SPATIAL AND FUNCTIONAL HETEROGENEITY OF SPHINGOLIPID-RICH MEMBRANE DOMAINS*

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Little is known about the organization of lipids in biomembranes. Lipid rafts are defined as sphingolipid- and cholesterol-rich clusters in the membrane. Details of the lipid distribution of lipid rafts are not well characterized, mainly because of a lack of appropriate probes. Ganglioside GM1-specific protein, cholera toxin, has long been the only lipid probe of lipid rafts. Recently it was shown that earthworm toxin, lysenin, specifically recognizes sphingomyelin-rich membrane domains. Binding of lysenin to sphingomyelin is accompanied by the oligomerization of the toxin that leads to pore formation in the target membrane. In this study, we generated a truncated lysenin mutant that does not oligomerize and thus is non-toxic. Using this mutant lysenin, we showed that plasma membrane sphingomyelin-rich domains are spatially distinct from ganglioside GM1-rich membrane domains in Jurkat T cells. Like T-cell receptor (TCR) activation and cross-linking of GM1, cross-linking of sphingomyelin induces calcium influx and ERK phosphorylation in the cell. However, unlike CD3 or GM1, cross-linking of sphingomyelin did not induce significant protein tyrosine phosphorylation. Combination of lysenin and sphingomyelinase treatment suggested the involvement of G-protein coupled receptor in sphingomyelin-mediated signal transduction. These results thus suggest that the sphingomyelin-rich domain provides a functional signal cascade platform that is distinct from those provided by TCR or GM1. Our study therefore elucidates the spatial and functional heterogeneity of lipid rafts.

Whereas the bilayer organization of biomembranes can be reconstituted in artificial liposomes with a simple lipid composition, biological membranes contain thousands of different lipid species, whose cellular distribution is stringently controlled (1). This complex distribution of lipids suggests that the targeting of lipids is highly regulated and that cells require a complex lipid supramolecular organization within their membranes. In the plasma membrane, most of the sphingolipids reside in the outer leaflet whereas phospholipids containing the amino groups, phosphatidylethanolamine and phosphatidylserine are located in the cytoplasmic surface (2, 3). Lateral segregation of lipids on the plasma membrane is also reported. A typical
example is a lipid heterogeneity between the apical and the basolateral membranes of epithelial cells (4). Lipid diffusion barrier between axons and dendrites (5, 6) is another example.

The existence of submicron scale heterogeneity of plasma membrane lipids is a matter of debate. In particular, the proposed existence of specific types of microdomains, called lipid rafts that are enriched in cholesterol and sphingolipids attracts much attention (7, 8). Lipid rafts are suggested to play important roles in a number of cellular processes as diverse as signal transduction, membrane traffic and pathogen entry (9). Detergent insolubility has been an operational definition of lipid rafts in cell membranes (10). However, recent results indicate that detergent resistance does not correlate with the existence of lipid domains (11). Therefore, a more direct approach is required to understand the detailed organization of membrane lipids. At present, this approach is hindered by the lack of appropriate probes.

Sphingomyelin (SM) is a major sphingolipid of mammalian cells. Previously it was shown that the earthworm toxin lysenin specifically recognizes SM (12-14). Recently we have revealed that lysenin recognizes SM only when SM forms aggregates or domains (15, 16). Using these characteristics, we showed that SM exists as aggregates of at least a few lipid molecules in most of the membranes. We have also shown that the distribution of SM is different among different cell types as well as between different membrane domains in the same cell. One big problem of using lysenin as an SM probe is its toxicity. In the present study, we have developed a non-toxic mutant of lysenin that recognizes SM. This mutant lysenin made it possible to examine the distribution of SM in living cells. Using mutant lysenin and sphingomyelinase, we suggest that SM-rich domains in the plasma membrane are spatially and functionally segregated from the ganglioside GM1-rich membrane domains.

MATERIALS AND METHODS

Antibodies and Reagents - Anti maltose-binding protein (MBP) antiseras, pC2X vector, amylose resin were purchased from New England Biolabs. Polyclonal anti-Glutathione S-Transferase (GST) antibody, pGEX vectors, GST sepharose 4B were purchased from Roche. Monoclonal antibody against GST was a gift from Dr. A. Kojima (17) (NIID, Japan). Alexa Fluor™ 488-conjugated antibody was prepared using protein labeling kit (Molecular Probes). Sheep anti-mouse immunoglobulin and goat anti-choleragenoid (cholera toxin B subunit) were from Chemicon and List Biological Lab, respectively. Secondary antibodies and Cholera toxin B subunit (CTxB) conjugated with various Alexa Fluor fluorophores were from Molecular Probes. Sphingomyelinase (SMase) from Bacillus cereus was purchased from Higetashoyu and Sigma. CTxB, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lysophosphatidic acid (LPA) were from Sigma. Anti-panERK and PKC antibodies were purchased from Transduction Lab. Anti-LAT and phosphor- specific antibodies were from Cellular Signaling. Anti-CD3 antibody was from Pharmingen. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoaziazol-4-yl) (N-NBD-PE), Brain sphingomyelin (SM), egg phosphatidylcholine (PC), liver phosphatidylethanolamine (PE), brain phosphatidylserine, liver phosphatidylinositol, phosphatidic acid, brain ceramide and brain cerebroside were purchased from Avanti Polar Lipids. Sphingosine, D-erythro-sphingosine-1-phosphate and sphingosylphosphorylcholine were from Biomol.
Research Laboratories, Inc. PD985059 was from Promega. U73122 and phorbol myristate acetate (PMA) were from CalBiochem.

Expression and Isolation of Recombinant Lysenins - The cDNA of lysenin and polyclonal anti-lysenin antibody were generous gifts from Drs. Y. Sekizawa and H. Kobayashi of Zenyaku Kogyo Inc (18). The cDNA fragments were amplified by the polymerase chain reaction (PCR) and subcloned into pC2X or pGEX-4T. The recombinant proteins fused to MBP or GST were expressed in Escherichia coli (E. coli) JM 109 or BL 21, purified using amylose resin or GST-sepharose 4B according to the manufacturer’s instruction. For deletion mutants, forward-primers containing BamHI site and reverse primers containing HindIII site were used for PCR, and the obtained fragments were cloned into pC2X or pGEX after digesting with the corresponding enzymes. Amplified cDNA sequences from wild type and mutants were confirmed using BigDye Terminator and Genetic Analyzer ABI Prism™ 310 (PE Applied Biosystems). pRSET-Venus was a gift from T. Nagai and A. Miyawaki (RIKEN, Japan) (19). To obtain recombinant His-Venus-non toxic lysenin (HV-NT-Lys), PCR-amplified cDNA fragment corresponding to aa161-297 was inserted into the 3’-terminus of the Venus sequence. The monomer-Venus was prepared by replacing amino acid A206 to K as described previously (20). His-tagged proteins were expressed in E.coli, and lysed and purified by AKTAprime (Pharmacia) according to the manufacturer’s protocol.

ELISA (Enzyme-linked immuno-sorbent assay) - ELISA was performed as described (21).

Kinetic analysis of GST-NT-Lys binding to SM – Binding of GST-NT-Lys to SM was quantified using a BIAcore™ system instrument (Pharmacia Biosensor AB) as described (12).

Liposome binding assay - Multilamellar liposomes were prepared as described (12, 13) and incubated with recombinant proteins at 37 °C for 30 min. 0.5 ml of the suspension was mixed with 1ml of 2.1 M sucrose in 10mM Hepes (pH 7.2) and loaded at the bottom of an ultracentrifuge tube, and overlaid sequentially with 1.5 ml 1.2 M sucrose and 1 ml 0.8 M sucrose. The gradient was centrifuged for 20 h at 90,000 x g at 4°C using Beckman-Coulter Optima™ MAX-E Ultracentrifuge. Fractions (0.6 ml each) were collected from the top of the tube and were subjected to Western blotting. Fluorescence intensity of N-NBD-PE was also measured to monitor the position of liposomes in the gradient.

Cells and cell culture - Niemann-Pick type A (NPA) fibroblasts and Jurkat cell line were gifts from Drs. Hitoshi Sakuraba and Michiyuki Matsuda, respectively. NPA cells were maintained in Ham-F10 medium plus 10% fetal bovine serum (FBS, GIBCO-BRL) and penicillin/streptomycin. The mutant Jurkat cell lines were purchased from ATCC. The wild type and mutant Jurkat cell lines were maintained in RPMI1640 (Sigma) supplemented with 10% FBS and penicillin/streptomycin.

Immunofluorescence - NPA fibroblasts were plated onto cover glass. After 24-48 hours, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, permeabilized with 50 µg/ml digitonin for 10 min, pre-incubated with 0.2 % gelatin in PBS for 20 min at room temperature, and then incubated with the recombinant protein for 60 min at 4°C. Cells were fixed again, incubated with anti-GST antibody for 30 min followed by the addition of anti-rabbit antibody conjugated to Alexa 488 and mounted in Mowiol. For SMase treatment, NPA cells were incubated with 10 mU/ml of recombinant Bacillus cereus sphingomyeliasise in PBS for 1 h at 37 °C.

Jurkat cells were incubated with 50 µg/ml of His-Venus-lysenin (HV-NT-Lys) or
His-monomer VenusA206K-non toxic lysenin (HmV-NT-Lys), in the presence of 10 µg/ml of CTxB conjugated with Alexa594 on ice for 30 min. Cells were washed with PBS and re-suspended in OPTI-MEM (Gibco-BRL). Cells were then placed onto a glass-bottom culture dish pre-coated with poly-L-lysine (MatTEK). Confocal images were obtained using an LSM510 confocal microscope equipped with Plan-Apochromat 100x (1.4 n. a.) objective, and processed with an LSM Image Browser (Zeiss).

**MTT assay** - Cell viability was assessed by the MTT assay (22). 1 x 10^6 Jurkat cells were washed with PBS twice, incubated with various concentrations of GST or GST-NT-Lys for 30 min at room temperature, resuspended in RPMI1640 without sera, and then cultured in the presence of 0.5 mg/ml of MTT at 37 ºC. After 4 h incubation, MTT solution was removed and cells were disrupted with DMSO. The absorbance at 595 nm was measured with a spectrophotometer.

**Fluorescence-activated cell sorter (FACS) analysis** - In Fig.2, 1 x 10^6 Jurkat cells were washed twice with PBS, incubated with 50 µg/ml of GST or GST-NT-Lys for 30 min at room temperature, washed and further incubated with or without 10 µg/ml of monoclonal anti-GST antibody, followed by goat anti-mouse IgG antibody conjugated with Alexa-488. In Fig.3, cells were labeled with Alexa 488-conjugated CTxB or HmV-NT-Lys or HmRFP-NT-Lys at 4 ºC to avoid rapid internalization of CTxB. HmRFP-NT-Lys was prepared from monomeric red fluorescent protein (mRFP) (23) and specific binding to SM was confirmed (data not shown). Flow cytometry was performed on a Beckman Coulter EPICS XL. Data analyses were performed using the software package EXPO32.

**Electron microscopy** - Plasma membrane was prepared from Jurkat cells as described previously (24, 25). Briefly, cells were first labeled with HmV-NT-Lys and biotinylated cholera toxin B fragments at 4 ºC, and then fixed with 4% paraformaldehyde and 0.02% glutaraldehyde for 10 min at 4 ºC. The fixed cells were quenched with 0.1M NH₄Cl and blocked with 2% bovine serum albumin. The fixed cells were labeled with anti-GFP rabbit polyclonal antibody at 4 ºC, followed by labeling with goat anti-rabbit IgG-5nm gold and goat anti-biotin IgG-10nm gold at 4 ºC. The labeled cells were attached to nickel EM grids that had been coated with Formvar and carbon and treated with poly-L-lysine (0.8 mg/ml for 30 min, followed by 10 s dH₂O rinse and air drying). The cell-attached EM grids were covered with poly-L-lysine coated coverslip and pressure was applied to the coverslip for 20 s by bearing down with a rubber cork. The coverslips were lifted, leaving fragments of the cell membranes adherent to the grids. Membranes were fixed in 2% glutaraldehyde for 10 min and further fixed for 10 min with 1% OsO₄ in dH₂O. Samples were then processed for 10 min in 1% aqueous tannic acid, followed by two 5-min rinses with dH₂O, 10 min with 1% aqueous uranyl acetate and two 1-min rinses with dH₂O. The grids were air-dried and examined and photographed at x50,000 using a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan) at 80kV.

**Image analysis** - Twenty EM negatives were digitized with an image scanner (EPSON GT-X700). The scanned images were contrast-adjusted and trimmed at 1500x1500nm with Photoshop 6.0 software. The x-y coordinates of each gold particle were obtained with ImageJ public domain software. Obtained data were analyzed with Ripley’s K-function using SPSS 2.0 software.

**Intracellular free Ca²⁺ measurement** - Jurkat cells were harvested from the growth medium and 1 x10^7 cells were resuspended in 1ml of RPMI1640 containing 0.5% BSA. Fura-2AM (Dojin Kagaku)
was added to the cells at the final concentration of 3 μM. After 30 min at 35 °C, the cells were washed twice with Fura2-solution [0.1% BSA, 25mM Hepes (pH7.0), 140 mM NaCl, 5 mM KCl, 1mM NaHPO₄, 0.1% glucose, 0.5 mM MgCl₂, and 1 mM CaCl₂], and resuspended in 3 ml of the same solution. The ratio of the light intensity emitted at 505 nm upon dye excitation at the two wavelengths, 340 and 380 nm at 35 °C was measured every other second using a FP-6500 spectrofluorometer equipped with a thermostatic cell holder (Jasco, Tokyo, Japan). When indicated as EGTA buffer, EGTA (pH 8.0) was added to the buffer at the final concentration 1 mM. For SMase treatment, SMase (1.25 U/mL) was added during Fura-2AM loading.

Sphingomyelin cross-linking and Western-blotting - 3 x 10⁶ Jurkat cells were washed with PBS twice, incubated with 50 μg/ml of GST or GST-NT-Lys for 30 min at room temperature, then incubated with or without 10 μg/ml of monoclonal anti-GST antibody. Cells were suspended in RPMI medium and replated onto 12-well plates pre-coated with anti-mouse Ig antibody as described previously (26). After the indicated period, an equal volume of cell lysis buffer containing 10 mM Tris-hydrochloride (pH 7.5), 10 mM EDTA, 150 mM NaCl, 2mM Na₃VO₄, 20 mM NaF, 2% TritonX-100 was added. Samples were subjected to SDS-PAGE and Western-blotting as described (27).

Transfection - 3-5x 10⁶ cells were suspended in Nucleofector™ solution V and then 3μg of pLM21-H-Ras N17, which coded for a dominant negative form of H-Ras with mRFP under IRES sequence, was introduced using program C-16 of the nucleofector device (Amaxa). Seventy-two hours later, the cells were utilized for analysis.

RESULTS

Characterization of deletion mutants of lysenin - In the previous study, we employed native lysenin or full-length recombinant lysenin to characterize the organization of sphingomyelin (SM) on plasma membranes (13, 15). One drawback of using full-length lysenin is its toxicity. Recently we examined the role of tryptophan residues of the protein on the recognition of SM and the cell toxicity (14). Systematic tryptophan to alanine mutation resulted in either the loss of both SM recognition and cell killing activities or no significant alteration of both activities. In the present study we prepared a series of deletion mutants of lysenin and measured their SM-binding and hemolytic activities. Fig. 1A indicates the list of these deletion mutants. Mutants were tagged with maltose-binding protein (MBP) at their N-termini and expressed in E. coli. The cytotoxicity of the MBP-conjugated mutants was analyzed by measuring hemolysis against sheep erythrocytes whereas the recognition of SM was assessed by immunofluorescence labeling of fibroblasts from a Niemann-Pick type A (NPA) patient (14). NPA cells are deficient in acid sphingomyelinase (SMase) activity and therefore accumulate SM in late endosomes/lysosomes. As shown in Fig. 1A, deletion of C-terminus amino acids diminished the recognition of SM by lysenin. In contrast, lysenin could bind SM even after removal of N-terminus amino acids. The minimal fragment that could recognize SM contained amino acids 161-297 of lysenin. Hereafter we refer this minimal peptide as non-toxic lysenin (NT-Lys). It is noteworthy that all deletion mutants lost their hemolytic activity.

Changing the tag from MBP to glutathione-S-transferase (GST) did not alter the activity of the lysenin mutants. In Fig. 1B and C, the binding specificity of GST-lysenin and the deletion mutant GST-NT-Lys was compared by ELISA. As shown in Fig. 1B, GST-NT-Lys has
lower affinity than GST-lysenin. However, both GST-lysenin and GST-NT-Lys selectively bound SM. Slightly positive signals were obtained with sphingosine 1-phosphate (SIP) and sphingosylphosphorylcholine (SPC) in both GST-lysenin and GST-NT-Lys. Other phospholipids, sphingolipids, or glycolipids bound neither GST-lysenin nor GST-NT-Lys. GST alone did not bind to any lipids. The kinetic parameters of GST-NT-Lys binding to SM were then determined using BIAcore™ system and the results were compared to those published for earthworm lysenin (12) (Table I). GST-NT-Lys and native lysenin showed comparable on-rate of binding to SM. In contrast, dissociation of GST-NT-Lys was 100 times faster than that of native lysenin. This gives 36 times difference of overall $K_D$. To overcome this difference, we used relatively high concentrations of NT-lysenins compared to native lysenin throughout experiments. In Fig. 1D, we examined the selective binding of GST-NT-Lys to SM using artificial membranes. Multilamellar liposomes composed of SM/phosphatidylcholine (PC)/cholesterol (Chol), SIP/PC/chol, or SPC/PC/Chol were prepared. All liposomes contained fluorescent phosphatidylethanolamine as a non-exchangeable lipid marker. After incubation with GST or GST-NT-Lys, liposomes were separated from unbound proteins by a flotation gradient. GST-NT-Lys was floated up together with SM/PC/Chol liposomes whereas GST alone remained at the bottom of the gradient. The GST-NT-Lys probe weakly bound to SIP/PC/Chol liposomes. GST-NT-Lys did not float up when SPC/PC/Chol liposomes were employed. These results indicate that GST-NT-Lys selectively binds SM-containing membranes. Native and full-length lysenins oligomerize in the presence of sphingomyelin (13). The oligomerization is accompanied by pore formation in target membranes. Unlike native lysenin, SM did not induce oligomerization of GST-NT-Lys as shown in Fig. 1E. The recognition of cellular SM by GST-NT-Lys was then examined by measuring the binding of GST-NT-Lys to fixed and permeabilized NPA fibroblasts. Like native and non-truncated lysenin, GST-NT-Lys labeled intracellular organelles that are co-localized with tetraspanin CD63, which is enriched in lysosomes/late endosomes (28-30) (Fig. 1F). GST alone did not stain the cells.

In Fig. 2A, the toxicity of mutant lysenin against Jurkat cells was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Although GST conjugated wild type lysenin showed toxicity at as low as 0.2 µg/ml, GST-NT-Lys did not significantly affect viability of cells up to 50 µg/ml. This result, together with the result of Fig. 1, indicates that GST-NT-Lys is not toxic to nucleated cells as well as red blood cells. Low toxicity of NT-Lys mutant made it possible to specifically label cell surface SM of living cells. In Fig. 2B, living Jurkat cells were labeled with GST-NT-Lys, followed by incubation with anti-GST antibody and corresponding Alexa 488 conjugated secondary antibody. FACS analysis indicated a significant increase of fluorescence intensity under these conditions (Fig. 2B, GST-NT-Lys). In contrast, cells were not significantly labeled when anti-GST alone and fluorescence-conjugated secondary antibodies were added (Fig. 2B, Ab alone) or when GST was used instead of GST-NT-Lys (Fig. 2B, GST). Fluorescence was also abolished when cells were pretreated with bacterial SMase. These results indicate that GST-NT-Lys recognizes cell surface SM in living Jurkat cells.

**Mutant lysenin reveals the spatial heterogeneity of cell surface lipid raft components** - Cholera toxin subunit B (CTxB) is a well-known lipid marker for...
labeled GM1 and SM by head group (not shown) and the distribution of lipids was strongly enhanced by pretreatment with NT-Lys (Fig. 3D). Cells were stained with HmRFP-NT-Lys and biotinylated CTxB at low temperature followed by fixation with paraformaldehyde and glutaraldehyde. Cells were further incubated with anti-GFP antibody and anti-NT-Lys IgG (5 nm-gold) to visualize NT-Lys-rich membranes, and anti-biotin IgG (10 nm-gold) to mark GM1. Both SM and GM1 were distributed in a co-localized manner (Fig. 3D). The prior binding of cholera toxin did not affect the binding of GM1 and SM. These results, together with our previous observation, indicate that whereas lysenin recognizes specific subset of SM molecules, CTxB recognizes GM1 irrespective of the distribution of the lipids. Since pretreatment of one toxin did not affect the binding of the other, either they do not compete binding or the lipids are spatially segregated.

Our results indicate that while non-toxic, NT-Lys keeps the characteristics of earthworm lysenin and efficiently labels cell surface SM-rich membrane domains if appropriate concentration is employed. When plasma membranes of living Jurkat cells were doubly labeled either with NT-Lys and CTxB or with monomeric HmRFP-iNT-Lys and CTxB, cells were evenly stained with both green and red fluorescence (Fig. 4A), supporting the idea that the size of the lipid domains are below the resolution of the fluorescence microscope. Distribution of GM1 and SM were further examined on fixed-2D sheets of plasma membrane, ripped off from cells directly onto EM grids (24,25, 33-35) (Fig. 4B). Membrane fragments trapped on the EM grids are composed largely of flat area of cell membranes. They are easily distinguished from contaminated structure containing ER or Golgi membrane or cytoskeleton under electron microscope. We selected the flat area without contamination for the present image analysis (25, 35). Cells were doubly labeled with HmRFP-NT-Lys and biotinylated CTxB at low temperature followed by fixation with paraformaldehyde and glutaraldehyde. Cells were further incubated with anti-GFP antibody and anti-rabbit IgG (5 nm-gold) to visualize SM-rich membranes, and anti-biotin IgG (10 nm-gold) to mark GM1. Both SM and GM1 were distributed in a co-localized manner (Fig. 3D). The prior binding of cholera toxin did not affect the binding of GM1 and SM. These results, together with our previous observation, indicate that whereas lysenin recognizes specific subset of SM molecules, CTxB recognizes GM1 irrespective of the distribution of the lipids. Since pretreatment of one toxin did not affect the binding of the other, either they do not compete binding or the lipids are spatially segregated.

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over the whole membrane. The gold patterns were further analyzed by using Ripley’s K-function (24, 25). Ripley’s K-function evaluates all interparticle distances over the study area and compares the observed distribution of gold particles with that expected from complete spatial randomness (CSR). This analysis focuses the second-order properties of gold distribution and has the advantage that it analyzes spatial structure at multiple ranges simultaneously. The solid horizontal line at 0 in Fig. 4C gives the expected value for L(r)-r under CSR. The blue lines (sim(max) and sim(min)) give a 99% confidence envelope for CSR from 100 simulations. In Fig. 4C, the experimental values for L(r) – r (red lines) are plotted relative to reference values (blue lines). K-function analysis of SM distribution shows that the gold pattern is clustered, i.e. the curve shows significant positive deviation from the blue lines. The maximum deviation occurs at a radius of 60-80 nm, indicating that SM forms domains with radius of 60-80 nm. GM1 also forms domains with similar radius. In contrast, the pair-wise values for SM and GM1 fall within the blue lines (Fig. 4C, SM/GM1), indicating that colocalization of SM-rich domains and GM1-rich domains is not significant. Our results indicate that plasma membrane SM-rich domains are spatially distinct from ganglioside GM1-rich membrane domains.

**Sphingomyelinase treatment reveals existence of SM-related and non-related signal pathways** - It is well established that upon binding of antigen to lymphocyte receptors, the calcium release from intracellular stores is up-regulated, followed by the induction of influx via calcium channels on the plasma membrane (36, 37). Cross-linking of T cell receptor (TCR)-CD3 complex with anti-CD3 antibody mimics the presentation of antigen. Similarly, the binding of CTxB to Jurkat cells induces both the release of Ca^{2+} from the intracellular stores and a Ca^{2+} influx from extracellular spaces (38). Binding of the CTxB pentamer induces the clustering of five GM1 molecules (39). Antibody against CTxB enhances this clustering (40). Our results indicate that SM-rich domains are spatially distinct from GM1-rich domains on the plasma membranes of Jurkat cells. We asked whether SM-rich domains are functionally related to GM1-rich domains. To examine this possibility, Jurkat cells were treated with Bacillus cereus sphingomyelinase (SMase) to deplete cell surface SM followed by various stimulation. As shown in Fig. 5A, SMase treatment did not significantly affect the Ca^{2+} increase induced by anti-CD3 antibody or by cross-linking of GM1. Lysophosphatidic acid (LPA) is reported to increase intracellular calcium and ERK activation through its receptor, LPA1/2/3, a member of G-protein coupled receptor (GPCR) followed by activation of phospholipase C (PLC) β via G-protein, Gq (41). In contrast to CD3 and GM1 stimulation, LPA-induced calcium increase was dramatically decreased by SMase treatment.

ERK1/2 are key regulators of cytokine production in T cells (42). Both ERK1 (p44 MAP kinase) and ERK2 (p42 MAP kinase) belong to a family of protein serine/threonine tyrosine kinases that are activated by a variety of growth factors, as well as by other extracellular stimuli. Activation of ERK1/2 occurs through phosphorylation of threonine and tyrosine (202 and 204) by a MAPK kinase (MAPKK or MEK), which is activated by a MAPKK or MEK kinase (MAPKKK or Raf family). Anti-CD3 antibody, CTxB as well as LPA treatment of Jurkat cells induce ERK phosphorylation. Fig. 5B and C indicates that SMase treatment selectively inhibits LPA-mediated ERK phosphorylation. Our results indicate that SM is important for signaling through LPA. In contrast, SM is dispensable for the signal transduction initiated by cross-linking of TCR or GM1.
Cross-linking of SM-rich domain provides a signaling platform that is distinct from those induced by cross-linking of TCR or GM1. We then asked whether cross-linking of SM-rich membrane domains provides a signaling platform as observed in cross-linking of TCR or GM1. In Fig. 6A, Jurkat cells were loaded with calcium indicator Fura-2AM, and intracellular Ca\(^{2+}\) was monitored both in the presence and absence of Ca\(^{2+}\) in the media. Stimulation of TCR-CD3 by a specific antibody increased intracellular Ca\(^{2+}\) both in the presence and absence of extracellular Ca\(^{2+}\) under these conditions (Fig. 6A, upper left). Addition of GST-NT-Lys did not significantly affect intracellular Ca\(^{2+}\). GST had no effect (Fig. 6A, upper right). Next, cells were labeled with GST-NT-Lys on ice, loaded by Fura-2AM at 35°C, and stimulated with anti-GST antibody (Fig. 6A, lower left). A sustained increase of Ca\(^{2+}\) was observed in the presence of exogenous Ca\(^{2+}\). However, unlike CD3 stimulation, Ca\(^{2+}\) release was not significantly observed in the absence of extracellular Ca\(^{2+}\). We then labeled cells with GST-NT-Lys and anti-GST antibody on ice, loaded by Fura-2AM at 35°C, and stimulated with corresponding secondary antibody. Cross-linking was further enhanced by the addition of a secondary antibody (Fig. 6A, lower right). Under these conditions, intracellular calcium was increased both in the absence and the presence of EGTA. Anti-CD3-induced calcium influx from extracellular space was inhibited by nifedipine, a blocker for L-type Ca\(^{2+}\) channel, to the similar extent to calcium chelating (Fig 6B, left). In case of GST-NT-Lys and anti-GST antibody-induced calcium influx, treatment of DMSO, a solvent of nifedipine, slightly inhibited calcium influx. However, the addition of nifedipine significantly blocked calcium influx (Fig 6B, right). In Fig. 6C, phosphorylation of ERK was examined after cross-linking of SM-rich domains. In this experiment, Jurkat cells incubated with GST-NT-Lys were replated onto the plates coated with anti-GST antibody (left panel). Alternatively, cells were incubated with GST-NT-Lys and anti-GST antibody followed by replating onto the plates coated with the corresponding secondary antibody (right panel). Under this condition, cells attached to the plates slowly thus gave low ERK signal after 3 min incubation because of the detachment of the cells. Phosphorylation of ERK was observed in both cases in the presence of GST-NT-Lys. Our results thus indicate that the cross-linking of SM-rich domain provides a calcium signaling and ERK activation platform.

Upon anti-CD3 antibody stimulation, ERK1/2 phosphorylation is induced via tyrosine kinase cascade (43). It is also reported that the cross-linking of GM1 with CTxB and its corresponding antibody induces local accumulation of protein tyrosine phosphorylation (40). We asked whether tyrosine phosphorylation was involved in signal transduction mediated by the cross-linking of SM. While anti-CD3 antibody induced tyrosine phosphorylation, cross-linking of SM by GST-NT-Lys did not significantly affect phosphorylation (Fig. 7A). Both anti-CD3 antibody and GST-NT-Lys activated ERK to the similar extent (Fig. 7C, the 4th and 5th rows). As previously described, GM1 cross-linking with CTxB and anti-CTxB antibody induced similar pattern of tyrosine phosphorylation to that induced by anti-CD3 antibody stimulation (Fig 7B).

Linker for activation of T cells (LAT) is an adaptor protein that performs a critical function in TCR-mediated signal transduction (44). LAT is reported to be tyrosine phosphorylated by ZAP-70 upon TCR/CD3 stimulation or CTxB engagement to GM1 in T cells (38). LAT was immunoprecipitated from the cell lysates after treatment of Jurkat cells with anti-CD3 antibody or with GST-NT-Lys. The samples were blotted
against phosphorylated-tyrosine specific antibody after SDS-gel electrophoresis (Fig 7C, the second row). Whereas anti-CD3 antibody treatment induced tyrosine phosphorylation of LAT, only a slight upregulation of phosphorylation of LAT was observed after GST-NT-Lys treatment (Fig 7C, second row). This observation was confirmed by using anti-phosphotyrosine antibody for aa191 of LAT protein (Fig 7C, first row). These results indicate that unlike CD3 or GM1, cross-linking of SM-rich domain very weakly induce protein tyrosine phosphorylation.

Signalling molecules related to Lck are necessary for ERK activation by cross-linking SM-rich domains - Lck is the key regulator for TCR-mediated signaling events. Tyrosine phosphorylation of ZAP-70 and PLCγ-1 by activated Lck up-regulates their activities (43, 45). Chemical mutagenesis generated mutant Jurkat cell lines, JCaM1.6 (46), J.gamma1 (47), P116 (48), and J45.01 (49), that are defective in Lck, PLCγ, ZAP-70, and CD45 expression, respectively. We examined phosphorylation of ERK in these cell lines after different stimulation. As shown in Fig 8A, ERK phosphorylation induced by cross-linking of SM-rich domains was severely blocked in Lck-, ZAP-70-, and CD45-defective mutants, and around 40% of inhibition was observed in PLCγ1-defective mutants. CD45 is a transmembrane tyrosine phosphatase which positively regulates Lck activity (49), and ZAP-70 is a protein kinase located in the downstream of Lck (48). These results indicate that CD45-Lck-ZAP70 cascade is necessary for ERK phosphorylation by SM aggregation. Although PLCγ1 is positively regulated by tyrosine phosphorylation by Lck (45), its role for ERK activation by SM cross-linking was limited. LPA-induced ERK phosphorylation was partially inhibited in the mutants whereas Lck was required for anti-CD3 dependent ERK phosphorylation.

PKC is required for SM-cross-linking induced ERK activation - The mechanism of phosphorylation of ERK by cross-linking of SM was further examined. It is well established that intracellular calcium levels regulate the activity of classical and novel types of protein kinase C (PKC), and that long-term treatment with phorbol-12-myristate-13-acetate (PMA) decreases their expression (50). To examine the role of PKC upon ERK activation, Jurkat cells were treated with PMA for 48 h, followed by cross-linking of SM. As shown in Fig 8B, PMA treatment prevented the expression of PKCθ. In these cells, ERK activation by SM cross-linking was severely inhibited. We confirmed the expression of α, β, δ, and θ types of PKC in Jurkat cells and depletion of their expression by PMA treatment (data not shown).

In Fig. 8C, we examined the effect of various inhibitors on ERK-phosphorylation mediated by cross-linking SM-rich domains. The degree of phosphorylation 10 min after GST-NT-Lys cross-linking was taken as one (Fig8C, the sixth bar from the left). PD98059, an inhibitor for MEK (51) significantly reduced ERK activation. The result from cells depleted of PKC by PMA, was also quantified. H89 is known as the inhibitor for PKA at low (50 nM), and several kinases including calmodulin kinase II (CaMKII), casein kinase, MLCK, and PKC at high (10 μM) concentration (52). Treatment of H89 at high concentration severely inhibits ERK phosphorylation, while partial inhibition was observed at low concentration. PLC inhibitor U73122 (53), which acts both on β and γ types of PLC, inhibited phosphorylation down to the level of non-treated cells. These results suggest the involvement of a PLC-PKC and/or calmodulin modulated CaMKII pathways in SM cross-linking dependent signal transduction. Gαi/o specific inhibitor, pertussis toxin (PTX), also inhibited
ERK phosphorylation. Ras has been implicated in an Lck-ZAP70-LAT-PLCγ-1 cascade (54). Expression of a flag-tagged dominant negative form of H-Ras, which is expressed two-five fold compared to endogenous Ras (data not shown), did not suppress ERK phosphorylation.

DISCUSSION

Using a non-toxic sphingomyelin (SM)-rich domain specific probe, we have examined the distribution and function of SM-rich membrane domains in living cells. Our results indicate that SM-rich membrane domains are spatially and functionally different from those enriched with glycolipid GM1. Our results thus indicate the spatial and functional heterogeneity of lipid rafts. *Non-toxic lysenin, a unique probe to monitor sphingomyelin-rich membrane domains in living cells* - Lysenin is an SM-specific toxin isolated from the coelomic fluid of the earthworm *Eisenia fetida*. Lysenin comprises a family of proteins together with lysenin-related protein 1 (LRP-1) and LRP-2 (14, 16, 55). Recently we have shown that lysenin recognizes SM in a distribution dependent manner (15). Thus, this toxin binds SM only when SM forms aggregates or domains. Therefore, lysenin is a probe for SM-rich membrane domains. Upon binding to SM, lysenin assembles into SDS-resistant oligomers, leading to the formation of pores with a hydrodynamic diameter of approximately 3 nm (13). This toxicity of lysenin hindered the characterization of SM-rich domains in living cells. In the present study, we have prepared a series of deletion mutants of lysenin. Our results indicate that the N-terminus of this protein is not required for recognition of SM but is required for cytotoxicity. The presence of SM did not induce oligomerization of GST-NT-Lys, suggesting the requirement of the toxin oligomers for the cytotoxicity of lysenin. Lysenin contains 6 tryptophan residues of which 5 are conserved in LRP-1 and -2 (14). Our recent data suggest the importance of the conserved tryptophan residues for the toxic functions. Two conserved tryptophans reside in the N-terminus of lysenin. Since these tryptophans are not required for the binding of SM, they are not directly involved in the recognition of SM. Deletion of N-terminus of lysenin did not affect on-rate of the protein binding to SM. In contrast, dissociation of the protein was accelerated in the mutant lysenin. Since GST-NT-lysenin does not oligomerize, this result suggests that oligomerization of lysenin stabilizes the binding of the protein to SM-containing membranes. Our results using model membranes indicate that while non-toxic, NT-Lys keeps the characteristics of earthworm lysenin and efficiently labels cell surface SM-rich membrane domains if appropriate concentration is employed.

*Spatial heterogeneity of the lipid rafts* - Lipid rafts are defined as membrane microdomains enriched with sphingolipids and cholesterol. Micrometer scale heterogeneity of lipid raft components has been reported in migrating T cells and T cells after antigen stimulation (56, 57). These heterogeneities could be observed under a fluorescent microscope. However, the accumulating evidence suggests that, in steady state, the size of lipid rafts in biomembranes is much smaller (32, 58). Indeed, when living Jurkat cells were labeled with mutant lysenin or CTxB, fluorescence was evenly distributed along the cell surface (Fig. 4B). Biophysical methods detecting sub-micron scale interaction or specific probes that recognize lipid clusters are required to study small domains. Using fluorescence resonance transfer (FRET) of fluorescently labeled CTxB, Kenworthy et al. showed that clustering of GM1 does not occur on plasma membranes (31). Recently, Nichols...
employed the same procedure and found that at least part of the GM1 molecules exist as clusters (32). Using a combination of lysenin and SMase, we have shown that the organization of SM differs between different cell types and between different membrane domains within the same cell (15). Our results also support the idea of the existence of small condensed SM complexes consisting of just a few lipid molecules in living cells. Little is known about sub-micron scale heterogeneous organization of membrane lipids in biomembranes (59). Here, we compared the distribution of SM-rich domains with those of GM1-rich domains. Our results indicate that two lipid domains were differently distributed on the plasma membrane, indicating a spatial heterogeneity of SM-rich domains from GM1-rich membrane domains. It should be noted that GM1 inhibits the binding of lysenin to SM. Therefore “by definition” lysenin-labeled domains are devoid of GM1 in molecular level. However, this does not exclude the possibility that SM-rich domain and GM1-rich domain form mixed domains in the area of diameter 60-80 nm. Our statistical analysis indicated the presence of sub-micron scale SM-rich domains and GM1-rich domains. However, our results revealed a lack of colocalization of SM-rich domains and GM1-rich domains. Since both SM and GM1 are well recognized as lipid raft markers, our results indicate heterogeneity of lipid rafts on a sub-micron scale.

**Functional heterogeneity of lipid rafts** - Current view of raft domains is that they contain few raft molecules and exchange their components on a sub-millisecond scale (60). It is proposed that the cross-linking of raft components leads to a coalescence of rafts and that these stabilized raft domains function as signaling centers (7). Like T-cell receptor activation and cross-linking of GM1, cross-linking of SM induces calcium influx and ERK phosphorylation in Jurkat cells. However, unlike CD3 or GM1, cross-linking of SM did not induce significant protein tyrosine phosphorylation. These results suggest that SM provides a functional signal cascade platform that is distinct from those provided by TCR or GM1. This idea was supported by our observation that SMase treatment of Jurkat cells abolished LPA-mediated but not TCR-dependent signal transduction. These results indicate that LPA-mediated signal transduction is functionally related to SM-rich membrane domains and is distinct from those mediated by anti-CD3 antibody or cross-linking of GM1. LPA mediates signal transduction via its receptor, LPA1, 2, and 3, members of the so-called ‘endothelial differentiation gene (EDG)’ family of GPCR, and were previously called EDG2, 4, and 7, respectively (61). Gαi/o specific inhibitor, PTX inhibited ERK phosphorylation by cross-linking of SM-rich domains, suggesting that SM-rich domain mediated signaling pathways pass through or reside after GPCR signaling.

Inhibitor experiments suggest the importance of intracellular calcium on ERK activation induced by the cross-linking of SM-rich domains. Calcium regulating calmodulin-CaMKII (62) and PKC-Raf activation pathways (63-65) are reported to be utilized in ERK activation. Slight inhibition by expression of dominant negative H-Ras suggests that tyrosine kinase-mediated Ras activation pathway is not the main pathway initiated by SM clustering. Although cross-linking of SM-rich domains did not induce significant protein tyrosine phosphorylation, studies using somatic mutants indicate the importance of CD45-Lck-ZAP70 tyrosine kinase cascade in NT-Lys triggered ERK activation. One could speculate that small amount of tyrosine phosphorylated proteins are sufficient to induce ERK phosphorylation by cross-linking SM-rich domains. Alternatively, these molecules may work as scaffolding molecules as observed in
the interaction with integrin (66). The involvement of tyrosine kinases in GPCR pathway has been proposed. The Gαi and Gαs families of proteins can be tyrosine phosphorylated in vitro, when they were immunoprecipitated by their antibodies (67). In vivo, upon carbachol stimulation and overexpression of Fyn, Gαq/11 are tyrosine phosphorylated.

In conclusion, our results indicate that sphingomyelin-rich domains are spatially distinct from ganglioside GM1-rich membrane domains. Our results also suggest that the sphingomyelin-rich domain provides a functional signal cascade platform that is distinct from those provided by TCR or GM1. Our study therefore elucidates the spatial and functional heterogeneity of lipid rafts.

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FOOTNOTES

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The abbreviations used are: SM, sphingomyelin; Chol, cholesterol; CTxB, cholera toxin B subunit; *E. Coli*, *Escherichia coli*; ERK, extracellular signal regulated kinase; GPCR, G-protein coupled receptor; LAT, linker for activated T cell; LPA, lysophosphatidic acid; MBP, maltose-binding protein; N-NBD-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); NPA, Niemann-Pick type A; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SMase, sphingomyelinase; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; TCR, T cell receptor.

**FIGURE LEGENDS**

**Fig. 1.** Characterization of non-toxic probe for SM. A, Schematic representation of deletion mutants of lysenin. WT (wild type) and number of amino acids of lysenin coded by mutants are indicated at the left. The bars corresponding to mutants are indicated at the middle. Niemann-Pick type A (NPA) cells were incubated at a concentration of 10 µg/ml for WT and 50 µg/ml for mutant lysenins. Positive (+) or negative (-) labeling of NPA cells with these mutants are indicated at the right side of the lines. Positive (+) or negative (-) hemolytic activities of these mutant proteins at the concentration of 50 µg/ml are indicated at the right. B, C, Binding of GST-tagged lysenin and GST-NT-Lys to various lipids measured by ELISA. The microtiter wells were coated with 50 µl of 10 µM various lipids. Wells were then incubated with 100 µl of various concentrations of GST tagged wild type lysenin, GST-NT-Lys or GST (B) or 100 µl of 10 µg/ml GST tagged wild type lysenin or 25 µg/ml GST-NT-Lys or GST (C). After washing, the wells were further incubated with anti-GST antibody and corresponding secondary antibody conjugated with biotin. The binding of the secondary antibody was detected at 490 nm as signal and 630 nm as reference (21). 300 nM of protein corresponds to 20 µg/ml GST-Lys, 13 µg/ml GST-NT-Lys and 7.8 µg/ml GST. SM; sphingomyelin, PC; phosphatidylcholine, PE; phosphatidylethanolamine, PS; phosphatidylinerine, PI; phosphatidylinositol, PA; phosphatidic acid, Cer; Ceramide, Sph; Sphingosine, S1P; sphingosine 1-phosphate, SPC; sphingosyl phosphorylcholine, GalCer; galactosylceramide, GlcCer; glucosylceramide, LacCer; lactosylceramide. In B, triangles show binding to SM whereas squares indicate binding to PC. Insert in B shows the expansion of the area of the curve between 0-40 nM. D, Binding of GST-tagged truncated lysenin to liposomes containing various sphingolipids. The molar ratio of sphingolipid, phosphatidylcholine (PC), cholesterol (Chol) and N-NBD-PE in the liposome was 1.5:3:5:0.5. The liposomes were incubated with 10 µg/ml of GST or GST-NT-Lys. The mixture was then subjected to a sucrose gradient centrifugation and fractionated from the top (1-9). The same volume from each fraction was subjected to SDS-PAGE and blotted against anti-GST antibody. Fluorescence intensity of N-NBD-PE was also measured to monitor the position of liposomes in the gradient. E, GST-NT-Lys does not form oligomers. The Liposomes composed of SM/ Chol/N-NBD-PE (4:5:1) were incubated with 6.6 µg/ml of GST-lysenin or 10 µg/ml of GST-NT-Lys. The mixture was then subjected to a sucrose gradient centrifugation and fractionated from the top (1-9). The same volume from each fraction was subjected to SDS-PAGE and blotted against anti-GST antibody. O and M indicate the oligomer and monomer form of GST-lysenin, respectively. F, Immunofluorescence detection of the recognition of cellular SM by
GST-NT-Lys. NPA fibroblasts were fixed, permeabilized and incubated with GST alone or GST-NT-Lys, followed by treatment with anti-GST and -CD63 antibodies and the corresponding secondary antibodies conjugated with Alexa488 and 546, respectively. Bar, 20 µm

Fig. 2. Recognition of SM by GST-NT-Lys in living cells. A, MTT assay of Jurkat cells. 1 x 10^6 Jurkat cells were washed twice with phosphate-buffered saline (PBS), incubated with various concentrations of GST (open circles), GST-NT-Lys (closed triangles), GST-Lysenin (closed squares) for 30 min at room temperature, re-suspended in RPMI1640 without sera, and cultured in the presence of 0.5 mg/ml of MTT at 37 °C. After 4 hours, MTT solution was removed and cells were disrupted by the addition of DMSO. The absorbance at 595 nm was measured with a spectrophotometer. B, FACS analysis of Jurkat cells. 1 x 10^6 Jurkat cells were washed with PBS twice, incubated with 50 µg/ml of GST or GST-NT-Lys for 30 min at room temperature, incubated with or without 10 µg/ml of monoclonal anti-GST antibody, followed by goat anti-mouse IgG antibody conjugated with Alexa-488. For SMase treatment, cells were treated SMase at 37 °C for 30 min prior to incubation with GST-NT-Lys. Insert, Immunofluorescent image of GST-NT-Lys labeled cell. Jurkat cells were incubated with GST-NT-Lys, anti-GST and Alexa-488 conjugated anti-mouse antibody as described above. Living cell images were obtained by confocal microscopy as described in MATERIALS AND METHODS. Bar, 10 µm.

Fig. 3. Recognition of SM and GM1 by lysenin and CTxB. A, Both HV-NT-Lys and HmV-NT-Lys specifically recognize SM. The binding to various lipids was examined by ELISA as described in MATERIALS AND METHODS. Twenty-five µg/ml protein was employed. B, The presence of GM1 inhibits the binding of HmV-NT-Lys to SM-containing liposomes. Left, Liposomes composed of SM/PC/GM1/N,NBD-PE (1:4:0:0.25, SM+ GM1- or 1:4:1:0.5, SM+ GM1+) incubated with HmV-NT-Lys were fractionated by sucrose density gradient as described in the legend for Fig. 1D. The bound protein was quantitated by SDS-PAGE and western blotting using anti-GFP antibody. Right, Liposomes composed of SM/PC/GM1/N,NBD-PE (0:4:1:0.25, SM- GM1+ or 1:4:1:0.5, SM+ GM1+) incubated with CTxB was fractionated and quantitated by dot blotting against anti-CTxB antibody. Representative results from two independent experiments are shown. C, SMase treatment did not affect CTxB binding to Jurkat cells. Cells were incubated with or without SMase for 30 min, followed by the incubation with Alexa 488-conjugated CTxB. The labeling of CTxB was monitored by FACS. D, Pretreatment of Jurkat cells with one lipid probe did not affect the binding of the other. Cells were incubated with either CTxB or HmRFP-NT-Lys (denoted as 1) CTxB or 1) HmRFP-NT-Lys) for 30 min on ice followed by 30 min treatment with HmV-NT-Lys or Alexa 488-conjugated CTxB on ice (denoted as 2) HmV-NT-Lys or 2) CTxB488). As a control, cells were incubated without any probe (denoted as 1) None). The fluorescence was monitored by FACS as described in the legend for Fig. 2B.

Fig. 4. Cell surface distribution of SM-rich domains and GM1-rich domains. A, Immunofluorescent images of Venus tagged NT-Lys. Jurkat cells were incubated with HV-NT-Lys or HmV-NT-Lys, in the presence of CTxB-conjugated with Alexa594. Living cell images were obtained by confocal microscopy as described in MATERIALS AND METHODS. Bar, 10 µm. B, Distribution of SM-rich domains and GM1-rich domains on 2D sheets of plasma membrane. Jurkat cells were first labeled with HmV-NT-Lys and
biotinylated cholera toxin B fragments at 4 °C, and fixed with 4% paraformaldehyde and 0.02% glutaraldehyde for 10 min at 4 °C. The fixed cells were labeled with anti-GFP rabbit polyclonal antibody at 4 °C, followed by labeling with goat anti-rabbit IgG-5 nm gold and goat anti-biotin IgG-10 nm gold at 4 °C. The distribution of gold particles on the plasma membrane was examined under electron microscope after ripping off as described in MATERIALS AND METHODS. Bar, 100 nm. In the right panel, distribution of SM (5 nm gold) was colored in red whereas those of GM1 (10 nm gold) were in blue. C, Analysis of the distribution of SM-rich domains and GM1-rich domains using Ripley’s K-function. EM images were analyzed as described in MATERIALS AND METHODS. Both SM-rich domains and GM1-rich domains form clusters whereas the pair-wise values for SM and GM1 falls within blue lines that represent the range of values expected for pairs of different particles whose distribution are random.

Fig. 5. Sphingomyelinase (SMase) treatment reveals existence of SM-related and non-related signal pathways. A, Cells were pretreated with (open squares) or without (closed squares) SMase in the presence of Fura-2AM indicator for 30 min at 35 °C. Cells were washed with calcium-containing buffer and stimulated with 1 µg/ml of anti-CD3 antibody or 20 µM of LPA for 10 min. For GM1 cross-linking (GM1-XL), cells were labeled with CTxB followed by the incubation with anti-CTxB antibody for 15 min. Intracellular calcium was measured as described in MATERIALS AND METHODS. B, Cells were pretreated with or without SMase and stimulated with indicated reagents. The phosphorylated ERK was visualized using phospho-specific antibody. In C, Relative ERK activation was calculated as (phospho-ERK with SMase treatment) / (phospho-ERK without SMase treatment).

Fig. 6. Induction of calcium influx and ERK activation by cross-linking of SM-rich domains. A, Cross-linking of SM-rich domains induces calcium influx. Jurkat cells were loaded with Fura-2AM as described in MATERIALS AND METHODS and the proteins were added at 30 sec (indicated by arrows). (Upper left) Red squares show the result in the presence of 1 mM calcium buffer, while blue triangles are in the presence of 1 mM EGTA. One µg/ml of anti-CD3 antibody was added. The remaining panels; red squares and blue triangles are results from cells incubated with GST-NT-Lys, and measured in the presence of calcium or EGTA, respectively. Pink squares and black circles are results from cells incubated with GST, and measured in the presence of calcium or EGTA, respectively. (Upper right) GST or GST-NT-Lys were added at 30 sec after loading Fura-2AM. (Lower left) Cells were first labeled with GST or GST-NT-Lys on ice for 30 min, loaded by Fura-2AM, and anti-GST antibody was added as indicated by the arrow. Results shown here are representative data from at least two experiments. B, Calcium channel blocker inhibits calcium influx triggered by cross-linking of SM-rich domains. (Left) Jurkat cells were loaded with Fura-2AM in the absence of DMSO (red squares), in the presence of DMSO (pink squares) and in the presence of 200 µM of nifedipine in DMSO (blue triangles). One µg/ml of anti-CD3 antibody was added at 30 sec as indicated by the arrow. (Right) Cells were incubated with GST-NT-Lys on ice for 30 min in the absence of DMSO (red squares), in the presence of DMSO (pink squares) and in the presence of 200 µM of nifedipine in DMSO (blue triangles). Fura-2AM was then loaded and cells were activated according to the protocol of Fig. 6 A lower left. Results shown here are representative data from at least two experiments. C, Cross-linking of SM-rich domain induces
ERK phosphorylation. Jurkat cells were incubated with 50 µg/ml of GST or GST-NT-Lys or without any addition (no prt), and replated onto plates coated with anti-GST antibody (left panel), or cells were further incubated with anti GST antibody and replated onto plates coated with the corresponding secondary antibody (right panel). After the indicated periods, cells were lysed and subjected to SDS-PAGE and Western blotting. Upper row: blotted against anti phosphor-specific antibody; Lower row: anti-ERK antibody.

Fig. 7. Cross-linking of SM-rich domains only slightly induces protein tyrosine phosphorylation. A, Jurkat cells were stimulated for 10 min according to the protocol described in the legend of lower left panel of Fig. 6A. Cell lysates were separated by SDS-PAGE and blotted against anti-phosphotyrosine antibody as described in MATERIALS AND METHODS. As a positive control, cells were stimulated with anti-CD3 antibody without cross-linking for three min. An arrow indicates the band corresponding to LAT designated by the molecular weight. B, Jurkat cells were labeled with CTxB on ice for 30 min, and followed by the cross-linking (XL) with anti-CTxB antibody at 37 °C for 10 min. As a control, cells were stimulated with anti-CD3 antibody as described above. C, Cells were stimulated as described in A. Cell lysates were separated by SDS-PAGE and blotted as indicated. To immunoprecipitate (IP) LAT protein, cell lysates were incubated with anti-LAT antibody, followed by incubation with agarose beads conjugated with proteinA. Immunoprecipitants were separated by SDS-PAGE and blotted against anti-phosphotyrosine antibody. Arrows indicates bands corresponding to LAT protein.

Fig. 8. Effect of inhibitors on ERK activation induced by cross-linking of SM-rich domains. A, Mutants of Jurkat cells were stimulated as described in the legend for the right panel in Fig 6C. Intensity of phosphorylated ERK was measured by LAS system (Fuji film) and analyzed by Image Gauge (Fuji Film). The relative activation of ERK was calculated as (stimulation of ERK-phosphorylation in mutant)/(stimulation of ERK-phosphorylation in wild type). B, Jurkat cells were pretreated with 200 nM of PMA for 48h, and stimulated and analyzed as described in the legend of right panel of Fig 6C. C, Jurkat cells were treated in the presence or the absence of various inhibitors for 60 min in the medium at 37 °C. Concentrations of inhibitors were: PD985059 (20 µM) U73122 (10 µM), H89 high (10 µM), H89 low (50 nM), and PTX (100 ng/ml for 16 hours). To express a dominant-negative form of H-Ras (RasDN), a plasmid was introduced by amaxa transfection apparatus, and cultured for 72 hrs. In parallel experiments, a plasmid coding GFP or YFP was introduced to monitor the transfection. Transfection efficiency was around 75-90 %. Expression of RasDN was also confirmed by Western blotting with anti-Ras antibody (data not shown). Cells were labeled with GST-NT-Lys followed by incubation with anti-GST antibody, and stimulated for 10 min by plating onto a dish precoated with the secondary antibody. The relative activation of ERK was calculated as (phosphor-ERK with inhibitor treatment)/(phosphor-ERK without inhibitor treatment). Results are the average of three independent experiments.
Kinetic analysis of GST-NT-Lys binding to sphingomyelin

Kinetic analysis of GST-NT-Lys binding to immobilized phospholipid membranes was performed as described (12). SM/dipalmitoylphosphatidylcholine (DPPC) (8:2) liposome was immobilized to the sensor chip HPA. Liposome consisted of DPPC alone was taken as a control. Various concentrations of GST-NT-Lys was injected over the immobilized lipid membrane surface. The binding kinetics were analyzed according to the manual of the software BIAevaluation 3.0.

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Fig. 2

A

![Graph showing OD 595 vs. Protein conc. (µg/ml)]

B

![Cell count histograms for Ab alone, GST, GST-NT-Lys, and SMase GST-NT-Lys]
**Fig. 4**

A

![Image of CTxB and Merged channels for HmV-NT-Lys](image)

B

![Image showing SM/GM1 and data](image)

C

![Graphs comparing SM, GM1, SM/GM1](image)
Fig. 5

A

GM1-XL

CD3

LPA

Relative ERK phosphorylation

B

SMase

GM1-XL  CD3  LPA

-  +  -  +  -  +

pERK

ERK

C

Relative ERK phosphorylation

GM1-XL  CD3  LPA
Fig. 6

A

B

C

No prt | GST | GST-NT-Lys | No prt | GST | GST-NT-Lys | No prt | GST | GST-NT-Lys | No prt | GST | GST-NT-Lys | No prt | GST | GST-NT-Lys

pERK

0 min | 3 min | 10 min

ERK

0 min | 3 min | 10 min
Fig. 7

A

B

C

None
CD3 1µg/mL
CD3 3µg/mL
GST-NT-Lys(0 min)
GST
GST-NT-Lys

None
CD3 1µg/mL
CD3 3µg/mL
GST-NT-Lys(0 min)
GST
GST-NT-Lys

None
CD3 1µg/mL
CD3 3µg/mL
GST-NT-Lys(0 min)
GST
GST-NT-Lys

Total, Blot: pTyr

Total, Blot: pY191LAT

Total, ERK
Fig. 8

A

Relative phosphorylation

WT  J.CaM1.6  P116  J.gamma1  CD45.01

B

No treatment  PMA treatment

No prt  GST  GST-NT-Lys  No prt  GST  GST-NT-Lys  No prt  GST  GST-NT-Lys  No prt  GST  GST-NT-Lys

ERK  pERK  PKC

0 min  10 min  0 min  10 min

C

Relative phosphorylation

No prt  GST  GST161-297  No prt  GST  none  PD98059  PMA  H89 (high)  H89 (low)  U73122  PTX  RasDN

0 min  10 min  GST-NT-Lys, 10 min
Spatial and functional heterogeneity of sphingolipid-rich membrane domains
Etsuko Kiyokawa, Takeshi Baba, Naomi Otsuka, Asami Makino, Shinichi Ohno and Toshihide Kobayashi

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