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Intracellular distribution and mobilization of unesterified cholesterol in adipocytes: triglyceride droplets are surrounded by cholesterol-rich ER-like surface layer structures

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SUMMARY

In addition to their central role in triglyceride storage, fat cells are a primary depot of unesterified cholesterol (FC) in the body. In comparison, peripheral cells contain very little FC. This difference in adipocytes versus peripheral tissues is inconsistent with the current theory of cholesterol homeostasis. Attempting to resolve this discrepancy, we examined intracellular storage sites of FC in murine 3T3-F442A adipocytes. Using the cholesterol-binding antibiotic, filipin, in combination with high resolution fluorescence microscopy, intense fluorescent staining characteristically decorated the periphery of triglyceride droplets (TGD) as well as the plasma membrane (PM) of fat cells. Filipin-staining was not visible inside the lipid droplets. Purification of TGD by subcellular fractionation demonstrated that the rise in total FC content of adipocytes upon differentiation was attributable to an increase in TGD-FC, which contributed up to one third of the total cellular FC. The protein component of purified TGD from cultured adipocytes as well as from murine adipocytes obtained from fresh tissues contained the lumenal endoplasmic reticulum (ER) immunoglobulin binding protein (BiP) and the integral ER membrane protein calnexin. Efflux experiments using the extracellular FC acceptors β-cyclodextrin or apolipoprotein A-I demonstrated that TGD-associated FC was releasable from TGD. Whereas FC efflux from adipocytes was unaffected in the presence of brefeldin A or monensin, the secretion of a control protein, lipoprotein lipase, was effectively reduced.

In summary, our findings identify the TGD surface layer as primary intracellular storage site for FC within adipocytes. We suggest that the structural role of ER-resident proteins in this adipocyte TGD envelope has been previously neglected. Our findings support the suggestion that an ER-like structure, albeit of modified lipid composition, constitutes the lipid droplets' surface layer. Finally, the efflux process of FC from adipocytes upon extracellular stimulation with β-cyclodextrin provides evidence for an energy-dependent intracellular trafficking route between the TGD-FC pool and the PM-FC sites which is distinct from the secretory pathway of proteins.

Key words: Adipocyte, Free and esterified cholesterol, Cholesterol efflux, Lipid droplets, BiP, Fluorescence microscopy

INTRODUCTION

Adipose tissue normally represents 10-20% of the total body mass in humans, and fat cells (adipocytes) are the major cell type of this organ (Ailhaud et al., 1992). In addition to the central role of adipose tissue in mammalian nutrient storage and energy balance (Flier, 1995; Bernlohr and Simpson, 1996), it is also the body's largest pool for free (unesterified) cholesterol (FC) (Krause and Hartman, 1984; Kraemer et al., 1994).

A delicate balance between uptake, synthesis, and storage tightly controls the abundance of FC in peripheral cells (Brown and Goldstein, 1997). The sterol-regulatory element binding-protein-1 (SREBP-1) (Brown and Goldstein, 1997), a transcription factor and member of the basic helix-loop-helix leucine zipper family, has been shown to be implicated in this process, which prevents FC overaccumulation that would otherwise lead to cellular necrosis (Tabas et al., 1996). Interestingly, not only the cholesterol content of membranes, but also adipogenesis seems to be regulated accordingly, since several genes related to adipocyte differentiation such as fatty acid synthase, acetyl-CoA carboxylase, and glycerol-3-phosphate acyltransferase have been identified as targets of ADD1 (adipocyte determination and differentiation-dependent factor-1), the rat homologue (Kim and Spiegelman, 1996) of human SREBP-1. Therefore, a general housekeeping function of SREBP-1/ADD1 in regulating the lipid composition of animal cell membranes has been proposed (Shimomura et al., 1998).

Most cells and tissues are able to store small pools of cholesterol in esterified form. Cells specialized for the production of steroid hormones, such as adrenal cells (Servetnick et al., 1995), have the capacity to accumulate considerable quantities of excess cholesteryl esters. In comparison, differentiated adipocytes are the only cell type that harbour large amounts of unesterified cholesterol (Farkas et al., 1973), and accordingly, cholesteryl ester synthesis has been reported to be very low (Barbaras et al., 1985). Most notably, during the differentiation process in tissue culture (Zechner et al., 1991), the capacity of fat cells to store FC parallels the increase in neutral lipids. Consistent with this observation, early studies have proposed that fat cell size might influence the body's fat cell cholesterol content (Kovanen et al., 1975; Krause and Hartman, 1976).

Under certain fasting conditions, a decrease of intracellular cholesterol content and a concomitant increase in LDL-cholesterol levels have been observed in experimental animals (Kraemer et al., 1994; Kovanen et al., 1975; Krause et al., 1981). Conversely, an elevation of the adipocyte cholesterol amount has been reported in diet-induced hypercholesterolemia (Krause and Hartman, 1984; Smith et al., 1976; Goodmann et al., 1980; Arner, 1997). Taken together, considerable evidence suggests that a dynamic equilibrium exists between serum lipoprotein cholesterol and adipocyte FC pools. Accordingly, adipose tissue has been proposed to perform a 'buffer' function for serum cholesterol (Krause and Hartman, 1984). Despite the significant physiological importance of FC, especially with regard to the reported association between obesity and mortality from cardiovascular diseases (Hubert et al., 1983; Després, 1991), the localization of intracellular FC pools within adipocytes is, as yet, an unresolved question. Moreover, the mechanisms facilitating communication between the fat tissue FC depot and the blood cholesterol pool are still poorly understood.

Thus, the objective of the present study was to obtain new insights into the nature of intracellular depots for unesterified cholesterol in adipocytes and to probe the possibility that FC can be mobilized from these cells when stimulated with extracellular cholesterol acceptors. Moreover, an as yet undescribed role for ER proteins as components of the adipocyte TGD surface layer was revealed.

MATERIALS AND METHODS

Chemicals and cell culture materials

Tissue culture flasks, dishes, and 6-well-plates were obtained from Iwaki (Bertoni/Vienna, Austria), or from Corning/Costar Glass Works (Scandic, Vienna, Austria); cover slides (22×22 mm) from Roth (Karlsruhe, Germany); DMEM (high glucose), penicillin/streptomycin, L-glutamine, heat-inactivated fetal bovine serum (FBS) and trypsin-EDTA from Life Technologies (Vienna, Austria); glycerol from Merck (Vienna, Austria); *p*-formaldehyde from Fluka, (Vienna, Austria); L-α-lecithin (from egg yolk) from Avanti Polar Lipids (Birmingham, USA); 1,2,^{3}H(N)-cholesterol and ^{3}H-triolein from NEN Life Science Products (Brussels, Belgium); See Blue pre-stained molecular weight standard was from Novex-ICT (Vienna, Austria). All other chemicals were obtained from Sigma (Vienna, Austria).

Cell culture conditions and differentiation of adipocytes

3T3-F442A mouse preadipocytes (Green and Kehinde, 1976) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (standard medium) in a humidified incubator (95%) at 37°C with 5% CO_2. Cells were differentiated in standard medium containing 17 nM insulin, 2 nM triiodothyronine, 0.8 mg/ml biotin, and 0.4 mg/ml pantothenic acid (day 0 of differentiation) (Aubert et al., 1996). The medium was changed every other day. If not otherwise indicated, cells were grown in the presence of 10% FBS.

Isolation of the TGD fraction from adipocytes

Differentiated adipocytes (at various stages of differentiation), grown in 78 cm^2 dishes, were washed twice with ice-cold PBS and gently homogenized in 2 ml of hypotonic PBS (dilution 1:10 in dist. water) containing protease inhibitors (Steyrer et al., 1990). TGD were isolated by ultracentrifugation according to a protocol established for isolation of cytoplasmic lipid droplets from mammary epithelial cells (Dylewski et al., 1984). Briefly, ultracentrifugation was performed at 4°C and 100,000 ***g*** in 0.25 M sucrose dissolved in TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, and 5 mM $MgCl_2$) for 60 minutes in a final volume of 12 ml using a Beckman SW-41 rotor. The supernating fraction was collected, and the pellet fraction was washed twice with 0.8 ml of TKM buffer each in a Beckman TLA-120 ultracentrifuge.

Extraction of lipids and enzymatic assays

Lipids were extracted with hexane:isopropanol (3:2, v/v) from whole cells (5 ml of solvent per 78 cm^2 dish) or from fractions after separation of TGD. 200 μl-aliquots of lipid extracts were mixed with 40 μl of the detergent Thesit (Van Veldhoven et al., 1998) (20% w/v; in chloroform) and evaporated to dryness. The oily residue was redissolved in 50 μl of dist. water. FC concentrations were then determined enzymatically from lipid extracts of duplicate dishes according to the manufacturer's protocol (F-CHOL; Boehringer Mannheim, Germany).

LDH activity was assayed using a test kit from Boehringer Mannheim (Germany).

Prelabeling of cells using ^{3}H-FC, determination of FC efflux, and preparation of PC vesicles

Large unilamellar phosphatidylcholine (PC) vesicles (LUV) were prepared as previously described (Rodrigueza et al., 1997). Cells grown to confluency in 3.8 cm^2 dishes were differentiated and radiolabeled by incubation with DMEM containing antibiotics, 10% FBS and 0.1 μCi/ml ^{3}H-FC for 24 hours. For equilibration of cellular FC pools, cells were incubated for another 24 hours with DMEM supplemented with 0.2% BSA. AcylCoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz-compound 58-035 (1 μg/ml, 0.1% DMSO final concentration) (Rodrigueza et al., 1997) was included in both the prelabeling and equilibration media to prevent cholesterol esterification. For FC efflux experiments (Christian et al., 1997), cells were washed twice with PBS at 20°C, and DMEM efflux medium containing 10 mM β-cyclodextrin (β-CD) plus 0.6 mM LUV as indicated was added. FC efflux was performed in an incubator at 37°C for the indicated times. FC efflux experiments at 15°C were performed in a water bath placed in a cold room. Media were collected and cells were rinsed with PBS. Cells were lysed with 1 ml of 0.3 M NaOH for 3 hours on a rotary shaker on ice. Media and cell lysates were mixed with Ready Safe Liquid Scintillation Cocktail (Beckman Instruments, Vienna, Austria), incubated overnight and counted in a TRI-CARB 2700TR Analyzer (Packard/Vienna, Austria).

For determination of FC efflux in the presence of inhibitors, adipocytes were labeled with ^{3}H-FC as described above and preincubated for 30 minutes with 0.5 ml DMEM containing brefeldin A (5 μg/ml) or monensin (20 μg/ml). Cells were further incubated for another 60 minutes with fresh DMEM containing inhibitors in the presence or absence of β-CD/LUV. Media from duplicate dishes were collected and the amount of ^{3}H-FC was determined.

Preparation of apoA-I:PC proteoliposomes, and apolipoprotein-mediated FC efflux

Purified human apolipoprotein A-I (ApoA-I) was kindly provided by Dr Ernst Malle, Department of Medical Biochemistry and Medical Molecular Biology, University of Graz. ApoA-I:PC proteoliposomes were prepared as previously described (Steyrer and Kostner, 1988) at 1:1 (w/w) of apoA-I and L-α-phosphatidylcholine, β-palmitoyl-γ-oleoyl (POPC). 3T3-F442A cells (day 11 of differentiation) were prelabeled in 78 cm^2 dishes with ^{3}H-FC for 24 hours as indicated and incubated with apoA-I or apoA-I:POPC proteoliposomes as described in the legends to Fig. 6C. After incubation, aliquots of cell media were removed, PM and TGD were isolated by subcellular fractionation, and radioactivity of different compartments was determined by liquid scintillation counting.

Inhibition of intracellular vesicle transport and determination of lipoprotein lipase (LPL) activity

Adipocytes were treated with 5 U/ml heparin (1 ml/well) for 30 minutes to remove LPL attached to the cell surface. After washing with DMEM, cells were incubated for 75 minutes with fresh DMEM containing either brefeldin A (5 μg/ml) or monensin (20 μg/ml) in the presence of heparin (5 U/ml). Then, cells received fresh DMEM including inhibitors and were further incubated as indicated in the legends. Culture media were collected from duplicate dishes, and LPL activity in cell medium was determined as previously described (Haubenwallner et al., 1993).

Protein quantitation

Cells were dissolved in 1 ml of 0.3 M NaOH (for 3.8 cm^2 dishes) and the protein concentration was determined by the method of Lowry (Steyrer et al., 1990).

Fluorescence microscopy

3T3-F442A preadipocytes and differentiated adipocytes were grown in 3.8 cm^2 culture dishes on micro slides, washed with PBS and switched to DMEM-efflux media as described above. After the indicated times, cells were rinsed with PBS and fixed for 20 minutes with 4% *p*-formaldehyde/PBS at room temperature (Berger, 1986). FC was stained with filipin (dissolved first in 20 μl of DMSO before dilution with PBS to 0.2 mg/ml) for 3 hours on a rotary shaker in the dark. Filipin:FC fluorescence was recorded at 380 nm (excitation)/510 nm (emission) (Butler et al., 1992) using high resolution image analysis with a deconvolution microscope (Nikon Eclipse TE 300, Nikon, Vienna, Austria) as recently described (Graier et al., 1998). Images were collected with a CFI Plan Fluor ×40 (NA1.3) and ×100 (NA 1.3) oil immersion objective and the slice interval was 0.5 and 0.3 μm, respectively. Out-of-focus fluorescence was removed using Micro Tome® (VayTek, Inc., Fairfield Imaging, Turnbridge Wells, UK). Cells were stored in the dark as a micro slide sandwich sealed with glycerol.

For experiments with a Cy2-labeled secondary antibody (dilution 1:200), fat cells were permeabilized with 0.6% Triton-X-100 (in PBS) for 30 minutes at 20°C. After blocking for 30 minutes in 5% BSA (in PBS) and incubation with primary and secondary antibody for 60 minutes each, fluorescence signals were analyzed on a confocal laser scanning microscope (Leica TCS NT, Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with an argon-krypton laser. A simultaneous 2-channel-detection with an ×100 Planapo objective was performed using an excitation wavelength of 488 nm, with a 580 nm short-pass and a 530 nm band-pass filter together with a differential interference contrast. Negative controls were performed by replacing the primary antibody with rabbit non-immune serum (dilution 1: 200). The scanning field dimension (xy) was 100 μm.

Cryosections

3T3-F442A adipocytes (day 10 of differentiation), cultured in 78 cm^2 dishes, were washed three times with 37°C PBS, and scraped with 1.5 ml of PBS. After transfer into an Eppendorf vial, cells were carefully pelleted at 2,000 rpm in an Eppendorf centrifuge for 10 minutes, and supernatant PBS was removed by aspiration. The following procedures of embedding and cryosectioning of adipocytes were performed with the CryoJane System® (Instrumedics, Hackensack, NJ, USA). Using a 1,000 μl plastic pipette tip, pelleted cells were carefully aspirated, injected directly into approx. 2 ml of viscous CryoGel® (20°C) placed in a rubber mold, and immediately frozen to −70°C using the snap-freezing system®. This frozen block was mounted onto a prechilled cryostat blockholder. A cold adhesive tape® was adhered onto the block surface to capture the section, and 5 μm sections were collected. The cryostat chamber (Reichert-Jung Frigocut 2000) temperature was −25°C. The tape was then transferred to a cold glass microscope slide (adhesively-coated slide®, CS4x), where the tape was carefully removed. Sections were anchored by UV-light polymerization (ultraviolet light flash chamber®) at −25°C. Slides were then fixed for 30 seconds in 10% glutaraldehyde diluted with aqueous buffer®, washed in water, followed by staining of polar lipids with Oil Red-O solution (0.3% in 60% isopropanol) (Denk et al., 1998) for 10 minutes. Note that all washing steps with isopropanol were omitted at this stage to minimize loss of lipids. After washing in distilled water, nuclei were counterstained for 2 minutes in Mayer's hemalum. After blueing in 2.5% ammonium water, sections were mounted in Kaiser's glycerol gelatin (Merck, Vienna, Austria).

Amplification of a BiP cDNA fragment

Total RNA was isolated from differentiated 3T3-F442A cells (day 9) with TRI REAGENT (Molecular Research Center, Inc.) (Chomczynski, 1993). Reverse transcription was performed using 1.5 μg total RNA following the protocol of a 3′ RACE system (Life Technologies). A 600 bp BiP cDNA fragment was amplified with Ampli Taq Gold (PE Biosystems) at recommended conditions using 2 μl of the reverse transcription reaction (forward primer: 5′-CCC-GGCATGATGAAGTTCAC-3′, reverse primer: 5′-TCCTCATGAC-ATTCAGTCCAGC-3′). Amplification was performed with an initial denaturing step at 95°C for 9 minutes followed by 38 cycles of a step program (94°C for 30 seconds, 56°C for 45 seconds, 72°C for 50 seconds) and a 10 minutes final extension step at 72°C. The fragment was cloned into vector pSTBLUE-1 (Novagen) and BiP cDNA identity was confirmed by DNA sequence analysis (Sanger et al., 1977).

Northern hybridization

Electrophoretic separation of total mRNA (20 μg per lane) of 3T3-F442A cells at different stages of differentiation was performed in a 1% agarose gel (#15510-019, GibcoBRL) under standard conditions (Davis et al., 1986). RNA was transferred to a nylon membrane (Biodyne Plus Membrane, PALL), and probed with the ^{32}P-labeled BiP fragment (random primer labeling kit, Promega). Prehybridization of the blot was carried out in a solution containing 1% SDS and 0.1 M NaCl for at least 30 minutes. The blot was further incubated for 24 hours at 42°C in hybridization solution HS 114F (Molecular Research Center, Inc.) containing denatured BIP probe at 2×10^6 dpm per ml. After the standard washing procedure, the blot was exposed 3.5 hours for autoradiography.

Isolation of mature adipocytes by collagenase-treatment

Epididymal fat pads were isolated from two 6 month old mice and transferred into a Petri dish. The following procedure has been described in detail elsewhere (Néchand et al., 1983) Blood vessels were removed, and fat was meticulously minced. 3 ml of DMEM containing 2 mg/ml of collagenase A from *Clostridium histolyticum* (Boehringer Mannheim, Germany) and 20 mg BSA/ml were added to the fat tissue in a plastic tube. The suspension was incubated at 37°C in a shaking water bath for 20 minutes. Subsequently, 20 ml of DMEM was added to the incubation mixture, and the suspension was filtered through a 100 μm Falcon® cell strainer (Laevosan, Vienna, Austria) to remove non-digested fat clots. The filtrate was centrifuged at 1,000

g in a table top centrifuge for 10 minutes at 20°C. The top-floating adipocyte fraction was collected, 10 ml of DMEM were added, and the centrifugation procedure was repeated two more times.

Antibodies

The following antibodies, as described in the text, were used: rabbit polyclonal anti-BiP antibody, directed against amino acids 624-636 (PIGEEDTSEKDEL) of the hamster protein (Ting et al., 1987) plus an additional N-terminal Cysteine (sequence identical in mouse); rabbit polyclonal anti-calnexin antibody, directed against amino acids 555-573 (AEEDEILNRSPRNRKPRRE) of the mature dog protein (Wada et al., 1991) plus an additional N-terminal cysteine (sequence identical in mouse). Both antibodies were kindly provided by Dr N. Erwin Ivessa, Department of Medical Biochemistry, University and Biocenter of Vienna, Austria. Rabbit anti-human fibronectin was obtained from DAKO (Glostrup, Denmark), goat-anti-rabbit-Cy2 was from Amersham/Pharmacia (Vienna, Austria).

RESULTS

Localization of FC sites within adipocytes

The typical size of differentiated 3T3-F442A adipocytes was between 35 and 45 μm. The size of TGD, as observed morphologically, varied widely depending on the differentiation status (Fig. 1, insert). We used filipin:FC fluorescence microscopy to localize unesterified cholesterol stores in adipocytes. Deconvolution software was applied to remove out-of focus fluorescence. Intense fluorescence staining was detectable, as expected, in the plasma membrane (PM) of fat cells (Fig. 1; white arrowheads). Remarkably, substantial filipin:FC fluorescence also decorated the periphery of TGD (Fig. 1; yellow arrowheads). This particular image was consistently observed in different cell depths and with various cell preparations. As demonstrated in Fig. 1, FC was not detectable in the core of TGD.

Association of FC with TGD

To examine the possibility that the areas of intense FC-fluorescence around TGD were directly associated with the lipid droplets, fat cells were lysed in hypotonic buffer, and TGD were separated by subcellular fractionation. After resuspending TGD in PBS, the resulting supernatant fractions were stable for several weeks at 4°C and exhibited physicochemical and ultrastructural features of a phospholipid:TG micro-emulsion (S. Prattes et al., unpublished). The lipid composition of these TGD was examined by thin layer chromatography (TLC). We observed that the TG:phospholipid ratio increased, as expected, during differentiation (data not shown). As determined with a commercially available FC quantitation kit, FC concentrations of whole fat cells also increased during differentiation (Fig. 2A) and were higher in adipocytes grown in FC-supplemented medium (Fig. 2A, lanes 1-3) compared to the cholesterol-depleted status (lanes 4-6). Upon separation of TGD from residual cell components of day-8 adipocytes by ultracentrifugation, up to one third of the total cellular FC content was detected in the TGD-fraction (Fig. 2A, lane 7), two thirds were in the ‘pellet’ fraction including the PM. The presence of remarkable amounts of FC in the TGD fraction upon ultracentrifugation suggested a tight association between FC and TGD, reminiscent of lipoprotein particles.

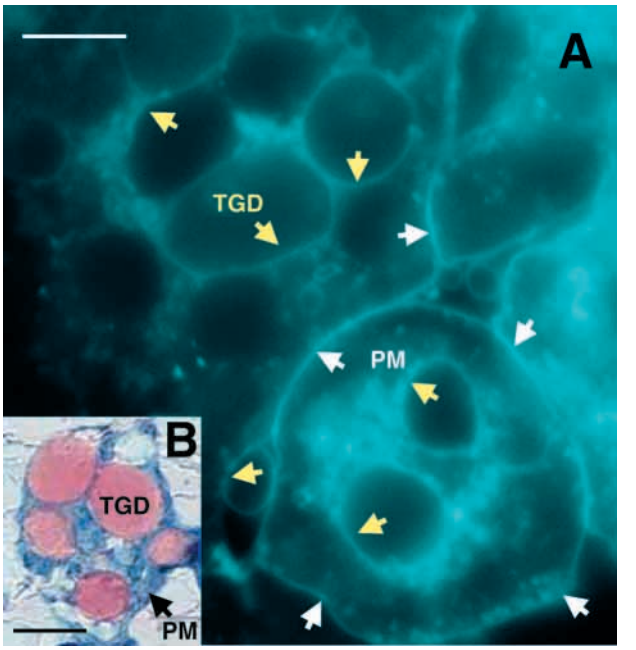

Fig. 1. Localization of intracellular FC stores. (A) Differentiated 3T3-F442A adipocytes (day 10 of differentiation) were fixed and incubated with filipin as indicated. The relative distribution of FC in the middle depth of differentiated adipocytes was recorded using deconvolution microsopy as indicated. Bar, 10 μm. (B) Differentiated adipocytes (day 10 of differentiation) were harvested, frozen in CryoGel®, and 5 μm slices were collected and mounted onto an Adhesively-coated slide® as described in Materials and Methods. Neutral lipid staining was performed with Oil Red-O, hemalum-staining was preformed to visualize cytosol (blue). Pictures were taken with an Axioplan 2 Microscope (Zeiss, Oberkochen, Germany), intrumental magnification was ×63. TGD, triglyceride droplet(s). White arrowheads, plasma membrane (PM); yellow arrowheads, TGD surface layer. N, nucleus. Bar, 20 μm.

In addition, the distribution of FC in isolated TGD using filipin:FC deconvolution fluorescence microscopy was demonstrated directly. Specific structures of various sizes with fluorescently highlighted areas at their periphery could be visualized in isolated TGD (Fig. 2B, Filipin). We also found that TGD particles of various sizes isolated from mouse fat biopsies (data not shown) exhibited a fluorescent gleam, indicative of the presence of FC. Thus FC abundance in the outer layer of TGD appears to be a general phenomenon within fat cells.

The TGD surface layer contains ER proteins

Milk lipid droplets secreted from mammary epithelial cells are functionally unique, yet compositionally related to TGD particles of adipocytes with regard to lipid composition (Dylewski et al., 1984). The former have been reported to contain reticuloplasmins in their outer surface layer (Ghosal et al., 1994). To our knowledge, the possibility that TGD of fat cells also might contain ER proteins in their outer shell had not been examined so far. To test this hypothesis, the presence of marker proteins in TGD derived from fat cells was probed immunochemically. TGD fractions isolated from day-10 adipocytes (Fig. 3A) and a corresponding top-floating fraction

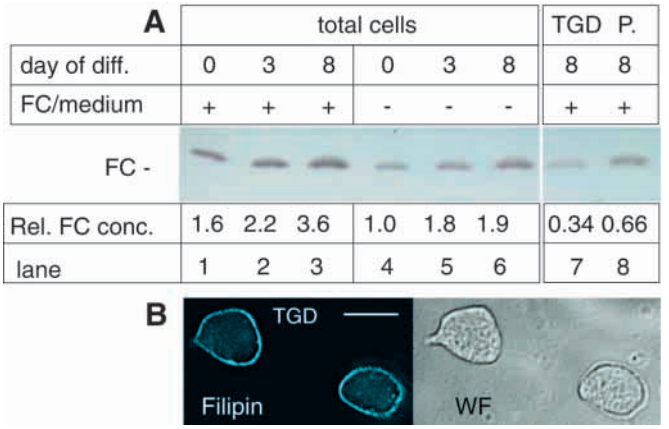

Fig. 2. Relative FC contents of (pre)adipocytes and TGD at various stages of differentiation. (Pre)adipocytes were cultured in the presence (+) or absence (–) of FC and harvested on the days indicated. (A) Extraction of lipids and thin layer chromatography (petrol ether: diethylether: acetic acid; 70:30:1, v/v/v) was performed as previously described (Zechner et al., 1991) using Silica gel-60 coated TLC aluminium sheets (Merck, Darmstadt, Germany). Lanes 1-6, lipid extracts (hexane:isopropanol; 3:2, v/v) from total cells. For the experiment of lanes 7 and 8, TGD and the cell pellet fraction (P.) were isolated by ultracentrifugation from day-8 adipocytes as described and then subjected to lipid extraction and thin layer chromatography as above. FC contents, normalized per well, were determined enzymatically and are expressed as relative concentration (Rel. FC conc.) (reference value, lane 4, corresponds to 29.6 μg FC/78 cm^2 dish). B. Purified TGD were transferred to microscope slides and incubated with filipin as described in Materials and Methods. Fluorescence signals were recorded as indicated in Fig. 1B. Instrumental magnification was ×40. WF, differential interphase contrast. Bar, 10 μm.

from preadipocytes, which do not contain TGD, (Fig. 3B) were carefully separated from other cellular organelles by subcellular fractionation and repeated washing steps. Proteins of these fractions were used for immunoblotting experiments with a specific anti-peptide antibody against BiP, a lumenal ER protein. This antibody recognizes the hamster, mouse and rat protein (N. E. Ivessa, personal communication). We detected in the TGD protein fraction of differentiated adipocytes a single protein band at approx. 78 kDa (Fig. 3A, TGD), consistent with the published molecular mass of BiP (GRP78) (Haas, 1994). As expected, the same protein of 78-kDa was visualized in the fat cell pellet fraction containing all other cell organelles, including also the microsome fraction (Fig. 3A, pellet). Importantly, the TGD fraction did not contain specific marker proteins of the PM such as fibronectin (Fig. 3A and B) or LDL receptor (not shown), an integral PM protein (Brown and Goldstein, 1997) in adipocytes (Kraemer et al., 1994). Fibronectin, which is tightly associated with β_1-integrin (Guilherme and Czech, 1998), was detected in the fat cell pellet fraction and in whole cell lysates (Fig. 3A, total), yet was absent from the TGD fraction. In control experiments, we tested for the presence of BiP in preadipocytes, morphologically characterized by the absence of microscopically visible lipid particles. There, BiP was absent from the top-floating fractions after subcellular fractionation (Fig. 3B, Top). These findings imply that BiP from differentiated adipocytes was present in the TGD fraction by virtue of its association with lipid particles.

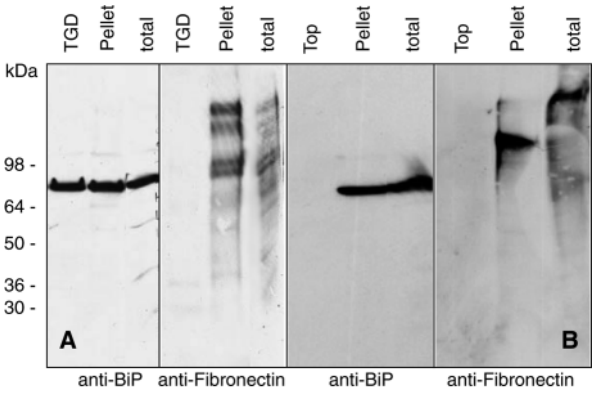

Fig. 3. Characterization of TGD particles. TGD were isolated by ultracentrifugation, and lipids were removed by extraction. Aliquots of the sucrose fractions were solubilized in electrophoresis loading buffer. Then proteins were subjected to SDS electrophoresis in 4.5-18% polyacrylamide gels and transferred onto nitrocellulose as described (Steyrer et al., 1990). Immunoblotting was performed as previously described (Steyrer et al., 1994). Samples for fractionation were obtained from day-10 adipocytes (A), or from preadipocytes (B). Dilution was 1: 2,000 for anti-BiP antiserum, and 1: 1,000 for anti-fibronectin antiserum. Bands were visualized using horseradish-peroxidase-labeled goat-anti-rabbit IgG and ECL detection. Molecular mass standards are indicated at left.

Increased expression of ER proteins during adipocyte differentiation

Hypothesizing that BiP is localized at the periphery of TGD, an increase of BiP expression during TGD growth is expectable. BiP expression was analyzed during adipocyte differentiation by western and northern blotting experiments (Fig. 4A). A gradual increase in the amount of the 78-kDa BiP protein between day 0 and day 10 was observed upon immunoblotting. A similar increase was also detectable when BiP mRNA levels were determined by northern blotting (Fig. 4B). BiP mRNA increased with the differentiated state of 3T3-F442A cells. Both the protein amount as well as the mRNA levels in the course of differentiation are indicative of a correlation between TGD growth and BiP expression.

Next, we asked whether *other* ER proteins such as calnexin might also be constituents of the TGD surface envelope. Using a specific antibody directed against calnexin (Fig. 4C) in immunoblotting experiments, the presence of this integral ER membrane protein could be demonstrated in isolated TGD of differentiated fat cells as well as in whole cells. In differentiated adipocytes (Fig. 4C, day 10), the concentration of calnexin (molecular mass of 90 kDa; Wada et al., 1991) was approximately twofold higher than that in the undifferentiated status (Fig. 4C, day 0 vs day 10). The 90-kDa species was not detected using nonimmune serum (Fig. 4C, N.) Taken together, these findings support the idea that TGD are enfolded by ER elements (Murphy and Vance, 1999). In addition, our data raise the possibility that ER proteins are constituents of the TGD, most likely of their surface layer, and our results imply a tight association of TGD with ER or ER-like elements.

BiP is present in the outer surface layer of TGD

The localization and distribution of BiP was investigated in isolated TGD by Laser scanning microscopy. BiP was detected

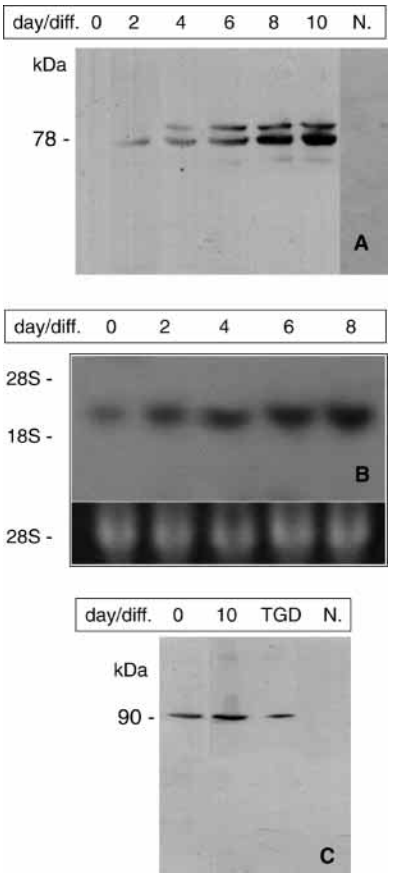

Fig. 4. Expression of ER proteins in adipocytes during differentiation. (A and C) (Pre)adipocytes were harvested at the indicated time points and lysed. Proteins (40 μg/lane) were electrophoretically separated in 4.5-18% polyacrylamide gels and electroblotted to nitrocellulose as indicated in Materials and Methods. The N.-lane contained proteins as in day 10, yet was incubated with rabbit nonimmune IgG. Visualization of bands was as in Fig. 3. (A) Immunoblotting using a rabbit anti-BiP antibody was performed as in Fig. 3. (B) Northern hybridization of total RNA (20 μg/lane) which was isolated at the indicated time points, using a ^{32}P-labeled 600 bp BiP-fragment as described in Materials and Methods. Exposure time was 3.5 hours. The position of 18S and 28S rRNA is indicated (top panel). Lower panel, section of the ethidium bromide-stained RNA-agarose gel (28S) to demonstrate identical RNA loading. (C) Immunoblotting using a rabbit anti-calnexin antibody antibody (dilution 1:2,000).

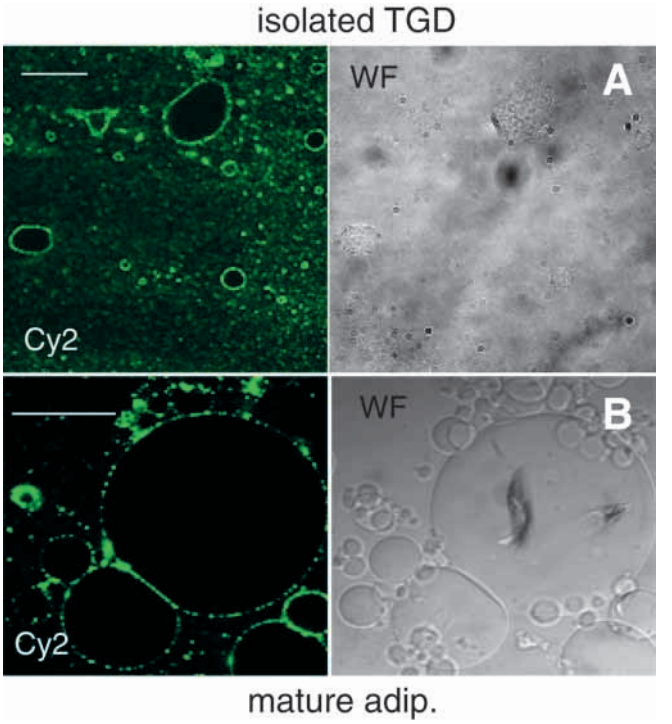

Fig. 5. Visualization of BiP at the periphery of TGD. TGD (A) or mature adipocytes from mouse adipose tissue (B) were isolated and transferred onto microscope cover slides as indicated in Materials and Methods. After permeabilization, slides were incubated with rabbit anti-BiP, followed by Cy2-labeled goat anti-rabbit IgG as indicated in Materials and Methods. Laser scanning microscopy was performed for fluorescence and differential interphase contrast with a simultaneous 2-channel detection. Excitation was at 488 nm, detection at 530 nm. Instrumental magnification was ×100. WF, differential interphase contrast. The scanning field dimension (xy) was 100 μm. Bars, 20 μm.

by immunofluorescence in differentiated 3T3-F442A adipocytes, mature adipocytes from fat biopsies, and in TGD fractions. The TGD preparations consistently contained particles of varying size, as observed previously (Fig. 5A, WF). Using a BiP-specific antibody, the periphery of many of these particles was characteristically decorated by a fluorescence signal, indicating the presence of BiP in their outer surface layer (Fig. 5A, Cy2). Very similar pictures were obtained using 3T3-L1 adipocytes (data not shown).

To exclude the possibility that the specific BiP pattern might reflect features of particular cell lines, adipose tissue samples from the epididymal fat pad of adult mice were analyzed. After collagenase treatment of adipose tissue to release adipocytes from the connective tissue, differentiated fat cells were isolated by flotation and immediately transferred to microscope cover slips. Laser scanning fluorescence microscopy revealed the presence of substantial amounts of BiP at the periphery of large and small triglyceride droplets (Fig. 5B, Cy2). This indicated that the specific BiP distribution pattern in fat cells is not restricted to cultured cell lines, but rather reflects a general structural element of TGD.

Efflux of FC from adipocytes

Next, we probed the possibility that TGD-associated FC could be mobilized upon extracellular stimulation of FC efflux. Adipocytes were preincubated with ^{3}H-FC at 37°C for 24 hours. Efflux media containing β-cyclodextrin (β-CD) (Christian et al., 1997) in the presence or absence of large unilamellar vesicles (LUV) were used to extract FC from adipocytes. Efflux experiments were conducted at 37°C and at 15°C. The lower temperature is known to diminish intracellular FC exchange processes (Kaplan and Simoni, 1985). After incubation for a maximum of 60 minutes, LDH release in cell medium revealed that less than 10% of the cells were necrotic (data not shown), consistent with earlier findings (Kilsdonk et al., 1995). As expected, β-CD in the presence of LUV at 37°C induced FC release most effectively

(Fig. 6A), and a 15 minute incubation period was sufficient to extract the majority of cellular ^{3}H-FC. The efflux potential of β-CD alone was only approx. 50% that of β-CD in the presence of LUV. LUV in the absence of β-CD, or DMEM alone, did not induce FC release. Importantly, FC efflux rates at 15°C were only 65% of the corresponding values obtained at 37°C under identical acceptor conditions. However, when prelabeled fat cells were kept at 15°C for 30 minutes, a subsequent switch of the incubation temperature to 37°C allowed an additional fraction of labeled FC to reach the cell medium via the PM in a time dependent manner. Maximum release of FC was observed upon further incubation of adipocytes for 30 minutes at 37°C (Fig. 6A, dotted line), and the amount of FC that could finally be extracted was comparable to the value obtained by incubation of cells for 60 minutes at 37°C. This indicated that despite preincubation at 15°C, almost identical amounts of total FC were eventually detectable in cell medium, most likely because TGD can make available their FC pool for release only at temperatures higher than 15°C.

Fig. 6B displays the residual ^{3}H-FC at two different temperatures in the TGD and the remaining denser cell compartments ('pellet'), respectively. This experimental set-up allowed us to determine the fate of the TGD-FC pool. Subcellular fractionation was used to separate TGD from the 'pellet' fraction before or after incubation of adipocytes with β-CD. A rapid loss of labeled FC from the 'pellet' pool contrasted with a minor decrease in TGD-FC at both temperatures in the initial phase of the experiment. After 10 minutes at 37°C (Fig. 6B, solid lines), the 'pellet' compartment had already lost 50% of its FC, whereas TGD had lost only 26%. However, when efflux proceeded further, the release characteristics of both FC-compartments changed, and after 60 minutes, the 'pellet' compartment only lost a further 10% of FC, while TGD released 35% of the initial label. As expected, at 15°C these effects were less pronounced, but showed the same trend. It is important to note that in a series of 3 experiments, the difference for TGD-FC efflux at 15°C vs 37°C was highly significant (P<0.005).

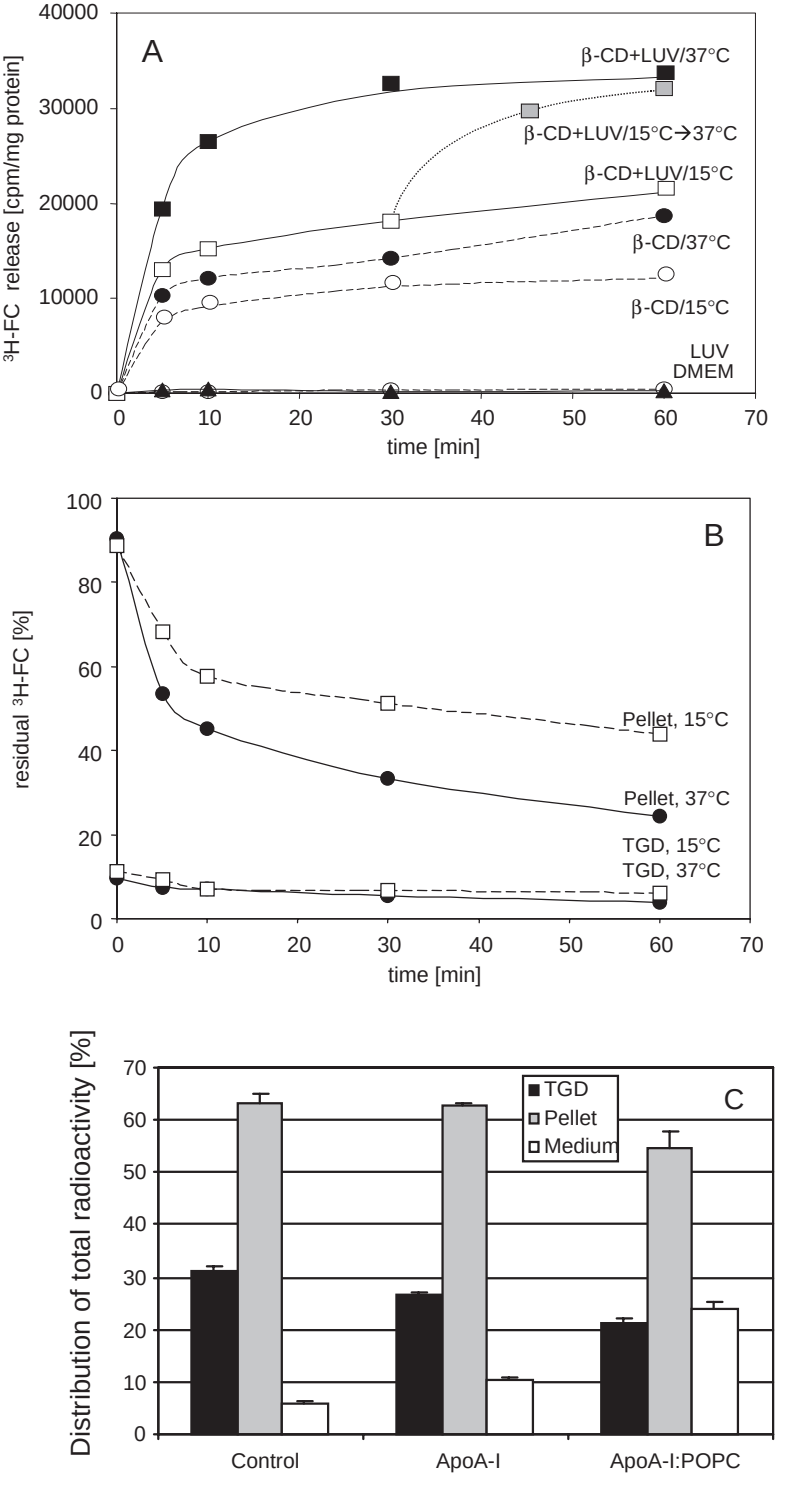

Fig. 6. Efflux of FC from adipocytes. Adipocytes (day 11 of differentiation) were prelabeled with ^{3}H-FC, FC-efflux experiments in the presence of β-CD (A and B) or apoA-I (C) as described in Materials and Methods. Mean values from duplicate dishes were expressed as cpm/mg protein (A and B). The experiments were repeated three times (A and B), or twice (C), with similar results. One representative experiment is shown. (A) Filled symbols, incubation at 37°C, open symbols, 15°C. Full lines, incubation in the presence of LUV; broken lines, absence of LUV. The dotted line indicates the temperature switch from 15°C to 37°C. (B) At time points indicated in the figure, adipocytes were lysed as described in Materials and Methods, and both the TGD- and the pellet-fraction were obtained by ultracentrifugation. Incubation conditions: filled symbols, incubation at 37°C, open symbols, 15°C. (C) FC efflux from adipocytes was performed in the presence of either purified human apoA-I, or apoA-I:POPC proteoliposomes (both at 100 μg/ml). Control cells were incubated in the absence of FC acceptors. The sum of radioactivity recovery in TGD, pellet, and medium after 5 hours represents 100%. The mean values for duplicate dishes were expressed as distribution of total radioactivity ± S.D.

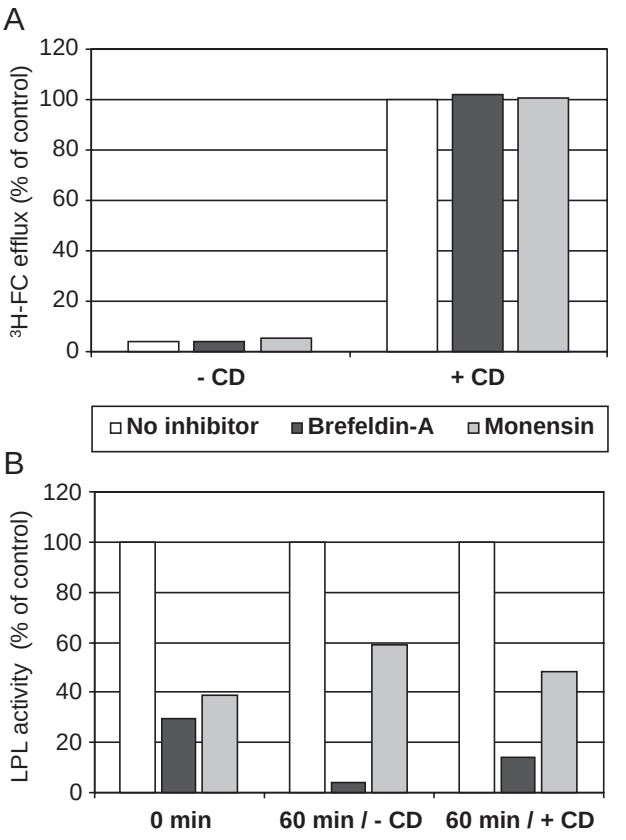

Fig. 7. Inhibition of protein secretion in adipocytes and cholesterol efflux. (A) F442A cells were differentiated and labeled with ^{3}H-FC as described in Materials and Methods. Incubation of adipocytes for 30 minutes in the absence (no inhibitor) or presence of brefeldin A (5 μg/ml) or monensin (20 μg/ml) was followed by the standard FC-efflux procedure performed for 60 minutes in absence or presence of inhibitors. The values of ^{3}H-FC release were compared to the condition lacking inhibitors in the presence of efflux agents and expressed as % of control. (B) Adipocytes were preincubated for 30 minutes in the presence of heparin (5 U/ml) and inhibitors as described above. Cells were washed twice with PBS and further incubated for 75 minutes. Media were collected, and LPL activity was determined (0 minutes). Again, medium was changed to efflux conditions in absence or presence of inhibitors. Heparin was present throughout the whole procedure. After 60 minutes, efflux media were collected. LPL activity was determined and compared to values obtained in the absence of inhibitors (expressed as % of control).

Apolipoprotein-mediated FC efflux

FC efflux from differentiated adipocytes was further characterized using either purified apoA-I or reconstituted apoA-I:POPC proteoliposomes. Compared to the control cells, a 13.7% reduction in TGD-associated FC was seen after 5 hours in the presence of free apoA-I, whereas only a 1.2% reduction in the FC content of dense cellular compartments ('pellet') was observed. In the presence of reconstituted apoA-I (apoA-I:POPC), the decrease in FC was 31.3% for TGD and 13.6% for the 'pellet' fraction (Fig. 6C). Additionally, the amount of ^{3}H-FC was determined in the cell medium after 5 hours of incubation. ApoA-I proteoliposomes induced the highest efflux (23.9% of the total ^{3}H-FC-label), while 10.6% of radioactivity was released into the medium in the presence of free apoA-I. Only 4.5% of ^{3}H-FC was detectable in the cell medium after 5 hours in the absence of cholesterol acceptors.

Effect of inhibition of intracellular vesicle transport on FC release

Among other possibilities, this putative FC exchange process between TGD and PM might be attributable to the constitutive secretory pathway of proteins. This was tested by FC efflux experiments upon inhibition of intracellular vesicle transport to the Golgi compartment from the ER, which has previously been observed in close vicinity to TGD using electron microscopy (Novikoff et al., 1980). After preincubation of adipocytes in the presence or absence of either brefeldin A or monensin (Dinter and Berger, 1998; Shiao and Vance, 1993), the activity of lipoprotein lipase, representative of protein secretion, was determined in the medium of adipocytes (Fig. 7B). Brefeldin A inhibited LPL secretion by more than 90% compared to the control experiment without brefeldin A. The effect of monensin was less pronounced, but still an inhibition of approx. 50% was observed. These results were not affected whether or not β-CD was present in the medium. In contrast to the results on protein secretion, no effect at all on FC efflux was found under identical experimental conditions (Fig. 7A). As expected, no FC was released when β-CD was absent from the incubation medium.

As a second possibility, we investigated the effect of inhibition of the cytosolic heat-shock protein-caveolin-chaperone complex-mediated FC transport route (Uittenbogaard et al., 1998) in adipocytes. Using rapamycin, which disrupts this FC transport complex, the FC efflux was unaffected (data not shown). In a third approach, we evaluated the role of microtubuli disruption on caveolin-mediated FC traffic from Golgi to the PM (Conrad et al., 1995). FC release from fat cells (data not shown) was also unaffected by nocodazole, which depolymerizes microtubuli and thus interferes with the constitutive cycling of caveolin from Golgi to PM.

Tracking intracellular FC sites upon β-CD induced FC efflux

In search of the actual efflux route of FC from TGD to the PM, FC release from adipocytes was induced by β-CD in the presence of LUV (Fig. 8). FC:filipin fluorescence microscopy was used to track residual FC sites within the cells at the indicated time points. As is apparent in Fig. 8, a significant shift of specific surface layer fluorescence of TGD in the form of a distinct punctuated staining was observed from the interior of fat cells towards regions proximal to the PM, which had already lost the majority of filipin staining (Fig. 8, 10 minutes). This effect was observed consistently and appeared to increase with duration of β-CD stimulation (Fig. 8, 30 minutes). After 60 minutes of incubation with β-CD in the presence of LUV, both the specific PM and TGD staining had largely disappeared. Additional highlighted areas most likely represented ER, the site of de novo FC biosynthesis. These data clearly demonstrated that a reduction of the FC content of the PM induced by extracellular acceptors stimulated an anterograde flux of FC from the major intracellular FC depot within adipocytes, the TGD/ER compartment, towards the PM.

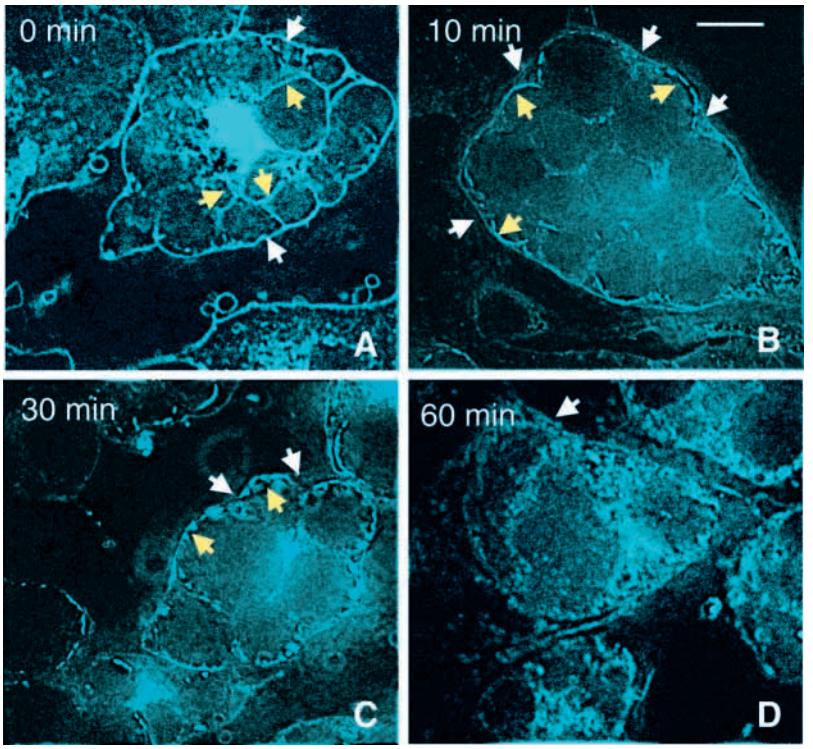

Fig. 8. Tracking intracellular FC sites upon β-CD induced FC efflux. FC release was triggered from differentiated fat cells using β-CD and LUV at 37°C for the indicated time points as described in Materials and Methods. (A) 0 minutes, control cells in the absence of β-CD/LUV. The relative distribution of FC in the middle depth of differentiated adipocytes was recorded using deconvolution microsopy as indicated. Instrumental magnification was ×40. Bar, 10 μm. White arrowheads, plasma membrane; yellow arrowheads, TGD membrane. Note the punctuated FC:filipin staining of TGD surface structures near the PM.

DISCUSSION

The identification and characterization of intracellular, metabolically active, FC sites in mammalian cells poses a biochemical challenge. Due to its presumed role as the body's primary depot for unesterified cholesterol (Krause and Hartman, 1984), adipose tissue has attracted much attention. It has been shown that the rise in triglyceride content within fat cells during their differentiation is paralleled by a substantial accumulation of FC (Zechner et al., 1991). At first glance, this appears physiologically inconceivable and seems to challenge the current cholesterol-homeostasis paradigm (Brown and Goldstein, 1986) which excludes the accumulation of toxic amounts of FC (Tangirala et al., 1993; Tabas, 1997). To date, the existence of distinct intracellular FC pools in adipocytes has not been conclusively demonstrated. The central goal of our work, therefore, was the attempt to visualize and characterize this putative intracellular 'FC-depot' within fat cells and to study its mobilization.

In a direct experimental approach, which to our knowledge has not been applied to adipocytes so far, we used filipin-fluorescence microscopy to localize these FC stores. Filipin, a fluorescent polyene antibiotic, has previously been used to localize sites of FC abundance in peripheral cells and macrophages (e.g. Blanchette-Mackie et al., 1988; Tabas et al., 1994; Gu et al., 1997; Watari et al., 1999). We observed in adipocytes considerable filipin:FC fluorescence of the PM and at the periphery of, but not inside, the TGD. Our observation, however, substantiates the proposal that FC is associated with TGD (Kraemer et al., 1994).

To exclude the possibility of procedural artifacts, we pursued additional independent biochemical evidence for TGD-FC association. To this end, TGD from adipocytes at different stages of differentiation were isolated by subcellular fractionation. Using TLC for qualitative analysis and an enzymatic FC quantitation method, the presence of FC could be demonstrated in TGD fractions. In addition, filipin fluorescence microscopy revealed the presence of FC at the periphery of purified TGD. These data suggested that the rise in total FC content of adipocytes upon differentiation observed previously (Zechner et al., 1991) was most likely attributable to an increase in TGD-associated FC. We showed that this compartment harbours up to one third of adipocytes' unesterified cholesterol. This finding is remarkable, because intracellular compartments or organelles are generally poor in FC compared to the PM which contains approx. 90% of total unesterified cholesterol of peripheral cells (Gu et al., 1997; Lange et al., 1989). Our results indicating such massive accumulation of unesterified cholesterol in a distinct subcellular compartment has, to our knowledge, no precedent in any other peripheral cell.

Having clearly localized the primary sites of FC abundance in adipocytes, we further characterized the protein envelope of TGD. Vimentin (Lieber and Evans, 1996; Franke et al., 1987), perilipin (Blanchette-Mackie et al., 1995), ADRP/adipophilin (Ye and Serrero, 1998) and lipotransin (Syu and Saltiel, 1999) have been identified as structural components of adipocyte TGD. In a functionally and histologically completely unrelated cell type, the milk-producing mammary epithelial cells (Valivullah et al., 1988), BiP was previously shown to be associated with intracellular milk lipid droplets of various sizes (Ghosal et al., 1994). BiP is a major peptide-binding chaperone which has been shown to function in immunoglobulin folding and transport (Haas, 1994). Here, we demonstrated for the first time that BiP is also a component of the adipocyte TGD surface layer. Not only BiP, the expression of which increases during the differentiation process, but also calnexin, an integral ER membrane protein, could be identified in purified TGD. These

findings raise the possibility that constituents of the ER (Murphy and Vance, 1999), where triglyceride synthesis takes place (Cases et al., 1998), may generally contribute to the biogenesis of TGD. The nature of their structural and/or functional role remains undefined. It is of importance to note that BiP (Nakai et al., 1995) may undergo phosphorylation like perilipin (Clifford et al., 1998), or lipotransin, recently characterized as another 'cage protein' of TGD and as a docking protein for hormone sensitive lipase (Syu and Saltiel, 1999), respectively.

The presence of TGD in adipocytes is strongly dependent on the nutritional status and energy homeostasis of an individual. During fasting, fat mobilization and triglyceride catabolism are essential to maintain the availability of energy subtrates. The association of marked amounts of FC in TGD raises the possibility of FC comobilization under certain metabolic conditions (Bennion and Grundy, 1975; Swaner and Connor, 1975). The questions of whether or not, and to what extent, this TGD FC-pool of fat cells might be mobilizable with extracellular acceptors is intriguing. Addressing these questions should provide insights regarding the putative 'crosstalk' between adipocyte FC-pools with the serum FC-pool and its pivotal role in atherosclerosis.

To investigate this possibility, β-CD methodology was applied to release FC from adipocytes. β-CD, in combination with LUV, induces FC efflux rates from cells far in excess of those resulting from physiological acceptors like high density lipoproteins (HDL) (Christian et al., 1997; Jonas et al., 1994; Yokoyama, 1998). This rapid removal of FC from adipocytes minimized the possibility for de novo FC biosynthesis within the observed time period, and FC release was recorded in a time- and temperature-dependent way. At 15°C, intracellular vesicle transport does not take place (Liscum and Munn, 1999), while FC de novo biosynthesis can still occur to a minor degree (Liscum and Munn, 1999). Under these experimental conditions, two thirds of cellular FC was released from fat cells within 30 minutes compared to the value obtained at 37°C. The fact that an additional mobilizable pool of FC representing approximately 30% of totally releasable FC could be detected upon shifting the incubation temperature from 15°C to 37°C can be interpreted in two ways. First, the donor behaviour of the PM and/or the characteristics of the acceptor differ with changing incubation temperatures. Second, the intracellular TGD-FC pool is more readily mobilized at 37°C than at 15°C. While we can not rule out the first proposal, we provided experimental evidence for the latter explanation. A relatively higher, significant decrease in the amount of TGD-associated FC was demonstrated at 37°C compared to 15°C. This finding can not be attributed to differences in donor characteristics of the PM and/or acceptor behaviour of β-CD and favours the proposal of an energy-dependent intracellular FC trafficking process.

To prevent misinterpretation of the above result, the absolute labeling state of TGD has to be considered. Upon prelabeling of adipocytes with ^{3}H-FC, a maximum of 13% of total ^{3}H-FC was TGD associated. The most likely explanation for this phenomenon is that the majority of extracellularly supplemented ^{3}H-FC is readily integrated into the PM. Redistribution may not result in subcellular labeling that reflects the physiological state. Accordingly, this parameter has to be considered in proportional terms with respect to interpretation of absolute FC flows. Therefore, the minor, yet highly significant, effect in TGD-FC decrease in fact represents an approximately threefold larger absolute change in TGD-FC concentration.

β-CD is a very potent, yet non-physiological acceptor of FC. In vivo, high density lipoproteins (HDL) serve as primary vehicle for reverse cholesterol transport (Yokoyama, 1998) aimed at shuttling excess of free cholesterol from peripheral tissues back to the liver (Glomset, 1968). Since apoA-I is the most abundant protein component of HDL, purified apoA-I as well as lipid-reconstituted apoA-I was used for efflux experiments. Generally, many water-soluble apolipoproteins with multiple amphiphilic helix motifs, in phospholipid-reconstituted as well as in their free form, have been found to induce FC efflux from a variety of cells (Yokoyama, 1998). It is believed that the mechanisms for FC removal differ between lipid-free (poor) apoA-I and intact (lipidated) HDL (Rothblat et al., 1999). The former directly interacts with the plasma membrane, removes FC and serves as FC shuttle from the PM to larger lipoproteins (e.g. HDL). The latter stimulates FC efflux via a scavenger receptor-B-I (SR-BI)-mediated mechanism. Our data demonstrate that both apoA-I in free and reconstituted form can mediate FC efflux from fat cells. The fact that the relative reduction in TGD-associated FC was higher than that in denser cellular compartments including the PM is in line with the view that the PM serves as an intermediate acceptor of TGD-derived FC on its way from intracellular TGD-depots to extracellular acceptors.

FC acceptors gain access to the cell only at the PM (Christian et al., 1997). However, these compounds were also able to mediate the release of FC from intracellular stores, which implied an exchange process between the FC pools of the PM and TGD/ER. First, we investigated whether or not the constitutive secretory pathway (Gaynor et al., 1998) might be involved in this process. Incubation of adipocytes in the presence of brefeldin A or monensin had no effect at all on FC release, whereas protein secretion was substantially inhibited. This finding is consistent with previous reports (e.g. Shiao and Vance, 1993; Mendez and Uint, 1996), which demonstrated in other cells that FC or sphingomyelin trafficking from ER to the PM might bypass the Golgi apparatus. Additional experiments revealed that a role of the recently described cytosolic heat-shock protein-caveolin-chaperone complex (Uittenbogaard et al., 1998; Smart et al., 1996) can be disregarded in adipocytes under these experimental conditions. This putative complex is involved in trafficking of newly synthesized FC from the ER to the PM. In our experiments, rapamycin which disrupts the transport complex (Uittenbogaard et al., 1998), had no effect on FC efflux. We have also evaluated the possibility that FC trafficking en route to the PM might occur in a microtubule-dependent pathway. Conrad and colleagues (1995) had demonstrated previously that nocodazole, a drug known to disrupt microtubuli, is able to prevent the constitutive cycling of caveolin from Golgi to PM. Again, FC release was virtually unaffected by nocodazole. This result suggested a microtubuli-independent efflux process of FC from fat cells.

While we were not able to demonstrate any inhibitory effect of several drugs on FC trafficking along distinct intracellular routes (e.g. ER to Golgi, Golgi to PM, or cytosolic FC transport), tracking the residual TGD-FC by filipin:FC fluorescence microscopy upon β-CD stimulation provided a

clue. These observations favored the view that FC might be shutteled from TGD/ER to the PM by unidirectional lateral diffusion along a FC concentration gradient to TGD surface structures which are in tight contact with the PM. There, FC could easily be incorporated into the PM bilayer, from where a further release from the fat cell is feasible.

Previously it had been shown that lipids like long-chain fatty acids and monoacylglycerol can reversibly flow by lateral movement in an interfacial membrane continuum. This transport process describes the flux of lipids from the outer leaflet of the plasma membrane of capillary endothelial cells to intracellular membranes of adipocytes, where the TGD surface and/or mitochondrial membranes had been clearly identified in biochemical and histological studies as acceptor compartments (reviewed by Scow and Blanchette-Mackie, 1991). Although this interfacial membrane continuum flux has not yet been demonstrated for free cholesterol, such a mechanism might represent a general lipid transport phenomenon.

A functionally similar, yet distinct process had been proposed by Kruth and collegues (1999) in macrophages. This laboratory provided evidence that a labyrinth of membrane-bound compartments, connected to the cell surface (so-called surface-connected compartments, SCC) contributes to the uptake of microcrystalline FC and aggregated low density lipoproteins. Accordingly, it is feasible that this process might shuttle FC – although in the opposite direction but also along a concentration gradient – from TGD surface layers to the PM. A related process of vectorial lipid transport via contact sites of donor (ER) and acceptor (mitochondria) organelles has recently been proposed (Daum and Vance, 1997). It remains to be determined whether this process of FC exchange in adipocytes might occur also in vivo and whether sphingolipid-FC rafts play a role (Simons and Ikonen, 1997; Pralle et al., 2000).

In summary, we have provided experimental evidence for a model in which the surface layer of adipocyte TGD contains, besides well-established components like vimentin, perilipin, ADRP/adipophilin, or lipotransin, additional constituents such as ER-resident proteins and unesterified cholesterol. With regard to FC, perhaps the most straightforward interpretation of our results is that FC represents a structural component of the TGD surface layer. This hypothesis could explain the discrepancy between reports which point towards the existence of ‘intracellular pools’ of FC within fat cells (Krause and Hartman, 1984), and the well-established paradigm (Brown and Goldstein, 1986) which theoretically excludes this possibility. The organization of triglyceride stores with a PC monolayer containing FC at the interphase between the hydrophobic droplet and the cytosol is, from a structural point of view, feasible and is reminiscent of the supramolecular lipid assembly of plasma lipoproteins. One important iss_ue related to our study but not addressed herein refers to the question of whether or not FC is an *essential* structural component of TGD. Experiments currently performed in our laboratory are focussing on this point.

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