Sphingosine Activation of Protein Kinases in Jurkat T Cells

IN VITRO PHOSPHORYLATION OF ENDOGENOUS PROTEIN SUBSTRATES AND SPECIFICITY OF ACTION*

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Sphingosine displays multiple biochemical and biological effects, in particular inhibition and activation of protein kinases. To determine the predominant interaction of sphingosine with cellular kinases, the effects of sphingosine on endogenous protein phosphorylation in Jurkat T lymphoblastic cells were investigated in vitro. Sphingosine was found to cause prominent phosphorylation of a number of cytosolic proteins ranging in molecular mass from 18 to 165 kDa. Phosphorylation was calcium-independent. Phosphorylation of substrates was increased in response to concentrations of sphingosine as low as 10 μM and peaked at concentrations of 20–200 μM. Multiple lines of evidence suggested that sphingosine activated more than one protein kinase: 1) the concentration dependence on sphingosine differed from substrate to substrate, 2) phosphorylation of one group of substrates required ATP as the phosphate donor, whereas a second group showed no preference between ATP and GTP, and 3) phosphorylation of some substrates was inhibited by heparin, whereas other substrates were resistant. Activation of these kinases demonstrated a very specific requirement for D-erythro-sphingoid bases. DL-erythro-dihydrosphingosine was partially active, whereas DL-threo-dihydrosphingosine was not. Other related molecules such as stearylamine, sphingomyelin, and C2-ceramide were not active. Sphingosine-activated kinase(s) were distinct from protein kinase C, cyclic nucleotide-activated kinases, and calcium-dependent kinases. These observations demonstrate the existence of multiple sphingosine-activated protein kinases with high specificity for D-erythro-sphingosine, suggesting physiologic regulation of protein phosphorylation by sphingosine.

A role for sphingolipids as precursors to cell regulatory molecules is increasingly appreciated through the identification of biologic and biochemical activities of sphingolipid-derived products (1–3). This is best illustrated with the recent discovery of a "sphingomyelin cycle" whereby the action of tumor necrosis factor α (4), γ-interferon (4), or 1α,25-dihydroxyvitamin D3 (5) results in activation of a neutral sphingomyelinase. This causes the hydrolysis of membrane sphingomyelin and the generation of ceramide. In HL-60 human leukemia cells, ceramide appears to mediate the effects of tumor necrosis factor and 1α,25-dihydroxyvitamin D3 on cell proliferation and cell differentiation (4, 6). Thus, a cell regulatory pathway is being defined with ceramide as a key intracellular mediator.

Another sphingolipid-derived breakdown product, sphingosine, is a critical and defining component of sphingolipids (7). Sphingosine has multiple biologic activities such as inhibition of human platelet aggregation (8, 9), inhibition of neutrophil activation (10), inhibition of cell differentiation (11), inhibition of blood coagulation (12), stimulation of mitogenesis (13), and many other effects (for review see Ref. 1 and 2). At the biochemical level, sphingosine is a potent and reversible inhibitor of protein kinase C (8). This activity appears to mediate many of the biologic effects of sphingosine (1, 2). Sphingosine also exhibits protein kinase C-independent activities such as inhibition of tissue factor (12), inhibition of calmodulin-dependent kinases (14), inhibition of insulin receptor tyrosine kinase (15), biphasic effects on diacylglycerol kinase (16), inhibition of phosphatidic acid phosphohydrolase (17, 18), inhibition of CTP:phosphocholine cytidylyltransferase (19), and activation of phospholipase D (20, 21).

In addition, sphingosine has been shown to enhance phosphorylation of the EGF receptor through protein kinase C-independent mechanisms (22) and to activate the tyrosine kinase domain of the epidermal growth factor receptor (23). Recently, it was also shown that sphingosine directly activates purified casein kinase II (24), thus indicating that sphingosine may have positive direct effects on protein kinases.

Since sphingosine appears as a bifunctional molecule with the ability to activate some kinases and inhibit others, it became important to determine the predominant effects of sphingosine on cellular protein phosphorylation and to examine the specificity of sphingosine action. In this study, the effects of sphingosine on phosphorylation of endogenous proteins in Jurkat T cells was investigated in vitro. We show that sphingosine causes activation of endogenous cytosolic protein kinases resulting in the phosphorylation of a number of endogenous protein substrates. Sphingosine shows very high stereo and structural specificities. The implications of these studies on the possible roles of sphingosine as an endogenous modulator of protein kinases are discussed.

EXPERIMENTAL PROCEDURES

Materials

D-Erythro-sphingosine (from sphingomyelin), DL-erythro-sphinganine, DL-threo-sphinganine, polylysine, stearylamine, spermine,
and spermidine were from Sigma. C2-ceramide was synthesized as described (6). Sphingomyelin and phosphatidylserine were from Avanti Polar Lipids. Psychosine was from Serdari Research Laboratory. Chlorpromazine, heparin, and H7 (1-(5-isouquinolinylsulfonyl)-2-methylpipеразине) were from Sigma. Genistein was from Calbiochem. Dihleoleoylglycerol was synthesized as described (25). Reagents for SDS-PAGE, and standard protein molecular mass markers were obtained from Bio-Rad. [γ-32P]ATP (specific activity 3000 Ci/mmol) and [γ-32P]GTP (specific activity 6000 Ci/mmol) were from Du Pont-New England Nuclear.

Methods

Tissue Culture—Jurkat T cells were grown in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum (GIBCO) in 5% CO2 at 37 °C. Cells were maintained at a density between 4 × 105 and 1.2 × 106 cells/ml.

Preparation of Extracts—Cells were washed two times with cold phosphate-buffered saline and once with extraction buffer (80 mM β-glycerophosphate, pH 7.6, 20 mM EGTA, 15 mM MgCl2, 1 mM Na3VO4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin). Cells were resuspended in extraction buffer (4 × 106 cells/ml) and homogenized by sonication twice for 1 min at room temperature in a bath sonicator.

Preparation of Sphingosine—Sphingosine was prepared as a 10% solution in 10% ethanol in 0.5 M NaCl by sonication twice for 1 min at room temperature in a bath sonicator.

Phosphorylation Studies—Assay mixtures (total volume of 50 μl) contained 20 μl of cytosol (15-40 μg of protein), 5 μl of sphingosine (or 10% ethanol in 0.5 M NaCl), 5 μl of aqueous solution of different effectors, and 20 μl of buffer (100 mM Tris-HCl, pH 7.6, 25 mM MgCl2, 1.25 mM ATP, 4-5 μCi of [γ-32P]ATP) which was added last to start the reaction. Reactions were carried out for 10 min at 30 °C and were terminated with 50 μl of 2× sample buffer for SDS-PAGE. The samples were heated in a boiling water bath for 5 min. Proteins were separated on 10% SDS-PAGE (26). Gels were stained with Coomassie Brilliant Blue R-250 in 10% acetic acid and 30% methanol, destained, and subjected to autoradiography with X-Omat (Kodak) films using intensifier screens. Exposures were usually overnight. Phosphorylation was quantitated by excising protein bands from the dried gels and counting in scintillation fluid in a β-scintillation counter (Pharmacia LKB Biotechnology Inc.).

RESULTS

Sphingosine Induces Phosphorylation of Endogenous Substrates in Jurkat T Cells—Incubation of cytosol and membranes from Jurkat T cells with Mg2+ and ATP resulted in the phosphorylation of a number of endogenous substrates in both fractions (Fig. 1). Addition of 0.4 mM sphingosine to cytosol resulted in significant phosphorylation of a number of proteins of molecular masses ranging from 18 to greater than 165 kDa with prominent phosphorylation of proteins of apparent molecular masses of 18, 30, 32, 35, 42, 46, 55, 60, 94, 130, and 165 kDa. Sphingosine did not appear to modify phosphorylation of membrane proteins in vitro (Fig. 1B). Moreover, sphingosine-induced phosphorylation of substrates was independent of calcium (data not shown). These studies suggest that sphingosine activates endogenous protein kinase(s) present in Jurkat cytosol.

Characterization of Sphingosine-activated Kinase(s): Evidence for the Involvement of Multiple Protein Kinases—Sphingosine caused phosphorylation in a dose-dependent manner with phosphorylation of some proteins observed at a sphingosine concentration of 10 μM (Fig. 2). However, the dose dependence on sphingosine was clearly substrate-dependent such that some substrates showed near maximal phosphorylation in response to 20 μM sphingosine (32 and 46 kDa), whereas phosphorylation of other substrates peaked at sphingosine concentrations of 40 μM (35 kDa), and other substrates required concentrations of 200 μM (18, 55, and 94 kDa). Thus, sphingosine appears to be a potent activator of cytosolic protein kinase(s). Also, these results suggest that either sphingosine activates multiple protein kinases, or enhancement of phosphorylation by sphingosine may be related directly to substrate interactions.

The sphingosine activated kinase(s) appeared to utilize both ATP and GTP (Fig. 3). However, there were notable differences in the phosphorylated polypeptides when GTP was used as the substrate. In the presence of GTP, sphingo-
Sphingosine-activated kinase(s) did not phosphorylate the 30- and 65-kDa substrates (Fig. 3). These results suggest that sphingosine activates at least two different kinases: one that is capable of equally utilizing ATP and GTP, whereas the other exhibits selectivity for ATP as the nucleotide substrate.

Additional support for the activation of multiple kinases by sphingosine was provided by studies examining the effects of heparin on sphingosine-induced phosphorylation. Heparin, which modulates multiple protein kinases (27-31), exhibited distinct effects on sphingosine-induced phosphorylations (Fig. 4A). Thus heparin (20-80 μg/ml) caused significant inhibition of phosphorylation of 18-, 32-, and 90-kDa polypeptides in response to sphingosine (Fig. 4A). The phosphorylation of other substrates (e.g. 60 kDa), however, was not inhibited by heparin. Moreover, heparin appeared to augment the phosphorylation of some substrates in response to sphingosine, especially the 72- and 80-kDa polypeptides (Fig. 4A), with heparin alone having no effect on phosphorylation of these substrates (data not shown). These opposing effects of heparin are quantitated in Fig. 4B which shows that heparin increases, inhibits, or has no effect on phosphorylation of p72/p80, p18, and p60, respectively. These distinct effects of heparin strongly suggest the activation of multiple kinases by sphingosine.

Relationship of Sphingosine-activated Kinase(s) to Established Kinases with Allosteric Regulators—Because sphingosine has been shown to activate purified casein kinase II in vitro (24), the effects of polylysine, a casein kinase II activator (32, 33), were also investigated. Polylysine (0.4 μg/ml) was found to cause significant phosphorylation of a number of Jurkat cytosolic proteins that were distinct from those induced by sphingosine (Fig. 1A). The most prominent substrates migrated with apparent molecular masses of 66 and 100 kDa. Thus, both sphingosine and polylysine showed distinct phosphorylation patterns with overlap. These studies suggest that either sphingosine activates a kinase distinct from casein kinase II or sphingosine and polylysine induce casein kinase II to phosphorylate different substrates (see "Discussion").

Sphingosine-induced phosphorylations were distinct from those induced by Ca²⁺/phosphatidylserine/diolein, Ca²⁺, cAMP, cGMP, polyamines (Fig. 5), and polylysine (Fig. 1A). These results suggest that sphingosine-activated kinase(s) are distinct from known allosterically regulated kinases (protein kinase C, calmodulin-dependent kinases, cyclic nucleotide-dependent kinases, and casein kinase II, respectively).

In order to determine further the relationship of sphingosine-activated kinase(s) to other kinases, the effects of multiple kinase inhibitors were investigated. Genistein, a tyrosine kinase inhibitor (34), failed to inhibit sphingosine-induced phosphorylation (data not shown). H7, an isoquinolinesulfonamide which inhibits serine/threonine kinases with some specificity toward protein kinase C and cyclic AMP-dependent protein kinase (35), did not inhibit sphingosine-induced phosphorylation at concentrations up to 400 μM (data not shown). Chlorpromazine, a calmodulin-dependent kinase inhibitor (36, 37) and a protein kinase C inhibitor (38), failed to inhibit phosphorylation induced by sphingosine at concentrations up to 200 μM (data not shown).

Specificity of Sphingosine-induced Phosphorylations—Although sphingosine has been shown to have multiple in vitro biochemical targets, the specificities of these actions have not been well examined except for the inhibition of protein kinase C. Inhibition of protein kinase C by sphingosine requires the free amine and the hydrophobic hydrocarbon tail but shows
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little stereospecificity and no requirement for the hydroxyls at positions 1 and 3 (8, 39). Studies on epidermal growth factor receptor phosphorylation by sphingosine have not been evaluated for structural specificity and show conflicting results with the effects of ceramides (22, 40). Therefore, the ability of aliphatic amines and other sphingosine-related molecules to induce phosphorylation in Jurkat T cells were investigated.

Although sphingosine caused phosphorylation of multiple cytosolic substrates, stearylamine, a long chain amino base, did not cause any significant phosphorylation (Fig. 6). The closely related sphingolipids, sphingomyelin and C2-ceramide (N-acetyl-sphingosine) also did not cause any significant phosphorylation (Fig. 6). Psychosine (galactosylsphingosine), which differs from sphingosine in having a galactose in glycosidic linkage at the C-1 hydroxyl, induced modest phosphorylation that mimicked some, but not all, sphingosine effects (Fig. 6).

The more interesting and specific results were obtained with the stereoisomers of dihydrosphingosine (sphinganine). DL-Erythro-dihydrosphingosine was able to mimic the effects of sphingosine, although with less potency, whereas DL-threo-dihydrosphingosine was largely inactive (Fig. 6). Moreover, DL-erythro-dihydrosphingosine was significantly less potent than D-erythro-sphingosine, whereas DL-threo-dihydrosphingosine showed little activity over a wide concentration range (10–400 μM) (Fig. 7). These studies argue against nonspecific effects of sphingosine such as detergent effects or nonspecific lipid-lipid or lipid-protein interactions. These results demonstrate the specificity and stereoselectivity of sphingosine-induced phosphorylation.

**DISCUSSION**

The results from this study suggest two important conclusions. First, sphingosine appears to induce in vitro phosphorylation of endogenous substrates in cytosol of Jurkat T cells. Similar effects of sphingosine were observed in HL-60 cells (data not shown). Thus, sphingosine appears to activate protein kinase(s), although at this point it could not be determined whether these are direct or indirect effects. Second, the effects of sphingosine appear to be very specific, thus suggesting possible physiologic functions for sphingosine.

The nature of substrates phosphorylated by sphingosine is undetermined at this point. The 18-kDa substrate may be related to the recently cloned (41) 18-kDa phosphoprotein involved in leukemia proliferation and signal transduction. The identification of substrates should enhance the characterization and purification of sphingosine-activated kinases.

Multiple lines of evidence suggest that sphingosine may activate more than one protein kinase. First, some of the phosphorylations induced by sphingosine appear to be caused by protein kinase(s) with selectivity for ATP, whereas other phosphorylations appear to be caused by protein kinase(s)
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that can equally utilize ATP or GTP.\(^2\) Thus, these results suggest the involvement of at least two distinct protein kinases. Second, the dose response of phosphorylation by sphingosine is significantly different depending on the individual substrates. This is most consistent with the activation of different kinases that differ in their responsiveness to sphingosine. A less likely possibility would be differential interaction of sphingosine with the different substrates rendering them more susceptible to phosphorylation by the same kinase. This latter possibility is not consistent with the differences in phosphorylation obtained with ATP and GTP. Third, heparin shows differential effects in inhibiting and stimulating sphingosine-induced phosphorylation.

One of the kinases activated by sphingosine in Jurkat T cells may be related to casein kinase II. Jurkat T cells contain casein kinase II activity as determined by the ability of cytosol to phosphorylate the RRREEETEEE polypeptide substrates which has been proposed as a specific substrate for casein kinase II (42). Sphingosine was found to enhance this activity.\(^3\) That some of the phosphorylations induced by sphingosine may be mediated by casein kinase II is suggested by a number of features. First, casein kinase II utilizes equally ATP and GTP (43), and a subgroup of sphingosine-induced phosphorylation occurs equally with ATP and GTP. Second, casein kinase II is sensitive to heparin (27) as are some of the phosphorylations induced by sphingosine. On the other hand, polylysine, which is an in vitro activator of casein kinase II (32), caused phosphorylation of different substrates in Jurkat T cells. Polyamines, which also activate casein kinase II (44), did not induce any phosphorylation. This may indicate that sphingosine, polylysine, and polyamines activate different kinases or that they induce the same kinase to phosphorylate different sets of proteins. The latter possibility is consistent with the inability of polyamines to induce phosphorylation of the RRREEETEEE peptide (24) and with the selective activation of casein kinase II by polylysine to activate some, but not all, substrates (32, 33). Further studies (for example with immunoprecipitating antibodies) are required to resolve this issue.

The protein kinases activated by sphingosine (other than casein kinase II) appear to be distinct from established kinases with known activators. Phosphorylations induced by sphingosine are different from those induced by calcium, calcium PS/dioleoylglycerol, cyclic AMP, or cyclic GMP (Fig. 5). This suggests that sphingosine-activated kinases are distinct from calcium/calmodulin kinases, protein kinase C, and cyclic AMP- and cyclic GMP-dependent protein kinases. Characterization of these kinases would require availability of appropriate substrates to allow purification and identification. Whether sphingosine activates other kinases (such as mitogen-activated protein kinases and raf-1) is under investigation.

The other major implication of these studies originates with the strict structural requirements for induction of phosphorylation by d-erythro-sphingosine. These phosphorylations appear to be mimicked only by d-erythro-dihydrosphingosine. The stereoisomer, dL-three-dihydrosphingosine, is not active; neither are stearylamine, C\(_2\)-ceramide, or other sphingolipids, with the exception of psychosine (galactosylsphingosine) which only partially mimics sphingosine-induced phosphorylation. This high structural specificity is not shared by other targets for the actions of sphingosine identified so far, although such structural studies have been extensively performed only with protein kinase C (39).

These results suggest a possible physiologic role for d-erythro-sphingosine as an activator of endogenous protein kinase. This is supported by the high structural specificity and by the ability of sphingosine to cause phosphorylation of endogenous substrates. Moreover, in preliminary studies, we find that sphingosine is able to induce early phosphorylation in intact Jurkat cells.\(^3\) Although sphingosine is active at relatively low concentrations (10–20 \(\mu\)M), it is not known at this point how this corresponds to endogenous levels of sphingosine, since little is known about changes in sphingosine levels during signal transduction processes. Resting cellular levels of sphingosine appear to be in the range of 1 \(\mu\)M (2), therefore, significant elevations of sphingosine during signal transduction may be sufficient to activate endogenous protein kinases. Furthermore, it remains to be determined whether sphingosine activates the above cytosolic protein kinases in a soluble form or on a membrane surface in analogy with protein kinase C activation by diacylglycerol. Thus, these kinases may require additional cofactors for optimal activation by sphingosine, and therefore, the most effective concentrations of sphingosine are yet to be determined. Future studies at the biochemical level should allow further analysis of these questions.

The inability of threeo-sphinganine to mimic the actions of sphingosine and erythro-sphinganine provides a powerful tool for exploring mechanisms of signal transduction involving sphingosine-activated kinases. For example, effects that occur equally well with the threo- and erythro-sphinganines would be more likely to be caused by inhibition of protein kinase C or other effects, whereas effects specific to the erythro-isomer may be mediated by activation of sphingosine-activated protein kinases.

The lack of effect of ceramide on protein phosphorylation suggests that sphingosine is involved in a different signaling pathway than ceramide. These results are consistent with the previous observation that sphingosine, but not C\(_2\)-ceramide, can enhance phosphorylation of threonine 669 in the epidermal growth factor receptor (22). Although another study found that both sphingosine and C\(_8\)-ceramide equally phosphorylate threonine 669 in membrane fractions (40), these studies did not rule out the generation of sphingosine from C\(_2\)-ceramide in that system.

The ability of sphingosine to modulate the activity of various biochemical targets raises the intriguing possibility that sphingosine is a pleiotropic intracellular signaling molecule. A similar role has been established for calmodulin which regulates the activity of multiple enzymes. Such a role for sphingosine is consistent with its unique property as the only known positively charged lipid in the cell. Sphinganine (di-hydrosphingosine) exists in much smaller amounts (2), and other lysosphingolipids have been only detected in various sphingolipidosis but not in normal cells (1). Thus, sphingosine may emerge as a multifunctional intracellular regulator that is able to modulate the activity of many targets such as protein kinases, phosphatidate phosphohydrolase, diacylglycerol kinase, phospholipase D, and possibly other enzymes involved in cell regulation.

In conclusion these studies disclose specific biochemical effects of sphingosine and identify sphingosine-activated kinases. Our results strongly suggest a possible physiologic function for sphingosine in modulating the activity of multiple protein kinases.

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\(^2\) Partially purified sphingosine-activated protein kinases also show similar results with ATP and GTP (data not shown).

\(^3\) M. Yu. Pushkareva and Y. A. Hannun, unpublished observations.
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