Three Types of Low Density Lipoprotein Receptor-deficient Mutant Have Pleiotropic Defects in the Synthesis of N-linked, O-linked, and Lipid-linked Carbohydrate Chains

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Abstract. Biochemical, immunological, and genetic techniques were used to investigate the genetic defects in three types of low density lipoprotein (LDL) receptor-deficient hamster cells. The previously isolated *ldlB*, *ldlC*, and *ldlD* mutants all synthesized essentially normal amounts of a 125,000-D precursor form of the LDL receptor, but were unable to process this receptor to the mature form of 155,000 D. Instead, these mutants produced abnormally small, heterogeneous receptors that reached the cell surface but were rapidly degraded thereafter. The abnormal sizes of the LDL receptors in these cells were due to defective processing of the LDL receptor's N- and O-linked carbohydrate chains. Processing defects in these cells appeared to be general since the *ldlB*, *ldlC*, and *ldlD* mutants also showed defective glycosylation of a viral glycoprotein, alterations in glycolipid synthesis, and changes in resistance to several toxic lectins. Preliminary structural studies suggested that these cells had defects in multiple stages of the Golgi-associated processing reactions responsible for synthesis of glycolipids and in the N-linked and O-linked carbohydrate chains of glycoproteins. Comparisons between the *ldl* mutants and a large number of previously isolated CHO glycosylation defective mutants showed that the genetic defects in *ldlB*, *ldlC*, and *ldlD* cells were unique and that only very specific types of carbohydrate alteration could dramatically affect LDL receptor function.

We previously have isolated a large number of mutant Chinese hamster ovary (CHO)[1] cells that do not express active cell surface receptors for low density lipoprotein (LDL), the principal cholesterol transport protein of human plasma (reviewed in Krieger et al., 1985). Although all of these mutants have an LDL receptor-deficient phenotype (i.e., inability to bind significant amounts of ^{125}I-labeled LDL), genetic studies have shown that these mutants can be divided into four distinct complementation groups: *ldlA*, *ldlB*, *ldlC*, and *ldlD* (Krieger, 1983; Kingsley and Krieger, 1984). This suggests that at least four distinct genes are required for expression of LDL receptor activity.

The recent isolation of antibodies that recognize hamster LDL receptors has allowed us to examine the different classes of *ldl* mutants in detail. In the accompanying paper, we showed that different *ldlA* mutants synthesize different mutant forms of the LDL receptor protein (Kozarsky et al., 1986). These experiments, in conjuction with direct analysis of gene structure using cloned probes (Sege, R. S., K. F. Kozarsky, and M. Krieger, manuscript in preparation), have confirmed the conclusion from earlier genetic experiments that *ldlA* mutants have defects in the structural gene for the LDL receptor (Kingsley and Krieger, 1984; Sege et al., 1984) and are thus analogous to cells derived from familial hypercholesterolemia patients (Goldstein and Brown, 1983). In the current studies, we have used anti-receptor antibodies to investigate the nature of the genetic defects in *ldlB*, *ldlC*, and *ldlD* mutants. Biochemical and genetic studies suggest that the LDL receptor deficiencies in these mutants are associated with general defects in processing N-linked, O-linked, and lipid-linked carbohydrate chains.

1. *Abbreviations used in this paper*: CHO, Chinese hamster ovary; LDL, low density lipoprotein; Lec, lectin-resistant; VSV, vesicular stomatitis virus; WGA, wheat germ agglutinin.

Materials and Methods

Materials

Human and newborn calf lipoprotein-deficient sera, human LDL, and ^{125}I-labeled LDL were prepared as previously described (Krieger, 1983). Ham's F-12 medium (30 μM methionine), methionine-free Ham's F-12 medium, minimal essential medium, and alpha minimal essential medium were obtained from Gibco Laboratories (Grand Island, NY) or KC Biological Inc. (Lenexa, KS). [^{35}S]Methionine (>800 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Endoglycosidase H was provided by Phillips Robbins and Catherine Hubbard (Massachusetts Institute of Technology [MIT]). Sialidase and pronase were purchased from Calbiochem/Behring Diagnostics Corp. (La Jolla, CA). Lectins and all other reagents and supplies were purchased from

Sigma Chemical Co. (St. Louis, MO), or were obtained as previously described (Krieger, 1983).

Cell Culture and Selection of Somatic Cell Hybrids

Proline requiring (Pro^-) parental and mutant CHO-K1 cells were grown in medium I [Ham's F-12 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM)] supplemented with 5% (vol/vol) fetal bovine serum (medium II). The *ldlA*, *ldlB*, *ldlC*, and *ldlD* mutants used in this paper refer to mutant 7, mutant 11, mutant 475, and mutant 14-1a, respectively (Krieger, et al., 1981; Kingsley and Krieger, 1984). Previously isolated lectin-resistant (Lec) CHO mutants and their corresponding parental cells were obtained from Dr. Pamela Stanley (Albert Einstein College of Medicine, New York). The properties of these cells have been reviewed recently (Stanley, 1984, 1985*a*, *b*). Pro^- Lec mutants representing recessive complementation groups 1, 1A, 2, 2B, 3, 4, 5, 8, 13, 13A, and 15; a double Lec1.Lec2 mutant; and the dominant LEC mutants 10, 11, 12, 14, and 17 were maintained in medium II. The Lec1.Lec2 mutant was originally isolated by Dr. Carlos Hirschberg (St. Louis University, St. Louis), who provided us with corresponding parental cells. Glycine, adenosine, and thymidine requiring (Gat^-) Lec mutants representing recessive complementation groups 1, 2, 3, and 8; the double mutants Lec1.Lec6 and Lec2.Lec7; and the dominant LEC16 mutant were maintained in medium II formulated with alpha minimal essential medium instead of Ham's F-12. Hybrids between (Gat^-) Lec mutants representing complementation groups 1, 2, 3, and 8, and the (Pro^-) *ldl* mutants representing complementation groups B, C, and D were isolated by cell fusion (Kingsley and Krieger, 1984) and growth in minimal essential medium lacking proline, glycine, adenosine, and thymidine which was supplemented with 3% (vol/vol) newborn calf lipoprotein-deficient serum. All incubations were at 37°C in a humidified 5% CO_2 incubator unless otherwise indicated. Stocks of Lec5, Lec9, and LEC16 cells were maintained at 34°C.

Wheat Germ Agglutinin Selections

Forty million CHO cells were plated into 40 100-mm dishes in medium I supplemented with 10% (vol/vol) newborn calf serum and wheat germ agglutinin (WGA; 20 μg protein/ml). After 7 d, cells were washed once with PBS and refed medium III (medium I supplemented with 3% [vol/vol] newborn calf lipoprotein-deficient serum) to induce LDL receptor activity. 2 d later, all surviving colonies were screened in situ for expression of LDL receptor activity using DiI-LDL, (LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine iodide; Pitas et al., 1981; Kingsley and Krieger, 1984). Colonies that failed to accumulate fluorescence were isolated and tested for complementation with existing *ldl* mutants as previously described (Kingsley and Krieger, 1984).

Viral Infection and Metabolic Labeling

Stocks of vesicular stomatitis virus (VSV) (Indiana Serotype) were grown and titered on wild-type CHO cells (Krieger et al., 1983). For metabolic labeling experiments 300,000–400,000 cells/well were seeded into the wells of a six-well dish in 3 ml of medium III. 2 d later, cells were infected with 5–10 plaque-forming units of VSV per cell in 0.5 ml of medium III for 1 h then refed with medium III. 3–4 h later, cells were washed twice with methionine-free medium III, pulse-labeled for 5–10 min with [^{35}S]methionine (20 μCi/ml in methionine-free medium III), and chased for 1 h in complete medium III. Cells were then washed twice with PBS and lysed in 0.25 ml of either buffer A (PBS without calcium and magnesium and supplemented with 1% [vol/vol] Triton X-100, 1% [vol/vol] Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) or buffer B (150 mM Tris, pH 8.8, 1% [vol/vol] SDS, 1% [vol/vol] β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride).

Immunoprecipitations

The conditions for setting cells, labeling with [^{35}S]methionine, treating with tunicamycin or pronase, harvesting, and immunoprecipitating LDL receptors with the anti-C polyclonal antibody are described in the accompanying paper (Kozarsky et al., 1986). Endoglycosidase H and sialidase digestions also were performed as described (Kozarsky et al., 1986).

Polyacrylamide Gel Electrophoresis

Immunoprecipitates of LDL receptors and lysates of VSV-infected cells were analyzed in 6 or 10% SDS-polyacrylamide gels by the method of Laemmli (1970; Kozarsky et al., 1986).

Glycolipid Analysis

Glycolipids were isolated from cells grown in medium III and analyzed by thin-layer chromatography, using minor modifications of previously described methods (Stanley et al., 1980; Kundo, 1981). Glycolipid standards were gifts from Dr. Gary Schwarting (E. K. Shriver Center, Waltham, MA).

Other Assays

The sensitivity of cells to toxic lectins was determined by seeding cells on day 0 into 96-well dishes at a concentration of 2,000 cells/well in 0.1 ml of medium III. On day 1, various concentrations of lectins were added to the cells in medium III (0.1 ml/well). On day 3, cells were washed, fixed, and stained (Krieger et al., 1981). Lectin sensitivities are expressed as the lowest lectin concentration that reduced cell density to 0–10% of that seen in wells without lectins (Stanley, 1981).

The receptor-mediated uptake and degradation of ^{125}I-labeled LDL was measured at 34°C (Lec9 cells) or 37°C (all other cells) as previously described (Krieger, 1983). The values presented are the differences between determinations made in the absence (duplicate determinations) and presence (single determinations) of excess unlabeled LDL. For experiments with the Lec9 cells and the Gat^- cell types, alpha minimal essential medium was used instead of Ham's F-12. The protein concentrations of cells and lipoproteins were determined by the method of Lowry et al. (1951).

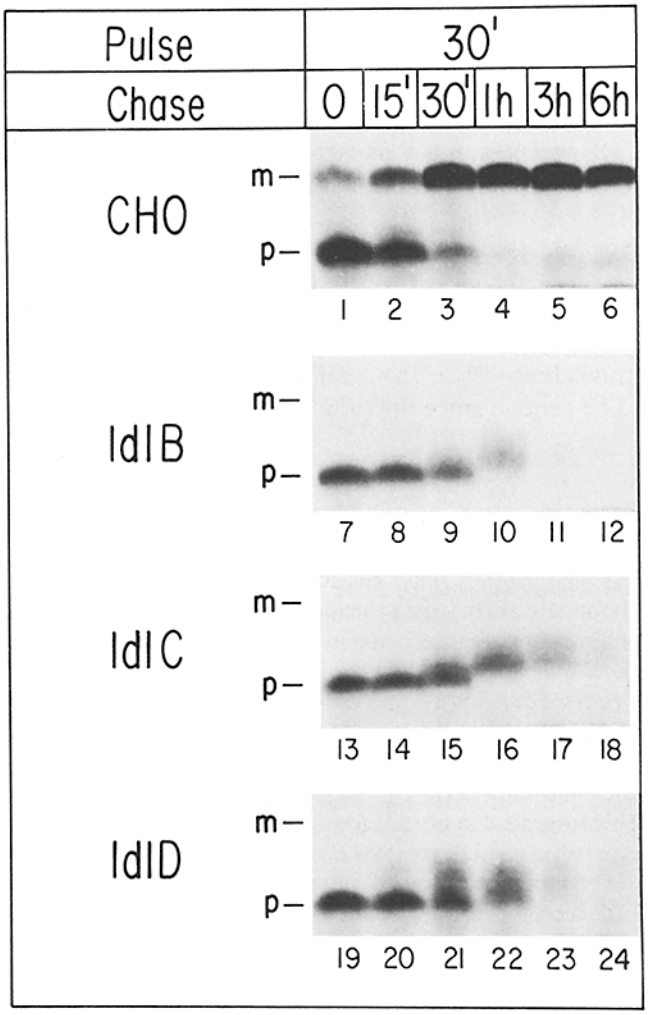

Figure 1. Structure and processing of LDL receptors in wild-type and *ldl* mutant cells. The indicated cell types were pulse-labeled with 180 μCi/ml of [^{35}S]methionine for 30 min, chased for the indicated times in medium III, and subjected to immunoprecipitation, electrophoresis, and autoradiography as described in Materials and Methods. The letters *p* and *m* refer to the 125,000-D precursor and the 155,000-D mature form of the LDL receptor protein, respectively.

Results

Altered LDL Receptors in ldlB, ldlC, and ldlD Mutants

An anti-LDL receptor antibody (see accompanying paper, Kozarsky et al., 1986) was used to compare the synthesis and structure of LDL receptors in wild-type and *ldl* mutant cells. Cells were pulse-labeled with [^{35}S]-methionine, chased for various lengths of time, and analyzed by immunoprecipitation, electrophoresis, and autoradiography (Fig. 1). Wild-type cells synthesized a 125,000-D precursor form (*p*) of the LDL receptor that was rapidly converted to a mature form (*m*) of 155,000 D (Fig. 1, lanes *1–6*). This mature form was stable for at least 6 h and could still be detected in wild-type cells after chase periods as long as 20–30 h (Kozarsky et al., 1986). The *ldlB*, *ldlC*, and *ldlD* cells all produced approximately normal amounts of a 125,000-D precursor form of the LDL receptor but failed to process these receptors to the mature form (Fig. 1, lanes *7–24*). Instead these mutants produced small, heterogeneous LDL receptors that were almost completely degraded within 3–6 h of synthesis.

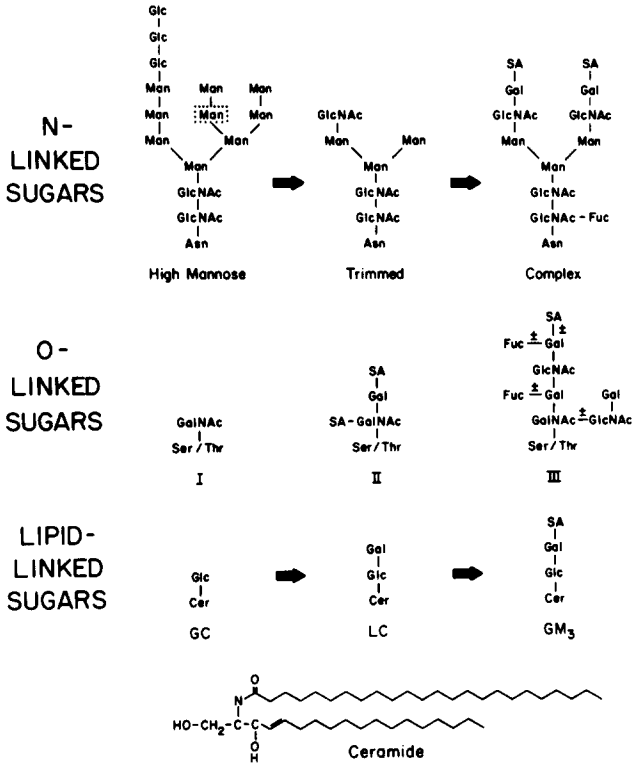

Figure 2. Structure and synthesis of different classes of glycoconjugates. (*top*) High mannose N-linked carbohydrate chains are added cotranslationally to glycoproteins in the rough endoplasmic reticulum. These chains can be trimmed and subsequently converted to the complex type by a series of Golgi processing steps (Hubbard and Ivatt, 1981). The partially processed structure in the middle is one of the earliest processing intermediates that should be resistant to digestion with endoglycosidase H (Tai et al., 1977). The complex structure shown is the major form of N-linked carbohydrate found on mature VSV G protein in CHO cells (Stanley, 1985*a*). The N-linked chains of the mature LDL receptor are also of the complex type (Cummings et al., 1983; Kozarsky et al., 1986). (*middle*) A wide variety of O-linked oligosaccharides attached to serine or threonine side chains has been observed (Kornfeld and Kornfeld, 1980). In most O-linked chains determined to date, an *N*-acetylgalactosamine residue is linked directly to the hydroxyl group of the serine or threonine side chains. Structures I and II have been observed on the precursor and mature forms, respectively, of the LDL receptor in human A431 cells in culture (Cummings et al., 1983). (*bottom*) The pathway of glycolipid synthesis in CHO cells involves the sequential addition of three different monosaccharides to ceramide (Yogeeswaran et al., 1974; Briles et al., 1977; Stanley et al., 1980, Stanley, 1980). *abbreviations*: Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; SA, sialic acid; Asn, asparagine; Ser/Thr, serine or threonine; cer, ceramide; GC, glucosylceramide; LC, lactosylceramide; GM_3, sialyl-galactosyl-glucosylceramide.

Previous experiments have shown that the conversion of the 125,000-D precursor form of the LDL receptor to a mature form of 155,000 D is the result of extensive processing of N- and O-linked carbohydrate chains (Kozarsky et al., 1986; Cummings et al., 1983; see Fig. 2 for review of these processing pathways). When cells were treated with tunicamycin to block addition of N-linked chains (Hubbard and Ivatt, 1981), the *ldlB*, *ldlC*, and *ldlD* cells still produced receptors which were substantially smaller and more heterogeneous than receptors in wild-type cells (Fig. 3). This suggests that the production of abnormal forms of the LDL receptor in *ldlB*, *ldlC*, and *ldlD* cells is at least in part due to defective synthesis of O-linked chains.

The LDL receptors made by *ldlB*, *ldlC*, and *ldlD* cells were more heterogeneous in size than receptors produced by wild-type cells. Most of this size heterogeneity was eliminated when LDL receptors from *ldlB*, *ldlC*, and *ldlD* mutants were treated with sialidase after immunoprecipitation (Fig. 4). Heterogeneity in sialic acid content thus appears to be a major source of the heterogeneous mobility of LDL receptors from *ldlB*, *ldlC*, and *ldlD* cells. In addition, sialidase-treated LDL receptors from tunicamycin-treated *ldl* mutants were substantially smaller than receptors from similarly treated wild-type cells (Fig. 4, compare lanes *10*, *12*, *14*, and *16*). These results suggest that *ldlB*, *ldlC*, and *ldlD* mutants have alterations in O-linked chains that are more extensive than simple variability in sialic acid content.

A pronase assay was used to determine if abnormal glycosylation of the LDL receptor affected its localization in *ldlB*, *ldlC*, and *ldlD* cells (Kozarsky et al., 1986). When wild-type and *ldl* mutant cells were pulse-labeled with [^{35}S]methionine for 30 min and immediately treated with pronase, a substantial fraction of the LDL receptors were resistant to digestion

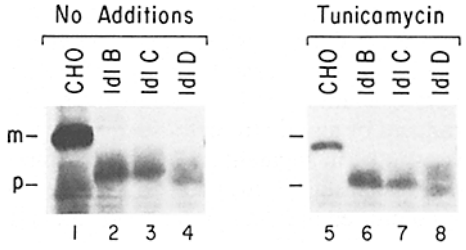

Figure 3. Effect of tunicamycin on LDL receptor structure in wild-type and *ldl* mutant cells. The indicated cell types were pulse-labeled with 200 μCi/ml [^{35}S]methionine for 1 h, chased for 1 h, and subjected to immunoprecipitation, electrophoresis, and autoradiography as described in Materials and Methods. Tunicamycin (2 μg/ml) was present continuously in the preincubation, labeling, and chase media for the cells shown in lanes *5–8*.

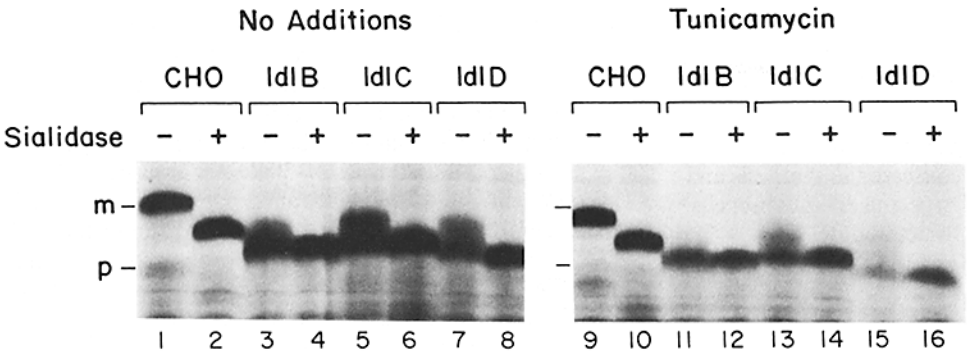

Figure 4. Sialidase sensitivity of LDL receptors in wild-type and *ldl* mutant cells. The indicated cell types were pulse-labeled with 200 μCi/ml [^{35}S]methionine for 30 min, chased for 1 h, and subjected to immunoprecipitation, sialidase treatment, electrophoresis, and autoradiography as described in Materials and Methods. Tunicamycin (2 μg/ml) was present continuously in the preincubation, labeling, and chase media for the cells shown in lanes *9–16*.

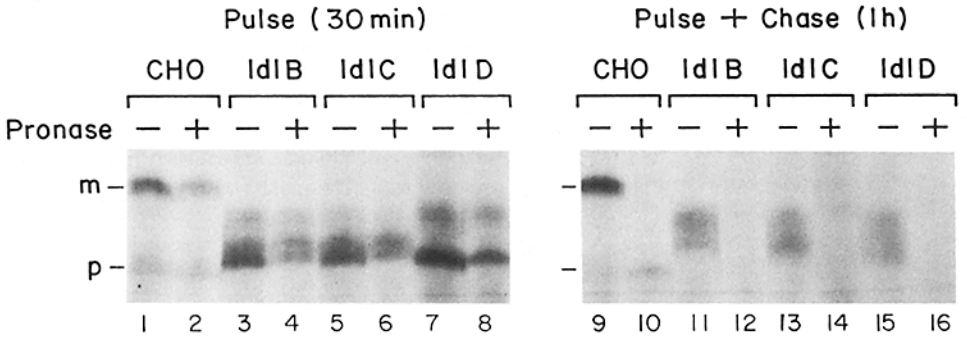

Figure 5. Pronase treatment of LDL receptors in cell monolayers. The indicated cell types were labeled with 200 μCi/ml [^{35}S]methionine for 30 min. Cells in lanes *1–8* were incubated immediately in the presence or absence of pronase (20 μg/ml in Hepes-buffered saline, 20 min). Cells in lanes *9–16* were chased for 1 h before incubation in the presence or absence of pronase. All cells were then washed and subjected to immunoprecipitation, electrophoresis, and autoradiography as described in Materials and Methods.

(Fig. 5, lanes *1–8*). When pulse-labeled cells were chased for 1 h and then treated with pronase, almost all of the LDL receptors in both wild-type and mutant cells were accessible to pronase digestion (Fig. 5, lanes *9–16*). Similar pronase sensitivities were observed after labeling for 4.5 h (data not shown). This suggests that the abnormal LDL receptors in *ldlB*, *ldlC*, and *ldlD* mutants reach the cell surface.

Altered VSV G Protein in ldlB, ldlC, and ldlD Mutants

To determine if other membrane glycoproteins were abnormally processed in the *ldl* mutants, we examined the synthesis of the N-glycosylated G protein of VSV. Wild-type and mutant cells were infected with VSV, labeled with [^{35}S]methionine, and the proteins in detergent lysates were separated by SDS-gel electrophoresis and visualized by autoradiography (Fig. 6). In all cell types, the unglycosylated L and N virus-encoded proteins exhibited identical electrophoretic mobilities. In wild-type cells, the VSV G protein appeared as a prominent 69,000-D band (G form) whose apparent size was reduced to ~60,000 D (G_0 form) after treatment with tunicamycin (Fig. 6, compare lanes *2* and *3*). As anticipated, the VSV G protein produced by an *ldlA* (receptor structural gene) mutant (lane *4*) was similar in apparent size to that produced by wild-type cells. However, the electrophoretic mobilities of the VSV G proteins produced in *ldlB*, *ldlC*, and *ldlD* mutants were greater than the mobility of G protein made in wild-type cells (Fig. 6, lanes *5–7*). Treatment with tunicamycin eliminated these differences (data not shown), suggesting that N-linked carbohydrate processing was abnormal in these mutants.

The VSV G proteins produced by wild-type and *ldl* mutant cells during a short pulse labeling with [^{35}S]methionine all showed identical electrophoretic mobilities and were all sensitive to endoglycosidase H digestion (Fig. 7, lanes *1–8*). Similar endoglycosidase H results were seen for the apparently normal precursor forms of the LDL receptor produced by all of the *ldl* mutants (data not shown). These results suggest that all of the mutants were able to assemble high mannose N-linked carbohydrate chains and to transfer them to proteins normally (Fig. 2).

During a 1-h chase, the apparent molecular weight of the G protein in wild-type cells increased (Fig. 7, compare lanes *1* and *9*). Simultaneously, the G protein became resistant to digestion with endoglycosidase H (Fig. 7, lanes *9* and *10*) and sensitive to treatment with sialidase, an enzyme that removes sialic acid residues (Fig. 8, lanes *1* and *2*). This behavior is typical of glycoproteins whose high mannose N-linked carbohydrate chains have been trimmed and then processed to a complex form (Fig. 2). In contrast, the G proteins in *ldlB*, *ldlC*, and *ldlD* cells showed a slight decrease in apparent size during a 1-h chase (Fig. 7, and data not shown) and did not show further changes after 2 h of chase (data not shown). Unlike the mature G protein in wild-type cells, the mature forms in these mutants were resistant to sialidase (Fig. 8, lanes *3–8*), indicating that *ldlB*, *ldlC*, and *ldlD* cells cannot completely process high mannose chains to the fully complex form. The different mutants apparently process the high mannose oligosaccharides to different extents. In *ldlD* cells the mature G protein was fully resistant to endoglycosidase H (Fig. 7, lanes *15* and *16*), whereas in *ldlB* and *ldlC* cells they retained partial endoglycosidase H sensitivity (Fig. 7, lanes *11–14*). This partial sensitivity was not altered by chase times as long as 2 h or by more extensive treatment with the enzyme (data not shown).

A variety of assays were used to determine if abnormal

glycosylation of the VSV G protein affected virus production. Pronase digestion showed that the abnormal forms of the VSV G protein in *ldlB*, *ldlC*, and *ldlD* reached the cell surface (data not shown). All of the mutants developed similar numbers of viral plaques when monolayers were exposed to dilute VSV stocks (Kingsley and Krieger, 1984; and data not shown). Plaques on *ldlC* and *ldlD* cells were larger than those on wild-type cells; plaques on *ldlB* were smaller. All of the mutants produced amounts of infectious VSV during an 8-h period after initial VSV infection that were equal to or greater than that produced by wild-type cells (data not shown).

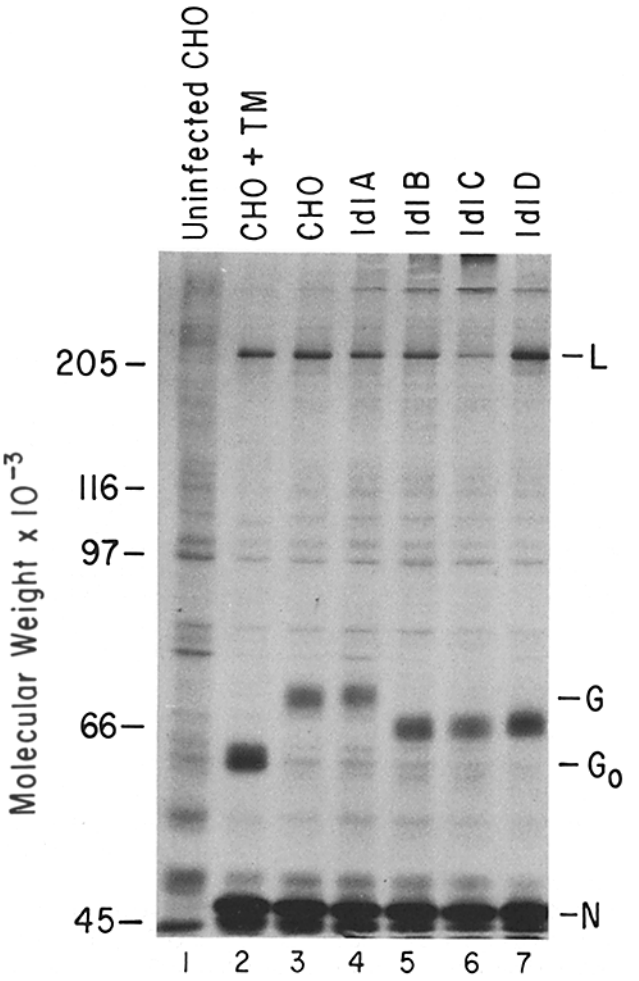

Figure 6. Synthesis of vesicular stomatitis viral proteins by cells from different *ldl* complementation groups. The indicated cell types were seeded into six-well dishes, infected (lanes *2–7*) or mock infected (lane *1*) with VSV, labeled with [^{35}S]methionine for 7.5 min, and chased for 1 h as described in Materials and Methods. Tunicamycin (2 μg/ml) was continuously present in the infection, labeling, and chase media for the cells shown in lane *2*. Proteins in buffer A lysates of the cells were analyzed by electrophoresis and autoradiography as described in Materials and Methods. G and G_0 represent the glycosylated and unglycosylated forms of the VSV glycoprotein. L and N are unglycosylated viral proteins.

Altered Glycolipids and Lectin-resistance in ldlB, ldlC, and ldlD Mutants

To determine if the defects in *ldlB*, *ldlC*, and *ldlD* cells affected additional classes of glycoconjugates, glycolipids were extracted from wild-type and *ldl* mutant cells and analyzed by thin-layer chromatography (Fig. 9). Previous studies (Yogeeswaran et al., 1974; Briles et al., 1977; Stanley et al., 1980, Stanley, 1980) have shown that the predominant glycolipid species made in CHO cells is sialyl-galactosyl-glucosylceramide (Fig. 2). Sialyl-galactosyl-glucosylceramide was present in wild-type CHO cells but was strikingly reduced in *ldlB*, *ldlC*, and *ldlD* mutants (Fig. 9). Lactose ceramide was also present in wild-type cells and the *ldlB* and *ldlC* mutants but was reduced in the *ldlD* mutant. The *ldlC* and *ldlD* cells contained slightly more material that comigrated with a glucosylceramide standard than did wild-type cells. The glycolipids in an *ldlA* mutant were indistinguishable from those in wild-type cells (data not shown). These studies suggest that the defects in *ldlB*, *ldlC*, and *ldlD* cells affect addition of galactose and sialic acid residues to glycolipids.

To detect possible changes in a broad spectrum of glycoconjugates in the *ldl* mutants, we examined the sensitivity of various cells to five different toxic plant lectins that bind to cell surfaces with different sugar specificities (Table I). Mutants in the *ldlB*, *ldlC*, and *ldlD* complementation groups showed up to 200-fold changes in sensitivity to these lectins relative to wild-type cells. For example, the *ldlB*, *ldlC*, and *ldlD* mutants all show marked resistance to WGA (Table I), a lectin that binds to sialic acid and *N*-acetylglucosamine residues (Goldstein and Hayes, 1978; Bhavanandan and Katlic, 1979). In contrast, a mutant in the *ldlA* complementation group showed no significant differences from wild-type CHO cells.

Comparison of ldl Mutants and Previously Isolated Glycosylation Mutants

At least 17 different types of CHO glycosylation mutant have been isolated by other laboratories, primarily on the basis of changes in sensitivity to toxic plant lectins (reviewed by

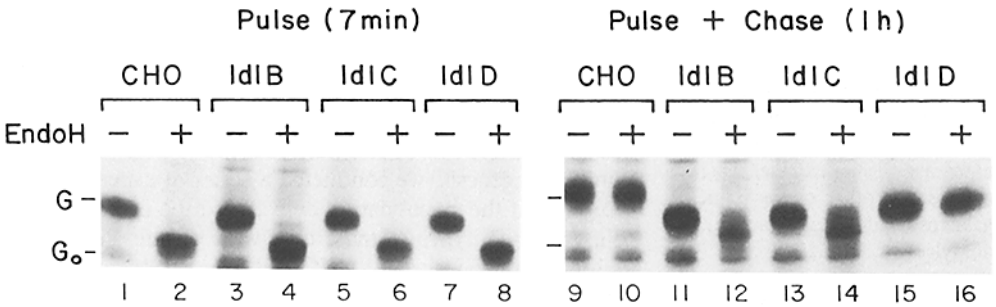

Figure 7. Endoglycosidase H sensitivity of precursor and mature forms of VSV G protein made in wild-type and *ldl* mutant cells. The indicated cell types were infected with VSV and labeled with [^{35}S]methionine for 7 min. Some of the cells (lanes *1–8*) were then lysed immediately in buffer B. The remaining cells (lanes *9–16*) were chased for 1 h before lysis in buffer B. Cell extracts were incubated in the presence or absence of endoglycosidase H (1.3 μg/ml) for 17 h at 37°C, as previously described (Robbins et al., 1977). Acetone precipitates (Robbins et al., 1977) of the samples were dissolved in sample buffer, and analyzed by electrophoresis and autoradiography as described in Materials and Methods.

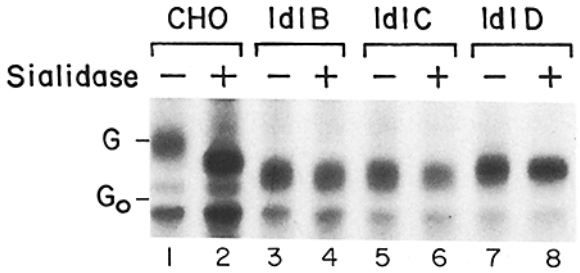

Figure 8. Sialidase treatment of VSV G protein made in wild-type and *ldl* mutant cells. The indicated cell types were infected with VSV and labeled with [^{35}S]methionine for 5 min. After a 1.5-h chase, cells were washed with Hepes-buffered saline and refed either 0.5 ml of Hepes-buffered saline (lanes *1*, *3*, *5*, and *7*) or 0.5 ml of Hepes-buffered saline containing sialidase (0.1 U/ml) (lanes *2*, *4*, *6*, and *8*). After 20 min at 37°C, cells were washed, lysed in 0.25 ml of buffer A, and analyzed by electrophoresis and autoradiography as described in Materials and Methods. Control experiments with a pronase assay (Kozarsky et al., 1986) showed that all of the radiolabeled G protein present in the cells after a 1.5-h chase was on the cell surface (data not shown).

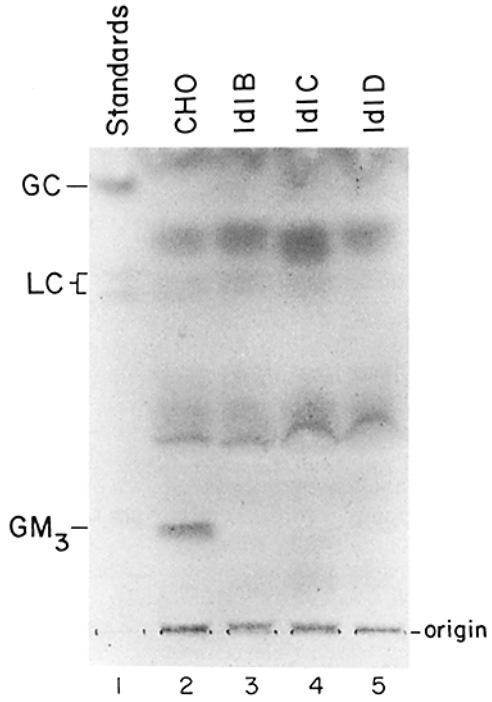

Figure 9. Comparison of glycolipids in wild type and *ldl* mutant cells. Chloroform/methanol extracts from ~30 × 10^6 cells of the indicated cell type were separated by thin-layer chromatography on a Bakerflex silica gel plate in chloroform/methanol/water (60:30:4.5). The dried plate was sprayed with 0.3% (wt/vol) napthol in 95% (vol/vol) ethanol followed by 50% (vol/vol) sulfuric acid. The positions of glycolipid standards are also indicated. The glycolipid standards, the cellular material that comigrated with these standards, and the material remaining at the origin of the chromatogram all stained blue/purple, indicating the presence of sugar residues. All other bands stained yellow, red, or gray. GM_3, sialyl-galactosyl-glucosylceramide; LC, galactosyl-glucosylceramide; GC, glucosylceramide (Fig. 2).

Stanley, 1984, 1985*a*, *b*). The patterns of lectin sensitivities in four of these previously described mutants (Lec1, Lec2, Lec3, and Lec8 cells) were somewhat similar to those seen in *ldlB*, *ldlC*, and *ldlD* mutants (Table I and Stanley, 1985*a*).

Table I. Differences in Lectin Sensitivities between Wild-Type and Mutant CHO Cells

Cells	Lectin sensitivity (LD_{10})				
	WGA	RIC	PHA	Con A	LCA
	μg/ml	*ng/ml*	*μg/ml*	*μg/ml*	*μg/ml*
CHO	3	240	15	25	60
ldlA	3	240	20	30	60
ldlB	120	5	>240	5	>240
ldlC	30	1	>240	5	120
ldlD	30	120	240	10	8
WGA^r-2	180	5	>240	5	60
Lec1	60	>240	>240	5	>240
Lec2	30	<1	20	25	30
Lec8	150	10	>240	15	15

The lectin concentrations required to reduce cell density to 0–10% of that seen for untreated cells were determined by a semiquantitative 3-d growth test as described in Materials and Methods. The relative lectin sensitivities of the wild-type cells and the Lec1, Lec2, and Lec8 mutants are for the most part similar to those previously reported (Stanley, 1985*a*). Differences between absolute lectin sensitivities in these and previous experiments, as well as a substantially higher relative sensitivity of Lec8 cells to ricin in our experiments, may be due to the different growth conditions, serum sources and concentrations, and lectin preparations used in different laboratories. Although the binding specificities of lectins are complex and incompletely understood, the following carbohydrate structures are likely components of the surface binding sites for the five lectins shown in this table: WGA, terminal sialic acid or *N*-acetylglucosamine residues; ricin (RIC), terminal galactose or *N*-acetylgalactosamine residues; phytohemagglutinin (PHA), galactose in β1–4 branches of complex N-linked carbohydrate chains; concanavalin A (Con A), mannose residues; lens culinaris agglutinin (LCA), mannose residues in fucosylated N-linked carbohydrate chains (Goldstein and Hayes, 1978; Stanley, 1985*a*).

Somatic cell hybrids were isolated between these four Lec mutants and the *ldlB*, *ldlC*, and *ldlD* cells. Unlike all of the original mutant cells, all of the hybrids produced VSV G proteins with wild-type electrophoretic mobilities (data not shown). Thus, these Lec and *ldl* mutants represent distinct genetic complementation groups.

Although *ldlB*, *ldlC*, and *ldlD* mutants expressed only 5–10% of wild-type LDL receptor activity, all of the 17 previously classified Lec mutants expressed 38–170% of the receptor activity in their respective parental cells, Pro$^-$5 or Gat$^-$2 (Table II). In addition, other isolates of Lec mutants (Lec1A, Lec2B, Lec13A, and Lec1.Lec2 cells) were examined and found to express 71–102% of parental LDL receptor activity. Taken together, these data indicated that the *ldlB*, *ldlC*, and *ldlD* mutants were unrelated to previously described CHO mutants. Four of the Lec mutants were examined by immunoprecipitation with the anti-C peptide antibody (Lec1, Lec2, Lec3, and Lec8 cells). Each of these mutants synthesized an abnormal form of the LDL receptor protein that differed in size or heterogeneity from the receptors observed in *ldlB*, *ldlC*, and *ldlD* cells (data not shown).

Genetic Linkage between Structural and Functional Phenotypes

Because the *ldlB*, *ldlC*, and *ldlD* mutants were isolated after heavy mutagenesis, we conducted a series of experiments to determine if the glycosylation defects and the LDL receptor-deficient phenotypes of these cells were due to single or to multiple mutations. First, CHO cells (no mutagen) were incubated with concentrations of WGA (20 μg/ml) that are toxic to wild-type cells but not the *ldlB*, *ldlC*, or *ldlD* cells. WGA-resistant colonies appeared at a frequency of 5 × 10^{-6} (45 million cells tested in two independent experiments).

Table II. Comparison of ldl and Lec Mutants

Cells‡	Established carbohydrate defects*			LDL receptor activity
	Asn-linked	Ser/Thr-linked	Lipid-linked	
				% control§
Wild-type CHO				100
ldlA				<1
ldlB	X	X	X	4
ldlC	X	X	X	8
ldlD	X	X	X	7
Pro⁻ Lec mutants				
Wild-type Pro⁻ 5				170
Lec1	X			149¶
Lec2	X	X	X	118¶
Lec3	X		X	92¶
Lec4	X			123
Lec5	X			110
Lec8	X	X	X	104¶
Lec9	X			127
LEC10	X			144
LEC11	X			96
LEC12	X			288
Lec13	X			158
LEC14				165
Lec15	X			157
LEC17				137
Gat⁻ Lec mutants				
Wild-type Gat⁻2				100‖
Lec1.Lec6	X			43‖
Lec2.Lec7	X	X	X	38‖
LEC16	X			90‖

* O-linked carbohydrate defects in Lec2 and Lec8 cells were confirmed by analysis of LDL receptor sizes in the presence and absence of tunicamycin (data not shown). All of the other Lec mutant carbohydrate defects listed in the table have been reviewed elsewhere (Stanley, 1985*a*).

‡ The wild-type CHO cells are the strain of Pro⁻ CHO-K1 cells used for isolation of the various *ldl* mutants and for all of the structural experiments in this paper. Most of the Lec mutants were isolated from other strains of Pro⁻ CHO cells, for example, Pro⁻5 cells (Stanley, 1985*a*). The Lec1.Lec6, Lec2.Lec7, and LEC16 mutants were isolated from a Gat⁻ cell line, Gat⁻2 (Stanley, 1985*a*).

§ High affinity degradation of ^{125}I-LDL relative to CHO cells in the same experiment unless otherwise noted. 100% control values ranged from 1,664–4,094 ng ^{125}I-LDL degraded per 5 h per mg cell protein in eight separate experiments.

¶ The Lec mutants that had defects in several types of glycoconjugate or that had lectin sensitivities somewhat similar to those of the *ldl* mutants were fused to *ldlB*, *ldlC*, and *ldlD* cells for genetic complementation tests. Analysis of cell hybrids indicated that the mutations in Lec1, Lec2, Lec3, and Lec8 cells were distinct from those in *ldlB*, *ldlC*, and *ldlD* cells (see text).

‖ High affinity degradation of ^{125}I-LDL relative to wild-type Gat⁻2 cells in the same experiment. 100% control value: 2,418 ng ^{125}I-LDL degraded per 5 h per mg cell protein.

Approximately 1% of the WGA-resistant (WGA^r) colonies (3 of 215) proved to have a dramatic deficiency in LDL receptor activity based on the uptake of fluorescent LDL and the degradation of ^{125}I-LDL (see below and data not shown). The overall frequency of isolating WGA-resistant, LDL receptor-negative colonies in these experiments (7×10^{-8}) is at least five orders of magnitude higher than the frequency expected if WGA resistance and LDL receptor deficiency are due to defects in two independent genes (overall frequency estimated as the product of the spontaneous frequency of each event, 5×10^{-6} [WGA resistance, this paper] $\times$ $<1 \times 10^{-7}$ [LDL receptor deficiency, Krieger et al., 1981 and 1983] = $<5 \times 10^{-13}$). Complementation tests (Kingsley and Krieger, 1984)

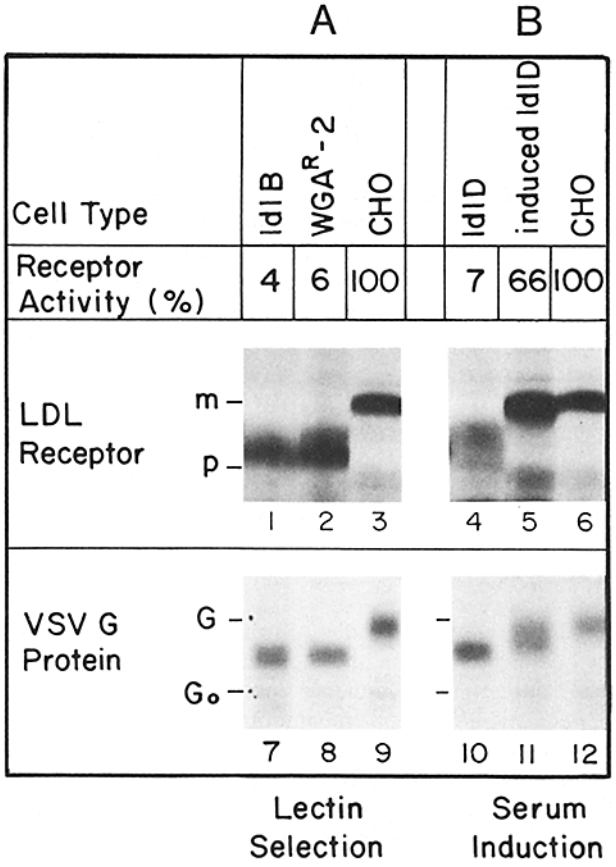

Figure 10. Genetic linkage between altered LDL receptor activity and altered protein processing in *ldl* mutants. (*A*) A mutant isolated by direct selection for resistance to WGA (WGA^r-2 cells) was deficient in LDL receptor activity and had defects in synthesis of both LDL receptors and VSV G protein. (*B*) Treatment of *ldlD* cells with high concentrations of serum induced LDL receptor activity and partially corrected defects in glycoprotein processing. *LDL receptor activity.* On day 0, 60,000 cells/well of the indicated cell types were seeded into the wells of a 24-well dish in medium III supplemented with 50% (vol/vol) human lipoprotein-deficient serum (induced *ldlD* cells) or in medium III alone (all other cells). On day 2, all cells were incubated in 0.5 ml of medium I supplemented with 5% (vol/vol) human lipoprotein-deficient serum and ^{125}I-labeled LDL (10 μg protein/ml, 85 cpm/ng). After 5 h at 37°C, the amounts of specifically degraded ^{125}I-LDL products released into the medium were determined as described in Materials and Methods. Values are expressed as percent of control wild-type cells (100% = 4,044 ng ^{125}I-LDL degraded/5 h per mg cell protein). *LDL receptor structure.* On day 0, 300,000 cells/dish of the indicated cell types were seeded into 60-mm dishes in 3 ml of medium III or in 3 ml of medium I supplemented with 50% (vol/vol) human lipoprotein-deficient serum (induced *ldlD* cells). On day 2, cells were labeled with [^{35}S]methionine (85 μCi/ml) for 5 h in medium III or in medium III supplemented with 50% (vol/vol) human lipoprotein-deficient serum (induced *ldlD* cells). Immunoprecipitates of the labeled cells were analyzed by electrophoresis and autoradiography as described in Materials and Methods. *VSV G protein structure.* On day 0, 300,000 cells of the indicated cell types were seeded into six-well dishes in 3 ml of medium III or in 3 ml of medium III supplemented with 50% (vol/vol) human lipoprotein-deficient serum (induced *ldlD* cells). On day 2, cells were infected with VSV, pulse-labeled with [^{35}S]methionine for 7 min, and chased for 1 h. Proteins in buffer A lysates were analyzed by electrophoresis and autoradiography as described in Materials and Methods.

established that the three new isolates were members of the *ldlB* complementation group (data not shown). The LDL receptor activity, LDL receptor structure, VSV G protein structure, and lectin sensitivity of one of the WGA-selected colonies (WGA^r-2) were tested and found to be essentially identical to that seen for *ldlB* cells (Fig. 10, lectin-selection, *A*; and Table I).

Functional linkage between the altered processing and altered receptor activity in the *ldl* mutants was also tested by a biochemical reversion test. LDL receptor activity in *ldlD* cells can be partially restored by cocultivation with other cells or by treatment of *ldlD* cells with high concentrations of serum (Krieger, 1983). The electrophoretic mobilities of both the LDL receptor and the VSV G protein produced in *ldlD* cells were also restored to nearly wild-type mobilities after the cells had been treated with high concentrations of serum (Fig. 10, serum induction, *B*). These and other recent data (Kingsley et al., 1986; see below) show that the various functional and structural phenotypes of *ldlD* cells are due to a single defect.

Discussion

Anti-LDL receptor antibodies (Kozarsky et al., 1986) have helped define the nature of the genetic defect in three types of CHO mutant. These mutants from the previously defined *ldlB*, *ldlC*, and *ldlD* complementation groups are deficient in LDL receptor activity but are genetically distinct from the *ldlA* mutants that have defects in the structural gene for the LDL receptor (Kingsley and Krieger, 1984). In wild-type CHO cells the LDL receptor is initially synthesized as a 125,000-D precursor that is subsequently processed to a mature form of 155,000 D by extensive glycosylation (Kozarsky et al., 1986). The *ldlB*, *ldlC*, and *ldlD* mutants all produce substantial amounts of a 125,000-D precursor form of the LDL receptor protein. Therefore these mutants do not have defects in the production, accumulation, or translation of receptor messenger RNA. The *ldlB*, *ldlC*, and *ldlD* mutants produce mature forms of the LDL receptor that are abnormally small and heterogeneous in size. The abnormal sizes of the LDL receptors in these cells are due to defects in processing of the LDL receptor's N- and O-linked carbohydrate chains. Glycosylation defects in these cells also affect the N-linked chains of the VSV G protein and the lipid-linked carbohydrates of cellular glycolipids. The carbohydrate structures of many different cell surface molecules are probably altered in the *ldlB*, *ldlC*, and *ldlD* cells since all of these mutants show dramatic changes in sensitivity to five different toxic plant lectins.

Our results and previous experiments suggest that glycosylation mutants can make up a significant fraction of the mutants that are isolated after selection for altered function of a particular surface molecule (reviewed by Stanley, 1985*a*,*b*). A CHO mutant that was isolated because of decreased levels of mannose-6-phosphate receptor activity (Robbins et al., 1981) has been shown to have a general defect in synthesis of high mannose N-linked carbohydrate chains. This defect results from a marked deficiency in the enzyme dolichol-mannose-phosphate-synthetase (Stoll et al., 1982). In addition, four of five types of mutant lymphoma cells isolated because of decreased expression of Thy-1 antigen show altered glycosylation of this molecule (Trowbridge et al., 1978). One of these classes of mutant, Thy-1E, has a defect in dolichol-mannose-phosphate synthetase activity that is very similar to that in the mannose-6-phosphate receptor defective CHO mutant (Chapman et al., 1980). The close associations between glycosylation defects and altered expression of the LDL receptor, the mannose-6-phosphate receptor, and the Thy-1 antigen emphasize the importance of carbohydrate chains for the expression and function of some cell surface glycoproteins.

Although we have seen a strong association between altered glycosylation and altered LDL receptor activity in *ldlB*, *ldlC*, and *ldlD* mutants, there are many carbohydrate alterations that appear to have relatively little effect on LDL receptor function. For example, previous studies have shown that treatment of LDL receptors with sialidase has little effect on LDL binding activity (Schneider et al., 1982). In addition, we have shown that 17 different types of previously isolated CHO glycosylation mutant have substantial levels of LDL receptor activity. One of these mutants (Lec15) is the mannose-6-phosphate receptor-deficient CHO mutant described above. A different mannose-6-phosphate receptor-deficient CHO mutant with alterations in endosome acidification, Golgi glycosylation patterns, and lectin sensitivity (Robbins et al., 1984) has been reported to have essentially normal LDL receptor activity (Robbins et al., 1983). The finding of essentially normal LDL receptor activity in a large number of different glycosylation mutants suggests that only very specific types of glycosylation defects can dramatically affect LDL receptor function. The alterations in O-linked carbohydrate chains in *ldlB*, *ldlC*, and *ldlD* cells may be particularly important in this regard (see below). Conversely, the glycosylation defects that affect LDL receptor function do not necessarily affect the expression of other glycoproteins. For example, previous studies have shown that both *ldlB* and *ldlD* mutants express significant levels of mannose-6-phosphate receptor activity (Leichtner and Krieger, 1984). These results emphasize the variety of effects that particular carbohydrate alterations can have on the functions of different glycoproteins.

The current experiments provide insight into the nature of the glycosylation defects in *ldlB* and *ldlC* mutants, but the molecular bases for these defects are not yet known. The structural alterations in these two mutants are strikingly similar. Both mutants are able to add normal high mannose N-linked carbohydrate chains to the precursor forms of the VSV G protein and the LDL receptor. Neither mutant can convert the VSV G chains to a completely endoglycosidase H-resistant form and neither mutant appears to subsequently add galactose (unpublished data) or sialic acid residues to these chains. Both mutants produce O-linked chains on the LDL receptor that are only partially processed and that show heterogeneity in sialic acid content. Both mutants can add glucose and some galactose to glycolipids but do not add significant amounts of sialic acid. Thus both the *ldlB* and *ldlC* mutants appear to have defects in a number of different processing reactions that previously have been shown to take place primarily in the Golgi apparatus (Dunphy and Rothman, 1985). The heterogeneous defects in N-linked trimming are consistent with disruptions of *cis* or *medial* Golgi mannosidase or *N*-acetylglucosamine transferase activities (Dunphy et al., 1985). The heterogeneity or absence of sialic acid residues in glycolipids and the N-linked and O-linked chains of glycoproteins suggests additional defects in activities, probably associated with the *trans* Golgi (Bennett and O'Shaughnessy, 1981). Overall

transport between different Golgi compartments is probably not disrupted in the *ldlB* and *ldlC* mutants since both the VSV G protein and the LDL receptor protein reach the cell surface.

Because of the complicated nature of the processing defects in *ldlB* and *ldlC* cells, we presume that both of these mutants have alterations that affect the regulation, compartmentalization, or activity of several different Golgi enzymes or enzyme substrates. Nevertheless, genetic evidence strongly suggests that the various processing changes seen in *ldlB* cells are due to defects in a single gene. Independent isolates of *ldlB* mutants show similar phenotypes, new *ldlB* mutants can be isolated in the absence of mutagen treatment by direct selection for changes in lectin resistance (see Results), and DNA mediated-reversion of the LDL receptor defects in *ldlB* mutants is always accompanied by the simultaneous correction of the glycosylation abnormalities (Kingsley, D., R. S. Sege, K. F. Kozarsky, and M. Krieger, manuscript in preparation). We have not yet identified the *ldlB* defect, but its consequences are very similar to those of an independent defect in *ldlC* cells. Although the structural phenotypes of the *ldlB* and *ldlC* mutants are almost indistinguishable, the two mutants belong to distinct complementation groups and hybrids between the mutants show essentially normal levels of LDL receptor activity and essentially normal patterns of glycoprotein processing (unpublished data). This suggests either that defects in two distinct genes give rise to a very similar processing phenotype or that complementation between *ldlB* and *ldlC* is actually intra-allelic.

In contrast to the complicated structural changes in *ldlB* and *ldlC* cells, most of the structural changes in *ldlD* cells can be accounted for by a general defect in addition of galactose and *N*-acetylgalactosamine (GalNAc) residues to glycoconjugates. We have recently shown that this is due to a severe deficiency in UDP-Gal/UDP-GalNAc 4-epimerase activity (Kingsley et al., 1986). Without this enzyme, *ldlD* cells are unable to synthesize normal amounts of UDP-galactose and UDP-*N*-acetylgalactosamine from their corresponding glucose precursors. Addition of galactose and GalNAc to the culture medium allows the *ldlD* mutant to synthesize these UDP-sugars via salvage pathways and fully corrects all of the structural and functional defects in the cells. Galactose alone fully corrects N-linked but not O-linked processing defects and does not induce LDL receptor activity. In contrast, *N*-acetylgalactosamine alone partially corrects O-linked but not N-linked processing and substantially increases LDL receptor activity (Kingsley et al., 1986). It thus appears that the defects in O-linked processing may be the primary cause of the LDL receptor-deficient phenotype of the *ldlD* mutant. We do not yet know if this is also true for the *ldlB* and *ldlC* mutants, but both of these mutants do have severe defects in O-linked processing, and in this respect are different from many of the previously described N-linked CHO glycosylation mutants which express substantial LDL receptor activity (Table II).

The fate of the abnormally glycosylated LDL receptors is quite similar in the *ldlB*, *ldlC*, and *ldlD* cells. In each of these mutants, LDL receptors reach the cell surface but are degraded at least 5–10-fold more quickly than receptors in wild-type cells. It appears that the altered stability or function of LDL receptors in *ldl* mutants can largely account for their LDL receptor-deficient phenotype. Given approximately equal rates of receptor synthesis, a 5–10-fold reduction in receptor stability should lead to a 5–10-fold reduction in the steady-state levels of LDL receptors in the mutant cells. Actual residual levels of LDL receptor activity in *ldlB*, *ldlC*, and *ldlD* cells are ~5–10% of normal (Table II and Krieger et al., 1981).

Enhanced degradation of LDL receptors in *ldlB*, *ldlC*, and *ldlD* mutants could be the direct result of the altered structure of the LDL receptors in these cells. Alternatively, enhanced degradation of LDL receptors could be an indirect result of changes in the structure or function of other components in the *ldlB*, *ldlC*, and *ldlD* mutants. Studies with other systems have shown that some carbohydrate alterations can grossly disrupt the conformation, solubility, and intracellular transport of certain glycoproteins (e.g., the VSV G protein, Gibson et al., 1980). It seems unlikely that the glycosylation defects in *ldlB*, *ldlC*, and *ldlD* mutants have such a drastic effect on the structure and solubility of LDL receptors. A significant fraction of LDL receptors in these cells reaches the cell surface, and these mutants express small but detectable levels of LDL binding and internalization activity. The carbohydrates that are normally found on wild-type LDL receptors may help stabilize the receptor during multiple rounds of endocytosis. For example, these oligosaccharides may help protect the protein from denaturation in the acidic environment of endosomes (Tyko and Maxfield, 1982). Alternatively, carbohydrate chains on LDL receptors or other molecules may be essential for some aspect of LDL receptors' recycling to the cell surface after endocytosis. We are currently investigating these possibilities and are also attempting to identify the nature of the cellular components responsible for rapid degradation of the altered LDL receptors in *ldlB*, *ldlC*, and *ldlD* mutants.

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