore, when a gly CHO cell line that lacks mSHMT activity (32) was analyzed for the presence of the mimosine binding activity, the major radioactive band at 50 kDa was greatly reduced (Fig. 6).

Several additional lines of evidence suggest that p50 corresponds to SHMT. For example, all known eukaryotic SHMT enzymes (whether mitochondrial or cytosolic) migrate between 50 and 53 kDa on denaturing SDS-polyacrylamide gels and, like p50 (20), most have isoelectric points near 7.0 (20, 42). Not surprisingly, each of these enzymes is purified by separation methods very similar to those employed here (33, 43–45). Furthermore, $[^3H]$mimosine-labeled p50 elutes from a nondenaturing gel filtration column as a multimer with an estimated size of 200–250 kDa, as does purified rabbit mSHMT (33, 43–45). Thus, there is little doubt that mimosine-labeled p50 corresponds to SHMT.

The two forms of SHMT (cytosolic and mitochondrial) from several mammalian species are readily distinguishable by kinetic parameters and by heat lability (32). They have been shown to co-purify through biochemical separation techniques very similar to those employed in the present study (e.g. Ref. 31), and the two forms in rabbit co-migrate at 53 kDa on one-dimensional SDS-PAGE gels (31). The amino acid sequences of each form are highly conserved across species, but within a species the two forms are more divergent. For example, 97% of the residues are identical in rabbit and human mSHMT (30), while human cSHMT and mSHMT are –63% similar to one another (30).

The question whether mimosine inhibits the cytosolic or the mitochondrial form of SHMT, or both, is a difficult one to answer. Although the proteins have distinguishing turnover numbers and heat sensitivities (31), the mitochondrial form is in such great excess over the cytosolic form in the Chinese hamster (32) that we were not able to study cSHMT in isolation. It is therefore not surprising that mSHMT is the form from which we obtained sequence information, regardless of whether one or both forms bind to mimosine. Furthermore, the polyclonal antibodies prepared against rabbit cSHMT and used here to precipitate the $[^3H]$mimosine-p50 complex recognize both forms in the rabbit approximately equally.

The gly CHO cell line, which lacks mSHMT activity but retains cSHMT activity (32), displays only a very faint 50-kDa mimosine-binding species in autoradiograms that may represent cSHMT (Fig. 6). Since this cell line retains sensitivity to mimosine, it is probable that both forms are inhibited by the enzyme. This suggestion is consistent with the presence of a prominent $[^3H]$mimosine-labeled 50-kDa polypeptide in monkey and rabbit cells, in which cSHMT is the predominant enzyme activity (43).

It is paradoxical, therefore, that mimosine had no obvious effect on SHMT activity when added directly to CHO cell extracts (mostly mSHMT) or to purified cSHMT isolated from rabbits. One possible explanation is that mimosine binding activity (but not SHMT enzyme activity) is quite labile, especially at low temperature: when the CHO lysate was incubated with $[^3H]$mimosine on ice for as little as 8 min prior to photo-cross-linking, the bulk of the mimosine binding activity was lost. Obviously, the rabbit cSHMT enzyme preparation encountered these conditions during purification. The CHO cell extracts were not subjected to this treatment, and still retain both enzyme and mimosine binding activity. However, cell extracts prepared for enzyme activity measurements are 10-20-fold more dilute than those used in cross-linking studies, and we have observed that the more dilute the extracts are, the...
more difficult it is to obtain reproducible cross-linking. In addition, the assay is performed in the presence of 12 mM dithiothreitol to maintain the tetrahydrofolate in a reduced state; this high concentration of reducing agent could affect the mimose-mSHMT interaction significantly (although 1 mM dithiothreitol had only a marginal effect; Fig. 7). It is also possible that the high levels of tetrahydrofolate utilized in this assay could affect the binding of mimose to SHMT.

The observation that the gly−CHO cell line that lacks mSHMT activity has also lost most of its mimose binding ability suggests that mimose either binds to the active site of the enzyme or to a site critical for enzyme function in vivo. In addition, when the Schiff base that binds pyridoxal phosphate to SHMT was reduced, cross-linking to mimose was specifically prevented (Fig. 7). This result suggests that mimose binds to SHMT either through a free Schiff base or a free pyridoxal phosphate in or near the active site.

Many enzymes, particularly those involved in amino acid metabolism, require pyridoxal phosphate as a cofactor for activity (46). It is interesting to note that an antibody against pyridoxal phosphate detects low levels of many different proteins in both normal and tumor cell extracts, but the signal from a 50-kDa polypeptide with a pI of ~7.0 is greatly amplified in tumor cells (38). Similarly, a large spectrum of faint [3H]mimosine binding bands is detected in extracts from normal rat tissues, while in extracts from cell lines, all of which are transformed to some degree, the 50-kDa species predominates (Figs. 1 and 6). If SHMT is, indeed, overexpressed in transformed cell lines, and if its overexpression is required to maintain the tumorigenic state, SHMT could represent an important target for chemotherapeutic protocols.

The actual effects of mimose on cycling mammalian cells are undoubtedly complex and almost certainly depend upon drug concentration. We believe that the primary in vivo effect at the minimal effective concentration (50–100 μM for CHO cells) is probably to inhibit DNA replication by interacting with SHMT in an as yet undefined way. This raises the question why mimose affects only dATP and dGTP pools without affecting dTTP pools (16), since methylenetetrahydrofolate is directly involved in the thymidylate synthase reaction. As discussed below, all of our cross-linking studies were performed at 100 μM mimosine; in contrast, the deoxynucleotide pools measurements were performed in the presence of 400 μM (16), which may, indeed, be high enough to inhibit ribonucleotide reductase. In this regard, it is interesting to note that hydroxyurea would be expected to lower pool levels of all four deoxyribonucleotides; in fact, hydroxyurea lowers only dATP and dGTP pools, while dCTP and dTTP are actually increased (7).

Since we have not yet been able to demonstrate a direct effect on enzyme activity in vitro, the interesting possibility arises that mimose might disrupt an allosteric interaction within a proposed multienzyme replication complex (47). This could lead to an inhibition of replication fork formation when added to cells entering the S period, as well as to the loss of existing forks when added to S phase cells, both of which we have previously observed (12, 14). An alternative and intriguing possibility is that mimose affects the function of a proposed purine-synthesizing multienzyme complex, for which there is both in vitro and genetic evidence in various eukaryotic systems (see Ref. 23 for review).

This would explain why dATP and dGTP pools are selectively affected by mimose, but only after exposure to mimose for at least 4 h (16). Inhibition of complex formation (or slow disruption of the complex) could also explain why it takes 4 h for mimose to reduce purine deoxyribonucleotide pool levels to zero, while hydroxyurea exerts its effects within minutes (7). It would have to be additionally supposed that a subset of these complexes are dedicated to providing (compartmentalizing) precursors for DNA synthesis, however, since we do not observe a significant effect of mimose on RNA synthesis for at least 4 h after mimose addition to cultured cells.

At mimose concentrations below 100 μM, some chelation of metal ions (e.g. iron and copper) may occur, but chelation is probably not the critical factor preventing DNA replication at low concentrations by the following argument. Although we were able to isolate cell lines that were marginally resistant to 1 mM mimose (presumably because of a point mutation that destroys binding ability), no resistant variants could be isolated at higher drug concentrations. This result would be expected if low levels of mimose inhibit SHMT and can be overridden by a point mutation that affects binding affinity, but higher levels act as a general chelating agent. In fact, in the cell line selected for resistance to 1 mM mimose, labeled p50 was almost undetectable in extracts exposed to 100 μM [3H]mimosine; while DNA replication could still be inhibited by 100 μM deferoxamine, a specific iron chelator (48). Furthermore, in wild-type cells, the effect of 100 μM mimose on [3H]thymidine incorporation into DNA could be completely overridden with the substrate, serine, or the end product, glycine, both of which are capable of forming a Schiff base with SHMT, while inhibition by 100 μM deferoxamine was not affected by these amino acids. However, at concentrations above 100 μM mimose, glycine or serine could not restore DNA replication to control values. These findings may indicate that mimose does, in fact, inhibit ribonucleotide reductase and/or other enzymes by metal chelation, and could explain why others have observed changes in deoxynucleotide pools in CHO cells treated with 400 μM mimose (16).

Our initial interest in mimose stemmed from its utility as an efficacious and economical synchronizing agent which, when added to cells approaching the S period, seems to prevent the formation of replication forks at early-firing origins (12, 14). However, we believe that mimose also deserves attention as a potential anti-cancer agent. Not only is the drug capable of completely preventing replication fork progression for extended intervals (11), but we were unable to isolate cell lines displaying more than 5-10-fold higher resistance to mimose than the starting cell line, and then only after a selection regimen lasting more than 18 months (20). Although there are a few older reports describing mimose’s effects on tumors in rat models (49, 50), perhaps this interesting drug should be reevaluated in light of its possible effects on SHMT.

It is interesting to note that inhibitors of dihydrofolate reductase (methotrexate) and thymidylate synthase (5-fluorodeoxyuridine) have been available for years and are used routinely in chemotherapeutic mixtures. However, no compounds other than mimose have been shown to target SHMT, which is the third enzyme in the deoxymethyldihydrofolate synthetic pathway (see Fig. 5). Although we do not understand the mechanism by which mimose disrupts the function of SHMT in vivo, it will be important to investigate its potential as a chemotherapeutic agent for cancer, alone or in combination with methotrexate and/or 5-fluorouracil.

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