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Identification of major proteins in the lipid droplet-enriched fraction isolated from the human hepatocyte cell line HuH7

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Abstract

Recent studies have revealed the presence of intracellular lipid droplets in wide variety of species. In mammalian cells, there exist proteins specifically localize in lipid droplets. However, the protein profile in the droplet remains yet to be clarified. In this study, a fraction enriched with lipid droplets was isolated from a human hepatocyte cell line HuH7 using sucrose density gradient centrifugation, and 17 major proteins in the fraction were identified using nano LC-MS/MS techniques. Adipose differentiation-related protein (ADRP) was the most abundant protein in the fraction. The secondary abundant proteins were identified to be acyl-CoA synthetase 3 (ACS3) and 17β-hydroxysteroid dehydrogenase 11 (17βHSD11). Included in the identified proteins were five lipid-metabolizing enzymes as well as two lipid droplet-specific proteins. When HuH7 cell lysate was fractionated by a density gradient, most of 17βHSD11 was found in the droplet-enriched fraction. In immunocytochemical analysis, 17βHSD11 showed ring-shaped images which overlapped with those for ADRP. These results suggest that a specific set of proteins is enriched in the lipid droplet-enriched fraction and that 17βHSD11 localizes specifically in the fraction.
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Keywords: Lipid droplet; HuH7; LC-MS/MS; Immunocytochemistry; Lipid-metabolizing enzyme

1. Introduction

Many kinds of eukaryotic cells have ability to accumulate neutral lipid molecules such as triacylglycerol (TG) and cholesteryl ester (CE). The majority of these neutral lipids are stored in subcellular compartments called lipid droplets (also called lipid storage droplets, lipid body or lipid particles). The lipid droplets consist of a hydrophobic core of neutral lipids surrounded by a monolayer of phospholipids. The lipid droplets are found in a variety of species including yeasts [1], plants [2] and mammals [3].

In mammals, marked storage of neutral lipids is observed in cells such as adipocytes, hepatocytes, steroidogenic cells and macrophages. In adipocytes, excess energy can be stored in the lipid droplets and released in starvation, providing a great advantage in survival. Neutral lipids pooled in hepatocytes are reconstituted into lipoproteins (VLDL) and distributed throughout the body. A pool of CE in steroidogenic cells (adrenal cortical and Leydig cells) is used for the synthesis of steroid hormones.

Increased accumulation of the neutral lipids in these cells often causes diseases and disorders. Excess food intake results in the development of lipid droplets in adipocytes and hepatocytes, resulting in obesity and fatty liver [4]. Formation of the lipid droplets also occurs in macrophages when they take up modified lipoproteins [5]. These lipid-accumulating macrophages, also called foam cells, are of fundamental importance in the development of atherosclerosis. It is thus important to understand the mechanism of

Abbreviations: ACS, acyl-CoA synthetase; ADRP, adipose differentiation-related protein; CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; HSD, hydroxysteroid dehydrogenase; LDH, lactose dehydrogenase; MAM, mitochondria-associated membranes; NSDHL, NAD(P)-dependent steroid dehydrogenase like protein; PBS, phosphate buffered saline; SDR, short chain dehydrogenase/reductase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; TG, triacylglycerol

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deposition and mobilization of cellular neutral lipids. Also, proteins specifically associated with lipid droplets appear to participate in the formation and function of the lipid droplets.

In mammalian cells, several proteins are known to specifically associate with lipid droplets; these include PAT family proteins [6,7] and 160-kDa capsule protein [8]. The PAT protein family includes three main varieties of lipid droplet-associating proteins; perilipin [9], adipose differentiation-related protein (ADRP) [10] and TIP47 [11]. These proteins specifically associate with lipid droplets [9,12–15], and appear to relate to diseases such as fatty liver and atheroscrerosis [16–18]. Functions of these proteins have recently been discussed in studies utilizing cell biological and reverse genetical approaches [19–23]. However, despite such progress, it remains unclear whether the PAT protein family are sufficient for regulation of lipid droplet metabolism, or whether any other proteins are necessary.

It is reasonable to assume that lipid metabolism is closely coupled with the formation and maintenance of the lipid droplets. However, lipid-metabolizing activities, such as cholesterol synthesis, TG synthesis or β-oxidation, are thought to be based in the endoplasmic reticulum (ER), mitochondria or peroxisomes. No member of the PAT family proteins or the 160-kDa capsule protein is shown to be a lipid-metabolizing enzyme, and no enzyme specifically associated with lipid droplets has yet been reported.

These factors motivated us to search for proteins in mammalian lipid droplets in a systematic way. For this, we first isolated a subcellular fraction enriched with lipid droplets from a human hepatocyte cell line, and then tried to identify the major proteins in the fraction using nano LC-MS/MS techniques. We clarified the entire protein profile of the fraction, and found that one of the proteins, 17β-hydroxysteroid dehydrogenase 11 (17βHSD11), was highly concentrated in the fraction.

2. Materials and methods

2.1. Materials

HuH7, a human hepatoma cell line, was obtained from Health Science Research Resources Bank (cell no. JCRB0403; Osaka Japan). Anti-ADRP monoclonal antibody was purchased from PROGEN (Germany). Anti-calnexin, anti-VLA-2α integrin and anti-GS28 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Peroxidase-, rhodamine- and fluorescein-conjugated goat anti-IgG antibodies were purchased from Biosource International (Camarillo, California). The bacterial expression vector pGEX-6P3 and ECL Western Blotting detection system were purchased from Amersham Pharmacia Biotech (UK).

2.2. Culture and Oil red O staining of HuH7 cells

HuH7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For Oil red O staining experiments, HuH7 cells were inoculated at 7.5×10^4 cells/well in four-well culture slides (Falcon, USA) and incubated at 37 °C overnight. Following incubation, cells were fixed and stained with Oil red O by the method described by Mori et al. [5].

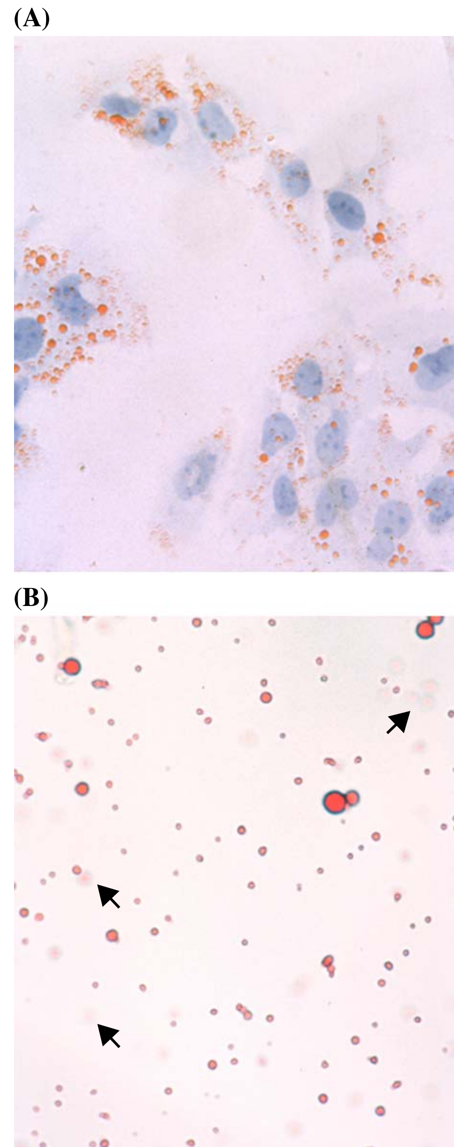

Fig. 1. Detection of intracellular lipid droplets in HuH7 cells. (A) Human hepatocyte cell line HuH7, cultured in DMEM supplemented with 10% FBS, was fixed and stained with Oil red O and Mayer's hematoxylin. Intracellular lipid droplets were detected as red spheres in the cells. Nuclei are stained in blue. (B) Lipid droplet enriched fraction isolated from HuH7 cells (see text and Fig. 2) was fixed and stained with Oil red O. Some droplets are out of focus (arrows). Original magnification, ×400.

2.3. Isolation of lipid droplets from HuH7

Confluent monolayers of HuH7 cells were collected after treatment with trypsin (0.2% trypsin, 0.02% EDTA and 0.2% glucose in PBS). The cells were disrupted by homogenization in buffer A (3 mM EDTA in 10 mM tricine (pH 7.4), containing 20μg/ml phenylmethylsulfonylfluoride, 10μg/ml antipine, pepstatin, leupeptin and chymostatin) in 250 mM sucrose using a Dounce type glass-Teflon homogenizer. The lysate was centrifuged for 10 min at 1000 × *g* at 4 °C. The sucrose concentration of the post-nuclear supernatant (PNS) was adjusted to 26% by adding buffer A containing 70% sucrose (26% sucrose PNS). In a 16-ml ultracentrifuge tube (16PA, Hitachi, Japan), 1.0 ml of buffer A containing 51% sucrose and 1.6 ml of buffer A containing 43% and 35% sucrose were layered sequentially. Then, 4.8 ml of 26% sucrose PNS was layered on this. Subsequently, 1.6 ml of buffer A containing 18% and 10% sucrose were layered sequentially on the PNS fraction. Finally, 3.6 ml of 1 in 5 diluted buffer A containing 2% sucrose was loaded on the top. The step-wise gradient was centrifuged at 24,000 rpm, at 4 °C, for 3 h using SRP28-SA rotor and 70P-72 ultracentrifuge (Hitachi). Following centrifugation, the samples were fractionated from the top (0.8 ml each). Lactose

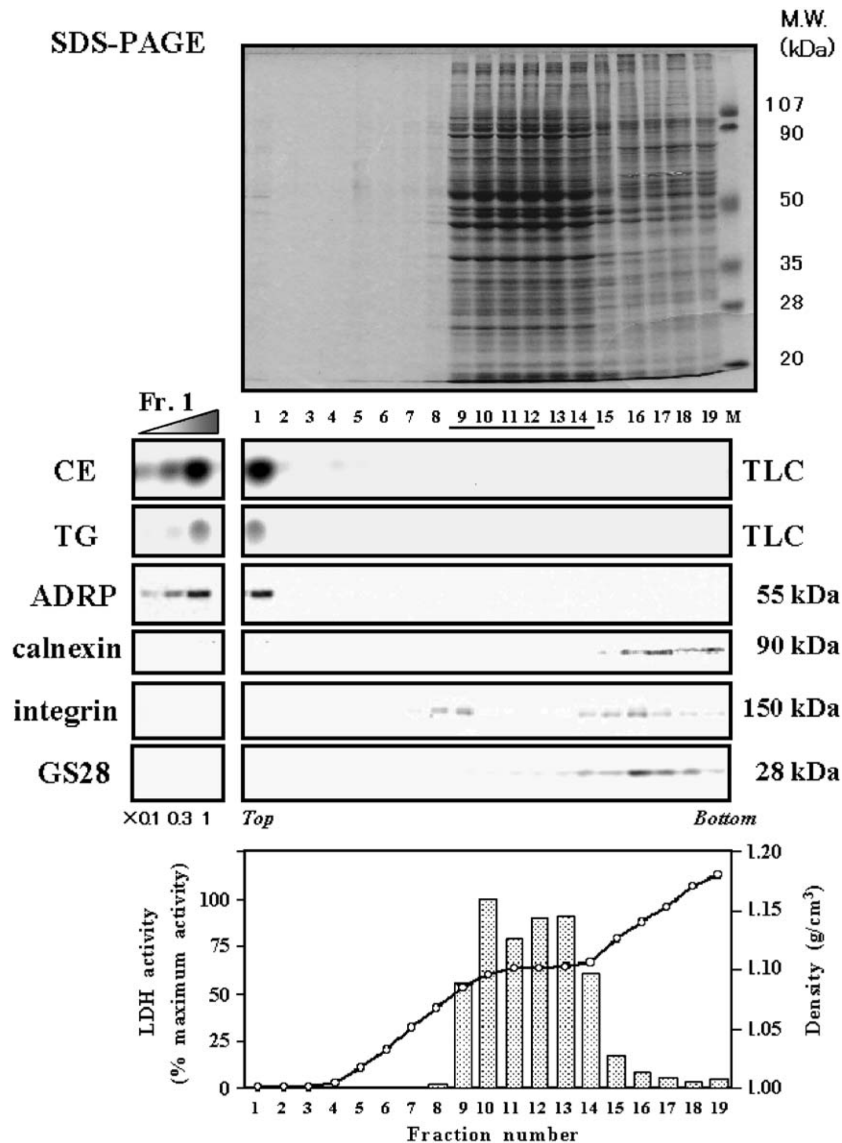

Fig. 2. Fractionation of HuH7 cells by sucrose density gradient centrifugation. HuH7 cells were cultured in DMEM supplemented with 10% FBS and then harvested. Post-nuclear supernatant from 1.7 ml of the cells was fractionated into 19 fractions (0.8 ml each) after sucrose density gradient centrifugation. Upper panel: Proteins in 3 μl of each fraction were separated by SDS-PAGE (9.5% acrylamide gel) and stained with Coomassie blue. The top and the bottom fractions are fraction 1 and 19, respectively. The underline below the fraction numbers indicates the position where the PNS sample was loaded. Middle panels: TG and CE in each fraction were analyzed by TLC. ADRP, calnexin, integrin and GS28 in each fraction were detected by immunoblot analysis. The left side small panels indicate the amounts of TG, CE and proteins in fraction 1 when diluted at × 1, × 0.3 and × 0.1. Lower panels: LDH activity (bars) and density (circles) were determined in each fraction.

dehydrogenase (LDH) activity in each fraction was assayed using LDH-UV Test kit (Wako, Japan).

To verify the purity of the lipid droplet-enriched fraction by re-centrifugation analysis, sucrose concentration of the lipid droplet-enriched fraction isolated as above was adjusted to 26% by adding buffer A containing 70% sucrose (final volume was 2.2 ml). This sample solution was loaded between 18% and 35% sucrose layers of stepwise sucrose density gradient as mentioned above. Volumes of 2% and 51% sucrose layers were 3.2 and 1.5 ml, respectively, while the other layers were 2.2 ml. The step-wise gradient was centrifuged under the same condition as above.

The isolated lipid droplet-enriched fraction was treated with 10% formaldehyde in PBS and stained with 0.12% Oil red O in 60% 2-propanol. Then stained lipid droplets were recovered by centrifugation in the presence of 2–26% sucrose in 1.5-ml plastic tubes and microscopically examined.

2.4. Lipid analysis by thin layer chromatography (TLC)

After sucrose density gradient centrifugation, lipids were extracted from each fraction using the method of Bligh and Dyer [24]. The extracted lipids were developed on a TLC plate (Silica gel 60, Merck) with hexane/chloroform/acetic acid (80:20:1 by volume). The TLC plate was soaked in 8% phosphoric acid containing 3% cupric acetate, and then the lipids were visualized by heating at 130 °C [25].

2.5. In-gel digestion of proteins

Proteins in the isolated lipid droplet were separated by 9.5% SDS-PAGE. Each protein band staining with Coomassie blue was excised using clean razor blades. In-gel trypsin digestion was performed according to the procedure described by Jeno et al. [26] Wilm et al. [27] Shevchenko et al. [28] with minor modifications. Briefly, protein bands were washed with 50% acetonitrile in 50 mM ammonium bicarbonate and dried in a Speedvac evaporator. Proteins were reduced in 10 mM dithiothreitol/50 mM ammonium bicarbonate for 1 h at 56 °C, and alkylated in 50 mM iodoacetamide/50 mM ammonium bicarbonate for 30 min at room temperature. The bands were then washed several times with 50 mM ammonium bicarbonate and 100% acetonitrile, dried in a Speedvac evaporator, and re-swollen with 12.5 ng/μl trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate. Digestion was allowed to proceed overnight at 37 °C. After digestion, products were recovered by sequential extractions with 25 mM ammonium bicarbonate and 100% acetonitrile. Extracts were dried in a Speedvac evaporator and re-suspended in a 10 μl of 0.1% TFA.

2.6. LC-MS/MS analysis and database searching

Chromatographic separation of samples was carried out using a HP1100 HPLC system equipped with a 75-μm PepMap column (LC Packing) (flow rate: 200 nl/min, mobile phase A: 0.1% acetic acid/water, B: 0.1% acetic acid/ethanol). MS/MS analysis was performed using LCQ ion trap mass spectrometry (ThermoQuest). For protein identification, MS/MS data were searched using Mascot software (Matrix Science Ltd., UK), a program for matching mass spectrometric information with sequence databases. Proteins were identified based on matching the MS/MS data with mass values calculated for selected ion series of peptides.

2.7. Determination of amino acid sequences of tryptic fragments by Edman degradation

Tryptic digestion, extraction of peptides and separation of peptides were performed using the method of Tempst et al. [29] with slight modifications. The proteins in isolated

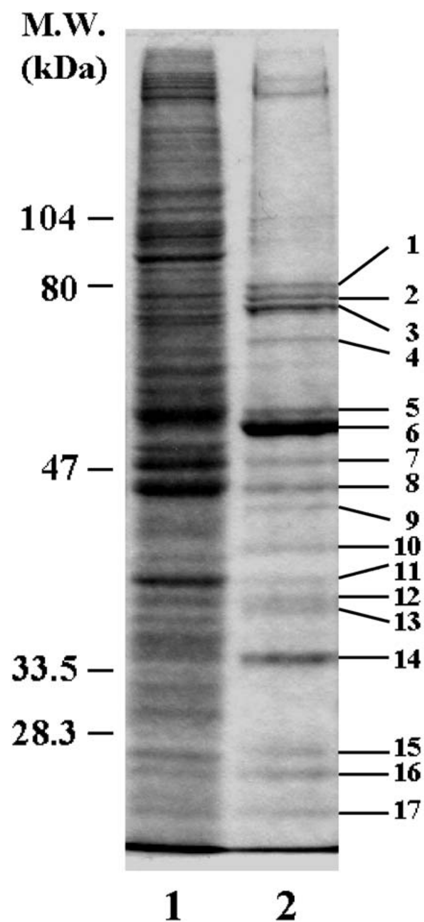

Fig. 3. SDS-PAGE analysis of the lipid droplet proteins. The protein composition of the lipid droplets (lane 2) was compared to that of HuH7 cell total lysate (lane 1). The proteins were stained with Coomassie blue after separation by SDS-PAGE (9.5% acrylamide gel). The amount of the lipid droplet proteins loaded in the lane 2 was 20 times that of the fraction 1 in Fig. 2. The molecular weights deduced from the marker proteins are indicated in the left. The bands numbered on the right side were further subjected to nano LC-MS/MS analysis. The numbers correspond to those in the Table 1.

lipid droplets were separated by SDS-PAGE (9.5% acrylamide gel) and electrophoretically transferred to nitrocellulose membranes (0.2 μm, Schleicher & Schuell). The membranes were soaked in 1% acetic acid containing 0.1% Ponceau S, followed by washing with 1% acetic acid, and bands of proteins were excised by clean razor blades. After tryptic digestion, extracted peptides were separated on an HPLC system (LC10, Shimadzu, Japan) equipped with ODS column (TSKgel ODS-80Ts, 4.6 × 250 mm, TOSOH, Japan). Amino acid sequences were determined using a PPSQ21 automated sequencer (Shimadzu).

2.8. Preparation of antisera against human ACS3 and human 17βHSD11

For the immunization of rabbits, a recombinant peptide corresponding to a region of acyl-CoA synthetase 3 (ACS3) (amino acid residues 70–259) was prepared as an antigen by inserting a cDNA fragment into *Sma*I site of the expression vector pGEX-6P3. The peptide was expressed and partially purified by the method of Fujino et al. [30] and used as an antigen. In the same way, another recombinant peptide corresponding to a region of 17βHSD11 (amino acid residues 175–300) was prepared and used as an antigen.

2.9. Immunoblot analysis

Proteins were separated by SDS-PAGE (9.5% acrylamide gel) and then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were incubated with anti-17βHSD11 antiserum (dilution of 1:8000) or anti-ACS3 antiserum (dilution of 1:3000), followed by peroxidase conjugated goat anti-(rabbit IgG) antibody (dilution of 1:20,000). ADRP, calnexin and integrin were detected by treatment of the membranes with monoclonal antibodies against ADRP (dilution of 1:100), calnexin (dilution of 1:1500), VLA-2α integrin (dilution of 1:500) and GS-28 (dilution of 1:1000), respectively, followed by peroxidase conjugated goat anti-(mouse IgG) antibody (dilution of

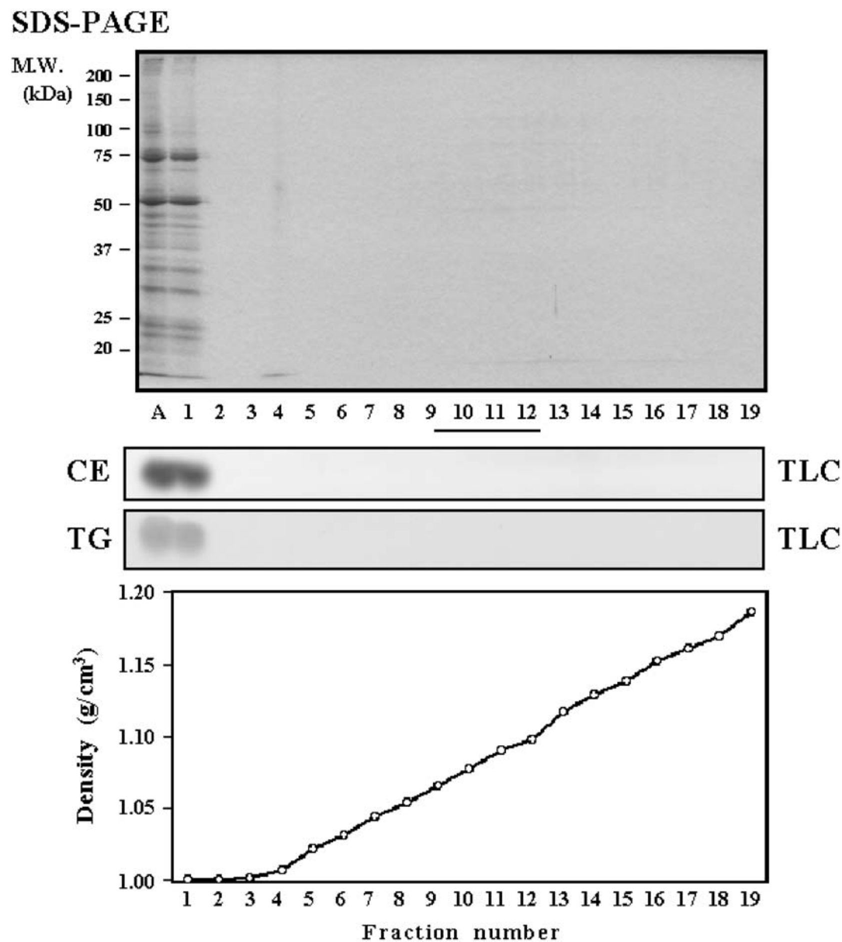

Fig. 4. Sucrose density gradient re-centrifugation analysis of the isolated lipid droplet-enriched fraction. The lipid droplet-enriched fraction, isolated from HuH7 cells as shown in Fig. 2, was applied to re-centrifugation analysis and fractionated into 19 fractions (0.8 ml each). Proteins in each fraction were separated by SDS-PAGE (9.5% acrylamide gel) and stained with Coomassie blue. TG and CE in each fraction were analyzed by TLC. Lane 'A' indicates original lipid droplet-enriched fraction applied to the re-centrifugation analysis. An underline below the fraction numbers indicates the position where the droplet-sample was loaded. Density of each fraction was shown below.

Table 1
The results of the identification of the proteins in the lipid droplet-enriched fraction isolated from HuH7 cells

Number in Fig. 3	Identification	ID in the data base	Characters
1	acyl-CoA synthetase 4	O60488	a subtype of acyl-CoA synthetase*
2	lanosterol synthetase	P48449	enzyme in cholesterol synthesis cascade*
3	acyl-CoA synthetase 3	O95573	a subtype of acyl-CoA synthetase*
4	P63	S33377	ER membrane protein
5	protein for IMAGE:3942891	BC006145	unknown
6	ADRP	Q99541	lipid droplet specific protein**
7	Cargo selection protein TIP47	O60664	subcellular transporter/lipid droplet protein**
8	CGI49	AF151807	unknown
9	FLJ14497	AK027403	unknown
10	NSDHL	Q15738	3β-hydroxysteroid dehydrogenase*
11	GAPDH, liver	P04406	cytosolic enzyme
12	NADH-cytochrome *B5* reductase	P00387	ER enzyme
13	annexin II	P07355	phospholipid binding protein**
14	17βHSD11	AF126780	SDR*
15	Rab 5C (or Rab 5A)	P51148 (or P18066)	Ras family small GTPase
16	B-cell receptor associated protein 31	P51572	membrane IgD association protein
17	Ras-related protein Rap 1B	P09526	Ras family small GTPase

The numbers indicated here correspond to those in Fig. 3. *: Lipid metabolizing enzymes. **: Lipid-associating proteins. Rab 5C and Rab 5A could not be distinguished completely because of their highly conserved amino acid sequences.

1:2000). Finally, the antigens were visualized using ECL Western Blotting detection system.

2.10. Immunocytochemical analysis

HuH7 cells were inoculated at 7.5×10^4 cells/well in four-well culture slides (Falcon), incubated at 37 °C for overnight and cultured for 1 more day in the presence or absence of 0.6 mM oleic acid [15]. The cells were fixed and stained by the method of Wollins et al. [15]. The cells were incubated with anti-ADRP monoclonal antibody (dilution of 1:2), anti-17βHSD11 antiserum (dilution of 1:400) or anti-ACS3 antiserum (dilution of 1:300). Rhodamine-labeled goat anti-(mouse IgG) antibody (dilution of 1:8000) or rhodamine-labeled goat anti-(rabbit IgG) antibody (dilution of 1:6000) were used as secondary antibody. For double staining experiments, a mixture of anti-ADRP monoclonal antibody (dilution of 1:2) and anti-17βHSD11 antiserum (dilution of 1:400) was used for primary incubation, and a mixture of rhodamine-labeled goat anti-(mouse IgG) (dilution of 1:8000) and fluorescein-labeled goat anti-(rabbit IgG) (dilution of 1:5000) antibodies was used for secondary incubation. The antibodies and the antisera were diluted with PBS containing 0.05% BSA, 0.05% goat serum, 0.05% sodium azide and 0.1% saponin.

3. Results

3.1. Detection of intracellular lipid droplets in HuH7 cells

In our previous study, it was shown that a human hepatoma cell line HuH7 synthesized and secreted lipoproteins containing apolipoprotein B [31,32], suggesting that lipid metabolism is active in HuH7 cells. When HuH7 cells, cultured in the presence of 10% FBS, were stained with Oil red O, a dye specific for neutral lipids, we found a number of red-colored particles in the cytosol (Fig. 1A). This observation suggests that HuH7 cells have lipid droplets, and thus we attempted to isolate lipid droplets from these cells.

3.2. Isolation of lipid droplet-enriched fraction from HuH7 cells

In order to isolate a fraction enriched with lipid droplets, HuH7 cells were disrupted and the resulting extract was fractionated using sucrose density gradient centrifugation. Following centrifugation, a layer of white matter was observed at the top surface of the gradient. When the top fraction was stained with Oil red O, a number of red-colored spheres were observed under a microscope (Fig. 1B). Lipids were extracted from each fraction and analysed on TLC. As shown in Fig. 2, almost all the TG and CE were recovered in the top fraction (fraction 1), which was the least dense fraction. The distribution of ADRP (also known as adipophilin), a marker protein for the lipid droplets [10,14], was determined using Western blot analysis. ADRP was detected

Table 2
Amino acid sequences of tryptic fragments of ADRP, ACS3 and 17βHSD11 determined by Edman degradation

Number of protein	Amino acid sequence	Identification	Positions in the entire protein (residues)
3	LKDIVSLVPR	ACS3	221–230
	HIITVDGKPPTWSDFPK		233–249
	SLLGGNIR		439–446
	GGAPLSATTQ		451–460
	NWEEGGYFNTDKPHPR		510–525
6	MMQLVSSGVENALTK	ADRP	168–182
	SQQTISQLHSTVHLIEFAR		246–264
14	AEIGDVSILVNNAGVVYTSDLFATQ	17β HSD11	109–133
	TFEVNVLAHFWTTK		140–153
	FVNTGFIK		220–227
	NPSTSLGPTLEPEEVVNR		228–245

only in the top fraction (Fig. 2). These data show that the lipid droplets were enriched in the top fraction.

Judging from the protein profile stained with Coomassie blue, the majority of the cellular proteins was present in the middle fractions and the denser fractions (Fig. 2, fractions 9–19), and the top fraction contained only a very small amount of protein. When a larger amount of the top fraction was loaded, more than 17 protein bands were detected, where three proteins with molecular mass of 55, 75 and 35 kDa were abundant (Fig. 3, lane 2, bands #6, 3 and 14). This protein composition was clearly distinct from that of the total cell lysate (Fig. 3, lane 1), suggesting that the proteins in the top fraction were the specific components of the fraction. This unique protein profile was obtained in a highly reproducible manner in more than 10 experiments. LDH activity, a cytosolic marker, was not present in the top fraction, while it mainly located in the middle fractions where the PNS sample was applied and to a lesser extent in the denser fractions (Fig. 2, lower panel). Calnexin, integrin (VLA-2α) and GS28, markers for ER membranes, plasma membranes and Golgi membranes, respectively, were also hardly detected in the top fraction (Fig. 2). Thus, it is unlikely that the proteins in the top fraction were contaminants 'accidentally' migrating from the denser fractions to the top fraction.

We tested the stability of the interaction of proteins and droplets. The lipid droplet-enriched fraction was loaded in the middle position of a sucrose density gradient and re-centrifugation analysis was performed. Again, most of the proteins were recovered in the top fraction together with CE and TG (Fig. 4). Thus, it is likely that association of the proteins with lipid droplets is reasonably stable. At this stage, however, it is not clear whether these proteins associated with the droplets directly or indirectly.

3.3. Identification of the proteins in the lipid droplet-enriched fraction

To identify lipid droplet proteins, the protein in each gel band was digested with trypsin, and the extracted peptides were analyzed by nano LC-MS/MS. Proteins were identified by matching the MS/MS data with mass values calculated for selected ion series of theoretical tryptic fragments of mammalian proteins in sequence databases. The results of the identification were summarized in Table 1. The most abundant protein (#6) of the lipid droplets appeared to be ADRP. The other two prominent proteins (#3 and 14) were identified as ACS3 [30] and 17βHSD11 (also known as Pan1b or retSDR2) [33], respectively. Such a protein composition seems specific for the lipid droplets since the condensation of these three proteins in any other particular organelle has not yet been reported.

Among the 17 identified proteins are two lipid droplet-associating proteins (ADRP and TIP47) that have previously been reported [14,15]. In addition, we found one phospholipid binding protein (annexin II) and five lipid metabolizing enzymes (17βHSD11, ACS3, ACS4, lanosterol synthetase and NAD(P)-dependent steroid dehydrogenase-like protein (NSDHL)).

Although some ER proteins or endosome proteins (i.e. P63, NADH cytochrome *B*5 reductase, Rab5C and Rap1B) were present in the identified proteins, the protein composition of the lipid droplets is still different from that of

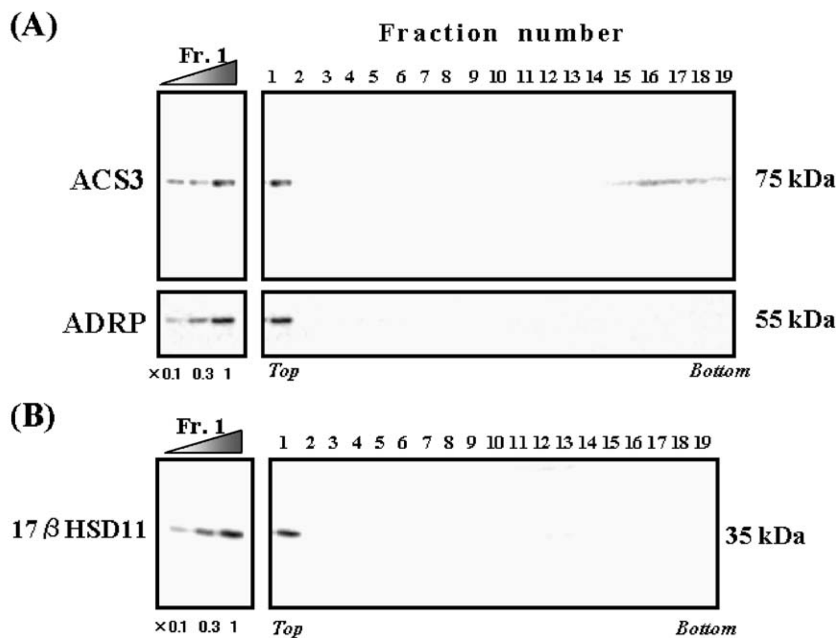

Fig. 5. Distribution of ACS3 and 17βHSD11 in subcellular fractions separated by sucrose density gradient centrifugation. Subcellular fractionation of HuH7 cells by sucrose density gradient centrifugation was performed under the same conditions as described in Fig. 2. ACS3, ADRP and 17βHSD11 in each fraction were detected by immunoblot analysis (A and B). The small panels on the left side in (A) and (B) indicate ×1, ×0.3 and ×0.1 dilution of the fraction 1.

typical ER. For example, cytochrome P450, which is one of the most abundant proteins in the liver ER [34], is not detected in the lipid droplets. Neither mitochondrial nor nuclear proteins were detected in the identified proteins. However, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a cytosolic protein, was found among the identified proteins. It is noteworthy that GAPDH has previously been reported to be present in the lipid droplets of *Saccharomyces cerevisiae* [35]. It may be possible that specific interactions between GAPDH and the lipid droplets exist.

The three major proteins (#3, 6 and 14 in Fig. 3) were excised from SDS-PAGE gel, and tryptic fragments were separated on a reverse-phase HPLC. The identification of these three major proteins was confirmed by determination of amino acid sequences of the tryptic fragments using Edman degradation. Table 2 shows the sequences of the peptides derived from the bands #3, 6 and 14. All of the amino acid sequences for the tryptic fragments were identified in the sequence of ADRP, ACS3 and 17βHSD11. These results are well in accordance with those from the

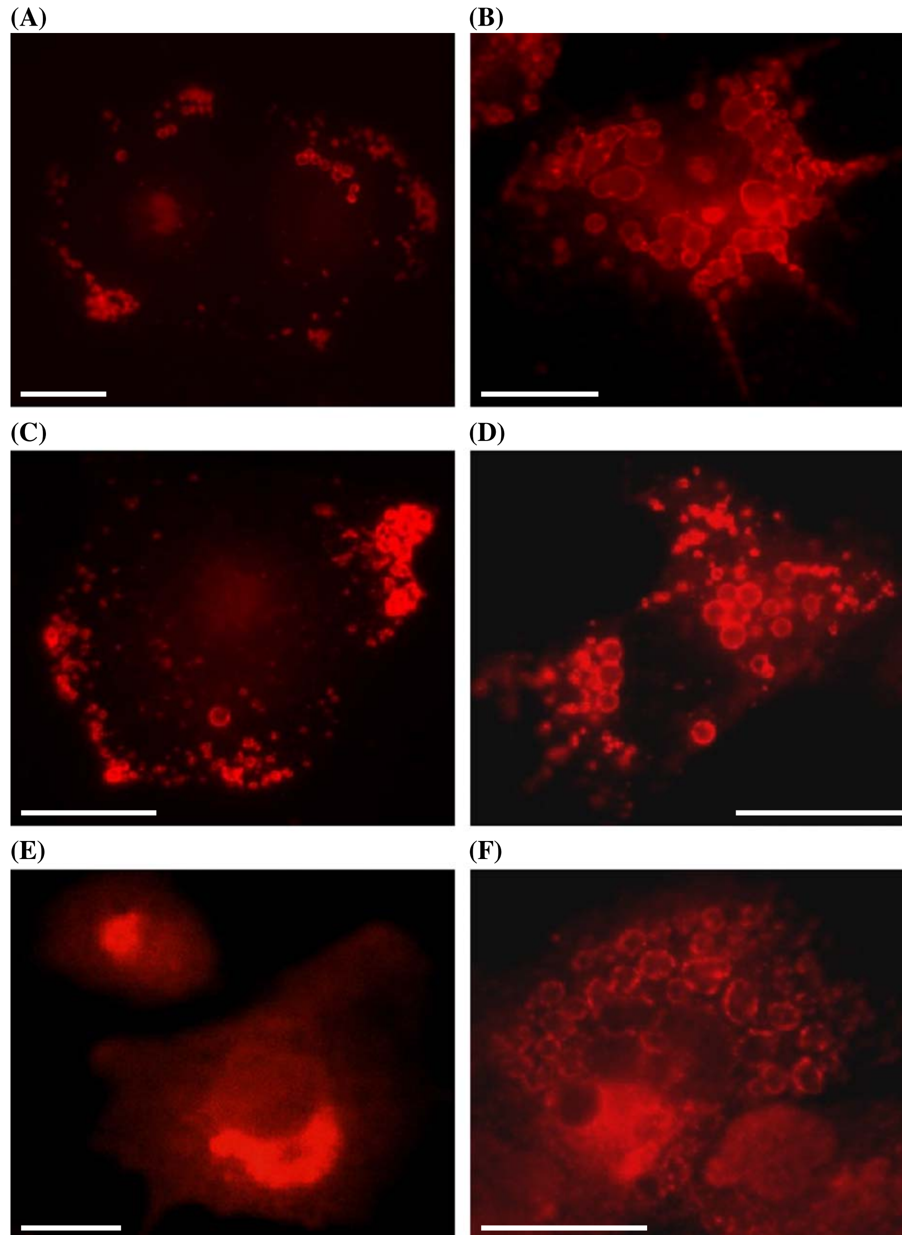

Fig. 6. Immunocytochemical analysis of ADRP, 17βHSD11 and ACS3. The three lipid droplet proteins in HuH7 cells were detected by indirect immunofluorescent analysis using anti-ADRP (A, B) monoclonal antibody or antisera against 17βHSD11 (C, D) and ACS3 (E, F) followed by rhodamine-labeled secondary antibodies. In (A), (C) and (E), cells were cultured with DMEM supplemented with 10% FBS. In (B), (D) and (F), 0.6 mM oleic acid was added in the medium for 1 day before the immunological staining for enlargement of the lipid droplets. In (E), cells containing very few droplets are shown. The bars indicate 25 μm.

nano LC-MS/MS analysis (Table 1), supporting the results obtained from this analysis.

3.4. Analysis of subcellular localization of 17βHSD11 and ACS3

Intracellular localization of ACS3 and 17βHSD11 has not been previously reported in the literature. Antisera against these proteins were prepared by immunizing rabbits with recombinant peptides. Subcellular localization of 17βHSD11 and ACS3 was examined using immunoblot analysis and immunocytochemistry. HuH7 cells were fractionated by a sucrose density gradient centrifugation, and the distributions of the two proteins were examined. It was shown that most of 17βHSD11 localized in the droplet-enriched fraction (Fig. 5B, fraction 1). About 40% of ACS3

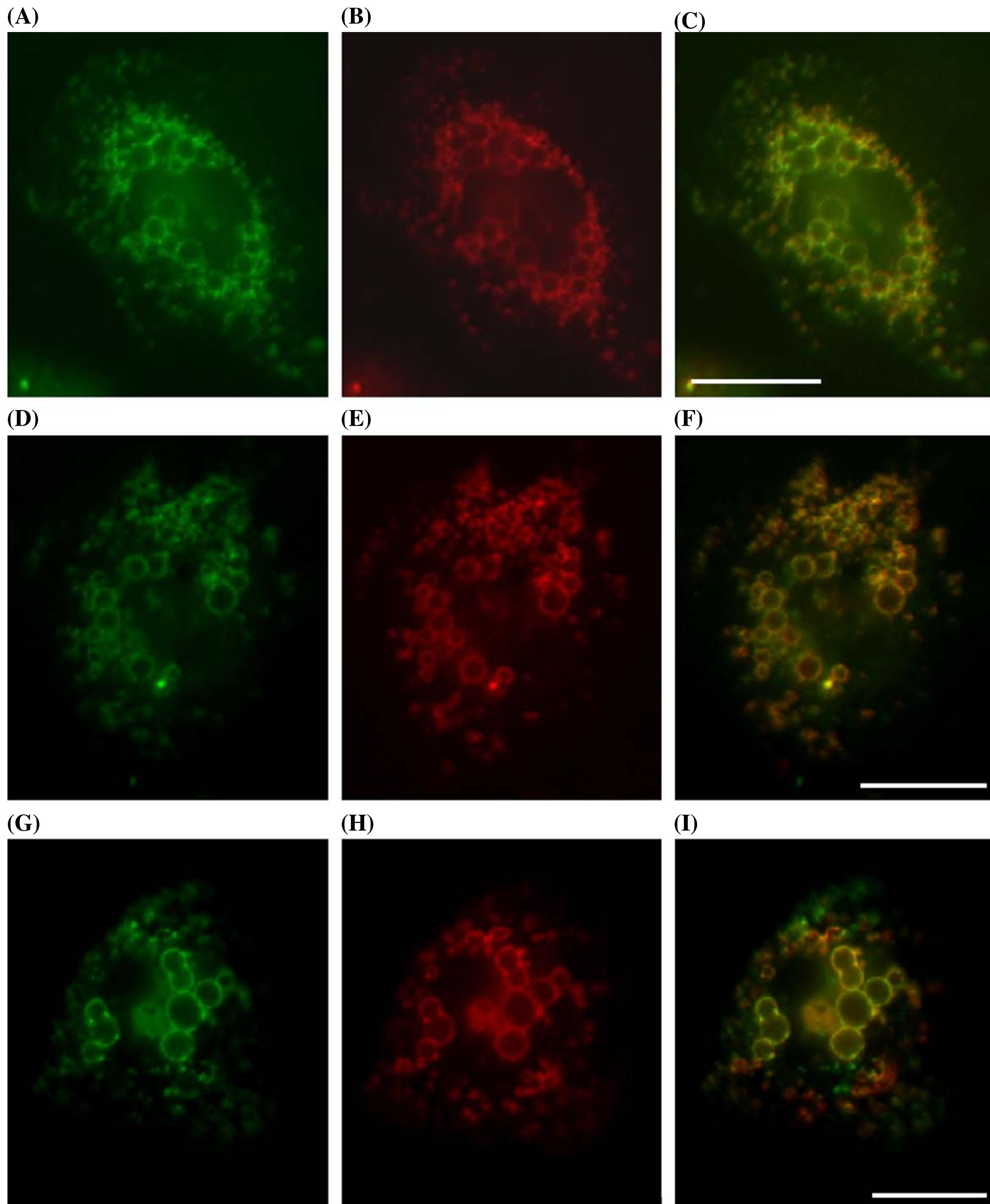

Fig. 7. Double staining of ADRP and 17βHSD11. HuH7 cells cultured in DMEM supplemented with 10% FBS and 0.6 mM oleic acid were simultaneously stained with anti-ADRP monoclonal antibody and anti-17βHSD11 antiserum. 17βHSD11 (A, D and G) and ADRP (B, E and H) were detected by fluorescein- and rhodamine-labeled secondary antibodies, respectively, and images were merged (C, F and I). Three different cells were indicated. The bars indicate 25 μm.

associated with the lipid droplet fraction, while the remaining ACS3 were recovered in the fractions #15–19 (Fig. 5A). Calnexin, a marker protein for ER membranes, was also recovered in the fraction #15–19 (see Fig. 2), suggesting that about 60% of ACS3 localized in particulate fractions including organelle membranes.

The subcellular distributions of these two proteins were further studied by immunocytochemistry. When cells were cultured in a conventional medium (DMEM supplemented with 10% FBS), a number of small fluorescent rings were detected in the periphery of the cells by anti-ADRP monoclonal antibody (Fig. 6A). When cells were cultured in the medium containing 0.6 mM oleic acid, fluorescent rings were much larger than those without oleic acid, and they were distributed broadly in the cells (Fig. 6B). These results agree well with the intracellular distribution of ADRP reported previously [7,14]. By the anti-17βHSD11 antiserum, fluorescent rings similar to those detected by anti-ADRP antibody were observed (Fig. 6C and D). Again, the sizes of fluorescent rings were larger when cells were cultured in the presence of oleic acid (Fig. 6D). Co-localization of these two proteins was further examined by double staining experiments. Localization of 17βHSD11 (Fig. 7, green) was clearly overlapped with that of ADRP (Fig. 7, red), though they are not identical (see Fig. 7I), suggesting that 17βHSD11 localizes on the droplet. This result is well in accordance with that of the immunoblot analysis (Fig. 5B). ACS3 was also detected as ring-shaped fluorescent signals; however, perinuclear staining for ACS3 in the same cell was also apparent (Fig. 6F). This observation corresponds with the dual distribution of this enzyme as shown in Fig. 5A. In cells containing very few droplets (a small part of cells obtained by culturing without oleic acid), fluorescence by anti-ACS3 antiserum was mainly detected in the perinuclear region (Fig. 6E). This suggests the possibility that ACS3 may migrate from some organelle in the perinuclear region to the periphery of droplets during the course of droplet formation.

4. Discussion

In this report, we isolated a subcellular fraction enriched with intracellular lipid droplets from a human hepatocyte cell line, HuH7, using sucrose density gradient ultracentrifugation and the resulting protein profile was characterized by identifying the 17 major proteins. Three proteins, ADRP, ACS3 and 17βHSD11, were highly enriched in the fraction, revealing a unique protein composition. In this study, the nano LC-MS/MS technique is very effective and enabling us to identify thoroughly the proteins found in the fraction. The reliability of the analysis was verified, at least in part, by the direct sequencing of tryptic fragments using Edman degradation.

The lipid droplet-enriched fraction isolated in the present study seemed to consist of a distinct subcellular constituent, since (i) almost all the TG, CE and ADRP were recovered in the fraction, (ii) the fraction had a highly reproducible protein composition which was distinct from those of other fractions, (iii) the cytosolic maker LDH, the plasma membrane marker integrin, the ER marker calnexin and the Golgi marker GS28 were hardly detected in the fraction, and (iv) proteins from other subcellular compartments such as cytosol, mitochondria and nuclei were scarcely detected among the identified proteins. These properties indicate enrichment of lipid droplets in the fraction and little contribution of other subcellular fractions. A couple of reports suggest that lipid droplets interact with membranous structures [12,36]. Thus, it is possible that our droplet-enriched fraction may contain some parts of cellular membranes. It is not clear at this stage whether the 17 proteins identified in this study are associated directly to the droplet surface. Immunoelectron microscopic studies will be necessary to answer this issue.

In the droplet-enriched fraction from HuH7 cells, the most abundant protein was found to be ADRP, one of the well-known lipid droplet-associating proteins. This agrees well to the idea that ADRP may be a shell-forming protein in lipid droplets [6]. Although elucidation of the function of ADRP is still in progress, ADRP seems to be involved in the formation and stabilization of lipid droplets, since (i) ADRP is induced when the lipid droplets accumulate [37]; (ii) ADRP is detected in lesions of lipid storage disorders such as hepatic steatosis and atherosclerosis [16–18]; and (iii) overexpression of ADRP in COS-7 cells facilitates cellular uptake of fatty acids [20]. When lipid droplets were isolated from foam cells derived from murine macrophage J774, ADRP was again found to be the most abundant proteins (unpublished data). Thus, ADRP seems to play an important role in a wide variety of cell types and species. It should be noted that ADRP is rather a minor component of lipid droplets in adipocytes [14]. However, our data suggest that ADRP might be the primary lipid droplet-associating protein in cells that do not express perilipins.

Apart from ADRP, another lipid droplet-associating protein, TIP47, a member of PAT protein family, was also found in the droplet-enriched fraction. Based on its sequence similarity to the known droplet proteins, Wolins et al. showed a specific interaction of TIP47 with the lipid droplets [7,15]. However, Barbero et al. disagreed with the result [38], who discovered the protein as a carrier involved in the sorting of mannose-6-phosphate receptor [11]. Our results support the presence of TIP47 in lipid droplets. The members of PAT family are known to associate with lipid droplets, however, there are some differences in their tissue specificities. Expression of perilipin is restricted to adipocytes and steroidogenic cells, while ADRP and TIP47 are expressed ubiquitously. In our study, perilipin was not present among the major proteins found in the droplet-enriched fraction from HuH7 cells, which are derived from human liver.

In addition to these lipid droplet-associating proteins, we also identified five kinds of lipid metabolizing enzymes,

with ACS3 and 17βHSD11 appearing to be two of the major proteins. It may be speculated that these function in the deposition and/or mobilization of intracellular neutral lipids via the metabolism of lipid molecules in the lipid droplets. For example, ACS3 is an enzyme producing acyl-CoA, which can be utilized in the synthesis of TG and CE. Or, the enzyme may participate in utilization of fatty acids produced by hydrolysis of TG and CE by derivatizing them to acyl-CoA. The physiological function of 17βHSD11 remains unclear, although it showed dehydrogenase activity toward hydroxysteroids at the 17β position when overexpressed in CHO cells [33].

By preparing specific antisera, we strongly suggested that 17βHSD11 localizes in the peripheral regions of the droplet surface. The apparent distribution of 17βHSD11 does not change either in the presence or absence of exogenous oleic acid. Although there are many members in short chain dehydrogenase/reductase (SDR) family, 17βHSD11 is the first to be specifically detected around the lipid droplets. It was also shown that as much as 40% of ACS3 was detected in the lipid droplet-enriched fraction. At least six subtypes of ACS have been reported in mammals (ACS1–5 and very long chain acyl-CoA synthetase). Of these, ACS1 mainly localize in ER and cytoplasm [39]. ACS4 is a subtype specific for mitochondria-associated membranes (MAM) [39], which are ER-like membranes enriched with several lipid biosynthetic enzyme activities [40], and ACS5 is the mitochondria-specific subtype [39]. Subcellular localization of ACS2 and ACS3 remains unknown. Our result suggested that ACS3 is, at least in part, a lipid droplet-related subtype of ACS. In immunocytological studies, ACS3 was detected in perinuclear regions in cells having very few lipid droplets while it appears as ring-shaped images in cells loaded with oleic acid. ACS3 may relocalize from the perinuclear regions to the perifery of the droplets during the course of the droplet formation.

The other three enzymes found in the droplet-enriched fraction were ACS4, lanosterol synthetase and NSDHL. The presence of ACS4 in the fraction suggests a possibility that the droplets interact with MAM, while the content of ACS4 in the droplets was much lower than that of ACS3 (Fig. 3, bands #1 and 3). However, other enzymes enriched in MAM, such as phosphatidylserine synthetase [41] and phosphatidylethanolamine *N*-methyltransferase [42], were not detected in our droplet fraction. Thus, it is unlikely that the lipid droplet-enriched fraction contains MAM itself. Lanosterol synthetase is the enzyme which catalyzes the conversion of (3*S*)-2, 3-oxidosqualene to lanosterol. Presence of the enzyme in lipid droplets suggests that this may be one of the sites where synthesis of lanosterol occurs. Interestingly, in *S. cerevisiae*, lanosterol synthetase localizes almost exclusively in lipid droplets [43]. NSDHL is a member of 3β-hydroxysteroid dehydrogenase, which is a subfamily of SDR enzyme family. The gene encoding this protein is responsible for mouse mutants bare patches (Bpa)/striated (Str) [44] and a human genetic disorder congenital hemidysplasia, ichthyosis and lim defects (CHILD) syndrome [45]. These diseases were X-chromosome-linked, dominant and male lethal. The phenotype of affected female mice is characterized by patchy hyperkeratonic skin eruptions that induce bare patches (*Bpa*) or striations of coat (*Str*). In human disorder, ichtyosiform inflammatory nevi and limb defects are observed. This disease seems to be caused by a defect in cholesterol biosynthesis [44].

Although some ER proteins were detected in the droplet-enriched fraction, its protein composition is different from that of typical ER. Cytochrome P450 was not detected in the droplet. In subcellular fractionation experiments, distribution of ADRP, 17βHSD11 and ACS3 was clearly distinct from that of calnexin, an ER marker. The meaning of the presence of Ras-related proteins such as Rab 5C and Rap1b in the lipid droplets remains unclear. However, as these proteins function in subcellular transport through targeting and fusion of transport vesicles [46–48], it is possible that some vesicles may interact with the lipid droplets [12].

Until now, vimentin [36], 160-kDa capsule protein [8], TG hydrolase [49], phosphatidylethanolamine *N*-methyltransferase [42], caveolins [50] and α-synuclein [51] were also identified as lipid droplet-associating proteins. In our results, these proteins were not found to be major proteins in the droplets from HuH7 cells. There are two possible explanations for this inconsistency: First, the difference seems to be caused by the different cell types and cell conditions. For example, α-synuclein is a neuronal protein and interaction of α-synuclein and the droplets is detected in HeLa cells by the transfection of α-synuclein gene [51]. In the hepatocyte cell line HepG2, localization of caveolin-2 in the lipid droplets can be detected only after the transfection of the gene, since this cell line lacks caveolins [50]. The localization of vimentin seems dependent on the stage of the droplets [12]. Second, most of these previously reported proteins are not originally discovered in lipid droplets and thus their subcellular distribution is not restricted to the droplets. Hence, the amount of these proteins localizing in the droplets may be too small to be detected in our study.

At present, the study of protein components in the lipid droplets is most advanced in *S. cerevisiae*. Sixteen proteins have been identified as the major proteins of the yeast droplets [35]. Surprisingly, comparing of our results with those of the yeast study shows that several droplet proteins are conserved in both the yeast and human samples. These include two kinds of acyl-CoA synthetases, a member of SDR family, lanosterol synthetase and GAPDH [35]. This suggests functional conservation of these droplet enzymes in a wide variety of eukaryotic species. Conversely, there are some differences between the protein compositions of the yeast and human droplets. The most significant difference is that a homologue of PAT family is not found in yeast droplets [35]. The most abundant protein of the yeast lipid droplets is not an ADRP homologue, but rather, sterol-Δ^{24}-methyltransferase [35]. Since members of PAT family are found in *Drosophila* and *Dictyostelium* [7], PAT family

proteins appear to emerge after the evolutionary bifurcation of yeasts and ancestors of *Drosophila* and *Dictyostelium*.

In conclusion, we have demonstrated in this study that the lipid droplet-enriched fraction isolated from human hepatic cells contain not only ADRP but also many kinds of proteins including lipid-associating proteins and lipid-metabolizing enzymes. These proteins are candidates for novel components of lipid droplets or structures associated with them. These results help to provide a basis for the understanding of the mechanism of storage of neutral lipids.

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