σ-1 Receptors (σ1 Binding Sites) Form Raft-Like Microdomains and Target Lipid Droplets on the Endoplasmic Reticulum: Roles in Endoplasmic Reticulum Lipid Compartmentalization and Export

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ABSTRACT

The brain σ1 receptors can bind neuropeptides and psychotropic drugs, including neuroleptics and cocaine and are implicated in schizophrenia, depression, and drug dependence. In this study, we found that σ1 receptors specifically target lipid storage sites (lipid droplets) on the endoplasmic reticulum by forming a distinct class of lipid microdomains. Both endogenously expressing σ1 receptors and transfected C-terminally enhanced yellow fluorescent protein (EYFP)-tagged σ1 receptors (Sig-1R-EYFP) target unique “ring-like” structures associated with endoplasmic reticulum reticular networks in NG108-15 cells. The ring-like structures contain neutral lipids and are enlarged by the oleate treatment, indicating that they are endoplasmic reticulum-associated lipid droplets (ER-LDs). σ1 receptors colocalize with caveolin-2, a cholesterol-binding protein in lipid rafts on the ER-LDs, but not with adipocyte differentiation-related protein (ADRP), a cytosolic lipid droplet (c-LD)-specific protein. When the double-arginine ER retention signal on the N terminus of σ1 receptors is truncated, σ1 receptors no longer exist on ER-LDs, but predominantly target c-LDs, which contain ADRP. σ1 receptors on ER-LDs form detergent-resistant raft-like lipid microdomains, the buoyancy of which is different from that of plasma membrane lipid rafts. (+)-Pentazocine causes σ1 receptors to disappear from the microdomains. N-Terminally EYFP-tagged σ1 receptors (EYFP-Sig-1R) failed to target ER-LDs. EYFP-Sig-1R-transfected cells showed an unrestricted distribution of neutral lipids all over the endoplasmic reticulum network, decreases in c-LDs and cholesterol in plasma membranes, and the bulboous aggregation of endoplasmic reticulum. Thus, σ1 receptors are unique endoplasmic reticulum proteins that regulate the compartmentalization of lipids on the endoplasmic reticulum and their export from the endoplasmic reticulum to plasma membrane and c-LDs.

The σ receptors were originally proposed as a subtype of opioid receptors because some psychomimetic benzomorphans bind to these sites (Martin et al., 1976). However, σ receptors are insensitive to a universal opioid receptor antagonist naltrexone (Su, 1982) and are enriched in microsomal fractions (McCann and Su, 1990; Hayashi and Su, 2001). Now, it is known that σ receptors are unique nonopioid receptor, nonphencyclidine receptor brain proteins (Su, 1982; Bowen et al., 1989; Snyder and Largent, 1989). The σ receptors are implicated in certain psychiatric disorders, including schizophrenia, depression, and drug dependence (Snyder and Largent, 1989; Ujike et al., 1996; Matsumoto et al., 2001). σ receptors consist of two subtypes: σ1 and σ2 receptors (Bowen et al., 1989). σ1 receptors have been cloned (Hanner et al., 1996; Seth et al., 1997), whereas the sequence of σ2 receptors remains unknown. σ1 receptors bind endogenous steroid hormones such as progesterone, pregnenolone-sulfate, and testosterone (Su et al., 1988). They also bind diverse classes of compounds such as haloperidol (Su, 1982), a most commonly used neuroleptic, tricyclic antidepressants (Su, 1982), selective serotonin reuptake inhibitors (Narita et al., 1996), and cocaine (Sharkey et al., 1988; Matsumoto et al., 2001). Recently, the physiological roles of σ1 receptors have been extensively investigated. In vitro, σ1 receptors show a modulatory action on the Kv 1.4 potassium channel

ABBREVIATIONS: ER-LD, endoplasmic reticulum-associated lipid droplet; c-LD, cytosolic lipid droplet; CYP450R, NADPH-cytochrome P450 reductase; CTx-B, cholea toxin subunit-B; GFP, green fluorescent protein; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; EYFP, enhanced yellow fluorescent protein; EYFP-Sig-1R, N-terminally EYFP-tagged σ1 receptor; Sig-1R-EYFP, C-terminally EYFP-tagged σ1 receptor; ADRP, adipocyte differentiation-related protein; Cav, caveolin.
(Aydar et al., 2002). α-1 receptors also modulate inositol 1,3,5-trisphosphate receptors at the endoplasmic reticulum (Hayashi et al., 2000; Hayashi and Su, 2001). α-1 receptor agonists modulate N-methyl-D-aspartate-induced neuronal firing in the CA3 region of hippocampus (Monnet et al., 1990) as well as the N-methyl-D-aspartate-induced dopamine release in the striatal slices (Nawyshid and Werling, 2003). Behaviorally, α-1 receptor agonists were found to improve learning and memory and cognition in animal models of amnesia (Maurice and Lockhart, 1997). α-1 receptor agonists also exhibit antidepressant-like activities (Urani et al., 2001). However, the basic molecular mechanisms underlying these actions of α-1 receptors are still unclear at present.

α-1 receptors have one putative transmembrane domain and two hydrophobic stretches, and possess a double arginine endoplasmic reticulum retention signal at the N terminus (Hanner et al., 1996; Seth et al., 1997). A study recently proposed a two-transmembrane domain model for α-1 receptors (Aydar et al., 2002). The sequence of α-1 receptors exhibits no homology to any other mammalian protein; however, it has a 30.3% identity to the fungal sterol C8-C7 isomerase (Hanner et al., 1996). More strikingly, about 75% of amino acids on the hydrophobic domain in the center of α-1 receptors are identical to the sterol binding pocket in fungal sterol C8-C7 isomerase (Keon et al., 1994). These suggest that α-1 receptors might bear certain relation to sterols. However, α-1 receptors lack the enzymatic activity as a sterol isomerase (Labbit-Le Bouteiller et al., 1998), and the cloned mammalian C8-C7 sterol isomerase (Braverman et al., 1999) is totally different from α-1 receptors. The biological action of α-1 receptors on sterols, if any, has never been reported.

We reported recently that α-1 receptors localize on smooth endoplasmic reticulum and form a dimeric complex with a cytoskeletal adaptor protein ankyrin-B (Hayashi and Su, 2001). Interestingly, upon stimulation by psychoactive drugs, α-1 receptors translocate to plasmalemmal and nuclear membranes (Morin-Surun et al., 1999; Hayashi et al., 2000; Hayashi and Su, 2001). Here, in a series of investigations using immunochemistry and transfection of green fluorescent protein-tagged α-1 receptors (invitrogen, Carlsbad, CA). Purified vectors were digested by EcoRI and BamHI (sites underlined) to yield α-1 receptors cDNAs for ligation into pEYFP-N1 or pEYFP-C1 vector (BD Biosciences Clontech) for expression of Sig-1R-EYFP and EYFP-Sig-1R, respectively. Italics indicated mutated nucleotides to avoid dimerization. Vectors were transfected by using LipofectAMINE-2000 (Invitrogen). Filipin and Nile Red Fluorescence Stainings. NG108 cells were fixed and incubated for 3 h in PBS containing 1% bovine serum albumin and 125 μg/ml filipin (Polysciences, Warrington, PA). Images were captured by a confocal microscopy with an UV laser (365 nm). For Nile red (Sigma-Aldrich) staining, fixed cells were washed in 50% glycerol/PBS containing 0.001% Nile red for 10 min. Dual capturing of both Nile red and EYFP images in fixed cells, Nile red image was captured first (no crossover of EYFP to a red channel) and then the EYFP image was captured after the Nile red photobleach. Labeling GM1 Gangliosides with CTx-B. For detection of intracellular GM1 ganglioside (Fig. 6c), fixed and permeabilized (0.5% Triton X-100 at 4°C) cells were incubated with 5 μg/ml Alexa conjugated-CTx-B (4°C, 30 min). Cells were washed with PBS containing 0.2% Triton X-100 and 0.1% sodium deoxycholate. For Western blotting of CTx-B directed against plasma membrane raft GM1 gangliosides (Fig. 6d), NG108 cells were incubated with 5 μg/ml CTx-B (4°C, 30 min) in HBSS, solubilized with 0.5% Triton X-100 (4°C, 30 min), and subjected to flotation assay gradient and SDS-polyacrylamide gel electrophoresis. Boiling of samples before application to SDS-polyacrylamide gel electrophoresis was omitted to maintain the pentameric structure of GM1 ganglioside-binding CTx-B. Protein bands were detected by anti-CTx-B antibodies. Purification of Detergent-Insoluble Lipid Microdomains. NG108 cells were extracted for 30 min in TNE buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2% sucrose; pH 7.2) containing 0.5% Triton X-100 (4°C). Triton X-100 extracts were adjusted to 40% sucrose (10 ml), placed in a ultracentrifuge tube, overlaid with sucrose gradients (35%, 12.5 ml; 15%, 2.5 ml; 5%, 2.5 ml; and 0%, 2.5 ml), and centrifuged at 141,000g (4°C, 24 h) in a SW28 rotor. Thirteen fractions (2.5 ml each from top) were collected.

Enzymatic Assays for Cholesterol. After fractionation of NG108 cell membranes, lipids in each fraction were extracted according to methods described elsewhere (Corvera et al., 2000). Total protein contents in each fraction were measured by bicinchoninic acid kit (Pierce Chemical, Rockford, IL). Lipid extracts dissolved in isopropanol were assayed enzymatically for total cholesterol (Wako Bioproducts, Richmond, VA).
Results

Endogenously Expressed α-1 Receptors Exhibit a Unique Pattern of Localization in NG108 Cells. Endogenously expressed α-1 receptors exhibit a unique pattern of localization when examined by immunocytochemistry in combination with an antigen retrieval method (see Materials and Methods). α-1 receptors localized in perinuclear areas as dense clusters (Fig. 1a). Higher magnification indicated that α-1 receptors accumulated predominantly as ring structures and a few accompanying tubular elements (Fig. 1b, inset). The size of the ring structure varied, ranging between 1 and 2.5 μm in a diameter.

α-1 Receptors Target ER-LDs: An Examination Using C-Terminally EYFP-Tagged α-1 Receptors. To further examine the intracellular localization of α-1 receptors, four different constructs of EYFP-tagged α-1 receptors were transfected in NG108 cells (Fig. 2). C-terminally EYFP-tagged α-1 receptors (Sig-1R-EYFP) showed the same localization pattern as that of endogenously expressed α-1 receptors (Fig. 3a). Sig-1R-EYFPs were highly concentrated on the ring structures and accompanying tubular elements (Fig. 3b, arrows). In highly overexpressed cells, a portion of Sig-1R-EYFP occurred on nuclear envelope and apparently increased the size of ring structures. Although Sig-1R-EYFP-containing ring structures were negative to CYP450R (a smooth endoplasmic reticulum resident protein) staining, they were always connected to CYP450R-containing endoplasmic reticulum reticular networks (Fig. 3c). Moreover, tubular elements associated with ring structures could be stained with CYP450R, indicating that they are integral endoplasmic reticulum structures (Fig. 3d, arrows). Together, these results suggest that the α-1 receptor-containing ring structure is a part of the smooth endoplasmic reticulum. The α-1 receptor-containing ring structures were negative to stainings by all tested organelle markers [EEA-1 (endosome), Mito Tracker and bcl-2 (mitochondria), Lyso Tracker (lysosome), synapsin II (synaptic vesicles), Fas and CTx-B (plasma membranes), and GM130 (Golgi)] and were negative as well to stainings by other endoplasmic reticulum-associated proteins, including Bip/GRP78, SRP54, presenilin-1, and calnexin. The Sig-1R-EYFP-containing ring structures, however, could be stained with Nile red, which is known to stain neutral lipid-enriched LDs (Fig. 3e). The Nile red result suggests that α-1 receptor reside specifically at the ER-LD.

To confirm this speculation, we used the oleic acid treatment to see whether α-1 receptor-containing ring structures might be enlarged by this treatment. Oleic acid (300 μM for 6 h), which facilitates neutral lipid formation, caused a significant enlargement of the α-1 receptor-containing ring structures and retention of neutral lipids therein (Fig. 3f). Thus, α-1 receptors or Sig-1R-EYFPs localize specifically on the ER-LDs.

α-1 Receptors Are Unique Proteins Specifically Targeting ER-LDs. So far, only a limited number of proteins are known to be associated with lipid droplets such as adipocyte differentiation-related protein (ADRP), perilipin (Brasaemle et al., 1997; Londos et al., 1999), and caveolins (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001). We set forth to examine whether any of those proteins may also be specifically localized, like α-1 receptors, at the ER-LD. ADRP was found to localize on c-LDs in NG108 cells, but not on ER-LD (Fig. 3g). ADRP has been suggested as being translated on free ribosomes and then recruited to nascent c-LD from the cytosol (Brasaemle et al., 1997; Londos et al., 1999). Interestingly, some, usually one to two, ADRP-positive c-LDs were in proximity to a single α-1 receptors-containing ER-LD (Fig. 3g, inset). Thus, in NG108 cells, ADRP might be recruited to nascent c-LDs during the budding stage of c-LDs from ER-LDs. Isoforms and mutants of caveolin, a cholesterol-binding protein found in caveola type of cholesterol-rich lipid rafts (Anderson, 1998), were reported to accumulate on LDs (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001). NG108 cells express Cav-1, Cav-2, but not caveolin-3 (Fig. 3h). By using monoclonal anti-caveolin-2 antibodies, we detected the caveolin-2-like immunoreactivity on Sig-1R-EYFP-containing ER-LDs (Fig. 3i). Caveolin-2-like immunoreactivity was also seen in cytosolic small vesicles. Most caveolin-1 localized at Golgi and the plasma membrane, but not on ER-LDs (Fig. 3j). Among the isomers of caveolin-2, β isoform (Cav-2β) was reported to target LDs. However, we transiently transfected GFP-tagged Cav-2β into NG108 cells and found that they targeted mostly c-LDs rather than ER-LDs (Fig. 3k). Thus, endogenously expressed caveolin-2 isoform in NG108 cells may not be caveolin-2β. Together, these results indicated that the only proteins known to target ER-LDs are α-1 receptors and an unknown isomer of caveolin-2.

α-1 Receptors Lacking an Endoplasmic Reticulum Retention Signal Target c-LDs. Structural requirement of α-1 receptors targeting the ER-LDs was examined. Similar to the KKXX motif, the double-arginine motif is known to direct retention of membrane proteins to the endoplasmic reticulum via a retrograde transport pathway (Schutze et al., 1994). The truncation of N-terminal seven amino acids containing a double-arginine endoplasmic reticulum retention signal from Sig-1R-EYFP (Δ2-8-Sig-1R-EYFP) caused α-1 re-
ceptors to occur predominantly on c-LDs (Fig. 4a). Δ2-8-Sig-1R-EYFP and ADRP colocalized on some c-LDs. Some c-LDs, however, contained only ADRP or only Δ2-8-Sig-1R-EYFP (Fig. 4b, arrows). Removal of a longer stretch of N-terminal amino acids from Sig-1R-EYFP (Δ2-29-Sig-1R-EYFP) caused Δ-1 receptors to localize solely on c-LDs. Also, a coalescence of c-LDs was observed (Fig. 4c). Live-cell imaging revealed that small c-LDs fused to become a large c-LDs (data not shown). A few large c-LDs thus formed were congregated by small c-LDs containing ADRP and/or Δ2-28-Sig-1R-EYFP (Fig. 4d).

**N-Terminal EYFP-Tagged Δ-1 Receptors Fail to Target ER-LDs.** In contrast to C-terminally EYFP-tagged Δ-1 receptors (Sig-1R-EYFP), N-terminally EYFP-tagged Δ-1 receptors (EYFP-Sig-1R) were found to exist at the endoplasmic reticulum reticular network, but failed to target ER-LDs (Fig. 5a). EYFP-Sig-1R-transfected cells showed not only significant decrease in number of all lipid droplets but also, importantly, the unrestricted distribution of neutral lipids all over the endoplasmic reticulum network, suggesting a lack of ER-LDs in EYFP-Sig-1R-overexpressing cells (a lower cell in Fig. 5b). In 38% of EYFP-Sig-1R-transfected cells, a bulbous aggregation of the CYP450R-positive endoplasmic reticulum network was seen (Fig. 5c). Neutral lipids and free cholesterol (filipin staining) were retained in these bulbous aggregations (Fig. 5, d and e). In EYFP-Sig-1R-transfected cells with these peculiar ER aggregations, free cholesterol levels were significantly reduced in Golgi and plasma membrane (a lower cell in Fig. 5e). Although, as mentioned above, C-terminally EYFP-tagged Δ-1 receptors (Sig-1R-EYFP) specifically target ER-LDs, the Sig-1R-EYFP failed to target ER-LD in the presence of brefeldin-A (0.5 μg/ml for 14 h), a forward inhibitor of the endoplasmic reticulum-to-Golgi network (Fig. 5f). Interestingly, as a result of the brefeldin-A treatment, Sig-1R-EYFP showed the same distribution pattern as that of EYFP-Sig-1R. These results suggest that protein sorting through the endoplasmic reticulum-to-Golgi network...
secretory pathway is required for targeting of endogenous \( \text{H}9268 \)-1 receptors to ER-LDs. On the contrary, EYFP-Sig-1R may fail to enter the pathway.

\( \text{H}9268 \)-1 Receptors Form Raft-Like Microdomains on LDs.

Because caveolins are known to form detergent-resistant cholesterol-rich microdomains, at least on caveolae (Anderson, 1998; Simons and Toomre, 2000), and because our data show that \( \sigma \)-1 receptors colocalize with caveolin-2 on ER-LDs (Fig. 3i), we tested whether the ER-LD membrane may contain raft-like microdomains and whether \( \sigma \)-1 receptors might exist in those domains. Because lipid rafts are known to be resistant to Triton X-100 at low temperature (Simons and Toomre, 2000), Sig-1R-EYFP-expressing cells were, at first, in situ extracted at 4°C in HBSS containing 0.5% Triton X-100 and examined for the presence of detergent-insoluble \( \sigma \)-1 receptors-containing structures. ER-LD membranes, but not endoplasmic reticulum-tubular elements, were resistant to 0.5% Triton X-100 at 4°C (Fig. 6a). The renowned classic lipid rafts are known to be composed of cholesterol and glycosphingolipids such as GM1 ganglioside (Simons and Toomre, 2000). Although containing relatively high amount of free cholesterol (stained with filipin), the ER-LD membranes observed in the present study, however, contained no detectable GM1 ganglioside (stained with CTx-B) (Fig. 6, b and c).

\( \sigma \)-1 receptor-containing detergent-resistant microdomains were further characterized by the Triton X-100-floatation sucrose gradient centrifugation, which is conventionally used for preparation of lipid rafts. Detergent-resistant lipid rafts are known to float during centrifugation in the presence of Triton X-100 at low temperature and can be separated from detergent-soluble proteins that remain on the bottom of the centrifuge tube (Simons and Toomre, 2000). Plasma membrane raft markers Src and CTx-B-bound GM1 gangliosides (under Materials and Methods) existed in high-buoyancy fractions (2–4; Fig. 6d) with accompanying high levels of cholesterol. Interestingly, both \( \sigma \)-1 receptor- and Cav-2-containing microdomains were less buoyant than plasma membrane lipid-rafts (seen in fractions 4 and 5; Fig. 6e). \( \sigma \)-1 receptors were also present in denser fractions 6–8 and an increase of \( \sigma \)-1 receptor-containing microdomains to higher buoyancy (fraction 3; Fig. 6f). This result suggests that buoyancy of \( \sigma \)-1 receptor-containing microdomains is rendered lower, at least in part, by tight interactions with actin cytoskeletons. Disappearance of \( \sigma \)-1 receptor-containing microdomains by the treatment of cells with digitonin, a cholesterol-bound deter-
Western blottings for EYFP-tagged EYFP- and EYFP-Sig-1R-expressing cells in low cell density culture. Membrane raft markers (src, CTx-B-bound GM1 ganglioside; under containing presented in percentage. e, buoyancy differences of microdomains containing cholesterol is an important constituent for α-1 receptor-containing microdomains on ER-LD (Fig. 6f). (+)-Pentazocine (100 nM for 10 min) decreased α-1 receptor-containing microdomains, but not that of Src- or caveolin-2 (Cav-2)-containing rafts (Fig. 6f). These results suggest either that caveolin-2 may not in the same microdomains as those containing α-1 receptors or that (+)-pentazocine can only mobilize α-1 receptors and not caveolin-2 from the same microdomains. Thus, α-1 receptors form unique cytoskeleton-associated ER-LD microdomains that differ from classical well recognized GM1 ganglioside-containing lipid rafts on the plasma membrane (Simons and Toomre, 2000).

Like endogenous α-1 receptors, Sig-1R-EYFP formed detergent-resistant microdomains (Fig. 6g). Δ4-8-Sig-1R-EYFP, which predominantly target c-LDs (Fig. 4, a and b), formed substantial amount of detergent-resistant microdomains. This result suggests that α-1 receptor-deletion mutants can form rafts on the c-LD membrane that is known to consist of phospholipid monolayer (Fujimoto et al., 2001; Tauchi-Sato et al., 2002). EYFP-Sig-1R failed to form the microdomains (Fig. 6g). In EYFP-Sig-1R-transfected cells, no endogenous α-1 receptor-containing rafts could be detected (Fig. 6g, bottom panels). EYFP-Sig-1R might thus act as functionally dominant negative proteins for α-1 receptors.

Discussion

Most eukaryotic cells store neutral lipids such as triglycerides and cholesteryl esters in lipid droplets. Lipid droplets are formed by coalescence of neutral lipids inside discs inside the bilayer of the endoplasmic reticulum (i.e., ER-LDs). c-LDs are known to bud from ER-LDs (Murphy and Vance, 1999). Lipid droplets play an important role in energy storage and/or transport of lipids. Malfunctions in neutral lipid storage are implicated in certain human diseases (Londos et al., 1999; Murphy and Vance, 1999). However, the regulations of lipid droplets and associated neutral lipids are not well understood. Specific proteins such as oleosin (in plant seeds), perilipin (in mammalian adipocytes), and ADRP (in most mammalian cells) can target and regulate biogenesis, storage capacity, and maturation of c-LDs (Brasaemle et al., 1997; Londos et al., 1999). Recently, caveolins, specifically caveolin-2β (Fujimoto et al., 2001), have been shown to accumulate on c-LDs under certain conditions. Although N-terminally truncated dominant negative mutant of caveolin-1, caveolin-2, or caveolin-3 can associate with c-LDs or ER-LDs, respectively (Ostermeyer et al., 2001; Pol et al., 2001), no naturally existing protein has been so far described to specifically target ER-LDs. Thus, our present study constitutes the first report demonstrating α-1 receptors as the first protein known to target ER-LDs under the physiological condition.

Topology of α-1 receptors on membrane is not fully understood, but a recent report (Aydar et al., 2002) and our data (unpublished results) indicate that both N and C termini of α-1 receptors are directed toward the cytoplasm. Although the N-terminal hydrophobic domain was originally proposed as a transmembrane domain in the first cloning study of α-1 receptors (Hanner et al., 1996), several recent reports confirm that the hydrophobic domain on the center of α-1 receptors, at least, can serve as a membrane-spanning region (Seth et al., 1997; Yamamoto et al., 1999; Aydar et al., 2002).
Interestingly, the topology of σ-1 receptors is similar to that of olesin or caveolin: a central hydrophobic domain flanked by two hydrophilic cytoplasmic domains. For caveolins and olesins, the central hydrophobic domain seems to embed into the phospholipid monolayer of lipid droplets, and possibly into the neutral lipid core (Murphy and Vance, 1999). Because lipid droplets are filled with lipids, transmembrane proteins that have hydrophilic domains on the lumen side cannot accumulate on lipid droplets. Therefore, it is very likely that σ-1 receptors, at least on lipid droplets, lack a luminal hydrophilic domain and thus form a hair-pin structure on surfaces of lipid droplet membranes in a manner similar to that of caveolin or olesin. The central hydrophobic domain of σ-1 receptors has a high homology (>70% identity) to the sterol binding pocket of fungal C8-C7 sterol isomerase. Therefore, σ-1 receptors might be able to bind tightly to lipids on surface and/or in the core of lipid droplets using this central hydrophobic domain.

Ostermeyer et al. (2001) showed that both KKSL (endoplasmic reticulum retrieval motif)-tagged caveolin-1 (Cav-KKSL) and caveolin-1 mutant lacking the N-terminal hydrophobic domain (Cav-ΔN2) are both present on ADRP-positive c-LDs. These two mutants should theoretically stay at the endoplasmic reticulum according to the well known endoplasmic reticulum-to-Golgi pathway for caveolins. These suggest a possibility that “ER-LD to c-LD” may represent a newly recognized pathway with different structural requirements for exporting caveolins, or by extension, neutral lipids, out of the endoplasmic reticulum. Thus, caveolins either with a KKXX signal on C terminus or with a truncated N-terminal stretch can still be exported via the ER-LD to c-LD pathway. Our results with deletion mutant of σ-1 receptors indicate that the double arginine endoplasmic reticulum retention signal on the N terminus of a protein can be an important determinant to keep that protein (e.g., σ-1 receptors) on ER-LDs. Deletion of the double arginine endoplasmic reticulum retention signal on σ-1 receptors causes export of almost all σ-1 receptors to c-LD, just like Cav-KKSL and Cav-ΔN2 (Fig. 5, a and b). In addition, although the molecular mechanism is unclear at present, our results suggest that the N-terminal hydrophobic stretch of σ-1 receptors seems to be involved in the membrane fusion of lipid droplets (Fig. 4, c and d). Further investigation is required to understand what controls the protein secretion in this ER-LD to c-LD pathway.

In this study, we found that σ-1 receptors form detergent-resistant lipid microdomains on ER-LD. Interestingly, a recent study shows that σ-2 receptors can also form lipid rafts (Gebreselassie and Bowen, 2002). The σ-1 receptor-containing lipid microdomains, however, possess different features from those of plasma membrane lipid rafts: 1) σ-1 receptor-containing microdomains have lower buoyancy in Triton X-100 sucrose floatation; 2) the buoyancy of σ-1 receptor-containing rafts is significantly affected by cytochalasin-D and σ-1 receptor ligands; and 3) σ-1 receptor-containing microdomains contain cholesterol, but not GM1 ganglioside. Glycosphingolipids are the well known components in plasma membrane lipid rafts (Simons and Toomre, 2000). Furthermore, concentrations of glycosphingolipids are known to be extremely low at the endoplasmic reticulum compared with those at the Golgi or the plasma membrane (van Meer and Holthuis, 2000). Together, these suggest that other as yet unidentified types of sphingolipids must participate in the formation of σ-1 receptor-containing detergent-resistant microdomains on ER-LDs. It would warrant very much to investigate what sphingolipid, if any, corroborates with cholesterol to form σ-1 receptor-containing detergent-resistant microdomains on the ER-LDs and how exactly σ-1 receptor ligands may affect this unique lipid assembly.

Our results showing that tagging EYFP to the N terminus of σ-1 receptors (i.e., EYFP-Sig-1R) renders σ-1 receptors functionally negative suggests that the resultant mutant fails to enter ER-Golgi secretory pathway and/or fails to acquire a post-translational modification at the Golgi. In EYFP-Sig-1R-transfected cells, several abnormalities have been observed: 1) retention of neutral lipids over entire ER network; 2) no compartmentalization of neutral lipids into ER-LDs; 3) a significant decrease in c-LDs; 4) formation of bulbous aggregations of endoplasmic reticulum that contain substantial amounts of neutral lipids and free cholesterol; and 5) decreases in free cholesterol in Golgi and plasma membrane. Neutral lipids such as cholesteryl esters and triglycerides are known to be synthesized in the entire endoplasmic reticulum network and are thought to be concentrated thereafter to specific loci (e.g., ER-LDs) (Tauchi-Sato et al., 2002). Therefore, we propose that σ-1 receptors may demarcate ER-LD surface from the bulk ER membrane and thus compartmentalize lipids inside the ER-LDs. Neutral lipids are known to be exported from the endoplasmic reticulum to c-LDs, and free cholesterol from the endoplasmic reticulum to Golgi and plasma membrane. Therefore, results mentioned above in 3 to 5 strongly suggest that EYFP-Sig-1R severely disturbs exports of lipids from the endoplasmic reticulum to expected destinations. It is thus plausible that σ-1 receptors serving to compartmentalize lipids in specialized loci on the endoplasmic reticulum can regulate export of lipids from the endoplasmic reticulum to c-LDs or to plasma membrane.

σ-1 receptors are present in high levels in the brain. In addition to certain steroid hormones, diverse kinds of psychotropic compounds, including neuroleptics, antidepressants, and psychostimulants can bind to σ-1 receptors (Su, 1982; Sharkey et al., 1988; Su et al., 1988; Narita et al., 1996). Neuroleptics, especially haloperidol, are known to function as σ-1 receptor antagonists, whereas cocaine and antidepressants as agonists (Hayashi and Su, 2001; Matsumoto et al., 2001; Takebayashi et al., 2002). σ-1 receptors are implicated in several physiological and pharmacological functions in the brain such as neuronal firing (Monnet et al., 1990), neurotransmitter release (Nuwayhid and Werling, 2003), psychostimulant sensitization (Ujike et al., 1996), and learning and memory (Maurice and Lockhart, 1997). How σ-1 receptors can affect such diverse functions in the brain is unknown. The brain is a lipid-enriched organ and contains approximately one-fourth of total cholesterol in the body (Dietschy and Turley, 2001). Brain lipids are mostly supplied via de novo synthesis and are used for energy consumption (highest proportion among organs), synaptic vesicle recycling and synapticogenesis (Dietschy and Turley, 2001). Thus, σ-1 receptor’s regulation of lipid compartmentalization at and lipid export from the endoplasmic reticulum, as proposed in this study, may represent a novel mechanism whereby energy source and/or lipid membrane materials are distributed to certain parts of neurons in the brain. σ-1 receptors may therefore be related to certain brain disorders with altered...
cholesterol metabolism and malfunctions in brain repair mechanisms. Apparently, psychoactive drugs such as cocaine may affect membrane compositions via \( \sigma \)-1 receptors.

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