Immunolocalization of Acyl-Coenzyme A:Cholesterol O-Acyltransferase in Macrophages

(Received for publication, November 20, 1997, and in revised form, January 29, 1998)

Nadia Khelef§§, Xavier Buton¶, Nanda Beating, Hongxing Wang, Vardiella Meiner**‡‡, Ta-Yuan Chang§§, Robert V. Farese, Jr.**, Frederick R. Maxfield‡, and Ira Tabas†††

From the §Department of Biochemistry, Cornell University Medical School, New York, New York 10021, the §Institut Pasteur, 75724 Paris Cedex 15, France, the Departments of Medicine and *Anatomy and Cell Biology, Columbia University, New York, New York 10032, the ‡Department of Medicine, Mt. Sinai School of Medicine, New York, New York 10029, the **Gladstone Foundation for Cardiovascular Research and University of California, San Francisco, California 94141, the †††Department of Genetics, Hadassah University Hospital, Jerusalem 91120, Israel, and the §§Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755

Macrophages in atherosclerotic lesions accumulate large amounts of cholesteryl-fatty acyl esters ("foam cell" formation) through the intracellular esterification of cholesterol by acyl-coenzyme A:cholesterol O-acyltransferase (ACAT). In this study, we sought to determine the subcellular localization of ACAT in macrophages. Using mouse peritoneal macrophages and immunofluorescence microscopy, we found that a major portion of ACAT was in a dense reticular cytoplasmic network and in the nuclear membrane that colocalized with the luminal endoplasmic reticulum marker protein-disulfide isomerase (PDI) and that was in a similar distribution as the membrane-bound endoplasmic reticulum marker ribophorin. Remarkably, another portion of the macrophage ACAT pattern did not overlap with PDI or ribophorin, but was found in as yet unidentified cytoplasmic structures that were juxtaposed to the nucleus. Compartments containing labeled β-very low density lipoprotein, an atherogenic lipoprotein, did not overlap with the ACAT label, but rather were embedded in the dense reticular network of ACAT. Furthermore, cell-surface biotinylation experiments revealed that freshly harvested, non-attached macrophages, but not those attached to tissue culture dishes, contained ∼10–15% of ACAT on the cell surface. In summary, ACAT was found in several sites in macrophages: a cytoplasmic reticular/nuclear membrane site that overlaps with PDI and ribophorin and has the characteristics of the endoplasmic reticulum, a perinuclear cytoplasmic site that does not overlap with PDI or ribophorin and may be another cytoplasmic structure or possibly a unique subcompartment of the endoplasmic reticulum, and a cell-surface site in non-attached macrophages. Understanding possible physiological differences of ACAT in these locations may reveal an important component of ACAT regulation and macrophage foam cell formation.

* This work was supported by NHLBI Grant HL-57560 from the National Institutes of Health (to I. T. and F. R. M.) and by Grant-in-Aid 95-239 from the American Heart Association (to R. V. F.). The Columbia University Confocal Microscope Facility used for this study was established by NIH Shared Instrument Grant #S10 RR10506 and is supported by NIH Grant #5 P30 CA13696 as part of the Herbert Irving Cancer Center at Columbia University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. || To whom correspondence and reprint requests should be addressed: Dept. of Medicine, Columbia University, 630 West 168th St., New York, NY 10032. Tel.: 212-305-9430; Fax: 212-305-5052; E-mail: iat1@columbia.edu.

Macrophages enter atherosclerotic lesions at an early stage and are found at all stages of lesion development thereafter (1). Several studies have provided evidence that macrophages play an important role in both lesion initiation and late lesion complications (2, 3). A major property of lesion macrophages is their tendency to become massively loaded with cholesteryl ester, a process known as foam cell formation because the numerous cholesteryl ester droplets give the cells a foamy appearance (1). The pathway by which macrophages accumulate cholesteryl ester is complex and not completely understood. Data from several laboratories suggest that macrophages internalize cholesterol through the uptake of certain "atherogenic" lipoproteins or by direct uptake of lipoprotein cholesterol. When the cellular cholesterol content increases to a certain threshold level, mixed pools of lipoprotein-derived and cellular cholesterol gain access to an integral membrane enzyme called ACAT, which then catalyzes the esterification of the cholesterol to fatty acid via a fatty acyl-CoA intermediate.

Given the importance of macrophage foam cells in atherosclerosis, there is much interest in how the cholesterol esterification pathway is regulated. Cholesterol esterification by ACAT increases manyfold when macrophages are incubated with atherogenic lipoproteins, yet ACAT mRNA and protein do not increase under these conditions (5–7). Rather, the major regulatory mechanism appears to involve access of membrane-bound ACAT to its hydrophobic substrate cholesterol (4, 7). When access to cholesterol is provided, ACAT activity increases by an allosteric mechanism as well as by substrate availability (4, 7). Therefore, understanding how intracellular pools of cholesterol contact ACAT is an important goal in elucidating the regulation of the cholesterol esterification pathway and foam cell formation.

To understand how ACAT gains access to cholesterol, it is necessary to determine the cellular location of ACAT. Studies have shown that plasma membrane cholesterol is a major source of substrate for ACAT (8, 9) and that plasma membrane vesiculation, perhaps as a means of transporting cholesterol to ACAT, is necessary for the stimulation of the cholesterol esterification pathway by atherogenic lipoproteins (10, 11). Biochemical studies employing subcellular fractionation of rat liver concluded that ACAT is located in the rough ER (12), and Chang et al. (13) published an image of melanoma cells in

1 The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol O-acyltransferase; ER, endoplasmic reticulum; PDI, protein-disulfide isomerase; β-VLDL, β-very low density lipoprotein; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay.
which the immunofluorescence pattern obtained with an anti-ACAT antibody appeared similar to the ER-like component of the pattern obtained with DioC6, which stains mitochondria and ER. Given the inherent flaws of subcellular fractionation studies and the importance of the ACAT pathway in macrophages, we undertook a detailed examination of ACAT localization in macrophages. Herein, we report that much of ACAT in macrophages is in a dense reticular cytoplasmic network and in the nuclear membrane that colocalized with the luminal endoplasmic reticulum marker PDI and that was in a similar distribution as the membrane-bound endoplasmic reticulum marker ribophorin. A noticeable portion of ACAT in macrophages, however, resides in a perinuclear cytoplasmic site that does not overlap with PDI or ribophorin. Furthermore, using a cell-surface biotinylation protocol, we found that a portion of ACAT was on the cell surface of freshly harvested, non-attached peritoneal macrophages. These findings may have implications regarding the regulation of the cholesterol esterification pathway and macrophage foam cell formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Falcon and Corning tissue culture plasticware was purchased from Fisher. Tissue culture media and reagents and calf serum were obtained from Life Technologies, Inc., and fetal bovine serum was purchased from Gemini Bioproducts (Calabasas, CA). Mouse IgG2a and anti-mouse CD12/CD32 Fc receptor antibody were from Chemicon International, Inc. (Temecula, CA) and Pharmingen (San Diego, CA), respectively. The anti-ACAT antiserum used in our immunofluorescence studies was made by injecting rabbits with a fusion protein consisting of the amino-terminal 120 residues of murine ACAT and glutathione S-transferase (14). For the immunoblot experiments displayed in Fig. 5, we used an antisera that was raised in rabbits against a synthetic peptide corresponding to the 40 C-terminal amino acids of human ACAT (cf. Ref. 15). Chicken anti-PDI serum was a generous gift from Dr. Ron Raines (Department of Biochemistry, University of Wisconsin, Madison, WI), and rabbit anti-ribophorin serum was kindly provided by Dr. Gert Kreibich (Department of Cell Biology, New York University Medical School, New York, NY). Rhodamine-conjugated anti-rabbit and anti-chicken IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and Oregon green-conjugated anti-rabbit IgG was from Molecular Probes, Inc. (Eugene, OR). β-VLDL was prepared from the sera of rabbits fed a high-cholesterol diet (2% cholesterol and 10% soy bean oil) as described previously (16) and conjugated to Texas Red (Molecular Probes, Inc.) according to the manufacturer’s instructions. All other reagents were purchased from Sigma.

**Cells**—Resident macrophages were harvested from the peritonea of 25–30-g female ICR mice, C57BL/6J/129 hybrid mice from Jackson Laboratories (Bar Harbor, ME), or ACAT knockout (ACAT−/−) mice in the same genetic background (17) as described previously (18). Macrophages were isolated by peritoneal lavage using Hanks’ balanced salt solution (HBSS) and resuspended in RPMI 1640 medium (Welgene, Inc., Danbury, CT) in PBS containing 0.5 mg/ml NHS-S-S-biotin (Pierce) for 40 min at 4 °C with gentle rotation, centrifuged at 1700 rpm for 5 min, and resuspended in PBS. The cells were pelleted and then resuspended in 50 ml ammonium acetate in PBS and incubated for 10 min at 4 °C. After washing twice with PBS, the cells were resuspended in RIPA buffer, 1% Triton X-100, 20 mM Tris, 150 mM NaCl, and 5 mM EDTA, pH 8 containing 0.2% bovine serum albumin and protease inhibitors. After incubation for 30 min, the lysate was centrifuged (13,000 × g for 10 min). The supernatant was precleared with unconjugated agaroose (CL-6B-200, Sigma), which had been prewashed three times with RIPA buffer, 2 h with rotating. The agarose was pelleted (13,000 × g for 10 s), and the supernatant was added to immuno pure immobilized streptavidin (Pierce), which had been prewashed with RIPA buffer, and incubated with gentle rotation for 18 h. The precipitates were then centrifuged at 13,000 × g for 10 s; the supernatants were removed; and the agarose was resuspended in RIPA buffer. This washing step was repeated twice with RIPA buffer, twice with RIPA buffer containing 500 mM NaCl, and three times with PBS. The agarose was resuspended with an appropriate volume of buffer containing 1% SDS and 10 mM dithiothreitol, heated to 37 °C for 30 min, centrifuged at 13,000 × g for 45 s, and subjected to immunoblot analysis.

*Immunofluorescence of Adherent Macrophages*—Adherent peritoneal macrophages from wild-type (A and B) or ACAT−/− (C and D) mice in the same genetic background were fixed, permeabilized, and incubated with 1:250 rabbit preimmune serum (A and C) or anti-ACAT N-terminal serum (B and D). Rhodamine-conjugated anti-rabbit IgG was used to detect the presence of anti-ACAT antibodies. Images were obtained by confocal microscopy at a succession of 12 focal planes (0.8-μm step size) using a Zeiss LSM 410 confocal microscope and a 100× objective. The intensity value corresponding to the background fluorescence (outside the cell) was subtracted from each image, and pictures are presented as a summation projection of these corrected images.

For colocalization studies involving β-VLDL, macrophages were treated with 5 μg/ml Texas Red-conjugated β-VLDL in Dulbecco’s modified Eagle’s medium and 0.2% bovine serum albumin for 10 min at 37 °C, fixed and permeabilized with cold methanol (−20 °C) on ice, and labeled with the rabbit anti-ACAT serum. The cells were observed with a Zeiss LSM 410 confocal microscope using an argon-krypton laser (488 and 568 nm excitation) and a 63× objective (NA 1.4) or with a Bio-Rad MRC 600 microscope using an argon laser (514 nm excitation) and a 63× objective (NA 1.4). The images were processed with Metamorph (Universal Imaging Corp.) and Photoshop (Adobe) software.

**Cell-surface Biotinylation Experiments**—We utilized a modification of the procedure of Rodriguez-Boulan and co-workers (20). Resident peritoneal macrophages were freshly harvested from 14 female ICR mice (see above) and washed once with PBS by low speed centrifugation. One-half of the cells were incubated with 0.5 mg/ml NHS-SS-biotin (Pierce) for 40 min at 4 °C with gentle rotation, centrifuged at 1700 rpm for 5 min, and resuspended in PBS. The cells were pelleted and then resuspended in 50 ml ammonium acetate in PBS and incubated for 10 min at 4 °C. After washing twice with PBS, the cells were resuspended in RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 20 mM Tris, 150 mM NaCl, and 5 mM EDTA, pH 8) containing 0.2% bovine serum albumin and protease inhibitors. After incubation for 30 min, the lysate was centrifuged (13,000 × g for 10 min). The supernatant was precleared with unconjugated agaroose (CL-6B-200, Sigma), which had been prewashed three times with RIPA buffer, 2 h with rotating. The agarose was pelleted (13,000 × g for 10 s), and the supernatant was added to immuno pure immobilized streptavidin (Pierce), which had been prewashed with RIPA buffer, and incubated with gentle rotation for 18 h. The precipitates were then centrifuged at 13,000 × g for 10 s; the supernatants were removed; and the agarose was resuspended in RIPA buffer. This washing step was repeated twice with RIPA buffer, twice with RIPA buffer containing 500 mM NaCl, and three times with PBS. The agarose was resuspended with an appropriate volume of buffer containing 1% SDS and 10 mM dithiothreitol, heated to 37 °C for 30 min, centrifuged at 13,000 × g for 45 s, and subjected to immunoblot analysis.
FIG. 2. Immunolocalization of ACAT, PDI, and ribophorin in mouse peritoneal macrophages. A–I, macrophages were fixed and permeabilized with cold methanol and then incubated with 1:200 rabbit anti-ACAT serum followed by 1:100 chicken anti-PDI antibody as described under “Experimental Procedures.” Oregon green-conjugated anti-rabbit IgG and rhodamine-conjugated anti-chicken IgG were used to detect the presence of the anti-ACAT (A, D, and G) and anti-PDI (B, E, and H) antibodies, respectively. J and K, macrophages were fixed and permeabilized with cold methanol and then incubated with 1:200 rabbit anti-ribophorin serum followed by 1:200 rhodamine-conjugated anti-rabbit IgG. All
network throughout the cell (Fig. 1B). In contrast, the staining of these cells with preimmune serum generated a very dim and diffuse fluorescent signal (Fig. 1A). The data for ACAT/−/− macrophages are shown in Fig. 1 (C (preimmune) and D (immune)). In both cases, the staining was dim and diffuse, similar to the preimmune staining of wild-type macrophages. These results show that the anti-ACAT serum is specific for the native ACAT protein and can be used in immunofluorescence experiments to determine the localization of ACAT.

The pattern of ACAT staining in Fig. 1B was consistent with the cellular distribution of an ER-associated protein. To further examine this point, we compared the localization of ACAT with that of PDI, a protein known to reside in the lumen of almost all of the subcompartments of the ER (22, 23). Fig. 2 (A–I) shows single confocal slices of macrophages double-labeled with anti-ACAT (green) and anti-PDI (red) antibodies. In a minority of cells, the two markers colocalized perfectly; there was a complete overlap of the nuclear envelope and the reticular network patterns (Fig. 2, A–C). In most of the cells, however, the comparison between the ACAT and PDI distributions was more complex. Although many structures labeled with the anti-ACAT (Fig. 2, D and G) and anti-PDI (Fig. 2, E and H) antibodies, including the nuclear envelope and part of the reticular network, were identical, there were regions of non-overlap. In particular, there was prominent ACAT labeling near the nucleus that did not overlap with the PDI distribution (Fig. 2, F and I, arrows). Similar data were obtained using the J774 murine macrophage cell line (data not shown). Macrophages immunofluorescently stained for ribophorin, a membrane-bound ER marker (24), are also shown (Fig. 2, J and K). The distribution of ribophorin looked similar to that of PDI, namely, a cytoplasmic reticular pattern without evidence of “nuclear” staining.

Although the non-overlapping ACAT staining sometimes appeared to overlap the nucleus in single optical sections, analysis of stacks of optical sections showed that the ACAT-positive structures were in cytoplasmic protrusions that sometimes projected into the nucleus of macrophages (Fig. 3, arrows in A and C show one cell in which such structures can be observed). Therefore, while much of the ACAT pattern in adherent macrophages is similar to that of PDI and ribophorin, consistent with localization in the ER, a noticeable portion of ACAT resides in a perinuclear cytoplasmic site that does not overlap with PDI or ribophorin. Whether this site represents another cytoplasmic organelle or possibly a unique specialized subcompartment of the ER remains to be determined (see “Discussion”).

Localization of ACAT and β-VLDL in Macrophages—In previous work, we showed that a portion of the atherogenic lipoprotein β-VLDL resides in deep, wide cell-surface invaginations, called STEMs (surface tubules for entry into macrophages), when incubated with macrophages for 5–20 min (25). Another foam cell-inducing lipoprotein, acetyl low density lipoprotein, also demonstrates prolonged association with the surface of macrophages (26, 27). Localization of β-VLDL in STEMs and possibly acetyl low density lipoproteins in their cell-surface sites may be important for the ability of these lipoproteins to stimulate the cholesterol esterification pathway in macrophages (28). To determine if β-VLDL-containing structures in macrophages are in close proximity to ACAT, we performed double-label fluorescence microscopy. Macrophages were incubated with Texas Red-conjugated β-VLDL for 10 min.
before fixing the cells for the immunolocalization of ACAT. We found no overlap of the ACAT label (Fig. 4, A and D) with the β-VLDL label (Fig. 4, B and E), which appeared to be contained in discrete structures (Fig. 4, C and F); in fact, the β-VLDL-containing structures often appeared as holes in the ACAT labeling (G, arrows). Nonetheless, labeled β-VLDL particles were often found in close proximity to the reticular structures containing ACAT (Fig. 4, C and F). Thus, while there was no specific concentration of ACAT in or around the β-VLDL structure, the dense network of the ACAT-containing ER surrounds β-VLDL-containing STEMs and endosomes.

Evidence That a Portion of ACAT is on the Cell Surface in Freshly Harvested, Non-attached Macrophages—Plasma membrane cholesterol is a major source of substrate for ACAT (8, 9), and foam cell-inducing lipoproteins demonstrate prolonged association with the surface of macrophages (see above). Therefore, we sought to determine whether a portion of ACAT might be localized on the cell surface. Initial immunofluorescence studies in which the antibodies were added to non-fixed and non-permeabilized macrophages at 4 °C were difficult to interpret due to a high level of nonspecific staining (i.e. staining found in ACAT−/− cells). To rectify this problem, we conducted a series of cell-surface biotinylation/immunoblot experiments (cf. Ref. 20). In this protocol, intact cells are incubated with a biotinylation reagent at 4 °C to derivatize cell-surface, but not intracellular, proteins. After the biotinylation reagent is quenched, the cells are then solubilized with detergent, precipitated with immobilized streptavidin, and subjected to immunoblot analysis. Most important, the immunoblot analysis includes antibodies against known intracellular proteins to prove that the biotinylation reagent did not have access to the intracellular compartment. As a means of comparison, we also performed immunoblot analysis of biotinylated membranes from a total cell homogenate; these membranes were biotinylated to control for any differences in immunoreactivity due to antigen derivatization by biotin. For some biotinylation experiments, we used the ACAT N-terminal antibody employed in our immunofluorescence experiments (see above), although we found that biotinylation of ACAT caused a decrease in immunoreactivity with this antibody. Another antibody that was raised against a C-terminal peptide of ACAT, however, reacted equally well with underivatized and biotinylated ACAT (data not shown), and so we used this antiserum for the experiments described below.

We initially conducted these experiments using macrophages plated on cell culture dishes. There was no cell-surface biotinylation of ACAT; a positive control, the cell-surface protein β1-integrin, was amply biotinylated (data not shown). Similar results were obtained when adherent macrophages were examined 1 h after plating or when they were plated on Milli-cell-CM 0.4-μm culture plate inserts placed in 35-mm wells, followed by addition of the biotinylation reagent to both the

![Fig. 4. Localization of ACAT and β-VLDL in macrophages.](image-url) Peritoneal macrophages were incubated for 10 min with Texas Red-conjugated β-VLDL, fixed and permeabilized with cold methanol, and incubated with rabbit anti-ACAT serum followed by Oregon green-conjugated anti-rabbit IgG. Oregon green fluorescence (ACAT) is shown in A and D, and Texas Red (β-VLDL) fluorescence is shown in B and E. C and F show color overlays of ACAT (green) and Texas Red-conjugated β-VLDL (red). G–I (insets in A–C) show a 3-fold magnification of the intracellular area depicted by the box in C; the arrows in G and H point out β-VLDL-containing structures that excluded the ACAT label. Images were acquired simultaneously by confocal microscopy as a single 1-μm-thick optical section using a Zeiss LSM 410 confocal microscope and a 100× objective. The intensity value corresponding to the background fluorescence (outside the cells) was subtracted from each image. The intensity corresponding to the Oregon green fluorescence leaking into the Texas Red channel (crossover) was mathematically determined and subtracted from the Texas Red image.
The ACAT pathway in macrophages plays a key role in foam cell formation during atherogenesis, and previous work from our laboratories and others has suggested that determining the intracellular location of ACAT in macrophages may provide important clues to its regulation. Herein, we show that much of ACAT is in a dense, widespread reticular network throughout the cytoplasm that overlaps with the luminal ER marker PDI and that has a pattern similar to that of the membrane ER marker ribophorin. In addition, our studies have demonstrated two surprising observations. First, a portion of ACAT in most of the cells we observed was present in a perinuclear cytoplasmic site that does not overlap with PDI or ribophorin. Second, 10–15% of ACAT in non-attached macrophages was present on the cell surface.

The finding that much of ACAT is in the ER of macrophages allows us to focus our hypotheses on how atherogenic lipoproteins stimulate the cholesterol esterification pathway. ACAT esterifies mostly plasma membrane-derived cholesterol after cellular cholesterol reaches a threshold level (30), and vesicular transport is important in this process (10, 11). Taken together, these observations suggest a model in which plasma membrane-derived vesicles “percolate” through the dense network of the cholesterol-poor, ACAT-containing ER and, if the cholesterol content of these vesicles is high enough (i.e. above threshold), cholesterol is transferred to ACAT down a concentration gradient.

The nature of the ACAT-containing perinuclear site that is distinct from PDI and ribophorin remains to be identified. Although this site could be a specialization of the ER, almost all subcompartments of the ER, including ACAT-rich mitochondrion-associated membranes (31), contain PDI (23). Therefore, it is possible that the site is one of several non-ER organelles that concentrate near the nucleus, such as the Golgi apparatus, the endocytic recycling compartment, late endosomes, or lysosomes. As mentioned above, most of the cholesterol substrate for ACAT is derived from the plasma membrane; recent data, however, indicate that a smaller portion may be delivered directly from lysosomes or late endosomes following the endocytosis of lipoproteins (32, 33). It is therefore possible that perinuclear ACAT, which is in close proximity to perinuclear lysosomes, may be a subpopulation of the enzyme that esterifies lysosomal free cholesterol. This idea and other possible functions of ACAT in the perinuclear site will be further evaluated once this compartment is definitively identified.

The finding that a portion of cellular ACAT is on the cell surface of non-attached macrophages, which may be consistent with the findings of Green et al. (34) using Xenopus oocytes transfected with ACAT cDNA, raises two important questions: what is the mechanism whereby cell-surface ACAT disappears upon macrophage attachment, and what is the functional importance of ACAT in this site? The disappearance of cell-surface ACAT upon attachment is not due to redistribution of the protein to the basal surface of the cell (see “Results”). One possibility is that one or more signaling pathways known to be activated by cellular attachment to the matrix (e.g. Ref. 35) lead to the internalization of cell-surface ACAT. In terms of function, it will be important to determine if ACAT stimulation by a variety of stimuli (cf. Refs. 10 and 36) differs between attached and non-attached macrophages. Another critical issue related to our finding is whether the active site of ACAT faces the intracellular or extracellular space; studies in rat liver led to the conclusion that the active site of microsomal ACAT faces the cytoplasm (37). Although the proportion of cell-surface ACAT is only 10–15% in non-attached macrophages, ACAT in this site may be functionally important since most of the cholesterol esterified by ACAT is derived from plasma membrane pools (see above), and thus, cell-surface ACAT may provide a

---

**Immunolocalization of ACAT in Macrophages**

**Fig. 5.** Immunoblots of biotinylated total cellular membrane proteins and cell-surface proteins from freshly harvested, non-attached peritoneal macrophages. Biotinylated total membrane proteins (TM) and cell-surface proteins were subjected to immunoblot analysis using antibodies against a C-terminal peptide of ACAT (~50 kDa) (A), ribophorin (~60 kDa) (B), or β1-integrin (~115 kDa) (C) as described under “Experimental Procedures.” 2.5x, 5x, and 10x refer to 2.5-, 5-, and 10-fold the number of cells used to prepare the cell-surface proteins relative to that used for the total membrane proteins (see “Results”).

**Upper and lower chambers** to make sure there was access to the adherent lower surface (see Ref. 20). We then conducted this protocol on freshly harvested macrophages in suspension. Fig. 5A shows the immunoblots using the anti-ACAT antibody. The lane labeled TM refers to biotinylated total membranes from the cell homogenates (i.e. total cellular ACAT) derived from a given number of cells; the -fold notations under the three cell-surface lanes refer to cell-surface material derived from 2.5-, 5-, and 10-fold the number of cells that were used for the total membrane lane, respectively. In contrast to our finding with attached cells, there was an amount of cell-surface ACAT in the freshly harvested, non-attached macrophages equivalent to between one-fifth and one-tenth of total cellular ACAT. In five repeat experiments, we found that cell-surface ACAT averaged between 10 and 15% of total cellular ACAT, and similar results were found when we used the N-terminal antibody with the J774 macrophage cell line (data not shown). No band was seen in either total membranes or cell-surface material when freshly harvested macrophages from ACAT−/− mice were subjected to this protocol (data not shown). As mentioned above, a critical control for these experiments is to determine access of the biotinylation reagent to the intracellular compartment. As shown in Fig. 5B, the rough ER membrane-bound protein ribophorin (24) shows a very faint cell-surface signal at 10-fold in comparison with a very bright total membrane signal. Densitometry revealed <1% biotinylation of ribophorin, and similar data were obtained with PDI (data not shown). Fig. 5C shows data for β1-integrin, a protein known to be heavily concentrated on the cell surface (29); as expected, ~50% of β1-integrin is on the surface of macrophages. In summary, ~10–15% of ACAT in freshly harvested, non-attached macrophages is exposed to the extracellular space.

**DISCUSSION**

**The ACAT pathway in macrophages plays** a key role in foam cell formation during atherogenesis, and previous work from our laboratories and others has suggested that determining the
direct route for cholesteryl ester synthesis under certain conditions.

Several studies from our (16, 25, 26) and other (27) laboratories have shown that foam cell-inducing lipoproteins undergo prolonged contact with the surface of macrophages, an event that may be important in the stimulation of the cholesterol esterification pathway (28). In this report, we examined the relationship in location between one such lipoprotein, β-VLDL, and ACAT. We know from previous work that a 10-min incubation of mouse peritoneal macrophages with β-VLDL results in much of the β-VLDL being contained in deep, wide cell-surface invaginations called STEMs (25). Herein, we show that “reticular” ACAT (see above) is not contained in these β-VLDL-containing structures, but rather seems to “wrap” around them. From these data, it is tempting to speculate that β-VLDL-derived free cholesterol may be transferred from these structures to nearby ACAT, analogous to and perhaps complementary with the plasma membrane-derived vesicle hypothesis mentioned above. Future fluorescence studies will be directed at determining the trafficking of cholesterol derived from β-VLDL and other atherogenic lipoproteins to ACAT in the cellular sites described in this work.

Acknowledgments—We thank Dr. Ron Raines for the anti-PDI antibody and Dr. Gert Kreibich for the anti-ribophorin antiserum.

REFERENCES

1. Ross, R. (1995) Annu. Rev. Physiol. 57, 791–804
2. Smith, J. D., Trogan, E., Ginosberg, M., Grigioni, C., Tian, J., and Miyata, M. (1995) Proc. Natl. Acad. Sci. USA 92, 8284–8288
3. Libby, P., and Clinton, S. K. (1993) Curr. Opin. Lipidol. 4, 355–363
4. Tabas, I. (1995) Curr. Opin. Lipidol. 6, 260–268
5. Wang, H., Germain, S. J., Benfield, P. P., and Gillies, P. J. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 809–814
6. Tabas, I., and Boykow, G. C. (1987) J. Biol. Chem. 262, 12175–12181
7. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) Curr. Opin. Lipidol. 8, 613–638
8. Tabas, I., Rosoff, W. J., and Boykow, G. C. (1988) J. Biol. Chem. 263, 1266–1272
9. Lange, Y., Strebel, F., and Steck, T. L. (1993) J. Biol. Chem. 268, 13838–13843
10. Skiba, P. J., Zha, X., Maxfield, F. R., Schissel, S. L., and Tabas, I. (1996) J. Biol. Chem. 271, 13392–13400
11. Lange, Y., Ye, J., and Strebel, F. (1995) J. Lipid Res. 36, 1092–1097
12. Balarasver, S., Kaukesi, S., Mitropoulos, K. A., and Peters, T. J. (1978) Biochem. J. 174, 863–872
13. Chang, C. C. Y., Chen, J., Thomas, M. A., Cheng, D., Del Priore, V. A., Newton, R. S., Pape, M. E., and Chang, T. (1995) J. Biol. Chem. 270, 29532–29540
14. Meiner, V., Cases, S., Myers, H. M., Sande, E. R., Bellosta, S., Schambelan, M., Pitas, R. E., McGuire, J., Herz, J., and Farese, R. V., Jr. (1996) Proc. Natl. Acad. Sci. USA 93, 14041–14046
15. Chang, C. C. Y., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) J. Biol. Chem. 268, 20747–20755
16. Tabas, I., Lim, S., Xu, X., and Maxfield, F. R. (1990) J. Cell Biol. 111, 929–940
17. Meiner, V., Cases, S., Myers, H. M., Sande, E. R., Bellisota, S., Schambelan, M., Pitas, R. E., McGuire, J., Herz, J., and Farese, R. V., Jr. (1996) Proc. Natl. Acad. Sci. USA 93, 14041–14046
18. Tabas, I., Boykow, G. C., and Tall, A. R. (1987) J. Clin. Invest. 79, 415–426
19. Saltzman, N. H., and Maxfield, F. R. (1989) J. Cell Biol. 109, 2057–2104
20. Sargiacomo, M., Lisanti, M., Grawe, L., Le Bivic, A., and Rodriguez-Boulan, E. (1989) J. Membr. Biol. 107, 277–286
21. Marathe, S., Schissel, S. L., Yellin, M. J., Beattini, N., Mintzer, R., Williams, K. J., and Tabas, I. (1988) J. Biol. Chem. 263, 4081–4088
22. Naiva, R., and Lennarz, W. J. (1992) J. Biol. Chem. 267, 3553–3556
23. Sita, R., and Meldolesi, J. (1992) Mol. Biol. Cell 3, 1007–1072
24. Kreibich, G., Czako-Graham, M., Grebenu, R., Mok, W., Rodriguez-Boulan, E., and Sahatschin, D. D. (1987) J. Supramol. Struct. 8, 279–302
25. Myers, N., Tabas, I., Jones, N. L., and Maxfield, F. R. (1995) J. Cell Biol. 123, 1389–1402
26. Zha, X., Tabas, I., Leopold, P. L., Jones, N. L., and Maxfield, F. R. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 20747–20755
27. Kruth, H. S., Skarlatos, S. I., Lilly, K., Chang, J., and Irim, I. (1995) J. Cell Biol. 129, 133–145
28. Tabas, I., Myers, J. N., Innerarity, T. L., Xu, X., Arnold, K., Boyles, J., and Maxfield, F. R. (1996) J. Biol. Chem. 271, 1928–1933
29. Hynes, R. O. (1987) Cell 48, 549–554
30. Xu, X. Y., and Tabas, I. (1991) J. Biol. Chem. 266, 17040–17048
31. Rusinol, E. E., Cui, Z., Chen, M., and Vace, E. J. (1994) J. Biol. Chem. 269, 27494–27502
32. Neufeld, E. B., Cooney, A. M., Pitas, R. E., McGuire, J., Herz, J., and Farese, R. V., Jr. (1996) J. Biol. Chem. 271, 4266–4274
33. Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) J. Biol. Chem. 271, 21604–21613
34. Underwood, K. W., Jacobs, N. L., Howley, A., and Liscum, L. (1998) J. Biol. Chem. 273, 4266–4274
35. Green, S., Steinberg, D., and Quesenbenger, O. (1996) Biochem. Biophys. Res. Commun. 218, 924–929
36. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Dev. Biol. 11, 549–599
37. Schissel, S. L., Beattini, N., Zha, X., Maxfield, F. R., and Tabas, I. (1995) Biochemistry 34, 10463–10473
38. Lichtenstein, A. H., and Brecher, P. (1980) J. Biol. Chem. 255, 9098–9104