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Involvement of sphingolipids metabolites in cellular proliferation modulated by ganglioside GM1‡

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The B subunit of cholera toxin, which binds specifically to ganglioside GM1, is mitogenic for quiescent Swiss 3T3 fibroblasts. Recently, sphingolipids metabolites, ceramide, sphingosine and sphingosine-1-phosphate, have been implicated as second messengers in cell growth regulation and differentiation. In this paper, we examined the possibility that interaction of the B subunit with membrane GM1 leads to alterations in metabolism of glycosphingolipids and that increased levels of sphingolipids metabolites may mediate the biological effects of the B subunit. While the B subunit did not induce a change in the level of ceramide or sphingosine, the level of sphingosine-1-phosphate was rapidly and transiently increased. The B subunit also transiently activated cytosolic sphingosine kinase activity, which catalyzes the phosphorylation of the primary hydroxyl group of sphingosine to produce sphingosine-1-phosphate. To determine whether the increase in sphingosine-1-phosphate level plays a role in B subunit-induced mitogenicity, we used a competitive inhibitor of sphingosine kinase, D,L-*threo*-dihydrosphingosine. D,L-*threo*-Dihydrosphingosine not only inhibited B subunit-induced DNA synthesis by 26%, it also reduced its ability to stimulate DNA-binding activity of the transcription factor AP-1. This sphingosine kinase inhibitor also inhibited B subunit-induced increases in the activity of cell cycle-regulated, cyclin-dependent serine/threonine kinases, cdk2 and $p34^{cdc2}$. These findings suggest that sphingosine-1-phosphate may play a role in the signal transduction pathways activated by binding of the B subunit to endogenous ganglioside GM1.

Keywords: ganglioside GM1, sphingolipids metabolites, B subunit of cholera toxin, cell growth

Abbreviations: CT, cholera toxin; BSA, bovine serum albumin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; SPP, sphingosine-1-phosphate; TPA, 12-O-tetradecanoylphorbol 13-acetate; EGF, epidermal growth factor; PDGF, platelet derived growth factor; AP-1, activator protein-1.

Introduction

Gangliosides, a diverse family of sialic acid-containing glycosphingolipids, have been implicated in regulation of cell growth and differentiation [reviewed in 1–3]. However, the intracellular mechanisms responsible for their effects are not yet well understood. Because the B subunit of cholera toxin (CT) binds exclusively and with high affinity to only one type of ganglioside, ganglioside GM1 [4, 5], this interaction has proved useful in the study of the molecular mechanisms leading to GM1-mediated cellular events [reviewed in 6]. The binding of the B subunit to endogenous ganglioside GM1 does not elicit the classical intracellular second messenger systems, such as cAMP, diacylglycerol (an endogenous activator of protein kinase C), or inositol (1,4,5)-trisphosphate (which mobilizes calcium from internal stores) [7–10]. However, the B subunit mediated a rapid increase of intracellular free calcium resulting from a net influx from extracellular sources in many cell types, including thymocytes [10], Swiss 3T3 fibroblasts [7], astrocytes [11], human B lymphocytes [12], and neurons and neuroblastoma cells [2, 13]. Although we have demonstrated recently that in Swiss 3T3 fibroblasts there is a strong correlation between cellular proliferation modulated by binding of the B

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‡Dedicated to Dr Sen-itiroh Hakomori on the occasion of his 65th birthday.

subunit to cell surface ganglioside GM1, calcium influx, and nuclear expression of transcription factor activator protein-1 (AP-1) activity [14], the rise in $[Ca^{2+}]_i$ by itself may not be sufficient to explain the entire effects of the B subunit. Calcium ionophores, in contrast to the B subunit, did not stimulate DNA synthesis in quiescent 3T3 fibroblasts [7] and did not increase the synthesis of numatrin, a nuclear protein whose synthesis is closely correlated to cellular commitment for mitogenesis [15]. It could be that, in addition to calcium, other signalling pathways may also be required.

Recent results from various laboratories suggest alternate mechanisms that could explain the actions of gangliosides in cell growth regulation [6, 16, 17]. There is abundant evidence that, in analogy with a glycerophospholipid cycle providing intermediates with second messenger functions, sphingolipids metabolites also have important biological functions (reviewed in [16–21]). Several cytokines, including TNF-α, γ-interferon, interleukin-1, stimulate the activity of sphingomyelinase, an enzyme which catalyzes sphingomyelin degradation resulting in the formation of ceramide. Ceramide, in turn has been shown to play previously unrecognized roles in both cellular differentiation and apoptosis (reviewed in [19–21]). Studies from our laboratory raise the possibility that ceramide may not only play a role in cellular differentiation but may also be involved in cell growth regulation [22]. In quiescent fibroblasts, ceramide enhances DNA synthesis and cellular proliferation [22, 23]. In addition, CD28 which is a co-stimulatory signal for the T cell receptor, has recently been shown to stimulate sphingomyelin hydrolysis in human Jurkat T cells [24]. Further metabolites of ceramide, sphingosine (formed from ceramide by the action of ceramidase) and its phosphorylated derivative, sphingosine-1-phosphate (SPP), have also been shown to be mitogenic for Swiss 3T3 fibroblasts [25, 26]. More importantly, the potent mitogens platelet-derived growth factor (PDGF) and serum, induced a rapid and transient increase in cellular levels of sphingosine and SPP in quiescent fibroblasts and also stimulated the activity of sphingosine kinase, the enzyme that catalyzes the phosphorylation of sphingosine to form SPP [27]. PDGF also increased sphingosine levels in vascular smooth muscle cells with a concomitant decrease in ceramide levels [28]. These responses are specific for certain growth promoting agents, since epidermal growth factor (EGF), bombesin or bradykinin did not induce significant changes [27]. In view of the diverse cellular actions of sphingosine and SPP on calcium mobilization [26, 29, 30], activation of phospholipase D [31], the Raf/MKK/MAP kinase [32] and transcription factor AP-1 [33], the increase in cellular levels of sphingosine and SPP appears to have significant roles in the signalling pathways leading to cellular proliferation. Therefore, it was of interest to determine whether the mitogenic effect of the B subunit, which bind to ganglioside GM1, was mediated by increases in the levels of the sphingolipids metabolites, ceramide, sphingosine or SPP.

Materials and methods

Materials

The B subunit of cholera toxin was purchased from Schwarz/Mann Biotech (Cleveland, OH) or from List Biological Labs (Campbell, CA). Insulin, and transferrin were from Collaborative Research (Lexington, MA). Sphingosine and D,L-*threo*-dihydrosphingosine were obtained from Matreya (Pleasant Gap, PA). Ceramides (bovine brain, type III) and DETAPAC (diethylenetriaminepentaacetic acid) were from Sigma Chemical Co. (St. Louis, MO). *E. coli* diacylglycerol kinase was from Lipidex, Inc. (Westfield, NJ). The various standard phospholipids and cardiolipin were from Avanti Polar Lipids (Birmingham, AL). Octyl-β-D-glucopyranoside was purchased from Calbiochem (La Jolla, CA). Calf serum was from Colorado Serum Co. (Denver, CO). [methyl^{3}H]Thymidine (55 Ci mmol^{-1}), [^{32}P]orthophosphate (carrier-free), [^{3}H]serine (32 Ci mmol^{-1}), and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) were purchased from Amersham (Arlington Heights, IL). Poly (dI-dC) was obtained from Pharmacia LKB (Piscataway, NJ). T4 polynucleotide kinase was from New England BioLabs (Beverly, MA). Oligonucleotides containing the consensus sequence of AP-1, SP-1 and NF1/CTF were from Stratagene (La Jolla, CA). Anti-cdk2 kinase antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY) and anti-cdc2 kinase antibodies were obtained from Transduction Laboratories (Lexington, KY). Sphingosine-1-phosphate was prepared by enzymatic digestion of sphingosylphosphorylcholine with phospholipase D as previously described [26].

Cell culture

Swiss 3T3 cells were from the American Type Culture Collection (CCL 92) and were cultured as previously described [8]. For measurement of DNA synthesis, lipid analysis and nuclear extract analysis, cells were subcultured at a density of 1.5×10^4 cells cm^{-2} in DMEM supplemented with 2 mM glutamine, penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), and 10% calf serum, refed with the same medium after 2 days, and used 5 days later when the cells were confluent and quiescent [8].

Mass measurement of ceramide

The mass amounts of ceramide in cellular extracts were measured by the DAG kinase enzymatic method [22]. Briefly, cells were treated with vehicle or the B subunit for various incubation periods and the reactions were

terminated by placing the dishes on ice and rapidly removing the medium. Cells were scraped from the dish in 1 ml of methanol, the lipids were extracted with chloroform/methanol/water (1:1:1, V/V) and phases separated [22]. An aliquot was taken from the organic phase and dried under nitrogen. The cellular lipids were then suspended in 7.5% octyl-β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETAPAC and freeze-thawed followed by sonication for 10 min. Mixed micelle preparations were suspended in 100 µl of buffer (100 mM imidazole-HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, 2 mM EGTA) and then 20 µl of 20 mM DTT, 20 µl of diacylglycerol kinase (5–15 units ml^{-1}) and 20 µl of [γ^{32}P]ATP (10 mM, 2.5×10^5 cpm $nmol^{-1}$ ATP) were added. After incubation at room temperature for 2 h, lipids were extracted by the addition of 1 ml of chloroform/methanol/conc. HCl (100:200:1, V/V) and 0.2 ml of balanced salt solution containing 10 mM EDTA [34]. Phosphatidic acid and ceramide-1-phosphate were resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, V/V).

Sphingolipids analysis

Confluent cultures were prelabeled with [^{3}H]serine (20–30 µCi ml^{-1}) for 12–15 h. Inhibitors of pyridoxal phosphate-dependent sphingosine-1-phosphate lyase, L-canaline (100 µM) and deoxypyridoxine (500 µM), were included in the labeling media. Cells were washed twice with DMEM and incubated in DMEM/Waymouth (1:1) supplemented with 20 µg ml^{-1} BSA and 5 µg ml^{-1} transferrin. Cells were then rinsed with serum-free medium and treated with the B subunit or vehicle for various time periods. Cells were scraped from the dish in 1 ml of 0.1 N HCl, 4 ml of chloroform/methanol (2:1, V/V) was added and phases separated exactly as described [25], except that unlabelled sphingosine-1-phosphate and sphingosine were added to improve recovery. The aqueous phase was washed with 2 ml of chloroform/methanol (88:12, V/V) and the lipids in the combined organic phases were analyzed by two-dimensional TLC. The plates were developed in chloroform/methanol/water (66:33:8, V/V) in the first dimension and butanol/ethanol/acetic acid/water (80:20:10:20, V/V) in the second dimension. Lipids were visualized with iodine vapors and the radioactivity quantified by liquid scintillation counting of the corresponding silica gel areas.

Measurement of sphingosine kinase activity

After various treatments, cells were scraped from dishes in 0.1 M phosphate buffer (pH 7.2) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM Na_3VO_4, 15 mM NaF, 10 mg ml^{-1} leupeptin and aprotinin, 1 mM PMSF and 0.5 mM 4-deoxypyridoxine, and lysed by freeze-thawing. After centrifugation at 2000 × g, cytosol was prepared by ultracentrifugation at 105 000 × g for 90 min. Sphingosine kinase activity was measured in supernatants by incubating with saturating concentrations of sphingosine (50 µM), added as a BSA complex [27], 10 mM $MgCl_2$, and [γ^{32}P]ATP (1 mM, 0.1–0.2 Ci $mmol^{-1}$) for 30 min at 37 °C. Labelled lipids were extracted, separated by TLC, and labelled SPP was visualized by autoradiography, scraped from the plate and counted in a scintillation counter [27].

DNA synthesis

DNA synthesis was measured by [^{3}H]thymidine incorporation [7].

Electrophoresis gel shift assays

Nuclear extracts and DNA-binding reactions for gel mobility-shift assays were performed exactly as previously described [14].

Measurements of cdk2 and p34^{cdc2} (cdc2) kinase activities in immune complexes

Cells were lysed in a buffer consisting of 50 mM Hepes, pH 7.4/0.1% Triton X-100/150 mM NaCl/1 mM EDTA/1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride/50 mM NaF/80 mM β-glycerophosphate/0.1 mM Na_3VO_4/with leupeptin and aprotinin (2.5 µg ml^{-1} each). After centrifugation at 14 500 × g, 250 µg aliquots of supernatant proteins were incubated with 2.5 µg of anti-cdk2 kinase or anti-cdc2 kinase antibodies for 1 h at 4 °C, followed by the addition of 50 µl of protein A-Sepharose CL-4B [35]. After an additional hour at 4 °C, the immune complexes bound to Sepharose were recovered by centrifugation, washed twice with 50 mM Hepes, pH 7.4/150 mM NaCl/0.1% Triton X-100 and twice with kinase buffer (50 mM Hepes, pH 7.4/1 mM dithiothreitol), and incubated with 30 µl of kinase reaction mixture (40 µg histone H1, 25 µM ATP (2.5 µCi [γ-^{32}P]ATP) and 10 mM $MgCl_2$ in kinase buffer) for 15 min at room temperature. An aliquot (15 µl) was spotted on phosphocellulose disks, washed extensively with 1% phosphoric acid, twice with water, and the radioactivity on disks measured by liquid scintillation spectrometry.

Results

B subunit and anti-GM1 antibodies induced DNA synthesis

We have previously shown that binding of the B subunit of CT to plasma membrane ganglioside GM1 stimulates DNA synthesis and cell division in quiescent cultures of Swiss 3T3 fibroblasts grown in chemically defined medium containing BSA, transferrin, and insulin [7]. In agreement, the B subunit caused a dose-dependent increase in DNA synthesis with a maximum response at a concentration of 1 µg ml^{-1}. This value was 33% of that

achieved by stimulation of the cells with 10% FBS (data not shown). To rule out the possibility that these effects were mediated by the B subunit itself rather than due to interaction with ganglioside GM1, we examined the effect of another ganglioside GM1 binding protein, anti-GM1 antibodies. Anti-GM1 antibodies specifically mimicked the mitogenic effects of the B subunit (Fig. 1). However, anti-GM1 antibodies were less potent and stimulated DNA synthesis to a lesser extent than the B subunit. Similar mitogenic effects of such specific, but completely different, GM1 binding proteins provide evidence that endogenous ganglioside GM1 is the key molecular target involved in the phenomena of cell growth regulation.

Effect of the B subunit on ceramide levels

Results from this laboratory raised the possibility that ceramides may not only play a role in cell differentiation and apoptosis but also may be involved in the regulation of cellular proliferation [6]. Thus, it was of interest to determine whether B subunit-stimulated cellular proliferation was accompanied by increases in cellular ceramide levels. As shown in Fig. 2, when quiescent 3T3 fibroblasts were placed in serum-free medium, ceramide levels increased by 50% after 3–4 h. Addition of the B subunit did not alter the response.

Effect of the B subunit on sphingosine and sphingosine-1-phosphate

To examine the effect of the B subunit on other sphingolipids metabolites, cells were metabolically labelled with [^{3}H]serine, a precursor of cellular sphingolipids. Similar to our previous observations with PDGF and serum, the B subunit induced a rapid and transient increase in [^{3}H]sphingosine-1-phosphate reaching a maximum of 1.58 ± 0.08 ($n = 6$) fold after 2–4 min (Fig. 3A). This was followed by a rapid decline to levels below basal values. However, in contrast to PDGF and serum, the B subunit did not induce significant changes in [^{3}H]sphingosine in the same time period (Fig. 3B). Furthermore, levels of [^{3}H]sphingomyelin, which constitutes a much greater proportion of the total [^{3}H]serine incorporated into lipids (12.0 ± 2.2%) than either free sphingoid bases (0.26 ± 0.01%) or sphingosine-1-phosphate (0.1 ± 0.02%), were not significantly altered (Fig. 3B). In agreement our previous studies, the B subunit did not have any significant effects on the levels of other phospholipids, including phosphatidylcholine, phosphatidylserine, phosphatidylinositol, or phosphatidylethanolamine (Fig. 3B). In addition, the B subunit did not induce changes in the levels of second messengers derived from glycerophospholipids, such as diacylglycerol, phosphatidic acid, and arachidonic acid (data not shown).

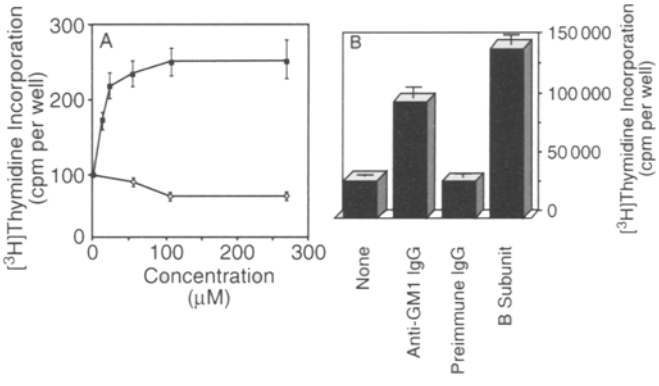

Figure 1. Effects of anti-GM1 antibodies and the B subunit on DNA synthesis. (A) Confluent quiescent cultures of Swiss 3T3 cells were incubated in DMEM-Waymouth (1:1) supplemented with BSA (20 μg ml^{-1}), transferrin (5 μg ml^{-1}), insulin (1 μg ml^{-1}), treated with the various concentrations of anti-GM1 IgG (■) or pre-immune IgG (○) and [^{3}H]thymidine incorporation (mean ± SD; $n = 3$) was measured as described in Materials and Methods. The data are expressed as percent of untreated control. (B) Cells were treated with anti-GM1 IgG (50 μg μl^{-1}), pre-immune IgG, or the B subunit (1 μg ml^{-1}) and [^{3}H]thymidine incorporation (mean ± SD; $n = 3$) was measured.

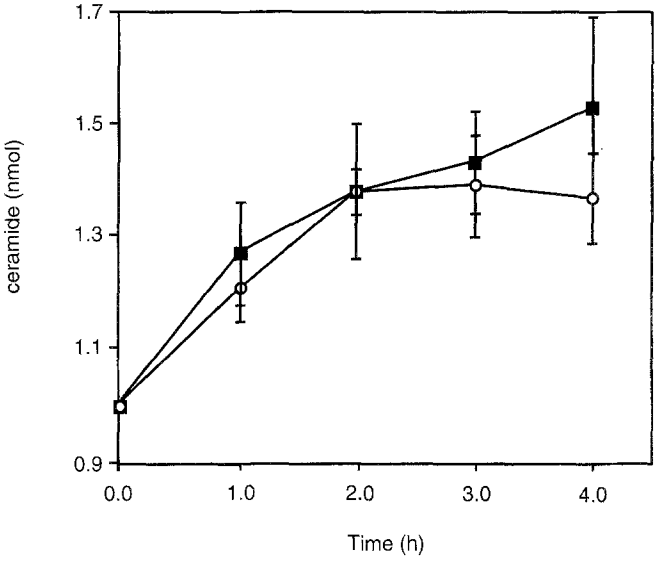

Figure 2. Changes in ceramide mass. Quiescent Swiss 3T3 cells were treated without (■) or with 2 μg ml^{-1} B subunit (○). At the indicated times, lipids were extracted and ceramide was quantitated using the DAG kinase method as described in Materials and Methods. The values are from a representative experiment and are expressed as nmol of ceramide present in 100 nmol phopholipids. Similar results were obtained in several independent experiments.

The B subunit activates sphingosine kinase

The level of SPP in cells is determined by the relative contributions of its formation from sphingosine, catalysed by sphingosine kinase [36], and its degradation, mediated by SPP lyase [37]. Because the B subunit did not increase levels of sphingosine, it was of interest to determine whether the increase in SPP was due to activation of

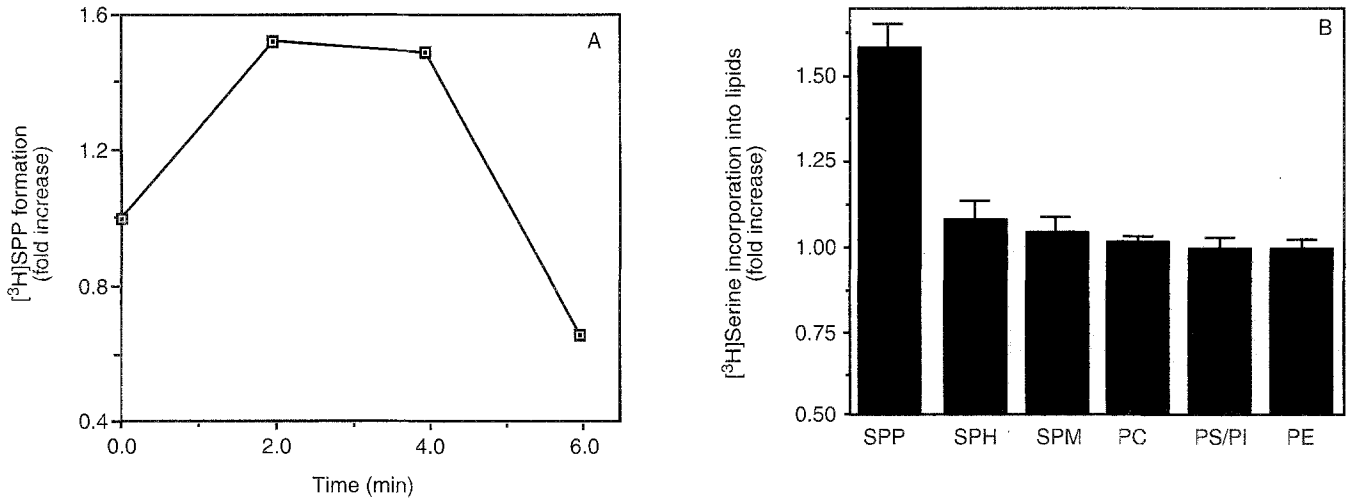

Figure 3. The B subunit increases the levels of sphingosine-1-phosphate. Confluent and quiescent cultures of Swiss 3T3 fibroblasts were labelled with [^{3}H]serine (20 μCi ml^{-1}) for 15 h in media supplemented with 0.1 mM L-canaline and 0.5 mM 4-deoxypyridoxine. Cells were treated with the B subunit for the indicated times. The lipids were extracted and separated by TLC as described in Materials and Methods. The results are presented as fold increase relative to untreated controls. (A) Kinetics of [^{3}H]SPP formation. Each value is the mean of triplicate determinations from a representative experiment. Similar results were obtained in four additional experiments. (B) Effect of the B subunit on the levels of other [^{3}H]serine-labelled lipids. Each value is the mean ± SE from six different experiments. The proportion of [^{3}H]serine incorporated into various lipids compared to that incorporated into total lipids: sphingosine-1-phosphate (SPP), 0.1 ± 0.02%; free sphingoid bases (SPH), 0.26 ± 0.01%; [^{3}H]sphingomyelin (SPM), 12.0 ± 2.2%; phosphatidylcholine (PC), 4.35 ± 0.64%; phosphatidylserine and phosphatidylinositol (PS/PI), 48.65 ± 3.25%; phosphatidylethanolamine (PE), 22.26 ± 0.41%.

sphingosine kinase. The majority of sphingosine kinase activity is present in the cytosol of Swiss 3T3 fibroblasts and only a small fraction is present as membrane bound forms. Cytosolic sphingosine kinase activity was dependent on the concentration of protein and sphingosine, showing typical Michaelis-Menten kinetics (data not shown). Similar to PDGF and serum [27], treatment of 3T3 fibroblasts with the B subunit, in the presence or absence of insulin, also induced activation of sphingosine kinase (Table 1). After 10 min, the B subunit stimulated the activity of the kinase by 1.6-fold, reaching a maximum within 10 min and declining thereafter to basal activity or below within 30 min (data not shown). Moreover, Ni^{2+}, which we recently showed inhibits B subunit-induced calcium influx [14], not only completely eliminated cellular proliferation induced by the B subunit, it also markedly reduced sphingosine kinase activity stimulated by the B subunit (Table 1). Similar to observations in other cell types [38], TPA but not TNFα, also stimulated sphingosine kinase activity in Swiss 3T3 fibroblasts (Table 1).

Table 1. The B subunit stimulates sphingosine kinase activity.

Treatment	*Sphingosine kinase activity (pmol min^{-1} mg^{-1})*	*(fold increase)*
A		
None	35.09 ± 1.6	1
Serum	52.99 ± 5.3	1.5
B subunit	49.14 ± 1.9	1.4
TPA	50.89 ± 3.7	1.5
TNFα	33.40 ± 3.7	0.9
B		
None	21.0 ± 1.1	1
B subunit	32.2 ± 1.8	1.6
Nickel +B subunit	22.2 ± 0.5	1.1

In A, quiescent Swiss 3T3 fibroblasts were incubated in serum-free DMEM and then stimulated with the growth promoting agents for 10 min. Cytosolic fractions were prepared and assayed for sphingosine kinase activity as described in Materials and Methods. The concentrations of the mitogenic agents were as follows: Serum (10%), EGF (20 ng ml^{-1}), PDGF (10 ng ml^{-1}), B subunit (5 μg ml^{-1}) and TPA (100 nM). In B, the effect of nickel on B subunit stimulation of sphingosine kinase was examined. In this set of experiments, Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.6 mM glucose, 160 μg ml^{-1} BSA and 20 mM HEPES, pH 7.4) was used due to the low solubility of nickel in DMEM [14]. Confluent and quiescent Swiss 3T3 fibroblasts were washed with Locke's solution and then stimulated in the same solution with 5 μg ml^{-1} B subunit in the absence or presence of $NiCl_2$ (1 mM).

Involvement of sphingosine-1-phosphate in cellular proliferation induced by the B subunit

Because SPP was the only sphingolipid metabolite whose level was significantly changed after binding of the B subunit to endogenous ganglioside GM1, we investigated the involvement of SPP in cellular proliferation induced by the B subunit. To this end, DNA synthesis was determined in the presence of a competitive inhibitor of sphingosine kinase, D,L-*threo*-dihydrosphingosine [39, 40]. D,L-*threo*-Dihydrosphingosine completely eliminated the increase in cellular levels of SPP elicited by the B subunit. In agreement with our previous results [27], this sphingosine kinase inhibitor inhibited DNA synthesis

induced by PDGF and serum by 45% and 55% respectively, but had no effect on EGF-induced cellular proliferation (Fig. 4). The effect on cellular proliferation induced by the B subunit was less dramatic and DNA synthesis was only inhibited by 26% (Fig. 4).

Of the known transcription factors, activation of AP-1, which consists of homo or heterodimers of Jun and Fos, is an important link in signalling pathways culminating in cellular proliferation [41]. Recently, we demonstrated that there was a strong correlation between cellular proliferation modulated by binding of the B subunit to GM1 and DNA binding activity of AP-1 [14]. Therefore, we also examined the effect of D,L-*threo*-dihydrosphingosine on B subunit-induced activation of AP-1. Consistent with our previous study [14], treatment of Swiss 3T3 fibroblasts with the B subunit for 3 h resulted in increased AP-1 binding activity (Fig. 5, lane 4) which was highly specific for the AP-1 consensus sequence (data not shown). Pretreatment with D,L-*threo*-dihydrosphingosine not only inhibited DNA synthesis induced by the B subunit by 26%, it also reduced the B subunit-induced stimulation of DNA binding activity of AP-1 (Fig. 5, lane 5). In sharp contrast, this sphingosine kinase inhibitor, had no effect on DNA-binding activity of AP-1 induced by TPA (Fig. 5, lanes 1, 2), demonstrating the specificity of the inhibitory effect.

D,L-threo-Dihydrosphingosine also inhibits B subunit-induced increases in cdk2 kinase and p34^{cdc2} kinase activities

The binding of the B subunit to ganglioside GM1 not only increased DNA binding activity of AP-1 and stimulated DNA synthesis, it also induced an increase in cell division. Major cell cycle check points occur at the G1/S and G2/M transitions and activation of one or more cell cycle-regulated, cyclin-dependent serine/threonine kinases regulates these transition points [42]. The activation of cdk2, which associates with cyclins A and E, is maximal at G1/S transition [43], whereas activation of p34^{cdc2} kinase (cdc2) is critical for progression of cells into mitosis [44]. Treatment with the B subunit induced increases of 1.4- and 3-fold in the activities of cdk2 and cdc2 kinases, respectively (Fig. 6A, B) effects which were

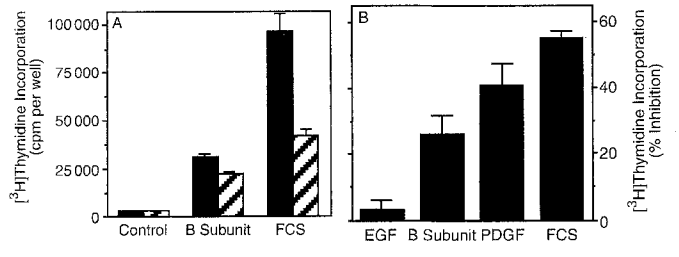

Figure 4. Inhibitory effects of D,L-*threo*-dihydrosphingosine on DNA synthesis induced by the B subunit and other growth promoting agents. [^{3}H]Thymidine incorporation was measured in quiescent cultures of Swiss 3T3 cells exposed to the indicated growth factors in the absence (filled bars) or presence of 10 μM D,L-*threo*-dihydrosphingosine (hatched bars). In (A), one representative experiment out of four which yielded similar results is included. In (B) data are expressed as percent inhibition relative to cells cultured in the absence of the inhibitor. Each value is the mean ± SD of triplicate determinations from at least three experiments. The concentration of the mitogenic agents were as follows: Serum (FBS) (10%); PDGF (20 ng ml^{-1}); EGF (20 ng ml^{-1}) in the presence of insulin (2 μg ml^{-1}); B subunit (2 μg ml^{-1}).

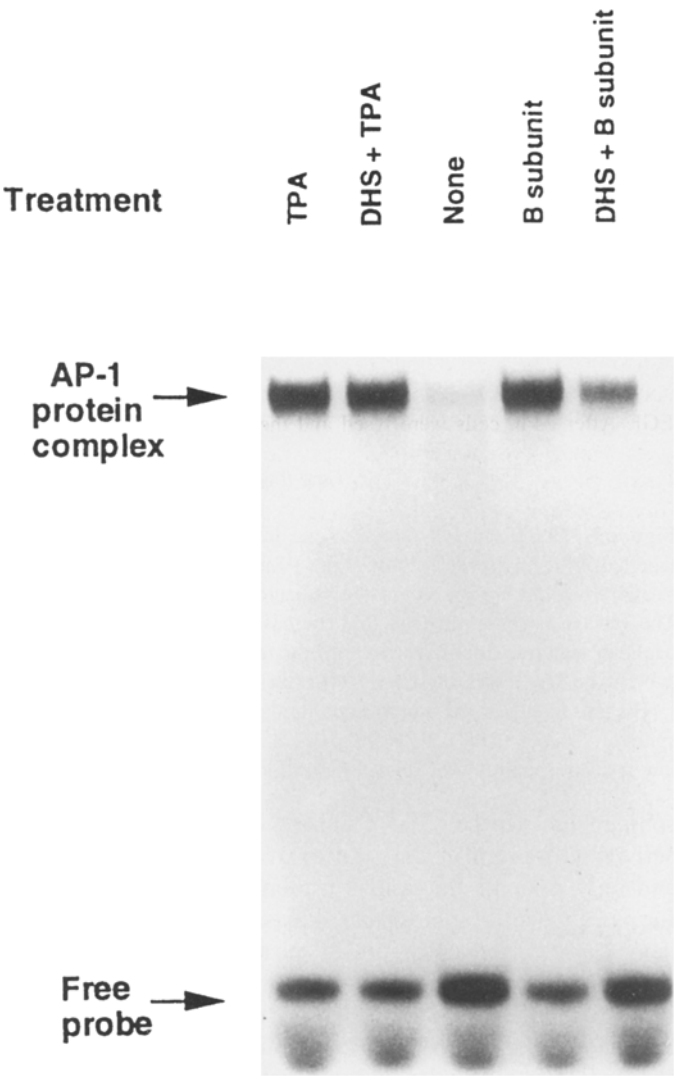

Figure 5. D,L-*threo*-Dihydrosphingosine inhibits DNA binding activity of AP-1 induced by the B subunit. Quiescent Swiss 3T3 fibroblasts were treated for 3 h without (lane 3), with 50 nM TPA (lanes 1,2), or with 2 μg ml^{-1} B subunit (lanes 4,5), in the absence (lanes 1,4) or presence (lanes 2,5) of 10 μM D,L-*threo*-dihydrosphingosine (DHS). Nuclear extracts were then prepared and DNA-binding activity of AP-1 analyzed by gel shift mobility assays. Where indicated, DHS was added 5 min prior to the addition of the mitogens and was present during the entire treatment.

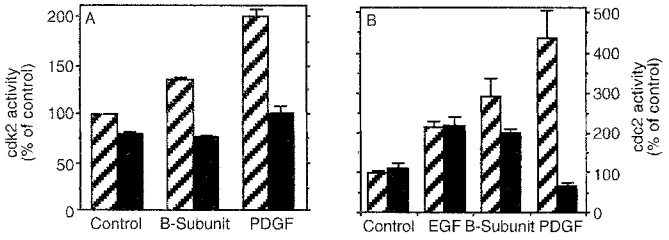

Figure 6. D,L-*threo*-Dihydrosphingosine inhibits cyclin-dependent kinases activities induced by the B subunit. Quiescent Swiss 3T3 fibroblasts were treated without (hatched bars) or with (solid bars) 10 μM D,L-*threo*-dihydrosphingosine for 10 min prior to the addition of 2 μg ml^{-1} B subunit, 50 ng ml^{-1} PDGF or 200 ng ml^{-1} EGF. After 24 h, cells were lysed and the cyclin-dependent kinases were immunoprecipitated with specific antibodies against cdk2 kinase (A) or cdc2 kinase (B) and the activities of kinases in the immune complexes were determined as described in Materials and Methods.

slightly greater than the stimulations induced by EGF, but less than the responses to PDGF. In agreement with previous results, D,L-*threo*-dihydrosphingosine markedly inhibits the activation of these kinases by PDGF, but not by EGF (Rani *et al.* unpublished). D,L-*threo*-Dihydrosphingosine also inhibits B subunit-induced increases in cdk2 kinase and p34^{cdc2} kinase activities, without affecting the response to EGF (Fig. 6A, B).

Discussion

Recent evidence suggests that sphingolipids metabolites may function as a new class of intracellular second messengers involved in cell growth regulation, differentiation, and apoptosis [reviewed in 6, 16–21]. Several cytokines, such as TNF-α, Fas and interleukin-1, stimulate sphingomyelinase leading to an increase in ceramide levels. Ceramide in turn arrests cell growth and induces apoptosis [19–21]. In contrast, growth factors, such as serum and PDGF, stimulate sphingosine kinase leading to an increase in sphingosine-1-phosphate levels, which then stimulates growth [27]. It is intriguing to note that the B subunit of CT has a similar bimodal growth effect as that of TNF-α. Both stimulate proliferation of quiescent 3T3 fibroblasts, while inhibiting proliferation of the same fibroblasts when they are rapidly growing [45–47]. In addition, both agents have opposite effects on normal versus transformed cells [46, 48]. Despite this similarity, TNF-α increases the levels of ceramide [19–21], whereas the B subunit has no detectable effects on the levels of ceramide. Interestingly, replacement of conditioned medium with fresh medium, induced a continuous increase in ceramide levels. In agreement, it has recently been reported for other cell types that there is an increase in ceramide level after removal of serum from the medium [49]. Furthermore, large changes in free sphingosine and sphinganine have been found in cells after cultures are changed to fresh medium [50, 51]. This burst results from an increased de novo synthesis of sphinganine and release of sphingosine due to enhanced sphingolipid turnover [51]. In J774A1 cells, free sphingosine is derived primarily from hydrolysis of sphingomyelin in an acidic compartment(s), perhaps reflecting the endosomal and/or lysosomal turnover of sphingolipids [51]. Ganglioside turnover may also contribute to the maintenance of the intracellular levels of free sphingosine and ceramide. Formation of free sphingosine and ceramide from exogenous ganglioside [^{3}H-sphingosine]GM1 was observed in cerebellar granule cells and increased with differentiation [52].

Binding of the B subunit to ganglioside GM1 did not increase the levels of sphingosine or ceramide, rather it induced a rapid and transient increase in SPP and also stimulated the activity of sphingosine kinase. In agreement with previous studies, the sphingosine kinase inhibitor D,L-*threo*-dihydrosphingosine reduced DNA synthesis induced by PDGF and serum [27]. It was found that while cell proliferation induced by FBS was markedly reduced, that induced by the B subunit was inhibited by only 26%. Although this appears to be rather small inhibition of DNA synthesis, this sphingosine kinase inhibitor also specifically inhibited B subunit-stimulated cyclin-dependent kinases and AP-1 DNA-binding activities to a greater extent. However, these assays are done at different time points and measure different processes whose contributions to the regulation of DNA synthesis are still not known. Collectively these results suggest that SPP may play a role in the proliferative response induced by binding of the B subunit to ganglioside GM1. Serum and PDGF induced 2.2- and 2.9-fold increases in the levels of endogenous SPP [27], respectively, whereas the B subunit caused a 1.5-fold increase, suggesting that SPP may play a more important role in the serum- and PDGF-induced mitogenic effects than in those induced by the B subunit. In agreement, a sphingosine kinase inhibitor more drastically reduced PDGF-induced increases in cell cycle-regulated, cyclin-dependent serine/threonine kinases, cdk2 kinase and p34^{cdc2} kinase in comparison to its effects on the response to the B subunit. Contrary to the effects observed with the B subunit, it has been shown that serum and PDGF increase the levels of phosphatidic acid, observations consistent with those effects being triggered by SPP [31]. Perhaps the larger increase in SPP levels induced by serum and PDGF, compared to the B subunit, may partially account for the release of intracellular calcium and phosphatidic acid formation in response to these mitogens, while the increase of SPP induced by the B subunit may not be sufficient to trigger any of these events.

By what mechanism does binding of the B subunit to ganglioside GM1 stimulate sphingosine kinase activity?

Although a definite answer is not yet known, two possibilities exist. First, the influx of calcium induced by the B subunit could stimulate the activity of calcium/calmodulin-dependent protein kinases leading to phosphorylation and activation of sphingosine kinase. In support of this hypothesis, Ni^{2+}, which has been shown to inhibit B subunit-induced calcium influx [14], completely eliminated the stimulatory effect of the B subunit on sphingosine kinase activity and also completely eliminated cellular proliferation induced by the B subunit.

The second possibility involves the specific localization of ganglioside GM1 in caveolae, flask-shaped invaginations of the plasma membrane, which are highly enriched with caveolin [53]. These unusual structures were isolated based on resistance to solubilization by nonionic detergents and contain, in addition to ganglioside GM1 and GPI-linked proteins, many signal transduction-linked proteins, including the src family of kinases and heterotrimeric guanine triphosphate-binding proteins. Such specialized distinct microdomains may exist to organize signaling molecules and to process surface-bound ligands differentially [54]. Binding of the B subunit increased the amount of GM1 in caveolae [53]. This might influence the architecture of the caveolae leading to activation of enzymes important for signal transduction. In this regard, recently it has been shown that ceramide production in response to the cytokine IL-1β is compartmentalized to the caveolae [55].

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