The Surface of Lipid Droplets Is a Phospholipid Monolayer with a Unique Fatty Acid Composition*

We found that caveolin-2 is targeted to the surface of lipid droplets (Fujimoto, T., Kogo, H., Ishiguro, K., Tauchi, K., and Nomura, R. (2001) J. Cell Biol. 152, 1079–1085) and hypothesized that the lipid droplet surface is a kind of membrane. To elucidate the characteristics of the lipid droplet surface, we isolated lipid droplets from HepG2 cells and analyzed them by cryoelectron microscopy and by mass spectrometry. By use of cryoelectron microscopy at the stage temperature of 4.2 K, the lipid droplet surface was observed as a single line without any fixation or staining, indicating the presence of a single layer of phospholipids. This result appeared consistent with the hypothesis that the lipid droplet surface is derived from the cytoplasmic leaflet of the endoplasmic reticulum membrane and may be continuous to it. However, mass spectrometry revealed that the fatty acid composition of phosphatidylcholine and lysophosphatidylcholine in lipid droplets is different from that of the rough endoplasmic reticulum. The ample presence of free cholesterol in lipid droplets also suggests that their surface is differentiated from the bulk endoplasmic reticulum membrane. On the other hand, although caveolin-2 and adipose differentiation-related protein, both localizing in lipid droplets, were enriched in the low density floating fraction, the fatty acid composition of the fraction was distinct from lipid droplets. Collectively, the result indicates that the lipid droplet surface is a hemi-membrane or a phospholipid monolayer containing cholesterol but is compositionally different from the endoplasmic reticulum membrane or the sphingolipid/cholesterol-rich microdomain.

Lipid droplets have been regarded as a depot of neutral lipids. They exist most abundantly in adipose cells and steroid-producing cells but can be found in virtually any kind of cell. The core of lipid droplets is occupied by triacylglycerol and cholesterol ester in various ratios depending on the cell type (1), but information on the lipid droplet surface has been scarce. Recently we as well as others showed that caveolins can exist in the lipid droplet surface (2–4). Caveolins, i.e. caveolin-1, 2, 3, are membrane proteins that are incorporated to the sphingolipid/cholesterol-enriched membrane microdomain and form the framework of caveolae (5). Furthermore, lipid droplets were reported to contain other microdomain proteins, i.e. Lyn and mitogen-activated protein kinase, as well as abundant free cholesterol (6–8). These results suggest that the lipid droplet surface is a kind of membrane and that it might have some similarity to the microdomain.

However, electron microscopy of conventional resin-embedded ultrathin sections cannot visualize any membranous structure around the lipid droplet. In the ultrathin section of specimens fixed by aldehydes and then by osmium tetroxide, the lipid droplet content appears vacant, and its periphery is usually seen as a thin intermittent line. In many diagrams, the lipid droplet surface has been depicted as a phospholipid monolayer with the hydrophilic headgroup facing the cytoplasm and the hydrophobic acyl chains extending into the lipid droplet content (9, 10). It has also been assumed that lipid droplets form by accumulation of neutral lipids between the two leaflets of the endoplasmic reticulum (ER) membrane. However, evidence to support the above assumptions is scarce. Only freeze-fracture electron microscopy showed that the fracture plane along the lipid droplet surface is occasionally continuous with the cytoplasmic leaflet of the ER membrane (11, 12).

We wanted to examine whether the lipid droplet surface is really a phospholipid monolayer. Because it is difficult to retain lipid-rich structures by conventional morphological methods, we took advantage of cryoelectron microscopy that can observe biological specimens at the atomic resolution without fixation or staining (13). Furthermore, to gain information about the lipid droplet phospholipids, we analyzed the fatty acid composition of phospholipids by mass spectrometry. The microscopy showed that the lipid droplet surface is indeed a hemi-membrane, or a phospholipid monolayer, and mass spectrometry revealed that the fatty acid composition of the lipid droplet phospholipids is distinct from that of rough ER and cholesterol/sphingolipid-rich microdomain. The result not only questions the current hypothesis on the mechanism of lipid droplet formation but also provides a firm basis for further studies on the physiological function of lipid droplets.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse anti-caveolin-2 antibody (BD Transduction Laboratories), mouse anti-adipose differentiation-related protein (ADRP) antibody (Progen), and colloidal gold-conjugated secondary antibody (Amersham Biosciences) were obtained from respective suppliers.

Isolation of Lipid Droplets by Subcellular Fractionation—HepG2 cells were grown in Dulbecco’s modified Eagle’s medium added with 10% fetal calf serum. A HepG2 cell line stably expressing human caveolin-2 (clone A-8) (2), kept in the presence of 200 μg/ml G418, was also used for some experiments. Cells were disrupted by nitrogen cav-
Cryoelectron microscopy of isolated lipid droplets. A, a low magnification micrograph of lipid droplets on a microgrid. Lipid droplets (arrow) are embedded in vitreous ice in microgrid holes (the rim of a hole is indicated by an arrowhead). Bar, 1 μm. As shown in B, a lipid droplet is observed as a round structure with a content of low electron density. Bar, 50 nm. C, a high magnification view of the rectangular portion in panel B. A single electron-dense line is clearly seen in the rim of the lipid droplet. Bar, 10 nm. D, three lipid droplets with a rim of a single line. Bar, 50 nm. As shown in E, a small structure (arrow) with a slightly higher electron density than lipid droplets is lined with two parallel electron-dense lines. A lipid droplet with multiple parallel lines is also shown (arrowheads). Bar, 50 nm.

RESULTS

Cryoelectron Microscopy of Lipid Droplets—Conventional electron microscopy of resin-embedded sections did not reveal membrane structure in the rim of lipid droplets in HepG2 cells (data not shown). To directly visualize the lipid droplet surface structure, we adopted cryoelectron microscopy that can visualize ultrastructures at high resolution. Liposomal membrane, or a phospholipid bilayer, was observed as two parallel lines by cryoelectron microscopy of resin-embedded sections did not reveal the high resolution of the lipid droplet surface structure. lipid droplets were subjected to DEAE-cellulose column chromatography to separate acidic and non-acidic lipids (23). They were chromatographed on HPTLC plates (Silica Gel 60, Merck) by chloroform-methanol-acetic acid-formic acid-water (35:15:6:2:1) and then by hexanediisopropyl ether-acetic acid (65:35:2), and charred by cupric acetate-phosphoric acid. Free cholesterol and total phospholipids in lipid extracts were quantified, and the relative molar ratio was obtained (24, 25).

Capillary Liquid Chromatography/Electrospray Ionization (Cap-LC/ESI) Mass Spectrometry—The lipids extracted from isolated lipid droplets were subjected to silica gel column to reduce the content of neutral lipids, which disturbs the analysis of phospholipids by Cap-LC/ESI mass spectrometry. The Cap-LC/ESI mass spectrometry analysis was done as described previously (26). Briefly, after chromatographic separation of phospholipids by normal-phase Cap-LC column (Deversoil S60, Nomura Chemicals), the sample was analyzed by a Quattro II triple-stage quadrupole mass spectrometer (Micromass) equipped with an electrospray ion source. Identification of individual molecular species of each phospholipid class was performed by the theoretical mass data.

TLC and Quantification of Lipids—The total lipids were extracted from lipid droplets and crude microsome (22). For TLC, the sample from lipid droplets was subjected to DEAE-cellulose column chromatography to separate acidic and non-acidic lipids (23). They were chromatographed on HPTLC plates (Silica Gel 60, Merck) by chloroform-methanol-acetic acid-formic acid-water (35:15:6:2:1) and then by hexanediisopropyl ether-acetic acid (65:35:2), and charred by cupric acetate-phosphoric acid. Free cholesterol and total phospholipids in lipid extracts were quantified, and the relative molar ratio was obtained (24, 25).

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electron microscopy. The difference indicates that lipid droplets were disrupted into small fragments during isolation.

Lipid droplets were observed as round structures (Fig. 1, B and D); the content was of low electron density, but the rim was observed as a single electron-dense line of about 2–2.5 nm in width (Fig. 1C). The result directly demonstrates that a single row of phosphorus atoms, or a phospholipid monolayer, covers the lipid droplet surface. A small number of structures, whose content showed a higher electron density than lipid droplets, were delineated with two parallel lines of the same width (Fig. 1E); they are thought to be a contaminating membrane organelle having a bilayer membrane.

Some lipid droplets were seen to have concentric parallel lines spaced regularly (Fig. 1D). The outermost line was the thickest, whereas the inner lines became thinner gradually and finally became invisible. A structure that seems to correspond to the concentric lines was seen by freeze-fracture of isolated lipid droplets; among lipid droplets with simple homogenous content, some showed multiple fracture faces or onion skin-like morphology (Fig. 2A). Furthermore, anti-caveolin-2 antibody labeled not only the outermost fracture face but also the inner fracture planes (Fig. 2B). Initially we thought that the lipid droplets with the concentric rings represent a unique population; we assumed that they may be folded phospholipid monolayers made upon the hydrolysis of lipid esters (28, 29). However, in contrast to membrane whorls observed by conventional electron microscopy, the concentric lines are aligned with complete regularity without any intervening spaces, and in numerous layers. Moreover, when unfixed intact cells were rapidly frozen and freeze-fractured, the onion skin-like morphology was found only very infrequently (data not shown). Thus we currently surmise that the multiple concentric lines might be derived from the cytoplasmic leaflet of the ER membrane. If the lipid droplet surface is continuous with the ER membrane, composition of phospholipids in the lipid droplet surface may be similar to that of the ER. As an alternative possibility, accumulation of caveolin-2β and its recovery in low density fractions, might occur because the lipid droplet surface is similar to the sphingolipid/cholesterol microdomain. To examine these possibilities, we compared the fatty acid composition of PC and lyso-PC in lipid droplets, rough microsome (representing rough ER), and TIFF (representing the sphingolipid/cholesterol microdomain) by Cap-LC/ESI mass spectrometry.

Lipid droplets have been hypothesized to occur by accumulation of neutral lipids between the two leaflets of the ER membrane (9); they may either detach from the ER or remain connected to it. The surface of lipid droplet is thus assumed to be derived from the cytoplasmic leaflet of the ER membrane. If the lipid droplet surface is continuous with the ER membrane, composition of phospholipids in the lipid droplet surface may be similar to that of the ER. As an alternative possibility, accumulation of caveolin-2β and its recovery in low density fractions, might occur because the lipid droplet surface is similar to the sphingolipid/cholesterol microdomain. To examine these possibilities, we compared the fatty acid composition of PC and lyso-PC in lipid droplets, rough microsome (representing rough ER), and TIFF (representing the sphingolipid/cholesterol microdomain) by Cap-LC/ESI mass spectrometry.

The fatty acid pattern of PC obtained by a low ionization voltage (30 V) was similar between lipid droplet and rough microsome: molecular species of mass/charge (m/z) 786.4 (dialcyl 36:2, e.g. 18:0–18:2), 760.4 (dialcyl 34:1, e.g. 16:0–18:1), and 732.4 (dialcyl 32:1, e.g. 16:0–16:1) were observed as major peaks (Fig. 3A). In contrast, PC in TIFF was enriched with long fatty acids with a high degree of saturation: m/z 818.3 (dialcyl 38:0, e.g. 18:0–20:0), 788.4 (dialcyl 36:1, e.g. 18:0–18:1), and 760.4 (dialcyl 34:1, e.g. 16:0–18:1) were prominent. The data obtained by this mode represent the mass of whole PC molecules and do not resolve individual fatty acids. On the other hand, by using a high ionization voltage (90 V), acyl chains are detached from

**Fig. 2. Freeze-fracture electron microscopy of isolated lipid droplets.** As shown in A, many lipid droplets show a single surface layer (arrows), whereas some are fractured in multiple layers (arrowhead). As shown in B, by immunogold labeling of the freeze-fracture replica of lipid droplets, caveolin-2 was labeled not only in the outermost layer but also in several other fracture planes (arrowheads). Bar, 500 nm.

**Fig. 3. Concentration of ADRP and caveolin-2β to low density floating fractions.** HepG2 expressing caveolin-2β was homogenized in either 500 mM sodium carbonate (pH 11) or 0.025% Triton X-100 and then subjected to sucrose density gradient ultracentrifugation. By both procedures, ADRP and caveolin-2β were floated to low density fractions.
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PC by in-source collision, and each chain can be resolved as a unique peak. By this analysis, lipid droplets and rough microsome showed distinct patterns (Fig. 3B): for example, the peak of \( m/z \) 281.2 (18:1) stood out for lipid droplets, but the peaks of \( m/z \) 281.1 (18:1) and 279.0 (18:0) were seen for rough microsome. The result indicates that the major portion of \( m/z \) 786.4 was PC of 18:1-18:1 in lipid droplets, whereas the same peak in rough microsome contained a considerable amount of PC (18:0-18:2). By the same token, the relative abundance of \( m/z \) 253.2 (16:1) implies that the peak of \( m/z \) 758.4 in lipid droplets may contain more PC (16:1-18:1) than in rough microsome, whereas the latter may be enriched with PC (16:0-18:2). Furthermore, lyso-PC analyzed by the 30-V mode also gave distinct patterns for lipid droplets and rough microsome (Fig. 3C): peaks of \( m/z \) 522.1 (acyl 18:1) and 494.1 (acyl 16:1) were high in lipid droplets, whereas they were low or invisible in rough microsome and TIFF, and \( m/z \) 524.2 (acyl 18:0) and 496.3 (acyl 16:0) were prominent in the same region. Collectively these data indicate that the lipid droplet surface is distinct from the rough ER membrane, nor is it similar to the sphingolipid/cholesterol-rich micromdomain.

DISCUSSION

The surface of lipid droplets has been speculated to be a phospholipid monolayer because hydrophobic lipid esters may exist stably in the aqueous cytoplasm only when covered with amphiphilic molecules with hydrophilic moiety facing outward. Yet most likely, the only experimental result that supports the speculation has been a few freeze-fracture pictures showing the continuity of a putative ER membrane leaflet and the lipid droplet surface (11, 12). In the present study, we employed a cryoelectron microscope, which is able to visualize biological specimens with minimal damage and has been used extensively to decipher three-dimensional molecular structures at atomic resolution (e.g. Ref. 32). The cryoelectron microscopy has shown previously that the envelope of the influenza A virus is a phospholipid monolayer underlain with a protein layer (39). By this technique, a single electron-dense line representing a row of phosphorus atoms was observed in the surface of isolated lipid droplets. It contrasts with two parallel lines seen in the liposomal membrane (27), which is a phospholipid bilayer, and thus proves for the first time that the lipid droplet surface is a phospholipid monolayer.

The presence of a phospholipid monolayer in the surface is consistent with the current model that lipid droplets are formed by lipid ester deposition between the two leaflets of the ER membrane and may remain connected to it (9, 10). Distribution of acetyl-CoA:cholesterol acyltransferase-1, a major enzyme that synthesizes cholesterol ester, in the entire ER (Ref. 34 and data not shown) seems to indicate that lipid droplets may bud anywhere along the membrane. However, Cap-LC/ESI mass spectrometry showed that fatty acid moieties of PC and lyso-PC in lipid droplets are distinct from those in rough microsome, which is thought to be equivalent to the rough ER. The result does not deny the possibility that the lipid droplet surface is generated from the ER membrane but indicates that the former is a highly differentiated domain. Mature lipid droplets may possibly exist as a structure independent of the ER; alternatively, the lipid droplet may be connected to the ER, but some molecular mechanism may demarcate the lipid droplet surface from the bulk ER membrane as postulated for other ER domains (35). Whichever possibility is the case, we suppose that esters synthesized in wide areas of the ER do not deposit indiscriminately but are concentrated to loci specialized to make lipid droplets. ADRP or other lipid droplet-associated proteins may be involved in the process.

A peculiar feature of lipid droplets revealed by Cap-LC/ESI mass spectrometry is the abundance of unsaturated fatty acids in lyso-PC. How and where the unique lyso-PC was generated is not known, but phospholipase A\(_2\), reported to exist in lipid droplets of leukocytes (6), may be involved. If so, lyso-PC in lipid droplets is derived from a rather rare population of PC with an unsaturated acyl chain in position 1. The relative abundance of PC with two mono-unsaturated acyl chains (i.e. 18:1-18:1 and 16:1-18:1) in lipid droplets (Fig. 4B) is in line with the speculation. The origin of the rare PC species is also unknown. They may be synthesized in the ER and sequestered to lipid droplets; interaction with neutral lipids might be involved in the accumulation of unsaturated acyl chains. However, it is also possible that the unique species were newly formed in lipid droplets; in this case, the continuous turnover of lipid esters and the resultant release and incorporation of fatty acids are thought to be closely related to the surface composition.

Major contamination in the lipid droplet fraction is excluded by the absence of marker enzymes of other organelles (2), but a possibility of minor contamination by phospholipids may need to be considered. However, using the same cell homogenate as a starting material, PCs with two mono-unsaturated acyl chains are found abundantly only in the lipid droplet and far less so in the microsome or TIFF. Furthermore, using the Cap-LC/ESI mass spectrometric technique, PC obtained from several cell and membrane preparations did not show the characteristics found with the lipid droplet. These results make it unlikely that the PCs found in the lipid droplet occur densely in a soluble phase and adhere to any existing membrane promiscuously.

In previous studies, sequestration of caveolins to lipid droplets was found to be induced or increased dramatically when cells were treated with brefeldin A (2, 3). The result was interpreted to indicate that caveolins first concentrated in the ER membrane and then moved to the lipid droplet by lateral diffusion through the membrane continuity. However, two questions were raised concerning this interpretation: one was why caveolins could be concentrated markedly in lipid droplets and the other was why caveolins sequestered to lipid droplets were not chased away even after brefeldin A was discontinued. The latter question may be answered readily by assuming that the lipid droplet surface is not continuous to the ER membrane. Yet, then, the former question can be answered only by supposing a specific transport mechanism because brefeldin A-induced concentration of caveolins appears to occur even in pre-existing lipid droplets. Interestingly, a recent study showed that newly synthesized cholesterol esters are incorporated to pre-existing droplets containing triglycerides (36). It is plausible that lipid esters, possibly along with phospholipids, may be transported from the ER to lipid droplets by an unknown mechanism, and caveolins might exploit the machinery under some circumstances.

Cap-LC/ESI mass spectrometry also showed that the lipid droplet surface is different from the sphingolipid/cholesterol-rich micromdomain in the fatty acid composition. Interestingly, TIFF contained a higher proportion of long saturated fatty acids than rough microsome, whereas lipid droplet did not. The result indicates that not only interaction between sphingolipid and cholesterol but also interaction between saturated phospholipid and cholesterol are important in forming the micromdomain, as predicted from model membrane studies (37). Based on the finding of caveolin sequestration, we speculated previously that the lipid droplet surface might have a property similar to the micromdomain (2). However, the present result

\(^2\) R. Taguchi, unpublished data.
**Fig. 4** Cap-LC/ESI mass spectrometry of phospholipids in lipid droplets, rough microsome, and TIFF, all of which were isolated from HepG2 cells. Only the data obtained by the positive ion mode are shown, although the negative ion mode data were also collected and confirmed the positive ion mode data. A, PC analyzed by the low voltage (30 V) mode. Lipid droplets and rough microsome showed a similar profile, whereas TIFF produced a distinct result; fatty acids in TIFF were longer and with a higher degree of saturation than those in the other two samples. B, PC analyzed by the high voltage (90 V) mode. In-source collision revealed that the fatty acid composition of lipid droplets and rough microsome is different in several aspects. C, lyso-PC analyzed by the low voltage (30-V) mode. Lipid droplets showed a unique composition of fatty acids distinct from rough microsome and TIFF.
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does not support the supposition in terms of the fatty acid composition. Furthermore, TLC showed that the relative amount of sphingomyelin and free cholesterol in comparison with PC is far less in lipid droplets than in TIF (data not shown). Thus lipid composition does not appear to be the cause of caveolin sequestration to lipid droplets. As far as HepG2 cells are concerned, caveolin-2β was found almost exclusively around lipid droplets, but caveolin-2α was mostly found in the Golgi and only minimally in lipid droplets; furthermore, caveolin-1 was not recruited to lipid droplets unless treated with brefeldin A (2). The differential behavior of caveolins may provide a clue to elucidate the mechanism of transport to lipid droplets.

TIF obtained by the 1% Triton X-100 method has been thought as the in vitro correlate of the sphingolipid/cholesterol-rich microdomain (38). We found that caveolin-2β in the lipid droplet was completely solubilized with 1% Triton X-100 in the cold but floated to low density fractions when 500 mM sodium carbonate or 0.025% Triton X-100 was used in cell homogenization. The low density fractions obtained by the sodium carbonate and 0.025% Triton X-100 method were presumed to contain the sphingolipid/cholesterol-rich microdomain (15). The present result showed that although the lipid composition is distinct, molecules in the lipid droplet surface are enriched in the same fraction as the sphingolipid/cholesterol-rich microdomain. It awaits further studies to determine whether the result indicates some similarity in the property of the two domains or simply indicates that the low density fractions obtained by the sodium carbonate and 0.025% Triton X-100 protocols could contain non-microdomain constituents.

In summary, the present study showed that the lipid droplet surface is a phospholipid monolayer with a unique fatty acid composition. Lipid droplets had been regarded as a simple reservoir of neutral lipids and as rather an inert structure. However, in view of recent findings indicating accumulation of proteins related to various diseases (39, 40), it is imperative to study how lipid droplets are formed, modified, and regulated. The properties revealed here should give a firm basis for the forthcoming studies.

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