review

Intracellular cholesterol transport

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Abstract Recent data on the roles of vesicle- and 'raft'-mediated pathways in intracellular free cholesterol (FC) transport are reviewed. Cholesterol internalized from plasma lipoproteins is transferred via endocytic vesicles to the *trans*-Golgi network (TGN), consistent with prior data indicating a key role for this organelle in protein and lipid sorting and transport. Newly synthesized and lipoprotein-derived FC are returned to the cell surface by a common raft-dependent pathway. Intracellular FC transport promotes the delivery of GPI-anchored proteins to the cell surface; it is also an additional mechanism to regulate cell FC content. Many peripheral cells express caveolin, an FC-binding protein localized to plasma membrane caveolae. FC delivery to cell surface caveolae is accelerated by caveolin. Caveolar FC becomes targeted to small, lipid-poor (prebeta-) high density lipoprotein particles. Caveolin may protect quiescent cells, regulating FC efflux more efficiently in response to changing medium lipoprotein concentrations. Overall, these recent findings suggest that cell FC content can be regulated at the levels of both influx and efflux, and indicate key roles for the TGN and in cells expressing caveolin, cell-surface caveolae.—**Fielding, C. J., and P. E. Fielding.** Intracellular cholesterol transport. *J. Lipid Res.* 1997. **38:** 1503–1521.

Supplementary key words *trans*-Golgi network • caveolae • cholesterol–sphingolipid rafts • GPI-anchored proteins

In mammalian cells, most (>85%) of cell free cholesterol (FC) is in the plasma membrane (1). Of this, only a small part (3–5%) is in the exofacial leaflet of the membrane, and so directly accessible to the extracellular medium. The remainder is restricted to the cytofacial half of the bilayer. This asymmetry was reflected in the kinetics of FC efflux in several peripheral cell lines, using the fluorescent FC analog ergostatraenol (2). The 'fast' FC pool identified by this technique possibly represents the exofacial pool of plasma membrane FC; an intermediate pool may define transfer of cytofacial FC across the membrane bilayer, and a 'slow' pool represents FC within intracellular organelles. FC in the plasma membrane (PM) plays a key role in regulating the activity of membrane proteins including receptors, transmembrane transport proteins, and enzymes. This is achieved in some cases by modifying the physical properties of FC within the bilayer, in particular by the compression of phospholipid acyl chains. In others, FC may serve as an essential cofactor of catalytic activity (3). Most intracellular organelles contain little FC. An exception is the *trans*-Golgi network (TGN) distal to the Golgi stacks, whose FC content may approach that of the plasma membrane (4).

There is convincing evidence that differences between the FC content of organelles are maintained dynamically. Although FC is transferred rapidly between intracellular membranes, its asymmetric distribution within the cell remains unchanged. Cytoplasmic filaments and tubules represent a barrier to passive exchange (5) and it is likely that little FC transport is the result of simple diffusion. Major pathways contributing to FC homeostasis in mammalian cells are shown in **Fig. 1.** All cells can internalize cholesterol from the extracellular medium. Mechanisms include: *1*) internalization of intact lipoprotein particles, most notably via the high affinity low density lipoprotein (LDL) receptor; *2*) selective uptake of FC from plasma lipoproteins, particularly LDL; and *3*) selective uptake of cholesteryl ester (CE) from LDL and high density lipoprotein (HDL) (6–9). All nucleated cells synthesize FC de novo although most do so only at a low rate, relative to the flux of FC through the cell. All mammalian cells can transfer FC from the PM to extracellular acceptors, such as plasma lipoproteins and albumin. Intracellular FC can also be esterified by acyl CoA:cholesterol acyltransferase (ACAT), and released from the ester storage pool by the action of neutral cholesterol esterase. In most cells the CE storage pool is quite small. This reflects the efficiency with which cells coordinate the influx, efflux,

Abbreviations: FC, free cholesterol; CE, cholesteryl esters; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; PM, plasma membrane; HDL, high density lipoprotein; LDL, low density lipoprotein; apoA-I, apolipoprotein A-I; SCP_2, SCP_x, 14 kD and 58 kD forms of sterol carrier protein-2; GPI, glycosyl-phosphatidylinositol.

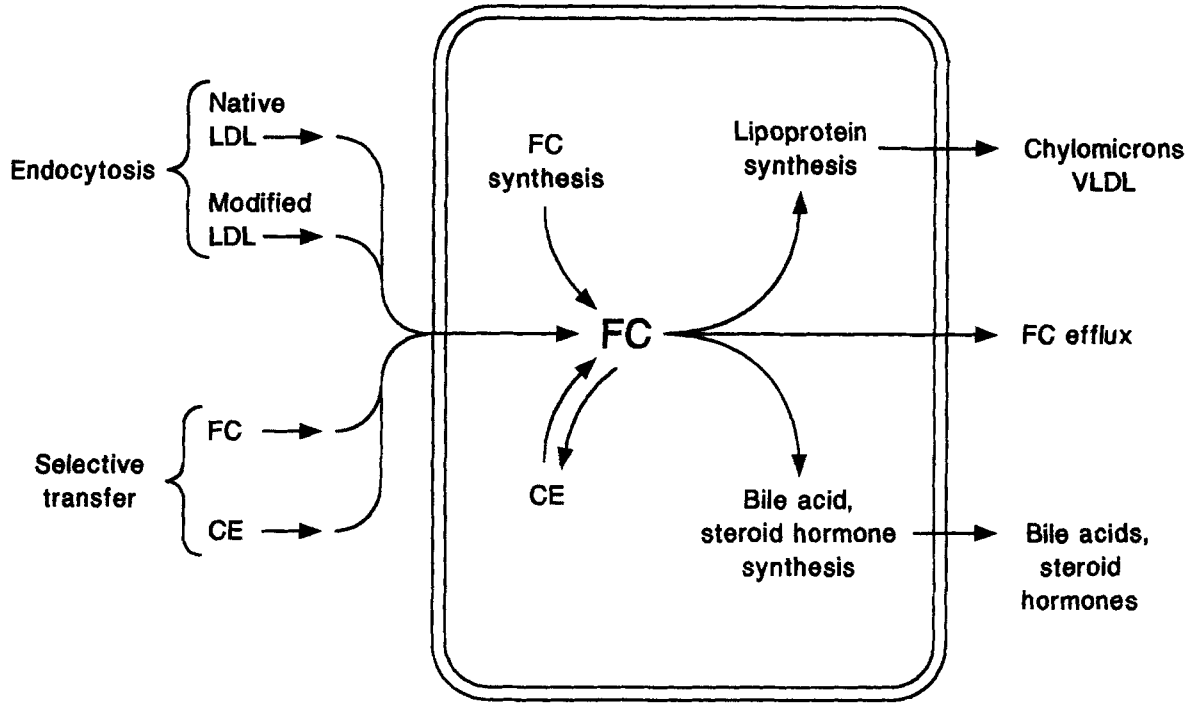

Fig. 1. Major pathways of FC homeostasis in mammalian cells. The endocytosis of intact LDL (ref. 6) can be carried out by most nucleated cells; the pathway is strongly down-regulated except in liver and adrenal tissues. Chemically modified LDL is internalized by scavenger receptors (ref. 44) in macrophages and endothelial cells. The pattern of selective uptake of CE (ref. 8) is comparable to that of LDL receptors. The selective transfer of FC appears to be of most consequence in quiescent peripheral cells including fibroblasts, vascular smooth muscle, and endothelial cells (ref. 7). Chylomicrons and VLDL are secreted by enterocytes and hepatocytes, respectively, bile acids only by hepatocytes, and steroid hormones by adrenal and gonadal cells. All cells studied can transfer FC to the extracellular medium.

synthesis, and metabolism of FC, which is toxic in excess (10).

In some specialized cells, additional mechanisms contribute to cellular FC homeostasis. Hepatocytes and enterocytes secrete lipoproteins (very low density lipoproteins and chylomicrons, respectively) which contain both FC and CE. Uniquely, hepatocytes also catabolize FC to bile acids, and secrete both bile acids and FC into bile. Adrenal and gonadal cells use FC as substrate for steroid hormone production. FC mass is normally maintained within narrow limits. This implies the presence of a central regulatory pool.

The properties of FC transport were recognized to differ fundamentally from those of the vesicular pathway which carries newly synthesized proteins and small solutes from Golgi vesicles to the cell surface (reviews: 11, 12). Within the last 2 years, cell and molecular biology techniques have provided new insights into the molecular mechanisms of intracellular FC transport.

MICRODOMAINS OF THE PLASMA MEMBRANE

The PM turns over rapidly (half the cell surface may be internalized h^{-1} in fibroblasts) (13). Proteins and lipids adsorbed nonspecifically to the cell surface are internalized as part of lipid vesicles, together with solutes present in extracellular medium and trapped during vesicle formation. Plasma membrane surface area of the cell is conserved by retrograde transport of lipid vesicles to the cell surface. This type of endocytosis seems to be largely unregulated. Studies with horseradish peroxidase, a marker for nonspecific endocytosis, showed that once this enzyme was internalized, it was rapidly returned to the cell surface, without transfer to other intracellular compartments (14).

Two specialized structures have been implicated in the specific or regulated transport of individual solutes (including FC) across the plasma membrane:

Clathrin-coated pits

Coated pits are invaginations of the plasma membrane stabilized on the cytoplasmic surface by interlocking trimers ('triskelions') of clathrin heavy- and light-chain heterodimers which in turn are stabilized by adaptor proteins AP-1 and AP-2 (**Fig. 2**). Compared to the rest of the PM, coated pits contain little FC or sphingolipid (15). Clathrin-coated pits are the site from which many integral proteins of the plasma membrane are internalized. These include membrane-spanning re-

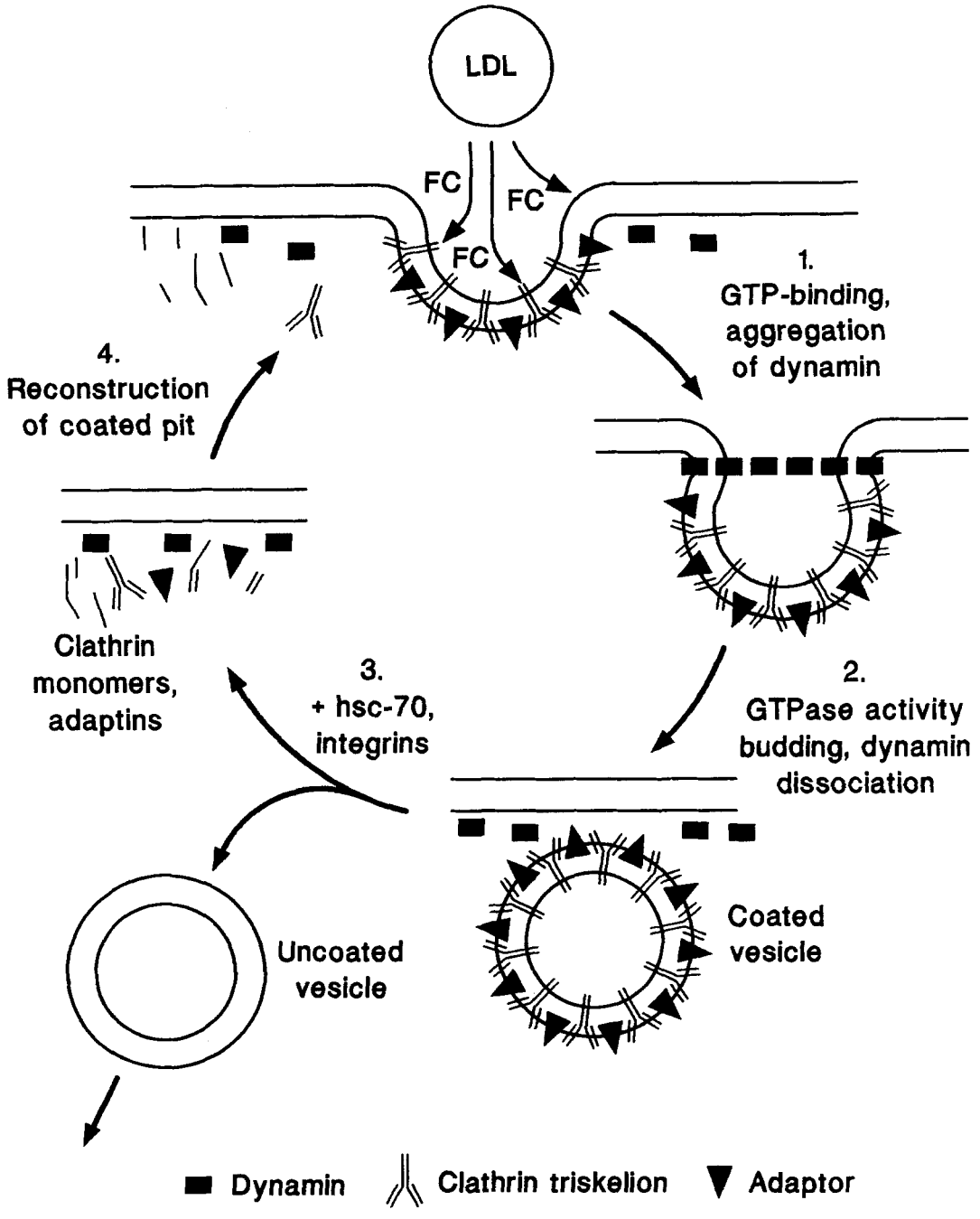

Fig. 2. Endocytosis of FC from cell surface coated pits. Steps shown represent common intermediates and mechanisms for the internalization of ligands utilizing this pathway (ref. 16). Formation of coated vesicles from coated pits involves the sequential activity of several ATPases and GTPases as well as roles for ATP- and GTP-binding proteins (ref. 17). The dissociation of clathrin and formation of uncoated vesicles involves auxilins as well as the uncoating ATPase hsc70 (refs. 18, 19). Steps involved in the realignment of dissociated clathrin at the cell surface are presently not well understood. The uptake of LDL-FC by selective transfer is illustrated (ref. 41).

ceptor proteins, which bind solutes from the extracellular medium. In some cases free receptors are localized to the coated pits. Other receptor proteins accumulate there only after formation of a receptor–ligand complex.

Formation of clathrin coated pits de novo is not required as part of internalization of receptor–ligand complexes, nor is budding a direct response to the presence of receptor proteins within the pit (16). An early step of vesicle formation involves the self-assembly of a restricting annulus of dynamin subunits in response to GTP binding (17). Pinching off of dynamin-limited vesicles also depends on the activity of one or more N-ethyl-

maleimide-dependent ATPases. Uncoating of clathrin is essential for the further metabolism of primary coated endosomes. An ATP-binding chaperone ('uncoating ATPase', hsc70) promotes the disassembly of clathrin. A protein cofactor (auxilin in neuronal cells) is also required. Auxilins and the beta-subunits of AP-1 and -2 bind to independent sites on the heavy chain of clathrin (18). Auxilin has an attachment site for hsc70 and also promotes the attachment of hsc70 to the clathrin heavy chain (19). The product of these reactions is an uncoated vesicle, from which dissociated clathrin monomers return to the cell surface for assembly into new coated pits (20, 21). It is possible that phosphorylation of one or more structural proteins of the coated pit initiates endocytosis. Changes in the lipid composition of the bilayer membrane within the pit may also contribute.

Caveolae

Caveolae are clathrin-free invaginations of the plasma membrane. A thin membrane over the opening of caveolae has been described, but was not observed by most investigators. Caveolae are rich in FC and sphingolipids (22). The budding of intracellular vesicles from caveolae has been reported (23) but the balance of evidence does not yet support such a mechanism. On the contrary, caveolae, unlike clathrin-coated vesicles, may represent semi-permanent features of the cell surface. The number of caveolae was decreased when protein kinase C activity was up-regulated, for example by phorbol esters (24). It is not known whether this represents a significant regulatory mechanism under physiological conditions.

Caveolae contain one or more of a family of FC-binding proteins (caveolins) (25, 26). Transfection of cells lacking caveolae with caveolin cDNA led to the appearance of microscopically authentic caveolar invaginations (27, 28). These data indicate that caveolin is required for formation of caveolae at the cell surface.

The primary sequence of caveolins includes a well conserved central region. This includes a highly hydrophobic 24 amino acid region bounded by proline residues. Antibodies directed to its N- and C-termini both have access to caveolin from the cytoplasmic side [29]. This suggests that the central region of caveolin penetrates, but does not traverse, the plasma membrane (**Fig. 3**). A G-protein binding site has been identified immediately N-terminal to the hydrophobic 'intramembrane' sequence (30). N-terminal to this, 1–2 short amphipathic helical regions may help anchor the protein to the cytoplasmic face of the bilayer. C-terminal to the hydrophobic sequence, all caveolins contain 4–5 cysteine residues. At least in caveolin-1, best characterized and most widely distributed of this protein family, these residues are palmitoylated (31). It is unknown whether this covalent modification is reversible under physiological conditions. The domain including these palmitoylated residues may function as an additional anchor fastening caveolin to the cytoplasmic face of the plasma membrane.

Enrichment of FC and glycolipids within caveolae in intact cells has been confirmed by electron microscopy (22). Proteins in addition to caveolin are present in purified caveolae. These include factors implicated in the vesicular transport of newly synthesized proteins to the basolateral surface of polarized cells, such as N-ethylmaleimide sensitive factor (NSF), soluble NSF accessory proteins (SNAPs), and cellubrevins (32). The list of other proteins co-purifying with caveolae includes (but is not limited to) GPI-anchored proteins, such as alkaline phosphatase, *thy-1* and folate receptor protein. Ca^{2+}-ATPase, annexins, and the epidermal growth factor receptor protein also co-purify with caveolae in vitro. It seems unlikely that all these proteins are part of caveolae in intact cells. GPI-anchored proteins may form a loosely associated 'rim' around a 'bowl' made up of FC, glycolipids, and caveolin (33) although significant levels of these proteins are present in the plasma membrane outside the caveolae (34). In fact, GPI-anchored proteins are probably quite mobile within the lateral plane of the plasma membrane, and also transfer between the plasma membranes of different cells (35).

Caveolae are largely or completely absent in hepatocytes, enterocytes, adrenal cells, and oncogenically transformed cell lines. Cells with active lipoprotein receptors such as hepatocytes, adrenal and gonadal cells have few caveolae (Type A cells, **Table 1**). When fibroblasts were oncogenically transformed, LDL receptors were overexpressed (36) while caveolae were reduced or disappeared completely (37). Cells with high levels of caveolae such as endothelial and vascular smooth muscle cells, pulmonary cells, adipocytes, and fibroblasts, usually express few high-affinity lipoprotein receptors under the same conditions (Type B cells, Table 1).

In cells without caveolae, microdomains consisting of GPI-anchored proteins, sphingolipids, and FC are scattered over the PM (38). These patches have a diameter of 50–100 nm (39), similar to that of caveolae, but differ in that they are flat and lack caveolin.

CHOLESTEROL INFLUX

In vivo, cells are in equilibrium with levels of lipoprotein cholesterol (relative to plasma) from as high as 100%, for hepatocytes and endothelial cells, to 5–10%,

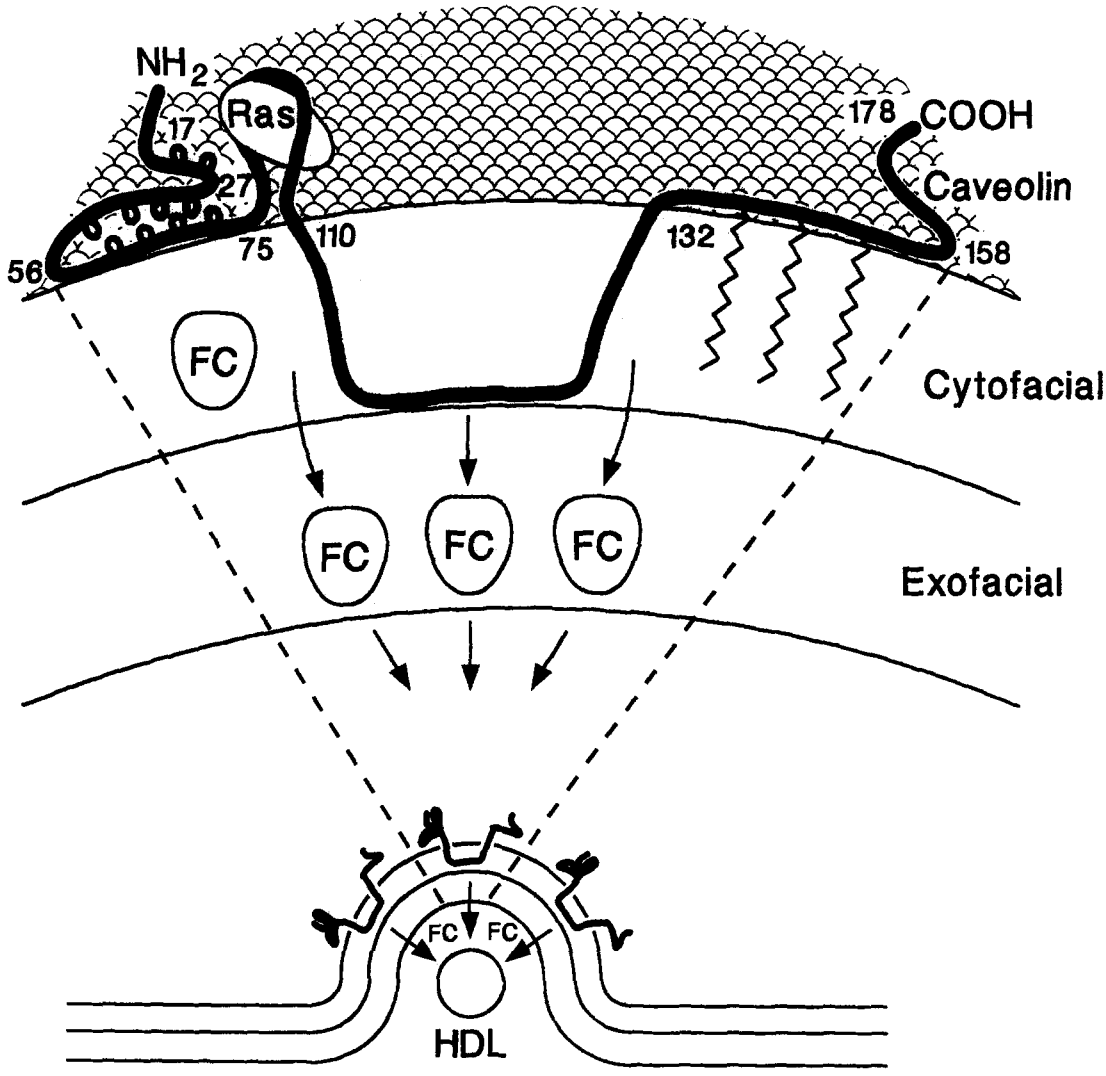

Fig. 3. FC efflux from caveolae. The model shown is based on published data concerning orientation of caveolin within the membrane bilayer (ref. 29), the concentration of FC within the caveolar invagination (refs. 22, 26), and the selective efflux of FC to HDL (ref. 73). Numerals beside the caveolin polypeptide chain reflect the sequence of amino acids within the caveolin polypeptide and correspond to the limits of predicted domains (refs. 28, 30). S-palmitoyl chains are shown between positions 132 and 158 (ref. 31).

TABLE 1. LDL receptors and caveolae in mammalian cells

	Type A Cells[a]	Type B Cells[b]
LDL, SRB-1 or scavenger receptors	many	few/none
Caveolae	few/none	many
Regulation of FC homeostasis[c]	influx	efflux

Relative LDL receptor expression was measured via mRNA levels in vivo, from the uptake of ^{125}I-labeled LDL in cells not up regulated with lipoprotein-deficient serum or with anti-LDL receptor antibodies. Caveolae were identified by electron microscopy or by expression of caveolin mRNA.

[a]Type A cells include hepatocytes, enterocytes, macrophages, and transformed cell lines.

[b]Type B cells include quiescent fibroblasts and endothelial and muscle cells.

[c]Receptor and caveolar expression are for cells equilibrated in media containing human or bovine serum as extracellular lipoprotein source.

for peripheral cells surrounded by filtered intracellular fluid (40).

Selective uptake of FC from LDL

In quiescent human fibroblasts, where the rate of receptor-mediated endocytosis of intact lipoproteins was low, FC was rapidly internalized from LDL by selective transfer, without concomitant internalization of LDL protein (7). Similar results were obtained with human vascular smooth muscle and endothelial cells (P. Fielding and C. Fielding, unpublished results). Rates of selective uptake of LDL-derived FC were similar in wild type and LDL-receptor-deficient fibroblasts (7). While a role for a cell-surface FC-binding protein ('receptor') has not been excluded, it seems more likely that FC transfers to the plasma membrane down a thermodynamic

TABLE 2. Parallel inhibition of receptor-mediated and selective endocytosis

Property/Inhibitor	% Baseline Activity Remaining	
	Receptor-Mediated Endocytosis[a]	FC Selective Uptake[b]
350 mOsm medium	27	34
N-ethylmaleimide (2 mM)	32	30
K^+-free medium	52	50

Activities shown are for normal fibroblasts.
[a]Determined for the uptake of ^{125}I-labeled transferrin.
[b]Determined for the uptake of [^{3}H]FC from LDL (see refs. 7 and 41).

concentration gradient. Selective uptake of FC from LDL was proportional to LDL concentration over the physiological concentration range. As cell cholesterol content was almost unchanged when LDL-FC concentration was increased 10-fold, FC levels in confluent fibroblasts, and possibly other peripheral cells with few active LDL receptors, must be regulated mainly by the rate of FC efflux.

Selective transfer of FC from LDL into the cell has the properties of endocytosis from coated pits (**Table 2**). The effects of cytochalasin and monensin, low K^+- and hyperosmotic media, and N-ethylmaleimide and NO_3^-, inhibitors of endocytosis via coated pits, were all similar in fibroblasts for the selective uptake of FC from LDL, and for the receptor-mediated endocytosis of transferrin, which is mediated exclusively by coated pits in these cells (7, 41).

The conclusion that uptake of LDL-derived FC involved coated pits was confirmed by the appearance of [^{3}H]FC originating from LDL in dense, clathrin-coated vesicles, when cells pulse-labeled with [^{3}H]FC-labeled LDL were fractionated on D_2O–Ficoll gradients (**Fig. 4**). The endocytosis of LDL-derived FC via coated pits was resistant to brefeldin A, vinblastine, and other inhibitors of transport from the *cis*- and *trans*-Golgi stacks [41].

The uptake of FC via coated pits has precedent in studies of the internalization of sphingomyelin (42, 43). This lipid, like FC, is deficient in coated pits. Also like FC, it was internalized in coated endosomes and delivered to lysosomes. Quantitative studies of sphingomyelin recycling suggest that about 5% of internalized sphingomyelin was degraded; the balance was recycled (43).

Endocytosis of native and modified LDL

The mechanism and significance of LDL receptor-mediated endocytosis are well established (6). The expression of LDL receptors is most evident in hepatic cells, although the adrenal gland and gonads are also important sites of LDL receptor expression. Interaction with LDL promotes the capping of LDL receptors into coated pits. Association of the LDL receptor with its lipoprotein ligand persists until uncoating of the vesicle activates H^+/ATPase activity. The major part of LDL receptor protein recycles back to the cell surface.

Uptake of cholesterol from LDL by receptor-mediated endocytosis is about 8-fold more efficient than by selective FC transfer (7). This is because only ~50% of LDL-FC was internalized per binding event during selective transfer and no detectable CE, which makes up about three quarters of total cholesterol in LDL. High levels of LDL receptors were induced in peripheral cells such as fibroblasts, vascular smooth muscle, and endothelial cells only in the presence of lipoprotein-deficient serum (6). Like FC selective transfer, the internalization of intact LDL particles by the coated pit mechanism was inhibited by cytochalasin and monensin.

Macrophages, endothelial cells, and (in some species) vascular smooth muscle cells express a 'scavenger' receptor active in the endocytosis of intact oxidatively modified LDL. These receptors are reported to be located within coated pits (44).

Selective uptake of HDL and LDL cholesteryl ester

CE selective uptake from HDL was first identified in adrenal cells (45). This mechanism was confirmed in gonadal cells for LDL (9) and in hepatocytes and other cell lines for HDL (46). A cell-surface receptor protein (SRB-1) catalyzes the selective uptake of CE (8). Its distribution, similar to that of up-regulated LDL receptors, identifies cells utilizing cholesterol for catabolism or steroid hormone synthesis. Selectively internalized CE, like CE taken in via intact LDL particles, down-regulates FC synthesis and promotes CE synthesis. This pathway is probably extralysosomal (47). The same mass of cholesterol delivered by selective transfer and the endocytosis of intact LDL particles had quantitatively similar effects on cellular cholesterol homeostasis (48).

INTRACELLULAR FC TRANSPORT TO LYSOSOMES AND THE *TRANS*-GOLGI NETWORK

A precursor–product relationship was identified between [^{3}H]FC from LDL in clathrin-coated vesicles, and in light, clathrin-free vesicles (d 1.06 g/ml) (41). The latter are likely to represent endosomes newly uncoated of clathrin. They copurify during density gradient ultracentrifugation with ^{125}I-labeled transferrin, which is present in secondary endosomes (Fig. 4). Unlike transferrin, LDL-derived FC was not recycled from secondary endosomes to the cell surface, but was recovered with protein markers of the *trans*-Golgi network (TGN) in vesicles of intermediate density (1.08–1.09 g/ml). It is not clear whether FC selectively internalized from LDL

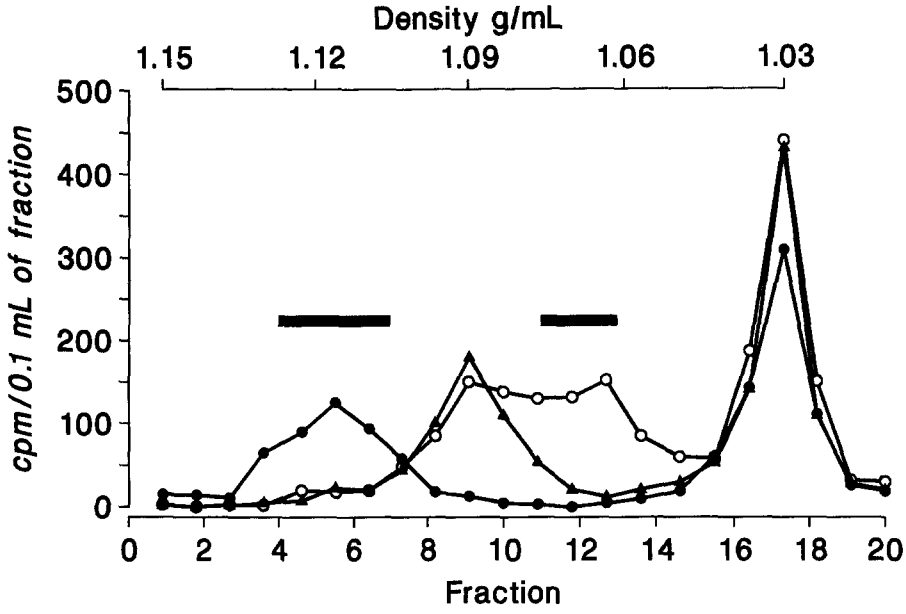

Fig. 4. Distribution of label among subcellular fractions of fibroblasts after the selective transfer of [^{3}H]FC from purified LDL. Closed circles: incubation for 2 h at 4°C, then for 0.5 min at 31°C after removal of LDL. Open circles: the same, but with 2 min incubation at 31°C in the absence of LDL. Closed triangles: the same, but after 15 min at 31°C in the absence of LDL. HDL was absent throughout the experiment to inhibit FC efflux. On these gradients, clathrin immunoreactivity co-migrated with transferrin (black bar) in dense (d 1.12 g/ml) primary endosomes. Transferrin was present (without clathrin) in light (d 1.07 g/ml) uncoated endosomes. On longer incubation, label was concentrated in an intermediate density (d 1.09 g/ml) fraction, together with TGN-specific protein markers (ref. 41).

transfers from primary endosomes through an acidic (lysosomal) compartment prior to reaching the TGN. In contrast, CE from HDL or LDL is hydrolyzed by lysosomal cholesterol esterase to FC, prior to further processing.

FC generated from LDL CE within lysosomes rapidly reappears in the plasma membrane at 37°C (49). It is also available for esterification by acyl CoA:cholesterol acyltransferase (ACAT) in the endoplasmic reticulum, without cycling through the plasma membrane (50). These reactions are inhibited by amphiphiles including plant sterols, intermediates of FC synthesis such as 6-dehydrocholesterol, progesterone, cholesterol-4-en-3-one, long chain fatty amines, and imipramine (51–54). FC accumulated in an intracellular membrane compartment that was identified with lysosomes on the basis of comigration at the top of density gradients with marker enzymes such as N-acetyl-β-glucosaminidase.

Amphiphiles block the transport of FC derived from LDL-CE as they do FC derived directly from LDL. This suggests a common transport mechanism. In Niemann-Pick C fibroblasts, whose phenotype strongly resembles normal fibroblasts treated with amphiphiles, the transport of FC from LDL-CE is inhibited. By electron microscopy, FC accumulated mainly in the TGN, with the balance within lysosomes (55). These data suggest that FC from LDL-CE, like LDL-FC, can be transferred to the TGN.

SORTING ROLE OF THE *TRANS*-GOLGI NETWORK (TGN)

The TGN is now recognized to play a key role in the sorting of newly synthesized and recycled proteins and lipids. The high level of FC in the TGN may represent the terminus of a standing gradient within the Golgi apparatus (4, 56, 57). The TGN generates at least three different transport vehicles (**Fig. 5**). *1*) Clathrin-coated TGN vesicles contain lysosomal enzymes, which fuse with endosomes containing lipids and proteins endocytosed from the cell surface (58). *2*) Clathrin-free vesicles stabilized by heteropolymeric coat proteins (COPs) move from the TGN to the cell surface (59). In polarized cells these vesicles carry proteins (including transmembrane proteins) and small solutes, such as neurotransmitters, to the basolateral surface. These transport vesicles are associated with an ATP-binding, N-ethylmaleimide sensitive factor (NSF) and one or more soluble NSF-accessory protein (SNAPs). Targeting of the vesicle to the plasma membrane is achieved by the interaction of a v-SNARE (cytobrevin) on the transport vesicle with a target protein (t-SNARE) on the recipient membrane. *3*) FC, sphingolipid-rich 'cytolipoprotins' (60) or 'rafts' (61) carry newly synthesized proteins, including GPI-anchored proteins, to the apical surface of polarized cells. Rafts are distinguished from other intracellular lipid complexes by their Triton-insolubility

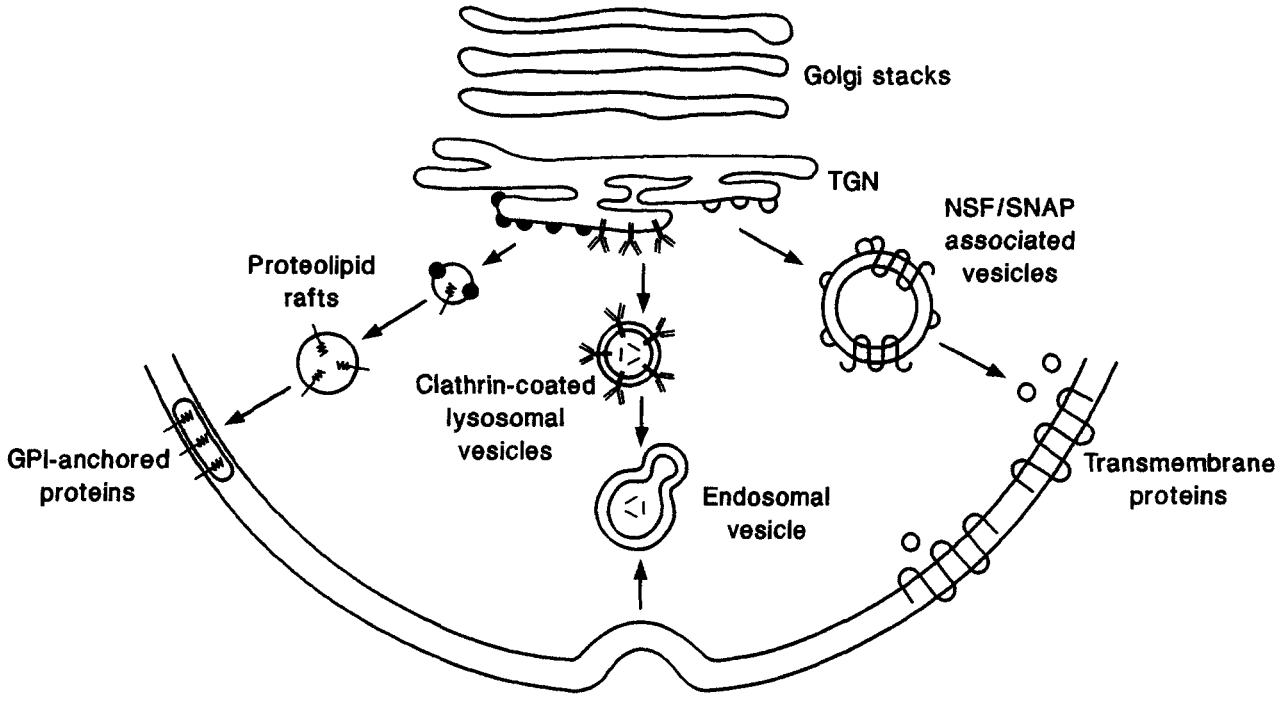

Fig. 5. Lipid and protein secretory pathways from the TGN. The figure shows the three secretory pathways that have been characterized. Clathrin-coated vesicles containing hydrolytic enzymes are targetted to the lysosomes to degrade endocytosed proteins and lipids (ref. 58). Transport vesicles associated with the NSF and SNAP proteins are targetted to the plasma membrane by cytobrevin (ref. 59). These vesicles carry transmembrane proteins to the basolateral surface of polarized cells. Proteolipid rafts containing sphingolipids, FC and GPI-anchored proteins are targeted to the apical surface of polarized cells, by factors as yet unidentified (ref. 61). Both pathways coexist in nonpolarized cells.

(62). In nonpolarized cells, both 'raft' and 'vesicle' pathways are present and functional (63). Microtubules may play a role in directing lipid rafts to the cell surface (64). Transport mediated by lipid rafts was not inhibited by antibodies to NSF or SNAP proteins in permeabilized cells (65). Several hydrophobic proteins ('proteolipids') including VIP17/MAL have been recovered in purified preparations of proteolipid rafts (66). While it is axiomatic that rafts are directed to some cell compartments (including the plasma membrane) and excluded from others (for example, endosomes), it is not clear whether any proteolipid yet described has a targeting function analogous to that of v-SNAREs (cytobrevins) of COP-coated transport vesicles. GPI-anchored proteins carried on glycolipid rafts to the cell surface can contribute to sorting these proteolipid complexes, as hybrids with the ectodomain of an apically directed protein, fused to a transmembrane protein normally sorted basolaterally, appeared at the apical surface (67).

Caveolin, the major structural protein of caveolae, was also present in purified lipid rafts in many cells (68). However, the raft-mediated transport pathway for GPI-anchored proteins was active in lymphoblastoma cells lacking caveolae (38). The mechanism of raft-mediated transport in polarized and nonpolarized cells appears to be the same (29, 63). This may indicate that caveolin is 'cargo' on plasma membrane-directed rafts in cells with caveolae. However, the possibility that caveolin plays a role in targeting lipid rafts to preexisting caveolae, as well as promoting FC concentration there, cannot be ruled out. These alternatives are discussed below.

FC TRANSPORT FROM THE TGN

Evidence of several kinds now makes it likely that FC normally moves from the TGN to the PM by 'raft' pathways also carrying sphingolipids and GPI-anchored proteins to the same target membrane.

Transfer of LDL-derived FC to the plasma membrane

When transfer of [^{3}H]FC from LDL to normal skin fibroblasts occurred at low temperature (<19°C), label accumulated in the intermediate density/TGN fraction (41). The mechanism of 'forward' transport of this accumulated lipid to the plasma membrane was determined by transferring the cells to 37°C. As shown in **Table 3,** movement of FC from the TGN to the plasma membrane was inhibited by amphiphiles including progesterone, cholesterol epoxide, and probucol. FC trans-

TABLE 3. Properties of basolateral ('vesicle'), apical ('raft') and FC transport

Inhibitor	Vesicle-Mediated[a]	Raft-Mediated[b]	FC[c]
AlF_3	Yes	No	No[d]
GTP-γS	Yes	No	No[d]
Brefeldin A	Yes	No	No
Vinblastine	Yes	No	No
Nocodazole	No	Yes	Yes
15°C	No	Yes	Yes

[a]Measured as the transport of stomatitis virus G protein from the TGN to the plasma membrane (63, 65).

[b]Measured as the transport of influenza hermaglutinin from the TGN to the plasma membrane (63, 65).

[c]Measured as the transport of LDL-derived or newly synthesized FC from the TGN or endoplasmic reticulum to the plasma membrane (41, and C. J. Fielding and P. E. Fielding, unpublished data).

port was also inhibited by nocodazole (41), as was the transport of caveolin and sphingolipids carried in rafts (69). Nocodazole inhibits microtubule formation in cultured cells, although it may be acting less specifically, as an amphiphile, when reducing raft-mediated transport to the cell surface. These agents did not affect the sorting of rafts to the apical surface in polarized cells (70). The same agents had little effect on the selective uptake of FC from LDL via the coated pits or the transport of FC from primary endosomes to the TGN (41). In contrast to vesicle-dependent transport, transfer of FC to the cell was resistant to brefeldin A and vinblastine (41) inhibitors of transport between *cis*- and *trans*-Golgi stacks (71, 72). There was also little or no effect of GTP-γS, pertussin toxin or Al^{3+}, inhibitors of GTP-dependent vesicular transport (C. Fielding and P. Fielding, unpublished data).

FC carried from the TGN appeared first in cell-surface caveolae, from which it could be released by HDL, but not LDL (73). The association of this FC with caveolae was shown by its unique accessibility to cholesterol oxidase in unfixed cells; and by its copurification with caveolin in purified preparations of plasma membranes. FC not released to medium lipoprotein acceptors transferred from caveolae to other domains of the plasma membrane.

In cells cultured in medium from which LDL had been removed, the transport of GPI-anchored proteins to the PM was reduced (74). This result suggests that the LDL-FC cycle could play an important role in intracellular protein transport by the apical/raft pathway.

Transfer of newly synthesized FC to the plasma membrane

The transport of FC synthesized in the endoplasmic reticulum, like that internalized by selective transfer from LDL, had properties resembling those of raft-mediated transport (Table 3). It was inhibited below 19°C, but not by classical inhibitors of basolateral protein transport including brefeldin A, monensin, and vinblastine (11, 12, 75). It was inhibited by nocodazole and progesterone (52). Newly synthesized FC, like that originating by selective transfer from LDL, was also transported to caveolae (73, 76). In spite of these similarities, more evidence is needed before the identity of TGN-derived transport complexes directed to the plasma membrane and endoplasmic reticulum can be assumed.

One laboratory, using FC-loaded fibroblasts, found FC efflux to native and particularly, delipidated HDL, to be inhibited by brefeldin A and monensin (77, 78). This report contrasts to the other studies described above, which used unloaded cells. It is possible that when FC levels are normal, transport from the TGN to the PM is raft-dependent, while in FC-loaded cells, basolaterally directed vesicles contribute to carrying excess FC to the cell surface.

Role of caveolin in FC transport

Caveolae represent the terminus, in fibroblasts, of FC transferred to the cell surface from the TGN. Their expression requires the presence of caveolin, a FC-binding protein. Caveolin is a FC-dependent gene up-regulated by LDL-derived FC (79). The expression of caveolae increased the rate at which FC was delivered to the PM (76). When caveolae were present, FC was delivered from the plasma membrane mainly to HDL, particularly the prebeta-HDL fraction (7, 73) which is a major acceptor of cell-derived FC in the extracellular medium. Cellular FC efflux to HDL was associated with an approximately 8-fold increase in the rate at which cellular FC was esterified and thus retained in the extracellular medium (80). As a result, the presence of caveolae is associated with an increase in both the rate and efficiency with which FC from the TGN could be removed from the cell. It will be of interest to characterize this pathway in other quiescent cells rich in caveolae, such as vascular smooth muscle and endothelial cells.

The $t_{1/2}$ of caveolin has been reported as approximately 10 h (69). This is much longer than the transit time of FC between TGN and caveolae (1–2 min) (41). Consequently, if caveolin is a component of the lipid rafts moving from the TGN and cell surface caveolae, FC-depleted caveolin must be reused and cycle back to the TGN. Two models of the role of caveolin are shown. Caveolin might be delivered to the PM for incorporation into caveolae only in response to a need for increased FC efflux (**Fig. 6, left**). In this model, the functional role of caveolin would be limited to the cell surface, while a different protein (such as the NPC factor) (55) would target rafts to the caveolae. Alternatively, caveolin could act like a cytobrevin to promote the specific docking of rafts at preexisting caveolae (Fig. 6, right). In this case, the primary role of caveolin would

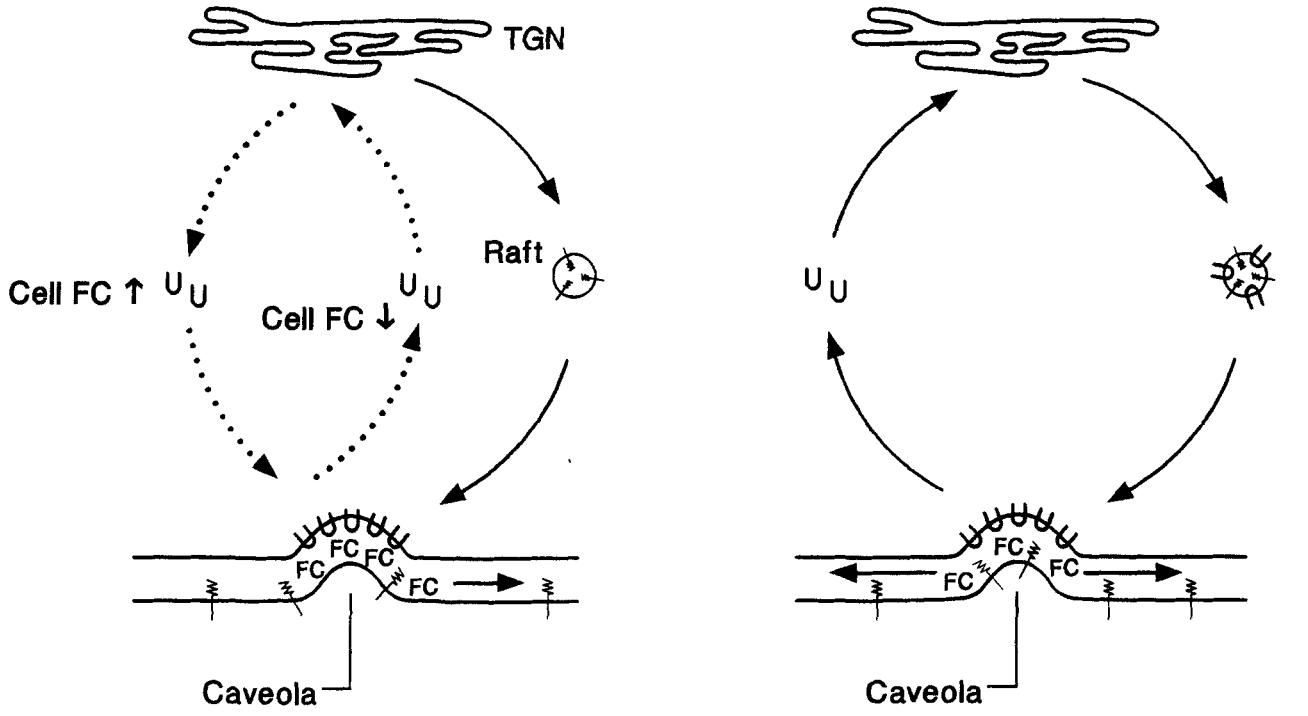

Fig. 6. Alternative models for the role of caveolin in the regulation of FC efflux. The left panel illustrates caveolin as a 'cholesterostat'. The model reflects the distribution of caveolin between cell surface and TGN pools according to cellular FC content. Caveolin would not be involved in targetting FC to the caveolae. It could be transported incidentally as 'cargo' on lipid rafts. The right panel shows caveolin acting as a 'cytobrevin' to target TGN-derived FC-sphingolipid rafts to the caveolae (ref. 76). After FC delivery, caveolin would dissociate and return to the TGN.

be as part of the raft-mediated shuttling of FC, sphingolipids, and GPI-anchored proteins between the TGN and cell surface. These alternative mechanisms cannot be distinguished on available data.

Our laboratory recently obtained evidence suggesting that the synthesis of caveolin responds to the rate at which LDL-FC enters the cell (79). Fibroblast monolayers selectively internalizing increased amounts of FC from LDL increased their caveolin mRNA levels and the rate of caveolin synthesis several-fold. This response was associated with a corresponding increase in FC efflux. As a result, cell levels of FC were maintained almost unchanged. Caveolin antisense DNA blocked the increase in caveolin mRNA levels induced by LDL, and also blocked the increase in FC efflux. These data suggest that the expression of caveolin can actively modulate FC efflux.

FC TRANSPORT TO THE ENDOPLASMIC RETICULUM

Acyl CoA:cholesterol acyltransferase (ACAT), localized to the endoplasmic reticulum, mediates all of cellular CE synthesis (review:81). CE is in rapid equilibrium with the regulatory pool of FC through the activity of neutral cholesterol esterase (82). Newly synthesized FC is preferentially utilized to form CE, although LDL-derived FC is also effectively incorporated (83). This selectivity is probably based on the spatial proximity of ACAT and enzymes of FC synthesis. Conversely, lipoprotein-derived FC was incorporated with greater efficiency into the plasma membrane than into CE. As cellular FC levels increased, ACAT was activated, but the level of ACAT mRNA was not increased. This finding indicates that ACAT activity is normally limited by the supply of FC (84, 85).

While not yet conclusive, available evidence suggests that FC moves from the TGN to the endoplasmic reticulum via raft-mediated, rather than vesicle-mediated transport. The pathway is resistant to brefeldin A (86). FC transport to the endoplasmic reticulum, like that to the PM, was inhibited by progesterone, as shown by the ability of this amphiphile to block the induction of CE synthesis which normally follows the receptor-mediated endocytosis of LDL cholesterol (87). Finally, several amphiphiles including progesterone, cholesterol α-epoxide, nocodazole, and probucol, inhibited the activation of caveolin gene transcription mediated by LDL-FC (79,

and P. E. Fielding, A. Bist, and C. J. Fielding, unpublished data). The targeting protein has not been identified, although this pathway is inhibited in Niemann Pick C disease (55).

The transcription of several FC-sensitive genes is now recognized to be mediated at the level of the synthesis and release of a family of nucleoproteins (sterol regulatory element binding proteins, SREBP) in the endoplasmic reticulum (88). A cysteine protease cleaves the SREBP transmembrane and cytoplasmic domains. The soluble cleavage product is directed to the nucleus. FC inhibits SREBP cleavage. SREBP binding to a sterol regulatory element (SRE) in the promoter region accelerates the transcription of FC sensitive genes including the LDL receptor protein (89), HMG-CoA reductase (90), and farnesyl pyrophosphate synthase (91). As a result these genes are down-regulated by FC.

The effects of LDL-derived FC on the expression of caveolin suggest a mechanism to explain FC homeostasis in quiescent cells such as fibroblasts. In these cells selective FC influx from LDL is largely unregulated. Nevertheless, the level of cellular FC is almost invariant over a wide range of medium concentrations of LDL. As a result, cellular FC content must be regulated by the rate of FC efflux. Transfer of FC from the TGN to the ER, and induction of a FC-sensitive protein other than SREBP, could explain the induction of caveolin mRNA levels in response to increased medium LDL levels, and compensatory promotion of FC efflux (79). Alternatively, SREBP itself could promote transcription of the LDL receptor and HMG-CoA reductase genes, while inhibiting caveolin gene transcription. A possible model to describe the role of caveolin in these effects is shown in **Fig. 7.**

RECYCLING AND EFFLUX OF FC

Mechanisms of FC efflux

In the presence of extracellular lipoprotein acceptors, particularly prebeta-HDL, a major part of FC reaching the caveolae from the TGN exits the cell from the caveolae (73). Homeostasis is maintained by the uptake of FC from LDL at the coated pits, such as that in confluent fibroblasts. Cellular FC remains constant within a 5% range despite a 10-fold change in extracellular LDL concentrations (7, 41). In lipoprotein-deficient medium, FC first enters the caveolae, then spreads laterally to other microdomains of the plasma membrane (76). GPI-anchored proteins and sphingolipids, reaching the cell surface in the same proteolipid rafts, may be dispersed in the same way, perhaps from a continuously growing 'rim' extending from the circumference of the caveolar 'bowl' (33).

There is now considerable evidence that two different mechanisms contribute to the transfer FC from the PM to the extracellular medium (reviews: 92–94). One, nonspecific, has kinetics consistent with passive diffusion. Desorption from the cell surface is considered rate-limiting, and may follow transfer of FC across the bilayer from the FC-rich cytofacial leaflet (1, 2). The second mechanism is protease-sensitive. It appears to reflect the interaction of prebeta-migrating HDL with FC within cell-surface caveolae in cells expressing these organelles and possibly, in the absence of caveolae, from FC-rich rafts within the exofacial leaflet of the PM. Based on this hypothesis, in the presence of normal lipoproteins, caveolae serve to increase the rate and specificity of FC efflux to HDL.

Studies of FC efflux from fibroblasts and macrophages have also been carried out using centrifugally isolated HDL or lipid-free HDL protein (apoA-I) (reviewed in 94). Protease-sensitive, 'specific' FC efflux to lipid-free apoA-I may correspond to efflux from rafts/caveolae on the exofacial leaflet of the plasma membrane bilayer. Based on this model, the genetic defect in apoA-I-mediated FC efflux found in fibroblasts from HDL-deficient (Tangier) fibroblasts (95) may involve a targeting protein of the 'raft' pathway between the TGN and the plasma membrane (Fig. 7). Nevertheless, significant differences in the acute regulation of FC efflux of this pathway have been reported, when native HDL or delipidated apoA-I was the extracellular acceptor. Phorbol esters and okadaic acid, which increase protein kinase C levels, inhibited FC efflux into native plasma (73, 96). Phorbol esters increased FC efflux to apoA-I under similar conditions (97).

Data obtained with lipid-free apoA-I have also been interpreted as evidence for direct interaction between delipidated apoA-I and an inducible cell-surface HDL-binding protein (94). Phospholipid efflux to delipidated apoA-I has been reported to precede FC efflux for both macrophages and fibroblasts (98). This suggests that interaction of cellular FC is with a partially lipidated, apoA-I–phospholipid complex. ApoA-I binding was greater to FC-rich than to control phospholipid vesicles (99). The 'induction' of apoA-I binding sites in cholesterol-enriched cells could be a reflection of this biophysical effect. Finally, an FC-inducible HDL-binding protein cloned earlier (100) has been identified as vigilin, a collagen-like protein expressed mainly in cartilage cells (101). While direct interaction of apoA-I with cell surface proteins cannot be ruled out, it remains possible that caveolae function mainly as a 'lens' to concentrate FC in the exofacial leaflet into microdomains of high concentration. Their narrow openings may pre-

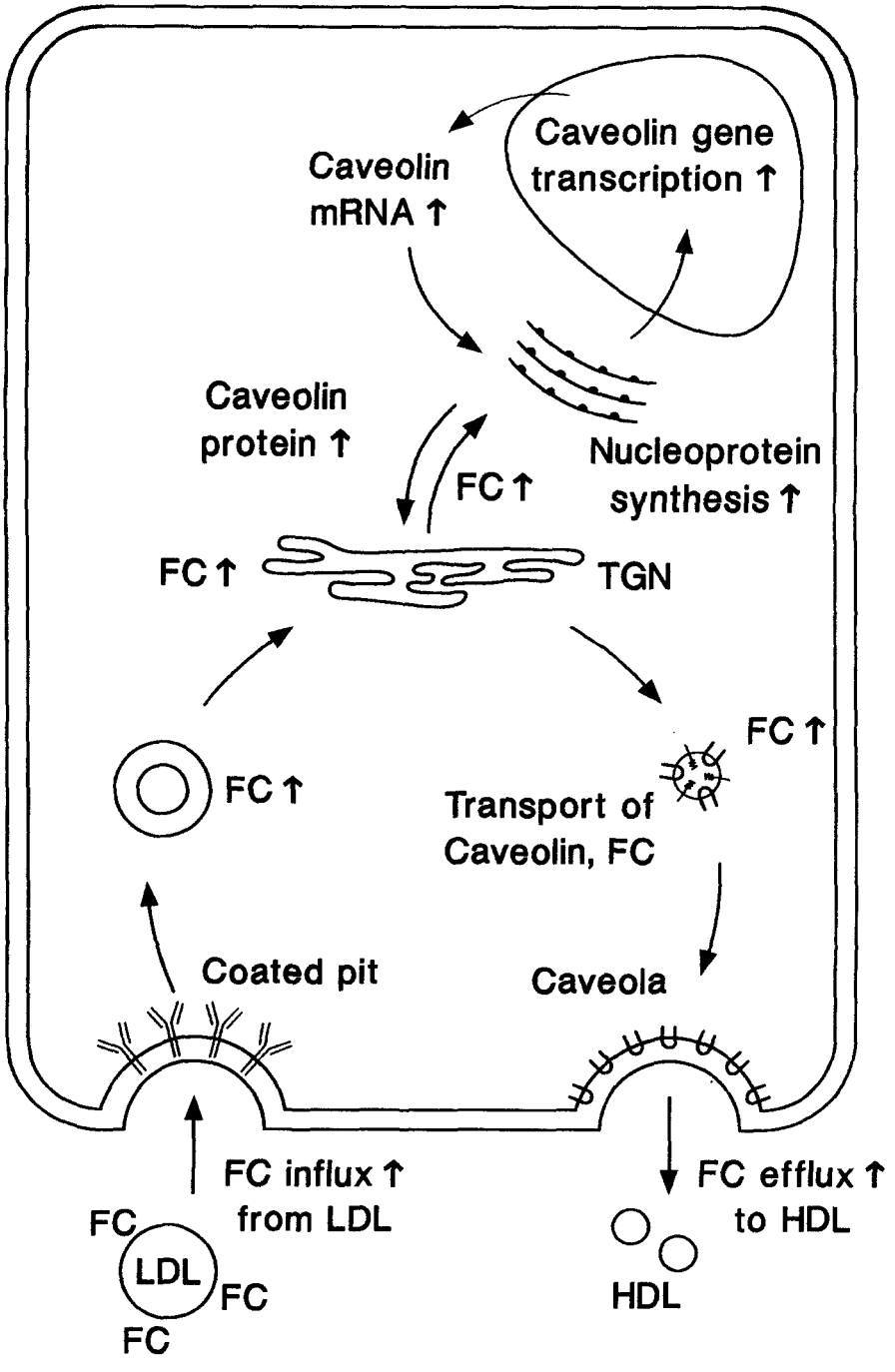

Fig. 7. A model to explain FC homeostasis in human fibroblast monolayers regulated by the expression of caveolin (ref. 79). After transfer of the cells to a medium containing increased LDL-FC concentration, selective uptake of LDL-FC would increase (ref. 9). This FC, transported to the TGN (ref. 41), would increase caveolin mRNA levels (ref. 79). We suggest a mechanism similar to that described for the LDL receptor protein (ref. 89) except that the regulatory nucleoprotein, possibly SREBP, would inhibit caveolin gene transcription, rather than activate as in the case of the LDL receptor gene. Increased synthesis would stimulate transfer of caveolin to the cell surface, promoting FC efflux (ref. 79). The symbols for clathrin (triskelion) and caveolin (U) are the same as in Figs. 2 and 6, respectively.

vent the access of larger lipoproteins. Together, these biophysical properties could provide a basis for the preferential interaction between prebeta-HDL (or apoA-I) and the cell surface.

Functions of FC efflux

Raft-dependent FC transfer to the plasma membrane maintains FC homeostasis in the presence of a wide range of medium FC concentrations, and is also the conduit for newly synthesized FC to the extracellular medium. Under physiological conditions, most cells are exposed to a fairly constant FC concentration, as part of intercellular fluid, lymph, or plasma. An additional function for FC efflux in normal metabolism is suggested from studies on the turnover of other raft components, GPI-anchored proteins and sphingolipids.

Lipid rafts, in the presence or absence of caveolin, deliver FC, sphingolipids, and GPI-anchored proteins to the cell surface. In physiological media, containing lipoprotein acceptors including prebeta-HDL, a major part of FC delivered to the PM is lost within a few minutes at 37°C. (73). The rate of efflux of sphingomyelin is lower by at least an order of magnitude (102) while little GPI-anchored protein or ganglioside is released into the extracellular medium (35). As a result, the sphingolipid-rich microdomains of the cell surface are likely to become increasingly FC-depeleted as they spread over the cell surface. In view of the recognized condensing effects of FC on phospholipid bilayers (1) it is possible that the loss of FC itself facilitates the spreading of GPI-anchored proteins (34) and possibly, their subsequent endocytosis. In any case, it seems clear that the rapid uptake of lipoprotein FC by the coated pits, its transfer through the cell, and its efflux via the caveolae or (in the absence of caveolae) from FC-rich rafts, may play an important role in the transport and distribution of sphingolipids and GPI-anchored proteins on the cell surface (74) in addition to the role of this pathway in FC homeostasis generally.

GPI-anchored proteins lack the sorting signals that direct transmembrane proteins to clathrin-coated pits. Their endocytosis from the PM takes place into noncoated vesicles (103). There has been controversy concerning the endocytosis of PM sphingolipids. Exogeneous sphingomyelin, like exogenenous FC, can be internalized via clathrin-coated pits (42, 104). On the other hand, PM gangliosides co-cap with GPI-anchored proteins such as thy-1 and are probably internalized together (105). Any FC remaining in rafts or caveolae after their interaction with extracellular lipoprotein acceptors may follow the same pathway. Potential mechanisms for recycling of FC, sphingolipid, and GPI-anchored proteins in the presence and absence of caveolae are shown in **Fig. 8.**

STEROL CARRIER PROTEIN (SCP) AND FC TRANSPORT

Sterol carrier proteins-2 and -x (SCP_2 and SCP_x) are lipid binding proteins normally present mainly in peroxisomes. SCPs are deficient or undetectable in peroxisome-deficient fibroblasts (106). Peroxisomes are a significant site of FC synthesis in hepatocytes and possibly other cell types (107). Both proteins are the products of a single gene; alternative translational start sites generate proteins of molecular weights 14 and 58 kD (108). Several effects of SCP-2 on FC transport and metabolism have been demonstrated.

In isolated cell fractions

When purified cell membrane fractions were incubated with SCP-2, the rate of FC exchange or transfer was in each case increased (109). Because an ATP-generating system was not present, this effect probably represents a facilitation of diffusion.

In intact cells

Transfection with SCP-2 cDNA in intact cells increased the proportion of cellular FC in the plasma membrane pool (110). There was a smaller effect of SCP-2 levels on FC influx (111). Peroxisome-deficient Zellweger syndrome fibroblasts, which contain little SCP_2 or SCP_x, showed a modest (−25%) reduction in FC efflux compared to normal cells (112).

Fibroblasts transfected with DNA antisense to SCP-2 showed delayed transfer of newly synthesized FC to the plasma membrane. This pathway, like that of raft-mediated FC transport under other conditions, was brefeldin-independent (113). Transfection with SCP-2 cDNA also increased the rate of steroid hormone production in steroidogenic cells (114). Also supporting a relationship between SCP-2 and FC transport, when cellular FC was increased in foam cells, SCP-2 mRNA and protein levels were increased several fold (115, 116). After fractionation of cholesterol-loaded cells, SCP_2 was recovered mainly in the cytoplasmic fraction (116). This finding contrasts with the peroxisomal origin of SCP_2 in unloaded cells.

In vivo

SCP-2 enhanced FC transfer to the plasma membrane (117). Rats injected with liposomes containing SCP-2 antisense DNA secreted 50% less FC into bile (118). As bile is secreted from the apical surface of the hepatocyte, this finding is consistent with a raft-mediated mechanism. Consistent with this, patients with cholesterol gallstones had enhanced hepatic SCP-2 levels (119). A majority of Zellweger patients showed evidence of impaired bile secretion (120) but as several steps of

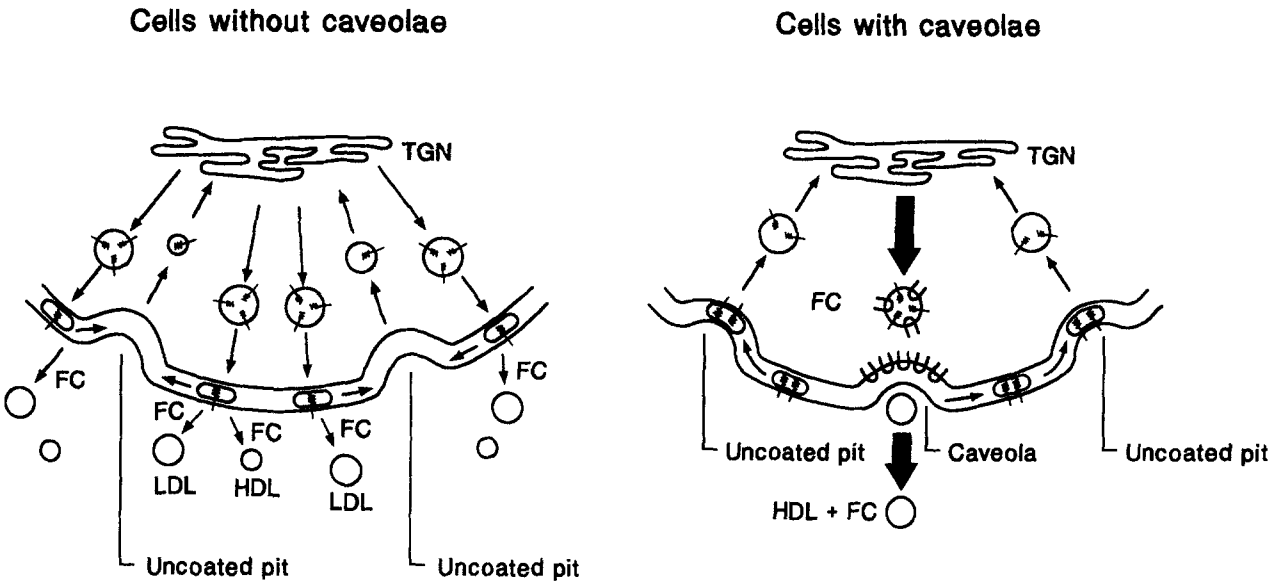

Fig. 8. A model to illustrate the role proposed for caveolae in accelerating FC transfer to the plasma membrane, and the targetting of caveolar FC to extracellular HDL (ref. 73). Transport between the TGN and cell surface in cells with or without caveolae is compared. Features shown are the recyclying of FC-depleted 'rafts' via uncoated pits (ref. 103) and the acceleration and selective desorption of cellular FC to HDL in the presence of caveolae (ref. 73).

bile acid synthesis are peroxisome-dependent, this finding may be unrelated to FC transport.

The balance of evidence suggests that SCP-2 can play a significant facilitating role in apical ('raft'-mediated) FC transport, particularly in hepatocytes, where peroxisomes are well developed. SCP-2 may facilitate FC incorporation into 'rafts' for transport to the plasma membrane or endoplasmic reticulum, possibly by increasing the effective concentration of FC in solution.

FC TRANSPORT IN DIFFERENTIATED CELLS

Most data on intracellular FC transport reviewed in preceding sections was obtained using fibroblasts or other poorly differentiated cells. To what extent do the conclusions drawn, particularly those concerning lipid rafts and caveolae, apply to specialized cells such as hepatocytes, macrophages, and vascular smooth muscle and endothelial cells?

Cells without caveolae: hepatocytes and macrophages

Hepatocytes are the major site of synthesis of plasma lipoproteins. These particles are secreted basolaterally. Hepatocytes are also the only site of catabolism of FC to bile acids. These are secreted apically to the bile canaliculi, along with FC and phospholipids. Hepatocytes represent a cell type whose cholesterol demand is high. Consistent with concepts put forward in earlier sections, these cells express a major portion of the high affinity LDL receptors detectable in vivo (121) and completely lack caveolin and caveolae. Transformed human hepatoma (HepG2) cells, which are imperfectly polarized, efflux FC to medium acceptors in vitro (122) possibly mainly from 'apical' plasma membrane domains.

Macrophages are specialized to scavenge and retain oxidized and aggregated cholesterol-rich lipoproteins and other particles. Consistent with this function, macrophages express high levels of scavenger receptors, which bind and internalize oxidatively modified LDL (8) but lack caveolin and caveolae (C. J. Fielding and P. E. Fielding, unpublished data). Because of the high level of peroxidases in macrophages, these cells contain relatively high levels of oxysterols. When macrophages internalize LDL, not only FC and cholesteryl esters but also oxysterols accumulate.

Oxysterols significantly decrease FC efflux from macrophages (79, 123) and the transfer of cellular FC to lipid-free or lipid-poor apoA-I (124). Macrophages retain increased levels of FC (125–128) despite the induction of ACAT (129).

Several mechanisms may contribute to the retention of FC within the macrophage. An induction of sphingomyelinase promoting the transfer of FC from the PM to the ER to stimulate ACAT activity (130) is inhibited by oxysterols (131). Oxysterols, possibly acting an amphiphiles, may also inhibit FC transport to the ER and

cell surface (51, 52, 79). The absence of caveolin from macrophages may prevent the induction of FC efflux that is associated with increased cellular FC in other cells (79).

Cells rich in caveolae: vascular smooth muscle and endothelial cells

Compared to macrophages or even fibroblasts, both smooth muscle and endothelial cells become cholesterol-enriched only with difficulty (132, 133). Human vascular endothelial cells took in little LDL via high-affinity receptors (132). Both smooth muscle and endothelial cells internalize FC from LDL by the selective pathway (C. J. Fielding and P. E. Fielding, unpublished data). Human endothelial cells were exceptionally active in effluxing FC to HDL in native plasma (134). In contrast, rat aortic smooth muscle cells are reported to efflux FC to apoA-I relatively slowly (97). The reason for this difference, and if it also applies to human smooth muscle cells, is not known.

In smooth muscle cells infected with herpes simplex virus, cholesteryl ester accumulated while FC efflux, which was otherwise rapid, decreased (96). This effect was mediated by viral protein kinase activity. Okadaic acid, which reduces protein phosphatase activity, also reduces the expression of caveolin and caveolae (24) and caveolar FC efflux (73). Both effects are opposed by staurosporine. It remains to be determined whether the effects of herpes virus on FC efflux are mediated by caveolin mRNA levels.

In summary, cells expressing many LDL receptors and few caveolae (Type A, Table 1) appear to be specialized to deliver cholesterol to cells. They regulate cholesterol at uptake, while FC efflux is passive or uncontrolled. In cells with few active LDL receptors and many caveolae (Type B, Table 1) cholesterol uptake seems to be poorly controlled, but FC efflux is regulated at the level of the caveolae. As a result, cells with many caveolae may be specialized to unload cholesterol.

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