

Sphingosylphosphocholine Reduces the Calcium Ion Requirement for Activating Tissue Transglutaminase*

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Tissue transglutaminase (tTG) catalyzes a Ca^{2+} -dependent transglutaminase reaction resulting in the formation of γ -glutamyl- ϵ -lysine bonds and is activated during apoptosis to catalyze the formation of apoptotic body. We investigate whether lipids that are membrane components and involved in cell signaling could modify the Ca^{2+} -dependent activation of tTG. We found that sphingosylphosphocholine (lyso-SM) was the only lipid to activate transglutaminase at low Ca^{2+} concentrations. In the presence of lyso-SM (125 μM), transglutaminase was detectable at 10 μM Ca^{2+} , whereas in the absence of lyso-SM, similar activity was obtained at 160 μM Ca^{2+} . Furthermore, in the presence of lipid vesicles lyso-SM retained the ability to enhance the Ca^{2+} -dependent activation of tTG. Lyso-SM did not significantly change the K_m for the glutamyl and primary amine substrates. However, the K_{act} for Ca^{2+} was reduced from 300 μM to 90 μM . Structure-function studies of lyso-SM analogs indicate that phosphocholine group on C1, the free amino group at C2 and a C4–C5 double bond are critical for the activation of transglutaminase activity. This is the first demonstration that a specific sphingolipid could enhance the activity of tTG and could play a role *in vivo* in activation of the tTG at physiologic Ca^{2+} levels.

Tissue transglutaminase (tTG)¹ is a calcium-dependent enzyme that plays an important role in many different intracellular and extracellular processes ranging from apoptosis to extracellular matrix formation (1–3). Calcium ions are required for the enzyme to adopt the proper conformation to bind glutamine and lysine residues within proteins or peptides (1–3). The intracellular levels of calcium are much lower than the

reported calcium levels required to obtain measurable transglutaminase (TGase) activity *in vitro*. This raises the question of whether there are intracellular cofactor(s) that can modify the sensitivity of tTG to Ca^{2+} . The tTG first forms a calcium-dependent thioester bond with select protein bound glutamines and releases ammonia (1–4). The thioester is a reactive enzyme-substrate intermediate that can then react rapidly with the primary amine group of either a lysine within a protein or a polyamine (1–4). The final reaction product contains an isopeptide bond that stabilizes inter- and intramolecular protein structure or generates a protein-polyamine conjugate that has unique biologic properties (1–4).

The binding of calcium ions reduces the affinity of tTG for GTP (5). GTP binding to tTG causes a conformational change in the protein which leads to a reduction in TGase activity (1–3). Based upon the affinity of the tTG for both calcium ions and GTP as well as the intracellular concentrations of these cofactors, the majority of the tTG *in vivo* is predicted to remain latent (1, 2, 6).

tTG is reported to associate with phospholipid bilayers *in vitro* (7, 8) and with cell membranes (9, 10). The recent discovery that a portion of the intracellular tTG is associated with the cytoplasmic domain of the cell membrane and can function as a G-protein coupled to the α_1 -adrenergic receptor suggests a role of membrane lipids in regulating tTG function (10). The C-terminal 8-amino acid residues (Leu⁶⁶⁵–Lys⁶⁷²) of tTG are involved in the interaction and activation of phospholipase C, which is critical for the completion of the α_1 -adrenergic receptor signaling event (11). More recently, it is reported that the association of tTG with membrane is stimulated by retinoic acid treatment (12). During apoptosis, the formation of the apoptotic envelope by tTG prevents the release of intracellular constituents, and recent studies demonstrate that apoptotic envelopes contain an increased content of isopeptide bonds and the tTG antigen (13–15).

The ability of tissue transglutaminases to bind phospholipids and the localization of tTG to the cell surface membrane compartment may play a role in cell receptor signaling and apoptosis. This led us to examine whether lipid cofactors could modulate the calcium-dependent cross-linking activity of tTG. We focused our attention on molecules of cell membrane components and molecules that are considered potential regulators of cell receptor function and calcium-dependent signaling events.

In this report, we demonstrate that sphingosylphosphocholine (lyso-SM) can serve as specific cofactor that reduces the calcium ion requirement for expression of intracellular tissue transglutaminase activity in the presence or absence of phospholipid vesicles. Lyso-SM is specific in its interaction with the tTG and induces a conformational change in the protein that selectively influences calcium binding, but does not influence

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¹ The abbreviation used are: tTG, tissue transglutaminase; lyso-SM, sphingosylphosphocholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; lyso-PC, lysophosphatidylcholine; lyso-PAF, lysophosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; TGase, the activity of tTG to carry out the calcium-dependent incorporation of [³H]putrescine or biotinylated pentylamine into *N,N*-dimethylcasein; BP, 5-biotin (amido) pentylamine.

the enzyme for its substrates. Lipid cofactors could play a major role in regulating tissue transglutaminase associated with the cytoplasmic domain of the cells surface. The specificity, biochemistry, and functional biologic role of the interaction of lyso-SM will be discussed.

EXPERIMENTAL PROCEDURES

Materials—Sodium salts of GTP were purchased from Sigma. [³H]Putrescine dihydrochloride (35.5 Ci/mmol) was purchased from NEN Life Science Products. Monoclonal antibody against guinea pig liver tTG (CUB 7401) was kindly provided by Dr. P. Birckbichler (16).

Lipids—Cholesterol, phosphatidylserine, phosphatidylethanolamine (PE, sodium salt), phosphatidylinositol (PI), phosphatidylcholine (PC), and sphingomyelin (bovine brain, SM) were purchased from Sigma. Lysophosphatidylcholine (lyso-PC) and lyso-platelet-activating factor (lyso-PAF) were purchased from Avanti. Sphingosine 1-phosphate was from Biomol, Plymouth Meeting, PA. Sphingosine stereoisomers were obtained in stereoselective synthesis starting from L- or D-serine, respectively (17). Dihydro sphingosine stereoisomers were prepared by hydrogenation over Pd/C (palladium on activated charcoal) of an adequate sphingosines. Ceramide stereoisomers were prepared by acylation of sphingosines with an adequate acid chlorides or anhydrides (18, 19). Lyso-SM was purchased from Sigma or Matreya Inc., Pleasant Gap, PA or was prepared by acid hydrolysis of bovine brain sphingomyelin, a procedure that has been established to yield ~7:3 D-erythro/L-threo mixture (20). D-Erythro and L-threo- stereoisomers of lyso-SM were kindly provided by Dr. Tigyi (21). Lyso-dihydro sphingomyelin (lyso-dihydro-SM) was prepared by hydrogenation of bovine brain lyso-SM and structure of the synthesized compounds was verified by mass spectroscopy and ¹H NMR analysis. All the lipids were dissolved in 95% ethanol except sphingosine 1-phosphate (in 80% methanol) and phosphatidylethanolamine (in chloroform).

Expression and Purification of Recombinant Human tTG—The conditions for growing *Escherichia coli* and the purification of the GST-tTG protein were performed as described previously (22). Protein concentrations were determined by Bradford's method (23) using bovine serum albumin as standards (Bio-Rad).

Effect of Lipids on Calcium-dependent Transglutaminase Activity—TGase activity was determined by quantitating the incorporation of 5-biotin (amido) pentylamine (BP) into *N,N'*-dimethylcasein-coated microtiter plate as described previously (24). The *K_m* for *N,N'*-dimethylcasein was determined using the [³H]putrescine incorporation assay (25). For the BP incorporation assay, lipids were added directly to the buffer containing 20 mM Tris-Cl, pH 7.5, 100 mM NaCl, and GST-tTG (2 μg/ml) to the desired concentration and applied to the *N,N'*-dimethylcasein coated microtiter plate, and the remaining procedures were the same as described previously (24). To determine the effect of lyso-SM on the activation of factor XIIIa, recombinant GST-factor XIII A-chains (4 μg/ml) were treated with 20 units/ml of thrombin at 37 °C for 15 min in a final volume of 50 μl to generate factor XIIIa as described previously (22). After the reaction, 10 μM thromstop (American Diagnostic, New York) was added and factor XIIIa generated was used immediately to test the effect of lyso-SM on TGase activity as described earlier. PC:PE (80:20) vesicles were generated immediately before use by mixing 8 mM PC and 2 mM PE and probe sonication on ice for 5 s for five times with a 20-s break in between.

Kinetic Analysis of the Effect of Lyso-SM on TGase Activity—To determine the *K_m* for the glutamine substrate, the concentration of *N,N'*-dimethylcasein was varied from 1.1 to 24.4 μM. To determine the *K_m* for primary amine substrate, the concentration of BP was varied from 16 to 1000 μM. The *K_{act}* for Ca²⁺ was determined using Ca²⁺ concentrations that varied from 40 to 5000 μM. The *K_m*, and *K_{act}* values were obtained using the Eadie-Hofstee plot (26).

Binding of Lyso-SM to tTG and Effect on Protease-dependent Degradation—The cleaved tTG was obtained by incubating GST-tTG with factor Xa (Hematologic Tech. Inc., Essex Jct., VT) (1%; w/w) overnight at 4 °C and applied to glutathione resins to remove the GST protein. The trypsin digestion experiment was performed by incubating 1 μg of cleaved tTG, 50 ng of trypsin (Calbiochem, high performance liquid chromatography-purified), and different concentrations of lyso-SM at 37 °C for 1 h. The reaction was stopped by the addition of SDS-PAGE loading buffer and products were separated on 9% SDS-PAGE and transferred to a nitrocellulose paper. The tTG antigen was probed using a monoclonal antibody against tTG (CUB 7401) and alkaline phosphatase-conjugated goat anti rabbit IgG and was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The intensity of full-length tTG was quantified using a scanning densitometer

TABLE I
Effects of lipids on the activation of transglutaminase activity at low calcium ion concentrations

	Transglutaminase activity ^a
tTG	1
Lyso-SM	22 ± 4
Cholesterol	1.4 ± 0.1
Phosphatidylserine	NA
PE	0.6 ± 0.1
Phosphatidylinositol	0.7 ± 0.1
PC	0.91 ± 0.04
D- <i>e</i> -Sphingosine	0.8 ± 0.8
L- <i>e</i> -Sphingosine	1.3 ± 0.6
L- <i>t</i> -Sphingosine	2.1 ± 1.3
D- <i>t</i> -Sphingosine	2.6 ± 1.4
D- <i>e</i> -Dihydro sphingosine	ppt
L- <i>e</i> -Dihydro sphingosine	0.6 ± 0.4
L- <i>t</i> -Dihydro sphingosine	0.13 ± 0.05
D- <i>e</i> -C ₂ ceramide	0.76 ± 0.06
DL- <i>t</i> -Dihydro C ₂ ceramide	1.9 ± 0.2
D- <i>e</i> -C ₆ ceramide	0.8 ± 0.1
D- <i>e</i> -C ₁₈ ceramide	0.3 ± 0.1
L- <i>e</i> -C ₁₈ ceramide	0.4 ± 0.1
L- <i>t</i> -C ₁₈ ceramide	0.4 ± 0.1
D- <i>t</i> -C ₁₈ ceramide	0.5 ± 0.1
Phytosphingosine	ppt
SM	1.0 ± 0.1
Arachidonic acid	2.1 ± 0.9

^a TGase activity of tTG at 100 μM Ca²⁺ was 5 mOD/min. All the transglutaminase activity measurements were performed at 100 μM Ca²⁺ and 125 μM lipids. NA, the chloroform that was used to dissolve PS inhibited the TGase activity. ppt, precipitates formed when these lipids were added to the reaction mixture.

(model GS300, Hoefer Scientific, San Francisco, CA). Control experiments using chromogenic substrate of trypsin, *N*-α-benzoyl-D-L-arginine *p*-nitroanilide (Sigma), that measured an increase in OD₄₀₅ reading in the presence of trypsin indicated that lyso-SM did not affect the trypsin activity at concentrations tested.

RESULTS

Effects of Lipids on Activation of TGase Activity at Low Calcium Ion Concentration—A wide variety of lipids, which are components of the cell membrane and are involved in all signaling, were examined to determine whether they altered the TGase activity of tTG at low calcium ion concentrations (Table I). In the initial screening, we tested the lipid's ability to activate latent tTG at suboptimum concentration of calcium ions (100 μM) with 1 mM lipids. The tTG showed <5% of maximum TGase activity at 100 μM calcium ions. We found that only lyso-SM produced a major increase in the activation of latent tTG. Therefore, we investigated the minimum concentration of lyso-SM required to activate the TGase activity of tTG (Fig. 1). We found that optimum TGase activity occurred at ~125 μM lyso-SM and then plateaued (Fig. 1). In the absence of lyso-SM, the maximum TGase activity was ~85% lower than that achieved with the addition of 125 μM lyso-SM to the reaction (Fig. 1). Table I summarizes the effects of the various lipids on calcium-dependent activation of the TGase activity. The results summarized in Table I at 125 μM lipids were similar to that obtained in the initial screening reaction with 1 mM amounts of the lipids.

We then examined the effect of varying the calcium ion concentration on TGase activity in the presence of these lipids. All lipids listed in Table I were tested and Fig. 2 shows representative of these results. We found that at 125 μM lyso-SM, measurable TGase activity occurred at 10 μM of Ca²⁺, whereas in the absence of lyso-SM there was no measurable TGase activity until Ca²⁺ was increased to 160 μM (Fig. 2). Furthermore, the TGase activity obtained at 160 μM of Ca²⁺ was ~16-fold lower than the activity measured in the presence of

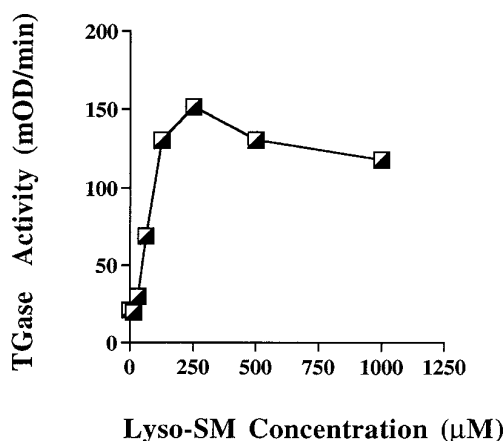


FIG. 1. Effects of lyso-SM on TGase activity of GST-tTG. GST-tTG (2 µg/ml) was incubated with 0–1000 µM lyso-SM, and the TGase activity was measured using BP incorporation assay in the presence of 200 µM CaCl₂ as described under “Experimental Procedures.”

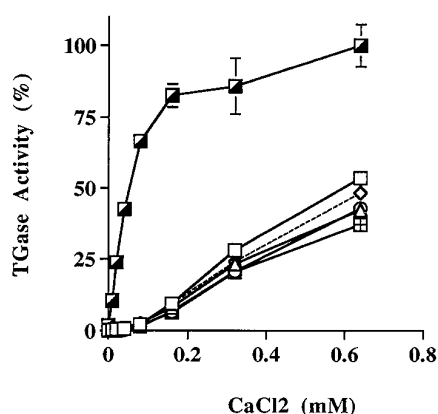


FIG. 2. Effect of Ca²⁺ and lyso-SM, D-e-C₂-ceramide, D-e-C₆-ceramide, phosphatidylcholine, and phosphatidylinositol on activation of TGase activity. GST-tTG (2 µg/ml) was incubated with 125 µM of lyso-SM, D-e-C₂-ceramide, D-e-C₆-ceramide, phosphatidylcholine, or phosphatidylinositol, and the TGase activity was measured using BP incorporation assay in the presence of 0 to 640 µM Ca²⁺ as described under “Experimental Procedures.” The 100% TGase activity represents 125 ± 15 mOD/min. □, no addition; ■, lyso-SM; △, D-e-C₂ ceramide; ▢, D-e-C₆-ceramide; ◇, phosphatidylcholine; ○, phosphatidylinositol.

lyso-SM (Fig. 2). The TGase activity obtained at 160 µM of Ca²⁺ was similar to the activity measured at 10 µM of Ca²⁺ in the presence of lyso-SM (Fig. 2). D-e-C₂ ceramide, D-e-C₆-ceramide and other lipids did not have effect on activation of TGase activity (Fig. 2).

Effects of Lyso-SM Analogs in Activating the TGase Activity of tTG—To examine the specificity of the lyso-SM effect further, other lyso-SM analogs, including lyso-PAF, lysophosphatidylcholine (lyso-PC), lyso-dihydro-SM, sphingosine 1-phosphate and lyso-SM stereoisomers (D-erythro- and L-threo-) were also tested (Table II and Fig. 3A). The results demonstrated that none of the lyso-SM analogs displayed any activation of tTG and the lyso-PAF had a slightly inhibitory effect on TGase activity (Fig. 3B). The ability of lyso-SM to serve as an activator of the tTG was lost when lyso-SM was replaced with sphingosine 1-phosphate in the calcium-dependent reaction, demonstrating that the choline group of lyso-SM was necessary in regulating the calcium dependent activation of the tissue TG. The free amino group was also necessary for the activation, since SM was not able to enhance the TGase activity (see Fig. 3A and Table II). Lyso-PC and lyso-PAF do not have free amino group at C2 and have different side chain at C3 and were not able to produce a calcium-dependent activation of

TABLE II
Specificity of lyso-SM on activation of tTG activity

	Transglutaminase ^a
tTG	1
L- <i>l</i> -Lyso-SM	19.6 ± 1.7
D- <i>e</i> -Lyso-SM	20.9 ± 0.2
Lysodihydrospingosylphosphocholine	2.2 ± 0.3
Lyso-PC	0.9 ± 0.1
Sphingosine 1-phosphate	0.7 ± 0.1
Lyso-PAF	0.2 ± 0.1
Glycerol 1-phosphate	0.7 ± 0.1
Phosphorylcholine	1 ± 0.5

^a TGase activity of tTG at 100 µM Ca²⁺ was 5 mOD/min. All the TGase measurements were performed at 100 µM CaCl₂ and 125 µM lipids.

the TGase activity. The double bond at C4–C5 was important for the reaction, since dihydrolyso-SM was not able to activate the TGase activity (Fig. 3B and Table II). Two stereoisomers of lyso-SM showed similar activation curve demonstrated that stereospecificity at C2, but not at C3, was important for the specificity (Table II). Glycerol 1-phosphate and phosphorylcholine were not able to activate the TGase activity, further demonstrating the specificity of lyso-SM in inducing the calcium-dependent activation of tTG (Table II).

Effects of Lyso-SM in Activating the TGase Activity in the Presence of PC:PE Vesicles—To examine whether lyso-SM retain an effect in activating the TGase activity in the presence of membrane lipids, TGase activity was measured in the presence of 100 µM of PC:PE (80:20) vesicles and 0–160 µM of lyso-SM (Fig. 4, A and B). We found that the TGase activity of tTG increased in the presence of increasing concentrations of lyso-SM. The activity was increased 310% by 160 µM lyso-SM (Fig. 4A). In addition, the calcium requirement to achieve half-maximal TGase activity was decreased in the presence of increasing concentrations of lyso-SM (Fig. 4B).

Effects of Lyso-SM on Activation of Recombinant Factor XIII A-Chains—The ability of lyso-SM to stimulate the calcium-dependent activation of the plasma transglutaminase, factor XIIIa, was also studied by the same assay as used for tTG. We found no enhancement in the calcium-dependent activation of plasma factor XIIIa using 125 µM of lyso-SM and Ca²⁺ concentration up to 1 mM.

Lyso-SM Reversed the Inhibition of TGase Activity by GTP—GTP is an important negative regulator of TGase activity and functions by binding to tTG (27). In the absence of lyso-SM, the IC₅₀ for GTP was found to be 4 µM (Fig. 5A). We found that lyso-SM could completely reverse GTP-dependent inhibition of TGase activity (Fig. 5B). When GTP was present (50 µM), the lyso-SM concentration required to achieve maximum calcium-dependent TGase activity was increased from 125 to 500 µM.

Analysis of Kinetic Properties—Kinetic analysis of the effect of lyso-SM on TGase function revealed that there was no significant effect of lyso-SM on the *K_m* for *N,N'*-dimethylcasein and BP. The *K_{act}* for calcium ions was reduced by ~3-fold (from 300 to 95 µM) in the presence of 125 µM of lyso-SM.

Analysis of tTG Conformational Changes by Trypsin Proteolysis—To demonstrate the binding of lyso-SM to tTG, the trypsin digestion pattern was analyzed by SDS-PAGE and immunoblotting using monoclonal antibody against tTG. There was a concentration-dependent protection of tTG from trypsin proteolysis when 31.5–500 µM lyso-SM was present in the reaction (Fig. 6). Control experiments using the chromogenic substrate of trypsin demonstrated that 31.5–500 µM lyso-SM did not inhibit the trypsin activity.

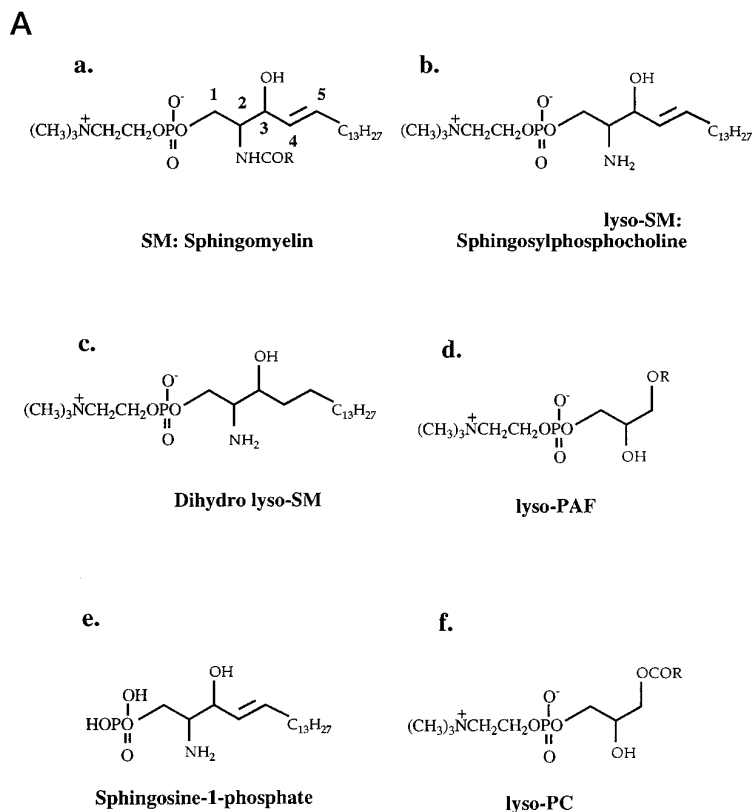
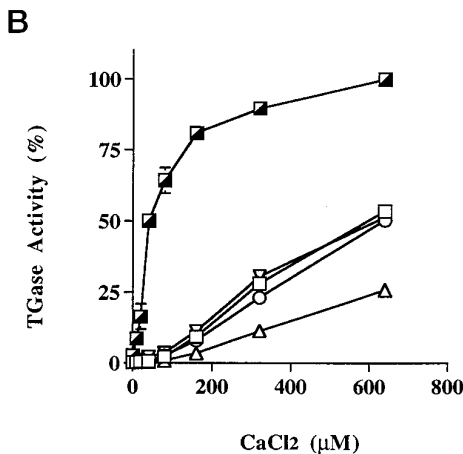


FIG. 3. A, structure of lyso-SM analogs. The following structures are shown: *panel a*, sphingomyelin (SM); *panel b*, lyso-SM; *panel c*, dihydro lyso-SM; *panel d*, lyso-PAF; *panel e*, sphingosine 1-phosphate; and *panel f*, lyso-PC. B, effect of Ca^{2+} on activation of TGase activity in the presence of lyso-SM analogs. GST-tTG ($2 \mu\text{g}/\text{ml}$) was preincubated with $125 \mu\text{M}$ lyso-SM, dihydrolyso-SM, lyso-PC, or lyso-PAF, and TGase activity was measured in the presence of 0 – $640 \mu\text{M}$ of Ca^{2+} . The 100% TGase activity represents 115 ± 5 mOD/min. \square , no addition; \blacksquare , lyso-SM; ∇ , dihydrolyso-SM; \triangle , lyso-PAF.



DISCUSSION

The TGase activity of tTG is considered to be an important intracellular and extracellular reaction during apoptosis, bone ossification, tissue repair, and tumor growth (1–3). The major proportion of tTG is found inside the cells, although it is also reported to exist in extracellular environment (1–3). Recently, tTG is also found to associate with the cell membrane and functions in intracellular signaling pathways (10, 28). The association of tTG with membrane suggests that lipid molecules could modulate enzymatic activities of tTG. In this study, we investigated the effect of membrane lipids that are involved in cell signaling on TGase activity.

Calcium ions are essential for tTG to serve as glutamine donor and lysyl acceptor in the transglutaminase reaction (1–4). The calcium ions promote the assembly of the active site pocket with cysteine 277 to form a thioester bond with the glutamyl-containing substrate (4). The second step of the reaction requires that the lysine residue fit into the active site pocket and align with the catalytic triad to catalyze the isopep-

ptide bond (4). Since lyso-SM could potentiate the ability of calcium ions to trigger TGase activity, we propose that lyso-SM binds specifically to the tTG and enhances the affinity of the enzyme for Ca^{2+} . This allows the enzyme to recognize glutamine substrates at lower calcium ion concentrations and increase in the reactivity of the thioester intermediate with the lysyl substrates. The binding of lyso-SM to tTG was demonstrated by changing the tTG from a protease sensitive to protease resistant conformation and the affinity of the enzyme for Ca^{2+} was increased by ~ 3 -fold.

Human plasma factor XIII A-chains share 39% amino acid identity with human tTG with the greatest identity regions in the active site and calcium binding site(s) regions (1–3). Despite similar, but not identical, structure homology, tTG and factor XIIIa are regulated by a unique set of cofactors. In the case of plasma factor XIII, fibrinogen plays an important role in the activation of factor XIIIa by reducing the Ca^{2+} concentration required for B-chain dissociation (29). For tTG, GTP, and Ca^{2+} are important regulators of TGase activity (27). The spec-

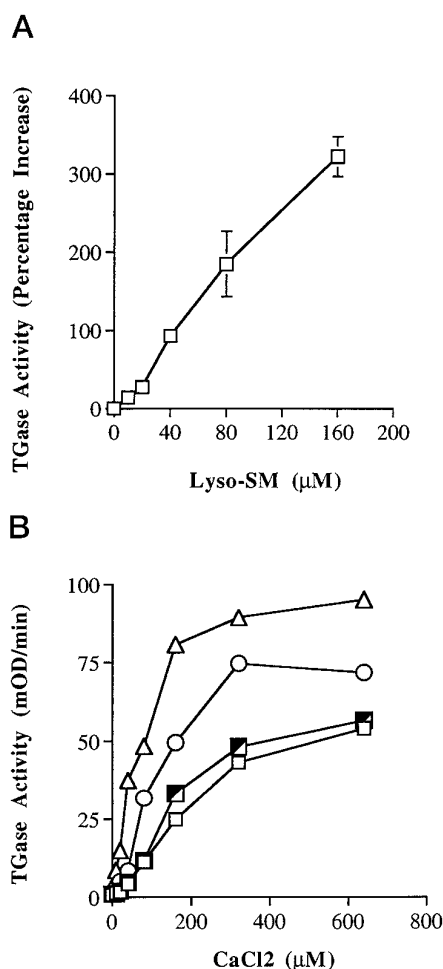


FIG. 4. Effect of lyso-SM on the activation of TGase activity in the presence of PC:PE vesicle. **A**, GST-tTG (2 μg/ml) was incubated with 0–160 μM of lyso-SM in the presence of 100 μM PC:PE (80:20) vesicle, and the TGase activity was measured using BP incorporation assay in the presence of 200 μM CaCl₂ as described under “Experimental Procedures.” □, TGase. **B**, GST-tTG (2 μg/ml) was incubated with 100 μM PC:PE (80:20) vesicle in the presence of 0, 5, 50, or 100 μM of lyso-SM, and TGase activity was measured in the presence of 0–640 μM of Ca²⁺. □, vesicles; ■, vesicles + 5 μM lyso-SM; ○, vesicles + 50 μM lyso-SM; △, vesicles + 100 μM lyso-SM.

ificity of lyso-SM for the tTG and not plasma factor XIII A-chains relates to their overall differences in protein structure and the fact that fibrin(ogen) functions as the extracellular cofactor that modifies the calcium-dependent activation of plasma factor XIII (29). Furthermore, since plasma factor XIII functions as an extracellular enzyme, there is no need for it to react with lyso-SM, since it can function at existing concentrations of extracellular calcium ions in the presence of fibrinogen and fibrin.

The specificity of lyso-SM binding to the tTG is documented by the unique ability of lyso-SM to promote calcium-dependent activation of the tTG. The lack of ability of dihydro lyso-SM and SM to activate TGase activity demonstrates the importance of the double bond (at C4–C5) and the free amino group (at C2), respectively. The stereospecificity at C2, but not at C3, of lyso-SM indicates that the nature occurring *erythro*-lyso-SM is equipotent as *L-threo*- diastereoisomer in the activation of TGase activity. The structure-activity studies demonstrated the requirement of phosphocholine group at C1, the free amino group at C2, and a C4–C5 double bond. There is no preference for *D-erythro*-(2*S*,3*R*) over *L-threo*-(2*S*,3*S*) stereoisomer, suggesting that stereospecificity at C3 is not important for activating TGase activity.

The lyso-SM could also interfere with GTP-dependent inhi-

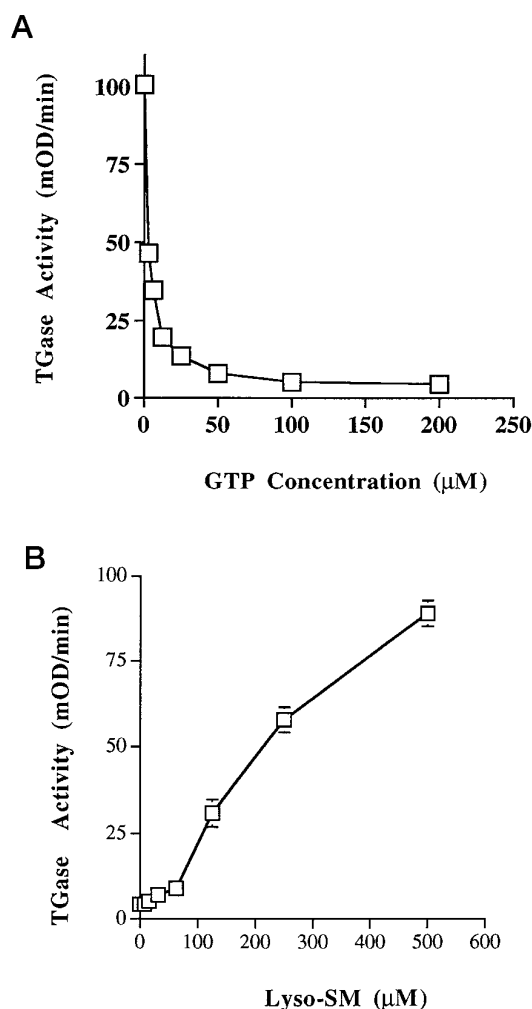


FIG. 5. **A**, effect of GTP on TGase activity. GST-tTG (2 μg/ml) was preincubated with 0–200 μM GTP, and the TGase activity was measured using BP incorporation assay in the presence of 1 mM Ca²⁺. **B**, effect of lyso-SM on TGase activity in the presence of GTP. GST-tTG (2 μg/ml) was preincubated with 50 μM GTP, and the TGase activity was measured in the presence of 0–500 μM lyso-SM and 1 mM Ca²⁺ using BP incorporation assay as described under “Experimental Procedures.”

hibition of TGase activity of tTG. Calcium ions reverse GTP inhibition by reducing the affinity of tTG to GTP (5). It is possible that lyso-SM binding allows the protein to adopt a conformation that has reduced affinity for GTP. This hypothesis is supported by the fact that the binding of lyso-SM increases the enzyme affinity for Ca²⁺.

The distribution and physiological concentration of lyso-SM inside the cells are not well established. Although we find that lyso-SM is very soluble in water (up to 0.5 M), its partitioning into membranes has not been determined. Importantly, in the presence of PC:PE vesicles, lyso-SM retained its effects in activating TGase activity of tTG. These results suggest that lyso-SM is capable of activating tTG in the context of membrane lipids. In preliminary studies, we found lyso-SM level was increased in cells undergoing apoptosis, suggesting that the intracellular lyso-SM concentration is also dependent on different physiological state of the cells.² The tTG is found in the membrane compartment as well as cytoplasm of mammalian cells (9–12). When the local concentration of lyso-SM reaches a critical concentration, it could promote the local activation of this latent intracellular enzyme.

² T.-S. Lai, A. Bielawska, Y. A. Hannun, and C. S. Greenberg, unpublished observations.

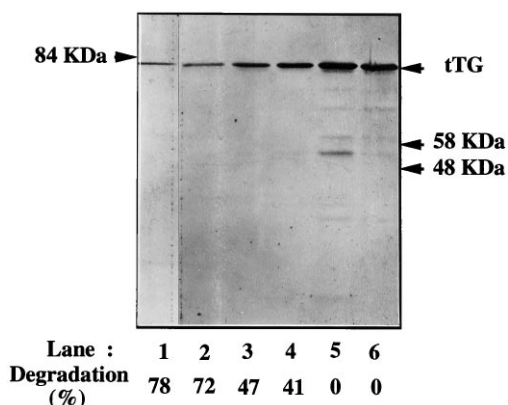


FIG. 6. Immunoblotting of tTG after trypsin proteolysis of tTG in the presence or absence of lyso-SM. The trypsin proteolysis was performed in the presence of tTG (1 μ g), 50 ng of trypsin, and varying concentrations (0–500 μ M) of lyso-SM at 37 $^{\circ}$ C for 1 h. The reaction products were separated by 9% SDS-PAGE, transferred to nitrocellulose paper, and the tTG was localized using monoclonal antibody against tTG as described under "Experimental Procedures." The concentration of lyso-SM used in lanes 1–6 were 0, 31.25, 62.5, 125, 250, and 500 μ M, respectively. The intensity of full-length tTG in each lane was scanned as described under "Experimental Procedures," and the intensity of tTG at lane 6 was used as 0% degradation.

The metabolic pathways that produce lyso-SM in mammalian cells are poorly defined (30). It is believed that lyso-SM is a metabolite of SM, possibly by the enzymatic activity of a sphingomyelin acylase, which is highly expressed in the stratum corneum from patients with atopic dermatitis (31). The same enzymatic activity was also found in *Pseudomonas sp.* TK4 and is called sphingolipid ceramide *N*-deacylase (32). SM is a ubiquitous constituent of plasma membrane and several metabolites derived from SM serve as intracellular second messengers are reported (33–35). Lyso-SM can induce an increase of Ca^{2+} concentration due to mobilization of Ca^{2+} from internal stores (36, 37). Recent studies by NMR spectroscopy indicate the existence of lyso-SM in normal and tumor tissues (38). It is proposed that lysosphingolipids play a pathogenic role in in-born metabolic disorders known as sphingolipidoses (39). Lyso-SM level was increased by 40-fold in spleen, liver, and brain of patients with Niemann-Pick type A disease who have sphingomyelinase deficiency, hepatosplenomegaly, and brain dysfunction (40). However, with the recognition of the sphingomyelin cycle (35, 41, 42) and the extensive lipid remodeling that occurs during apoptosis and cell receptor signaling events (35), it is interesting to speculate that a lyso-SM molecule could play a role in modulating the function of intracellular tissue transglutaminase in various biologic processes.

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REFERENCES

- Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) *FASEB J.* **5**, 3071–3077
- Aeschlimann, D., and Paulsson, M. (1994) *Thromb. Haemostasis* **71**, 402–415
- Ichinose, A., Bottenus, R. E., and Davie, E. W. (1990) *J. Biol. Chem.* **265**, 13411–13414
- Folk, J. E. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol.* **54**, 1–56
- Bergamini, C. M. (1988) *FEBS Lett.* **239**, 255–258
- Smethurst, P. A., and Griffin, M. (1996) *Biochem. J.* **313**, 803–808
- Harsfalvi, J., Arato, G., and Fesus, L. (1987) *Biochim. Biophys. Acta* **923**, 42–45
- Fesus, L., Horvath, A., and Harsfalvi, J. (1983) *FEBS Lett.* **155**, 1–5
- Korner, G., Schneider, D. E., Purdon, M. A., and Bjornsson, T. D. (1989) *Biochem. J.* **262**, 633–641
- Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M.-J., and Graham, R. M. (1994) *Science* **264**, 1593–1596
- Feng, J.-F., Rhee, S. G., and Im, M.-J. (1996) *J. Biol. Chem.* **271**, 16451–16454
- Singh, U. S., and Cerione, R. A. (1996) *J. Biol. Chem.* **271**, 27292–27298
- Nemes, Z., Friis, R. R., Aeschlimann, D., Saurer, S., Paulsson, M., and Fesus, L. (1996) *Eur. J. Cell Biol.* **70**, 125–133
- Fesus, L., Zsuzsa, S., and Uray, I. (1995) *J. Cell. Biochem. Suppl.* **22**, 151–161
- Fesus, L., Davies, P. J. A., and Piacentini, M. (1991) *Eur. J. Cell Biol.* **56**, 170–177
- Birckbichler, P. J., Upchurch, H. F., Patterson, M. K., Jr., and Conway, E. (1985) *Hybridoma* **4**, 179–86
- Harold, P. (1988) *Helv. Chim. Acta* **71**, 354–362
- Bielawska, A., Crane, H. M., Liotta, D., Obeid, L. M., and Hannun, Y. A. (1993) *J. Biol. Chem.* **268**, 26226–26232
- Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. M., and Hannun, Y. A. (1995) *J. Biol. Chem.* **270**, 2047–2052
- Van Veldhoven, P. P., Foglesong, R. J., and Bell, R. M. (1989) *J. Lipid Res.* **30**, 611–616
- Bunemann, M., Liliom, K., Brandts, B. K., Pott, L., Tseng, J. L., Desiderio, D. M., Sun, G., Miller, D., and Tigyi, G. (1996) *EMBO J.* **15**, 101–108
- Lai, T.-S., Achyuthan, K. E., Santiago, M. A., and Greenberg, G. S. (1994) *J. Biol. Chem.* **269**, 24596–24601
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Slaughter, T. F., Achyuthan, K. E., Lai, T. S., and Greenberg, C. S. (1992) *Anal. Biochem.* **205**, 166–171
- Miraglia, C. C., and Greenberg, C. S. (1985) *Anal. Biochem.* **144**, 165–171
- Eisenthal, R., and Danson, M. J. (1993) in *Enzyme Assays*, Oxford University Press, Walton Street, Oxford
- Achyuthan, K. E., and Greenberg, C. S. (1987) *J. Biol. Chem.* **262**, 1901–1906
- Singh, U. S., Erickson, J. W., and Cerione, R. A. (1995) *Biochemistry* **34**, 15863–15871
- Credo, R. B., Curtis, C. G., and Lorand, L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 4234–4237
- Bell, R. M., Hannun, Y. A., and Merrill, A. H., Jr. (eds) (1993) *Sphingolipids Part B: Regulation and Function of Metabolism*, Academic Press Inc., San Diego, CA
- Murata, Y., Ogata, J., Higaki, Y., Kawashima, M., Yada, Y., Higuchi, K., Tsuchiya, T., Kawaminami, S., and Imokawa, G. (1996) *J. Invest. Dermatol.* **106**, 1242–1249
- Ito, M., Kurita, T., and Kita, K. (1995) *J. Biol. Chem.* **270**, 24370–24374
- Hannun, Y. A., Obeid, L. M., and Wolff, R. A. (1993) *Adv. Lipid Res.* **25**, 43–64
- Hannun, Y. A. (1994) *J. Biol. Chem.* **269**, 3125–3128
- Hannun, Y. A., and Obeid, L. M. (1995) *Trends Biochem. Sci.* **20**, 73–77
- Seufferlein, T., and Rozengurt, E. (1995) *J. Biol. Chem.* **270**, 24343–24351
- Spiegel, S., and Milstien, S. (1995) *J. Membr. Biol.* **146**, 225–237
- Seijo, L., Merchant, T. E., van der Ven, L. T. M., Minsky, B. D., and Glonek, T. (1994) *Lipids* **29**, 359–364
- Hannun, Y. A., and Bell, R. M. (1987) *Science* **235**, 670–674
- Berger, A., Rosenthal, D., and Spiegel, S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5885–5889
- Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989) *J. Biol. Chem.* **264**, 19076–19080
- Mathias, S., Dressler, K. A., and Kolesnick, R. N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10009–10013