Compartmentation and Turnover of the Low Density Lipoprotein Receptor in Skin Fibroblasts*

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The low density lipoprotein receptor (LDLR) was immunoprecipitated from \[^{35}S\]methionine-labeled skin fibroblasts derivatized at 4 or 18°C with an impermeant biotinylating reagent. Separation of derivatized and undervatized receptor from immunoprecipitates by selective binding to streptavidin-agarose allowed assessment of receptor protein cellular compartmentation and rates of intercompartmental transfer. At both 4 and 18°C the amount of LDLR that is derivatized in cells labeled to near steady state saturates after 1-2 h of reaction at, respectively, 47 and 70% of total immunoprecipitable receptor protein. On the basis of temperature titration experiments, protein exposed only to the cell surface reacts at 4°C; raising the temperature to 18°C provides access to an additional pool of receptor protein. Remaining LDLR is derivatized at 37°C. LDLR unreactive at 18°C largely resides in membrane compartment(s) devoid of plasma membrane on the basis of its fractionation on Percoll gradients. While total cellular LDLR and \[^{4}C\]-labeled LDL receptor protein surface localization and do not permit measurement of rates of appearance and disappearance from different membrane compartments.

This study reports measurements of LDLR compartmentation and intercompartmental transfer obtained by using a new type of compartmentation assay. The assay is called the biotinylation/recovery assay and combines cell surface biotinylation using a cleavable reagent with immunoprecipitation (13). In agreement with another study (9), the results identify 39-49% of the receptor that is localized to the cell surface, the rest residing within the cell. In addition, variation of the derivatization temperature distinguishes between two different intracellular compartments as well as the surface compartment. Also, newly synthesized receptor protein is found to turn over more rapidly on the cell surface than in intracellular compartments. This latter result shows that receptor transiently appears at the cell surface before it distributes to other membranes.

MATERIALS AND METHODS

Cells and Cell Labeling Conditions—Normal skin fibroblasts were obtained from Dr. R. Illingworth, Department of Medicine, Oregon Health Sciences University, Portland, OR. The cells were originally outgrown from a skin biopsy from a patient with normal plasma LDL levels of LDL. The cells exhibited normal rates of \[^{35}S\]methionine-labeled LDL internalization. A frozen stock was obtained at passage 3 and experiments were conducted between passages 5 and 14. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (14) in culture dishes. 20-24 h prior to labeling, the medium was replaced with DMEM supplemented with 10% LDL-depleted human serum (14) in order to transcriptonally up-regulate receptor expression. All labeling experiments were done on cells at 50% of confluence. To label cellular DNA, cells were incubated in DMEM overnight with 1 \(\mu\)Ci \[^{3}H\]thymidine/ml. To pulse label the LDLR, the cell medium was replaced with 2.0 ml methionine-free, minimal essential medium supplemented with 10% LDL-depleted human serum. Fifteen minutes after the addition of methionine-free media, cultures were labeled with 200-400 \(\mu\)Ci/ml \[^{35}S\]methionine (Du Pont-New England Nuclear, 1000 Ci/mmol) for 1-2 h as indicated, after which the cultures were washed three times with phosphate-buffered saline (PBS) and then chased for different lengths of time in DMEM supplemented with 2 mM methionine and 10% fetal bovine serum or LDL-depleted human serum. To label the LDLR to steady state, cultures were labeled with 100 \(\mu\)Ci/ml \[^{35}S\]methionine in 99% methionine-free media 1% DMEM, each 10% in LDL-depleted human serum. After labeling and chasing, culture dishes were then washed three times with cold PBS and derivatized with 400 \(\mu\)g sulfosuccinimidy-
idyl 2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-ss-biotin, Pierce Chemical Co.) at the temperature indicated with constant swirling (50 revolutions/min). Cultures were then washed five times with PBS and the cells scraped off of the dishes with a rubber policeman in 1.0 ml of LDLR lysis buffer (15). The lysate was centrifuged at 10,000 x g for 5 min and frozen at -20°C. Collected lysates were then thawed, centrifuged at 10,000 x g for 5 min, and incubated with 25 µl of protein A-Sepharose with end-over-end rotation for 2 h at 4°C. After centrifugation the lysate for 15 min, 4 µg of monoclonal anti-LDLR antibody (IgG-C7, Amersham Corp.) were added to each lysate, and these were then rotated for 2 h before addition of 25 µl of washed protein A-Sepharose. After 30 min of rotation, LDLR adsorbed to protein A-Sepharose was centrifuged at 10,000 x g for 20 s and washed three times with lysis buffer, once with lysis buffer, 0.5 M in NaCl, once with 0.01 M Tris-HCl (pH 7.5), and bound proteins eluted in 0.01 M Tris-HCl (pH 7.0), and heating at 95°C for 3 min. Eluted, immunoprecipitated LDLR was diluted to 0.95 ml in distilled water to which 25 µl of washed streptavidin-agarose (Sigma) had been added. After overnight, end-over-end rotation at 4°C, agarose beads were centrifuged on a microcentrifuge for 20 s, the supernatant lyophilized to dryness, and the dried pellet solubilized in 50 µl of SDS sample buffer with no reducing agent (16). The agarose beads were washed three times in 50 µl of SDS sample buffer (10 with 10 µM dithiothreitol.

**Electrophoresis and Receptor Quantitation**—Samples were electrophoresed in 8% acrylamide, 0.21% bisacrylamide gels (16) or in Prosieve 6% modified agarose gels (FMC Corp., Marine Colloids Division). In the former case gels were treated with Fluoro-bane (RPI) autodissociator according to the manufacturers' directions, dried, and exposed to Kodak X-Omat film pretreated to an absorbance of 0.15. Developed film images were quantitated by silver grain extraction with NaOH and measurement of light scattering (17). In the case of proteins separated on modified agarose, the LDLR band was localized by its migration just behind a prestained protein A-Sepharose band and counting in a scintillation counter (see under “Materials and Methods” for details).

![Fig. 1. Degradation of LDLR in skin fibroblasts. Cell cultures were labeled for 2 h with [35S]methionine and then chased in the absence of radiolabeled methionine for different times in the presence of LDL-depleted human serum (●) or fetal bovine serum (□). Cell lysates were prepared and the LDLR immunoprecipitated at each interval. The LDLR band from SDS-polyacrylamide gel lanes was quantitated by extracting the radioactivity from dried gels and counting in a scintillation counter (see under “Materials and Methods” for details).](http://www.jbc.org/)

**TABLE 1**

| Temperature of biotinylation | Percent of LDL R bound to streptavidin-agarose |
|-----------------------------|-----------------------------------------------|
| °C                          |                                               |
| 4                           | 42 (± 5.7)²                                  |
| 8                           | 38                                            |
| 15                          | 49                                            |
| 18                          | 74 (± 4.0)²                                  |
| 20                          | 80                                            |
| 23                          | 95                                            |
| 26                          | 100                                           |
| 30                          | 95                                            |
| 37                          | 90¹                                           |

² Average (S.D.) from three experiments.

¹ Average from two experiments.

![Fig. 2. LDLR derivatization at 4°C (A) or 18°C (B) with 400 µg/ml NHS-ss-biotin for the indicated periods of time. From cell lysates LDLR was immunoprecipitated and streptavidinagarose bound and unbound LDLR determined. For underderivatized cells 64 cpm were recovered in the streptavidin-agarsone-bound lane and 1245 cpm recovered in the unbound lane. For cells derivatized for 3 h at 4°C, 454 cpm were removed in the bound lane, and 572 cpm were removed in the unbound lane. For cells derivatized for 3 h at 18°C, 889 cpm were recovered in the bound lane and 399 cpm in the unbound lane.](http://www.jbc.org/)

**RESULTS**

**Degradation of Total Cellular LDLR**—Monoclonal antibody (IgG-C7) against the LDLR immunoprecipitated a single prominent band at \( M_r = 130,000 \). In the presence of reducing agents, the band was more diffuse and migrated at a slightly lower mobility, a finding consistent with its high content of disulfide-bonded residues (1). To measure turnover of total cellular receptor, skin fibroblast cultures were pulse-labeled with [35S]methionine and chased for different intervals from...
2 to 50 h in the presence of excess cold methionine and LDL-depleted human serum or fetal bovine serum. In agreement with previous studies (22, 23) receptor in these confluent cells was degraded at a rate that was first order and unaffected by the type of serum present in the chase medium (Fig. 1). The measured half-life of 12–13 h is identical (23) to or slightly lower (22) than that seen by others in the same cell type. Unlike the case of other membrane proteins (24), receptor degradation is not slowed by lysosomotropic amines such as methylamine (23; results not shown). Some LDLR appears to be shed into the media in a shortened form as evidenced by the appearance of an immunoprecipitated protein in the media of cells pulse-labeled and chased for 24 h but not of those chased for 1 h.2 If related to LDLR, this media protein, however, accounts for only a small fraction of receptor lost between 1 and 24 h of chase. No measurable cell turnover was seen during the same time interval as detected by loss of [3H] thymidine labeled DNA (results not shown).

**Steady State Compartmentation of the LDLR**—Previous studies restricting access of ligands or labeling reactions to the cell surface have been carried out at 0–4°C. Thus at 4°C only surface-accessible amino groups should be available to derivatize. Temperatures between 12 and 20°C have been used to prevent access of ligands and chemically labeled plasma membrane proteins to late endosomes and lysosomes (25–28). To determine if the temperature of derivatization can selectively control access to different cellular compartments, cells, labeled for 36 h to near steady state, were derivatized with 400 ng/ml NHS-ss-biotin at different temperatures for 3 h. The results are summarized in Table I. At temperatures >23°C, 95% or more of the receptor protein becomes streptavidin-agarose-bound. This shows that there is little receptor that cannot be derivatized, providing its access to the biotinylating reagent is not restricted by membrane fusion events. At derivatizing temperatures from 4 to 8°C, 42% or less of the receptor protein becomes streptavidin-agarose-bound. This shows that there is only surface-accessible amino groups should be available to derivatize. Temperatures between 12 and 20°C have been used to prevent access of ligands and chemically labeled plasma membrane proteins to late endosomes and lysosomes (25–28). To determine if the temperature of derivatization can selectively control access to different cellular compartments, cells, labeled for 36 h to near steady state, were derivatized with 400 μg/ml NHS-ss-biotin at different temperatures for 3 h. The results are summarized in Table I. At temperatures >23°C, 95% or more of the receptor protein becomes streptavidin-agarose-bound. This shows that there is little receptor that cannot be derivatized, providing its access to the biotinylating reagent is not restricted by membrane fusion events. At derivatizing temperatures from 4 to 8°C, 42% or less of the immunoprecipitated LDLR is streptavidin-agarose-bound. These values in some experiments can be as low as 26%, the variability between experiments largely dependent on the method of detection. Values obtained from autoradiograms tend to be lower than those obtained by removing and counting the radioactivity directly from gels. The latter is likely to be the most accurate method of estimating differences between bound and unbound receptor pro-

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**Fig. 3. Fractionation on Percoll gradients of streptavidin-agarose bound or unbound LDLR after derivatizing cells at 18°C.** A, to mark cell surface proteins, cells were chemically labeled at 4°C with [3H]I as described under "Materials and Methods." Cells were then broken and fractionated on 15% Percoll gradients as described under "Materials and Methods" to identify fractions rich in this lysosomal marker. B, cells labeled at 18°C were fractionated as in (A), but the lysosomal enzyme, β-glucuronidase, was assayed as described under "Materials and Methods" and fractionated as in (A). C, cells were labeled for 36 h as described under "Materials and Methods" and fractionated as in (A). LDLR was immunoprecipitated from each fraction and then separated into streptavidin-agarose bound or unbound, resolved on SDS-polyacrylamide gels, and exposed to x-ray film. Arrows mark the position of the LDLR (Mr = 130,000) while the line marks the position of a putative degradation product of the LDLR that appears in the unbound fraction only.

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2 J. Hare, unpublished observations.
Methionine and 10% LDL-depleted human serum for the indicated times. At the end of the chase period, cells were derivatized with NHS-ss-biotin for 2 h at 4°C (A, C) or 18°C (B) and streptavidin-agarose bound and unbound receptor isolated as described under “Materials and Methods.” Arrows mark the position of a lane was applied one-half the total isolated receptor from each dish. Between derivatizing temperatures of 15 and 23°C, the percent of total receptor bound to streptavidin-agarose jumps from 49 to 95%. Between 18 and 20°C the percent of receptor derivatized is intermediate between that derivatized at lower temperatures and that derivatized at temperatures >23°C. These results taken together with previously published data suggest that cellular LDLR can be separated into three groups: that which is derivatized at 4°C, that which is not derivatized at 4°C but is at 18°C, and that which remains underivatized at 18°C. 4°C-derivatized receptor is likely to reside on the cell surface. Receptor that is derivatized at 13°C but not at 4°C resides in a compartment accessible only at 18°C. Receptor refractory to derivatization at 18°C represents receptor that is inaccessible to the derivatizing agent but still chemically reactive. In each case it is assumed that streptavidin-agarose-bound receptor is derivatized while streptavidin-agarose-unbound receptor is underivatized.

If temperature of derivatization serves to block access of the NHS-ss-biotin to intracellular compartments, increasing time of derivatization would be expected to saturate derivatizable receptor at any given temperature. In both the case of 4 and 18°C derivatization, the amount of receptor bound to streptavidin-agarose saturates by 1–2 h of reaction. As might be expected, at 4°C the amount of derivatized receptor bound to streptavidin-agarose saturates more rapidly than at 18°C; the additional time may be needed to access intracellular compartments. It is not clear in the case of 18°C-derivatized receptor whether the derivatizing agent reacts with cell surface-exposed proteins which then internalize or whether the agent actually gains access to an intracellular compartment.

To distinguish biochemically between compartments accessible at 4 and 18°C, and those inaccessible at 18°C, derivatized cell homogenates were fractionated on self-forming Percoll gradients. Such gradients can separate plasma membrane from lysosomes and dense endosomes (21). I found that, without DNase treatment, fibroblast membranes tend to aggregate at the top of the gradient. DNase treatment causes mitochondria and lysosomes to shift to the bottom fractions of the gradient (19). As shown in Fig. 3, protein labeled at the cell surface with [125I] at low temperature to mark cell surface-exposed protein appeared at the top of the gradient while fractions rich in β-glucuronidase (Fig. 3B) or N-acetylglucosaminidase (not shown) exhibited peaks both at the bottom (fraction 1) and the top (fractions 9, 10, and 11) of the gradient. This bimodal distribution of lysosome enzyme markers also seen in other studies (21, 29) may be attributed to the presence of lysosomal hydrolases in light endosomes (30), two forms of lysosomes (21), damage to some lysosomes during cell homogenization, or aggregation unaffected by DNase treatment. Displacement of the peak of β-glucuronidase activity one fraction heavier than that of the plasma membrane marker peak fraction, however, suggests that those light membrane compartments enriched in this activity are denser than plasma membrane. Cells were then labeled to steady state and chased for 1 h to dilute out newly synthesized receptor that had not reached the cell surface. LDLR from cells derivatized at 4°C (not shown) or 18°C (Fig. 3D) comigrated with [35S]-surface-labeled protein and thus could be localized to the plasma membrane or light endosomes. LDLR underivatized at 18°C (Fig. 3D), on the other hand, was distributed throughout the gradient with peaks in fractions 1 and 10. These results show that the bulk of underivatized receptor resides in compartments other than plasma membrane. Another immunoprecipitated band slightly smaller than LDLR appears only in fraction 1. The same band can also be seen in pulse-chase experiments but only after a pulse and chase of 4 h or longer (see Fig. 4). These two pieces of data argue for this band representing a proteolytically modified form of the receptor found exclusively in denser membrane compartments. Chemical characterization of the isolated polypeptides, however, would be required to substantiate this conclusion.

Turnover of the LDLR in Different Membrane Compartments—If receptor in different cellular compartments can be isolated by the protocol described, the rate of appearance and stability of newly synthesized, immunoprecipitated LDLR recovered in these compartments can also be evaluated. For this purpose fibroblast cultures were pulse-labeled for 1 h with [35S]methionine and chased for different intervals before being derivatized at 4 or 18°C. Both streptavidin-agarose-bound and -unbound receptor protein-specific activity was measured after each fraction was separated by SDS-polyacrylamide or agarose gel electrophoresis. When cells were derivatized at 4°C, newly synthesized LDLR appeared at highest specific activity in the bound fraction after 1 h of chase and then decreased in specific activity from 1 to 2 h, after which it remained at constant specific activity for up to 8 h (Figs. 4A and 5A). This same result was seen in four separate experiments regardless of whether pulse-labeled cells were chased in the presence of LDL-depleted human serum (Fig. 4A) or fetal bovine serum (Fig. 5A). The specific activity of unbound receptor peaked at 2 h of chase. In pulse-chase experiments a putative precursor form of the receptor was identified at slightly greater mobility (Mr, = 95,000) in the unbound fraction in the absence of chase. This protein was
always absent after 1 h of chase and was found to represent up to half of the unbound receptor pool in other experiments in the absence of chase. The increase in the total LDLR between 0 and 1 h of chase (Fig. 5, B and C) reflects continued conversion of precursor into mature protein, a process that takes 30 min to complete(15).

When pulse-chased cells were derivatized at 18°C, streptavidin-agarose-bound receptor turned over more slowly than that bound after 4°C derivatization (Fig. 4B). Thus the metabolic stability of LDLR in the 4°C pool is distinctly different from that in the 18°C pool. The specific activity of receptor in the unbound fraction after 18°C derivatization peaked at 2-6 h of chase before decaying at the same rate as bound receptor. These results are expressed in a quantitative form in Fig. 5C. The putative breakdown product of the receptor, also seen on Percoll gradients, is shown in Fig. 4B, lane 4, of the unbound protein.

If the transient appearance of LDLR in the 4°C pool reflects the behavior of newly synthesized protein, 4°C compartmentalized LDLR in steady state labeled cells should not exhibit the same rapid turnover as shown by pulse-labeled receptor. This is shown by the decay of radiolabeled receptor in the bound fractions from cells labeled for 36 h before a chase (Fig. 4C). Quantitative expression of these results (Fig. 5, A and D) shows that both bound and unbound receptor have about the same half-life ($\tau_{1/2} = 10$ h). Turnover of streptavidin-agarose-bound LDLR in cells derivatized at 18°C and labeled to steady state was no different from that seen in cells derivatized at 4°C (results not shown).

**FIG. 5. Quantitation of LDLR turnover data presented in Fig. 4.** A, streptavidin-agarose-bound LDLR bands from cells pulse-labeled, chased, and then derivatized at 4°C (○-○) and from cells labeled to steady state, chased, and then derivatized at 4°C (□-□) were eluted from modified agarose gels and the radioactivity recorded as cpm. B and C, the LDLR bands from streptavidin-agarose-bound (□-□) and unbound (○-○) fractions shown in autoradiograms in Fig. 3, A-C, were excised and the absorbance of eluted silver grains for each at 500 nm was recorded against a blank of the same size removed from the same lane. B, cells were pulse-labeled, chased, and treated with NHS-ss-biotin at 4°C before isolation of bound and unbound receptor. C, cells were pulse-labeled, chased, and treated with NHS-ss-biotin at 18°C before isolation of bound and unbound receptor. D, cells were labeled for 36 h before being chased and treated with NHS-ss-biotin at 4°C and isolation of bound and unbound receptor.

## Discussion

The biotinylation/recovery assay described herein allows the isolation of labeled protein appearing at the cell surface and measurement of its turnover. Although transiently high concentrations of newly synthesized proteins that chemically react with exogenous agents have been seen at the cell surface in other studies (10, 11), this is the first description of this cell surface behavior for a specific protein. The novel finding reported here is that a significant fraction of newly synthesized LDLR transiently localizes to the 4°C accessible compartment prior to its transfer to another compartment. Decrease in the specific activity of 4°C-accessible LDLR between 1 and 2 h of chase could be due to its release into the media (shedding) or internalization. Although only trace amounts of shed LDLR were recovered in the media by immunoprecipitation, it cannot be excluded that shed protein loses its immunoreactivity. On the other hand, LDLR unbound to streptavidin-agarose after derivatizing cells at 4°C briefly increased in specific activity between 1 and 2 h of chase before its subsequent decay. Receptor unbound after derivatizing cells at 18°C increased in specific activity between 2 and 6 h of chase. These results suggest movement of newly synthesized receptor from the cell surface to the 18°C accessible /4°C inaccessible and finally to 18°C inaccessible membrane compartments. The distinct difference in turnover of newly synthesized, streptavidin-agarose-bound receptor between 4°C-derivatized (Fig. 5B) and 18°C-derivatized (Fig. 5C) cells and between pulse-labeled and steady state labeled 4°C-ac-
cessible receptor (Fig. 5D) argues for the rapid transfer of a major portion of newly synthesized receptor from the cell surface to intracellular membranes. If the bulk of receptor that appears at the cell surface after a 1-h chase is shed into the medium, 18°C-derivatized protein, which includes cell-surface protein, would also exhibit rapidly decreasing specific activity between 1 and 2 h of chase, and this is not the case. The transient appearance of newly synthesized receptors at the cell surface prior to redistributing to other compartments implies that sorting decisions to determine membrane protein compartmentation must be made after their cell surface appearance. It is likely that this redistribution of newly synthesized receptor simply reflects its constitutive recycling between the cell surface and intracellular membrane compartments (31). Whether constitutive recycling receptors are those that segregate into clathrin-coated pits when they reach the cell surface is unclear. Similar measurements on cells expressing the internalization defective LDLR mutant (32) should provide the answer to this question.

Aside from the efficacious recovery of cell-surface proteins, another feature of the biotinylation/recovery assay is that intracellular plasma membrane protein can be conveniently separated into three cellular membrane compartments defined by access of derivatizing agent at different temperatures. Although it seems most probable that proteins inaccessible at 4°C but accessible at 18°C are found in recycling endosomes, it cannot be excluded that these proteins also lie on the cell surface, but for other reasons are unreactive at the lower temperature. While differentiation of 4- and 18°C-accessible compartments may only be possible by electron microscopy, 18°C-derivatized and -underivatized receptor can be biochemically distinguished on Percoll gradients. Percoll gradient separation of derivatized protein shows its co-localization with cell-surface and endosomal protein at the top of the gradient. Underivatized receptor is distributed differently on Percoll gradients than derivatized receptor. Its appearance in all gradient fractions and highest concentration in both the bottom and top fractions reflects its residence in vesicles of different density than plasma membrane. The retention of a third or less of underivatized receptor at the top of the gradient in plasma membrane-enriched fractions may have resulted from either imperfect fractionation or some plasma membrane localized receptor that remains underivatized or derivatized but not bound to the streptavidin-agarose. Incomplete derivatization or incomplete binding of derivatized protein to streptavidin-agarose may also explain some experimental variability in compartmentation studies as well as the appearance of more streptavidin-agarose unbound receptor in pulse-chase studies than expected (e.g., Fig. 5, B and C). For this reason it is advisable when doing compartmentation measurements to make triplicate determinations or carry out titrations as shown in Fig. 2.

Modifications of the biotinylated/recovery assay may allow the reappearance of intracellular protein at the cell surface (recycling) as well as transfer of protein in the 18°C-accessible compartment to the inaccessible compartment to be measured. The latter may be the rate-limiting step in membrane protein turnover. The kind of measurements reported in this study are currently being repeated with LDLR mutants to see the effect of sequence alterations on compartmentation and intercompartmental transfer. The likelihood that NHS-sulfo-biotin would react with other kinds of plasma membrane proteins is high as this reagent derivatizes a large number of plasma membrane proteins (11).

Acknowledgments—I thank Stephen Chen for technical assistance, Faye Hagomene and Dr. R. Illingworth for the cells and [125I]-labeled LDL internalization data, and Dr. David Kabat for critical reading of the manuscript.

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Compartmentation and turnover of the low density lipoprotein receptor in skin fibroblasts.
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J. Biol. Chem. 1990, 265:21758-21763.

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