Low density lipoprotein receptor degradation is influenced by a mediator protein(s) with a rapid turnover rate, but is unaffected by receptor up- or down-regulation

Livia A. F. Casciola, Deneys R. van der Westhuyzen, Wieland Gevers, and Gerhard A. Coetzee

MRC/UCT Research Unit for the Cell Biology of Atherosclerosis, Department of Medical Biochemistry, University of Cape Town Medical School, Cape Town, South Africa

Abstract Treatment of cultured human skin fibroblasts with cycloheximide retarded the down-regulation of low density lipoprotein (LDL) receptor activity caused by 25-hydroxycholesterol. The rate of LDL receptor degradation, measured directly by means of [^{35}S]methionine pulse-chase experiments, was also markedly inhibited by cycloheximide (or puromycin), suggesting that continuous synthesis of a short-lived mediator protein(s) was necessary for normal LDL receptor turnover. In the absence of cycloheximide, both the up- and down-regulation of LDL receptor activity took place with a half-time of approximately 12 hr. Pulse-chase measurements with [^{35}S]methionine yielded a receptor half-life ($t_{1/2}$) of 11.7 ± 2.2 hr (n = 10) in up-regulated cells; the $t_{1/2}$ in the partially down-regulated state was similar. The presence of LDL or 25-hydroxycholesterol did not alter this degradation rate. Regulation of LDL receptor activity under these various culture conditions therefore probably occurred solely as a result of changes in the rate of receptor synthesis. The cycloheximide-sensitive factor(s) that influences receptor turnover apparently did not play a regulatory role in the up- or down-regulation of the LDL receptor.—**Casciola, L. A. F., D. R. van der Westhuyzen, W. Gevers, and G. A. Coetzee.** Low density lipoprotein receptor degradation is influenced by a mediator protein(s) with a rapid turnover rate, but is unaffected by receptor up- or down-regulation. *J. Lipid Res.* 1988. **29:** 1481–1489.

Supplementary key words human skin fibroblasts • LDL receptor • cycloheximide • 25-hydroxycholesterol • degradation • turnover • up- and down-regulation

Cells are known to regulate their internal cholesterol supply by means of a coordinated set of responses (reviewed in 1). Briefly, these ensure that capture of cholesterol molecules from the environment (generally catalyzed by low density lipoprotein (LDL) receptors) and synthesis of new cholesterol molecules (catalyzed by a set of enzymes of which an important example is hydroxymethylglutaryl CoA (HMG-CoA) reductase), on the one hand, and temporary sequestration of the lipid in the form of cholesteryl esters (catalyzed by acyl CoA:cholesterol acyltransferase (ACAT)), on the other, are varied in a reciprocal manner by appropriate up-regulation and down-regulation mechanisms.

The processes underlying these homeostatic responses are poorly understood and neither the sensor molecules nor the mediators of their action have been identified. While various oxysterols (2), possibly formed by the action of mixed function oxidases of the endoplasmic reticulum (3, reviewed in 4), have been shown to bind with high affinity to a cytosolic protein (5), details of the repression effects required of such regulators are not yet available, except for information on likely control sequences upstream of the HMG-CoA reductase (6) and LDL receptor genes (7, 8). In the case of HMG-CoA reductase, enhanced degradation occurs concomitantly with gene repression during sterol oversupply or provision of an oxysterol such as 25-hydroxycholesterol (9, 10). Evidence gathered to date on the LDL receptor system is consistent with a transcriptional regulation of the same general kind (7, 8). Experiments reported by Brown and Goldstein in 1975 (11), based on measurements of LDL receptor activity, indicated similar half-lives for this protein in both the up- and down-regulated state, at least in the absence of ongoing protein synthesis (cycloheximide inhibition).

Abbreviations: LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; $t_{1/2}$, half-life; HMG-CoA, hydroxymethylglutaryl coenzyme A; ACAT, acyl coenzyme A:cholesterol acyltransferase; HEPES, N-2-hydroxylethylpiperazine-N′-2-ethanesulfonic acid.

A number of observations on the control of HMG-CoA reductase and ACAT in cultured Chinese hamster ovary cells and mouse peritoneal macrophages have indicated that at least two distinct, but as yet unidentified, short-lived proteins are important in the homeostatic mechanisms relating to cellular cholesterol supply. In the case of HMG-CoA reductase, the decreased activity associated with sterol oversupply or addition of 25-hydroxycholesterol is apparently related specifically to the induction of a short-lived protein(s) that enhances the degradation of the enzyme (12). In the case of ACAT, the decreased activity resulting from sterol depletion is thought to be due to the accumulation of a short-lived, reversible inhibitor of the enzyme (13–15). There is also evidence from the properties of a mutant cell line that a further single protein may be required for the coordinate regulation of both the LDL receptor and HMG-CoA reductase (16).

We have now addressed the following questions that relate specifically to the regulation of LDL receptors in cultured cells. *i)* Is there a short-lived mediator protein(s) that influences the LDL receptor turnover rate? *ii)* Is the half-life of LDL receptors different in states of sterol oversupply or starvation, specifically in the absence of protein synthesis inhibitors such as cycloheximide? Related to this question is whether a ligand-induced change in receptor degradation occurs in the LDL receptor system. Our approach has been to make use of [^{35}S]methionine pulse-chase protocols directly to measure LDL receptor turnover in the absence or presence of protein synthesis inhibitors.

MATERIALS AND METHODS

Materials

Human LDL (density 1.019–1.063 g/ml) and human lipoprotein-deficient serum (LPDS) (density > 1.25 g/ml) were prepared by ultracentrifugation and LDL was labeled with ^{125}I by the iodine monochloride method as previously described (17). Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (MEM), and methionine-free MEM were purchased from Flow Laboratories (Ayrshire, Scotland). Tran^{35}S-label™ (^{35}S-label) from ICN Radiochemicals (Irvine, CA) was the hydrolysate of *E. coli* grown in the presence of $H_2{}^{35}SO_4$ and contained [^{35}S]methionine, 70%; [^{35}S]cysteine, 20%; [^{35}S]methionine sulfoxide, 7%; [^{35}S]cysteic acid, 2%; and other ^{35}S-compounds, 1%. Goat anti-mouse IgG and goat anti-rabbit IgG (both heavy- and light-chain specific) were purchased from Cappel Laboratories (Malvern, PA). 25-Hydroxycholesterol was obtained from Research Plus, Inc. (Bayonne and Denville, NJ).

Cells

Human skin fibroblasts, strain GM 3348A, characterized as LDL receptor-normal, were obtained from the Human Genetic Cell Repository (Camden, NJ). Cultures were prepared according to the following standard protocol. On day 0, each Petri dish (60 × 14 mm) was seeded with 0.15×10^6 cells in 4 ml medium A (DMEM containing 60 μg/ml penicillin G and 100 μg/ml streptomycin sulfate), supplemented with 10% heat-inactivated fetal calf serum. When required, up-regulation of the LDL receptors was initiated on day 4 by incubating the cells with up-regulation medium (DMEM/LPDS; medium A supplemented with LPDS at 5 mg protein/ml).

Receptor antibodies

IgG-C7, a monoclonal antibody to the human LDL receptor, was obtained from Balb/c mice by intraperitoneal injection of clonal hybridoma cells that synthesize IgG-C7 (American Type Culture Collection, CRL 1691). IgG was purified from the ascites fluid by column chromatography on protein A–Sepharose CL-4B, as described (18). IgG-St, a control mouse monoclonal antibody directed against an irrelevant antigen (testosterone), was prepared analogously using hybridoma cells obtained as a gift from Bioclones (Stellenbosch, SA). B3/25, a mouse monoclonal antibody to the human transferrin receptor, was a gift from Dr. I. S. Trowbridge (The Salk Institute for Biological Studies, San Diego, CA). A rabbit polyclonal antibody to the entire bovine LDL receptor and rabbit nonimmune IgG were generous gifts from Drs. M. S. Brown and J. L. Goldstein (University of Texas Southwestern Medical Center at Dallas, TX).

Preformed immune complexes

The following immune complexes containing goat anti-mouse IgG and one of the following mouse monoclonal antibodies were prepared: IgG-C7, IgG-St, or B3/25. Immune complexes were also prepared using goat anti-rabbit IgG and either rabbit polyclonal antibody against the LDL receptor or rabbit nonimmune IgG. Immune complexes were produced essentially as described (19), except that the protein ratio of goat anti-mouse IgG to monoclonal antibody was always 10:1 (at this ratio, more than 85% of the added monoclonal antibodies as recovered in the immune complex).

^{35}S-Labeling and analysis of labeled LDL receptors

Cells were incubated with ^{35}S-label in methionine-free MEM/LPDS and chased in DMEM/LPDS contain-

ing 200 μM unlabeled methionine as indicated in the figure legends. Receptor solubilization and immunoprecipitation were performed essentially as described by Tolleshaug et al. (19). Following the pulse period, cell monolayers were washed once with phosphate-buffered saline (PBS) before adding complete DMEM/LPDS (containing 200 μM unlabeled methionine) in the absence or presence of various agents, as detailed in the legends to the figures. After the appropriate chase time, cells were washed and then lysed by the addition of 0.2 ml ice-cold buffer B containing 10 mM N-2-hydroxylethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 1% (v/v) Triton X-100. Cells were scraped and each dish was washed with a further 0.2 ml of buffer B. Pooled harvests from one dish (0.4 ml) were spun at 4°C for 10 min at 12 000 *g* in a Beckman microfuge (Beckman Instruments, La Jolla, CA). A portion (0.32 ml) of each supernatant was precipitated with 80 μl of the desired preformed immune complex. After immunoprecipitating for 1 hr at 4°C with gentle shaking, the mixture was spun briefly (2 min, 4°C, 5000 *g*), and the pellet was resuspended in 0.2 ml buffer B before being spun through a stepwise sucrose gradient (20). The immunoprecipitates were dissolved in 20 μl of a buffer containing 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 4.7% (w/v) SDS, and 150 mM Tris, pH 6.8, and 20 μl of a solution containing 8 M urea and 200 mM dithiothreitol was then added to each sample. Samples were heated at 90°C for 3 min prior to electrophoresis on 5–20% polyacrylamide slab gels in the presence of SDS (SDS-PAGE) (21). Myosin and low molecular weight markers (Pharmacia, Uppsala, Sweden) were used as molecular weight standards. The gels were treated with 1 M sodium salicylate as fluorographic enhancer for 30 min at room temperature (22), dried, and subjected to fluorography. Quantitation of the fluorograms was performed by excising the relevant fluorogram bands, extracting with 1 M NaOH, and measuring the absorbances on a spectrophotometer at 500 nm (23).

The immunoprecipitation of receptors was linear over an appropriate range of LDL receptor concentration in the standard immunoprecipitation procedure; the radioactivity was directly proportional to the LDL receptor content in the entire set of samples. No detectable radioactivity was found in the 160 kDa position when immune complexes containing the control antibody, IgG-St, were used. Care was taken to expose the fluorograms in such a manner that bands were below the plateau density of film darkening, and hence well within the linear response range of the film (absorbance readings were linear within the range 0.02–0.2 absorbance units, after subtraction of the film blank value).

Surface binding of ^{125}I-labeled LDL at 4°C

Fibroblast monolayers were chilled to 4°C and washed once with ice-cold PBS. The binding of ^{125}I-labeled LDL was then measured after incubating the cells at 4°C for 2 hr, using 10 μg/ml ^{125}I-labeled LDL either in the absence or presence of 200 μg/ml unlabeled LDL (24). High-affinity binding was determined by subtracting the values for ^{125}I-labeled LDL bound in the presence of excess unlabeled ligand from that bound in its absence. All values were determined in duplicate or triplicate dishes and never differed by more than 5% from the means.

Other assays

The extent of inhibition of protein synthesis by cycloheximide or puromycin was assessed by measuring the amount of [^{3}H]phenylalanine incorporated into total cell protein in the presence and absence of either of these agents. After 1 hr of treatment, protein synthesis was inhibited by >98% in each case, while the cells remained >95% viable, as determined by trypan blue dye exclusion. Cell protein was measured by the method of Lowry et al. (25) using bovine serum albumin as a standard.

Determination of $t_{1/2}$ and statistical analysis of data

The $t_{1/2}$ values were obtained from best fit, single exponential curves fitted to data points using a weighted, nonlinear regression program adapted from Duggelby (26), and the parameters thus derived were compared by Peritz' F test (27).

RESULTS

As in the early studies of Brown and Goldstein (11), we first obtained indirect estimates of the rate of turnover of LDL receptors in cultured fibroblasts from the rate of decrease of active cell surface receptors in the presence of cycloheximide. LDL receptors were assessed by the binding of ^{125}I-labeled LDL to cells that had been fully up-regulated and then treated for various times with the inhibitor (**Fig. 1**). In the presence of cycloheximide, receptor activity decreased gradually such that about 50% of activity was lost after 36 hr. Similar results were also obtained using puromycin, a protein synthesis inhibitor with a different mechanism (data not shown). In control cultures, however, receptor activity also decreased after the longer periods of incubation, indicating that a steady-state level of

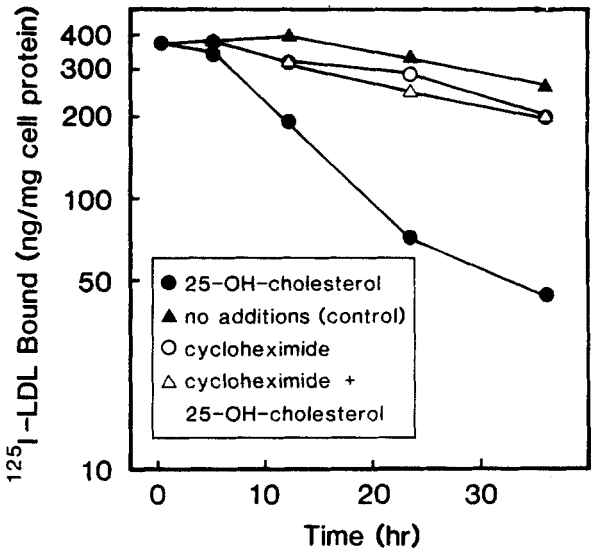

Fig. 1. The effect of cycloheximide on the disappearance of surface LDL receptors in cultures treated with 25-hydroxycholesterol. On day 7 of cell growth, after incubation for 40 hr in DMEM/LPDS, each monolayer received 2 ml of medium supplemented with : no additions (▲), 1 μg/ml 25-hydroxycholesterol (●), 100 μM cycloheximide (○) or 1 μg/ml 25-hydroxycholesterol + 100 μM cycloheximide (△). Cultures were maintained at 37°C for the indicated times, after which high-affinity binding of ^{125}I-labeled LDL was determined at 4°C. The results represent the means of duplicate dishes from a single experiment and are typical of several experiments performed.

receptors was not fully maintained throughout the incubation, possibly as a result of receptor regulation in response to changing cell proliferation and cell density (1). 25-Hydroxycholesterol, a sterol known to effectively down-regulate LDL receptors largely through the suppression of receptors at the transcriptional level (8), was also tested in this manner. In this case, down-regulation kinetics in the presence of this compound indicated a significantly faster LDL receptor degradation ($t_{1/2}$ = 12 hr), assuming that 25-hydroxycholesterol completely blocked new receptor synthesis. Simultaneous treatment with both agents caused a rate of receptor loss that was similar to that observed with cycloheximide alone. These results suggest either that cycloheximide inhibited the breakdown of existing receptors, possibly by blocking the synthesis of a short-lived protein(s) necessary for receptor degradation, or that 25-hydroxycholesterol enhanced the normal rate of receptor degradation.

The down-regulation of receptor activity caused by exposure of up-regulated cells to medium containing LDL was similarly studied by surface-binding assays. Following an initial lag period, receptor activity decreased rapidly with first order kinetics (**Fig. 2**). The half-life estimated from these values, assuming complete inhibition of receptor synthesis and a constant rate of degradation, was 11.4 hr. When down-regulated cells were switched to medium lacking lipoproteins, the single exponential curve depicting the increase in receptor activity mirrored the down-regulation curve (excluding the lag phase) and an estimated half-life of 12.6 hr was calculated assuming a constant rate of degradation. These estimates of receptor half-life were similar to the value obtained using 25-hydroxycholesterol (Fig. 1) and suggest that cycloheximide retarded receptor degradation.

To examine the breakdown of the LDL receptor more directly, and in the absence of cycloheximide, the loss of biosynthetically labeled ^{35}S-labeled LDL receptors was investigated. Antibodies were used to isolate LDL receptors as an intact 160 kDa species (19, 20) following a pulse-chase procedure. As shown in **Fig. 3,** the decay of labeled LDL receptors in up-regulated cells followed first order kinetics and yielded a receptor half-life of 11.7 ± 2.2 hr (mean ± SD of ten experiments). The amount of ^{35}S-labeled LDL receptor precursor (120 kDa) detected in these exper-

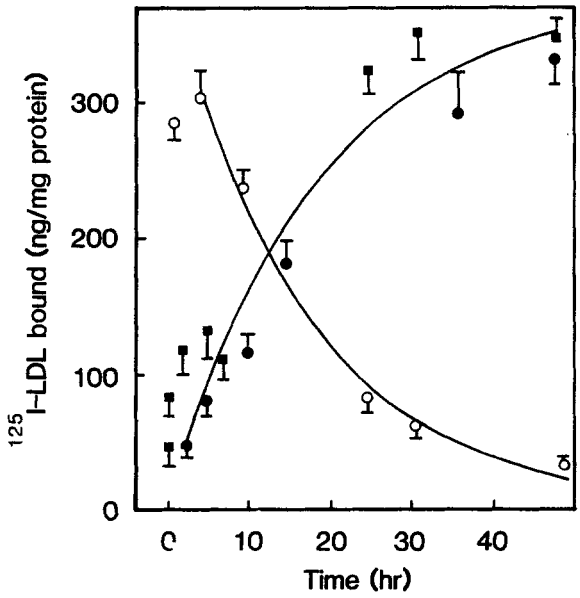

Fig. 2. A comparison of the up-regulation (●, ■) and down-regulation (○) kinetics of LDL receptors in fibroblast cultures. In the up-regulation protocol, cells grown for 5 days in normal growth medium were then incubated in DMEM/LPDS for the indicated times. High-affinity binding of ^{125}-labeled LDL was subsequently measured at 4°C. Two separate experiments were performed (●, ■), using different batches of ^{125}I-labeled LDL, and the best fit, single exponential curve from the pooled data was obtained. In the down-regulation protocol, cells were incubated for 48 hr in DMEM/LPDS (to maximally induce the LDL receptors), after which each dish received 2 ml of fresh DMEM/LPDS, supplemented with 40 μg/ml LDL. The cells were maintained at 37°C for the indicated times, and then incubated with DMEM/LPDS medium for 30 min at 37°C. The cultures were chilled to 4°C, and high-affinity binding was measured. A best fit, single exponential curve was plotted through all points, excluding the zero time point. Data points are mean ± SD obtained from triplicate dishes. Nonspecific binding was always <10% of total binding in the fully up-regulated state.

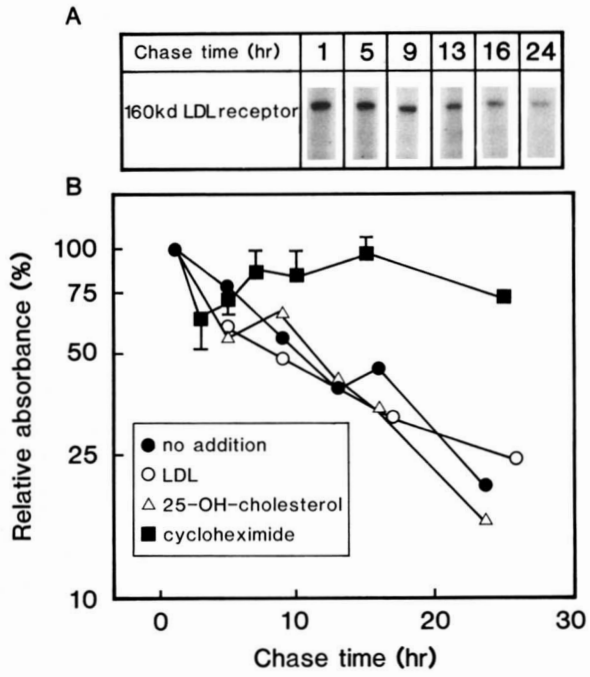

Fig. 3. Degradation of ^{35}S-labeled LDL receptors in up-regulated cells in the absence or presence of various agents. Cells were up-regulated (16 hr) and pulse-labeled for 8 hr with 70 μCi/ml of ^{35}S-label. The cells were then chased for the indicated times in DMEM/LPDS medium containing 200 μM unlabeled methionine and supplemented with : no further addition (●), 50 μg/ml LDL (○), 1 μg/ml 25-hydroxycholesterol (△), or 100 μM cycloheximide (■). The solubilized cell extracts were incubated with preformed immune complexes containing mouse monoclonal IgG-C7, and the labeled immunoprecipitates were subjected to SDS-PAGE and fluorography. (A) Fluorograms showing the 160 kDa LDL receptor at each chase time, after chases had been performed in DMEM/LPDS only. (B) Semilog plots of percentage absorbance (relative to the first chase point) versus chase time were obtained after extraction of the fluorograms. Values plotted as (○, ●, △) are all means obtained from duplicate incubations. Duplicates never differed by more than 15% from the means. The points plotted for cycloheximide (■) are the mean ± SD of results obtained from triplicates, except in the case of the time-point at 24 hr, which is the mean of a duplicate. Similar results were obtained in at least two other such experiments for (○, △, and ■). In the case of (●), these results typify those obtained in nine other separate experiments.

iments was very small in comparison with the 160 kDa mature form. (The 120 kDa precursor was almost undetectable in the fluorogram shown in Fig. 3). This is in line with the rapid conversion of the 120 kDa precursor to the mature receptor (50% conversion in about 15 min; references 19 and 20) and the relatively extended pulse-labeling period of 8 hr used. Precursor maturation, therefore, did not influence the measurement of the rate of degradation of the mature receptor.

We also examined the effects of cycloheximide, LDL, and 25-hydroxycholesterol on LDL receptor degradation. Cells that had been up-regulated, were pulse-labeled and then chased in lipoprotein-deficient medium supplemented with the various agents (Fig. 3). The viability of cells after incubations with either LDL or 25-hydroxycholesterol was checked by assaying the rate of total cellular protein synthesis; in each case this was found to be the same as in controls, indicating an absence of either cell toxicity or decreased cell viability caused by these agents. In the presence of saturating concentrations of LDL, the rate of receptor degradation was unchanged and no ligand-induced alteration in receptor breakdown was detectable. There was also no change resulting from 25-hydroxycholesterol treatment. In contrast, the presence of cycloheximide in the chase medium markedly decreased the rate of receptor degradation. (The apparent increase in the average values between the 4th and 10th hr of chase was not statistically significant.) These results indicate that cycloheximide exerted an inhibitory effect on receptor degradation. Puromycin produced the same effect (data not shown). By comparison, another plasma membrane protein, the transferrin receptor, was degraded in the same cells at a much slower rate (the $t_{1/2}$ was approximately 55 hr) than the LDL receptor, and its degradation was unaffected by cycloheximide.

We used a rabbit polyclonal antibody to test the possibility that we were measuring loss of antigenicity towards the IgG-C7 monoclonal antibody in the pulse-chase experiments, rather than actual degradation of LDL receptors. As with IgG-C7, the polyclonal antibody immunoprecipitated the 160 kDa LDL receptor (**Fig. 4**). In addition, a minor band of 120 kDa was detected with the polyclonal antibody. Neither of these proteins was detected when control nonimmune IgG was used. The 160 kDa band clearly represented the mature LDL receptor; it had a half-life of 13.7 hr (Fig. 4), which was not significantly different ($P > 0.05$) from the value obtained when the monoclonal antibody was used. These results provide evidence that the loss of the 160 kDa band in our pulse-chase experiments represented receptor degradation and not merely loss of immunoreactivity towards IgG-C7. The 120 kDa band may represent a high molecular weight intermediate of LDL receptor degradation as previously postulated by Lehrman et al. (28) and Kozarsky, Brush, and Krieger (29). This band remained relatively unchanged in amount throughout the chase period and thus seems likely to be a degradation product of the mature 160 kDa receptor in vivo, rather than the product of breakdown in vitro.

The results shown in Fig. 3 indicate that the presence of ligand (LDL) does not exert any direct effect on receptor degradation. To examine further whether receptor degradation may play a role in receptor regulation, cells were studied under two different conditions. In the first, cells were up-regulated to approximately 80–100% of maximal receptor activity by

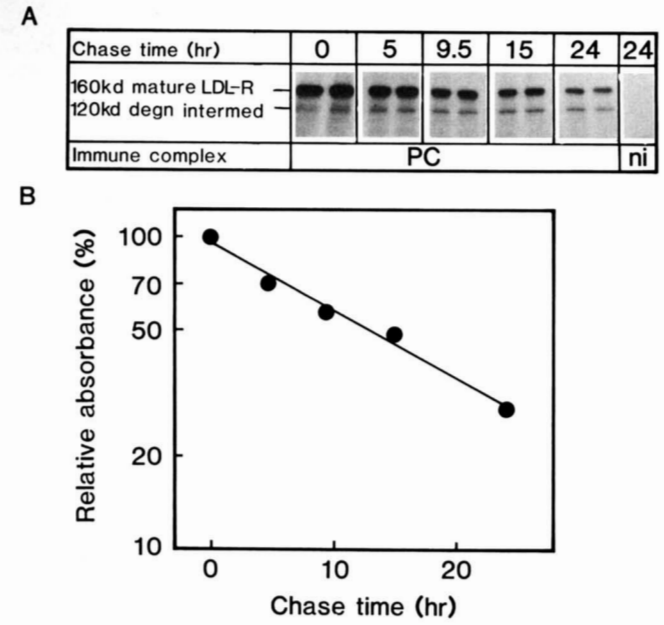

Fig. 4. Degradation of ^{35}S-labeled LDL receptors as measured using a polyclonal antibody. Fibroblasts were up-regulated (16 hr), pulse-labeled for 8 hr in methionine-free MEM-LPDS medium containing 100 μCi/ml ^{35}S-label, chased for the indicated times in DMEM/LPDS medium containing 200 μM unlabeled methionine, and then solubilized in buffer B. Cell extracts were incubated with preformed immune complexes containing either rabbit polyclonal antibody or rabbit non-immune IgG. The ^{35}S-labeled immunoprecipitates were subjected to SDS-PAGE and fluorography. Precipitations with the polyclonal antibody were performed in duplicate (values never differed by more than 7% from the means), while those with the nonimmune IgG were done on single dishes at each chase time. (A) Fluorogram showing the mature 160 kDa LDL receptor and the putative 120 kDa LDL receptor degradation intermediate at various chase times. The nonimmune IgG precipitated neither of these proteins at each chase time. (In the case of the nonimmune IgG, only the 24-hr time point is depicted). (B) Semilog plot of the relative percentage absorbance of the 160 kDa LDL receptor (expressed relative to the 160 kDa LDL receptor at zero hr) at each chase time. PC, immune complex containing rabbit polyclonal antibody to the LDL receptor + goat anti-rabbit IgG; ni, immune complex containing rabbit nonimmune IgG + goat anti-rabbit IgG.

exposure to lipoprotein-deficient media; during the subsequent chase period, the cells were maintained in the absence of lipoproteins. LDL receptors under these up-regulating conditions showed the expected half-life of about 12 hr (**Fig. 5**). Alternatively, cells were maintained in full growth medium containing lipoproteins prior to ^{35}S-labeling for 4–8 hr in lipoprotein-deficient medium. These conditions resulted in a lower level of LDL receptor expression (26–41% of fully upregulated receptor activity) and any possible increase in receptor number during the subsequent chase period was prevented by the presence of LDL in the medium. The half-life of labeled LDL receptors under these down-regulated conditions was approximately 10 hr, a value similar to that found in the up-regulated state. Although the quantitation of ^{35}S-labeled receptors became increasingly less accurate at the lower receptor levels, these results nevertheless argue against any significant differences in degradation rates between the two conditions.

DISCUSSION

We have determined that the half-life of LDL receptors on human skin fibroblasts is approximately 12 hr (Fig. 3), using a pulse-chase protocol in the absence of any added agents. This half-life is markedly shorter than that of the transferrin receptors on the same cells, which is about 55 hr. Most plasma membrane receptors that have been studied appear to have half-lives in the range of about 10–80 hr (reviewed in 30), and our results are consistent with the concept that transmembrane receptor proteins are not turned over as a unit in the plasma membrane; more likely there is a mechanism(s) that determines the unique rate at which

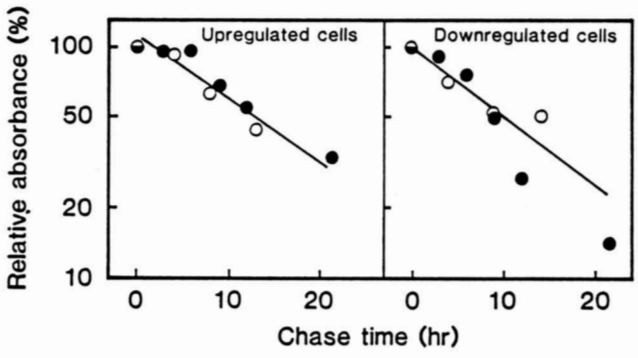

Fig. 5. Degradation of ^{35}S-labeled LDL receptors in up- and down-regulated cells. For the up-regulated cells, cultures were treated with DMEM/LPDS for 16 hr (○) or for 40 hr (●), pulse-labeled with medium containing 50 μCi/ml ^{35}S-label for 7 hr (○) or for 4 hr (●), and chased for the indicated times in DMEM/LPDS containing 200 μM unlabeled methionine. For the down-regulated cells, cultures were maintained in medium A supplemented with 10% fetal calf serum. Prior to initiating the pulse, the cells were incubated for 30 min with DMEM/LPDS at 37°C. The cells were then pulse-labeled in methionine-free MEM/LPDS containing 150 μCi/ml ^{35}S-label for 8 hr (○) or 200 μCi/ml ^{35}S-label for 4 hr (●), and subsequently chased for the indicated times in complete DMEM/LPDS supplemented with 10 μg/ml LDL. Following the chase period, detergent-solubilized cell extracts were incubated with preformed immune complexes containing monoclonal antibody to the LDL receptor (IgG-C7). Semilog plots of percentage absorbance (relative to the first chase point) versus chase time were obtained after extraction of the fluorograms. The data points were obtained from two separate, paired experiments [experiments 1, (●) and 2, (○)]. Cell surface receptor activity in the down-regulated cells was 26% (●) and 41% (○) of that in the corresponding up-regulated cells in these experiments. Points plotted are the means of duplicates (●) or triplicates (○). The $t_{1/2}$ values determined from the pooled data were 12.2 hr and 9.9 hr for the up-regulated and down-regulated cells, respectively.

each of the plasma membrane receptor types is degraded. The data presented here show the suitability of the LDL receptor as a model system for future studies of surface protein degradation, a process that is only poorly understood.

The decreased rate of LDL receptor breakdown that was observed in the presence of protein synthesis inhibitors (Figs. 1 and 3) suggests that a short-lived protein(s) influences the rate of degradation of the LDL receptor. Cessation of synthesis and subsequent depletion of the cellular pool of this putative short-lived protein would account for the severely decreased rate of receptor loss in the presence of the inhibitor. There would appear to be several general possibilities for the action(s) of such a mediator protein. Since degradation is first order under all conditions examined, the absolute rate of LDL receptor degradation is proportional both to the concentration of (homogeneous) receptors and to the effective activity of the degradation system; the latter may in turn reflect either the concentration of the catalytic entities concerned or the presence in the receptor population of molecules with enhanced susceptibility to degradation. The prolonged $t_{1/2}$ of the receptor caused by an absence of the mediator could thus arise at any of these potential loci of action.

The fact that a short-lived protein influences, or perhaps even mediates, the degradation of LDL receptors is remarkably analogous to the situation regarding HMG-CoA reductase turnover in Chinese hamster ovary cells (12). Since the rate of the LDL receptor degradation is unchanged during up- or down-regulation, while that of the reductase increases up to sixfold in states of sterol oversupply, it is clear that the two mediator proteins cannot be the same or, at least, that their site and/or mechanism of action must be very different. By the same token, it is unlikely that the protein postulated to mediate the coordinate expression of LDL receptors and HMG-CoA reductase (16) can be one of the mediators that appear to influence the degradation rates of these two proteins. The postulated short-lived inhibitor of ACAT that may accumulate during sterol starvation (13–15) must also be a distinct entity. The participation in the overall homeostatic system of further entities such as oxysterol-forming enzymes and oxysterol receptors, as well as the unknown enzyme systems responsible for the degradation of the key catalytic entities, illustrates the complexity of control and the opportunities for modulation of the responses by a wide variety of agents and circumstances.

We addressed the question of whether the binding of a ligand (LDL) exerts any direct influence on the rate of receptor breakdown. The pulse-chase experiments performed in the presence or absence of LDL (Fig. 3) are consistent with entirely independent routing of receptor and ligand towards degradation, since the presence of LDL did not significantly alter receptor half-life. This behavior of the recycling LDL receptor stands in marked contrast to certain other receptors, for example, the epidermal growth factor receptor, which is internalized and rapidly degraded together with its ligand (31). Since similar half-lives of the LDL receptor were obtained when either the C7 monoclonal or the rabbit polyclonal antibody was used, it is likely that the loss of radioactivity from the 160 kDa band in our pulse-chase experiments represented receptor degradation and not loss of receptor antigenicity.

Receptor breakdown was also assessed on the basis of cell surface receptor activity and the rate at which transition occurred between the up- and down-regulated states in response to the absence or presence of ligand (Fig. 2). The results were consistent with a receptor half-life of about 12 hr (similar to the value determined from the pulse-chase measurements) and a constant receptor degradation rate during the experimental period. The results indicate that the number of active surface receptors reflects the total receptor content in cells under the conditions examined, and that regulation is not associated with receptor redistribution between the cell surface and intracellular sites.

An important related question is whether the half-life of the LDL receptor differs between the up- or down-regulated states when a considerable difference in LDL receptor concentrations exists. The half-lives of LDL receptors remained the same at the two levels of receptor up- and down-regulation examined (Fig. 5). Our experiments, therefore, strongly suggest that up- and down-regulation of the LDL receptor is due only to changes in the rates of receptor synthesis.

In conclusion, we have shown that the half-life of LDL receptors is about 12 hr in human skin fibroblasts and that cycloheximide markedly retards receptor degradation, presumably blocking the synthesis of a short-lived mediator necessary for receptor breakdown. This mediator is apparently distinct from a number of other postulated short-lived proteins that help to regulate the various elements of the cellular homeostatic system for cholesterol. Receptor degradation is unaffected by the binding of ligand and the receptor half-life remains constant over a range of cellular receptor content, indicating that homeostatic regulation is exerted only at the level of receptor synthesis.

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