Intracellular Sterol Distribution in Transfected Mouse L-cell Fibroblasts Expressing Rat Liver Fatty Acid-binding Protein*

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The potential role of liver fatty acid binding protein (L-FABP) in modulating cellular sterol distribution was examined in mouse L-cell fibroblasts transfected with cDNA encoding L-FABP. L-cells were chosen because they contain only a small amount of endogenous FABP which does not bind [3H]cholesterol, does not enhance intermembrane sterol transfer, and whose content is unaltered by the expression of L-FABP. Transfected L-cells expressed 0.34% of cytosolic protein as L-FABP. Transfection alone with low expression of L-FABP (0.008% of cytosolic protein) had no effect on any of the parameters tested. Three aspects of cellular sterol transfer were examined. First, cellular sterol uptake, monitored by [3H]cholesterol and the fluorescent sterol, Δ-5,7,9(11),22-ergostatetraen-3β-ol, was increased 21.5 ± 2.8% (p < 0.001) in L-cells expressing L-FABP. This increase was not accounted for by increased sterol esterification in the cells expressing L-FABP. Inhibition of both cholesterol transfer and esterification with 3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide from Sandoz abolished the L-FABP related enhancement of both [3H]cholesterol uptake and esterification.

Second, plasma membrane transbilayer distribution of sterol, determined by fluorescence methods indicated that the majority of sterol was in the inner leaflet of the plasma membrane. In transfected cells expressing L-FABP, twice as much sterol (28 ± 4%) was present in the exofacial leaflet of the plasma membrane as compared to that of control cells (15 ± 2%). Third, expression of L-FABP enhanced sterol transfer from the plasma membrane to microsomes in intact cells. Treatment of [3H]cholesterol or [3H]oleate-loaded cells with sphingomyelinase resulted in increased formation of radiolabeled cholesterol ester, consistent with enhanced microsomal esterification of plasma membrane derived cholesterol. Concomitantly, plasma membrane [3H]cholesterol became less accessible to oxidation by cholesterol oxidase. Sphingomyelinase-stimulated cholesteryl esterification was 21 ± 3% greater in transfected cells. Concomitantly, accessibility of plasma membrane [3H]cholesterol to cholesterol oxidase was decreased 18 ± 3% in cells expressing L-FABP. These differences are consistent with the ability of L-FABP to influence sterol transport and plasma membrane transbilayer sterol distribution in intact cells.

The intracellular distribution of cholesterol is not uniform (1–3). It is generally agreed that the bulk of cellular cholesterol is found in the plasma membrane (4–7). Within the plasma membrane the sterol pool is asymmetrically distributed between the two leaflets of the lipid bilayer (reviewed in Ref. 8). In addition, evidence for the existence of an additional sterol asymmetry, lateral subdomains within these two transbilayer sterol populations, was presented for model membranes and biological membranes (reviewed in Ref. 9).

The mechanism(s) by which the cell generates and maintains the steady-state nonequilibrium distribution of sterol is not known. Recently, considerable attention has been given to one of these phenomena, namely the intracellular intermembrane cholesterol distribution. The equilibrium partitioning of cholesterol between various intracellular membranes cannot be explained in terms of thermodynamic stability (10) or in terms of preferential interaction of sterol with sphingomyelin alone (8, 11). Both vesicular transfer (12, 13) and cytoplasmic protein-mediated (8, 9, 14–19) cholesterol transfer processes have been proposed.

One of the cytoplasmic proteins, liver fatty acid-binding protein (L-FABP)† may function as both a fatty acid and a sterol carrier. L-FABP binds fatty acids (20) and was recently shown to enhance fatty acid exchange between membranes in vitro (21). On the basis of indirect evidence (reviewed in Ref. 15), it was proposed that L-FABP also interacts with cholesterol and should therefore be termed sterol carrier protein. Subsequently, direct evidence for L-FABP interacting with sterols in vitro was presented (16, 17, 22, 23). Consistent with a role in intermembrane sterol transfer, L-FABP enhanced cholesterol transfer from vesicles to microsomes and stimulated acyl CoA:cholesterol acyltransferase (ACAT) in vitro (23) and was effective in stimulating sterol transfer between L-cell plasma membranes (9) but not between model membranes (9, 14, 24). Thus, L-FABP can enhance fatty acid and cholesterol transfer between membranes in vitro. However, such an effect of L-FABP has heretofore not been demon-

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* This work was supported in part by Grants DK41402 (to F. S.) and AM3289 (to T. J. S.) from the United States Public Health Service National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by a postdoctoral fellowship from Lipid Research Training Grant HI07460.

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† The abbreviations used are: L-FABP, rat liver fatty acid-binding protein; SCP-2, sterol carrier protein; L-cells, mouse connective tissue, L spt "tk" mutant; ACAT, acyl-CoA:cholesterol acyltransferase; dehydroergosterol, Δ-5,7,9(11),22-ergostatetraen-3β-ol; Compound 58-035, 3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide; HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid.
strated in intact cells either for fatty acid or sterol transfer. Thus, a complete description of the physiological function of this protein is yet to be presented.

Herein, the possible role of L-FABP in sterol transfer between intracellular membranes of intact cells and in regulating the asymmetric distribution of sterol in the plasma membrane has been examined. Cultured mouse L-cell fibroblasts were transfected with cDNA encoding L-FABP. These cells expressed L-FABP and were used to investigate transfer of cholesterol from the plasma membrane to microsomes as well as to determine the effect of the protein on plasma membrane transbilayer sterol distribution. The use of clonal cell lines derived from mouse fibroblast L-cells which have been genetically altered through the technique of transfection to express L-FABP provides a means to investigate the function of this protein independent of other variables which might complicate the analysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse L-cells (L sprrt ‘tk’1) (25) was transfected with the expression vector pLP2-CM-PSMTS and selected on the ability to express L-FABP as described previously (26). [H]Cholesterol and [H]oleic acid were obtained from Amerham Corp. Cholesterol oxidase was obtained from Calbiochem Corp., La Jolla, CA. Spingomyelinase, glucolipase, tritribenzenesulphonilic acid (also called picrylsulphonic acid), Lipidex 1000 (hydroxylalkoxypyrrol dextran, Type VI) were obtained from Sigma. High performance liquid chromatography standards were obtained as follows: cholesterol and ergosterol from Sigma; cholesterol oleate from Eastman; desmosterol from Applied Science Corp., Tampa, FL; Δ,7,9(11),22-ergostatetraen-3- β-dol (dehydroergosterol) was synthesized and purified by high performance liquid chromatography as described previously (27, 28). Commercially available anti-SCP-2-specific IgG as described (26). Most of our experiments were performed at 4°C and does not penetrate to trinitrophenylate cytocatal transfer of lipids. The use of clonal cell lines derived from mouse fibroblast L-cells which have been genetically altered through the technique of transfection to express L-FABP provides a means to investigate the function of this protein independent of other variables which might complicate the analysis.

**Expression of Lipid Transfer Proteins in L-cells**—Recombinant rat liver L-FABP and endogenous FABP were used as antigens for generating polyclonal antisera from rabbits (26). Rat liver SCP-2 was used as antigen for producing polyclonal antisera, also in rabbits (29). The neutral antigen-specific IgG was prepared as described previously (26). SCP-2-specific IgG was further affinity purified as reported previously (30). Western blot assays performed as described earlier (26) demonstrated the absence of cross-reactivity between these antibodies and the respective antigens.

L-cells transfected with cDNA encoding rat liver L-FABP were obtained as described earlier (26). L-FABP expression in L-cells was determined by Western blotting also as described therein (26). Endogenous FABP in L-cells was quantitated in the same manner as L-FABP. Endogenous FABP was used as a standard, and rabbit antiendogenous FABP antibody was used as the antibody. L-cell endogenous SCP-2 was quantitated as follows: L-cells frozen at −70°C in phosphate-buffered saline were thawed and sedimented in an Eppendorf microcentrifuge at 14,000 x g for 10 minutes. The pellet was resuspended in 0.2 ml of phosphate-buffered saline, pH 7.2, vortexed, and transferred to a microhomogenizer (Micrometric Inc., Urbana, IL). The dehydroergosterol in the membranes or cells was extracted as described previously (17, 27, 28). The transbilayer sterol distribution was calculated from fluorescence quenching as described earlier (32, 33).

**Fluorescence Spectroscopy**—Samples for spectrophotometric analysis were prepared from membrane fractions or by dilution of freshly prepared washing buffer, and labeled cells (25 μg protein/ml). For measurements of whole cell suspensions, the cells were diluted to a concentration of ~0.5 × 10⁶ cells/ml so that the absorbance at 325 nm (the excitation wavelength) would be less than 0.16. The protein concentration of tritribenzenesulphonilic acid antigens was maintained. All fluorescence measurements were corrected for background cell fluorescence and light scatter by use of cells not containing dehydroergosterol. The transbilayer sterol distribution was calculated from fluorescence quenching as described earlier (32, 33).
zyed and averaged. Standard sterols were used to establish response curves. The observed retention times of dehydroergosterol, desmosterol, ergosterol, and cholesterol were 8.3, 11.5, 12, and 16 min, respectively. The individual response factors (area/weight in μg × 10⁻⁶) were 10.61, 0.77, 3.0, and 0.69, respectively, as determined with a 3390A integrating recorder (Hewlett-Packard). The average recovery of the internal standard ergosterol from 56 runs was 94%.

Lipid analysis of cells supplemented with radiolabeled lipids was performed using silica-coated plastic thin layer sheets (Eastman) developed in hexane/2-propanol/glacial acetic acid/water (130:30:2 v/v/v) (37). Radioactivity of ['H]-lipids was determined by liquid scintillation on a LS6000 counter (Beckman Instruments). Sphingomyelin content was determined as described earlier (38).

then rinsed with phosphate-buffered saline, fixed in 1% glutaraldehyde, and exposed to serum-free modified Higuchi medium (2) (without methyl cellulose and sodium dextran sulfate and the presence of 10% fetal calf serum) as described above for 4 days. The cells were then kept for 3 days in the modified Higuchi medium containing unesterified ['H]-cholesterol (0.1 μCi/ml) with or without 10% fetal calf serum. The cells were then incubated for 24 h in serum-free modified Higuchi medium. With this labeling procedure, cells contained less than 2% esterified ['H]-cholesterol at the start of the experiment.

['H]-Oleic acid was incorporated into cells basically as described for ['H]-cholesterol, except that after preincubation for 24 h in serum-free modified Higuchi medium, the cells were washed with phosphate-buffered saline, and exposed to serum-free modified Higuchi medium containing ['H]-oleic acid (694 nmol/ml at 0.42 μCi/ml) for 30 min at 37°C.

Cholesteryl Oxidase Treatment of Cells—Plasma membrane cholesterol in intact cells was oxidized by treatment of cells with cholesterol oxidase by the method of Slotte et al. (39). Briefly, the cells (prelabeled with ['H]-cholesterol or ['H]-oleate) were first rinsed with ice-cold phosphate-buffered saline, kept on ice, and fixed for 10 min (4°C) with 1% glutaraldehyde in phosphate-buffered saline (40, 41). The fixed cells were washed three times with 1% phosphate-buffered saline. The cells were then treated with cholesteryl oxidase (1 unit/ml) and sphenoglycineline (100 milliunits/ml) for 30 min at 37°C. The cells were then washed with phosphate-buffered saline, frozen, and the cell content of ['H]-cholesterol, ['H]-cholesternone, and ['H]-cholesterol ester or ['H]-oleic acid and cholesteryl-['H]-oleate was determined as described above.

Protein Purification and Functional Assays—Recombinant rat liver L-FABP was isolated from Escherichia coli host strains carrying plasmid pBl2.42). Endogenous mouse fibroblast FABP was isolated essentially in the same way except that cytosol was obtained from serum-free fibroblasts (2) instead of E. coli. SCP-2 was obtained as described previously (43). The Lipidex-1000 binding assay was used to measure ['H]-oleic acid binding to L-FABP and endogenous FABP (44). Ligand binding measured by such competitive assays can be dependent on whether these endogenous proteins can function in sterol binding and/or transfer, and 2) the cytosolic content of these proteins must not be altered in the transfected cells. Therefore, endogenous FABP was isolated, and its properties were compared to those of L-FABP and SCP-2. The following two sections indicate that these requirements were satisfied.

Ligand Specificity of Lipid Transfer Proteins—Four lines of evidence indicate that the endogenous FABP does not act as a sterol transfer protein.

1) A Lipidex-1000-binding assay was used to examine fatty acid binding by the three lipid transfer proteins. Endogenous FABP bound ['H]-oleic acid with a Kd of 1.6 μM and binding stoichiometry of 0.81/nmol of protein. L-FABP binds 2 mol of

TABLE I

Quantitation of cytosolic lipid transfer proteins in L-cells

Cell cytosols were prepared by centrifugation of cell homogenate and aliquots were subjected to polyacrylamide gel electrophoresis followed by Western blotting or to enzyme-linked immunosorbant assay as described under "Experimental Procedures." Values represent the mean ± S.E. (n = 3-4).

<table>
<thead>
<tr>
<th>L-cell line</th>
<th>L-FABP</th>
<th>Endogenous FABP</th>
<th>Endogenous SCP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Not detected</td>
<td>0.123 ± 0.025</td>
<td>0.0069 ± 0.0033</td>
</tr>
<tr>
<td>Transfected</td>
<td>0.340 ± 0.006</td>
<td>0.191 ± 0.0039</td>
<td>0.0031 ± 0.0003</td>
</tr>
</tbody>
</table>

1) L-FABP was determined using a specific antibody in a Western blotting assay (see "Experimental Procedures").

2) Endogenous FABP antibody did not cross-react with L-FABP or SCP-2.

3) Endogenous SCP-2 was determined with an enzyme-linked immunosorbant assay and affinity purified anti-rat liver SCP-2 antibody as described under "Experimental Procedures."
Sterol Distribution in Transfected L-cells

fatty acid/mol of protein, although with affinity similar to that observed for endogenous FABP (20). In contrast, SCP-2 is not a fatty acid-binding protein (29, 47).

2) The Lipidex-1000 binding assay was used to determine cholesterol binding by the lipid transfer proteins. L-FABP bound [3H]cholesterol with $K_d = 0.8 \pm 0.1 \mu M$ and stoichiometry near 1 (Fig. 1, top curve). This observation confirms earlier results obtained by this laboratory for binding fluorescent sterol, dehydroergosterol, to L-FABP (16, 17, 23). Rat liver SCP-2 binds dehydroergosterol with a $K_d = 1.3 \pm 0.2 \mu M$ and 1:1 stoichiometry, confirming earlier observations from this laboratory (14). Since the endogenous SCP-2 was present in very low concentration in L-cells (110-fold lower than L-FABP in transfected L-cells, Table I), it was not further purified. However, when a crude 15-16-kDa fraction from L-cell cytosol was used to test dehydroergosterol binding a small amount of dehydroergosterol binding was observed. This binding could be entirely accounted for by the 0.007% endogenous SCP-2 in the cell cytosol (Table I). In contrast, endogenous FABP did not bind either [3H]cholesterol or dehydroergosterol. Moreover, energy transfer from protein aromatic amino acids to bound dehydroergosterol was observed for L-FABP-dehydroergosterol (16, 17, 23) and SCP-2-dehydroergosterol (14), but not endogenous FABP-dehydroergosterol.

3) The effect of the lipid transfer proteins on sterol exchange between L-cell plasma membranes was examined. For this purpose, a dehydroergosterol fluorescence polarization exchange assay was used. The assay directly correlates polarization changes with molecular sterol transfer between model membranes (9, 11, 24) and between L-cell plasma membranes (9). Polarization of dehydroergosterol in the donor plasma membrane vesicles in the absence of acceptor (Fig. 2A, curve 1) is low (due to self-quenching of fluorescence) and stable over the time period of exchange. However, if acceptor plasma membrane vesicles (not containing dehydroergosterol) are added (arrow in Fig. 2A, curve 2) fluorescence polarization increases due to the release of self-quenching when the dehydroergosterol is transferred from the donor to the acceptor plasma membranes. Kinetic analysis of three separate exchange experiments indicated that spontaneous exchange of dehydroergosterol was best described by three components: a rapidly exchanging component with $t_{1/2} = 23 \pm 4$ min, a slowly exchangeable component $t_{1/2} = 129 \pm 11$ min, and a very slowly or nonexchangeable component. The fractional contributions of these components were $f_1 = 0.13 \pm 0.02$, $f_2 = 0.77 \pm 0.02$, and $f_3 = 0.10 \pm 0.02$. In the presence of 1.5 mM endogenous FABP (Fig. 2B), the polarization curve was essentially superimposable on that of the spontaneous exchange curve (Fig. 2A, curve 2). The kinetic parameters for three separate sterol exchange experiments in the presence of endogenous FABP were: $t_{1/2} = 15.7 \pm 4.0$ min, $t_{1/2} = 177 \pm 32$ min, and a very slowly or nonexchangeable component. The fractional contributions of these components were $f_1 = 0.19 \pm 0.09$, $f_2 = 0.69 \pm 0.09$, and $f_3 = 0.11 \pm 0.03$, respectively. No significant differences were noted in the half-times and fractional contributions as compared to spontaneous exchange shown above. In contrast, earlier data from this laboratory demonstrated that both L-FABP and SCP-2 stimulated the transfer of fluorescent dehydroergosterol between L-cell plasma membranes (9).

4) The ability of the three proteins to stimulate [3H]cholesterol transfer from donor membranes to microsomes and stimulate microsomal ACAT was determined. Endogenous FABP did not significantly stimulate ACAT at any of the concentrations tested (Fig. 3). In contrast, L-FABP stimulated ACAT significantly in a dose-dependent manner; maximal stimulation was 45% (Fig. 3). Under the same conditions maximal stimulation of ACAT by SCP-2 was 500% (14).

Expression of L-FABP in Transfected L-cells—Mouse L-cells were transfected with cDNA encoding L-FABP. Subsequently, stable transfectant cells expressing high amounts of L-FABP were isolated (Table I). L-FABP was not detected in the control cell line but was expressed as 0.34% of cytosolic protein in transfected cells. Expression of 0.54% of L-cell cytosol as L-FABP resulted in the following cellular lipid transfer protein changes. 1) The total amount of fatty acid-binding protein (L-FABP plus endogenous FABP) was increased from 0.123 to 0.441% of cell cytosol. 2) The total amount of sterol binding/transfer proteins (L-FABP plus SCP-2) was increased from 0.007 to 0.343% of cell cytosol (Table I). The high expression transfected cells had the same

![Fig. 1. [3H]Cholesterol binding to L-FABP. The binding of [3H]cholesterol to L-FABP (0.3 μM) was determined in the absence (circles) or presence (triangles) of Sandoz Compound 58-035 (0.1 μg/ml) as described under "Experimental Procedures."](image-url)
doubling time but higher maximal density (26). The transfection process and expression of L-FABP in L-cells did not alter the cytosolic content of the endogenous FABP or SCP.

Consequently, the expression of L-FABP in the L-cells appears to be responsible for the differences in cell sterol uptake noted herein. In the following sections the effect of L-FABP expression on sterol uptake, sterol distribution in the plasma membranes, and intracellular sterol redistribution are examined.

Uptake of \([^3H]\)cholesterol and the Fluorescent Sterol, Dehydroergosterol—The effect of L-FABP expression in L-cells on sterol uptake was measured in two ways: uptake of \([^3H]\)cholesterol and uptake of a fluorescent sterol, dehydroergosterol. L-cells are unable to synthesize cholesterol due to an inability to convert desmosterol into cholesterol. However, the cultured L-cells readily incorporate exogenous sterol provided in the culture medium and the endogenous synthesis of desmosterol is inhibited (~2% of total sterol). When the serum containing medium is supplemented with either \([^3H]\)cholesterol or the fluorescent sterol, dehydroergosterol, the labeled sterols are readily incorporated as is evidenced by appearance of radioabeled cholesterol or dehydroergosterol fluorescence in the L-cells. Three lines of evidence indicate that sterol uptake was significantly enhanced in L-cells expressing 0.34% of cytosolic protein as L-FABP.

1) When L-cells were cultured in serum-containing medium supplemented with cholesterol (10 \(\mu\)g/ml) and \([^3H]\)cholesterol, total sterol uptake, as measured by \([^3H]\)cholesterol incorporation was increased 21.0 \(\pm\) 2.7% (from 24.1 \(\pm\) 0.5 to 29.2 \(\pm\) 0.6 nmol/mg protein, \(p < 0.005\)) in transfected cells expressing 0.34% of cell cytosol as L-FABP. No difference in \([^3H]\)cholesterol uptake (24.1 \(\pm\) 0.5 versus 25.1 \(\pm\) 0.8 nmol/mg protein, \(p = \text{NS}\)) was observed for L-cells and an L-cell line expressing 0.008% of cytosol protein as L-FABP.

2) L-cell dehydroergosterol uptake, as evidenced by fluorescence, was proportional to the amount of fluorescent sterol added to the medium (Fig. 4). Under the conditions used herein, self-quench of the dehydroergosterol fluorescence was not observed. When supplemented with 10 \(\mu\)g of dehydroergosterol/ml culture medium, the relative fluorescence intensity/mg of cell protein of incorporated dehydroergosterol in control and transfected cells was increased 19 \(\pm\) 3% (from 76.9 \(\pm\) 3.1 to 91.5 \(\pm\) 3.8, \(p < 0.025\)). Also as shown in Fig. 4, relative slope of the concentration-dependent increase in dehydroergosterol fluorescence was significantly greater for transfected versus control L-cells (9.2 \(\pm\) 0.2 versus 7.9 \(\pm\) 0.1, \(p < 0.01\)).

3) HPLC of lipid extracts taken from cells cultured with serum containing medium and 10 \(\mu\)g of dehydroergosterol/ml confirmed that the increase in fluorescence noted above was due to increased dehydroergosterol uptake in the transfected cells expressing 0.34% of cell cytosol as L-FABP. The dehydroergosterol uptake was significantly increased by 22.0 \(\pm\) 2.7%. It should be noted that dehydroergosterol uptake was stimulated to the same extent as \([^3H]\)cholesterol.

Role of Sterol Esterification in Enhanced Sterol Uptake by Transfected L-cells—Fluorescence intensity increase in the cell or increase in radiolabel in the cell only provide information on total sterol uptake. Moreover, the stimulation of sterol uptake by L-FABP might not be due to increased transport but to enhanced esterification of sterol in the cell. L-FABP can significantly stimulate microsomal ACAT (Fig. 3). Therefore, the lipids from these cells were extracted and subjected to analysis by high performance thin layer chromatography or HPLC as described under "Experimental Procedures." Data from four separate experiments and utilizing radiolabeled sterol as well as fluorescent sterol were combined (Fig. 5). The total sterol uptake was stimulated from 24.2 \(\pm\) 0.4 nmol/mg of protein to 29.3 \(\pm\) 0.6 nmol/mg of protein (\(p < 0.001\)) in the transfected cells (Fig. 5A, -drug). Sterol esterification was enhanced 2-fold from 0.50 \(\pm\) 0.08 to 1.00 \(\pm\) 0.04 nmol/mg of protein (\(p < 0.005\)) in the transfected cells (Fig. 5C, -drug). However, the enhanced esterification did not account for the stimulation of total sterol uptake. Indeed free cholesterol uptake was stimulated from 23.6 \(\pm\) 0.4 to 28.3 \(\pm\) 0.5 nmol/mg of protein (\(p < 0.001\)).

Despite the above results demonstrating that the enhanced uptake of sterol in transfected L-cells expressing 0.34% of cell cytosol as L-FABP was not due to stimulation of esterification, it might be argued that the stimulation in sterol uptake was due to some other property of the cell or the transfection process itself. The following results indicate that this was not the case:

1) Faster growth of transfected cells could enhance sterol uptake. However, the doubling time near 1.5 days was not significantly altered in the transfected cells whether they expressed low (0.008% of cytosol) or high (0.34% of cytosol) levels of L-FABP.

2) The enhanced uptake of sterol might be associated with
increase in the content of cellular sphingomyelin, which has been postulated to bind sterol. The percent of phospholipid comprised of sphingomyelin was increased in both whole cells (from 8.2 ± 0.6 to 17.3 ± 2.3, p < 0.05) and isolated plasma membranes (Table II; from 13.9 ± 0.7 to 16.7 ± 0.8, p < 0.05) in L-cells expressing 0.34% of cytosolic protein as L-FABP. Cholesterol esters were not detected in significant quantities in the isolated membranes. Most important, the increased plasma membrane sphingomyelin content of transfected cells did not correlate with increased sterol. In fact, the total sterol content (cholesterol in top panel of Table II; or cholesterol + dehydroergosterol, in the case of cells supplemented with both serum and dehydroergosterol, lower panel Table II) of the plasma membranes was lower in the transfected cells.

3) Finally, sterol uptake enhancement in the transfected cells was examined with the Sandoz Compound 58-035. This drug has been shown to inhibit cholesterol esterification and/or cholesterol transport (48). As shown in Fig. 1, bottom curve, Compound 58-035 inhibited [3H]cholesterol binding to L-FABP. Thus, we would expect Compound 58-035 to inhibit L-FABP-mediated cholesterol uptake. The data show that this was indeed the case (Fig. 5A, +drug). Compound 58-035 abolished the L-FABP-associated enhancement of total sterol uptake (Fig. 5A, +drug), the L-FABP-associated stimulation of free sterol uptake (Fig. 5B, +drug), and the L-FABP-associated stimulation of ACAT (Fig. 5C, +drug). However, Compound 58-035 did not reduce sterol uptake in transfected L-cells to less than that observed in control L-cells. This basal level of sterol uptake was probably due to low density lipoprotein receptor-mediated sterol uptake, a pathway completely different from molecular sterol transfer mediated by L-FABP.

In summary, sterol uptake was enhanced in transfected L-cells expressing 0.34% of cell cytosol as L-FABP. The enhanced uptake was not due to the transfection process or to enhanced esterification.

**Lipid Composition of Plasma Membranes from L-cells Expressing L-FABP**—Because the above data are consistent with the interpretation that L-FABP enhances sterol transfer into the cell, we examined whether the expression of this protein might also cause intracellular redistribution of sterol. Therefore, cells were cultured with and without dehydroergosterol and plasma membranes were isolated. Three observations can be made from Table II. First, in cells grown with serum only the cholesterol content of plasma membranes from transfected cells was nearly 50% lower than in plasma membranes from control cells. Second, in cells grown with serum + dehydroergosterol the plasma membrane total sterol content was decreased 20%. Third, in cells grown with serum + dehydroergosterol the plasma membrane ratio of dehydroergosterol/cholesterol was increased significantly from 0.26 to 0.42 (p < 0.01). Enhanced uptake of dehydroergosterol by the transfected cells would be expected to result in increased amount of dehydroergosterol replacing cholesterol in the plasma membrane thereby increasing the ratio.

Thus, the data on fluorescent sterol uptake by whole cells and on the sterol content and composition of isolated plasma membranes are consistent with an enhanced transfer of sterol not only from the medium into the cell membrane but also from the cell membrane into intracellular sites in transfected as compared to control cells.

**Fluorescent Sterol Localization in Intact Cells**—In the preceding section the intracellular distribution of sterol was examined after cell homogenization; isolation of plasma membranes, and quantitation of sterol by HPLC of lipid extracts. Although sterol exchange between membranes is very slow at 4°C, the temperature of the isolation conditions, it is still possible that the isolation procedures in themselves could have caused some redistribution of sterol or differential contamination of plasma membranes with intracellular membranes in the transfected versus control cell lines. Therefore, a different approach using the fluorescent dehydroergosterol and selective quenching of dehydroergosterol in intact cells was taken to circumvent these potential difficulties. The extent of sterol exposure in the outer leaflet of plasma mem-
branes in intact cells was examined using fluorescent dehydroergosterol. Exposure was measured by determining the degree of dehydroergosterol fluorescence quenching in cells whose plasma membrane exofacial leaflet was labeled with trinitrobenzenesulfonic acid under nonpenetrating conditions (4 °C). The donor/acceptor pair dehydroergosterol/trinitrophenyl quench efficiently only when the two molecules are in very close proximity and are localized in the same leaflet. Thus, the fluorescence remaining in whole cells which are labeled with trinitrobenzenesulfonic acid under nonpenetrating conditions is due to the dehydroergosterol located in the plasma membrane cytofacial leaflet and in intracellular membranes. Quenching of dehydroergosterol fluorescence was determined in cells supplemented with dehydroergosterol (1–10 μg/ml medium) (Fig. 6). When the cells are supplemented with high concentrations of dehydroergosterol (10 μg/ml medium), large quantities of fluorescent sterol are incorporated and found inside the cells, thereby resulting in low percent quenching near 15% that was not significantly different between the cell lines. In contrast, the percent quenching at infinitely low dehydroergosterol content (obtained from extrapolation of the lines to the y axis) was 39.8 ± 0.5 and 43.5 ± 0.5% for control and transfected cells, respectively. Thus, less dehydroergosterol was in the outer leaflet of the plasma membrane of intact transfected cells as compared to control cells.

The above observation could result either from (a) a simple redistribution of sterol within the plasma membrane from the exofacial to the cytofacial leaflet of the transfected cells or (b) more dehydroergosterol distributed to intracellular sites in transfected cells. Two lines of evidence indicate that the latter possibility is more likely. First, the data in Table II indicate that the plasma membrane of transfected cells has less sterol than that of the control cells. If the concentration of exofacially localized dehydroergosterol increased then the percent quenching in the transfected cells should have been higher as compared to control cells. Thus, sterol was both lost from the plasma membrane to intracellular sites and redistributed within the plasma membrane of transfected cells as compared to control cells. Second, the transbilayer distribution of sterol in the isolated plasma membrane was determined using dehydroergosterol and the selective quenching as described under "Experimental Procedures." It is clear that in both control and in transfected cells the majority of sterol was located in the inner (cytofacial) leaflet (p < 0.01, Table III). However, the percent of plasma membrane sterol located in the exofacial leaflet of plasma membranes from transfected cells was 1.8-fold higher as compared to control cells (p < 0.05, Table III). Taken together with the data on total sterol content of plasma membranes (Table II), this suggests that in the transfected cells the majority of sterol is lost from the cytofacial rather than exofacial leaflet of the plasma membrane and transported to intracellular sites.

Transfer of Plasma Membrane Cholesterol to Microsomes: Cholesterol Esterification—The transfer of cholesterol from plasma membranes to microsomes was examined in intact cells using the sphingomyelinase method previously developed by Slotte et al. (37, 48). Sphingomyelinase treatment of a variety of intact cells releases cholesterol from the plasma membrane into the cell cytoplasm. The transfer of cholesterol from cell plasma membranes to microsomes of intact cells stimulates the esterification of cholesterol by the microsomal enzyme ACAT (37, 48). Therefore, [3H]cholesterol was incorporated into the L-cells as described under "Experimental Procedures." The radiolabeled cholesterol was rapidly incorporated into the cells. A small portion of the [3H]cholesterol was internalized within 8 h as indicated by appearance of [3H]cholesterol ester in control and transfected cells, 0.66 ± 0.09 versus 1.03 ± 0.02%, respectively. Thus, the rate of sterol transfer from the plasma membranes to the microsomes (as indicated by cholesterol esterification) was elevated in transfected cells.

Figs. 7 and 8 show the effect of sphingomyelinase treatment

<table>
<thead>
<tr>
<th>L-cell line</th>
<th>Exofacial leaflet</th>
<th>Cytofacial leaflet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.1 ± 1.7</td>
<td>84.9 ± 1.7*</td>
</tr>
<tr>
<td>Transfected</td>
<td>27.9 ± 4.3*</td>
<td>72.1 ± 4.3*†</td>
</tr>
</tbody>
</table>

Fig. 7. Esterification of [3H]oleate upon treatment of L-cells with sphingomyelinase. [3H]Oleic acid was incorporated into confluent L-cell fibroblasts as described under "Experimental Procedures." The cells were exposed to buffer with (circles) or without (triangles) sphingomyelinase for the indicated time; the reaction was terminated at the indicated time by freezing, and the incorporated [3H]oleate as [3H]cholesterol ester was determined from the cellular neutral lipid extract as described under "Experimental Procedures." Values represent the mean of determinations on duplicate dishes.
FIG. 8. Esterification of [3H]cholesterol into cholesterol esters upon treatment of L-cells with sphingomyelinase. All conditions were as described in legend to Fig. 7 except that the confluent fibroblasts were treated with [3H]cholesterol instead of [3H]oleate and allowed to continue growth for 24 h in serum-free medium.

Following treatment with sphingomyelinase for the indicated times, the cells were fixed in 1% glutaraldehyde, washed with phosphate-buffered saline (three times), and treated with cholesterol oxidase for 30 min at 37°C as described under "Experimental Procedures." The cells were then washed, frozen and the amount of [3H]cholesterol in cholestone, cholesterol, and cholesterol ester was determined from the neutral lipid extract also as described under "Experimental Procedures." Values represent the mean of determinations on duplicate dishes (n = 4).

The cellular localization of the unesterified cholesterol after sphingomyelin treatment was investigated with the use of the glutaraldehyde/cholesterol oxidase. Consecutive treatment of cells with sphingomyelinase followed by glutaraldehyde and cholesterol oxidase did not influence the amount of cholesterol ester formed (37, 48). Thus, this method provides an indication of the cholesterol remaining in the plasma membrane after sphingomyelinase treatment. In Fig. 9, the results of the treatment of [3H]cholesterol-fed cells (both control and L-FABP expressing) with sphingomyelinase and glutaraldehyde/cholesterol oxidase show that the amount of plasma membrane [3H]cholesterol which is available for oxidation by this enzyme is rapidly and maximally decreased within 30 min after treatment with sphingomyelinase.

Effect of L-FABP Expression on Cholesterol Transfer from Plasma Membranes to Microsomes in L-cells—The effect of L-FABP on the transfer of cholesterol to the microsomes was examined using sphingomyelinase-stimulated cholesterol redistribution as described above. The decrease in cholesterol oxidase accessibility noted with control cells above was significantly greater in transfected cells expressing L-FABP and persisted for the first 60 min of exposure to sphingomyelinase (Fig. 9). Thereafter, there was no significant difference between the response of the two cell lines. In Fig. 10, the rate and extent of cholesteryl ester formation as a result of sphingomyelinase treatment on both control cells and transfected cells containing either [3H]cholesterol or [3H]oleic acid was determined. The data for both radiolabels were similar so that they were combined in the figure. The cells which expressed L-FABP incorporated more radiolabeled precursor into cholesteryl ester than control. Since the enhancement of [3H]cholesterol ester formation is not yet maximal within 60 min (Fig. 10) while changes in cholesterol oxidase accessibility were maximal within 30 min (Fig. 9), the data are consistent with a precursor (plasma membrane cholesterol) product (microsome cholesteryl ester) relationship.

Membrane Transbilayer Sterol Distribution in Sphingomyelinase-treated Cells—In a similar experiment using cells which had been supplemented with dehydroergosterol, the effect of sphingomyelinase treatment had a notice-
able change on the transbilayer distribution of the sterol in the plasma membrane. Sphingomyelinase treatment of control L-cells for 120 min changed the distribution of plasma membrane sterol by 10.6% such that there was a smaller proportion of the sterol on the exofacial leaflet. In the case of the transfected cells expressing L-FABP, a similar although slightly larger change of 18.9% was observed in the percent sterol which was localized in the exofacial leaflet.

**DISCUSSION**

The mechanism(s) of intracellular cholesterol movement is(are) not known. Exogenous esterified cholesterol enters the cell via the low density lipoprotein-receptor-mediated endocytosis pathway (49). In contrast, exogenous nonesterified cholesterol is internalized either by the low density lipoprotein-receptor pathway or by molecular transfer (50–54). Although the low density lipoprotein-receptor-mediated pathway is believed to account for the majority of cellular cholesterol internalization, in certain instances as much as 65–90% of cholesterol accumulates via molecular transfer (51).

Intracellular molecular cholesterol transfer appears bidirectional. Transfer of cholesterol from endoplasmic reticulum to the plasma membrane is thought to be vesicular and occurs rapidly (10–60 min in Chinese hamster ovary cells) and 1–2 h in skin fibroblasts (12, 55). In contrast, the movement of cholesterol from the plasma membrane to the endoplasmic reticulum appears slow (56). Two possible mechanisms whereby cholesterol is transferred from the plasma membrane to intracellular organelles are 1) microvesiculation and 2) cytosolic cholesterol transfer proteins. The former possibility was precluded by Slotte et al. (54) who demonstrated that inhibition of pinocytosis by a variety of agents did not inhibit transfer of cholesterol from the plasma membrane to the endoplasmic reticulum. The possibility that cytosolic sterol carrier proteins may be involved was examined herein.

L-cells constitute an excellent model system for examining the role of L-FABP in sterol transfer. The major reasons for this choice are provided under “Results.” L-cells do not contain L-FABP. They have only low amounts of endogenous FABP (near 0.1%) which does not bind sterol, does not enhance sterol transfer, and does not stimulate ACAT. An endogenous sterol carrier protein does exist in L cells (17, 57, 58). This protein bound [3H]cholesterol (57) and dehydroergosterol (present work) but was present in very low amount (0.007% of cell cytosol). On the basis of preliminary evidence presented as an abstract by our laboratory, a partially purified sterol carrier protein obtained from L-cells was called SCP or sterol carrier protein (58). Unfortunately, the designation SCP had previously also been applied to liver fatty acid-binding protein, also called L-FABP (15, 19). The immunochmical data presented herein indicate that the endogenous FABP and the endogenous sterol carrier protein are completely different proteins. The latter reacts with anti-rat liver SCP-2 IgG (Table I), the former reacts with anti-endogenous FABP (Table I), and neither reacts with anti-rat liver L-FABP. Irrespective of nomenclature, the endogenous content of sterol transfer protein in L-cells is miniscule (0.003%) as compared to the expression of L-FABP in the transfected cells (0.340%) (Table I). Most important, the level of the endogenous SCP-2 was unaltered by the transfection process.

Liver fatty acid-binding protein (L-FABP, also called sterol carrier protein) is not overexpressed in the transfected cells. This protein represents 0.24–0.43% (15, 59–61), 2% (61), and 3–8% (61), respectively, of brain, intestine, and liver cytosolic protein. Thus, changes in sterol uptake and plasma membrane transbilayer redistribution of sterol are not due to overexpression of L-FABP or to conversion of L-cells into an unnatural state.

L-FABP binds sterol both in vitro and in intact cells (16, 17, 23). Thus, it could act as a carrier. To test this possibility L-cell fibroblasts (which do not contain L-FABP) were transfected with cDNA encoding L-FABP in order to determine if L-FABP may enhance sterol transfer from the plasma membrane to endoplasmic reticulum in intact cells. Three different types of experiments were designed to determine the effect of L-FABP on sterol movement in the intact cells.

First, the measured uptake of both [3H]cholesterol and dehydroergosterol was significantly enhanced in L-cells expressing 0.34% of cytosolic protein as L-FABP.

Second, the rate of intracellular transfer of radiolabeled [3H]cholesterol and of the fluorescent sterol, dehydroergosterol, was determined in control and transfected L-cells. Transfer of [3H]cholesterol from the plasma membrane to the microsomes can be monitored by the ability of microsomes to esterify cholesterol that enters the cells by molecular transfer. In control L-cells the rate of this process was 0.06 ± 0.01%/h, quite slow. Several other cell types are also able to esterify cholesterol that enters the cells by molecular transfer (39, 46, 53, 54, 62). The process occurs slowly at a rate of 0.02–0.12%/h in human fibroblasts and arterial smooth muscle cells, respectively (48). In contrast, the transfected L-cells expressed L-FABP in their cytosols and the rate of transfer (0.15 ± 0.01%/h) was significantly higher than in control cells. Uptake of dehydroergosterol by transfected cells was also significantly higher than by control cells. Selective quenching data and isolation of plasma membrane fractions confirmed that the fluorescent sterol was not just taken up into the plasma membranes, but its internalization was enhanced in the transfected cells as compared to control cells. The enhanced uptake and internalization of sterol by the transfected cells also resulted in net accumulation of cholesterol esters. The total cholesterol ester content of the transfected cells was also higher than that in control cells, 6.0 ± 0.7 versus 3.9 ± 0.4 nmol/mg of cell protein, respectively. However, the uptake experiments indicate that the magnitude of enhanced sterol uptake could not be accounted for by increased esterification. 2) Inhibition of esterification and sterol binding/transfer by the Sandoz Compound 58-035 abolished the stimulation of sterol uptake observed in the transfected cells. These findings were not due to the transfection process since another transfected L-cell line expressing only 0.008% of cytosolic protein as L-FABP had essentially the same uptake of sterol as the control cells.

Third, the plasma membrane transbilayer sterol distribution was examined in order to determine from which leaflet (cytofacial versus exofacial) the sterol is lost in the transfected as compared to the control cells. For this purpose the fluorescent sterol, dehydroergosterol was used in conjunction with selective quenching to examine the transbilayer distribution of sterol in the plasma membrane of control and transfected cells. This methodology and the similarity of dehydroergosterol properties to cholesterol have been extensively documented (see “Experimental Procedures”). In the present study sterol was enriched in the cytofacial leaflet as compared to the exofacial leaflet of the plasma membrane. This observation is consistent with earlier observations with a variety of L-cell-derived cell lines (8, 32, 33, 35, 63). However, the transbilayer distribution of sterol in the plasma membranes of transfected cells was dramatically altered as compared to that of control cells. The presence of L-FABP increased the degree of asymmetry by increasing the proportion of sterol
which was accessible to quench from the exofacial side in comparison to control nontransfected cells. The exofacial leaflet of intact cells as well as of isolated plasma membranes became more enriched with sterol at the expense of the cytosolic leaflet. However, this enrichment of sterol in the exofacial leaflet was only apparent since the total sterol content of plasma membranes from transfected cells was lower than that of control cells. Taken together these data are consistent with a loss of sterol from the cytosolic leaflet of the plasma membrane from transfected cells without change in the exofacial leaflet.

Fourth, the enzyme sphingomyelinase was used to release cholesterol from the plasma membrane and its transfer to intracellular sites was monitored by determination of cholesterol esterification. Treatment of cultured cells with the enzyme sphingomyelinase has been shown to deplete the plasma membrane content of sphingomyelin by up to 90%, and as a result of these changes in the structure of the plasma membrane a very rapid movement of cholesterol away from the exofacial leaflet was only apparent since the total sterol content of sphingomyelin by up to 90%, and as a result of these changes in the structure of the plasma membrane a very rapid movement of cholesterol away from the exofacial leaflet was only apparent since the total sterol content of plasma membranes from transfected cells was lower than that of control cells. Taken together these data are consistent with a loss of sterol from the cytosolic leaflet of the plasma membrane from transfected cells without change in the exofacial leaflet.

Acknowledgment—The helpful technical assistance of Timothy Hubbell was much appreciated.

REFERENCES
Sterol Distribution in Transfected L-cells