Mechanism of the Growth-related Activation of the Low Density Lipoprotein Receptor Pathway*

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Growth activation of quiescent cells leads to enhanced low density lipoprotein (LDL) receptor expression at the cell surface. To determine the basis for this stimulated receptor activity, we measured LDL receptor activity, changes in receptor protein mass, and mRNA abundance in quiescent and growth-activated cultured human skin fibroblasts. Growth activation, using insulin or platelet-derived growth factor, led to dose-dependent increases in cellular LDL receptor mRNA level (average 5.2-fold increase at 10 ng/ml platelet-derived growth factor, 4.1-fold increase at 58 ng/ml insulin) and cell surface expression (average 3.5-fold increase at 10 ng/ml platelet-derived growth factor, 2.5-fold increase at 58 ng/ml insulin). Increased LDL receptor mRNA levels could be detected as early as 2 h after addition of growth factor (3.2-fold), whereas increased levels of LDL receptor binding and mass were not detected until after 4–8 h. Growth activation led to induction of LDL receptor gene transcription, and the increase of LDL receptor mRNA produced by addition of growth factor was completely prevented by actinomycin D. These observations indicate that growth-related activation of the LDL receptor pathway is accounted for, primarily, by growth-activated enhancement of LDL receptor gene transcription.

The low density lipoprotein (LDL) receptor is a widely distributed cellular endocytic receptor that functions to internalize cholesterol-containing LDL particles (1, 2). Lipoprotein cholesterol that is internalized via this receptor is required for optimal cell growth in culture (3) and may serve to provide cholesterol for synthesis of new plasma membrane, which contains the largest portion of cellular cholesterol (4). The function of this receptor is negatively regulated by cellular cholesterol content (1, 5, 6). Thus, sterol enrichment of cells has been shown to depress LDL receptor expression at the cell surface (as measured by 125I-LDL binding) and to decrease the cellular level of LDL receptor mRNA. On the other hand, cholesterol depletion of cells using lipoprotein-deficient serum or human high density lipoprotein-3 (both of which promote cholesterol efflux from cells) enhances LDL receptor expression. Growth stimuli such as insulin and platelet-derived growth factor also augment LDL receptor activity in fibroblasts and arterial smooth muscle cells (7–9). Analysis of binding kinetics has provided indirect evidence that this enhancement of LDL receptor activity is due to an increase in the number of LDL receptors at the cell surface. The mechanism for this increase has not been reported, but it has been shown for other proteins that the net positive effect of insulin on protein levels is due to combined inhibition of protein degradation and stimulation of protein synthesis (10–12). The latter effect for certain proteins may result from regulation at a translational level where insulin increases the rate of peptide chain initiation (13). For other proteins, insulin induction of protein synthesis occurs via increases in relevant mRNA species (14). Likewise, growth activation of quiescent cells with serum has been shown to positively modulate synthesis of certain proteins by modulating cognate mRNA species, whereas other proteins are stimulated only at the translational level (15).

In this study, we probe the mechanisms for humoral enhancement of LDL receptor activity. We report that growth activation of quiescent human skin fibroblasts leads to enhanced LDL receptor gene transcription. In addition, actinomycin D completely abolishes growth-related increases in LDL receptor mRNA. Increased levels of LDL receptor mRNA can be detected as early as 2 h after addition of growth factor. Increased LDL receptor mass or activity are not detected until at least 4 h after growth factor addition. These data indicate that the growth-related activation of the LDL receptor pathway occurs primarily at a translational locus.

MATERIALS AND METHODS

Cell Culture—Human skin fibroblasts were grown from explants of penile foreskin as previously described (16). Cells were used between the 7th and 18th subculture. Subcultured cells were fed twice weekly with 10% fetal bovine serum in DMEM. Cells were plated at 5 × 10^4 per 35-mm dish or 5 × 10^5 per 75-cm² flask. After 6 days cells were confluent at 3–4 × 10^6 per 35-mm dish and 3–4 × 10^6 per 75-cm² flask. In some experiments, cells were preincubated in 1% pooled human lipoprotein-deficient plasma-derived serum. The efficacy of such incubations in establishing quiescence was confirmed by the observation that subconfluent cells incubated in 1% lipoprotein-deficient plasma-derived serum showed no increase in cell number or protein over 3 days, whereas identically plated cells grown in 10% fetal bovine serum showed a doubling of both parameters. In addition, cells incubated in 1% lipoprotein-deficient plasma-derived serum incorporated 4.7 ± 0.5 × 10^6 dpm/10^6 cells of [3H]thymidine into DNA, which is similar to levels of incorporation reported in other studies of quiescent fibroblasts (17). Plasma-derived serum was prepared as described by Kazlauskas and DiCorleto (18) and lipoproteins were removed by ultracentrifugation in potassium bromide (d = 1.215 g/ml) as previously described (16). Partially purified PDGF (19) was
provided by E. Raines and R. Ross (University of Washington, Seattle, WA). The PDGF content of these preparations was determined by radioceptor assay (19) and this information was used to calculate PDGF concentration in culture media. A polyclonal antibody to PDGF inhibits 100% of the mitogenic activity of these PDGF preparations.

Lipoproteins—Human blood was drawn into EDTA-containing syringes (final concentration 1.5 mg/ml). After removal of red blood cells, phenylmethylsulfonyl fluoride (10 μM) and Trasylol (10 μg/ml) were added and LDL (d = 1.019-1.063 g/ml) was isolated by sequential centrifugation in potassium bromide (16). Lipoprotein fractions were dialyzed into 1 mM EDTA in phosphate-buffered saline and stored steriley at 4 °C under nitrogen. Lipoprotein fractions were checked for purity by agarose gel electrophoresis, and appropriate apoprotein composition was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). LDL was iodinated with [125I] by the iodine monochloride method as modified for lipoproteins to yield specific activities of 80-210 cpm/ng of LDL protein (21). Radioactivity in LDL tracer preparations was >96% precipitable in 10% trichloroacetic acid.

**LDL Receptor Binding and Mass Measurements**—Binding of [125I]-LDL to fibroblast monolayers was measured using displacement by dextran sulfate as described by Goldstein and Brown (22) except that preincubations prior to chiling cells to 4 °C were performed as described in the figure legends. [125I]-LDL was added to cells at a concentration of 5 μg of LDL protein/ml, plus or minus a 100-fold excess of unlabeled LDL. The binding of iodinated LDL that occurred in the presence of 100-fold excess of unlabeled LDL was subtracted from total binding to give a measure of high affinity receptor binding. Measurement of [125I]-LDL binding provides a measure of active cell surface receptors and not total cell receptor mass. Binding data were calculated as nanograms of ligand bound per milligram of cell protein.

LDL receptor mass was assayed by Western blot and slot blot hybridization. Fibroblasts were scraped into a buffer containing 10 mM Hepes (pH 7.4), 2.5 mM MgCl2, 200 mM NaCl, 2 mM CaCl2, 0.5 mM phenylmethyisulfonyl fluoride, 0.5 mg/ml leupeptin, 15 mg/ml soybean trypsin inhibitor, and 40 mM octyl glucoside. After 10 min at 4 °C, cell extracts were spun at 100,000 g for 20 min and the supernatant was recovered for measurement of protein. For Western blotting, electrophoresis and transfer to nitrocellulose were performed as described by Beisiegel et al. (23). After transfer, nitrocellulose was incubated for 1 h at room temperature in blocking buffer (10 mM Tris-HCl (pH 8), 50 mM NaCl, 2 mM CaCl2, 0.5% non-fat dry milk) and then washed quickly in reaction buffer (1% non-fat dry milk in phosphate-buffered saline). Filters were then incubated for 2 h at room temperature in this buffer containing 1 μl rabbit anti-BSA (acylated) at 1:10000 dilution against LDL receptor purified from bovine submaxillary gland (24). Following this incubation, nitrocellulose filters were washed three times for 15 min each in reaction buffer and again incubated in this buffer containing 0.4 μl of [125I]-protein A (Du Pont, 133 μCi/ml, 8.3 μCi/μg) for 2 h at room temperature. Filters were then washed quickly five times with reaction buffer and two times with phosphate-buffered saline, and dried for autoradiography. For slot blots, 50, 25, 12.5, and 6.25 μg of cell extract protein was applied to nitrocellulose paper using a slot blot apparatus and the filters were processed as described above. Autoradiograms of slot blots were quantitated as described for slot blot mRNA analyses.

**LDL Receptor mRNA Measurements**—For quantitation of LDL receptor mRNA, total cellular RNA was extracted in guanidine thiocyanate as previously described (20). Radiolabeled LDL receptor cDNA probe was prepared by random primed synthesis (25) and was hybridized to cellular RNA immobilized on nitrocellulose paper (20). Cloning plasmid pIC 119 contains a 119-base pair EcoRI subfragment of the 5.3-kilobase pair cDNA clone for the human LDL receptor (5) cloned into pSP64 and was generously provided by Dr. David Russell (University of Texas Health Science Center, Dallas, TX). A range of cellular RNA concentrations was applied to nitrocellulose using a slot blot apparatus (Schleicher & Schuell) and hybridization was measured by autoradiography using a slot blot hybridization signal with RNA load. Autoradiographs of the slot blot hybridizations were quantitated by scanning densitometry or by measuring the optical density at 500 nm of autoradiographic silver grains which were eluted in NaOH and rehydrated in 30% glycerol (20). Film exposures of varying duration were quantitated according to the lens response to the hybridization signal. Changes in the slope of the lines generated by either quantitation method reflect relative changes in LDL receptor mRNA abundance. For Northern blot hybridization, electrophoresis and transfer were performed as described by Maniatis et al. (26) and hybridization was performed as previously described (20).

Experiments using a human β-actin probe were performed as described above except that this probe was radiolabeled by nick translation (20). The β-actin cDNA clone has been previously described (27).

**Run-on Transcription Assay**—Human skin fibroblast nuclei were isolated and nuclear run-on transcription was performed as described by Greenblatt and Chojkier (28) except that the reaction buffer contained 10 mM MgCl2, 1 mM MnCl2, 6.7 mM creatine phosphate, and 1 mM ATP. Newly generated transcripts were isolated as described by Groudine et al. (29). In three experiments, final counts in isolated transcripts averaged 1 cpm/nucleus. Equal numbers of counts/minute from labeled transcripts were hybridized to 3 μg of linearized cDNA for the human LDL receptor (pLDL-RP/HH1) as described by Bremner and Chojkier (30). Dried nitrocellulose filters were exposed to x-ray film for 3-7 days.

**Other Assays**—DNA synthesis was measured as the incorporation of [3H]thymidine (2.5 μCi/ml) into DNA during a 2-h pulse (16). Protein determinations were performed by the method of Lowry et al. (31) using bovine serum albumin as standard.

### RESULTS

The addition of biosynthetic human insulin, in doses ranging from 0.58 to 58 ng/ml, led to enhanced DNA synthesis (24,982 ± 3,411 versus 74,201 ± 10,700 dpm [3H]thymidine incorporated into DNA per milligram of cell protein) and LDL receptor expression in cultured fibroblasts (Fig. 1A). Enhanced LDL receptor binding at the cell surface reflected the increased abundance of LDL receptor mRNA species (Fig. 1B). The increased DNA synthesis in response to insulin demonstrates that biosynthetic human insulin, at the concentrations used, acts as a mitogen for cultured human skin fibroblasts. This observation is in accord with observations by Conover et al. (32), demonstrating increased human skin fibroblast thymidine incorporation and cell number in response to similar insulin concentrations.

Growth activation using partially purified PDGF likewise augmented DNA synthesis (24,672 ± 5,958 versus 475,275 ± 73,133 dpm/mg), LDL receptor expression, and mRNA abundance in human fibroblasts (Fig. 2A and B). Relative changes in mRNA abundance exceeded changes in LDL receptor activity when compared over similar dose ranges for both insulin and PDGF. For example, from 0 to 58 ng/ml insulin there is a 4.5-fold increase in LDL receptor mRNA abundance but a 2.5-fold increase in LDL receptor cell surface expression. Comparing 2 ng/ml PDGF (the lowest concentration used in Fig. 2B) to 10 ng/ml PDGF, LDL receptor mRNA increases 3.7-fold, whereas binding increases 1.7-fold. These observations suggest that stimulation of LDL receptor activity by these two growth-promoting agents is accomplished predominantly at pretranslational levels. In three separate experiments similar to the one presented in Fig. 1B, 58 ng/ml insulin produced an average 4.1-fold increase in LDL receptor mRNA. In two additional experiments similar to that shown in Fig. 1B, PDGF at 10 ng/ml led to an average 5.2-fold increase in LDL receptor mRNA levels compared with cells incubated in serum-free medium alone. Wells incubated with 58 ng/ml insulin contained 18% more cell protein compared with control wells. PDGF (10 ng/ml)—containing wells showed a 26% increase in protein content, which is the largest increase we observed under our experimental conditions.

Fig. 3 provides time course information on PDGF induction of the LDL receptor pathway. Increased relative abundance of LDL receptor mRNA (Fig. 3, A and B) can be detected as early as 2 h after PDGF addition (3.2-fold). This increase precedes, by several hours, detectable increases in LDL receptor mass (Fig. 3C) or activity (Fig. 3D). LDL receptor protein

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2 E. Raines, personal communication.
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**FIG. 1. Insulin stimulation of LDL receptor expression.** Human skin fibroblasts were incubated in 0.2% bovine serum albumin in DMEM containing the indicated concentration of biosynthetic human insulin for 18 h. At that time, measurements of LDL receptor activity (A) or LDL receptor mRNA abundance (B), as described under "Materials and Methods," were performed. The data shown in B were quantitated by elution of silver grains as described in the text; LDL binding is reported as the mean ± S.D. of triplicate measurements. *R* values for the lines in B are: control, 0.97; insulin, 0.96 (11.6 ng/ml), 0.96 (29.0 ng/ml), and 0.99 (58.0 ng/ml).

**Fig. 2. PDGF stimulation of LDL receptor expression.** Fibroblasts were incubated for 48 h in DMEM containing 1% lipoprotein-deficient plasma-derived serum. Cultures were washed four times with serum-free medium and incubated for another 18 h in serum-free medium containing the indicated concentrations of PDGF. At that time, LDL receptor activity (A) and mRNA abundance (B) were quantitated as described in the legend to Fig. 1. Points shown for mRNA measurements in PDGF at 10 ng/ml represent analysis of RNA pooled from two separate flasks. *R* values for lines in B are PDGF, 0.99 (2 ng/ml), 0.92 (5 ng/ml), and 0.99 (10 ng/ml).

To examine the events accounting for PDGF induction of mRNA levels, nuclear run-on transcription analysis was performed on control and PDGF-treated fibroblasts. Each autoradiogram is representative of three separate analyses done on extracts from three separate experiments which show similar results. In each case, a single band of appropriate size is detected by the LDL receptor cDNA (Fig. 4A) or antisera (Fig. 4B). Also in each case, the degree of induction observed after PDGF stimulation is appropriate when compared with that observed by slot blot analysis.

**DISCUSSION**

In animal cells the pathways for internalization of cholesterol, via the LDL receptor, and synthesis of endogenous cholesterol are regulated in an integrated fashion to preserve cell cholesterol content (33). Cholesterol depletion enhances whereas cholesterol repletion suppresses the activity of each of these pathways. Activation of growth in quiescent cells also stimulates lipoprotein cholesterol internalization and cholesterol synthesis (7, 8). The importance of this growth-related enhancement of the LDL receptor pathway is demonstrated by the observation that cells fail to respond optimally to mitogens in absence of cholesterol-containing lipoproteins (3, 34).

Russell and co-workers (5, 6) have demonstrated that increased LDL receptor activity at the cell surface after cellular cholesterol depletion occurs via induction of its cognate fibroblasts with a labeled cDNA for human β-actin indicated that insulin, at 18 h, had no effect on β-actin mRNA levels, whereas PDGF increased these levels, as has been reported by others (28) (not shown).
Fig. 4. Northern and Western blot analyses of control and PDGF-treated cells. Fibroblasts were incubated as described in the legend to Fig. 3. Cells were harvested 2 h after PDGF (10 ng/ml) addition for Northern blot analysis (A) or at 16 h after PDGF (10 ng/ml) addition for Western blot analysis (B). For A, 10 μg of cellular RNA per lane was used for electrophoresis. For B, 75 μg of cellular protein per lane was used for electrophoresis. The position of a purified LDL receptor standard is shown on the right.

mRNA. In this report we examine, in detail, the mechanism for the growth-related increase in LDL receptor cell surface expression described by others (7-9). The concentration-dependent responses illustrated in Figs. 1 and 2 indicate that growth-induced increases of LDL receptor messenger RNA equal or exceed those of LDL receptor cell surface activity. The time course data presented in Fig. 3 indicate that PDGF-induced excursions of LDL receptor mRNA precede, by more than 2 h, excursions of LDL receptor mass or binding. Taken together, these data provide no support for a translational or post-translational locus for growth-related activation of the LDL receptor pathway. The data shown in Fig. 5A show that PDGF enhances transcription of the LDL receptor gene. This observation alone would not exclude additional mechanisms which could increase LDL receptor mRNA levels, such as mRNA stabilization. The data in Fig. 5B, however, indicate

DMEM alone for 18 h. PDGF (10 ng/ml) was added to appropriate cultures and cells were harvested for measurement of LDL receptor messenger RNA (A, B), protein (C), or binding (D), at the indicated times. For C and D, PDGF-induced changes are expressed as fold change in PDGF-treated cultures compared with control cultures. Points shown in C and D represent averages from triplicate cultures. For C, autoradiographs of slot blot hybridizations were quantitated by scanning densitometry as described under "Materials and Methods." Control values in A and B refer to values in control cells harvested at zero time unless otherwise labeled. R values for the lines in A and B are: zero time control, 0.99; 2-h control, 0.97; 24-h control, 0.97; 2-h PDGF, 0.99; 24-h PDGF, 0.99.
the LDL receptor mRNA is completely lost. We believe measurements made on nuclei prepared from cells grown in two separate experiments.

that, in the absence of ongoing transcription, PDGF induction of the LDL receptor mRNA is completely lost. We believe that a synthesis of the data presented in this manuscript makes it most likely that growth-related increases of LDL receptor activity are accounted for primarily by regulation of LDL receptor gene transcription.

A number of recent publications have examined regulatory sequences from the LDL receptor gene promoter in detail (35–37). Data obtained from stable transfection experiments utilizing portions of the human LDL receptor promoter fused to the bacterial chloramphenicol acetyltransferase gene identified a 177-bp sequence which extends 144 bp upstream from the major transcription initiation site as the most important sequence for LDL receptor gene basal expression and sterol regulation. Within this sequence there are three imperfect repeats, each 16 bp long, which are particularly important for expression and end-product repression by sterol. Repeats 2 and 3, which are located adjacent to each other with their 5' end at –68, have been studied in great detail using a hybrid promoter containing a 42-bp sequence from the LDL receptor promoter (which encompasses these repeats) inserted immediately upstream of the TATA box (position 32) in the HSV-TK gene promoter. Studies using this hybrid promoter fused to chloramphenicol acetyltransferase structural sequences have shown that repeat 3 is a strong constitutive positive transcription element which binds purified Sp1. Repeat 2 is responsible for strongly suppressing repeat 3 when sterols are present.

The mechanism by which LDL receptor gene transcription is facilitated by growth activation remains to be determined. Whether or not the promoter regulatory sequences responsible for enhanced transcription after growth initiation are encompassed within previously defined regulatory sequences will require further detailed experimentation. As noted previously, growth activation not only leads to increased LDL receptor expression, but also to increased endogenous cholesterol synthesis. This observation, taken together with the data indicating that lipoprotein cholesterol is required for optimal cell growth after mitogenic stimulation, strongly suggests that cell growth imposes an increased cellular requirement for cholesterol. This increased need for cholesterol (to be used, for example, for new membrane synthesis) may release sterol-mediated repression of the LDL receptor promoter and thereby account for enhanced transcription. This hypothesis requires that common sequences would transduce the regulatory activity of sterols and growth on the LDL receptor promoter. An alternative hypothesis would suggest that growth-enhanced LDL receptor gene transcription occurs, at least partially, independent of changes in cell cholesterol homeostasis. An example of the latter situation applies to the humoral regulation of steroidogenesis and LDL receptor expression in ovarian granulosa cells. In these cells Golos and Strauss (38) have shown that gonadotrophin enhancement of LDL receptor synthesis occurs even when normally suppressive concentrations of 25-hydroxycholesterol are included in the cell culture medium. Determining whether this model of regulation applies to the mechanism for growth-related activation of LDL receptor gene expression will require additional investigation.

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