Intracellular Cholesterol Transport in Synchronized Human Skin Fibroblasts†

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ABSTRACT: Normal human skin fibroblasts maintained in serum-containing medium were synchronized with aphidicolin. After removal of inhibitor, free cholesterol (FC) homeostasis was determined at intervals during the following cell cycle. FC mass per cell doubled following S-phase, and reached its maximum well before mitosis. This increase was mainly the result of stimulation of the rate of selective uptake of FC from medium lipoproteins, and reduction of FC efflux. Rates of cholesterol synthesis, endocytosis of intact low-density lipoprotein, and HDL receptor (CLA-1) activity were relatively low and little changed during the cell cycle. The expression of caveolin (structural protein of cell surface caveolae) and caveolar FC were decreased along with FC efflux. To test the hypothesis that regulation of caveolin expression could contribute to changes in FC efflux during cell division, cells were transfected with human caveolin cDNA, synchronized with aphidicolin, and then allowed to divide. In the transfected cells, caveolar FC and FC efflux were both increased. FC accumulation and entry into mitosis were markedly inhibited compared to controls. The contribution of transcriptional regulation to caveolin mRNA levels was determined with a 705 bp caveolin 5′-flanking sequence ligated to the pGL3 luciferase expression vector. Expression of the reporter gene was downregulated at S-phase of synchronized cells. Deletion of a hybrid E2F/ Sp1-like site between −139 and −150 bp abolished this downregulation. These data are consistent with a role for caveolin in cell cycle kinetics, which may be mediated, at least in part, at the transcriptional level.

Free cholesterol (FC)[1] is a major lipid component of the plasma membrane of mammalian cells. The plasma membrane normally contains >80% of total cell FC (*1*). In several peripheral cell lines, including fibroblasts, a major part of plasma membrane FC has been localized to the inner (cytofacial) leaflet of the membrane bilayer (*2*). Extracellular filipin, which binds selectively to FC, could access only a small part of plasma membrane FC. By electron microscopy, this FC pool was localized to caveolae, 60−80 nm invaginations at the cell surface of many peripheral cells (*3*). FC first transported to caveolae was subsequently distributed to other plasma membrane domains, or could be released to extracellular lipoprotein acceptors (*4*−*7*). Caveolae thus appear to represent cell surface microdomains where FC may be localized to the exofacial leaflet of the membrane bilayer, and portals through which intracellular FC transfers across the cell membrane prior to efflux.

In monolayers of quiescent peripheral cells in serum-containing media, FC homeostasis appears to be primarily maintained by FC which cycles between the cells and their extracellular medium (*8*). In confluent human skin fibroblasts, cell FC remained largely constant over a 10-fold range of medium FC. Under these conditions, the rate of FC influx, mainly from low-density lipoprotein (LDL), increased but FC efflux, mainly via the caveolae, increased in parallel. While quiescent fibroblasts have a very low net demand for FC, a different situation exists when these cells divide. To maintain FC content per cell in the expanding monolayer, total FC must double in the course of each mitotic cycle (24−28 h). The origin of this FC and the mechanism of its regulation have not been established. Several different pathways are available to supply the FC needed for rapid growth. FC synthesis and/or lipoprotein endocytosis could be transiently upregulated at one or more checkpoints during the cell cycle. The selective uptake of FC (*8*) or cholesteryl ester (CE)(*9*, *10*) could be increased. Finally, FC efflux from the cell might be decreased.

When unsynchronized human fibroblasts were virally transformed, and growth became unrestricted, the increased rate of cell division observed was associated with a reduction or disappearance of caveolae (*11*). In the present study, possible links between mitosis, the regulation of cellular FC transport, and the expression of caveolin were investigated using synchronized dividing human skin fibroblasts. The results obtained indicate that the regulation of caveolin expression, via its effects on FC efflux, may represent a major mechanism determining cellular FC content and mitotic rate in these cells.

MATERIALS AND METHODS

Cell Culture and Synchronization. Normal human skin fibroblasts and HeLa cells were maintained in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM). For individual experiments, cells were plated into

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[1] Abbrevations: FC, free cholesterol; CE, cholesteryl ester; LDL, low-density lipoprotein; HDL, high-density lipoprotein; GAPD, glyceraldehyde-3-phosphate dehydrogenase.

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3.5 cm dishes (1 mL of medium) at an initial density of (3−4) × 10^4 cells/dish, or with proportionately increased cell number and medium volume in 6 cm dishes. After 3 days, dishes were incubated with aphidicolin (4 μg/mL)(CalBiochem, San Diego, CA) to inhibit DNA polymerase, leading to cell arrest immediately prior to DNA synthesis (S-phase)-(*12*, *13*). After 18 h incubation with aphidicolin, inhibitor was removed for a further 8 h, and then restored at 8 μg/mL for a further 18 h. The second exposure to aphidicolin was previously shown to increase cell synchrony (*13*). After final removal of the inhibitor, the cells were incubated in 10% serum−DMEM for up to 32 h at 37 °C. Cell synchronization was assessed by flow cytometry using the cell-permeable stain Hoechst 33342 (Molecular Probes, Eugene, OR)(*14*). Cell suspensions were incubated at room temperature with 4 μg/mL Hoechst 33342 for 45 min. Samples were analyzed on a FACStar Plus (Becton Dickinson, San Jose, CA). Data were analyzed using Lysis II software (Becton Dickinson); 83.9 ± 3.1% of cells were in synchrony at the beginning of S-phase, consistent with literature values (*12*, *13*). DNA formation from [*methyl*-^{3}H]thymidine (NEN; 89 Ci mmol^{-1}; 10 μCi per 3.5 mL dish) was measured as the rate of synthesis of TCA-insoluble label over 30 min at 37 °C.

Determination of Cell FC and Protein Mass. Fibroblast monolayers were dissolved in 0.2 N NaOH, and then extracted with equal volumes of methanol and chloroform. Portions of the chloroform phase were dried under N_2 and then dispersed in 0.5% Triton X-100 in phosphate buffer (pH 7.4). FC mass was first determined with cholesterol oxidase using a fluorimetric assay (*15*). Esterified cholesterol mass was then determined from the fluorescence increment observed after addition of cholesterol esterase to the same solution. Esterified cholesterol was <5% of FC under the conditions of the present experiments. The FC content of undiluted fetal bovine serum was 65−69 μg mL^{-1}. Cell FC at the beginning of each experiment (that is, after final removal of aphidicolin) was 0.5−1.1 μg per 3.5 cm dish.

In some experiments, the cells were preequilibrated (24 h) with two changes of medium labeled with [1,2-^{3}H]FC (NEN, Boston, MA; 55−60 Ci/mmol). Briefly, labeled FC was dried under N_2, and dissolved in ethanol. The ethanol solution was injected during stirring into fetal bovine serum maintained at 37 °C in a proportion in most experiments of 5 μCi mL^{-1} medium. After equilibration (60 min, 37 °C), the serum was diluted into DMEM, and this medium was added to individual dishes of cells. Cellular FC specific activity was determined from FC mass, assayed as described above, and by liquid scintillation spectrometry. Cell and medium specific activity were both determined. These were consistent ±5% in the same experiment, and were (0.2−0.9) × 10^5 dpm μg^{-1} FC in different experiments.

Assay of FC Efflux and Influx. FC efflux was assayed as the transfer of [^{3}H]FC from labeled cells to 10% unlabeled serum−DMEM (*16*). Fetal bovine or human LDL was isolated by heparin−agarose affinity chromatography and by ultracentrifugation between density limits 1.02 and 1.050 g/mL. Equilibrium-labeled cells were washed (×3) in phosphate-buffered saline (PBS). They were then incubated for 10 min at 37 °C with unlabeled human LDL (50−80 μg/mL FC) to dissociate any labeled lipoprotein particles still adsorbed to the cell surface, representing <2% of cell cholesterol (<12 ng of FC, or <0.15% of LDL FC). This procedure was without effect on FC incorporated into the plasma membrane (*8*). After washing with PBS−human serum albumin (4 mg/mL, pH 7.4)(PBS−HSA) and 3 times with PBS, prewarmed unlabeled 10% plasma−DMEM was added for 3−5 min at 37 °C. Linearity of FC efflux under these conditions was established in preliminary experiments as previously described (*16*). In subsequent experiments, a 0.7 mL sample was collected, and chilled on ice. The sample was microfuged to pellet any cellular debris. Finally, a 0.5 mL portion of supernatant was taken for analysis of radioactivity. FC efflux was linear over the 5 min assay period. FC efflux was calculated from the rate of appearance of medium label and cell FC specific radioactivity.

FC influx was measured as the rate of uptake of [^{3}H]FC from labeled serum−DMEM medium (*8*). Unlabeled cells were washed with PBS−HSA and PBS, and then incubated for 5 min at 37 °C with [^{3}H]FC-labeled medium. Subsequently, the cells were incubated (10 min, 37 °C) with unlabeled LDL to exchange surface LDL, and then washed with PBS−HSA and PBS, prior to extraction with $CHCl_3$−methanol. Portions of $CHCl_3$ phase were collected for analysis. From these data, and the specific activity of plasma or LDL FC, the rate of FC influx was calculated. The contribution of changes in influx and efflux rates to the increase in cellular FC observed during the cell cycle was estimated by summing the hourly difference between these rates over 28 h.

Rate of FC Synthesis. [2-^{14}C]Acetate (NEN; 57 mCi mmol^{-1}) was incubated with arrested or dividing fibroblasts in 10% fetal bovine serum−DMEM at 37 °C. To establish conditions in which intracellular acetate pools were fully equilibrated, total FC synthesis rates over 28 h (one cell cycle) were assayed in the presence of 2 or 20 mM acetate [(1−5) × 10^6 cpm μmol^{-1}]. The cells were dissolved in 0.2 N NaOH. Cell lipids extracted into $CHCl_3$ were fractionated by thin-layer chromatography on silica gel plates developed in petroleum ether−diethyl ether−acetic acid 80/20/1 v/v. FC was then determined as previously described (*17*). Since synthesis rates were the same at both concentrations of acetate, subsequent incubations were carried out in 2 mM labeled acetate for 2−4 h at intervals during the cell cycle, using the same protocol.

Rate of LDL Endocytosis. Total endocytosis of LDL (sum of receptor-mediated and nonspecific uptake of intact lipoprotein particles) was estimated as the rate of production of TCA-soluble radioactivity generated from ^{125}I-protein-labeled LDL. LDL protein was labeled with ^{125}I by the iodine monochloride method (*18*) and then dialyzed overnight against PBS− 0.001 M disodium-EDTA, pH 7.4; >99% of label was precipitated with 10% w/v trichloroacetic acid. LDL protein specific radioactivity was 880−940 dpm/μg. Arrested or synchronized cells were washed 3 times with PBS, and then incubated with ^{125}I-labeled LDL in PBS−HSA for 1−2 h at 37 °C. Following incubation, protein was precipitated from the medium with TCA (final concentration 10% w/v). Supernatant (0.5 mL) was mixed with 5 μL of 40% w/v aqueous KI and 20 μL of 30% w/v H_2O_2. After extraction of free I with chloroform (1 mL), portions of the supernatant aqueous phase were assayed for ^{125}I radioactivity (*19*). The rate of endocytosis of LDL total cholesterol was estimated from the TCA-soluble ^{125}I-protein specific activity, multiplied by 1.33 to correct for the ratio between protein

(25.0% w/w), FC (8.6% w/w), and cholesteryl ester (41.9% w/w, equivalent to 25.2% sterol mass)(*20*).

Selective Uptake of HDL CE and Cholesterol Oleyl Ether. To determine the activity of the HDL receptor (CLA-1), fetal bovine or human HDL was isolated by centrifugal flotation between density limits 1.063 and 1.21 g/mL. Following dialysis into PBS−0.001 M EDTA, pH 7.4, HDL cholesteryl ester was labeled by exchange for 24 h at 37 °C with [^{3}H]-cholesteryl oleate (Amersham, Chicago, IL) adsorbed to Celite (*21*). More than 98% of cholesteryl ester label was recovered with HDL between the original density limits. The final specific activity was $(0.5-2.6) \times 10^5$ cpm/μg of CE. This HDL (final concentration 20−40 μg of protein/mL in PBS−HSA) was incubated with arrested or dividing cells for 1−2 h at 37 °C. Following incubation, surface-bound label was dissociated with unlabeled HDL (10 min, 37 °C) (*21*). Cell monolayers were washed with PBS−HSA and then 3 times with PBS. The cells were then assayed for CE label.

Selective uptake from HDL was also determined using the nonhydrolyzable labeled CE analogue [1α,2α-^{3}H]cholesteryl oleyl ether (Amersham Life Science, Arlington Heights, IL; 50.0 Ci/mmol)(*22*). The cholesteryl ether was incorporated into HDL as described above for cholesteryl oleate. The final specific activity was 3.2×10^4 to 2.7×10^5 cpm μg^{-1} HDL esterified cholesterol. Incubation with cell monolayers was for 2 h at 37 °C, followed by incubation with unlabeled HDL for 2 h.

Measurement of Cell Surface FC. Cell FC accessible to cholesterol oxidase was assayed in unfixed fibroblast monolayers which had been prelabeled to equilibrium with [^{3}H]-FC. Earlier studies by this laboratory (*4*, *5*) confirmed the original observation (*23*) that the caveolar fraction of plasma membrane FC is specifically modified under these conditions. Specifically, the cells were cooled on ice, and then washed with ice-cold PBS−HSA and then 3 times with PBS. Cholesterol oxidase (Boehringer-Mannheim, Indianopolis, IN) was added to a final concentration of 1.0 unit/mL. Incubation was carried out on ice for 4 h. Under these conditions, reactivity with cholesterol oxidase reached a maximum (*5*). No uptake of trypan blue was detected after 4 h incubation with cholesterol oxidase (<1 in 10^4 cells reactive). Cell permeability was also monitored as the appearance of lactate dehydrogenase (LDH) in the extracellular medium after 4 h with cholesterol oxidase. This was compared to the activity assayed in the same volume of cell lysate prepared with 0.5% sodium deoxycholate (*24*). LDH activity was assayed with a Sigma Diagnostics kit (Sigma, St. Louis, MO). Medium LDH activity was <0.1% that of total cell lysate, indicating negligible leakage of cell contents under the conditions used to measure cell surface FC. There was no difference in medium LDH levels in the presence or absence of cholesterol oxidase.

In individual experiments, cells which had been incubated with cholesterol oxidase were washed with ice-cold PBS, and solubilized overnight with 0.2 N NaOH. Following extraction with methanol and chloroform, portions of the chloroform phase were fractionated on silica gel layers on plastic sheets developed in petroleum ether−diethyl ether−acetic acid 80/20/1 v/v. FC (R_f 0.3) and its oxidation product cholest-4-en-3-one (R_f 0.45) were identified by their comigration with the corresponding lipid standards (Sigma). Lipid-containing areas, identified with iodine vapor, were excised, and ^{3}H-label was determined. >98% of the total ^{3}H label was recovered in the two fractions described.

Expression of Caveolin and CLA-1. Total RNA was purified with RNeasy kits (Qiagen, Chatsworth, CA). For Northern blotting, 4−10 μg of total RNA was applied to 1% agarose/formaldehyde gels. After transfer to nylon membranes, caveolin mRNA was identified using random-primed ^{32}P-labeled full-length caveolin cDNA (*26*) and quantitated from Kodak X-OMAT AR film with a computerized densitometer (*25*).

Caveolin protein was assayed from cell homogenates using 12% polyacrylamide gels. Separated proteins were electrotransferred to nitrocellulose sheets (0.2 μm pore size; S & S, Keene, NH). Following incubation with rabbit anti-human caveolin polyclonal antibody, blots were incubated with anti-rabbit IgG conjugated with horseradish peroxidase, and then visualized with SuperSignal CL-HRP substrate (Pierce, Rockford, IL). Following autoradiography, caveolin in different lanes was estimated by densitometry as described above.

The human cell-surface HDL receptor CLA-1 is homologous to the rodent receptor SR-BI (*27*). CLA-1 mRNA was probed with the oligonucleotide 5′-CAG AAT AGG CCT GAA TGG CCT CCT TAT CCT-3′, which corresponds to nucleotides 1514−1543 of the human CLA-1 cDNA sequence (*28*). Glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA (pHcGAP, ATCC/NIH Repository, Rockville, MD) was labeled with ^{32}P in the same way. The level of mRNA in each lane was estimated with a computerized densitometer as described above. Caveolin and CLA-1 mRNA levels during the cell cycle were expressed relative to GAPD in the same total RNA sample. In view of the low levels of CLA-1 previously detected in fibroblastic cells in published studies (*27*), an equivalent mass of total RNA from unsynchronized HeLa cells, which express ~10-fold higher levels of CLA-1, was assayed simultaneously.

Transfection with Caveolin cDNA. Fibroblasts were plated at a density of 1.7×10^5 cells per 6 cm dish. After 24 h, transfection was carried out with 8 μg/dish of wild-type caveolin cDNA subcloned into a pCDNA3 vector (InVitrogen, Carlsbad, CA). Transfection was carried out using the calcium phosphate coprecipitation method (Profectin; Promega, Madison, WI) in 10% fetal bovine serum−DMEM according to the manufacturer's instructions. After incubation for 16 h at 37 °C, the medium was changed, and aphidicolin was added using the protocol described above. Following removal of the inhibitor, total cell FC, FC reactive with cholesterol oxidase, and cell number were determined. Cells sham-transfected with empty vector were included in these experiments as controls. Transfection efficiency (47−54%) was determined using cells transfected with pSV-galactosidase.

Assay of Caveolin Gene Transcription Rates. A 705 bp *Bgl*II promoter fragment (*26*) was subcloned into the pGL3 luciferase vector (Promega) to generate pGL3-CAV. Human skin fibroblasts were cotransfected with pGL3-CAV, together with pSV-galactosidase to correct for any differences in transfection efficiency. The cells were then synchronized with aphidicolin according to the protocol described above. Immediately prior to the final removal of aphidicolin, and at intervals thereafter, individual dishes of cells were washed with PBS and lysed. Samples of lysate were assayed for

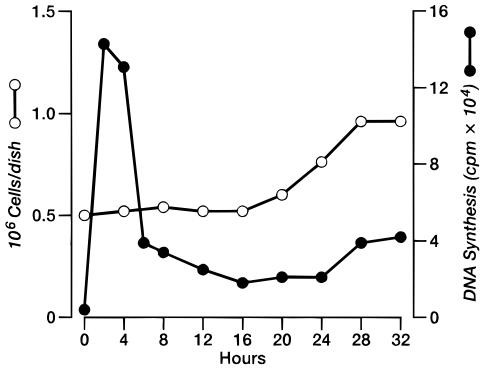

FIGURE 1: DNA synthesis and cell number as a function of time following removal of aphidicolin. Closed circles, incorporation of [^{3}H]thymidine into DNA. Open circles, cell number.

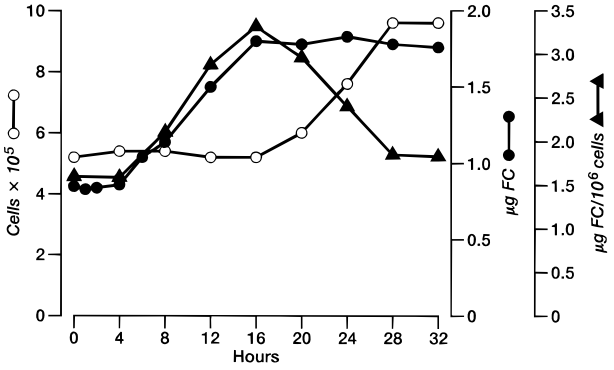

FIGURE 2: Cell FC content of human skin fibroblasts as a function of time following removal of aphidicolin. Cells in fetal bovine serum−DMEM were synchronized as described under Materials and Methods. Following removal of inhibitor, dishes were taken at intervals for determination of FC mass and cell number. Open circles, cell number/6 cm dish; closed circles, FC mass per 6 cm dish over the same time course; closed triangles, FC mass per 10^6 cells over the same time course.

luciferase activity with an analytical luminometer (Monolight; Analytical Luminescence Laboratories, San Diego). Galactosidase activity measured with X-Gal (CalBiochem, La Jolla, CA) was used as above as an internal control to correct for differences in transfection efficiency.

The caveolin promoter sequence TTGGCGGGCGGC at −139 to −150 bp contains overlapping E2F- and Sp1-like binding sequences (TTGGCGC and GGCGGGCGGC). Using the published caveolin gene 5′-flanking sequence (*26*), an oligonucleotide was synthesized of the sequence between −125 and −165 bp deleting residues −139 to −150 bp. Synthesis of mutant promoter DNA was carried out with a Stratagene QuickChange site-directed mutagenesis kit to generate the mutant promoter. Mutant and wild-type base sequences were reconfirmed prior to use in individual transfection experiments.

RESULTS

Sources of FC in Dividing Cells. Following removal of aphidicolin, the FC mass of synchronized fibroblast monolayers remained unchanged for 2−4 h. This interval corresponded to the peak of S-phase, determined from the rate of DNA synthesis with [^{3}H]thymidine (Figure 1). Total cell FC increased over the next 8−12 h. At the end of this period, FC content per cell had doubled. Over this interval, the increase in cell FC was approximately linear with time. FC then remained unchanged for the rest of the cell cycle. No increase in cell number was observed prior to 20−24 h, but it had doubled by 28 h. Following cell division, FC per cell returned to its original value (Figure 2). In cells maintained in aphidicolin over the same period, there was no activation of DNA synthesis, nor did FC increase. These data show that the increase in cellular FC preceded cell division by 10−12 h in dividing human skin fibroblasts.

Those pathways contributing to increased cell FC mass were identified by assaying the rates of FC synthesis, LDL endocytosis, the selective uptake of lipoprotein FC and CE, and FC efflux during the cell cycle. In the arrested cells, rates of influx and efflux were similar as expected (*8*)(Figure 3). In contrast, following the removal of aphidicolin, the rate of FC efflux decreased, and was not completely restored to base line values until 16 h had elapsed. Over a similar period, the selective uptake of FC from LDL increased. By 16 h, FC influx and efflux were once more equivalent.

Total FC synthesis was 22.4 ± 0.6 and 22.0 ± 2.2 ng over 28 h in the presence of 2 and 20 mM acetate,

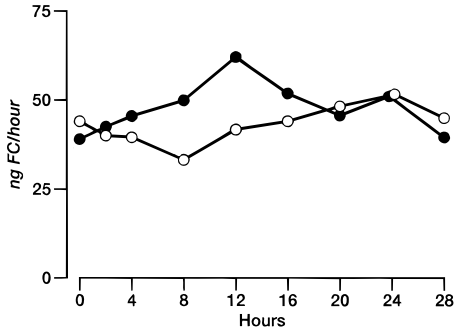

FIGURE 3: FC homeostasis in synchronized human fibroblasts. Open circles, FC efflux measured as the rate of transfer of [^{3}H]FC from labeled skin fibroblasts to unlabeled 10% fetal bovine serum medium. Cell FC specific activity was 0.69×10^5 dpm/μg. Closed circles, FC influx determined from the rate of uptake of radioactivity from [^{3}H]FC equilibrated 10% serum (specific activity 0.69×10^5 dpm/μg) into unlabeled fibroblast monolayers.

respectively (6 experiments, difference not significant), showing that cell acetate pools had been fully equilibrated. Over the same period, FC mass in the same dishes increased 760 ± 60 ng (6 experiments). These experiments showed that newly synthesized FC contributed about 3% of the total increase in FC mass which occurred during the cell cycle. FC synthesis was also measured in 2.0 mM acetate both in cells arrested with aphidicolin and at 2−4 h intervals following its removal. The rate of FC synthesis in the arrested cells (1.5 ± 0.6 ng h^{-1})(n = 5) was unchanged during the following 28 h. This result indicated that synthesis was not upregulated over the period during which cell FC mass increased.

Endocytosis of [^{125}I]LDL was determined under the same conditions of arrest and release into cell division as described above, at concentrations of fetal bovine or human LDL (6.5 μg of FC mL^{-1}) equivalent to those in 10% fetal bovine serum. In fetal bovine serum, like human serum, the major plasma lipoprotein is a FC-rich LDL (*29*, *30*). In arrested cell cultures, the rate of LDL protein degradation was 1.5 ± 0.5 ng h^{-1} (equivalent to 0.5 ng h^{-1} LDL FC). This rate rose slightly (+50%) after 2 h and then decreased to its original value at 4 h and for the remainder of the cell cycle. There was no difference in the rates of degradation of fetal bovine and human LDL. Total cholesterol entering the cell

Table 1: Contributions of FC Influx and FC Efflux to the Increment of Total Cell FC[a]

expt	ΔFC[b]	Δinflux[b]	Δinflux (μg of FC)$^{-1}$	Δefflux[b]	Δefflux (μg of FC)$^{-1}$
1	1.10	0.42	0.38	0.40	0.38
2	0.54	0.29	0.53	0.24	0.44
3	0.70	0.38	0.54	0.30	0.43
4	0.70	0.34	0.48	0.30	0.42
mean ± SD	0.76 ± 0.24	0.36 ± 0.05	0.47 ± 0.06	0.31 ± 0.07	0.42 ± 0.03

[a] Individual values are from three 3.5 cm dishes at each point. The increase in FC mass was determined fluorometrically. Influx was estimated from using [^{3}H]FC-labeled serum medium over 5 min at 37 °C, as described under Materials and Methods. Efflux was determined as the rate of transfer of [^{3}H]FC (5 min, 37 °C) from labeled cells to unlabeled medium as described under Materials and Methods. [b] As micrograms of FC.

by the endocytosis of LDL over 28 h was 30 ± 5 ng, representing about 4% of the total increase in cellular FC over the same period.

The contributions of changes in FC influx and FC efflux to the total increase in cell FC mass over the same period were calculated as described under Materials and Methods (Figure 3). Total decrease in FC efflux from base line rates over 16 h after initiation of the experiment averaged 310 ng (0.31 ± 0.07 μg, 4 experiments). Total increase in FC influx from base line rates under the same conditions was 360 ng (0.36 ± 0.05 μg, 4 experiments)(Table 1). There was no significant difference between influx and efflux rates over the remainder of the cell cycle. These data indicate that decreased FC efflux and increased selective uptake of FC from LDL were important mechanisms leading to FC accumulation during the cell cycle.

The rate at which CE was internalized from fetal bovine or human [^{3}H]CE-labeled HDL was also determined, using a lipoprotein FC concentration of 6.5 μg mL^{-1}. CE uptake under these conditions represents the sum of CE internalized by the endocytosis of intact HDL particles, and the selective uptake of HDL-CE. Recovery of label from the incubated cells was undetectable (<0.3 ng of FC h^{-1}) under the conditions described, both in arrested cells and following release into cell division.

This finding might be the result either of an effective absence of HDL receptor activity or of an efficient recycling of internalized CE, after hydrolysis, to the extracellular medium. To address this point, measurements were also made using HDL labeled with [^{3}H]cholesteryl oleyl ether, in place of [^{3}H]cholesteryl oleate. Using this tracer, uptake of label in arrested cells was equivalent to 3.0 ± 1.2 ng of FC h^{-1} (μg of cell FC)$^{-1}$ (4 experiments). Using the correction factor described under Materials and Methods, this represented about 6% of the total uptake of cholesterol by aphidicolin-inhibited cells. Uptake of ether label from HDL decreased to <2% of FC influx over the period (4−12 h) during which FC was accumulating in the cells, before returning toward base line after 16 h.

Regulation of FC Efflux during the Cell Cycle. Published studies have indicated that the expression of caveolin is linked to the level of intracellular cholesterol (*25*, *31*) and could mediate, at least in part, FC efflux from quiescent cells, by regulating the level of FC in cell surface caveolae (*4*, *5*). The relevance of this mechanism for synchronized dividing skin fibroblasts was determined by comparing caveolin expression and FC efflux over the cell cycle. Following removal of aphidicolin, there was a marked decrease in the expression of caveolin mRNA (Figure 4). Caveolin mRNA levels declined steeply at 1−2 h, and then recovered to base line over the next 8−12 h (Figure 5). In contrast, CLA-1

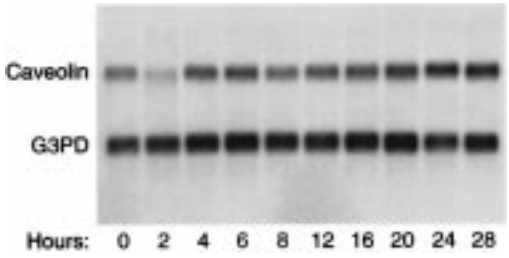

FIGURE 4: Caveolin mRNA from arrested and dividing human skin fibroblasts. After electrophoretic fractionation of total RNA and transfer to nylon screens, caveolin and GAPD mRNAs were visualized with ^{32}P-labeled hybridizing cDNAs as described under Materials and Methods.

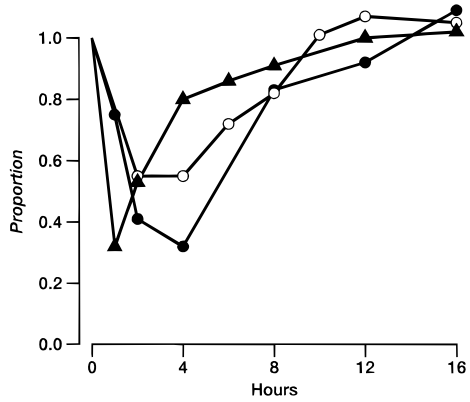

FIGURE 5: Parameters of caveolae expression in dividing human skin fibroblasts. Closed triangles, caveolin mRNA normalized to that of the housekeeping gene GAPD. Open circles, caveolin protein assayed with rabbit polyclonal anti-human caveolin antibody. Closed circles, cell surface FC assayed with cholesterol oxidase expressed as a ratio to unmodified cell FC. Measurements were made as described under Materials and Methods. Values are expressed relative to the same parameters in arrested cells (zero time).

mRNA levels, which were low in arrested cells (0.1−0.2 those in HeLa cells), were unchanged over the cell cycle. Caveolin was assayed in extracts from arrested and dividing cells over the same time course. A decrease in caveolin protein similar to that of caveolin mRNA was observed. Caveolar FC was estimated in terms of its selective reactivity with cholesterol oxidase in cells preequilibrated with [^{3}H]-FC (*5*, *23*). This parameter, like caveolin mRNA and protein, also decreased sharply at 2−4 h after removal of aphidicolin. It gradually returned to base line (arrested) levels over the next 8−12 h.

Effects of Caveolin Overexpression on FC Transport and Rate of Mitosis. The preceding experiments confirmed in dividing cells the correlation between FC efflux and caveolin expression reported earlier, but did not establish a causal relationship between these parameters. FC homeostasis was

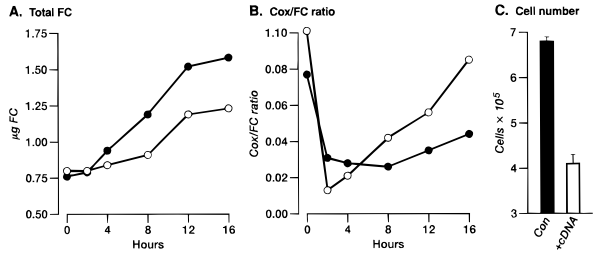

FIGURE 6: Effects of transfection with human caveolin cDNA on FC homeostasis and mitosis in human skin fibroblasts. Conditions of transfection and cell culture are described under Materials and Methods. (A) FC mass following release from aphidicolin-mediated cytostasis in sham-transfected cells (closed circles) and caveolin cDNA-transfected cells (open circles). (B) The proportion of total cell FC accessible to cholesterol oxidase under the same conditions in sham-transfected cells (closed circles) and caveolin cDNA-transfected cells (open circles). (C) Cell number 32 h following removal of aphidicolin. Initial cell number (zero time) was 3.4×10^5 cells. Con, sham-transfected cells; +cDNA, cells transfected with caveolin cDNA.

Table 2: FC Efflux from Synchronized Caveolin cDNA-Transfected and Control Cells

	0 h	12 h	24 h
caveolin transfected[a]	34.8 ± 2.6[c]	35.6 ± 17[c]	50.8 ± 2.2[d]
control[b]	26.0 ± 0.9	26.5 ± 0.9	30.3 ± 1.7

[a] Fibroblast monolayers transfected with caveolin cDNA in the correct orientation. [b] Fibroblast monolayers transfected with empty vector. Efflux per microgram of cell FC is expressed as nanograms of FC per hour transferred from monolayers equilibrium-labeled with [^{3}H]FC to serum−DMEM medium at 37 °C. Values shown are means ± one SD from three transfections. [c] $p < 0.05$. [d] $p < 0.02$.

therefore measured in synchronized cells which had been transfected with human caveolin cDNA prior to release from aphidicolin-mediated cytostasis. In [^{3}H]FC labeled aphidicolin-arrested cells and at intervals following removal of the inhibitor, total and caveolar FC and FC efflux were measured in transfected and control cultures. Cell number was also measured, at zero time when aphidicolin was first removed, and 32 h after removal of inhibitor. Control assays were carried out simultaneously in cells transfected with empty vector.

Transfection with caveolin cDNA reduced the accumulation of FC observed when synchronized cells entered the cell cycle. The increase in FC seen in transfected cells 16 h following removal of inhibitor was only 44 ± 4% of that measured in nontransfected cells (Figure 6A). Under the same conditions, FC efflux was 33−68% greater in transfected than in sham-transfected fibroblast cultures (Table 2). Transfection with empty vector was without effect on FC accumulation relative to nontransfected control cultures. Significant differences were also seen in cell surface FC reactive with cholesterol oxidase in cells transfected with caveolin cDNA, compared to controls. Caveolar FC measured with this assay was about 50% higher in arrested transfected cells compared to controls. Following entry into S-phase, there was a rapid decrease in caveolar FC in both transfected and control cells, but in the cells transfected with caveolin cDNA, recovery toward initial values was much more rapid, and by 24 h caveolar FC was double that measured in sham-transfected cells (Figure 6B). These data are consistent in indicating a stimulation of FC transport and efflux in transfected cells, particularly in later stages of the cell cycle. Finally, transfection with caveolin cDNA significantly reduced the rate of cell division at 32 h (Figure 6C). At 32 h after removal of aphidicolin, the increase in cell number in the caveolin transfected monolayers was only 15.7 ± 6.5% of the increase observed in control dishes (3 experiments).

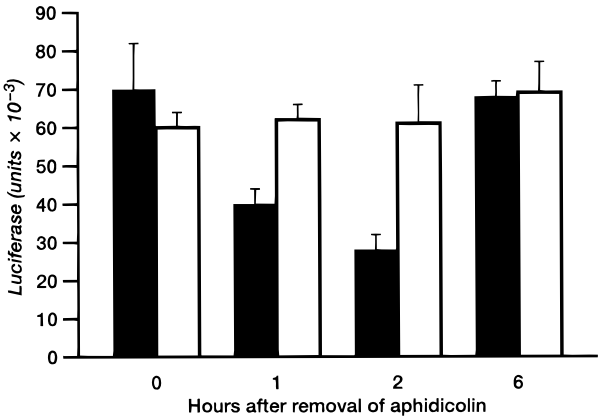

FIGURE 7: Regulation of caveolin gene transcription at S-phase. Cell monolayers were first transfected with pGL3 luciferase vector ligated to either the wild-type human caveolin promoter fragment (closed bars) or the mutant fragment from which −139 to −150 bp had been excised (open bars) as described under Materials and Methods. The cells were then synchronized with aphidicolin. Immediately before, and at the intervals shown after removal of inhibitor, the luciferase activity of cell lysates was determined. Values shown represented means ± one standard deviation (3 experiments).

Transcriptional Regulation of Caveolin Expression in Dividing Cells. The rate of caveolin gene transcription was determined in cells transfected with wild-type caveolin promoter ligated to the luciferase expression vector pGL3. The transfected cells were then synchronized using aphidicolin. Following removal of inhibitor, luciferase yield was assayed at intervals during the cell cycle. As shown in Figure 7, there was a marked decrease in the expression of the reporter gene during S-phase, with complete recovery by 6 h. In contrast, when the same measurements were carried out using the caveolin mutant promoter from which bp spanning −139 to −150 had been deleted, luciferase expression was constant over the cell cycle.

DISCUSSION

When confluent human fibroblasts are maintained as monolayers in serum-containing medium, the need for new FC is low. Nevertheless, under these conditions, FC continues to be selectively internalized from medium lipoproteins, particularly LDL. Homeostasis is maintained by efflux of FC at an equivalent rate to the extracellular medium (*8*). FC influx and efflux were modified in parallel over at least a 10-fold range of extracellular FC concentration. This balance was not the result of increased simple exchange at the cell surface because FC was taken up from LDL mainly via coated pits, while much of FC efflux occurred from the caveolae, mostly to HDL. Additionally, pulse−chase experiments identified intermediate steps in the intracellular transport of LDL-derived [^{3}H]FC to caveolae (*5*). In skin fibroblasts, FC homeostasis was mediated, at least in part, by the up- or downregulation of caveolin, the structural protein of the caveolae which terminated the FC cycle in

these cells. Changes in caveolin level were associated with parallel changes in the expression of caveolae at the cell surface (*31*). The 5′-flanking region of the caveolin gene includes FC-responsive elements that upregulate caveolin mRNA levels in response to an increase in cellular FC (*25*). These data appear to support the hypothesis (*32*) that changes in the expression of caveolin might regulate FC efflux by adjusting the number and FC content of cell surface caveolae. In the present research, the mechanisms by which the FC requirements of dividing human fibroblasts in culture are met were investigated.

Mechanisms of FC Accumulation in Dividing Cells. In cultures of exponentially dividing cells, homeostatic mechanisms controlling the FC content of quiescent cell monolayers must be modified to allow the FC content per cell to remain the same as the cell number increases. The present study shows that in dividing human skin fibroblasts which had been synchronized with aphidicolin, FC mass increased during a relatively short period (8−12 h) of the 24−28 h cycle. Additionally, this change was complete well before the end of the cell cycle (mitosis). Outside this period, cellular FC remained almost constant. While both cholesterogenesis and the endocytosis of LDL contributed slightly to the rise in cellular FC which was observed, the major contributors were an increase in the selective uptake of FC and a decrease in FC efflux. That is, in dividing cells, FC accumulated mainly as the result of a temporary imbalance between the influx and efflux pathways that stabilize the FC content of quiescent cells. Quantitative measurements indicated that on average about 93% of the doubling in cellular FC assayed during the cell cycle was contributed by these two pathways under the conditions described.

The significance of the HDL receptor (SR-BI, CLA-1) in the selective uptake of CE from HDL in cells utilizing FC for steroid hormone and bile acid production is now well established (*10*). In the present study, the activity of the HDL receptor in human skin fibroblasts was very low, consistent with previous measurements in fibroblastic cells (*22*). HDL receptor activity assayed with nonhydrolyzable cholesterol oleyl ether decreased as the cells entered S-phase, during the period in which cellular FC mass increased. Its expression was low but unchanged over the same period. It is of interest that exogeneous FC loading was previously shown to depress the selective uptake of cholesterol ether (*22*). The parallel changes in HDL receptor activity and caveolin expression may reflect, at least in part, the localization of the receptor to caveolae, previously reported in CHO cells (*33*). Like cholesterol synthesis and LDL receptor-mediated endocytosis, selective CE uptake appears not to play a major role in FC balance in these cells. While the proportions of FC contributed by different mechanisms are likely to differ in different cell lines, the influx of preformed extracellular FC could be a significant contributor to cellular FC homeostasis in vivo.

Mechanism of Inhibition of FC Efflux during Cell Division. The mechanism by which FC is selectively internalized from plasma lipoproteins is presently not fully understood, although the pathway shares kinetic properties with those of receptor proteins internalized via clathrin-coated pits (*5*). FC influx from LDL may be itself receptor-mediated, or the changes observed in FC uptake during cell division could reflect the rate of coated vesicle formation. In contrast, the inhibition of FC efflux observed over the same time period was associated with marked changes in the expression of caveolin, a protein now broadly implicated in intracellular FC transport and efflux (*4*−*7*). Caveolin antisense DNA downregulated both caveolin and FC efflux. The expression of caveolin and FC efflux were upregulated in parallel in the presence of increased levels of medium LDL, and caveolae and caveolin were downregulated as cell FC decreased. The intracellular transport of both recycling and newly synthesized FC to the caveolae has been demonstrated.

The present study identifies new links between these factors. Caveolin mRNA and protein levels were strongly downregulated shortly after entry into the cell cycle. Over a similar period, caveolar FC and FC efflux were decreased. Transfection with caveolin cDNA was associated with increases in caveolar FC and FC efflux, and an inhibition of FC accumulation. Transfection with caveolin cDNA was also associated with a significant lag in cell division. This is consistent with the decreased growth rate in anchorage-dependent, caveolin-transfected cells reported earlier (*34*). Taken together, these data provide strong evidence that the mechanism by which FC efflux is downregulated at S-phase depends on a corresponding decrease in the expression of caveolin.

Mechanism of Inhibition of Caveolin Expression during Cell Division. The expression of many genes important in cell cycle regulation is modified via the effects of transcription factors E2F and Sp1. Examples include cyclins and cyclin-dependent kinases, as well as enzymes of DNA synthesis such as polymerase D (*35*, *36*). The caveolin gene 5′-flanking sequence includes an unusual 'hybrid' site made up of overlapping E2F- and Sp1-like consensus sequences. Caveolin gene transcription was strongly downregulated at S-phase. This effect was absent following deletional mutagenesis of the −139 bp to −150 bp sequence, while 'baseline' (zero time) transcription rates were unaffected. The present study thus identifies a novel, additional mechanism which regulates caveolin transcription. Further research will of course be required to establish conditions under which this site is occupied at different points in the cell cycle.

Transcriptional regulation is unlikely to be the only mechanism by which caveolin levels can be regulated during the cell cycle. The decrease in caveolin mRNA and protein levels observed in this study is greater than would be predicted from the reported $t_{1/2}$ of caveolin protein (*37*), suggesting additional contributions for accelerated mRNA and protein turnover in the overall regulation of expression. Nevertheless, the identification of a transcriptional mechanism, dependent on the G/C-rich promoter sequence at −139 to −150 bp, suggests integration of the effects of caveolin with other, better-recognized cell cycle proteins.

In summary, the present study provides evidence that among its effects, caveolin can contribute significantly to cell cycle regulation via mechanisms mediated through FC homeostasis and transport.

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