Unusual Forms of Low Density Lipoprotein Receptors in Hamster Cell Mutants with Defects in the Receptor Structural Gene

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Abstract. The structure and processing of low density lipoprotein (LDL) receptors in wild-type and LDL receptor-deficient mutant Chinese hamster ovary cells was examined using polyclonal anti-receptor antibodies. As previously reported for human LDL receptors, the LDL receptors in wild-type Chinese hamster ovary cells were synthesized as precursors which were extensively processed by glycosylation to a mature form. In the course of normal receptor turnover, an apparently unglycosylated portion of the cysteine-rich N-terminal LDL binding domain of the receptor is proteolytically removed. The LDL receptor-deficient mutants fall into four complementation groups, *ldlA*, *ldlB*, *ldlC*, and *ldlD*; results of the analysis of *ldlB*, *ldlC*, and *ldlD* mutants are described in the accompanying paper (Kingsley, D. M., K. F. Kozarsky, M. Segal, and M. Krieger, 1986, *J. Cell. Biol*, 102:1576–1585). Analysis of *ldlA* cells has identified three classes of mutant alleles at the *ldlA* locus: null alleles, alleles that code for normally processed receptors that cannot bind LDL, and alleles that code for abnormally processed receptors. The abnormally processed receptors were continually converted to novel unstable intracellular intermediates. We also identified a compound-heterozygous mutant and a heterozygous revertant which indicate that the *ldlA* locus is diploid. In conjunction with other genetic and biochemical data, the finding of multiple mutant forms of the LDL receptor in *ldlA* mutants, some of which appeared together in the same cell, confirm that the *ldlA* locus is the structural gene for the LDL receptor.

THE low density lipoprotein (LDL)[1] pathway of receptor-mediated endocytosis has been one of the most thoroughly characterized endocytic systems. This is in large measure due to the extensive analysis of LDL metabolism in normal and mutant human fibroblasts by Brown, Goldstein, and their colleagues (Goldstein et al., 1979, 1985). The mutant fibroblasts were derived from individuals with familial hypercholesterolemia (FH), a relatively common autosomal codominant genetic disease which is caused by mutations in the LDL receptor structural gene. A variety of mutant alleles of the LDL receptor in human FH cells has been described, including defects affecting the synthesis, processing, and overall structure of the receptor (Goldstein et al., 1985). Analysis of these FH cells has been critically important in elucidating the role of receptors in endocytosis.

The biochemical and genetic analysis of these naturally occurring human mutations has been extraordinarily productive; however, there are inherent limits in the use of cells from human patients for the genetic analysis of endocytosis. Primary cultured cells do not lend themselves to manipulation by a number of somatic cell genetic techniques. In addition, the variety of naturally occurring human mutations is limited and these mutations must be compatible with the growth and survival of the entire organism. To circumvent these limitations, we and others have developed techniques for the isolation of cultured mammalian somatic cell mutants with defects in receptor-mediated endocytosis (for review, see Krieger et al., 1985).

We have developed two selection techniques for isolating mutants that exhibit defects in LDL endocytosis (Krieger et al., 1981, 1983) and a nutritional selection for revertants of such mutants (Sege et al., 1984; Kingsley and Krieger, 1984). These selections exploit the ability of native or modified LDLs to deliver substantial amounts of cholesterol or toxic or fluorescent cholesterol derivatives to cells via the LDL pathway (Krieger et al., 1979, 1981). Using these three selection techniques, we have isolated a large collection of LDL receptor-deficient Chinese hamster ovary (CHO) cell mutants and several revertants of these mutants. The mutants define at least four genetic complementation groups (*ldlA*, *ldlB*, *ldlC*, and *ldlD*; Kingsley and Krieger, 1984). These complementation groups probably represent four distinct genes required for normal LDL receptor function. Previous genetic studies suggested that the *ldlA* gene might be the structural gene for the LDL receptor whereas the *ldlB*, *ldlC*, and *ldlD* genes appear to be required for the expression or function of the

1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; FH, familial hypercholesterolemia; LDL, low density lipoprotein; PMSF, phenylmethylsulfonyl fluoride.

LDL receptor (Kingsley and Krieger, 1984; Kingsley et al., 1986; Sege et al., 1984).

To more fully define the defects in these mutant CHO cells, we have raised antibodies that recognize the CHO LDL receptor. In the current paper, we characterized these antibodies and compared the structure and processing of LDL receptors in human fibroblasts and in wild-type and *ldlA* mutant CHO cells. In the accompanying paper (Kingsley et al., 1986), we used these antibodies to examine the nature of the defects in the *ldlB*, *ldlC*, and *ldlD* mutants.

Materials and Methods

Materials

Newborn calf and human lipoprotein-deficient sera were prepared as previously described (Krieger, 1983). Methionine-free Ham's F-12 medium was obtained from K-C Biological Inc., Lenexa, KS. L-[^{35}S]Methionine (>800 Ci/mmol) was from New England Nuclear (Boston, MA). Freund's complete and incomplete adjuvants were purchased from Gibco Laboratories (Grand Island, NY). Protein A-Sepharose CL-4B was from Pharmacia Inc. (Piscataway, NJ). Sialidase (neuraminidase, No. 480717) and pronase (No. 53702) were from Calbiochem/Behring Diagnostics Corp. (La Jolla, CA). Peroxidase-conjugated and unconjugated goat anti-rabbit IgG antibodies were purchased from Cooper Biomedical, Inc. (Malvern, PA)/Cappel Laboratories Inc. (Cochranville, PA). Nitrocellulose paper (BA85) was obtained from Schleicher & Schuell, Inc. (Keene, NH). Phenylmethylsulfonyl fluoride (PMSF) and tunicamycin, purchased from Sigma Chemical Co. (St. Louis, MO), were dissolved in dimethylsulfoxide at concentrations of 400 mM and 2 mg/ml, respectively. Endoglycosidase H was provided by S. Catherine Hubbard and Phillips Robbins (MIT). All other reagents were from Sigma Chemical Co. or were obtained as previously described (Krieger, 1983).

Cell Culture

Parental and mutant CHO-Kl cells and normal human fibroblasts (S.J., GM3349) were grown as described (Krieger, 1983) except that stock cultures of CHO cells were grown in medium I (Ham's F-12 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) supplemented with 5% (vol/vol) fetal bovine serum (medium II). Mutant clone ldlA-7 refers to clone 7 and RevA7 refers to the revertant clone RevA7-1, which was derived from ldlA-7 (Krieger et al., 1983; Kingsley and Krieger, 1984). The other *ldlA* mutants were isolated from parental cells after treatment with 400 μg/ml of ethyl methanesulfonate by MeLoCo-Amphotericin B selection (Krieger et al., 1983; Kingsley and Krieger, 1984). The LDL receptor activities (percent of wild-type CHO) of some *ldlA* mutants as determined by [^{125}I]LDL degradation (Krieger, 1983) were: ldlA-3, 1.5%; ldlA-5, 9%; ldlA-7, 0.4%; ldlA-9, 2.3%; and ldlA-15, 0.8%. All mutants were members of the *ldlA* complementation group based on complementation assays (Kingsley and Krieger, 1984). LET-A-111 cells are secondary transfectants isolated by transfection of ldlA-7 cells with DNA from a primary transfectant and selection for LDL receptor expression (Sege et al., 1984). All incubations were at 37 °C in a humidified 5% CO_2/95% air incubator unless otherwise noted.

Antibodies

LDL receptor was partially purified from bovine adrenal cortex tissue as described by Schneider et al. (1982) through the DEAE-cellulose chromatography step and phosphatidylcholine/acetone precipitation (Schneider et al., 1980). A synthetic peptide corresponding to the C-terminal 14 amino acids of the bovine LDL receptor (Russell et al., 1984) was the gift of W. Schneider, M. Brown, and J. Goldstein (University of Texas Health Science Center, Dallas, TX).

Antibodies were raised in New Zealand White rabbits to either the partially purified receptor (anti-R antibodies) or the the C-terminal peptide crosslinked via a bridging N-terminal cysteine residue to hemocyanin (anti-C antibodies) using standard procedures (Beisiegel et al., 1981*a*; Russell et al., 1984). Anti-R primarily recognized the disulfide-rich, LDL binding, N-terminal domain of the receptor. For example, unlike anti-C, anti-R did not recognize the reduced form of the receptor, and anti-R inhibited cellular binding and subsequent degradation of [^{125}I]LDL (data not shown).

All experiments were performed using IgG fractions prepared using a slight modification of the method of Beisiegel et al. (1981*b*). IgG-C7, a mouse monoclonal antibody that recognizes the human but not the CHO LDL receptor (Beisiegel et al., 1981*b*; Sege et al., 1984), was provided by W. Schneider, Y. K. Ho, M. Brown, and J. Goldstein.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). The molecular mass standards (Sigma Chemical Co.) were: myosin (205 kd), β-galactosidase (116 kd), phosphorylase B (97.4 kd), and bovine albumin (66 kd). Gels were fixed, stained with Coomassie Blue, impregnated with Autofluor (National Diagnostics Inc., Somerville, NJ), dried, and exposed to pre-flashed (Laskey, 1980) Kodak XAR-5 X-ray film at −80°C.

Immunoblots

Cells were plated in 10 ml of medium III (medium I supplemented with 3% [vol/vol] newborn calf lipoprotein-deficient serum) in 100-mm dishes (1.5×10^6 cells/dish) and 2 d later were harvested with a rubber policeman. Solubilized cell membranes were prepared (Schneider et al., 1980, 1982) and were subjected to electrophoresis and transfer to nitrocellulose paper (Beisiegel et al., 1982). The blots were calibrated by staining the standards with india ink (Hancock and Tsang, 1983). For immunoblotting, the nitrocellulose paper was blocked with buffer A (5% [wt/vol] Carnation non-fat dry milk, 50 mM Tris-Cl, pH 8, 2 mM $CaCl_2$, 0.05% Tween-20, and 0.01% Antifoam A emulsion [Johnson and Elder, 1983; Russell et al., 1984]; 2 h, room temperature) and then incubated with 10 μg/ml anti-R IgG in buffer A for 1.5 h. The paper was washed in buffer A (Beisiegel et al., 1982), then incubated in buffer A containing peroxidase-conjugated goat anti-rabbit IgG (12 μg/ml of antibody protein; 1 h). The paper was washed once in buffer A and four times in PBS, pH 7.4; 5 min/wash) and developed with 4-chloro-1-naphthol (Hawkes et al., 1982; PBS substituted for Tris-buffered saline).

Cell Labeling

Parental and mutant CHO cells were plated on day 0 in 3 ml of medium III either in 60-mm dishes (300,000 cells/dish) or in six-well dishes (150,000 cells/well). On day 2, monolayers were washed with PBS, then incubated in methionine-free medium III (medium III prepared with methionine-free Ham's F-12) for 15 min, after which labeling medium (methionine-free medium III supplemented with [^{35}S]methionine) was added. For pulse-chase experiments, after labeling for 30 min, the monolayers were washed with complete Ham's F-12, and refed with 3 ml of medium III. In the detailed time-course experiments, the chase medium was supplemented with 1 mM unlabeled methionine. In some experiments 2 μg/ml tunicamycin was included in a 3-h pretreatment (medium III) and in the subsequent labeling and chase media.

Cells were lysed and the extracts prepared for immunoprecipitation as described by Tolleshaug et al. (1982) with the following exceptions. Lysis buffer contained PBS without calcium and magnesium, 1% Triton X-100, 1 mM methionine, 1 mM PMSF, and 0.1 mM leupeptin and the cell extracts were clarified by centrifugation at 4°C for 15 min in a microfuge (Beckman Instruments, Inc., Fullerton, CA).

Membrane fractions from labeled cells were prepared as described above for immunoblots. The first high speed supernatant was designated the nonmembrane-associated fraction.

Immunoprecipitation

Reagents were added as follows: 50 μl of 10 mg/ml BSA, 100 μl of cell extract (~0.25×10^6 cells), 150 μl of a detergent mixture (0.1 M Tris-Cl, pH 8, 1% Triton X-100, 1% [wt/vol] sodium deoxycholate, 0.5% [wt/vol] SDS, and 2 mM PMSF), and 9 μl (36 μg) of anti-R, anti-C, or preimmune IgG. The mixtures were incubated at 37°C for 1 h, then 30 μl (0.36 mg of antibody protein) of goat anti-rabbit IgG was added and samples were incubated at 4°C overnight. The immunoprecipitates were subjected to centrifugation (900 *g*, 10 min, 4°C) and the pellets washed three times in 2.8 ml of buffer B (0.5% Triton X-100, 0.5% sodium deoxycholate, 0.25% SDS, and 1 mM PMSF), followed by SDS-polyacrylamide gel electrophoresis.

Immunoprecipitations using IgG-C7 were performed as described above for cell extracts except that antibody was added as a preformed immune complex and incubations and washes were performed as previously described (Tolleshaug et al., 1983; Sege et al., 1984).

Endoglycosidase H and Sialidase Treatment

Endoglycosidase H. After washing, immunoprecipitates were resuspended in 20 μl of endo H buffer (30 mM sodium citrate, pH 5.5, 0.75% SDS, 2% (vol/

vol) β-mercaptoethanol, and 1 mM PMSF). The samples were boiled (5 min), cooled, sodium azide was added to a final concentration of 0.1% (wt/vol), and then 2 μl of 30 mM Na citrate (pH 5.5) with or without 0.3 μg/ml endoglycosidase H was added. Samples were incubated at 37°C overnight before electrophoresis.

Sialidase. After washing, immunoprecipitates were resuspended in 45 μl of sialidase buffer (50 mM sodium acetate, pH 5.5, 154 mM NaCl, 4 mM $CaCl_2$, 1 mM PMSF, and 0.1% sodium azide) in the absence or presence of 0.045 U of sialidase. Samples were incubated at 4°C overnight on a rotary shaker, then the precipitates were washed once in buffer B before electrophoresis. A specific sialidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acetyl-neuraminic acid (Boehringer Mannheim Diagnostics, Inc., Houston, TX) was used to confirm the specificity of the sialidase preparation (data not shown).

Other Assays

The amounts of [^{125}I]LDL degradation by cell monolayers at 37°C were measured as previously described (Krieger, 1983) and expressed as the percent of wild-type CHO activity measured in the same experiment. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard. Pronase treatment of labeled monolayers was carried out according to the method of Tolleshaug et al. (1983).

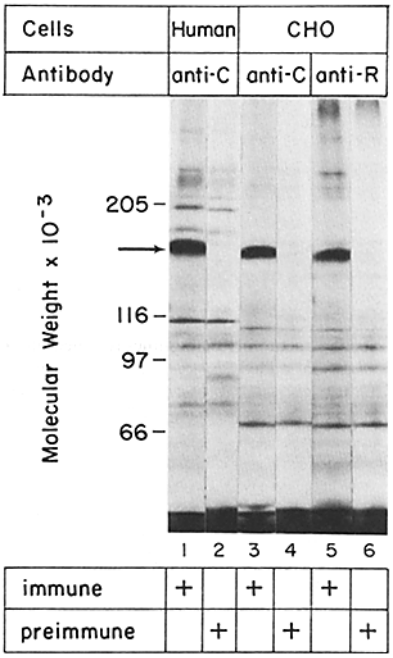

Figure 1. Immunoprecipitation of LDL receptors using anti-C and anti-R antibodies. On day 0, CHO cells (300,000 cells/dish) were plated in 3 ml of medium III and human fibroblasts (500,000 cells/dish) were plated in 3 ml of medium IV (medium I supplemented with 10% (vol/vol) human lipoprotein-deficient serum) in 60-mm dishes. On day 2, CHO cells were pulse-labeled for 30 min in methionine-free medium III and human cells were labeled in methionine-free medium IV, both containing 200 μCi/ml of [^{35}S]methionine. The cells were then washed and chased for 1 h in medium III (CHO cells) or medium IV (human cells). The cells were lysed by detergent and the extracts subjected to immunoprecipitation with one of the following antibodies: lanes *1* and *3*, anti-C (polyclonal anti-bovine LDL receptor C-terminal peptide); lanes *2* and *4*, preimmune control for anti-C; lane *5*, anti-R (polyclonal anti-bovine LDL receptor); lane *6*, preimmune control for anti-R. Proteins in the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography as described in Materials and Methods. The arrow indicates the mature form of the LDL receptor.

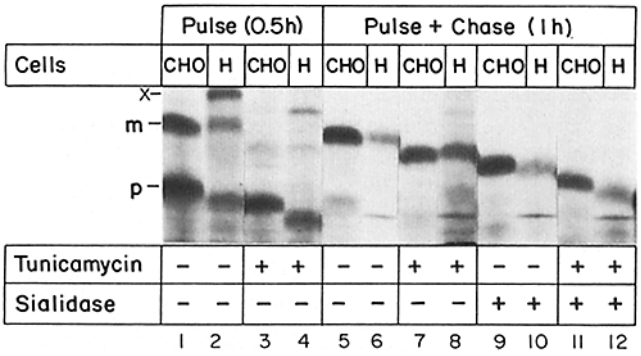

Figure 2. Comparison of LDL receptors from CHO and human fibroblast (H) cells. On day 0, CHO cells (300,000 cells/dish) and normal human fibroblasts (500,000 cells/dish) were plated in 3 ml of medium III in 60-mm dishes. On day 2, the cells were labeled with 200 μCi/ml of [^{35}S]methionine for 0.5 h, and immediately harvested or chased in medium III as indicated. To inhibit N-linked glycosylation, some of the cells (lanes *3*, *4*, *7*, *8*, *11*, and *12*) were treated with tunicamycin as described in Materials and Methods. After immunoprecipitation with the anti-C antibody, some of the immunoprecipitates were treated without (lanes *5–8*) or with (lanes *9–12*) sialidase as described in Materials and Methods. The precursor form of the CHO LDL receptor (125 kd) is indicated by *p*, and the mature form (155 kd) is indicated by *m*. The band labeled *x* represents a contaminant also present in control immunoprecipitations of human cells using preimmune antibody (see Fig. 1, lane *2*).

Results

Comparison of CHO and Human LDL Receptors

Fig. 1 compares LDL receptors immunoprecipitated from human and CHO cells using two different anti-LDL receptor antibodies, anti-C and anti-R. These antibodies recognize the receptor's C- and N-terminal domains, respectively. After a 30-min pulse-labeling with [^{35}S]methionine and a 1 h chase, the mature form (arrow) of the LDL receptor was specifically immunoprecipitated from human fibroblasts (lane *1*) by the anti-C antibody. IgG-C7, a monoclonal anti–receptor antibody that recognizes human but not CHO LDL receptors (Sege et al., 1984; Beisiegel et al., 1981 *b*), and anti-R specifically immunoprecipitated the same protein from these cells (data not shown). Anti-C (lane *3*) and anti-R (lane *5*) also immunoprecipitated the LDL receptor (155 kd) from CHO cells. The unidentified bands seen in the autoradiograms were present in immunoprecipitations using control preimmune IgG (lanes *2*, *4*, and *6*).

A detailed comparison of CHO and human receptors immunoprecipitated with the anti–C antibody is shown in Fig. 2. The human LDL receptor is synthesized as a precursor which is converted by extensive glycosylation to a mature form (Goldstein et al., 1985). After pulse-labeling for 30 min, both the precursor (p) and mature (m) forms of the receptors in CHO (Fig. 2, lane *1*) and human (H, lane *2*) cells were observed. A band of high molecular weight, designated x (lane *2*), was also present in control preimmune immunoprecipitations (Fig. 1, lane *2*) from labeled human cells and thus appears to be a nonspecific product of the immunoprecipitation procedure. The CHO precursor (125 kd) was ~5 kd larger than the human precursor (120 kd). Since inhibition of N-linked glycosylation with tunicamycin decreased the molecular masses of both precursors by ~8 kd (Fig. 2, lanes *3* and

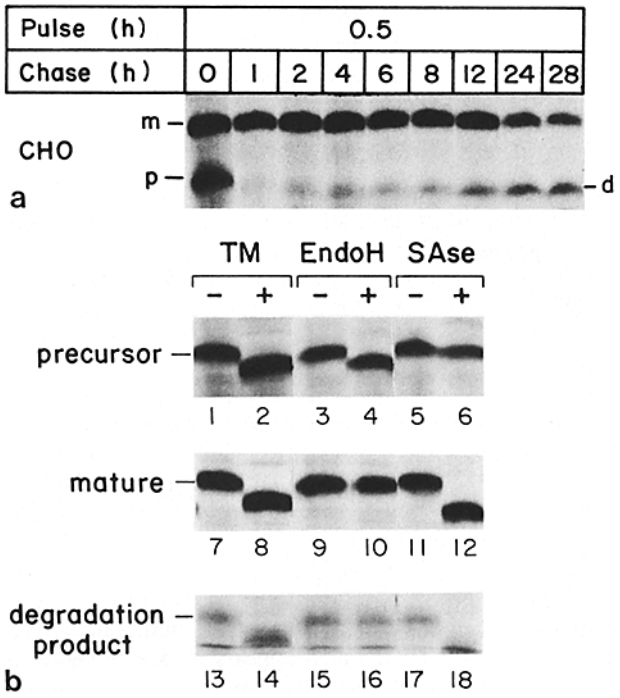

Figure 3. Processing of LDL receptors in wild-type CHO cells. (*a*) Kinetics of processing. CHO cells were pulse-labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for the indicated times, and subjected to immunoprecipitation (anti-C antibody), electrophoresis, and autoradiography as described in Materials and Methods. The degradation product of the LDL receptor (118 kd) is indicated by *d*. (*b*) Characterization of carbohydrates on the precursor, mature, and degradation forms. CHO cells were pulse-labeled with 200 μCi/ml of [^{35}S]methionine for 30 min and immediately harvested (lanes *1–6*) or chased for 6.4 h (lanes *7–18*). Some of the cells were treated with tunicamycin (*TM*, lanes *2*, *8*, and *14*). After immunoprecipitation (anti-C antibody), some of the immunoprecipitates were treated with endoglycosidase H (*Endo H*, lanes *4*, *10*, and *16*) or with sialidase (*SAse*, lanes *6*, *12*, and *18*) or subjected to control incubations without the enzymes (lanes *3*, *5*, *9*, *11*, *15*, and *17*) before electrophoresis and autoradiography as described in Materials and Methods.

4), the precursors apparently have similar amounts of N-linked oligosaccharides. As previously shown for human LDL receptors (Cummings et al., 1983), the precursor form of the CHO receptor was sensitive to endoglycosidase H (contains high mannose N-linked sugars) and resistant to sialidase (see Fig. 3*b* below). The different sizes of the human and CHO precursors may be due to differences in the primary structure or in the number or type of sugars added to the precursors (Cummings et al., 1983; Goldstein et al., 1985).

The mature forms of the receptors observed after a 1-h chase (Fig. 2, lanes *5* and *6*) had identical electrophoretic mobilities (155 kd) and identical sensitivities to tunicamycin (10 kd shift). They also were endoglycosidase H resistant (Cummings et al., 1983; Fig. 3*b*, lanes *9* and *10*). Thus, the mature form of the CHO receptor contained complex type N-linked glycans (Kornfeld and Kornfeld, 1980). Sialidase treatment, which removes terminal sialic acid residues from both N- and O-linked chains, reduced the size of the mature receptors by ~17 kd, although the effect on human receptors (Fig. 2, lane *10*) was somewhat greater than on CHO receptors (lane *9*). This difference between human and CHO receptors was also observed when N-linked sugar-deficient receptors from tunicamycin-treated cells were treated with sialidase (lanes *11* and *12*). Thus, as was the case for human receptors (Cummings et al., 1983), most of the shift in electrophoretic mobility from precursor to mature form was due to the processing of the O-linked sugars.

We have previously isolated and characterized CHO cells that were transfected with human DNA and contain and express functional human LDL receptor genes (Sege et al., 1984). The electrophoretic mobilities and glycosidase sensitivities of the precursor and mature forms of human LDL receptors in these transfected CHO cells (LET-A-111 cells) were essentially identical to those in human fibroblasts (data not shown). Therefore, the small differences in the structures of the human and CHO receptors must be due to small differences in the receptors themselves and not to differences in the availability of the types of sugars added or the activities of the enzymes responsible for posttranslational processing.

Taken together, these data show that the structures and the posttranslational processing of human and CHO LDL receptors are similar, that extensive N- and O-linked oligosaccharide processing is responsible for the precursor to mature transition, and that it is reasonable to interpret results from wild-type and mutant CHO cells based on the well-characterized human receptor (Goldstein et al., 1985).

Identification of a Third Form of the LDL Receptor

The pulse-chase experiment in Fig. 3*a* shows that in wild-type CHO cells the precursor form of the receptor was rapidly converted to the mature form. Subsequently, the mature form was slowly degraded (~50% remained after 15–20 h). After 2 h of chase, a third form of the receptor, ~118 kd (d), was detected. It seemed likely that this 118-kd protein was a degradation product of the mature form of the receptor.

To test this possibility, the oligosaccharide structure of the 118-kd form of the receptor was compared with the oligosaccharide structures of the precursor and mature forms of the receptor (Fig. 3*b*). The precursor form of the receptor (lanes *1–6*) was sensitive to tunicamycin or endoglycosidase H but resistant to sialidase, while the mature form (lanes *7–12*) was sensitive to tunicamycin or sialidase but resistant to endoglycosidase H. The oligosaccharides on the degraded form (118 kd) of the LDL receptor resembled those on the mature form. The apparent molecular weight of the degraded form was reduced by 10 kd after cells were treated with tunicamycin (lanes *13* and *14*) and by 13 kd after sialidase digestion (lanes *17* and *18*). The degraded form was resistant to endoglycosidase H treatment (lanes *15* and *16*). The 118-kd form of the receptor was readily detected using anti-C (Fig. 3, *a* and *b*) but not anti-R (data not shown). Since anti-R primarily recognizes the N-terminal region of the receptor (see Materials and Methods), it is likely that this protein was formed by the proteolytic removal of an apparently unglycosylated fragment from the N-terminus of the mature receptor.

Structure of Abnormal Receptors in ldlA Mutants

Previous experiments have indicated that the *ldlA* locus is the structural gene for the LDL receptor (Kingsley and Krieger, 1984; Sege et al., 1984). The effects of *ldlA* mutations on the structures of the LDL receptors in 15 receptor-deficient *ldlA* clones were examined using the anti-C and anti-R antibodies.

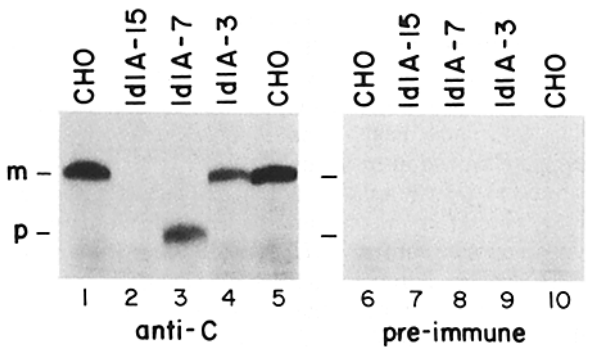

Figure 4. Comparison of LDL receptors from three classes of *ldlA* mutant. The indicated cells were labeled with 160 μCi/ml [^{35}S]-methionine for 5 h, subjected to immunoprecipitation with either the anti-C antibody (lanes *1–5*) or control preimmune antibody (lanes *6–10*) before electrophoresis and autoradiography as described in Materials and Methods.

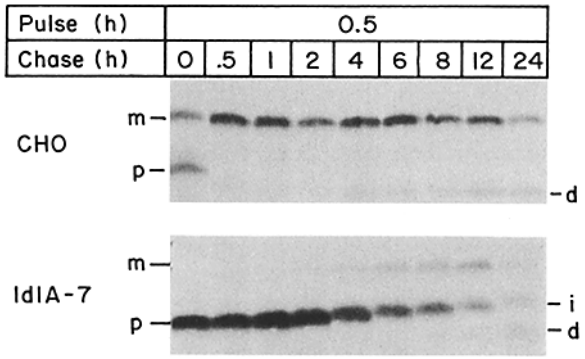

Figure 5. Kinetics of processing of LDL receptors in wild-type CHO cells and ldlA-7, a class 2 mutant. Cells were pulse-labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for the indicated times, and subjected to immunoprecipitation (anti-C antibody), electrophoresis, and autoradiography as described in Materials and Methods. Forms of the LDL receptor which appear to be intermediates in processing not seen in wild type cells are indicated by *i*.

Both the anti–C and anti–R antibodies yielded essentially identical results for all cell types examined (data for anti-R not shown for immunoprecipitation experiments). Three general classes of *ldlA* mutants were observed after labeling with [^{35}S]methionine for 5 h (Fig. 4). The mature form of the LDL receptor was observed in wild-type CHO cells (lanes *1* and *5*, preimmune controls for all cells are shown in lanes *6–10*). No LDL receptor was detected in nine class 1 mutants such as clone ldlA-15 (lane *2*). An abnormally small cross-reacting protein (125–130 kd) and very little mature LDL receptor was observed in five class 2 mutants such as clone ldlA-7 (lane *3*). A mature form with wild-type mobility was seen in the only class 3 mutant identified (clone ldlA-3, lane *4*). None of these mutants could bind or degrade significant amounts of [^{125}I]LDL (see Materials and Methods and data not shown). Each of the three clones in Fig. 4 was examined in greater detail.

ldlA-15, a Class 1 Mutant

Clone ldlA-15 cells were subjected to both short (30–45 min) and long (5 h) [^{35}S]methionine pulse-labeling protocols. In neither case did anti-C or anti-R detect cross-reacting material in the cells (e.g., Fig. 4, lane *2*). Since these antibodies recognize both the N- and C-termini of LDL receptors, it is likely that the *ldlA* mutation in this clone either blocks transcription, results in an unstable or untranslatable mRNA, or leads to a grossly abnormal protein product.

ldlA-7, a Class 2 Mutant

The synthesis and processing of the abnormal form of LDL receptor observed in clone ldlA-7 was examined in the pulse-chase experiment shown in Fig. 5. In these mutant cells, a precursor with an apparently wild-type mobility was detected immediately after the 30-min pulse-labeling (0 chase). In contrast to the rapid and complete processing to the mature form seen in wild-type CHO cells (top), the precursor in ldlA-7 cells was slowly and only partially converted to a mature form (bottom). This delayed processing was similar to that observed in some human FH mutants (Schneider et al., 1983). However, unlike these human mutants, most of the precursor in ldlA-7 cells was converted slowly and continually to slightly larger processing intermediates (indicated by i) which were not observed in wild-type cells. At the end of the 24-h chase, three forms of the receptor were observed: a form with mature receptor mobility (155 kd), the abnormal intermediate (~130 kd), and a 118-kd form which appears to be similar to the degraded form in wild-type cells. The LDL receptor in this clone was markedly less stable than receptors in wild-type cells. In contrast to the wild-type cells in which the LDL receptors diminished to 50% of maximum after 15–20 h of chase (see above), the receptors synthesized by ldlA-7 cells diminished to 50% of maximum after 4–6 h of chase.

To qualitatively assess the relative steady-state levels of receptor in ldlA-7 and wild-type cells, we used immunoblotting of unreduced specimens to visualize the receptors in unlabeled cells (Fig. 6). The apparent molecular weight of the wild-type LDL receptor (lanes *1* and *3*) was 130 kd. The difference in electrophoretic mobilities of the unreduced and reduced receptors (130–155 kd, compare Figs. 6 and 1) was probably due to the unfolding of the receptor after reduction of its many disulfide bonds (Daniel et al., 1983). LDL receptors in the ldlA-7 cells (Fig. 6, lane *4*) were essentially undetectable by immunoblotting. This low steady-state level of receptors in ldlA-7 cells was not due to abnormal secretion,

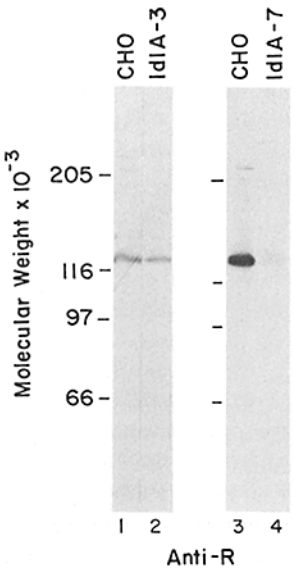

Figure 6. Immunoblot analysis of steady-state levels of LDL receptors. In two separate experiments, membranes were isolated from wild-type and ldlA-3 (class 3) or ldlA-7 (class 2) mutants as described in Materials and Methods and subjected to electrophoresis followed by transfer to nitrocellulose filters. The filters were then probed with the anti-R antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and visualization with 4-chloro-1-naphthol as described in Materials and Methods.

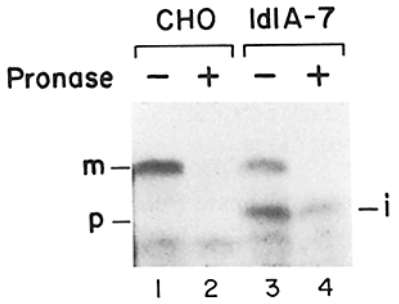

Figure 7. Effects of pronase treatment of cell monolayers on LDL receptors in CHO and ldlA-7 cells. Cells were labeled with 160 μCi/ml [^{35}S]methionine for 2 h, chased for 22 h, then treated with (lanes *2* and *4*) or without (lanes *1* and *3*) pronase (20 min) before harvesting, immunoprecipitation (anti-C antibody), electrophoresis, and autoradiography as described in Materials and Methods.

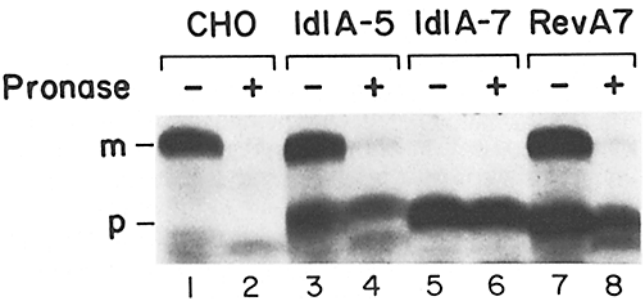

Figure 8. LDL receptors in heterozygous *ldlA* mutants: effects of pronase treatment. Wild-type, mutant (ldlA-7), heterozygous mutant (ldlA-5), and heterozygous revertant (RevA7, derived from mutant ldlA-7) cells were labeled with 100 μCi/ml [^{35}S]methionine for 2.25 h, and treated with (lanes *2*, *4*, *6*, and *8*) or without (lanes *1*, *3*, *5*, and *7*) pronase (20 min) before harvesting, immunoprecipitation (anti-C antibody), electrophoresis, and autoradiography as described in Materials and Methods.

as no receptor was detected in the culture medium (data not shown). In addition, in ldlA-7 cells, as in wild-type cells, the receptor was membrane associated (no significant receptor in the nonmembrane-associated fraction; data not shown).

In ldlA-7 cells, both the precursor form and an intermediate isolated after a 6.5-h chase were endoglycosidase H sensitive and sialidase resistant (data not shown). Therefore, some of the intermediate forms probably do not reach the *trans* region of the Golgi complex (Dunphy et al., 1981; Roth and Berger, 1982; Goldberg, and Kornfeld, 1983) or the cell surface. The latter possibility was tested by determining the location (surface or internal) of the various forms of the receptor in intact cells using an extracellular pronase sensitivity assay (Tolleshaug et al., 1983). When wild-type cells were pulse-labeled for short times to visualize the precursor form of the receptor, the precursor was resistant to pronase degradation (Kingsley et al., 1986). Fig. 7 shows the effects of pronase on cells that were labeled with [^{35}S]methionine for 2 h and then chased with unlabeled methionine for 22 h. Essentially all of the mature receptors in wild-type CHO cells were degraded by pronase (compare lanes *1* and *2*), indicating that under these conditions all of the wild-type receptors were accessible at the cell surface. In the ldlA-7 cells, little intermediate remained after this long chase (Fig. 5); however, the remaining intermediates were still resistant to pronase (Fig. 7, lanes *3* and *4*). Similar pronase resistance was observed immediately after labeling cells for 2.25 h (Fig. 8, lanes *5* and *6*). The mature (155 kd) form of the ldlA-7 receptor, however, was similar to the wild-type mature form in that it was degraded by extracellular pronase (Fig. 7, lanes *3* and *4*) and was endoglycosidase H resistant and sialidase sensitive (data not shown). Taken together, these experiments suggest that the receptor-deficient phenotype of ldlA-7 cells is due to the abnormal processing and instability of the LDL receptors and that the intermediates are primarily intracellular.

This abnormal processing of a glycoprotein in ldlA-7 cells was limited to the endogenous LDL receptors. For example, when ldlA-7 cells were infected with vesicular stomatitis virus, the N-linked oligosaccharide chains of the viral G glycoprotein were processed normally (Kingsley et al., 1986). Furthermore, transfected ldlA-7 cells which contain and express functional human LDL receptor genes (LET-A-111 cells) processed human LDL receptors normally while still expressing the mutant ldlA-7 allele (data not shown).

Four other independently isolated receptor-negative *ldlA* mutants also exhibited abnormal LDL receptor processing (data not shown), and along with ldlA-7, have been designated class 2 mutants. In these mutants, the conversion of precursor to mature receptor was slow. The processing of the precursor form to increasingly higher apparent molecular weight intermediates (125–139 kd), as with ldlA-7, was continuous. However, the rates of processing and electrophoretic mobilities of intermediates differed somewhat from those of ldlA-7 cells, suggesting that they represent different alleles. The bulk of the LDL receptor synthesized in these mutants was less stable than wild-type receptors. The pronase sensitivity of the intermediate form of the receptor in one of these mutants, ldlA-9, was examined. 4 h after labeling, the intermediate was pronase resistant (data not shown). Thus, as with ldlA-7 cells, the intermediate apparently remained intracellular.

ldlA-3, a Class 3 Mutant

The ldlA-3 cells synthesized mature LDL receptors which had the same electrophoretic mobility as wild-type receptors (Fig. 4, lane *4*). The structure of these mutant receptors was indistinguishable from wild-type receptors when analyzed under a variety of conditions. For example, the rate of conversion of precursor to mature form and the stability of [^{35}S]methionine-labeled receptor in pulse-chase experiments were essentially identical to those in wild-type cells (data not shown). The mature form of the receptor was endoglycosidase H resistant and sialidase sensitive and, in intact cells, the mature form was located on the cell surface (pronase sensitive, data not shown).

To assess the relative steady-state levels of receptor in ldlA-3 and wild-type cells, we used immunoblotting to visualize the receptors in unlabeled cells (Fig. 6). It is unlikely that the relatively modest reduction in steady state level (Fig. 6, lane *2*, compare with wild-type, lane *1*, and the undetectable levels of LDL receptor in the ldlA-7 cells, lane *4*) accounts for the extremely low levels of receptor activity in this mutant (1.5%). The ldlA-3 cells are probably receptor-deficient because the mature form of the receptor cannot bind LDL with high affinity.

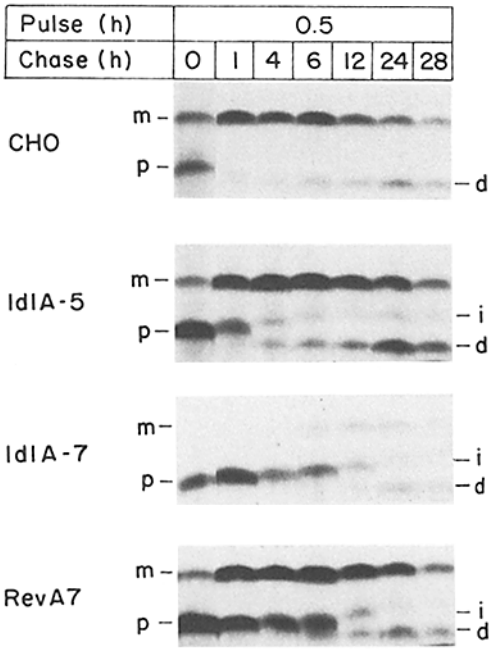

Figure 9. LDL receptor processing in a heterozygous *ldlA* mutant (*ldlA-5*) and a heterozygous *ldlA* revertant (*RevA7*). The indicated cells were labeled with 300 μCi/ml [^{35}S]methionine for 30 min, chased for the indicated times, and subjected to immunoprecipitation (anti-C antibody), electrophoresis, and autoradiography as described in Materials and Methods.

Heterozygous ldlA Mutants

After 2.25 h of continuous [^{35}S]methionine labeling, when only the mature form of the receptor was visible in wild-type CHO cells (Fig. 8, lane *1*), the ldlA-5 mutant contained two distinct immunoprecipitable forms of the LDL receptor (lane *3*). These forms resemble the wild-type mature form (lane *1*) and the abnormal intermediate form seen in ldlA-7 cells (lane *5*). As in ldlA-7 cells, the intermediate form in ldlA-5 cells was endoglycosidase H sensitive and sialidase resistant, while the mature form was endoglycosidase H resistant and sialidase sensitive (data not shown). The mature but not the intermediate form was sensitive to pronase treatment of intact cells (lane *4*). A small fraction of the pronase-treated receptor (lanes *2* and *4*) had an apparent molecular weight of 118 kd. Thus, this pronase-digestion product may have a structure similar to that of the degraded form (118 kd) observed during normal turnover of the receptor (see Fig. 3*a*, above).

The kinetics of synthesis and processing of both forms of receptor in the ldlA-5 cells was examined in the pulse-chase experiment shown in Fig. 9. During the chase period, some of the precursor form of the receptor was abnormally processed to an intermediate form similar to that in ldlA-7 cells while the remainder appeared to be processed normally and exhibited normal stability. In ldlA-5 cells, the steady-state concentration of the mature but not the abnormal intermediate form was sufficient to permit visualization in immunoblotting experiments (data not shown). Despite the presence of some LDL receptors with apparently normal molecular weight and stability, the ldlA-5 cells expressed little LDL receptor activity as determined by binding and degradation of [^{125}I]LDL (Materials and Methods and data not shown) or uptake and accumulation of fluorescently labeled LDL (data not shown; Kingsley and Krieger, 1984). Thus, the ldlA-5 mutant appeared to be a genetic compound with a class 2 allele similar to that in ldlA-7 cells and a second allele which codes for an abnormal receptor of essentially wild-type electrophoretic mobility (class 3).

Previous studies suggested that a spontaneous revertant, RevA7, derived from the ldlA-7 mutant, is also heterozygous at the *ldlA* locus (Kingsley and Krieger, 1984). The RevA7 cells have approximately half of normal LDL receptor activity. Fig. 8 (lane *7*) shows that these cells also synthesize two distinct forms of the receptor: an intermediate form and a mature form. The mature, but not the intermediate form, was readily accessible to extracellular pronase after labeling for 2.25 h (Fig. 8, lane *8*) and after a 4-h chase (data not shown). In addition, the mature form was endoglycosidase H resistant and sialidase sensitive (data not shown), and was as stable as the mature form in wild-type cells (Fig. 9). Receptor processing in RevA7 resembled that in ldlA-5 in that some of the precursor was rapidly converted to a mature form and some was converted to an unstable intermediate form as in ldlA-7 cells (Fig. 9). Synthesis of both forms of LDL receptors in the RevA7 cells was suppressed by the addition of either LDL or exogenous sterols to the medium (data not shown). Thus, the receptors were subject to normal sterol-mediated regulation, and as previously shown (Kingsley and Krieger, 1984), LDL could provide cholesterol to the RevA7 cells. In contrast, although receptor synthesis in ldlA-7 cells was suppressed by exogenous sterols, synthesis could not be suppressed by LDL (data not shown). Thus, the abnormally processed and inactive (<1% activity) LDL receptors in ldlA-7 cells were subject to sterol-mediated regulation.

Discussion

To examine the structure and processing of LDL receptors in wild-type and LDL receptor-deficient mutant CHO cells, we have prepared two distinct polyclonal antibodies. Using these two antibodies, we found that both the structure and the processing of the CHO LDL receptor were similar to those of the extensively characterized human LDL receptor (Tolleshaug et al., 1982, 1983; Goldstein et al., 1985). Both were synthesized as precursors (CHO, 125 kd; human, 120 kd) containing high mannose N-linked oligosaccharides. The precursors were processed rapidly without detectable intermediates to substantially larger mature forms (CHO and human, 155 kd) containing complex N-linked and O-linked oligosaccharides.

In the course of these studies, we observed a degraded form of the CHO LDL receptor which had not been characterized previously. This degraded form (118 kd) appeared to be an intermediate in normal receptor turnover which arose after the removal of an apparently unglycosylated portion of the receptor's cysteine-rich N-terminus. A C-terminal degraded form of the human LDL receptor in human fibroblasts has recently been observed (Lehrman et al., 1985) and is probably analogous to the degradation product characterized in this study.

The structure and processing of LDL receptors was also examined in a small sample of our collection of *ldlA* mutants. We found multiple mutant forms of the receptor in *ldlA*

mutants, suggesting that the *ldlA* locus is the structural gene for the LDL receptor. In addition, the observation that two independent forms of the LDL receptor can coexist in the same cells (clones ldlA-5 and RevA7) rules out the possibility that the *ldlA* mutants are abnormal because of pleiotropic processing defects. The assignment of the *ldlA* locus to the structural gene of the LDL receptor is consistent with conclusions from our previous genetic studies which included somatic cell complementation (Kingsley and Krieger, 1984) and gene transfer (Sege et al., 1984) experiments. The assignment of the *ldlA* locus to the structural gene of the LDL receptor has recently been verified directly using cloned LDL receptor cDNA and a genomic clone representing a portion of the hamster LDL receptor gene (Sege, R., K. Kozarsky, and M. Krieger, manuscript in preparation).

The characteristics of the mutant receptors in *ldlA* cells were similar although not identical to those of three of the four previously identified classes of mutant receptors in human FH cells (Goldstein et al., 1985): null alleles (class 1), alleles that code for abnormally processed receptors (class 2), and alleles that code for normally processed, cell surface receptors that do not bind LDL (class 3). We have recently characterized another mutant allele which encodes a truncated receptor which is abnormally processed and remains intracellular (Sege, R., K. Kozarsky, and M. Krieger, manuscript in preparation). We have not yet characterized mutant alleles in CHO cells that are analogous to the internalization defective alleles (class 4) in human FH cells.

The class 2 *ldlA* mutants differed somewhat from the class 2 human FH mutants (Schneider et al., 1983; Goldstein et al., 1985). In class 2 *ldlA* mutants, the 125-kd precursor form of the LDL receptor was apparently synthesized normally while the conversion to the mature form was abnormal. The rate at which the precursor was converted to the 155-kd mature form was dramatically slower than that for wild-type cells and only a small fraction of the precursor was fully processed to a mature form. As in wild-type cells, the 155-kd mature form resided on the cell surface. However, much of the precursor in these *ldlA* mutants was processed to unstable intracellular intermediate forms (125–139 kd) not previously observed in wild-type CHO cells. In contrast to the processing of precursors in wild-type CHO cells and in normal and naturally occurring class 2 FH mutant human cells (Goldstein et al., 1985), the processing of the precursors in several different class 2 mutants was continuous; in pulse-chase experiments, increasingly larger intermediates were observed at progressively later times of chase. We do not know whether the abnormal, intermediate receptor structures were the consequences or causes of the very slow processing and rapid degradation of the receptors in these mutants.

Several experiments were performed to clarify the molecular basis for the continual increases in apparent molecular weight of the abnormal intermediates in the class 2 ldlA-7 cells. Glycosidase treatment indicated that the increases were not due to the conversion of the high mannose N-linked sugars of the precursor to trimmed and complex oligosaccharides (for review see Kornfeld and Kornfeld, 1980). Additional, but incomplete, processing of O-linked oligosaccharides or other modifications such as sulfation, fatty acylation, phosphorylation, or abnormal N-linked glycosylation may be responsible for the processing observed. Abnormal posttranslational processing of glycoproteins has been observed in many other systems in which there are mutations in the structural genes for glycoproteins (Hercz and Harpaz, 1980; Rose and Bergman, 1983; Haguenauer-Tsapis and Hinnen, 1984; Doyle et al., 1985; Schauer et al., 1985). However, none of these has exhibited a similar type of continual processing to intermediate forms. The location of the intermediates within class 2 *ldlA* mutants and the site(s) of degradation have not yet been determined. The limited oligosaccharide structural data raise the possibility that the receptors may not gain access to the *medial* or *trans* regions of the Golgi complex (Dunphy et al., 1981; Roth and Berger, 1982; Goldberg and Kornfeld, 1983). Since the defects in class 2 *ldlA* mutants are in the structural gene for the receptor, these mutants are fundamentally different from other types of mutants in which the cellular apparatus associated with glycoprotein processing is disrupted (Novick et al., 1981; Robbins et al., 1984; Stanley, 1985; and Kingsley et al., 1986).

Two *ldlA* mutants each contained two different forms of the LDL receptor. One, ldlA-5, was directly isolated from mutagen-treated wild-type cells and the other, RevA7, was a spontaneous revertant from clone ldlA-7 and expressed ~50% of wild-type receptor activity (Kingsley and Krieger, 1984). These apparently heterozygous cells strongly support our conclusion from previous genetic studies (Kingsley and Krieger, 1984) that the *ldlA* locus is diploid in CHO cells. Clone ldlA-5 contained two distinct alleles which both code for inactive receptors while RevA7 contained one active and one inactive form of the receptor. The processing and the structures of the active receptors in RevA7 cells were similar to those of wild-type receptors.

The frequencies at which ethyl methanesulfonate-induced *ldlA* cells were isolated from wild-type cells ($1–25 \times 10^{-6}$; Kingsley and Krieger, 1984) and from RevA7 heterozygous revertants ($10^{-4}–10^{-3}$; Kingsley and Krieger, 1984) are consistent with the *ldlA* locus being diploid since mutagen-induced rates of mutation of $\sim 10^{-3}$ per haploid locus have been observed in a number of eukaryotes (e.g., Brenner, 1974; Bode, 1984). We presume that the mutants that appear to have only one form of the receptor protein (e.g., clones ldlA-7, -6, -9, and -3) either have two similar mutant alleles or are compound heterozygotes containing a null allele and the allele that codes for an inactive receptor protein. Recent experiments using a cloned fragment of the hamster LDL receptor gene have confirmed that the *ldlA* locus is diploid (Sege, R., K. Kozarsky, and M. Krieger, manuscript in preparation).

Because *ldlA* mutants can be isolated simply and efficiently after treating cells with a variety of mutagens (ethyl methanesulfonate, ICR-191, gamma rays), it may be possible to isolate additional classes of LDL receptor mutants. Since *ldlA* cells can be used as recipients for the introduction of exogenous LDL receptor genes (Sege et al., 1984) and since the LDL receptor gene has been cloned (Russell et al., 1984; Yamamoto et al., 1984), the consequences of specific in vitro mutagenesis of the receptor can be investigated readily using these cells. The study of mutations derived from somatic cell mutants or in vitro mutagenesis of the cloned gene undoubtedly will continue to provide the kinds of insights into the structure and function of the LDL receptor which have already begun to be derived from the study of naturally occurring human mutations (Goldstein et al., 1985).

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