Sphingosine Inhibits the Synthesis of RNA Primers by Primase *in Vitro*†

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Received December 6, 1993; Revised Manuscript Received May 12, 1994®

ABSTRACT: We have previously shown the presence of sphingomyelin and sphingomyelinase in cell nuclei, suggesting that they may play a role in the intranuclear production of sphingosine, a potent bioactive molecule modulating diverse cellular functions. In the present study, the direct effects of sphingosine ($C_{18:1}$) on the activity of DNA replication/repair polymerases were studied *in vitro*. Sphingosine had no effect on DNA polymerases α and β and slightly inhibited DNA polymerases γ, δ, and ϵ. In contrast, sphingosine strongly inhibited the activity of primase in a dose-dependent manner. On the other hand, dihydrosphingosine ($C_{18:0}$), glycolipids, sphingomyelin, and ceramide had no effect on primase activity. Sphingosine equally inhibited the activity of primase complexed with DNA polymerase α, as well as its free form, with a K_i value of 4 μM. A gel-retardation analysis showed that the binding of primase with ^{32}P-labeled template DNA was suppressed by sphingosine. Inhibition by sphingosine was competitive with the DNA template, but not with the substrate NTPs. After product analysis, a dose-dependent decrease in the amount of RNA primer products, consisting mainly of 10- and 11-mers, was observed in the presence of sphingosine, indicating that it inhibits the synthesis of RNA primers by primase. Sphingosine, however, had no effect on T7 RNA polymerase.

Sphingosine, a biologically active metabolite of membrane sphingolipids (Wilson et al., 1988), has been implicated as an endogenous modulator of diverse cellular functions [reviewed in Hannun et al. (1986) and Merrill and Steven (1989)]. When added exogenously, it has been shown to effect significant cellular responses, such as the inhibition of platelet and neutrophil responses and phorbol ester induced responses, as well as the modulation of growth factor action and receptor functions (Hannun et al., 1986; Malinow et al., 1988). It also suppresses the action of tumor promoters in various mammalian cells (Hannun et al., 1986; Merrill & Steven, 1989) and causes profound growth inhibition and cytotoxicity in some cell lines (Merrill et al., 1983) at high concentrations. These effects have been attributed to the fact that sphingosine is a specific, potent, and reversible inhibitor of protein kinase C (Hannun, 1986; Merrill et al., 1986; Malinow et al., 1988; Hannun & Bell, 1987; Nishizuka, 1988), thus acting as a negative modulator of signal transduction.

On the other hand, it was reported that, at low concentrations, sphingosine does not suppress cell growth and viability (Faucher et al., 1988; Davis et al., 1988) and in fact exhibits mitogenic effects in Swiss 3T3 cells in a protein kinase C independent manner, by increasing levels of phosphatidic acid (Zhang et al., 1990a,b) and sphingosine 1-phosphate (Zhang et al., 1991), both potent mitogens. Sphingosine likewise increases the affinity and activity of the EGF receptor, indicating that sphingosine may have pleiotrophic effects on cells (Faucher et al., 1988). These results, as well as recent reports that sphingosine also inhibits cell growth by specifically and potently inducing early dephosphorylation of the Rb protein (Chao et al., 1992), a tumor suppressor implicated in the regulation of progression through the cell cycle, raises the possibility that there may actually be other targets of sphingosine action aside from protein kinase C.

Endogenous free sphingosine mainly results from the hydrolysis of sphingomyelin and glycolipids (Wilson et al., 1988; Merrill et al., 1986). Although predominantly in the plasma membrane, the presence of sphingomyelin has also been reported in the nucleus and subnuclear fractions tightly associated with the chromatin fraction (Spangler et al., 1975) and composes one of the two major phospholipids in the nuclear matrix (Cocco et al., 1980). We have further demonstrated the presence of a neutral sphingomyelinase, which specifically hydrolyzes sphingomyelin into ceramide and phosphocholine, in the nuclear matrix of rat ascites hepatoma AH7974 cells (Tamiya-Koizumi et al., 1989). It was suggested that this nuclear sphingomyelinase may play some role in the regulation of nuclear function, probably in the intranuclear production of sphingosine from sphingomyelin, in cooperation with ceramidase. This would indicate that sphingosine may have an intranuclear target; thus, we examined the direct effects of sphingosine on the different DNA replication/repair polymerases, such as DNA polymerases α, β, γ, δ, and ϵ, as well as on primase associated with DNA polymerase α. The present study reports for the first time that primase may be an intranuclear target of sphingosine action, since sphingosine was found to be a specific, potent inhibitor of RNA primer synthesis by primase *in vitro*.

MATERIALS AND METHODS

Enzymes and Sphingolipids. Primase complexed with DNA polymerase α and free primase were purified from calf thymus by immunoaffinity column chromatography using an immunoaffinity Sepharose 4B column conjugated with mono-

† This work was supported in part by the Grant-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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® Abstract published in *Advance ACS Abstracts,* July 1, 1994.

[1] Abbreviation: SDS, sodium dodecyl sulfate.

0006-2960/94/0433-9007$04.50/0

clonal antibody directed against calf thymus DNA polymerase α, as described (Tamai et al., 1988), with some modifications. Successive elutions were performed with 3 M NaCl, 50% ethylene glycol, and 3.2 M $MgCl_2$ in base buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM 2-mercaptoethanol, and 10% glycerol. Free primase (two subunits: 47 and 52 kDa, 30 000 units/mg of protein) was recovered in the NaCl fractions (Suzuki et al., 1993a), while primase complexed with DNA polymerase α (four subunits: 140, 70, 52, and 47 kDa, 40000–60000 units/mg of protein) was recovered in ethylene glycol and $MgCl_2$ fractions. Calf thymus DNA polymerase β (Yoshida et al., 1979; 20 000 units/mg of protein) and DNA polymerase γ from bovine liver (Izuta et al., 1991; 15 000 units/mg of protein) were purified as reported previously, while DNA polymerases δ (3000 units/mg of protein) and ϵ (5000 units/mg of protein) were purified from low-salt extracts of calf thymus by successive column chromatography using DEAE cellulose, hydroxylapatite, Mono Q, and Mono S (will appear elsewhere). One unit of enzyme activity is defined as the amount that catalyzes the incorporation of 1 nmol of deoxynucleoside triphosphates into an acid-insoluble fraction in 60 min under the reaction conditions for each enzyme. DNA polymerase I (Klenow fragment) and T7 RNA polymerase were purchased from Takara (Kyoto, Japan) and Boehringer Mannheim, respectively. Sphingosine, dihydrosphingosine, sphingomyelin, ceramide, and sphingosylphosphocholine were purchased from Sigma, while the glycolipids GM1, GM3, SPG, and GD1a were purified as described (Taki et al., 1988).

Primase and DNA Polymerase Assays. Primase assays were performed as described (Yoshida et al., 1983), with the standard reaction mixture (25 μL) containing 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 1.6 mM GTP, 20 μM dGTP, 7.4 kBq of [^{3}H]dGTP, 2 mM $MgCl_2$, 1 μg of bovine serum albumin (BSA), 0.1 μg of poly(dC), 0.2 unit of *Escherichia coli* DNA polymerase I, and 0.05 unit of calf thymus DNA polymerase α–primase complex or free primase. After incubation at 37 °C for 60 min, the acid-insoluble radioactivity was measured. *E. coli* DNA polymerase I (Klenow fragment) was measured using the same reaction mixture as that used with DNA polymerase α using activated calf thymus DNA (Yoshida et al., 1981), while DNA polymerases β (Yoshida et al., 1979), δ (Syvaoja et al., 1990), and ϵ (Syvaoja & Linn, 1989) were assayed with poly(dA)/oligo(dT) under their optimal reaction conditions. DNA polymerase γ was measured with poly(A)/oligo(dT) (Izuta et al., 1991). T7 RNA polymerase activity was measured in a standard transcription assay according to the manufacturer's specifications.

Appropriate amounts of sphingosine, dihydrosphingosine, ceramide, sphingomyelin, and various glycolipids were suspended in water to make 1 mM stock solutions, which were then vortexed, heated at 60 °C, and sonicated for 20 s using a Kontes Sonifier (Vineland, USA). Sonicated samples were then added to primase (0.05 unit) or to other DNA polymerases at 0 °C, after which 20 μL of their appropriate reaction mixtures were added, and enzyme reactions were then performed as described above.

Product Analysis. The standard assay for RNA primer synthesis consisted of 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM ATP ([α-^{32}P]ATP, 37 kBq), 0.1 μg of poly(dT), and 0.05 unit of calf thymus DNA polymerase α–primase complex in a final volume of 25 μL, as previously described (Suzuki et al., 1993a). After incubation at 37 °C for 60 min, 1 μg of calf thymus DNA was added as carrier, followed by ethanol precipitation. The reaction products were then separated by denaturing gel electrophoresis at 2000 V for 4 h in a 20% polyacrylamide gel containing 8 M urea and then visualized and quantified using a Hamamatsu DVS 3000 image analyzer system.

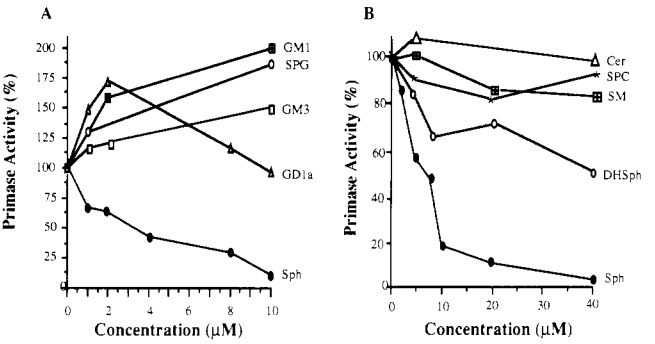

FIGURE 1: Sphingosine specifically and dose dependently inhibits primase activity *in vitro*. (A) Sonicated samples of sphingosine (Sph, ●) and complex sphingolipids such as the gangliosides GM1 (■), SPG (○), GM3 (□), and GD1a (△) were added in the indicated amounts to immunoaffinity-purified DNA polymerase α–primase complex (0.05 unit), followed by the addition of the primase reaction mixture. Incubation was then carried out for 60 min at 37 °C, and primase activity was measured as described in Materials and Methods. (B) Same as in A except that the indicated amounts of sphingosine (●), dihydrosphingosine (DHSp, ○), sphingomyelin (SM, ■), ceramide (Cer, △), or sphingosylphosphocholine (SPC, *) were added to the enzyme. dNMP incorporation in the coupled primase/DNA polymerase I assay (see Materials and Methods), in the absence of sphingosine or any sphingolipids, was taken as 100%, corresponding to 200 pmol/h, and the primase activity (%) was based on this control value. Each point is the mean of duplicate determinations, with the same results obtained in two independent experiments.

Gel-Retardation Assay. A DNA probe, CTPPS 1, was labeled as described previously (Suzuki et al., 1993b). One nanogram of ^{32}P-labeled CTPPS1 and 0.1 μg of DNA polymerase α–primase were incubated on ice for 30 min in 50 mM Tris-HCl (pH 8.0) and 1 mM dithiothreitol. Glutaraldehyde was then added in a final concentration of 0.05% and incubated at 37 °C for 5 min. The mixture was further incubated with 0.2% SDS at 90 °C for 1 min, and the [^{32}P]-DNA–protein complex was analyzed by electrophoresis on a 10% polyacrylamide gel containing SDS. Various amounts of sphingosine or 100 ng of DNA competitors was added at the same time as the ^{32}P-labeled CTPPS1 DNA probe.

RESULTS

Specific and Dose-Dependent Inhibition of Primase Activity by Sphingosine. We have previously found that the sialic acid-containing glycolipids, such as SPG, GM1, GM3, and GD1a, strongly inhibit DNA polymerase α activity, while sphingosine (Sph), their backbone moiety, had no effect on DNA polymerase α activity at all (Simbulan et al., 1994). On the other hand, the activity of primase associated with DNA polymerase α was specifically and dose dependently inhibited only by sphingosine and not by the other glycolipids (Figure 1A). Inhibition by sphingosine was dose dependent, with 50% inhibition observed at a dose of 4 μM (Figure 1A,B). The extent of inhibition was the same with or without preincubation of primase and sphingosine (data not shown). In contrast, its saturated form, dihydrosphingosine (sphinganine, DHSp), which differs from sphingosine only in its lack of a trans double bond, was not as effective, thus confirming the structural specificity of inhibition by sphingosine (Figure 1B). Furthermore, the two main sources of endogenous sphingosine, sphingomyelin and ceramide, also did not affect primase

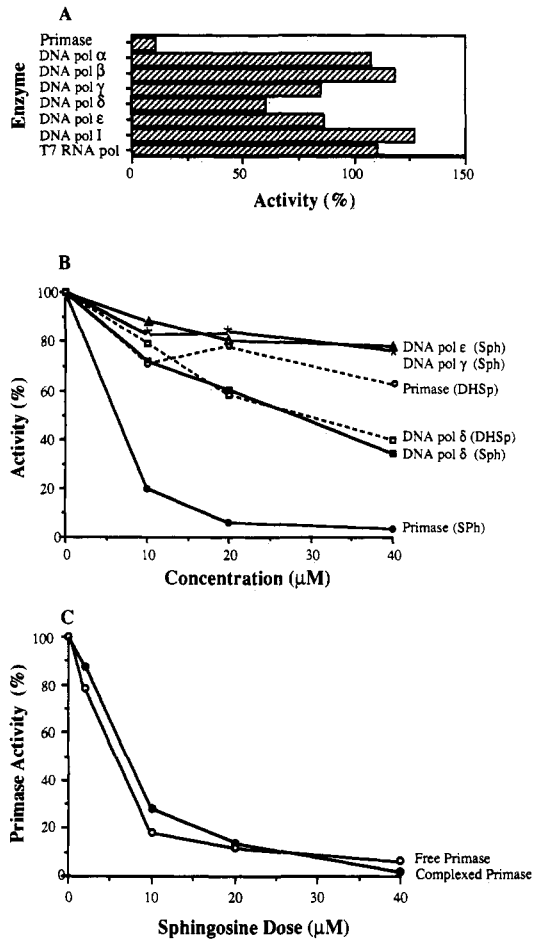

FIGURE 2: Enzyme specificity of the inhibition. (A) The comparative effects of sphingosine (20 μM) on the activities of various DNA polymerases α, β, γ, δ, and ϵ, DNA polymerase I (Klenow fragment), as well as T7 RNA polymerase, and primase associated with DNA polymerase α were investigated as described in Materials and Methods. 100% activity corresponds to the activities of the independent experiments. (B) Dose dependency of the inhibition was measured by adding the indicated concentrations of sphingosine (Sph) to the reaction mixture of primase (●), DNA polymerase γ (*), δ (■), and ϵ (△). The effects of dihydrosphingosine on primase (○) and DNA polymerase δ (□) were also measured. Reactions were carried out as described in Materials and Methods. (C) Indicated concentrations (from 0 to 40 μM) of sphingosine were added to either primase complexed with DNA polymerase α (●) or free primase (○), followed by measurement of primase activity.

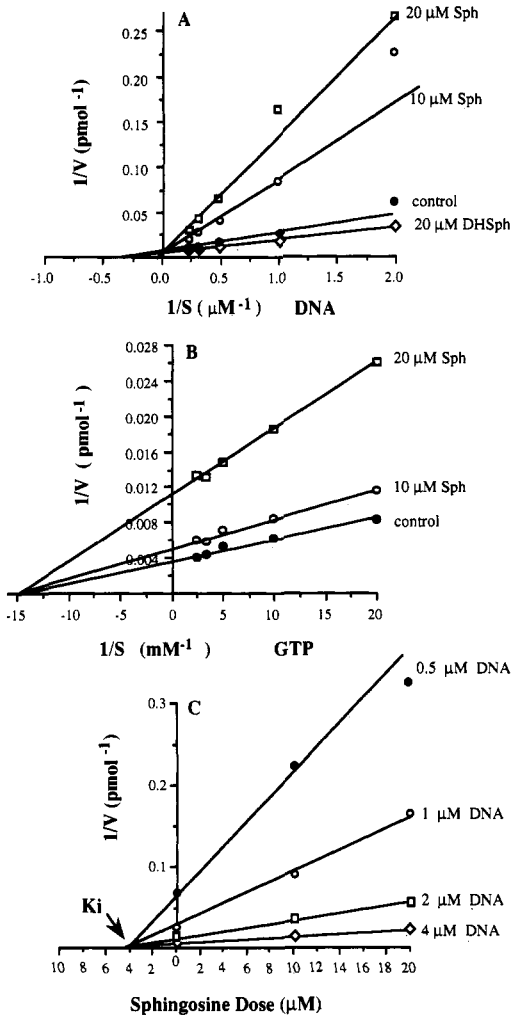

FIGURE 3: Kinetic analysis. (A) Primase activity was measured in the absence (●) or presence of 10 μM sphingosine (○), 20 μM sphingosine (□), or 20 μM dihydrosphingosine (DHSp, ◇) using the indicated concentrations of the DNA template (expressed as deoxynucleotide amounts of poly(dC)). A Lineweaver–Burk plot was then made on the basis of the data. Essentially the same results were obtained in three separate repetitions of the experiment. (B) Primase activity was assayed with the indicated concentrations of the GTP substrate in the presence of 10 (○) or 20 μM sphingosine (□) or in the absence of sphingosine (●). (C) The inhibition constant (K_i) was obtained at 4 μM from the Dixon plot made on the basis of the same data used for A.

activity at all. Sphingosylphosphocholine (SPC), also known as a potent bioactive sphingolipid (Sugiyama et al., 1993) and a deacylation product of sphingomyelin, similarly had no effect on primase activity.

Enzyme Specificity of Inhibition. As shown in Figure 2A, inhibition by sphingosine appears to be quite specific for primase, because it had no effect on the activities of DNA polymerases α and β and slightly inhibited polymerases γ, δ, and ϵ. Likewise, it had no effect on DNA polymerase I (Klenow fragment) alone, a component of the primase assay (Yoshida et al., 1983), confirming that sphingosine inhibits primase itself. It also did not suppress the activity of T7 RNA polymerase. However, sphingosine equally inhibited the activity of both primase complexed with DNA polymerase α and its free form, indicating that sphingosine directly interacts with and affects the primase subunit itself (Figure 2B). DNA polymerase δ was also dose dependently inhibited by sphingosine, but to a much lesser extent than primase (Figure 2B). Moreover, unlike primase, DNA polymerase δ was similarly inhibited by both sphingosine and dihydrosphingosine, suggesting that a different mechanism of inhibition may work for each enzyme.

Inhibition by Sphingosine Is Competitive with DNA, but Not with Substrate NTP. The extent of inhibition as a function of the DNA template dose and the GTP substrate concentration was studied to elucidate the mechanism of inhibition by sphingosine. Double-reciprocal plots indicated that inhibition was competitive with the DNA template, since there was a 5-fold increase in apparent K_m (from 2.67 to 13.3 μg/mL) while V_{max} was unchanged at 181.8 pmol/h (Figure 3A). On the other hand, since the apparent K_m for the substrate GTP was unchanged at 0.067 mM, inhibition by sphingosine is evidently noncompetitive with the substrate GTP (Figure 3B). Sphingosine may therefore interact with or affect the template binding sites on the enzyme, thereby decreasing its affinity for the template DNA. The K_i value, obtained from Dixon plots, was further found to be 4 μM, corresponding to the final concentration in the reaction mixture (Figure 3C).

Sphingosine Dose Dependently Inhibits RNA Primer Synthesis by Primase. Since the effect of sphingosine on

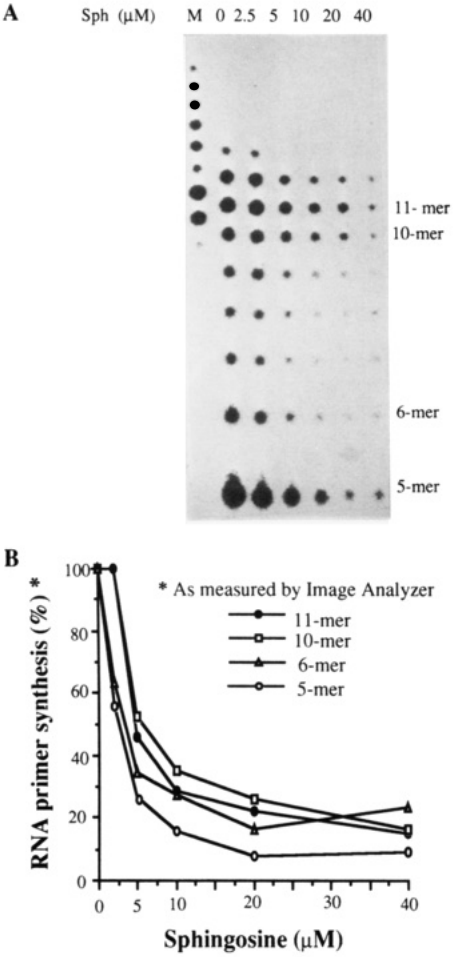

FIGURE 4: Product analysis: effect of sphingosine on RNA primer synthesis. (A) Synthesis of RNA primer products was performed using poly(dT) as DNA template and [α-^{32}P]ATP as substrate, as described in Materials and Methods, followed by separation of the products by denaturing gel electrophoresis. The RNA primer products of various lengths (from 5- to 12-mer) synthesized by primase in the presence of increasing concentrations of sphingosine (from 0 to 40 μM) were visualized by an image analyzer. Size markers (M) are shown on the left side. (B) Quantification of the product RNA of various sizes, i.e., 5-mer (○), 6-mer (△), 10-mer (□), and 11-mer (●), were made by the image analyzer. 100% RNA primer synthesis corresponds to the synthesis of RNA primer products in the absence of sphingosine.

primase activity so far has been tested in the coupled primase/DNA polymerase I (Klenow fragment) assay, product analysis was also performed to confirm whether sphingosine directly inhibits the synthesis of RNA primer products by primase. Primase complexed with DNA polymerase α and free primase have both been shown to similarly synthesize around a 10-mer of the RNA product, using poly(dT) as template. As is evident in Figure 4A, there was a dose-dependent decrease in the amount of RNA primer products due to the addition of increasing amounts of sphingosine, and various sizes of RNA products were equally decreased (Figure 4B), indicating that sphingosine decreases the frequency of priming events. In contrast, dihydrosphingosine, even at 40 μM, had no effect on the amount of RNA primer products (data not shown). Fifty percent inhibition of the synthesis of RNA primer products was observed at a dose of 4–5 μM sphingosine (Figure 4B), which is consistent with our previous data (Figures 1 and 2).

Sphingosine Inhibits the Physical Association of Primase with Template DNA. By a gel-retardation analysis, the physical association of primase with single-stranded template

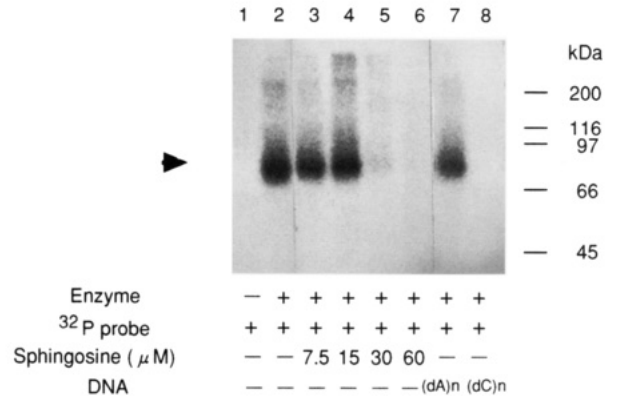

FIGURE 5: Effects of sphingosine and DNA competitors on the complex formation of DNA polymerase α–primase and ^{32}P-labeled DNA fragment CTPPS1. Various amounts of sphingosine were added to the mixture of DNA polymerase α–primase and the ^{32}P-labeled bovine DNA sequence, CTPPS1, and their effects on complex formation were examined by the gel-retardation assay as described in Materials and Methods: lane 1, only ^{32}P-labeled CTPPS1 without sphingosine; lane 2, DNA polymerase α–primase and ^{32}P-labeled CTPPS1 without sphingosine; lanes 3–6, DNA polymerase α–primase and ^{32}P-labeled CTPPS1 with 7.5, 15, 30, and 60 μM sphingosine, respectively; lanes 7 and 8, excessive amounts of poly(dA) and poly(dC) compared to CTPPS1 were added to the mixtures containing DNA polymerase α–primase and ^{32}P-labeled CTPPS1, respectively. The ^{32}P-labeled DNA fragment–protein complex was visualized using an image analyzer (Fujix BAS 2000 system).

DNA was measured in the presence of sphingosine, using the immunoaffinity-purified calf thymus DNA polymerase α–primase complex and a primase binding sequence, ^{32}P-labeled CTPPS1. After binding, protein and DNA were cross-linked and then enzyme subunits were partially dissociated in SDS, followed by SDS–polyacrylamide gel electrophoresis (Materials and Methods). As shown in Figure 5, the radioactive band corresponding to the primase subunit-conjugated DNA fragment disappeared by the addition of 30 μM sphingosine. Previously, we have shown that the binding of primase with DNA is strongly competed by poly(dC) but is resistant to poly(dA) (Suzuki et al., 1993a). As shown in Figure 5, the radioactive band corresponding to the molecular weight of the primase-conjugated DNA fragment (indicated by an arrow) was likewise competed by poly(dC) but was resistant to poly(dA).

DISCUSSION

Although the biological effects of sphingosine have been attributed to its inhibition of protein kinase C (Hannun et al., 1988), increasing evidence has shown that it may also have other biochemical targets, such as DAG kinase (Kanoh et al., 1990), phospholipase D (Zhang et al., 1990a,b, 1991; Lavie & Liscovitch, 1990), and phosphatase(s) associated with Rb dephosphorylation (Chao et al., 1992). Since endogenous sphingosine is mainly a product of the hydrolysis of sphingolipids, such as sphingomyelin (Merrill & Steven, 1989), the fact that sphingomyelin composes one of the two main phospholipids in the nuclear matrix (Cocco et al., 1980) and the presence of a neutral sphingomyelinase in the nucleus (Tamiya-Koizumi et al., 1989) both argue for an intranuclear target for sphingosine action. The present paper demonstrates that one such intranuclear target could be primase associated with DNA polymerase α. During the initiation events in DNA synthesis, eukaryotic primase is essential for the synthesis of RNA primers, which are consequently extended by the DNA polymerases α and δ. It exists as a complex with DNA polymerase α and primes the leading strand once and the

lagging strand repeatedly. Sphingosine apparently inhibits only primase (47 and 52 kDa) without having any effect on the DNA polymerase α catalytic subunit (Figure 2A). Inhibition of primase by sphingosine was quite specific because it had a much smaller effect on the other DNA polymerases α, β, γ, δ, and ϵ. Furthermore, unlike T-antigen, which has been shown to affect primase activity indirectly by binding to the DNA polymerase α subunit (Savoysky et al., 1993), sphingosine may directly interact with and affect the primase subunits, since the extent of inhibition was the same for primase in the presence or absence of the DNA polymerase α moiety (Figure 2B).

Kinetic analysis further showed that inhibition is competitive with the DNA template, but not with the substrate GTP (Figure 3A,B). Sphingosine may therefore inhibit primase activity by interacting with or binding to the primase template binding sites, consequently decreasing its affinity for the DNA template and decreasing the frequency of priming events. Consistently, it was clearly shown that the amounts of RNA primer products of various lengths were dose dependently decreased by sphingosine (Figure 4). Our results showing that T7 RNA polymerase was not inhibited by sphingosine (Figure 2A) suggested that inhibition of primase by sphingosine could not be correlated with general RNA synthesis. Preliminary results further showed that sphingosine inhibits HeLa cell growth and *de novo* DNA synthesis at concentrations that showed no effect on both RNA synthesis and protein synthesis (data not shown), indicating that mammalian RNA polymerases also were not inhibited by sphingosine. A gel-retardation assay confirmed that sphingosine inhibited the physical association of primase with DNA.

Free sphingosine is a natural constituent of cells, but its intracellular concentration is estimated to be low, i.e., in the micromolar range (Wilson et al., 1988; Merrill et al., 1986; Kobayashi et al., 1988), although it is comparable to the K_i for the inhibition of primase. This level, however, could be further regulated by certain physiological stimuli. For example, serum lipoproteins (Wilson et al., 1988) or diacylglycerol (Kolesnick, 1987) increases sphingosine levels mainly by the hydrolysis of sphingomyelin and glycolipids, while phorbol ester decreases sphingosine levels (Wilson et al., 1988). The activation of membrane sphingomyelinase, resulting in an elevation of sphingosine levels, has likewise been reported to occur concomitant with the differentiation of 3T3-L1 cells treated with dexamethasone (Ramachandran et al., 1990). Similarly, elevated sphingomyelin turnover has also been reported to be associated with cell differentiation induced by tumor necrosis factor α and interferon γ (Kim et al., 1991). Intranuclear levels of sphingomyelin have been shown to be significantly increased in proliferating cell nuclei, such as hepatoma cells (Spangler et al., 1975) and regenerating rat liver cells (Kobayashi et al., 1988), compared with resting liver. Sphingomyelin metabolism, therefore, also exists in nuclei, and the modulation of nuclear sphingomyelinase (Tamiya-Koizumi et al., 1989) may regulate the intranuclear levels of free sphingosine. Although it has been shown that exogenously added sphingosine and its analogues that enter cells are not further degraded, but are utilized intact for the synthesis of sphingomyelin and glycolipids (Ladenson et al., 1993), it is not clear whether exogenously added sphingosine can enter into the nucleus. However, it has been suggested that the facility by which sphingosine can undergo rapid movement across membranes and compartments (Hope & Cullis, 1987) allows it to easily relocate to other parts of the cell from its site of generation (Wilson et al., 1986).

Primase activity was inhibited by sphingosine but not by other complex sphingolipids, such as gangliosides GM3, GM1, GD1a, and SPG, as well as sphingomyelin and ceramide (Figure 1). The fact that dihydrosphingosine, the saturated form of sphingosine, had little effect on primase *in vitro* (Figure 1B) differs from results with protein kinase C (Merrill et al., 1989) and phospholipase D (Lavie & Liscovitch, 1990), which were both found to be sensitive to this derivative. This structural specificity of primase inhibition by sphingosine raises the possibility that it could be an important regulatory molecule and serve as a useful biochemical tool in the functional analysis of the roles of the different DNA polymerases in DNA replication and repair, as well as a useful pharmacological tool as a lead compound for the synthesis of derivatives that could be potent primase inhibitors.

ACKNOWLEDGMENT

The authors express their thanks to Dr. E. Savoysky for helpful discussions and also to Ms. T. Tomita and S. Furumoto for their excellent technical assistance.

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