Detergent-resistant microdomains determine the localization of sigma-1 receptors to the endoplasmic reticulum-mitochondria junction

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d) Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; BODIPY-Cer TR, N-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)sphingosine; BSA, bovine serum albumin; CHO cell, Chinese hamster ovary cell; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; COX 1, cytochrome c oxidase subunit I; CYP450R, cytochrome P450 reductase; DRM, detergent-resistant membranes; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; FB1, fumonisin B1; GalCer, galactosylceramide; GFP, green fluorescent protein; GlcCer, glucosylceramide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPTLC, high-performance thin layer chromatography; HRP, horseradish peroxidase; IP3R3, type-3 inositol 1,2,5-trisphosphate receptor; MAM, mitochondria-associated ER membrane; MβC, methyl-β-cyclodextrin; MEM, Minimum Essential Medium; NBD-Cer, 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine; PBS, phosphate buffered saline; PtChol, phosphatidylcholine; PtEt, phosphatidylethanolamine; PtSer, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate
polyacrylamide gel electrophoresis; Sig-1R, sigma-1 receptor; TNE, Tris-NaCl-EDTA buffer; TNC, Tris-NaCl-CHAPS buffer; Tx, Triton X-100.
**ABSTRACT**

Sigma-1 receptors (Sig-1R) that bind diverse synthetic and endogenous compounds have been implicated in the pathophysiology of several human diseases such as drug addiction, depression, neurodegenerative disorders, pain-related disorders, and cancer. Sig-1R are recently identified as novel ligand-operated molecular chaperones. Although Sig-1R are predominantly expressed at endoplasmic reticulum (ER) subdomains apposing mitochondria [i.e., the mitochondria-associated ER membrane (MAM)], they dynamically change the cellular distribution, thus regulating both MAM-specific and plasma membrane proteins. However, what determines the location of Sig-1R at the MAM and how the receptor translocation is initiated is unknown. Here we report that the detergent-resistant microdomains (DRM) play an important role in anchoring Sig-1R to the MAM. The MAM, which is highly capable of accumulating ceramides, is enriched with both cholesterol and simple sphingolipids, thus forming Triton X-114-resistant DRM. Sig-1R associate with MAM-derived DRM, but not with those from microsomes. A lipid overlay assay found that solubilized Sig-1R preferentially associate with simple sphingolipids such as ceramides. Disrupting DRM by lowering cholesterol or inhibiting *de novo* synthesis of ceramides at the ER largely decreases Sig-1R at DRM while causing translocation of Sig-1R from the MAM to ER cisternae. These findings suggest that the MAM, bearing cholesterol and ceramide-enriched microdomains at the ER, may utilize the microdomains to anchor Sig-1R to the location; thus it serves to stage Sig-1R at ER-mitochondria junctions.
The sigma receptor has been believed to serve as a novel target for therapeutic drugs. Although originally proposed as a subtype of opioid receptors, a series of recent studies has confirmed that the sigma receptor is a nonopioid intracellular protein involved in a variety of functions of the brain and other organs (Snyder and Largent, 1989; Hayashi and Su, 2008). Currently, there are two known subtypes: sigma-1 and sigma-2 (Bowen, 2000). The sigma-1 receptors (Sig-1R) were cloned, and the structure and molecular biological functions have just begun to be unveiled (Hanner et al., 1996; Hayashi and Su, 2008). Sig-1R have been implicated in the pathophysiology of certain human diseases such as psychiatric disorders, neurodegenerative diseases, pain-related disorders and cancer (Matsumoto et al., 2003; Maurice et al., 2002; Mei and Pasternak, 2007; Palmer et al., 2007). Recent evidence indicates potential neuroprotective and antidepressant-like actions for sigma-1 agonists (Maurice et al., 2002). In contrast, sigma-1 antagonists have been demonstrated to possess analgetic, anti-cancer, and anti-drug abuse actions (Matsumoto et al., 2003; Mei and Pasternak, 2007; Palmer et al., 2007).

The Sig-1R are the integral membrane protein with two transmembrane domains and a long C-terminus in the lumen of the ER (Hayashi and Su, 2007). A characteristic of Sig-1R is their moderate to high affinity for diverse synthetic and endogenous compounds including benzomorphans, sterols, sphingosine and trace amines (Bowen, 2000; Fontanilla et al., 2009; Ramachandran et al., 2009; Su et al., 1988). Sig-1R are recently identified as novel ligand-operated molecular chaperones expressed predominantly at the ER subdomain apposing mitochondria (the mitochondria-associated ER membrane, MAM) (Hayashi and Su, 2007). Thus, Sig-1R are positioned to regulate the crosstalk of signals between ER and mitochondria. For example, Sig-1R chaperones stabilize the conformation of MAM-residing type-3 inositol 1,4,5-trisphosphate receptors (IP3R3), thus regulating the Ca^{2+} influx from the ER to mitochondria.
(Hayashi et al., 2009). On the other hand, a number of studies demonstrate that Sig-1R also regulate cellular events at plasma membranes, such as neurotransmitter release, neurotrophic factor signaling, and opening of voltage-gated ion channels (Aydar et al., 2002; Hayashi and Su, 2008; Herrera et al., 2008; Nuwayhid and Werling, 2003). The tonic inhibition of the potassium Kv1.4 channel by Sig-1R involves the physical association between Sig-1R and Kv1.4 channels (Aydar et al., 2002). Although Sig-1R are predominantly expressed at the MAM, the proteins are highly dynamic under certain conditions, such as glucose deprivation and ligand treatment, which promote translocation of Sig-1R from the MAM to loci close to plasma membranes, e.g., plasmalemma (Hayashi and Su, 2003a; Hayashi and Su, 2007). The phenomenon is particularly important and relevant for understanding the Sig-1R’s regulatory role on both ER and plasma membrane proteins. The dynamic shift of the receptor distribution may be to switch the action site of Sig-1R from the MAM to the plasma membrane/plasmalemma (Hayashi and Su, 2008). What determines the location of Sig-1R at the MAM, and how the receptor translocation is initiated/restricted, however, remains largely unknown.

Lipid rafts composed of cholesterol and sphingolipids are small membrane domains that facilitate specificity and efficacy of signaling events by positioning involved molecules to the specific loci of the membrane (Jacobson et al., 2007; Simons and Toomre, 2000). Lipid rafts are resistant to extraction by non-ionic detergents such as Triton X-100 (Tx) at 4°C; therefore, lipid rafts can be purified as detergent-resistant membranes (DRM) by centrifugations (Simons and Toomre, 2000). We previously reported that Sig-1R are associated with DRM at cholesterol-rich ER subdomains (now identified as the MAM) in different types of cells (Hayashi and Su, 2003b; Hayashi and Su, 2004), and that Sig-1R ligands, which can cause translocation of Sig-1R from MAM to the bulk of ER membranes, also down-regulate Sig-1R at DRM (Hayashi and Su,
2003a; Hayashi and Su, 2003b). Because of DRM serving as platforms to cluster signaling molecules, these findings raise a potential hypothesis that ER-specific DRM may play a role in the targeting of Sig-1R at the ER-mitochondria junction. Thus, the objective of this study is examine whether DRM are involved in the localization of Sig-1R at the MAM and in the receptor translocation.
METHODS

Materials and Methods

Reagents for cell culture were purchased from Invitrogen (Carlsbad, CA). Sources of antibodies are anti-N-CAM from Parmingen; anti-IP3R3 and anti-BiP from BD Biosciences (San Jose, California); anti-Src, anti-cytochrome P450 reductase (CYP450R) and anti-calreticulin from Santa Cruz Biotechnology (Santa Cruz, CA); anti-ceramide from Sigma (St Louis, MO), anti-mitofusin-2 and anti-ERp57 from Abcam (Cambridge, MA); anti-ATP synthase inhibitor and anti-cytochrome c oxidase subunit I (COX1) from Invitrogen (Carlsbad, CA); and anti-green fluorescent protein (GFP) from Clontech (Mountain View, CA). Anti-Sig-1R antibodies were developed as described previously (Hayashi and Su, 2007). 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl)sphingosine (NBD-Cer) and N-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)sphingosine (BODIPY-Cer TR) were from Invitrogen. Chemicals and lipids were from Sigma or Avanti (Alabaster, AL). Expression vectors for Mito-DsRed, Mito-GFP, and KDEL-DsRed were purchased from Clontech (Mountain View, CA). The structures of lipids are provided in the supplemental figure.

Cell culture

Chinese hamster ovary (CHO; American Type Culture Collection, Manassas, VA) cells were maintained in Minimum Essential Medium (MEM)-alpha containing 2 mM GlutaMax (Invitrogen) and 10% heat-inactivated fetal calf serum at 37°C with 5% CO2. The vector encoding the mouse Sig-1R with an enhanced yellow fluorescent protein (EYFP)-tag on the C-terminus was constructed in the pEYFP-N1 vector (Clontech) as described previously (Hayashi and Su, 2003b). CHO cell lines stably expressing EYFP or Sig-1R-EYFP were established by
transiently transfecting pEYFP-N1 or pEYFP-Sig-1R vectors followed by colony selections with Geneticin (Invitrogen). Lipofectamine-2000 (Invitrogen) was used for gene transfection. CHO cells were treated with fumonisin B1 (FB1), methyl-β-cyclodextrin (MβC) or cholesterol-conjugated MβC in culture medium without serum. FB1 was dissolved in ethanol and used at 5 μg/ml. MβC was dissolved in phosphate buffered saline (PBS) and used at 5 mM with 5 μM of lovastatin. Cholesterol-conjugated MβC was prepared by rotating 1 ml of MβC solution (100 mM) with N2-dried cholesterol (4 mg) overnight at room temperature.

**DRM preparation**

For the sucrose gradient fractionation of DRM, CHO cells were cultured in a 10-cm dish at 80-90% confluency. CHO cells (2x10^6 cells) were lysed at 4°C in 0.5 ml of TNE-Tx buffer [10 mM Tris at pH 7.4, 150 mM NaCl, 1 mM phenylmethanesulphonylfluoride, 5 mM EDTA, 48 KIU/ml aprotinin, 0.5% Tx]. After being mixed with an equal volume of 80% sucrose, the cell lysate was subjected to sucrose gradient centrifugation at 100,000g, and a total of 13 fractions were collected from the top, as described before (Hayashi and Su, 2003b). The same volume from each fraction was applied to SDS-PAGE.

To prepare the Tx-insoluble pellet enriched with DRM and the Tx-soluble supernatant by differential centrifugations, cell membranes were incubated in TNE-Tx for 30 min at 4°C. After sonication (10 sec, 3 times), samples were centrifuged at 12,000g for 20 min. The supernatant was further centrifuged at 100,000g for 1 hr in a 50 Ti rotor. The pellet and the supernatant were collected as Tx-insoluble (P) and Tx-soluble (S) fractions, respectively.

**MAM fractionation**

The MAM fraction was prepared as previously described (Rusinol et al., 1994). In brief, CHO cells were grown on two 15-cm dishes at 90-100% confluency. Harvested cells were
homogenized by a glass Dounce homogenizer in a homogenization buffer (0.25 M sucrose, 10 mM HEPES/KOH, pH 7.4). The homogenate was centrifuged twice at 500g for 5 min to yield the P1 nuclear fraction. The supernatant was spun down at 10,300g for 20 min to yield crude mitochondria membranes as a pellet (P2 fraction). The supernatant was centrifuged at 100,000g for 1 hr in a 50 Ti rotor to obtain the P3 microsomal and cytosolic fractions. Crude mitochondrial membranes were suspended in 0.5 ml of isolation medium (250 mM mannitol, 5 mM HEPES/KOH pH 7.4, 0.5 mM EGTA/KOH), layered on Percoll solution [225 mM mannitol, 25 mM HEPES/KOH pH 7.4, 1 mM EGTA/KOH, 30% (v/v) Percoll (GE Healthcare, Piscataway, NJ)], and centrifuged at 95,000g for 30 min in an SW 55 Ti rotor. Purified mitochondrial and MAM fractions were collected and washed twice with an isolation medium by centrifugation.

**Immunocytochemistry and confocal microscopy**

Immunocytochemistry and confocal microscopy were performed as described earlier (Hayashi and Su, 2003b). Primary antibodies were used at 1:100 for anti-Sig-1R or 1:50 for anti-IP3R3. The filipin staining was performed according to the previous report (Hayashi and Su, 2003b).

**Lipid overlay assay**

CHO cells were grown in a 15-cm dish at 90% confluency. Cells were harvested in ice-cold PBS and centrifuged at 2,000g for 10 min. The cell pellet was suspended in 1 ml of 50 mM Tris (pH 7.4) containing 0.2% CHAPS. The cell lysate was rotated at 4°C for 2 hrs, followed by a centrifugation at 100,000g in a 50 Ti rotor for 1 hr. The supernatant was filtered by a Millipore 0.22-mm filter unit (Millipore, Billerica, MA). The total protein concentration was measured with a BCA protein assay kit (Thermo Scientific; Rockford, IL). The lysate was stored at –80°C until use.
Lipids were dissolved in a chloroform:methanol mixture (2:1) and spotted on a Hybond-C nitrocellulose membrane (GE Healthcare; 200 to 10,000 pmoles/spot). The membrane was air-dried for 1 hr at room temperature. The membrane was blocked with TNC buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.2% CHAPS) containing 30% non-fat dry milk and 5% chicken ovalbumin at 4°C for 6 hrs with gentle shaking. After washings twice with TNC buffer, the membrane was incubated with the CHAPS cell lysate (28-60 μg of proteins/cm²) in TNC containing 150 mM NaCl and 4% BSA at 4°C for 16 hrs with shaking at 200 rpm. After three washings with TNC containing 3% BSA (each for 10 min), the membrane was incubated with either anti-full length GFP antibodies (1:1500) or anti-Sig-1R antibodies (1:1000) in TNC with 3% BSA for 3 hrs at room temperature or 16 hrs at 4°C. After extensive washings with TNC containing 3% BSA, Sig-1R complexes associated with lipids were detected by horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by WestFemto chemiluminescence (Thermo Scientific; Rockford, IL). Images were captured with Kodak Image Station 440CF (Kodak, New Heaven, CT).

**Lipid assays**

Total lipids were extracted by the Bligh and Dyer method. Free cholesterol in the sample was measured enzymatically by the Free-Cholesterol E kit (Wako, Richmond, VA) or the Cholesterol Assay kit (Sigma). For high-performance thin layer chromatography (HPTLC; Merck, Darmstadt, Germany), lipid extracts dissolved in the chloroform:methanol mixture (2:1) were spotted on an HPTLC plate followed by resolution with the hexane:ether:acetic acid mixture (16:4:0.2) for cholesterol and neutral lipids and the chloroform:methanol:water mixture (13:5:0.5) for sphingolipids and phospholipids. Lipids were visualized on HPTLC plates using a ferric chloride spray (for cholesterol) or a diphenylamine-aniline spray (for neutral
glycosphingolipids). Phospholipids and ceramides were visualized under UV after spraying 0.2% 8-anilinonaphthalene-1-sulfonate (ANS) in ethanol. The ganglioside-enriched fraction was obtained by applying total lipid extracts to a silicic acid column fractionation followed by Dowex 50 (Na⁺ form) and Sep-Pak C18 column chromatography (Waters, Milford, MA). Gangliosides were resolved by HPTLC with chloroform:methanol:0.25% CaCl₂ (55:45:10) and visualized by resorcinol or diphenylamine-aniline spray. After separation from other lipids, the ceramides were extracted from a HPTLC plate (Merck) and spotted on a Hybond-C membrane (GE Healthcare). The membrane was blocked with 10% non-fat milk (Bio-Rad) in PBS, followed by incubation with ceramide antibodies (1:200) in the same solution at room temperature for 16 hrs. After five wash steps with PBS, ceramides on the membrane were visualized as described in the lipid overlay assay.

**SDS-PAGE and silver staining**

Samples were dissolved in a 2x Laemmli sample buffer after protein quantifications (BCA assay kit; Pierce). Proteins were separated by SDS-PAGE as described previously (Hayashi and Su, 2003b). Proteins in the acrylamide gel were electrically transblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) in a Towbeen buffer and detected by immunoblotting. Silver Stainings were performed according to the company’s instruction (Silver Stain Plus, Bio-Rad).

**Living cell labeling with fluorescent ceramides**

Fluorescent ceramides (NBD-Cer, BODIPY-Cer TR) were dissolved in the chloroform:methanol mixture (19:1) to a 1 mM concentration. After they were completely dried under N₂, the lipids were dissolved in 200 μl of ethanol. This ethanol solution was injected into 4 ml of Hank’s balanced salt solution supplemented with 10 mM HEPES (pH 7.4) and 12.5 μM of
BSA under vigorous vortexing. Yielded ceramide-BSA complexes were added at 4°C for 30 min to the culture medium at the final concentration of 1.25 μM, followed by washings with ice-cold PBS three times. In the chase experiment, Cer-labeled cells were returned to the culture incubator with a normal medium. Cells were fixed with 4% paraformaldehyde for 15 min, followed by two wash steps with ammonium chloride (5 mM) for 5 min twice and with PBS once.
RESULTS

ER proteins associate with DRM

We examined whether Sig-1R and other ER proteins could be found in DRM extracted from CHO cells. In agreement with previous studies using different types of cells (Hayashi and Su, 2003b; Hayashi and Su, 2004), the sucrose gradient centrifugation of the Tx lysate found that a portion of Sig-1R was indeed present in DRM fractions (Fig. 1A; fraction 4-7). As seen in NG108 cells (Hayashi and Su, 2003b), DRM associated with Sig-1R showed lower buoyancy in the sucrose gradient (fractions 4-7) than those of Src (fractions 2-5), the protein associated mainly with plasma membrane DRM. Some ER proteins that are enriched at the MAM including IP3R3 and BiP were also present in DRM fractions, although IP3R3 was present at a much lower level (Fig. 1A). Mitofusin-2, the protein tethering MAM to mitochondria (de Brito and Scorrano, 2008), was also detected in DRM (Fig. 1A). Some ER proteins such as ERp57 were completely excluded from DRM (Fig. 1A).

Unique detergent-solubility profile of DRM associating with Sig-1R

Although Sig-1R were consistently detected in DRM fractions in different cell types, the proportion of Sig-1R present in DRM fractions is only 10-20% of total Sig-1R proteins (Fig. 1A). On the other hand, at least 50% of Src or caveolin was fractionated in DRM (Fig. 1A-B). However, it is noteworthy that recent studies demonstrate that DRM at internal membranes have lower resistance to extraction by the prototypic detergent Tx, but exhibit somewhat higher resistance to other types of detergents (Marwali et al., 2003), implying that there are different subclasses of DRM. Thus, although the marginal level of Sig-1R was present in Tx-insoluble
DRM, the result may not merely imply few Sig-1R associated with DRM. We therefore conducted sucrose gradient centrifugations with different types of detergents. Over 50% of Src and caveolin consistently associated with DRM in the presence of any type of detergent tested (Fig. 1B). The highest detergent resistance for both proteins was seen with Tx. The detergent resistance was slightly decreased with Brij-35 or Lubrol WX (Fig. 1B). In contrast, detergent resistance of DRM associated with Sig-1R and IP3R3 dramatically changed depending on the type of detergent used. Among them, DRM associated with Sig-1R showed remarkable resistance against extraction with Triton X-114, where over 80% of Sig-1R was present in DRM fractions (Fig. 1B). Buoyancy of the DRM was significantly higher in Triton X-114 (fraction# 2-4) than that in Tx (Fig. 1B). DRM associated with IP3R3 exerted significantly higher resistance to extraction by CHAPS, Triton X-114, Brij-35, or Lubrol WX when compared to Tx (Fig. 1B). These suggest that the high proportion of Sig-1R may be associated with DRM at the ER, but those are likely much less resistant to extraction by Tx.

The MAM is the specialized ER subdomain accommodating DRM

We examined whether the Triton X-114-insoluble DRM associated with Sig-1R are derived from the MAM. Accordingly, MAM and microsomes were purified from CHO cells, and enrichment of Sig-1R-containing DRM and DRM-forming lipids was examined in those membranes. In agreement with a previous study (Hayashi and Su, 2007), Sig-1R were enriched in the MAM fraction (Fig. 2A). The levels of IP3R3 and certain ER chaperones such as BiP and calreticulin were also the highest in the MAM fraction (Fig. 2A). ER chaperones involved in vesicular transport of proteins were also detected in the cytosolic fraction (Fig. 2A). The ER chaperone ERp57 was more enriched in the microsomal fraction (Fig. 2A). The microsomal
protein CYP450R was present exclusively in microsomal and nucleus fractions (Fig. 2A). Mitofusin-2 was present both in mitochondrial and MAM fractions (Fig. 2A). Mitochondria-specific proteins were detected only in the mitochondrial fraction (Fig. 2A). The distribution patterns of these organelle markers thus verified the sufficiently high purity of MAM and microsomal membranes.

When each fraction was solubilized with Tx, a marginal, but still detectable level of Sig-1R was present in DRM from the MAM fraction, but not in those from microsomes (upper panel in Fig. 2B). As shown in Fig. 1B, a much higher level of Sig-1R was present in DRM extracted from the MAM with Triton X-114 when compared to the extract with Tx (lower panel in Fig. 2B). Sig-1R were present in DRM only from the MAM fraction (Fig. 2B), strongly indicating that the Sig-1R-associated DRM are mostly derived from the MAM. Further, the silver staining showed a much larger amount of proteins present in DRM derived from the MAM when compared to DRM from microsomes (Fig. 2C), suggesting that the MAM accommodates a higher level of DRM than the microsome. Indeed, HPTLC revealed that the MAM contains higher levels of DRM-forming lipids, such as cholesterol, ceramides, glucosylceramide (GlcCer), and sphingomyelin, when compared to microsomes (Fig. 2D). The higher level of ceramides at the MAM was further confirmed by the lipid overlay assay using anti-ceramide antibodies (bottom panel in Fig. 2D). Among phospholipids, phosphatidylserine (PtSer) and PtChol were present in both membranes at similar levels, but phosphatidylethanolamine (PtEt) was higher in the MAM fraction (Fig. 2D). Lactosylceramide and GM3, the major ganglioside in CHO cells, were not detected in either MAM or microsomes under our HPTLC conditions (data not shown).

The Sig-1R complex associates with specific sphingolipids
It is well known that DRM prepared from the plasma membrane mostly consist of cholesterol and complex sphingolipids such as gangliosides. In contrast, DRM from the MAM appear to be more enriched in simple sphingolipids such as ceramides and GlcCer (Fig. 2D). To examine whether the simple sphingolipids might contribute to the formation of DRM and the localization of Sig-1R at the MAM, we examined whether Sig-1R preferentially associate with those MAM-enriched lipids by using a modified lipid overlay assay. The assay is particularly advantageous in studies using highly hydrophobic molecules as ligands (e.g., lipids), because the assay avoids problematic insolubility/aggregation of ligands as well as partitioning of ligands into membranes. Since CHAPS (0.2%) solubilization is shown to maintain the ligand-binding property of Sig-1R well (McCann and Su, 1991), the assay employed CHAPS cell lysates. It should be noted, however, that the assay using the CHAPS whole cell lysate might detect the direct association between lipids and Sig-1R molecules as well as the association between lipids and protein complexes composed of Sig-1R (designated here as the “Sig-1R complex”). The commercially available SphingoStrips Array™ membrane (200 pmoles/spot; Invitrogen) demonstrated that Sig-1R-EYFP complexes from the CHAPS lysate of Sig-1R-EYFP-overexpressing CHO cells specifically associate with ceramide and sulfatide out of 15 different lipids immobilized on the array membrane (Fig. 3A). No association of Sig-1R-EYFP complexes with sphingosine, sphingomyelin or sphingosine-1-phosphate was observed under these conditions (Fig. 3A); however, the association between Sig-1R complexes and sphingosine was consistently detected when the lipid on the array was increased to 1 nmole/spot. Solubilized EYFP complexes obtained from CHO cells stably overexpressing EYFP did not associate with any lipids on the array (Fig. 3A).

To further examine lipids interacting with Sig-1R complexes, we prepared a
nitrocellulose array membrane with 30 different lipids immobilized (200 pmoles/spot). The assay found that Sig-1R-EYFP complexes associated preferentially with sphingolipids with few or no sugar moieties, but not with gangliosides [association potency: lactosylceramide > galactosylceramide > glucosylceramide > sulfatide > hydroxy or nonhydroxy ceramide (from bovine brains) >>> gangliosides] (Fig. 3B). The two oligodendrocyte-specific glycosphingolipids, GalCer and sulfatide, were both capable of associating with Sig-1R-EYFP complexes (Fig. 3B). Interestingly among sterols tested under this condition, only lathosterol showed a detectable, yet low, association with Sig-1R-EYFP complexes (Fig. 3B). This result is in agreement with the fact that the Sig-1R shares 66% similarity with a yeast C8-C7 sterol isomerase catalyzing the conversion of zymosterol to lathosterol (Hanner et al., 1996). Endogenous Sig-1R complexes also showed a similar profile in the lipid binding, but with lower signals, thus prohibiting detection of some interactions (Fig. 3C). This seemed to be mainly due to the lower protein level of endogenous Sig-1R complexes in the lysate. Increasing the total protein level in the lysate (from 28 μg to 60 μg of protein lysate/cm² of membrane), accompanied by increased lipids on the membrane (400 ≥ pmoles/spot), ensured the reproducible detection of endogenous Sig-1R complexes associated with ceramides or lathosterol (Fig. 3D).

It has been demonstrated that Sig-1R associate with certain steroids and cholesterol (Pal et al., 2007; Palmer et al., 2007; Su et al., 1988). Indeed, the assay detected the association of Sig-1R with some sterols (lathosterol >> progesterone ≥ testosterone >>> cholesterol), but much higher amounts of lipids (10 nmole/spot) were necessary, except for lathosterol, to obtain reliable signals (Fig. 3E).

D-erythro-sphingosine associated with Sig-1R complexes (Fig. 3F). However, in contrast to the previous report (Ramachandran et al., 2009), the interaction between the Sig-1R complex
and ceramide was consistently detected. Ceramides possessing a long fatty acid acyl chain (i.e., C16-18), which is naturally present, associated better with Sig-1R complexes, whereas synthetic ceramides with short acyl chains (e.g., C6, C2) failed to associate with Sig-1R-EYFP or Sig-1R complexes (Fig. 3B, F). The estimated order of potencies of ceramides/sphingosine in associating with Sig-1R complexes was C18-ceramide = C16-ceramide > D-erythro-sphingosine > C22-ceramide >>> C6-, C2- ceramides. The structures of these lipids are provided in the supplemental figure.

Since the MAM is shown to possess high ceramide biosynthesis activity (Bionda et al., 2004), we particularly focused in the following studies on ceramides to determine if the lipid may play a role in localization of Sig-1R to the MAM.

**MAM is highly capable of accumulating ceramides**

An early study demonstrates that exogenously applied fluorescent NBD-Cer distributes heterogeneously in living cells. NBD-Cer accumulate initially at the plasma membrane and ER and then at mitochondria in living cells when they are incubated at 2°C, a temperature at which energy-dependent vesicle transport of lipids is shut down (Lipsky and Pagano, 1983). This finding clearly illustrates that the intermembrane diffusion of ceramides from the ER to the mitochondrial membranes is restricted at the ER-mitochondria junction. Thus, the MAM might be highly capable of accumulating ceramides in order to serve as the checkpoint regulating the diffusion of ceramides from the ER to the mitochondria. To examine if the MAM accumulates ceramides, CHO cells were incubated with NBD-Cer at 2°C, and the cellular distribution was monitored by confocal microscopy. As expected, a striking accumulation of NBD-Cer was observed at MAM-like small structures (large arrows in Fig. 4A) juxtaposed to mitochondria that
bear the typical spaghetti-like structure (small arrows in Fig. 4A). NBD-Cer also accumulated at the cisternae of the ER (arrowheads in Fig. 4A) and the mitochondria, but at much lower levels. The observed NBD signals in living CHO cells appear to represent mostly the distribution of NBD-Cer, but not that of those metabolites, because few NBD-Cer were metabolized to GlcCer in the living cells under 4°C (see lane 1 in Fig. 4A). The structures accumulating NBD-Cer clearly apposed mitochondria that were visualized by expressing Mito-DsRed (Fig. 4B), and the staining pattern was identical to that of Sig-1R immunostaining (Fig. 4C). The other fluorescent ceramide (BODIPY-Cer TR) accumulated similarly at the MAM-like structures that colocalized with Sig-1R-EYFP (Fig. 4D). Membrane fractionation confirmed that the level of NBD-Cer is the highest in the MAM fraction (Fig. 4E).

To examine whether the accumulation of ceramides at the MAM is adversely affected by the low-temperature incubation, under which vesicular transports of lipids from ER to Golgi are stalled, cells labeled with NBD-Cer were returned to the normal culture condition at 37°C. Under these conditions, NBD-Cer accumulated at the Golgi apparatus while decreasing at the ER (Fig. 4F), indicating that the energy-dependent transport of ceramides from ER to Golgi was now resumed. Under these conditions, a portion of NBD-Cer was converted to GlcCer (see lane two of TLC in Fig. 4A); the step is known to be mainly catalyzed at the Golgi. Importantly, even under these conditions, the MAM still accommodated a significant level of NBD-Cer, suggesting that the MAM may possess innate ability to accumulate ceramides at the locus.

Although the distribution of NBD-Cer may not necessarily represent precise cellular dynamics of endogenous ceramides, the finding that MAM can attract NBD-Cer and accommodate the high level of endogenous ceramides (Fig. 2D) suggests that Sig-1R complexes that possess affinities for ceramides might be preferentially recruited to the ER subdomain.
Sig-1R require ceramides for their partitioning into DRM

To examine the importance of ceramides in localization of Sig-1R at the MAM, we employed pharmacological approaches that enable the manipulation of lipid contents and DRM integration at the ER membranes. MβC is a well-known compound that depletes free cholesterol in biological membranes, thus disrupting DRM (Simons and Toomre, 2000). Since water-soluble MβC does not readily penetrate plasma membranes, we assumed that MβC might extract cholesterol initially at the cell surface. On the other hand, the long exposure to MβC is known to cause a nearly complete depletion of cholesterol in the whole cell (Naslavsky et al., 1999). Accordingly, CHO cells were treated with MβC for different periods of time to preferentially destroy DRM either at the cell surface or in the entire cell. Incubation with MβC for 15 min depleted 30% of total cholesterol in CHO cells, while incubation for 60 min depleted approximately 80% of total cholesterol (Fig. 5A). Filipin staining confirmed that a short exposure to MβC reduced cholesterol mainly on the cell surface, but a longer exposure decreased cholesterol in the entire cell (Fig. 5B). Next, we examined the effect of MβC on partitioning of proteins to DRM. The treatment with MβC for 15 min significantly decreased Src associated with DRM, but not Sig-1R (Fig. 5E), suggesting that Sig-1R-residing DRM are intracellularly localized. On the other hand, a long exposure to MβC (for 60 min) significantly decreased Sig-1R in DRM (Fig. 5E), indicating that Sig-1R-residing microdomains also require cholesterol for stabilization/formation.

Fumonisin B1 (FB1), which blocks the conversion of sphinganine to dihydroceramide, is a potent inhibitor of the ceramide biosynthesis at the ER (Naslavsky et al., 1999). Although FB1 rapidly inhibits the synthesis of ceramides at the ER, FB1 requires prolonged incubation (e.g.,
24-72 hrs) in order to deplete gangliosides or sphingomyelin in the cell because of their slow turnover rates and/or the alternative synthesis pathways independent of \textit{de novo} synthesis of ceramides (Naslavsky et al., 1999). Since the MAM serves as the locus for ceramide biosynthesis (Bionda et al., 2004) and it is assumed that the major pool of ER ceramides relies on activity of the \textit{de novo} synthesis at the ER (Sprong et al., 2001), we expected that FB1 might efficiently and rapidly decrease the focal concentration of ceramides at the MAM. In fact, FB1, even with its treatment for 4 hrs, significantly reduced ceramides at the MAM without largely affecting total levels of ganglioside GM3 in CHO cells (Fig. 5C). As shown previously (Naslavsky et al., 1999), prolonged incubation with FB1 (24 hrs) significantly decreased GM3 in CHO cells (data not shown). Next, we examined whether the depletion of ceramides caused by FB1 might affect the association of Sig-1R with DRM. As shown in Fig. 5E, treatment with FB1 for 4 hrs significantly decreased Sig-1R associated with DRM. However, little effect on Src association with plasma membrane DRM was observed, suggesting that ceramides are important molecules for partitioning of Sig-1R to DRM.

FB1 may also reduce metabolites such as GlcCer, which might be components of DRM at the MAM, immediately downstream of the ceramide synthesis. GlcCer is synthesized by a GlcCer synthase at the cytoplasmic surface of the Golgi apparatus (Sprong et al., 2001). The GlcCer synthesis depends largely on ceramides supplied by the \textit{de novo} synthesis at the ER (Sprong et al., 2001). Thus, inhibition of the GlcCer synthesis by (±)-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol hydrochloride (PDMP) decreased GlcCer, while it concomitantly accumulated ceramides in the cell (Fig. 5D). We next examined whether PDMP affects the association of Sig-1R with DRM. Unexpectedly, PDMP (4 hrs) increased Sig-1R association with DRM (Fig. 6D), indicating that ceramides, but not GlcCer, appear to serve as a
positive regulator of Sig-1R for their partitioning into DRM.

**DRM anchor Sig-1R at the ER-mitochondria junction**

To assess the importance of DRM in MAM-specific distribution of Sig-1R, we tested whether disruption of DRM may affect cellular localization of Sig-1R. Treatment with MβC (60 min) or FB1 (4 hrs) caused a dramatic redistribution of Sig-1R-EYFP from MAM to ER cisternae (Fig. 6A), resulting in a significant reduction in Sig-1R-EYFP colocalization with Mito-DsRed (Fig. 6B). MβC pre-saturated with cholesterol (MβC-Chol), which provides free cholesterol to the cell, failed to promote translocation of Sig-1R-EYFP (Fig. 6A). MβC-Chol, however, did cause swelling of the mitochondria (Fig. 6A; round mitochondria shown in red), as previously reported (Colell et al., 2003). This redistribution appeared to be due to the translocation of Sig-1R-EYFP at ER membranes, but not due to structural disorganization of ER membranes caused by MβC or FB1, because the treatments did not significantly alter the overall structure of ER membranes or the spatial interaction between ER membranes and mitochondria at least under observations with confocal microscopy (Fig. 6C; also middle panels in D). The confocal analysis revealed that these treatments do not alter colocalization of fluorescence signals derived from Mito-GFP and KDEL-DsRed (Fig. 6C). The treatment with MβC or FB1 also caused a similar redistribution of IP3R3 originally present at the MAM (Fig. 6D). Thus, cholesterol and ceramides are important membrane components at the ER for anchoring Sig-1R at the MAM.
DISCUSSION

Our findings indicate that ER-residing DRM play an important role in the targeting of Sig-1R to the ER-mitochondria junction. A unique characteristic of the MAM shown in this study is the enrichment of DRM-forming lipids at the locus (e.g., cholesterol, ceramide), which are normally low at ER membranes (Sprong et al., 2001). Interestingly, Sig-1R-containing DRM formed at the MAM showed much higher resistance to Triton X-114 than to Tx. This characteristic is different from that of DRM derived from plasma membranes. On the other hand, sigma-2 receptors are shown to associate with DRM that share similar characteristics with those of plasma membrane DRM (e.g., high resistance to extraction by Tx) (Gebreselassie and Bowen, 2004). Therefore, it is uncertain whether the Sig-1R-containing DRM may serve as a subtype of so-called lipid rafts or whether they represent novel lipid microdomains biophysically dissimilar to lipid rafts.

The MAM plays a pivotal role at the ER by regulating ER-mitochondria communications (Hajnoczky et al., 2006; Rizzuto and Pozzan, 2006). Ca\(^{2+}\) influxed from the MAM to the mitochondria activates the tricarboxylic acid cycle (TCA) cycle, whereas overloading of Ca\(^{2+}\) in mitochondria causes apoptosis (Hajnoczky et al., 2006; Rizzuto and Pozzan, 2006). Several important metabolites are transported between the ER and the mitochondria via the MAM, including phospholipids, cholesterol, and ATP (Hayashi et al., 2009). To properly operate the interorganelle transport and signal transduction, the MAM is equipped with particular sets of proteins, such as Ca\(^{2+}\)-sensitive molecular chaperones, IP3 receptors, Ca\(^{2+}/\)ATPase, PtSer synthase, ubiquitin ligase, and mitofusin-2 (de Brito and Scorrano, 2008; Hayashi et al., 2009). Thus, defining the molecular mechanisms underlying the targeting of specific proteins to the
MAM is of utmost importance for understanding of the fundamental functions of the cell. Here we have demonstrated one potential mechanism by which the MAM can accommodate specific proteins at the locus. Namely, the MAM utilizes detergent-resistant lipid microdomains to recruit proteins to the ER-mitochondria junction. It is worth noting that DRM also play an important role in recruiting specific proteins at the junction between the plasma membrane and ER (Pani et al., 2008). A study demonstrates that caveolae-type DRM determine the targeting of STIM1 clusters to ER-plasma membrane junctions following depletion of ER Ca\(^{2+}\) (Pani et al., 2008). Therefore, although the sphingolipid components of DRM vary depending on the location, they might be utilized more commonly to recruit specific proteins to focal contacts of organelles.

It has been shown that Sig-1R bind sterols (Palmer et al., 2007; Su et al., 1988). Further, a recent study finds that D-erythro-sphingosine and sphinganine, but not sphingosine-1 phosphate, bind with Sig-1R in a manner competitive to the prototypic Sig-1R agonist (+)pentazocine (Ramachandran et al., 2009). In this study a lipid overlay assay demonstrated that the CHAPS-solubilized Sig-1R complex possesses affinities for ceramides and mono/di-glycosylated ceramides, but not for gangliosides and sphingomyelin. In agreement with the study done by Ramachandran et al., (2009), the lipid overlay assay demonstrated that the solubilized Sig-1R complex preferentially binds to sphingosine. However, a previous study reports no association between Sig-1R and ceramides using \textit{in vitro} binding assays (Ramachandran et al., 2009).

Potential explanations for this discrepancy are: 1) the association of ceramides with the Sig-1R complex might require the integrated oligomeric protein complex containing Sig-1R, thus the association may not be achieved with purified Sig-1R; 2) ceramides could be interacting with regions of Sig-1R that are not involved in ligand binding; 3) ceramides bearing a long acyl chain (> C16) are highly insoluble and easily aggregated in aqueous conditions if amphiphilic carrier
molecules such as albumin are absent (Siskind et al., 2006), thus aggregated ceramides might lose their affinity for Sig-1R in the binding assay; or 4) the length of the acyl chain of the ceramide used by Ramachandran et al., (2009) might be different from the one described here (the length is critical for the association with Sig-1R complexes; Fig. 3F). In contrast to sphingosine possessing the ionizable nitrogen atom, ceramides possess the nitrogen atom as an amide, thus not providing a possible interaction of the nitrogen head with its unshared electrons to interact directly with the Sig-1R-binding site. Therefore, the pharmacophore of these lipids utilized in association with Sig-1R might not be completely identical. Alternatively, it is also possible that the ceramides are regulating the distribution of the Sig-1R-containing microdomains by interacting with accessory proteins in the microdomain and not selectively with the Sig-1R.

Although sphingosine possesses an affinity for Sig-1R in the nM range, the lipid is not likely to be the constituent of Sig-1R-associated DRM at the MAM. As a metabolic intermediate of the rapid ceramide biosynthesis, sphingosine is kept at a considerably low level in the cell, especially under unstressed conditions (Spiegel and Milstien, 2003). Sphingosine is physicochemically categorized as a class III lipid (i.e., a swelling amphiphile that possesses strong polar groups of the aminoalcohol head) that renders the molecule soluble in water at low concentrations (Shen et al., 2001). Further, a previous study showed that sphingosine (at higher concentrations) is taken up by the membrane via a passive diffusion mechanism independent of lipid raft integrity (Garmy et al., 2005). Since sphingosine is a water-soluble lipid that quickly changes its concentration under cellular stress (e.g., via its phosphorylation) (Spiegel and Milstien, 2003), the lipid may rather serve as an important Sig-1R ligand that is called upon under pathological conditions. On the other hand, ceramides that are integrated in bilayer
membranes might serve as an anchor tethering Sig-1R complexes to the ER-mitochondria junction.

Our data suggested that ceramide is a sphingolipid component constituting Sig-1R-containing DRM at the MAM. PDMP that decreases GlcCer while concomitantly accumulating ceramides at the ER (Naslavsky et al., 1999) did increase partitioning of Sig-1R to DRM. However, one unsolved question arising from this finding is why GlcCer, which is enriched at the MAM (Fig. 2D) and possesses a strong affinity for Sig-1R complexes in vitro (Fig. 3B), failed to substitute for ceramides in partitioning Sig-1R to DRM. To explain this, we propose the asymmetric membrane model of the MAM where ceramides and GlcCer may be distributed at the inner and outer leaflets of the MAM, respectively. Although ceramides are synthesized at the cytoplasmic surface of ER membranes (Sprong et al., 2001), the rate of their spontaneous transbilayer movement is relatively rapid and less restricted because of the small polar head (Sprong et al., 2001). Further, ceramides remaining at the cytoplasmic leaflet might be readily transported to mitochondria via the intermembrane transport or to the Golgi via the ceramide transport protein (Sprong et al., 2001). Therefore, it is possible to speculate that ceramides and their containing DRM might be confined to the lumenal leaflet of the MAM. However, GlcCer is shown to function on the cytosolic surface of membranes to regulate the budding of transport vesicles (van Meer et al., 2003). Further, it is suggested that Sig-1R form a ligand-binding site at the lumenal surface of the ER membrane (Hayashi and Su, 2008; Pal et al., 2007), the region assumed to interact with prototypic Sig-1R ligands as well as with sphingosine and sterols (Palmer et al., 2007; Ramachandran et al., 2009). Therefore our asymmetry model of the MAM implies that GlcCer at the cytoplasmic leaflet of the MAM may have less probability of associating with the lipid-binding domain of Sig-1R in in vivo systems. In contrast, GalCer that
is synthesized at the ER lumen of oligodendrocytes may be able to associate with Sig-1R, as previously reported (Hayashi and Su, 2004).

In conclusion, our findings demonstrate that the MAM utilizes ceramide-rich lipid microdomains to anchor Sig-1R at the locus. The lipid microdomains at the organelle junction may therefore play crucial roles in positioning Sig-1R for cellular survival and mitochondrial bioenergetics. The most interesting question for future studies is whether and how Sig-1R agonists or antagonists alter the association of Sig-1R complexes with DRM, particularly with ceramides therein. It is unclear if Sig-1R ligands could promote the dissociation of Sig-1R from ceramides that are basically buried in membrane bilayers. On the other hand, it is known that cellular stress per se affects the metabolisms of ceramides (e.g., the breakdown of sphingomyelin to ceramide or ceramide to sphingosine) as well as membrane integrations including DRM (Spiegel and Milstien, 2003; Sprong et al., 2001). Therefore, another important question that awaits future study is whether stress-induced alterations of membrane lipids and their integration at DRM might represent the intrinsic mechanism by which translocation of Sig-1R is regulated in the cell. Since the dynamic redistribution is a key element for Sig-1R chaperones to determine their client proteins localized at either plasma membranes or the ER, elucidating molecular mechanisms by which the cell switches the action site of Sig-1R may help to further understand the function of Sig-1R but also to develop a new class of therapeutic drugs.
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REFERENCES


Hayashi T and Su TP (2003a) Intracellular dynamics of sigma-1 receptors (sigma(1) binding sites) in NG108-15 cells. *J Pharmacol Exp Ther* **306**(2):726-733.

Hayashi T and Su TP (2003b) Sigma-1 receptors (sigma(1) binding sites) form raft-like microdomains and target lipid droplets on the endoplasmic reticulum: roles in endoplasmic reticulum lipid compartmentalization and export. *J Pharmacol Exp Ther* **306**(2):718-725.


FOOTNOTES

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Legends for Figures

**Fig. 1. Detergent-insolubility profile of DRM associating with Sig-1R.**

A. ER proteins in DRM. Tx-cell lysates were fractionated (#1-13 from top to bottom) following sucrose gradient centrifugation. Fractions 1-7 represent DRM. Graphs represent fraction distributions of proteins where the sum of thirteen fractions was taken as 100% for each protein (mean of 2-3 experiments). B. Solubility of Sig-1R-containing DRM in different detergents. CHO cells (2x10^6 cells) were solubilized with different detergents (0.5% except for CHAPS at 20 mg/ml), followed by sucrose gradient centrifugation. Proteins enriched at plasma membrane rafts (Src and caveolin) or those at the MAM (Sig-1R and IP3R3) were detected by immunoblotting. Graphs represent fraction distributions of Src and Sig-1R (mean of 2 experiments).

**Fig. 2. MAM accommodates DRM associated with Sig-1R**

A. Purification of MAM and microsomal fractions from CHO cells. Nuclear (P1), mitochondrial (Mito), MAM, microsomal (P3) and cytosolic (Cyt) fractions were prepared by differential centrifugation combined with a Percoll gradient fractionation. Five μg of proteins were applied to SDS-PAGE, followed by immunoblotting. CYP450R, cytochrome P450 reductase; COX, cytochrome c oxidase subunit I. Asterisks indicate ER chaperones involved in vesicular transport. Graphs represent fraction distributions of proteins where the sum of five fractions was taken as 100% for each protein. B. Sig-1R-associated DRM in the MAM. MAM and microsomes (P3) (25 μg of total proteins in each) were extracted by 0.5% of Tx (Tx-100) or Triton X-114 at 4°C. DRM and
detergent-soluble supernatant (S) were prepared by differential centrifugations. The numbers represent the average of optical density (O.D.) measured in each protein band (N=3). **C.** Silver staining for total proteins associated with DRM in MAM and microsomal fractions. MAM and microsomes (25 μg of total proteins in each) were extracted in 0.5% Tx at 4°C and DRM and soluble supernatants (S) were prepared. Proteins were visualized by 13% SDS-PAGE, followed by silver staining. The numbers represent the average of optical density (O.D.) measured in each lane (N=3). MW: molecular weight of standard proteins. **D.** Lipid contents in MAM and microsomal fractions. Lipids were extracted and analyzed by HPTLC. Cholesterol (Chol) was detected using a ferric chloride spray; GlcCer using a diphenylamine-aniline spray. Lipids in the second panel were visualized under UV following an ANS spray. In the lipid overlay assay for ceramides (Cer at the bottom panel), ceramides extracted from HPTLC plates were immobilized on a nitrocellulose membrane followed by immunoblotting with anti-ceramide antibodies. SM, sphingomyelin.

**Fig. 3.** Association of simple sphingolipids with solubilized Sig-1R complexes

**A.** Lipid overlay assay using the SphingoStrips Array™ membrane (200 pmoles/spot). CHO cells stably expressing either EYFP or Sig-1R-EYFP were solubilized with 0.2% CHAPS. CHAPS cell lysates (28 μg of total proteins/cm²) were incubated with the array membrane followed by immuno-detections with anti-GFP antibodies. Blk: blank. Asterisks indicate lipids identified as being associated with Sig-1R-EYFP. **B.** Association of Sig-1R-EYFP complexes with simple sphingolipids. CHO cell lysates were incubated with Hybond-C Extra nitrocellulose membranes, 30 different lipids (200 pmoles/spot)
were immobilized. Cort, corticosterone; DHEA (-S), dehydroepiandrosterone (-sulfate); Preg (-S), pregnenolone (-sulfate); Prog, progesterone; Testo, testosterone; Eestra, estradiol; Chol, cholesterol; Cer, ceramide; NFA-Cer, Cer with non-hydroxy fatty acid; HFA, hydroxy fatty acid; Palmi, palmitate; Arachi, arachidonate. Asterisks indicate lipids identified as being associated with Sig-1R-EYFP. C. Association of endogenous Sig-1R complexes with lipids. Wild-type CHO cells were solubilized with 0.2% CHAPS, and the lysates incubated with the nitrocellulose membranes. The left membrane was developed with normal rabbit IgG and the right with specific anti-Sig-1R antibodies. D. Association of endogenous Sig-1R complexes with ceramides, lactosylceramides (LacCer) and lathosterol. Note that each spot contains 400 pmoles of lipid. The membrane was incubated with a cell lysate from wild-type CHO cells at a total protein concentration of 60 μg/cm². Sig-1R associated with the lipids were detected by anti-Sig-1R antibodies. E. Association of endogenous Sig-1R complexes with sterols. Each spot contains 10 nmoles of lipid. The membrane was incubated with a cell lysate from wild-type CHO cells at a total protein concentration of 60 μg/cm². F. Association of endogenous Sig-1R complexes with ceramides and sphingosine. D-erythro-sphingosine (Sph), C2-Cer, and C6-Cer are at 1 n mole/spot. Other ceramides are at 400 pmoles/spot. The protein concentration of the CHAPS lysate is 60 μg/cm² of membrane.

**Fig. 4. MAM accumulates ceramides**

A. Accumulation of NBD-ceramide (NBD-Cer) at subcellular membranes. CHO cells were incubated with NBD-ceramide (1.25 μM) at 4°C for 30 min. Confocal microscope (left panel). Arrowhead, ER cisternae; small arrow, mitochondria with a spaghetti-like
shape; large arrow, juxta-mitochondria structures accumulating NBD-Cer; N, nucleus. Bar=10 \( \mu \text{m} \). HPTLC (light panel), where lipid extracts from NBD-Cer-labeled CHO cells were visualized under UV. 1. the lipid extract from CHO cells incubated with NBD-Cer at 4\(^\circ\)C for 30 min; 2. the lipid extract from CHO cells incubated with NBD-Cer at 4\(^\circ\)C for 30 min, followed by washings and incubation at 37\(^\circ\)C for 30 min. Note the few conversions of NBD-Cer to NBD-GlcCer in lane 1. \textbf{B.} Accumulation of NBD-Cer at intracellular domains apposing mitochondria. NBD-Cer (upper) and mitochondria expressing Mito-DsRed (lower) were visualized in the same cell. The two panels were superimposed in inset (NBD-Cer in green, MitoDsRed in red). Bar=2.5 \( \mu \text{m} \). \textbf{C.} MAM-specific distribution of Sig-1R (upper panel; in green inset). The lower panel is for mitochondria in the same sample. Bar=2.5 \( \mu \text{m} \). Two panels were superimposed in inset. \textbf{D.} Colocalization between Sig-1R-EYFP and fluorescent ceramides. CHO cells expressing Sig-1R-EYFP (upper panel) were labeled with fluorescence ceramide BODIPY-Cer TR (1.25 \( \mu \text{M} \); lower panel) at 4\(^\circ\)C for 30 min. Two panels are superimposed in the inset (Sig-1R-EYFP in green, BODIPY-Cer TR in red). Bar=2.5 \( \mu \text{m} \). Colocalization represented by yellow in inset. The same results were obtained from 4 independent samples (\( > 5 \) fields/sample). \textbf{E.} MAM accumulates NBD-Cer. CHO cells labeled with NBD-Cer at 4\(^\circ\)C for 30 min were subjected to membrane fractionations. The level of NBD-Cer in each fraction was measured by a fluorescence microplate reader. Fluorescence intensities were normalized to protein concentrations in each sample. \textbf{F.} Retention of NBD-Cer at MAM. CHO cells were labeled with NBD-ceramides at 4\(^\circ\)C for 30 min (left image). After washings, cells were incubated in normal medium at 37\(^\circ\)C for 30 min (right image). Thin arrows: mitochondria, thick arrows: MAM. N: nucleus, G:
Fig. 5. Cholesterol and ceramide-dependent partitioning of Sig-1R into DRM

A. Time-dependent depletion of free cholesterol caused by MβC. CHO cells in a serum-free medium were incubated with 5 mM of MβC. Lipids were extracted, and free cholesterol was measured enzymatically. The graph represents mean±SEM from five results. B. Time-dependent depletion of free cholesterol caused by MβC. CHO cells treated with MβC for 15 or 60 min were fixed and stained with filipin. Bar=50 μm. Panels represent results from five independent experiments.

C. Depletion of ceramides at the MAM caused by FB1 (5 μg/ml, 4 hrs). Ceramides were extracted from MAM fractions. GM3 gangliosides were extracted from whole cells. The panels represent results from three independent HPTLC. Lipids were visualized by ANS (upper) and diphenylamine-aniline (lower) sprays, respectively. D. PDMP (25 μg/ml, 4 hrs) decreases GlcCer, but accumulates ceramides. Ceramides and GlcCer were extracted from whole cells. The panels represent results from three independent HPTLC. Lipids were visualized by ANS (upper) and diphenylamine-aniline (lower) sprays, respectively.

E. Effects of depletion of cholesterol or ceramide on partitioning of Src and Sig-1R to DRM. Src-containing DRM were extracted by 0.5% Tx while Sig-1R-containing DRM were extracted by 0.5% Triton X-114. CHO cells were treated with MβC, FB1 or PDMP under the same conditions in B-D.

Fig. 6. Relocation of MAM proteins caused by depletion of cholesterol or ceramides

A. Relocation of Sig-1R-EYFP from MAM to ER cisternae. CHO cells expressing Sig-
MOL #62539

1R-EYFP (in green) and Mito-DsRed (mitochondria in red) were treated with MβC (5 mM for 60 min), cholesterol-saturated MβC (MβC-chol, 5 mM for 60 min) or FB1 (5 μg/ml for 4 hrs). Cells were fixed and observed under a confocal microscope. Bar=10 μm. B. Quantitative analyses for Sig-1R-EYFP in the vicinity of mitochondrial membranes. The percentage of Mito-DsRed colocalizing with Sig-1R-EYFP was analyzed by the PerkinElmer UltraView system (N=6-7, mean±SEM). **p<0.01 compared to control by one-way ANOVA followed by Tukey post-hoc test. C. No effect of MβC (5 mM for 60 min) or FB1 (5 μg/ml for 4 hrs) on association between ER and mitochondrial membranes. Colocalization between KDEL-DsRed (ER membrane) and GFP-Mito (mitochondria membrane) was assessed as described in B. D. Relocation of IP3R3 from MAM to ER cisternae. Drugs were applied to CHO cells under the same conditions as in A. IP3R3 (in green) were immunostained with anti-IP3R3 antibodies in cells expressing KDEL-DsRed (in red) for ER. Bar=10 μm.