Intracellular Transport of Low Density Lipoprotein Derived Free Cholesterol Begins at Clathrin-Coated Pits and Terminates at Cell Surface Caveolae†

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Received June 5, 1996; Revised Manuscript Received September 12, 1996⊗

ABSTRACT: Free cholesterol (FC) is selectively internalized from low-density lipoprotein (LDL) by confluent fibroblast monolayers (Fielding & Fielding (1995) *Biochemistry 34*, 14237−14244). The kinetics of transport of LDL-derived ^{3}H-FC within the cell were studied by density-gradient ultracentrifugal fractionation and in terms of the effects of inhibitors of endocytosis and intracellular transport. By these criteria, the initial uptake of LDL-FC was mediated by the cell-surface clathrin-coated pits. FC label then appeared in clathrin-coated dense vesicles. Uncoating of clathrin from these vesicles led to the appearance of label in a light density fraction and, subsequently, in an intermediate density fraction coincident with protein markers of the trans-Golgi network in these cells. ^{3}H-FC was finally transported to the plasma membrane via a temperature-sensitive, probably microtubule-dependent pathway. These data are consistent with a role for the trans-Golgi network as an intermediate compartment in intracellular FC transport. They provide further evidence of a role for cell-surface caveolae in FC efflux.

The plasma membrane of mammalian cells is continuously recycled at rates that may exceed 50% of total cell surface h^{-1} (Steinman et al., 1976). Lipids and proteins, invaginated from the cell surface, first appear as membrane-limited primary endosomes. Some of the endosomal contents, such as transferrin and its receptor protein, are returned rapidly to the cell surface. Others remain permanently within the cell, or follow more complex recycling pathways that may include fusion with lysosomes or elements of the Golgi apparatus. While intermediates in these pathways are still incompletely identified, it is recognized that this traffic includes several kinds of vesicles (Rothman & Wieland, 1996). Among the best characterized are the clathrin-coated vesicles formed as a result of endocytosis from cell-surface coated pits (Pearse & Robinson, 1990). Proteins internalized by this mechanism include transferrin, epidermal growth factor, LDL,[1] and their high-affinity receptors. Once internalized, clathrin dissociates from the vesicles under the influence of a specific ATP-binding protein (heat shock cognate protein-70) to generate uncoated vesicles which mediate transport to different cell compartments.

Caveolae, clathrin-free cell-surface invaginations, represent a second class of plasma membrane microdomains implicated in transmembrane transport. Most caveolae are coated with caveolin, a 21 kDa lipid-binding protein.[2] Recycling of caveolin between the cell surface caveolae and the trans-Golgi network has been described (Dupree et al., 1993; Conrad et al., 1995). Data recently presented suggest that caveolae facilitate the exocytosis of FC (Fielding & Fielding, 1995b). This concept is consistent with the finding that caveolae contain relatively high levels of proteins implicated in transport to the cell surface (Schnitzer et al., 1995). Finally, some solutes are internalized by bulk- or fluid-phase endocytosis, probably through unspecialized membrane domains (Cupers et al., 1994).

Sorting of the contents of endocytic vesicles has been best characterized for proteins entering the cell via clathrin-coated pits. This occurs in a compartment of uncoupling of receptors and ligands (Pearse & Robinson, 1990). There is less information on the trafficking of endosomal lipids. Fluorescent analogs of natural sphingolipids were efficiently incorporated into intracellular vesicles which were rapidly returned to the cell surface (Martin & Pagano, 1994). Confluent fibroblasts selectively internalize FC from LDL by an LDL-receptor-independent, *N*-ethylmaleimide-sensitive pathway (Fielding & Fielding, 1995a). This FC later copurified in a plasma membrane fraction which contained caveolin (Fielding & Fielding, 1995b) and was selectively released into the medium by high-density lipoprotein (HDL) but not by LDL.

Intermediates of the intracellular transport of FC selectively derived from LDL have now been studied using selective transport inhibitors and ultracentrifugal density gradient fractionation.

EXPERIMENTAL METHODS

Preparation of ^{3}H-FC-Labeled LDL. Plasma was obtained from the blood of normal donors who had fasted overnight. LDL was isolated from plasma by affinity chromatography on heparin−agarose (Pharmacia-LKB, Piscataway, NJ) as previously described (Fielding & Fielding, 1986). LDL-FC was labeled to a final specific activity of $(2-6) \times 10^4$ cpm μg^{-1} by incubation (60 min, 37 °C) with agarose−human serum albumin covalent complex labeled with 1,2-[^{3}H]-FC

† Supported by the National Institutes of Health through Arteriosclerosis SCOR HL 14237.

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; FC, free (unesterified) cholesterol; CE, cholesteryl ester; TGN, trans-Golgi network.

[2] In this research, caveolae refers to the clathrin-free surface invaginations shown in intact cells by electron microscopy to be rich in FC, sphingolipids, and GPI-anchored proteins. Caveolar FC is uniquely accessible to cholesterol oxidase in unfixed cells. Caveolin, a lipid-binding protein, has been shown to be associated with caveolae in several cell lines, including fibroblasts, and in caveolar membrane fractions purified from these cells.

S0006-2960(96)01338-4 CCC: $12.00

(45−56 Ci mmol^{-1}; NEN, Boston, MA) (Miida et al., 1990). FC mass was measured fluorimetrically with cholesterol oxidase (Heider & Boyett, 1978). Phospholipid mass was measured colorimetrically as inorganic phosphate (Emmelot et al., 1964)

Cell Culture. Normal and LDL-receptor-deficient fibroblasts (GM2000 line, American Type Culture Collection, Rockville, MD) were grown to near confluence in plastic dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. The cells were transferred to media containing human plasma or LDL for individual experiments described below.

Uptake of FC from LDL. This assay measures the rate of selective internalization of ^{3}H-FC from LDL (Fielding & Fielding, 1995a). Cell monolayers were washed with Dulbecco's phosphate-buffered saline pH 7.4 (PBS) ($CaCl_2$, 0.1 g L^{-1}; KCl and KH_2PO_4, both 0.2 g L^{-1}; NaCl, 8 g L^{-1}; Na_2HPO_4, 1.15 g L^{-1}; $NaH_2PO_4{\cdot}7H_2O$). They were then incubated at the temperature indicated for 1−15 min with ^{3}H-FC-labeled LDL (50−200 μg mL^{-1} FC). Unbound labeled LDL was removed by washing with PBS, and adsorbed intact ^{3}H-LDL was then displaced with excess unlabeled LDL (60 min, 0−2 °C). The dishes were washed with PBS-recrystallized human serum albumin (5 mg mL^{-1}) and four times with PBS alone. The cells were solubilized with liquid scintillation cocktail (3a70b, RPI, Mount Prospect, IL), and the radioactivity was measured by liquid scintillation spectrometry.

In some experiments, the cells had been preincubated (60 min) with metabolic inhibitors affecting different steps of intracellular transport. ^{3}H-FC labeled LDL was then added, together with inhibitor. The experiment was then completed as described above. Cytochalasin D, monensin, brefeldin A, nocozadole, taxol, and vinblastine were purchased from CalBiochem, San Diego, CA. Trifluoperazine and *N*-ethylmaleimide were from Sigma Chemical Co., St Louis, MO. Bafilomycin A1 was obtained from Wako Chemical, Richmond, VA. These reagents were dissolved as a stock solution in dimethyl sulfoxide (DMSO) prior to dilution (>500-fold) in PBS or DME−0.01 M Hepes buffer (pH 7.4). There was no effect of carrier alone on the uptake of ^{3}H-FC label by the cells.

Transfer of FC between Intracellular Compartments. Early endosomal vesicles were labeled with LDL-derived ^{3}H-FC by a modification of procedures described by Woodman and Warren (1991). Fibroblast monolayers in 10 cm dishes were washed in ice-cold PBS. The cells were labeled by incubation (2 h, 4 °C) with ^{3}H-FC LDL. The monolayers were washed in PBS and brought to 31 °C for 0−15 min. The cells were then quickly chilled on ice, cold unlabeled LDL was added (15 min, 4 °C) to displace any remaining surface-bound labeled LDL, and all subsequent steps were carried out at 0−4 °C.

Cells from three 10 cm dishes were used for each gradient. Monolayers were washed with 140 mM sucrose, 0.5 mM $MgCl_2$, 1 mM EGTA, 20 mM 2-morpholinoethanesulfonic acid (MES), and 70 mM potassium acetate, pH 6.6 ("vesicle buffer") (Woodman & Warren, 1991). The cells were scraped from the dishes. Dithiothreitol (1 mM) and protease inhibitors (PMSF, 200 μg mL^{-1}; benzamidine, 0.5 mM; soybean trypsin inhibitor, 10 μg mL^{-1}; leupeptin, 1 μg mL^{-1}) were added. The cells were broken with a Dounce homogenizer (15 strokes), and the homogenate was centrifuged at 500*g* for 5 min. Ribonuclease A (50 μg mL^{-1}) was added. After 30 min, a second centrifugation was carried out (7000*g*, 30 min). Supernatant (∼2 mL) was layered on a 10 mL continuous gradient of 2% Ficoll−9% D_2O to 20% Ficoll−90% D_2O in vesicle buffer containing 1 mM dithiothreitol. Centrifugation in a Beckman SW41 rotor was carried out at 80000*g* for 16 h. Fractions (∼0.6 mL) were collected dropwise, and the distribution of FC label was determined. Because of slight variation in fraction size between gradients, data were normalized to 20 fractions for comparison between experiments. Solution density was determined gravimetrically using 100 μL portions of each fraction. The density of a given fraction was reproducible ±0.5% between experiments.

The identity of intracellular transport intermediates and the extent of any cross-contamination between fractions were established using specific antibodies to protein markers, labeled ligands of receptor proteins, and assays of enzyme proteins. Monoclonal antibody to human clathrin heavy chain was obtained from ICN Pharmaceuticals, Costa Mesa, CA. Anti-human caveolin polyclonal antibody was purchased from Transduction Laboratories, Lexington, KY. Antibody to mannose 6-phosphate receptor protein was the gift of Dr. Suzanne Pfeffer, Stanford University. For antibody assays, portions of gradient fractions were mixed with 0.1 mL of recrystallized human serum albumin (2 mg mL^{-1} in PBS) and brought to 1 mL with PBS. Protein was precipitated with trichloroacetic acid (final concentration 10% w/v). Following centrifugation (5000*g*, 15 min) the pellets were washed with 70% aqueous ethanol and dissolved in 20 μL of SDS gel sample buffer. After 12% SDS−polyacrylamide electrophoresis, proteins were transferred to nitrocellulose (0.2 μm pore size, S & S, Keene, NH). Following incubation with individual primary antibodies, blots were incubated with a second antibody (anti- mouse or rabbit IgG, Transduction Laboratories) conjugated with horse radish peroxidase and then visualized with Super-Signal CL-HRP substrate (Pierce, Rockford, IL). The distribution of antigen between different gradient fractions was determined with a computerized scanner (ImageQuant, Molecular Devices, Sunnyvale, CA).

Human transferrin (Sigma) was ^{125}I-labeled with chloramine T (Markwell, 1982). Labeled protein was incubated with the cell monolayers, lysate was prepared, and density gradient fractionation was carried out, as described for labeled LDL, except that unincorporated cell-surface transferrin was removed with desferroxamine (Woodman & Warren, 1991) rather than cold LDL.

Enzyme assays were carried out directly on samples of gradient fractions. Alkaline phosphatase was assayed by spectrophotometry at 420 nm after incubation with *p*-nitrophenyl phosphate (CalBiochem-BRL) according to the supplier's protocol. 5′-Nucleotidase was assayed as the rate of production of inorganic phosphate from 5′-AMP (Emmelot et al., 1964).

Transfer of Intracellular FC to the Cell Surface. This assay utilizes the finding (Smart et al., 1994; Fielding & Fielding, 1995b) that only FC in the caveolar fraction of the plasma membrane fraction is modified by cholesterol oxidase in unfixed fibroblast monolayers. The rate at which intracellular LDL-derived ^{3}H-FC became accessible at the cell surface was measured as follows. Cell monolayers were labeled with ^{3}H-FC LDL under the conditions shown for each experiment. Unbound and adsorbed LDL were removed as described above. In some experiments metabolic inhibitors

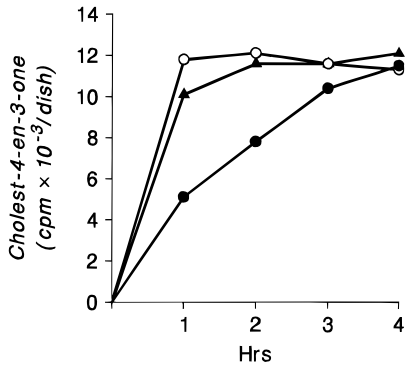

FIGURE 1: Temperature-dependence of the activity of cholesterol oxidase with cell-surface FC in unfixed fibroblast monolayers. The cells were prelabeled to equilibrium with ^{3}H-FC as described under Experimental Methods. The dishes were washed to remove soluble labeled LDL. Adsorbed ^{3}H-FC LDL was displaced with unlabeled LDL. The dishes were then washed again with PBS−albumin and PBS and incubated for the time indicated with cholesterol oxidase (final concentration 1 unit mL^{-1}). Closed circles: 4 °C. Closed triangles: 23 C°. Open circles: 31 °C. In each case the level of oxidized FC label was determined as ^{3}H-cholest-4-en-3-one following thin-layer chromatography as described under Experimental Methods.

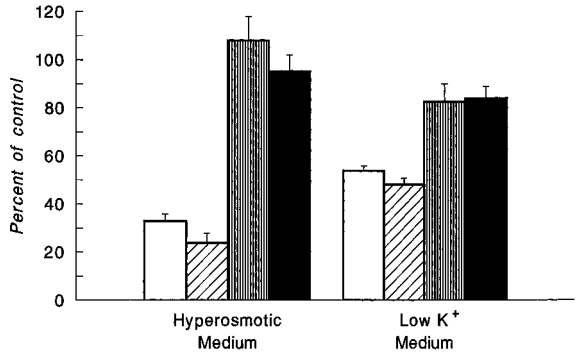

FIGURE 2: Uptake of ^{3}H-LDL-FC, [^{125}I]transferrin, [^{125}I]cholera toxin, or [^{125}I]peroxidase by fibroblast monolayers incubated in hyperosmotic or K^+-free media. Monolayers were preincubated (60 min, 37 °C) with PBS or with hyperosmotic or K^+-free medium. Incubation was then carried out with ^{3}H-labeled LDL or ^{125}I-labeled transferrin or cholera toxin in the same media for 30 min. Incubation with [^{125}I]peroxidase was for 5 min to minimize regurgitation of label. All rates were linear with time over the period of incubation. Cell-associated label in hyperosmotic or K^+-free media is expressed relative to the rate of uptake measured in PBS. Left to right for each panel: open bars, ^{3}H-FC LDL; diagonal bars, [^{125}I]transferrin; vertical bars, [^{125}Iperoxidase; black bars, [^{125}Icholera toxin.

of different transport steps were then added for 30 min at 15 °C. In each case, at the end of the experiment, the cells were washed with PBS containing recrystallized human serum albumin (5 mg mL^{-1}, pH 7.4) and then PBS. Cholesterol oxidase (Boehringer-Manheim, Indianapolis, IN) was added in PBS to a final concentration of 1 unit mL^{-1}. Incubation was for 4 h at 0 °C. The cells were washed with ice-cold PBS and extracted with 0.1 N NaOH. Cell total lipid was extracted with chloroform−methanol (1:1 v/v). Thin-layer chromatography of portions of $CHCl_3$ phase was carried out on silica gel layers (Whatman PE Sil G, Fisher Scientific, Pittsburg, PA) developed in petroleum ether−diethyl ether−acetic acid, 80:20:1 v/v. The yield of labeled cholest-4-en-3-one after 4 h at 0 °C did not differ significantly from that obtained after 1 h at 37 °C (Figure 1), conditions shown earlier to completely oxidize FC in the caveolar membrane fraction (Smart et al., 1994).

RESULTS

Transfer of ^{3}H-FC from LDL: Effects of Inhibitors. Horseradish peroxidase enters human fibroblasts exclusively by fluid-phase endocytosis (Steinman et al., 1976). Transferrin is internalized selectively via the clathrin-coated pits (Pearse & Robinson, 1990) while cholera toxin binds to ganglioside GM1 localized to the caveolae (Montesano et al., 1982).

Internalization via clathrin-coated pits is selectively reduced by PBS made hyperosmotic by inclusion of 350 mM in place of 150 mM NaCl (Larkin et al., 1983; Hansen et al., 1993; Cupers et al., 1994). The uptake of ^{3}H-FC from LDL was significantly inhibited under these conditions (Figure 2). A similar inhibition was observed with ^{125}I-labeled transferrin. There was no significant reduction in the uptake of labeled peroxidase or cholera toxin by hyperosmotic medium. Endocytosis via clathrin-coated pits is also reduced in PBS in which K^+ has been replaced isoosmotically by Na^+ (Cupers et al., 1994). The uptake of transferrin and ^{3}H-FC from LDL was reduced comparably in K^+-free medium. There was no significant effect on the uptake of peroxidase or cholera toxin under these conditions (Figure 2). Together the data suggested that the initial transfer of LDL-FC into the cell takes place via the coated pits.

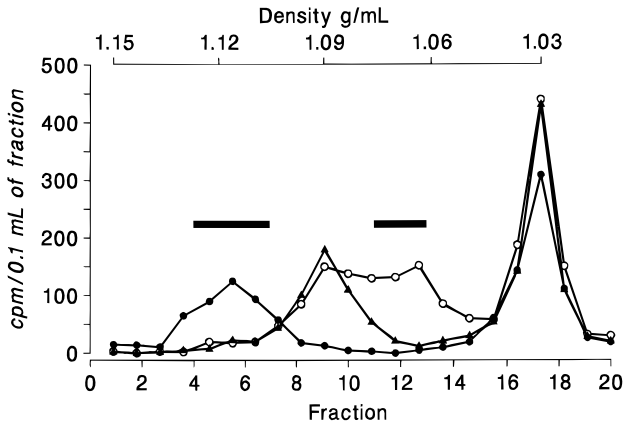

FIGURE 3: Distribution of ^{3}H-FC from LDL following D_2O−Ficoll density gradient centrifugation of fibroblast monolayers incubated with labeled LDL. Conditions of labeling and cell fractionation were as described under Experimental Methods. Closed circles: incubation with ^{3}H-FC LDL for 2 h at 4 °C and then 0.5 min at 31 °C in the absence of LDL. Open circles: the same, but with 2 min incubation at 31 °C in the absence of LDL. Closed triangles: the same, but with 15 min incubation at 31 °C in the absence of LDL. The distribution of [^{125}I]transferrin (collected at 0.5 and 2 min) is shown by solid bars; >95% of label was found within the fractions marked.

Formation and Subcellular Fractionation of FC-Labeled Vesicles. The early stages of intracellular FC transport were studied by density-gradient centrifugation of cell homogenates, under conditions maximizing the formation of clathrin-coated vesicles (Woodman & Warren, 1991). These were obtained from fibroblast monolayers which had been incubated (2 h, 4 °C) with ^{3}H-FC LDL, then brought to 31 °C for 0.5 min.

A peak of ^{3}H-FC was recovered within the gradient at a density 1.12 g mL^{-1} (fractions 4−6). The rest of the label was found at the top of the gradient (*d* 1.03 g ml-$^{-1}$) (Figure 3). A similar distribution was seen when the cells were incubated with [^{125}I]transferrin, or when LDL-receptor-deficient (GM2000) cells replaced normal cells in reaction

Table 1: Transfer of LDL-Derived ^{3}H-FC into Fibroblast Monolayers in the Presence and Absence of Metabolic Inhibitors[a]

inhibitor	concn, μM	influx of ^{3}H-FC: % remaining
none		100
cytochalasin D	40	38 ± 2
monensin	40	39 ± 6
nocodazole	60	101 ± 6
bafilomycin A1	45	70 ± 4

[a] Following incubation with PBS or inhibitor as described under Experimental Methods, incubation with ^{3}H-FC-labeled LDL was for 30 min at 37 °C. The influx rates shown are means (±1 SD) from three experiments.

with ^{3}H-FC-labeled LDL. After 0.5 min, 74% of transferrin label within the gradient was recovered in the density 1.12 g mL^{-1} fraction. Clathrin (96%) was identified immunologically at *d* 1.12 g mL^{-1} with the balance at the top of the gradient. Clathrin was undetectable in other fractions. Alkaline phosphatase, a GPI-anchored protein located in plasma membrane caveolae (Rothberg, 1995), and 5′-nucleotidase, an additional marker for plasma membrane domains (Emmelot et al., 1964), detected at the top of the gradient, but not elsewhere, under conditions where a 2% contaminant would have been detected. These data suggest that the initial appearance of ^{3}H-FC label within the cell is in the clathrin-coated vesicles formed from cell-surface coated pits.

Effects of Metabolic Inhibitors on the Uptake of LDL-FC. Several inhibitors reduce internalization of proteins through clathrin-coated pits (Pearse & Robinson, 1990). These agents were used initially at the highest concentration tested in the individual references given below. Where inhibition was found, a concentration curve was obtained over at least a 10-fold concentration range. Cytochalasin inhibits endocytosis from coated pits, probably by preventing the polymerization of actin in microfilaments required for effective invagination (Gottlieb et al., 1993). This agent reduced the uptake of ^{3}H-FC from LDL by an average of 65% at 40 μM (Table 1). No greater inhibition was obtained at 100 μM. Monensin inhibits the endocytosis of intact LDL particles through the coated pits (Goldstein et al., 1985). As shown in Table 1, it was also effective in reducing the selective uptake of FC from LDL. As with cytochalasin D, no increase in inhibition was found at concentrations up to 100 μM. Comparable results were obtained in LDL-receptor-deficient (GM2000) cells. Bafilomycin A1 (45 μM), which inhibits ATPase-driven acidification of endocytic vesicles (Furuchi et al., 1993), had a smaller maximal effect (−30%) in these cells. Inhibitors of Golgi-mediated protein transport (brefeldin A, vinblastine, taxol) (Ktistakis et al., 1992) were without effect at concentrations up to 60 μM on the uptake of ^{3}H-FC from LDL, as was nocodazole (up to 60 μM), which inhibits microtubule-dependent transport under these conditions (Thyberg & Moskalewski, 1992).

Intracellular Transport of ^{3}H-FC. Fibroblast monolayers were labeled as before (2 h, 4 °C). Cell-surface and soluble LDL were removed. The dishes were then brought to 31 °C for 2 min in the absence of LDL. Density-gradient ultracentrifugation and determination of label distribution and solvent density were carried out as described under Experimental Methods. Most of the ^{3}H-FC label had now disappeared from the *d* 1.12 g mL^{-1} fraction. Label within the gradient was now in a light vesicle fraction (*d* 1.07 g mL^{-1}) (centered on fraction 12) with significant radioactivity in a fraction of intermediate density (*d* 1.09 g mL^{-1}) centered on fractions 9−10. Following more extended incubation in the absence of ^{3}H-FC LDL (up to 15 min at 31 °C), most of the label within the gradient became concentrated in the fraction of intermediate density. The distribution of FC was compared with that of transferrin, which in fibroblasts enters the cell exclusively via the clathrin-coated pits (Woodman & Warren, 1991). [^{125}I]transferrin label was also found in the light vesicle fraction (Figure 3). In contrast to FC, none was found in the intermediate density fraction. Clathrin was not detected in either light or intermediate density fractions.

Under these conditions, the cellular origin of light and intermediate density fractions was investigated with antibodies to protein markers. The density of the light vesicles, the presence of [^{125}I]transferrin, and the absence of clathrin suggested that this fraction contained uncoated vesicles formed by the removal of clathrin by uncoating ATPase (Pearse & Robinson, 1990; Woodward & Warren, 1991). This conclusion was strengthened by an apparent precursor−product relationship between the dense (*d* 1.12 g mL^{-1}) and light vesicles (*d* 1.07 g mL^{-1}).

In cells incubated with LDL as described above (15 min, 37 °C) the FC−phospholipid molar ratio of the light vesicle fraction was 0.42 ± 0.02, while that of the intermediate density fraction was 0.65 ± 0.03. The density vesicle fraction (*d* 1.12 g mL^{-1}) had a FC−phospholipid molar ratio of 0.35 ± 0.04, similar to the value of 0.30 reported for adrenal cell coated vesicles (Pearse, 1976). The FC−phospholipid molar ratio for the fraction (d 1.03 g mL^{-1}) containing the plasma membrane markers was 0.68 ± 0.03, consistent with published data on this fraction (Cullis & Hope, 1991). Under the same conditions, 75% of caveolin antigen was present in the intermediate density vesicle fraction. The balance was recovered with the plasma membrane markers at the top of the gradient.

The absence of [^{125}I]transferrin from the intermediate density fraction (d 1.09 g mL^{-1}) suggested that transferrin and LDL-derived FC separated from each other in the endosomes, and returned to the cell surface by different pathways. This interpretation was also consistent with the kinetic data, which implied a precursor−product relationship between the light and intermediate density fractions. Antibodies to different vesicle-bound proteins were used to obtain better identification of the intermediate density fraction.

Mannose 6-phosphate receptor protein is localized mainly to the trans-Golgi network (TGN) (Pfeffer, 1991). Caveolin is present in the TGN as well as in the plasma membrane (Dupree et al., 1993). Both proteins are considered to recycle between the TGN and the cell surface. In cells preincubated (30 min) 75% of caveolin antigen was recovered with the whole of detectable mannose 6-phosphate receptor protein in the intermediate density fraction in cells exposed to LDL-FC. These data suggest that FC label in the intermediate density fraction comigrates during density-gradient fractionation with vesicles derived from the TGN. The lack of any detectable alkaline phosphatase or 5′-nucleotidase activity in the intermediate density fraction argues against contamination with plasma membrane material.

To determine if exchange or diffusion during fractionation contributed to the changing distribution of FC label shown in Figure 3, cells were labeled with ^{3}H-FC from LDL. Fractionation of the cell homogenate was carried out by density-gradient centrifugation as described under Experi-

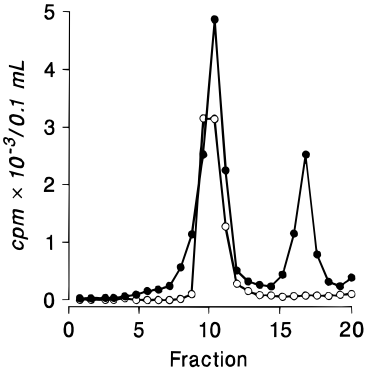

FIGURE 4: Reisolation of intermediate density fraction ^{3}H-FC label after mixing with whole cell homogenate from unlabeled fibroblasts. The intermediate density fraction (*d* 1.09 g mL^{-1}) was obtained following labeling for 15 min at 31 °C. A portion of this fraction was recentrifuged under identical conditions in a second D_2O−Ficoll gradient. Closed circles: density distribution of the first gradient. Open circles: density distribution of the peak fraction from the first gradient following recentrifugation.

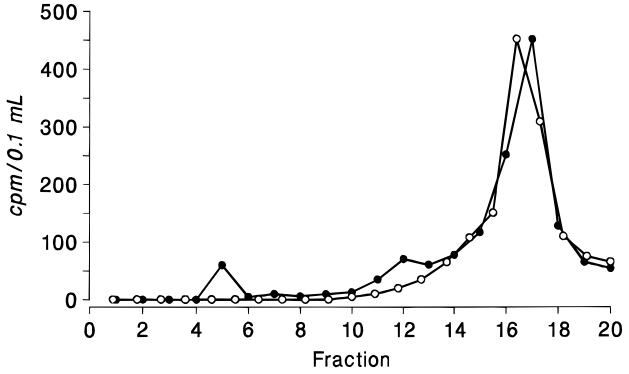

FIGURE 5: Effects of metabolic inhibitors on the distribution of LDL-derived ^{3}H-FC following density-gradient ultracentrifugation. Experiments were carried out as described in Figure 3, except that the cell monolayers had been preincubated (30 min, 37 °C) in either 2 mM *N*-ethylmaleimide (open circles) or 40 μM cytochalasin (closed triangles) before treatment with ^{3}H-LDL (2 h, 4 °C followed by 2 min at 31 °C).

mental Methods. Labeled fractions from the gradient were collected, mixed with homogenate from unlabeled cells, and recentrifuged as before. As shown in Figure 4, there was no significant redistribution of label. This finding indicates that the transfer of FC observed within the cell represents not an equilibration, but the orderly transport of this lipid between different cell compartments.

Effects of Metabolic Inhibitors on Intracellular Transport. *N*-Ethylmaleimide (2 mM) blocks selective uptake of FC from LDL (Fielding & Fielding, 1995a). In the presence of 2 mM NEM, no peak of FC label was present with clathrin at *d* 1.12 g mL^{-1}. Almost all was recovered with the plasma membrane fraction at the top of the gradient (Figure 5). A similar result was obtained in the presence of cytochalasin (Figure 5) or monensin (data not shown). The results indicate that these inhibitors of the selective transfer of LDL-FC into the cell inhibit the formation of clathrin-associated vesicles from the plasma membrane.

Return of Intracellular FC to the Cell Surface. In preliminary studies, fibroblast monolayers were pulse-labeled (2 min, 31 °C) with ^{3}H-FC transferred from LDL. The cells were then washed, and surface-bound labeled LDL was displaced, as described under Experimental Methods. The cells were incubated with cholesterol oxidase at 0−4 °C, and the oxidized fraction of FC was determined as a fraction

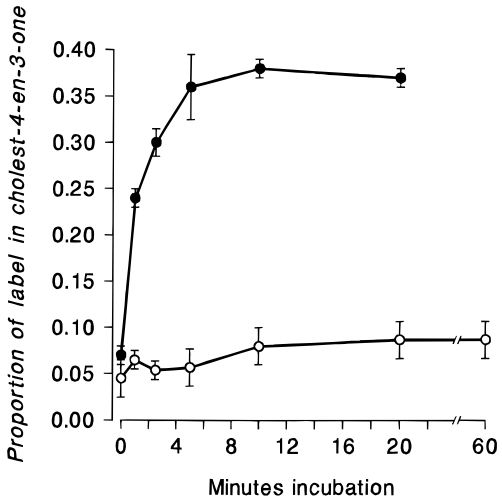

FIGURE 6: Rate of transfer of ^{3}H-FC to the cell surface as a function of temperature. Cell monolayers were prelabeled (2 min, 31 °C) with labeled LDL. Noninteriorized LDL was removed as described under Experimental Methods. The cells were then incubated in the absence of LDL at either 15 or 31 °C for the period indicated. The cells were then cooled on ice and incubated with cholesterol oxidase (1 unit mL^{-1}) for 4 h. The oxidized fraction of FC is expressed as a percent of total label. Open circles: 15 °C. Closed circles: 31 °C.

of total FC label (Smart et al., 1994). Under these conditions, the cholest-4-en-3-one fraction represented <1% of total label (4 experiments, 0.8 ± 0.2%). This result indicates that little or no ^{3}H-FC moves through the cell into the caveolae at 4 °C. Other cells labeled in the same way were brought to 15 °C for up to 60 min before incubation with cholesterol oxidase at 0−4 °C. There was only a slight increase in the amount of cell-surface FC label accessible to cholesterol oxidase (Figure 6). In contrast, when incubation was carried out at 31 °C, there was a rapid increase in oxidized FC ($t_{1/2}$ ∼2 min) reflecting the transfer of FC label from intracellular pools to the cell surface. Following the 2 min pulse label, about 40% of internalized FC became eventually localized to the oxidase-sensitive fraction, a proportion approximately 10-fold higher than that of cellular FC in this fraction in unloaded cells (Smart et al., 1994; Fielding & Fielding, 1995b).

In other experiments, cells were continuously labeled with ^{3}H-FC LDL at 31 °C in the absence of medium HDL, for a period of up to 60 min. Under these conditions FC accumulates in the cell. At intervals monolayers were cooled on ice, medium and cell-surface LDL removed, and the washed cells incubated at 0−4 °C with cholesterol oxidase. Oxidase-accessible FC was nearly maximal after ∼60 min at 37 °C. Total cell label (mainly unoxidized FC) continued to increase (Figure 7). FC label accumulated in an intracellular compartment not accessible to cholesterol oxidase. As already shown in Figure 4, the intracellular (oxidase-inaccessible) label under these conditions is recovered as a single major peak in the intermediate density fraction.

Fibroblast monolayers were prelabeled with FC-labeled LDL for 2 min at 31 °C. Medium and surface-bound labeled LDL were removed as described above. The cells were incubated with inhibitors of intracellular transport for 30 min at 15 °C. Movement of FC label to the cell surface remained negligible (Figure 6). The cells were brought to 31 °C for 15 min and then cooled on wet ice. ^{3}H-FC transfer to the cell surface (in the presence and absence of inhibitor) was

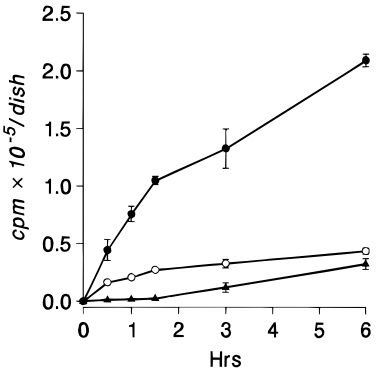

FIGURE 7: Evidence for the saturation of the caveolar FC compartment. Cells were incubated at 31 °C with ^{3}H-FC-labeled LDL. At the intervals shown, cells were cooled on ice and intact LDL particles removed as described under Experimental Methods. Cholesterol oxidase (1 unit ml^1) was added for 4 h at 4 °C. The level of label in CE, FC, and cholest-5-en-4-one was then determined following thin-layer chromatography as described under Experimental Methods. Open circles: cholest-4-en-3-one. Closed circles: FC. Closed triangles: CE.

Table 2: Effects of Metabolic Inhibitors on the Rate of Transfer of Intracellular ^{3}H-FC to the Cell Surface[a]

inhibitor	concn	% transfer[a]
N-ethylmaleimide	2 mM	50 ± 3
KNO_3	50 mM	70 ± 4
nocodazole	40 μM	40 ± 1
brefeldin A	40 μM	95 ± 5
vinblastine	40 μM	100 ± 2

[a]Relative to rate in the absence of inhibitor. Values are expressed in terms of ^{3}H-FC oxidized to cholest-5-en-4-one by cholesterol oxidase (1 unit mL^{-1}, 4 h at 0 °C) with cells pulse labeled with labeled LDL (2 min, 31 °C) and then incubated without LDL for 30 min (31 °C) in the presence of individual inhibitors (see Experimental Methods). Data are from triplicates of individual dishes, expressed as a percent of oxidized label recovered in the absence of inhibitor.

assayed with cholesterol oxidase as described under Experimental Methods.

The transport of intracellular ^{3}H-FC to the cell surface was reduced by nocodazole (Table 2). It was unaffected by vinblastine and by brefeldin A, which inhibit transport from the Golgi stacks but have little reported effect on vesicular transport from the TGN (Chege & Pfeffer, 1990). There was no effect of cytochalasin D or monensin on transport to the caveolae under conditions that inhibited initial uptake of FC from LDL (Table 1). *N*-Ethylmaleimide and NO_3^-, inhibitors of vesicle ATPases, which inhibited the initial endocytosis of LDL-FC, also inhibited the return of FC to the cell surface, but less effectively than did nocodazole.

Effect of Medium Lipoproteins on the Distribution of Intracellular FC. Fibroblast monolayers were incubated with ^{3}H-FC LDL for 30 min at 31 °C to predominantly label the intermediate density vesicle fraction. Cell-surface LDL was removed as described under Experimental Methods. The cells were then incubated (5 min, 31 °C) in the presence of human plasma or PBS. In the cells exposed to plasma there was a 12-fold decrease in the intermediate density fraction (Figure 8). There was a smaller decrease in the plasma membrane fraction. Since relatively little of total cell label was at the cell surface under these conditions (Figure 7), these data indicate that label in the intermediate density fraction can be quickly unloaded to the extracellular medium. It was shown earlier that FC was rapidly unloaded from these cells through the caveolae (Fielding & Fielding, 1995b).

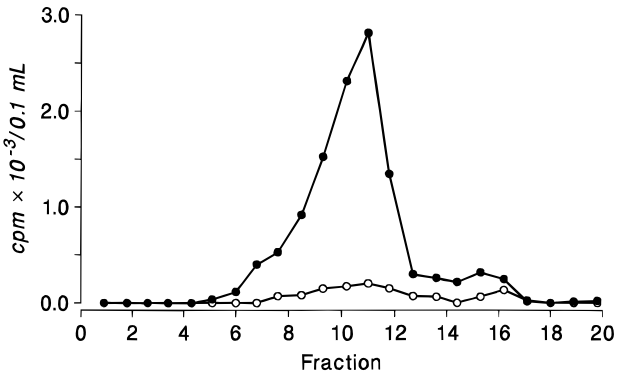

FIGURE 8: Unloading of LDL-derived FC to the extracellular medium in the presence of native plasma. Labeling of the cells was carried out as described in Figure 4. The labeled cells were then incubated with plasma or PBS for 5 min at 31 °C. The cells were then cooled on ice, homogenized, and fractionated by D_2O−Ficoll gradient. Closed circles: distribution after incubation in the presence of PBS. Open circles: distribution in the presence of plasma.

Consequently, FC in the intermediate density fraction must be transferred to the caveolae prior to unloading.

DISCUSSION

Cell-surface clathrin-coated pits are the portal by which many receptor-bound proteins enter the cell. The selective uptake of FC from LDL takes place without the internalization of LDL protein (Fielding & Fielding, 1995a). Nevertheless, the results of this study are consistent in indicating that selective FC transfer is also initiated via coated pits. Internalization of FC, like that of transferrin (Pearse & Robinson, 1990), was reduced by hyperosmotic and K^+-deficient media. Reduced FC uptake was also observed with other inhibitors of receptor-mediated endocytosis, including monensin and cytochalasin. Finally, LDL-derived FC was recovered in the same density-gradient fraction as [^{125}I]-transferrin and clathrin, and the appearance of label in these peaks was blocked by inhibitors along with selective FC transfer into the cell. The mechanism by which FC enters coated pits selectively from LDL must be distinct from that involving the high-affinity LDL receptor, since the rate of FC transfer is normal in LDL-receptor-deficient (GM2000) cells (Fielding & Fielding, 1995a). The FC−phospholipid ratio of isolated coated vesicles in this study was relatively low, compared to that of whole plasma membrane fraction or LDL itself (Fielding & Fielding, 1986) consistent with earlier data (Pearse, 1976). It is possible that FC transfers spontaneously to the outer leaflet of the coated pits and could help trigger the budding of the endocytic vesicle. The selective uptake of FC may be most relevant in cells where LDL receptors are mainly downregulated.

Vesicles formed from clathrin-coated pits are converted rapidly to a light vesicle fraction through the action of uncoating ATPase (Rothman & Schmit, 1986). The endosomal contents are retained in the vesicle. The first appearance of [^{125}I]transferrin and ^{3}H-FC in a light (*d* 1.07 g mL^{-1}) fraction is consistent with the expected kinetics of this conversion (Woodman & Warren, 1991). Shortly thereafter, FC and transferrin separated into different compartments. FC (but not transferrin) moved to an intermediate density fraction and finally appeared in a plasma membrane fraction enriched in caveolin (Fielding & Fielding, 1995b). Transferrin (but not FC) was returned to a noncaveolar

domain of the cell surface (Fielding and Fielding, unpublished observations). Earlier studies showed that FC selectively internalized from LDL became briefly inaccessible, before reappearing in the plasma membrane caveolar fraction, from which it could be released by HDL. These new data suggest that, as part of this transport process, FC transits through a vesicle fraction of intermediate density.

Several pieces of evidence now suggest that the TGN may be a significant component of this fraction. The mannose 6-phosphate receptor protein, identified in the intermediate density fraction, is present mainly in the TGN (Pfeffer, 1991). Caveolin, present in the intermediate density fraction in this study as well as in the plasma membranes, has been recognized previously as a protein component of the TGN (Dupree et al., 1993). Caveolin is believed to migrate between the TGN and cell-surface caveolae (Conrad et al., 1995), at least partially in response to cellular FC levels (Smart et al., 1994). Consistent with this relationship, the TGN was identified by electron microscopy in filipin-treated cells as the most FC-rich component of the Golgi stack (Coxey et al., 1993). In the present study, the FC−phospholipid ratio of the intermediate density vesicle fraction was higher than that of primary endocytic vesicles, and similar to that of the plasma membrane fraction. It was recently suggested that FC may play an important role in regulating the sorting activities of the Golgi apparatus, with the lowest levels of FC (relative to phospholipid) in the cis-Golgi vesicles and the highest in the trans-Golgi and TGN (Bretscher & Munro, 1993).

Two alternative models of the experimental data were also considered. In the first, FC entering the cell by the coated pits would partition immediately into two pools, one going to the TGN as proposed, the other returning directly to the plasma membrane. In this case, plasma membrane label at the earliest time point in Figure 3 would have already recirculated in the cell. However plasma membrane label was present even when endocytosis was inhibited with NEM (Figure 5; Fielding & Fielding, 1995a). At longer time points, FC label disappeared almost entirely from the plasma membrane fraction (Figure 8) even though this contains most of the cellular FC mass. These data indicate that plasma membrane label at the early time point represents FC not yet internalized, not FC already returned to the plasma membrane from within the cell. In a second alternative model, internalized FC would be transferred first to the caveolae, and only subsequently from the caveolae to the TGN. This model is incompatible with the data in Figures 6 and 8. At 15 °C , FC is readily internalized by the cell, but negligible FC reaches the caveolae. When these cells were brought to 31 °C, ^{3}H-FC was transferred to the caveolae (Figure 6) and became available for release to the medium (Figure 8). These data indicate that the major direction of FC transport at this step is from TGN to caveolae, rather than the reverse.

Further studies will be needed, but the data so far obtained are consistent with a model in which the intermediate density fraction, which colocalizes with material from the TGN on density gradients, represents a component of the intracellular transport of LDL-derived FC, and a reservoir from which excess FC can be transferred to the caveolae for release to plasma lipoproteins, particularly HDL.

ACKNOWLEDGMENT

The gift of antibody by Dr. Susanne Pfeffer is gratefully acknowledged. Wilma Norona provided expert technical assistance.

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BI9613382