Mannose 6-Phosphate Receptor-mediated Uptake of Modified Low Density Lipoprotein Results in Down Regulation of Hydroxymethylglutaryl-CoA Reductase in Normal and Familial Hypercholesterolemic Fibroblasts*

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Monophosphotetramannosyl-1-deoxymannitol-1-yllow density lipoprotein (Man-6-P-LDL) was prepared by covalent attachment of the pentasaccharide ω-(6-phospho-tetra(al-3)mannosyl(al-2)mannose to amino groups on low density lipoprotein. Normal human fibroblasts were shown to specifically bind, internalize, and degrade 125I-labeled Man-6-P-LDL. Specificity for the mannose 6-phosphate (Man-6-P) receptor was demonstrated by competitive displacement with cold Man-6-P-LDL, Man-6-P, or mannose. No displacement was seen with cold LDL. Kd is estimated to be 1 x 10⁻⁹ M. Degradation of 125I-labeled Man-6-P-LDL in familial hypercholesterolemic fibroblasts showed the same time course and specificity as observed in normal fibroblasts. Man-6-P-LDL was also able to deliver cholesterol to the cytosol where down regulation of the enzyme 3-hydroxy-3-methylglutaryl CoA reductase was observed in both normal and familial hypercholesterolemic fibroblasts. Down regulation could be blocked by Man-6-P in both cell lines. The possible uses of agents such as Man-6-P-LDL as research probes and therapeutic tools directed to specific cell types are discussed.

Studies on the receptor-mediated transport of a number of hormones, toxins, and other proteins into cells have revealed several common features which may have general application to all protein transport systems (for review see Ref. 1). In many cases binding to a cell surface receptor involves a discrete portion of the protein molecule spatially distinct from the site on the molecule eliciting a physiological response once entry has been achieved. This generalized bifunctional scheme may be applied not only to bacterial toxins and certain peptide hormones (where the response observed is due to some enzymatic or other action of a peptide (2-4), but also includes transport proteins in which the physiological effector is non-protein in nature (e.g. cholesterol transport by low density lipoproteins (5), vitamin B12 transport via transcobalamin II complex (6), etc.). Clear demonstration of the separation of functional components is often possible by physical separation (3, 7, 8), selective inhibitors (9, 10) or promoters (9), covalent modification (11), and genetic selection (12). If the functions are truly separate one should be able to modify receptor specificity by alterations on one portion without affecting the other. Utilizing this concept, a number of hybrid proteins have now been constructed with varying degrees of efficacy (13-19). The receptor-binding specificity of one protein is covalently attached to a second protein molecule bearing its own enzymatic or other biological specificity. These hybrid proteins may serve as a new class of cell type-specific pharmacological reagents (1, 13). In addition they may have utility for probing the biochemical steps in the entry process.

While the use of protein-protein hybrids as probes of transport mechanisms is a relatively new development, the ultimately more refined use of small ligands as the recognition moiety for binding has been exploited successfully for many years especially in the study of carbohydrate-specific catalytic systems for glycoproteins (20). The work reported here uses this technique of coupling an oligosaccharide chain to a protein as a means of specifying an alternate receptor-mediated route of entry. We report here the first use of this method to incorporate a new recognition signal into a transport protein such that receptor specificity is altered. Thus, it is possible to achieve transport via an unique receptor-mediated process utilizing a route independent of that normally used, while maintaining physiological effector function intact.

We have reported elsewhere the use of this method to produce a cell type-specific toxin, Man-6-P-ricin (21).

By using appropriate sugars to block the receptor-mediated uptake of a variety of glycoproteins, a number of carbohydrate recognition systems involved in uptake have been defined. Receptor-mediated endocytosis of glycoproteins involves primary recognition by the terminal sugar residue (nonreducing) in one of the oligosaccharide chains of the glycoprotein. Carbohydrate recognition systems have been described showing specificity for galactose (e.g. asialoligoceroproteins in rat and avian hepatocytes (22, 23)), N-acetylgalactosamine or mannose (avian hepatocytes, liver sinusoid cells, and macrophages (23-26)), fucose (lactoferrin in mouse hepatocytes (27)) and mannose 6-phosphate (lysosomal hydrolases in human fibroblasts (28, 29)). Rogers and Kornfeld (28) followed by Lee and coworkers (30, 31) and others (26) have constructed a number of semi-synthetic "neoglycoproteins" which have proved to be essential for a definition of the carbohydrate specificity of the hepatic clearance systems recognizing galactose or mannose/N-acetylgalactosamine. Further confirmation of the cell type specificity of these systems has been obtained by electron microscope autoradiographic studies (32).

Extensive study on the mechanism of uptake of low density lipoproteins into cultured human fibroblasts has resulted in a proposed description of the defect in type IIa hyperlipidemia (familial hypercholesterolemia) (for review see Ref. 5). This...
heritable disease apparently results from the absence of an LDL receptor on the surface of fibroblasts and a number of other cell types. The mutant cells are thus unable to bind LDL through the normal high affinity route of wild type fibroblasts. This inability to bind LDL specifically thus prevents internalization, degradation, and subsequent release of cholesterol and down regulation of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase.

In this report we show that the failure of familial hypercholesterolemic fibroblasts to take up LDL can be circumvented in tissue-cultured fibroblasts by supplying monophosphotetra-mannosyl-1-deoxymannitol-1-yl-LDL. By coupling LDL to a naturally occurring oligosaccharide, ω-(6-phospho)-tetra(α-L)-mannosyl-(β-2)mannose, uptake of LDL into the lysosome is achieved via the route normally used for the recapture of lysosomal hydrolases (as indicated by the ability to block uptake completely with Man-6-P and the inability of cold LDL to block entrance or degradation of [3H]labeled Man-6-P-LDL). Lipoprotein thus transported is resultantly degraded in both normal and familial hypercholesterolemic fibroblasts with a resultant down regulation of HMG-CoA reductase in both cell types.

Another chemically modified LDL derivative has been reported to elicit such response in fibroblasts, e.g. cationized LDL in which the lipoprotein was covalently linked with a large number of aminoethyl groups with N,N-dimethyl-1,3-diamine (33). The uptake of cationized LDL is a nonsaturable process over the reported concentration range (and hence may represent uptake as a result of low affinity binding to a very large number of surface membrane anionic sites). To contrast this with the present study, LDL coupled to a phosphomannan oligosaccharide has lost the ability to bind to the LDL receptor but has the newly acquired ability to bind specifically to a finite number of lysosomal hydrolase receptors.

We have thus modified a physiological protein, LDL, by attachment of mannose-6-phosphate. Man-6-P-LDL binds to a distinct physiological receptor, enters the cell, and is thus delivered to the lysosome independently of the LDL receptor. In the lysosome, degradation of the lipoprotein particle results in expression of the normal physiological function of LDL, namely down regulation of the enzyme HMG-CoA reductase.

**EXPERIMENTAL PROCEDURES**

**Isolation of LDL**

All LDL used in this study was between density 1.006 to 1.06 and was prepared by the density flotation method of Havel et al. (34). Fresh plasma collected in Na2/EDTA (4 mM) was centrifuged for 15 min at 4000 rpm to remove any cellular debris. The supernatant plasma was centrifuged at 4°C for 16 h at 50,000 rpm in a Beckman 60 Ti rotor after which lipoproteins of less than solenoid density (1.006) had concentrated in a layer at the top of the tube, with an intermediate clear zone and then a second layer of heavier lipoproteins. The infranatant was adjusted back to the original volume by addition of NaCl (0.85% w/v) and then the density was raised to 1.06 by addition of solid KBr (7.98 g/100 ml) and centrifuged as before. The tubes were sliced and the supernatant recovered, adjusted back to the original volume with KBr solution (density = 1.06), and centrifuged.

Again the tubes were sliced, and the LDL recovered in the supernatant was dialyzed for 24 h against four changes of 1 liter of saline/EDTA. Following dialysis LDL was concentrated on an Amicon UM 30 ultrafilter to approximately 30 mg of protein per ml and stored at 0–4°C. Maximum storage time was 2 months. Lipoprotein-depleted serum was obtained by density flotation of human serum at density 1.21. All lipoproteins were removed by slicing the supernatant layer. The infranatant was adjusted to pH 7.4 and dialyzed against three changes of 2 liters of saline/EDTA (pH 7.4) over a 24-h period. Serum thus obtained was filter sterilized and stored at -20°C.

**Preparation of ω-(6-Phospho)-tetra(α-1-3)mannosyl(α-1-2)mannose**

Phosphomannan obtained from Hansenula holsteii NRRL Y-2448 was a gift from Dr. Morey E. Slodki, Northern Regional Research Laboratories, Peoria, IL. Preparation of the phosphonylated penta-saccharide was essentially as previously described (35). The structure of this penta-saccharide has been shown to be 6-P-Man(α-1-3)Man(α-1-3)Man(α-1-2)Man (35, 36). Phosphomannan (5.4 g) was swollen in 290 ml of KCl by heating to 100°C on a hot plate for 30 min with stirring. After cooling the pH was adjusted to 2.5 by addition of 2 N HCl, and the solution was covered with a watch glass and placed in a boiling water bath for 6 h. After cooling the pH was adjusted to 6.5 with NaOH (1 N), and the faintly cloudy solution was centrifuged for 5 min at 9000 X g. The volume of the supernatant was readjusted to 200 ml by addition of water. Barium acetate (2 g) was then dissolved in the supernatant, and 1 N NaOH was added to pH 9.5. Addition of 20 ml of absolute ethanol followed by chilling on ice effected precipitation of unhydrolyzed phosphomannan and a high molecular weight acid-resistant core. After centrifugation as before, 400 ml of cold absolute ethanol was added to precipitate the barium salt of PMs, which was recovered by centrifugation as before. The precipitate so obtained was dissolved in water and submitted once again to ethanol fractionation. The final product was dissolved in water and lyophilized for storage as the Ba2+ salt.

Prior to use in coupling studies, 250 mg of PMs was dissolved in 2 ml of acetic acid (0.1 N) and chromatographed on Sephadex G-25 superfine (95 X 1.5 cm) with 0.1 N acetic acid as eluant. Fractions (2 ml each) were collected and analyzed for carbohydrate content. In a typical experiment 3 to 5% of the applied carbohydrate (elution volume 90 ml) eluted before the major peak. This was believed to be residual core phosphomannan and (PMs), resulting from incomplete hydrolysis. The major peak (elution volume 110 ml to 130 ml) was pooled and acetic acid removed on a rotary evaporator at 40°C. The Ba2+ salt was exchanged for Na+ by passing over a column of Dowex 50 (H+ form, 10 X 2 cm) and titrating with NaOH (1 N) to pH 6.5. PMs (Na+) so obtained was concentrated on a rotary evaporator at 40°C to approximately 400 mg/ml for use in the coupling experiment. Hexose:phosphate ratio of this peak was 5.0 to 5.5:1.0.

**Coupling of ω-(6-Phospho)-tetra(α-1-3)mannosyl(α-1-2)mannose to LDL**

Coupling of the oligosaccharide to LDL was by reductive amination essentially as reported by Schwartz and Gray (37). To 30 μl of LDL containing approximately 9 mg of LDL was added 100 μl of PMs, (Na+) (40 to 45 mg), 17 μl of 1:1 N,N-bis-hydroxy-2-ethynylglycine (bicine, final 0.05 M), and 25 μl of NaCNBH3 (final, 0.06 M). The reaction was incubated at 37°C for 4 h and then dialyzed against four changes of 1 liter of saline/EDTA, pH 7.4. Glc-LDL was prepared by substituting maltooltriose (final, 0.2 M) for PMs. Carbohydrate analysis of the product and control LDL incubated with NaCNBH3 but in the absence of exogenous carbohydrate revealed an increase of 40 to 50 PMs groups per 500,000 daltons of LDL protein or approximately to EDTA. For available lysine content, we reported 57 lysine per 100,000 molecular weight apo B (38). NaCNBH3 freshly obtained (Aldrich) was adequate for these purposes. Older stock was purified according to the method of Borch et al. (39). Ethyl acetate was substituted for ether as the solvent of choice in preparation of the dioxanate.

**Iodination of Man-6-P-LDL**

Labeling of Man-6-P-LDL with 125I was accomplished by the iodine monochloride method of Macfarlane (40). To Man-6-P-LDL (1 mg) in 130 μl of saline/EDTA in a 1.5-ml Eppendorf tube was added 80 μl of glycine/NaOH (1 M, pH 10) and 10 μl of carrier-free Na[I131] (1 μCi).
Man-6-P-LDL Regulates HMG-CoA Reductase via Man 6 P Receptor

mgI, Amersham/Searle); 20 μl of ICl (diluted to 244 μg/ml in 0.01 M NaCl) was then added, and the tube was rapidly capped and shaken vigorously. The product was freed of unbound label by passage over a Sephadex G-25 superfine column (30 cm x 1.5 cm) equilibrated with Tris-HCl (0.05 M), NaCl (0.015 M), bovine serum albumin (0.1%). Fractions were collected in tubes that had been preincubated with a 1% solution of bovine serum albumin prior to the experiment. Typically 20 to 30% of added [125I]-labeled LDL and [251]-labeled Man-6-P-LDL were displaced by assuming 100% recovery of protein from the column. The activity of various preparations ranged from 50 to 200 cpm/ng of protein.

Cell Cultures

Normal human fibroblasts were kindly supplied to us by Dr. Elizabeth Neufeld, National Institute of Arthritis and Metabolism, Bethesda, Md. GM2000 fibroblasts isolated from a familial hypercholesterolemic individual were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J. All cells were grown in monolayer and used before the 20th passage. Cell cultures were maintained in a 5% CO2 atmosphere at 37°C in 490 cm2 roller bottles (normal cell lines) or in 150 cm2 tissue culture flasks (GM 2000) containing 50 ml of Eagle's minimal essential medium with Earle's salts, modified to contain 1.6 g/liter of NaHCO3 and supplemented with nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml, Aureomycin (50 μg/ml), and Mrcosytin (100 units/ml). Normal medium contained 10% nonheat-inactivated fetal calf serum (Associated Biomedical Supplies), while lipoprotein-depleted medium contained 6% human lipoprotein-depleted serum (prepared as described). 1-Chloroamine was added to 7 ml immediately before use. Cells were dissociated from stock flasks with 0.075% trypsin in Puck's saline/EDTA (NaCl, 0.14 M, KCl, 5.4 mm, dextrose, 5.5 mm, NaCO3, 0.3 M, Tris-[HCl (0.015 M), pH 7.4), then 2 ml of a buffer containing 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mg/ml of heparin (Ca2+ salt, Sigma) and 50 mm NaCl, pH 7.4, was added, and the dish was incubated for 1 h at room temperature. This buffer then was removed for counting to determine heparin-releasable binding.

2. Binding—After removal of medium, the dish was washed rapidly with two 1-ml portions of Buffer A (Tris, 0.05 M, NaCl, 0.15 M, pH 7.4), then 2 ml of a buffer containing 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mg/ml of heparin (Ca2+ salt, Sigma) and 50 mm NaCl, pH 7.4, was added and the dish was incubated for 1 h at room temperature. This buffer then was removed for counting to determine heparin-releasable binding.

3. Uptake—Label remaining associated with the cells was determined next by addition of 2 ml of 0.1 M NaOH to each dish to solubilize cells. After 30 min at room temperature, the solution was removed using a Pasteur pipette, transferred to test tubes (12 x 75 mm), and vortexed. Part of the solution (1.5 ml) was reserved for counting, and 30 μl were used for determination of protein.

HMG-CoA Reductase

The assay for HMG-CoA reductase used was similar to that of Brown et al. (43) with the omission of detergent and the addition of a crude medium separation of products as described by Beg et al. (43). Cells were harvested by scraping cells from the dish with a rubber policeman into 1 ml of chilled Buffer A, transferring to 1.5-ml Eppendorf tubes, and then centrifuging at 900 x g for 2 min at room temperature. The pellet was then washed by resuspending in 1 ml of the same buffer and centrifuging as before. The pellets were frozen in liquid nitrogen and stored at -20°C until use (usually less than 24 h). Extracts were prepared by freezing and thawing the pellets (3 cycles of 1 min in liquid nitrogen, followed by 10 min at room temperature) and then resuspending them in 200 μl of Buffer B (KH2PO4, 150 mm, dithiothreitol (5 mm), Na2EDTA (5 mm), and KCI (0.2 M)). The suspension was mixed well using first a motor-driven glass pestle to disrupt any large aggregates and then vortexing. One-hundred microliters were dispensed into 1.5-ml Eppendorf tubes for assay, and 101 μl of the assay mix (101 gl) consisted of 20 μl of each of the following stock solutions: KH2PO4 (1 M), Glc-P (0.2 M), NADP (25 mm), dithiothreitol (40 mm), and t-3[3-glutaryl-3-'Cl]-hydroxy-3-methylglutaryl coenzyme A (0.3 mm, 7.67 Ci/mol (New England Nuclear)). and 1 μl of glucose 6-phosphate dehydrogenase (Sigma type XI) diluted to 700 units/ml. After incubation for 2 h at 37°C the reaction was stopped by addition of 25 μl of 5 M HCl followed by 25 μl of RS[5-3H(N)]-mevalonolactone (0.15 m, 0.038 Ci/mol (New England Nuclear)). The tubes were recapped, vortexed, and incubated for 15 to 30 min at 37°C to effect conversion of mevalonic acid to mevalonolactone. Samples were then passed over columns containing 1 ml of Bio-Rex 5 (100 to 200 mesh, Cl form) and eluted into scintillation vials with two 1-ml portions of distilled water. To determine heparin-releasable binding.

Analytical Methods

Protein concentrations were determined by the method of Lowry et al. (46). Carbohydrates were measured by the phenol-sulfuric acid method (47). Phosphate analysis was performed as described by Ames et al. (48).

Results and Discussion

Studies on Man-6-P-LDL in Normal Human Fibroblasts—[125I]-Labeled Man-6-P-LDL is shown to be rapidly bound to the surface of normal human fibroblasts, reaching a steady state after about 1 h (Fig. 1). Similarly, accumulation of [125I]-labeled Man-6-P-LDL in a nonheparin-releasable compartment begins immediately and then approaches steady state. Trichloroacetic acid soluble counts released to the extracellular medium show an initial lag of about 1 h and then a rapid increase indicating that the artificial hybrid constructed is rapidly degraded by normal human fibroblasts. Since degradation continues to increase with time while binding and internalization levels off we conclude the surface receptors are maintained at a steady state concentration during the course of internalization. Recycling of receptors or unmasking of existing receptors may account for this process.

The processes of binding, uptake, and degradation are shown to be both saturable and specific for the Man-6-P receptor (Fig. 2, A, B, C) since [125I]-labeled Man-6-P-LDL can be displaced by excess cold Man-6-P-LDL but not by LDL. Dependence on the Man-6-P receptor-mediated uptake is further shown by the displacement of label with both mannose 6-phosphate and mannose. Kd for mannose 6-phosphate is approximately 10-5 M and for mannose approximately 10-7 M. These values are in agreement with results obtained for inhibition of uptake of lysosomal hydrolases (28, 29). When Man-6-P-LDL was assayed for its ability to competitively inhibit uptake of α-t-iduronidase in fibroblast cultures, Kd was determined to be 2 x 10-7 M (49). While an accurate dissociation constant Kd cannot be obtained from our data, we estimate Kd ≤ 2 x 10-9 M. This is close to Kd = 10-8 M (50, 51) reported for various lysosomal hydrolases. The absence of displacement by cold LDL indicates that Man-6-P-LDL does not depend on the LDL pathway for any step in the process leading to degradation and must, therefore, be wholly dependent.

3 Since there was a sharp drop in [125I]-labeled Man-6-P-LDL binding with the lowest addition of cold man-6-P-LDL, we can only estimate Kd < 2 x 10-7 M, the concentration of cold Man-6-P-LDL resulting in a 50% loss in binding of label.
fibroblasts. In the experiment shown in Fig. 3, loss of 50% of the enzyme activity occurs at approximately 8 x 10^-5 M for Man-6-P-LDL compared to 1 x 10^-4 M for LDL. Various preparations of Man-6-P-LDL were able to down regulate the enzyme to varying extents. The best preparations of Man-6-P-LDL were equipotent with LDL in this assay. This variation in batches may reflect some loss of cholesterol from the lipoprotein particle as a result of the prolonged incubation at 37°C in the coupling reaction.

In the presence of 10^-2 M Man-6-P a shift in the dose response curve is seen for Man-6-P-LDL but not for native LDL. The ability of Man-6-P to provide almost complete blockade of the Man-6-P-LDL-mediated down regulation of HMG-CoA reductase is a further piece of evidence supporting our conclusion that Man-6-P-LDL can no longer be recognized by the receptor for native LDL but must now rely entirely on the recognition signal provided by the attached oligosaccharide.

LDL incubated with PMs, but in the absence of NaCNBH3, was shown to behave identically with native LDL (Table I) indicating that covalent attachment of the ligand is necessary to specify entrance. Glc-LDL, at a concentration predicted to reduce enzyme activity by 50%, was unable to effect the down regulation of HMG-CoA reductase in agreement with other reports that LDL modified in such a way as to lose a substantial proportion of its amino groups is no longer recognized by the LDL receptor (52).

Studies on Man-6-P-LDL in Familial Hypercholestero-

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**FIG. 1.** Time course of 125I-labeled Man-6-P-LDL binding, internalization, and degradation by normal human fibroblasts. Cells were plated at 2 x 10^5/dish in 60-mm culture plates as described under "Experimental Procedures" and grown for 6 days in complete medium (+10%, FCS). On Day 6 the medium was removed and replaced with 2 ml of medium containing 6% lipoprotein-depleted serum. After 24 h, the medium was replaced with 2 ml of fresh lipoprotein-depleted medium containing 0.7 μg/ml of 125I-labeled Man-6-P-LDL (75 cpm/ng, 39,000 cpm/dish) ± 100 μg/ml of Man-6-P-LDL and incubated at 37°C in a 5% CO2 atmosphere. Degradation, at various times the medium was removed and 125I-labeled trichloroacetic acid-soluble material was determined by precipitating with 5% trichloroacetic acid. Binding, after removal of medium, the dish was washed twice rapidly with 2-ml portions of cold Buffer A and then incubated for 1 h at room temperature with 2 ml of Buffer plus heparin (Hepes (10 mM), heparin (10 mg/ml), and NaCl (50 mM), pH 7.4). An aliquot (1 ml) of this buffer was used for determining heparin-releasable counts. Internalization, nonheparin-releasable material was released by dissolving the cell monolayer in 2 ml of NaOH (0.1 n). Values are corrected for cell protein. Correction for specific binding, internalization, and degradation was made by subtracting counts obtained in the presence of excess cold Man-6-P-LDL. At 24 h, nonspecific binding, internalization, and degradation were, respectively, 22, 9, and 10% of the total counts obtained. Lipoprotein concentrations are based on protein molecular weight of 500,000 per mol of LDL. Error bars indicate ± 1 S.D. —— binding (heparin-releasable counts); —— internalization (nonheparin-releasable counts); —— degradation (trichloroacetic acid-soluble counts).

**FIG. 2.** Competitive displacement of 125I-labeled Man-6-P-LDL binding, internalization, and degradation in normal human fibroblasts. A, binding; B, internalization, and C, degradation of 125I-labeled Man-6-P-LDL were determined as in Fig. 1. Incubations were for 4 h at 37°C with 0.6 μg/ml of 125I-labeled Man-6-P-LDL (52 cpm/ng, 31,000 cpm/dish) in the presence of the indicated amount of competitor. ——, Man-6-P-LDL; ——, LDL; ——, Man-6-P; Δ—Δ, mannose. Nonspecific counts were not subtracted.
LDL, and LDL* was used to determine a protein correction factor. An aliquot of the solubilized cell suspension was once more. Cells were ruptured by repeat cycles of freeze-thawing in liquid nitrogen. Buffer B was replaced with fresh lipoprotein-depleted medium containing the indicated amount of lipoprotein ± Man-6-P (10^{-5} M). After 10 h of incubation at 37°C in a 5% CO2 atmosphere, medium was removed, and the cells were scraped off into 1 ml of cold Buffer A, centrifuged, and then washed once more. Cells were disrupted by repeated cycles of freeze-thawing in liquid nitrogen. Buffer B (200 μl) was added, the suspension was mixed, and then 100 μl were incubated with the assay mix described under "Experimental Procedures." The enzyme activity was determined by converting [14C]mevalonic acid formed to the standard of [3H]mevalonolactone. An aliquot of the solubilized cell suspension was used to determine a protein correction factor. Man-6-P-LDL, LDL; without NaCNBH3.

FIG. 3. Down regulation of HMG-CoA reductase by Man-6-P-LDL (M6P-LDL) and LDL in normal human fibroblasts. Cells were plated and grown as described in Fig. 1. At time zero the medium was replaced with fresh lipoprotein-depleted medium containing the indicated amount of lipoprotein ± Man-6-P (10^{-5} M). After 10 h of incubation at 37°C in a 5% CO2 atmosphere, medium was removed, and the cells were scraped off into 1 ml of cold Buffer A, centrifuged, and then washed once more. Cells were disrupted by repeat cycles of freeze-thawing in liquid nitrogen. Buffer B (200 μl) was added, the suspension was mixed, and then 100 μl were incubated with the assay mix described under "Experimental Procedures." The enzyme activity was determined by converting [14C]mevalonic acid formed to the lactone and eluting from a column of Bio-Rex 5. Correction was made for recovery of [14C]mevalonolactone by including an internal standard of [3H]mevalonolactone. An aliquot of the solubilized cell suspension was used to determine a protein correction factor.

Man-6-P-LDL, LDL* was incubated for 40 h at 37°C in the presence of PM5 but without NaCNBH3.

FIG. 4. Time course of degradation of [125I]-labeled Man-6-P-LDL in normal and familial hypercholesterolemic fibroblasts. Cells were plated at 2 × 10^5 cells/dish in 2.5 ml of medium containing 6% lipoprotein-depleted serum. After 36 h of growth, medium was removed and replaced with fresh lipoprotein-depleted medium containing 0.2 μg/ml of [125I]-labeled Man-6-P-LDL (250,000 cpm) and the indicated competitor. Degradation to Trichloroacetic acid-soluble material was determined at various times as described in Fig. 1. All values were corrected by subtracting trichloroacetic acid-soluble counts from the input medium.

**TABLE I**

| Addition                               | HMG-CoA reductase activity (% control) |
|----------------------------------------|---------------------------------------|
|                                        | Without Man-6-P | With Man-6-P (10^{-5} M) |
| None                                   | 100 ± 14        | 106 ± 11                 |
| 25-Hydroxycholesterol (0.6 μg/ml) and cholesterol (15 μg/ml) | 5 ± 0.3         |                           |
| LDL                                    | 20 ± 2          | 38 ± 6                   |
| Man-6-P-LDL                            | 53 ± 10         | 107 ± 20                 |
| Glc-LDL                                | 101 ± 14        | 117 ± 2                  |
| LDL^{++}                               | 18 ± 2          | 34 ± 8                   |

All lipoproteins used were at a final concentration of 20 μg/ml.

LDL* was incubated for 40 h at 37°C in the presence of PM5, but without NaCNBH3.

Man-6-P-LDL for both normal and familial hypercholesterolemic fibroblasts. The results for these representative experiments show the down regulation in familial hypercholesterolemic fibroblasts which was always slightly less than that observed for normal fibroblasts assayed on the same day. The down regulation can be inhibited by 10^{-5} M Man-6-P and thus can be clearly seen to involve the Man-6-P receptor. (The higher Man-6-P concentration to inhibit this process is required to overcome the extremely large dose of Man-6-P-LDL (up to 100 μg/ml) used in these studies compared with the concentration of [125I]-labeled Man-6-P-LDL used in the tracer studies (0.1 to 0.5 μg/ml).)

Our data demonstrate the use of an oligosaccharide marker to covalently modify a protein with a known physiological role in such a way that transport to the interior of the cell is affected via an alternate receptor, leaving the physiology of the protein intact (as measured by intracellular response). The rate of internalization and degradation of [125I]-labeled Man-6-P-LDL is comparable to that reported by Brown and Goldstein for [125I]-labeled LDL (53). Thus we have created a functional hybrid lipoprotein capable of binding with high affinity to a surface receptor on fibroblasts followed by internalization and degradation. The resultant down regulation of HMG-CoA reductase confirms the delivery of lipoprotein cholesterol to the compartment in the cell where its physiological effector role is expressed.

The successful alteration of receptor specificity thus

| Time | [125I]-Labeled Man-6-P-LDL Degradated (pmol/g cell protein) |
|------|-----------------------------------------------------------|
| 12   |                                                |
| 18   |                                                |
| 24   |                                                |
| 36   |                                                |
| 48   |                                                |

**G. J. Murray and D. M. Neville, Jr., unpublished observations.**
Fig. 5. Down regulation of HMG-CoA reductase in normal and familial hypercholesterolemic fibroblasts by Man-6-P-LDL and LDL. Cells were plated and grown in medium containing 6% lipoprotein-depleted serum as in Fig. 4. After 36 h of growth medium was replaced with fresh lipoprotein-depleted medium containing 100 μg/ml of lipoprotein Ξ Man-6-P (10 mM), incubated for 10 h, and assayed for HMG-CoA reductase activity as described in Fig. 3. A, normal cells; B, familial hypercholesterolemia cells. A, effect of LDL on down regulation of HMG-CoA reductase. B, inhibition of Man-6-P-LDL-mediated down regulation of HMG-CoA reductase.

Achieved is fully predicted by the bifunctional model for transport proteins. What role, if any, the LDL portion of Man-6-P-LDL has in achieving eventual localization in the lysosome remains to be answered. Bifunctionality may extend beyond the transported protein to the receptor itself. Since there are clearly two functions, binding and transport, it will be of interest to determine whether these functions reside in the same molecules or require interaction between several membrane components. Other work in the laboratory using oligosaccharides linked to ricin may be useful in dissecting out these interrelationships between the multiple functions of a receptor and the transported protein (21).

Man-6-P-LDL or other lipoprotein hybrids designed to make use of this approach can be applied to the study of the various levels of control of lipoprotein synthesis and secretion. The rate of LDL-apo protein B synthesis has been shown to be significantly higher in familial hypercholesterolemic homozygotes than in normals while the fractional catabolic rate was lower (54). Observations on the synthesis of cholesterol in liver biopsies in familial hypercholesterolemic individuals indicate oversynthesis or defective suppression of synthesis in response to cholesterol feeding (55, 56). In contrast there is no increase in the incorporation of [14C]acetate into cholesterol when whole body synthesis is measured (57), nor is there increased synthesis of cholesterol in skin biopsies of familial hypercholesterolemic patients (58). The evidence points to a complex series of control mechanisms interacting to produce elevated plasma LDL in familial hypercholesterolemic individuals. Hybrid lipoproteins similar to Man-6-P-LDL can be constructed and may possibly cast some light on these relationships. Man-6-P-LDL could be used to deliver cholesterol to the fibroblasts of familial hypercholesterolemic individuals if this is found to be of therapeutic use. A similar means might be used to regulate hepatic cholesterol synthesis. In fact the hybrid approach permits the design of a variety of agents for delivery to many specific cell types.

We have demonstrated here one example of this new class of physiological reagents in which receptor specificity may be altered while effector function is maintained. This approach may prove useful in the construction of research probes and therapeutic agents for delivery to specific cell types.

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