LDLC Encodes a Brefeldin A-Sensitive, Peripheral Golgi Protein Required for Normal Golgi Function

Steven D. Podos, Pranhitha Reddy, John Ashkenas, and Monty Krieger

Department of Biology and the Whitaker College of Health Sciences, Technology, and Management, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Two genetically distinct classes of low density lipoprotein (LDL) receptor-deficient Chinese hamster ovary cell mutants, ldlB and ldlC, exhibit nearly identical pleiotropic defects in multiple *medial* and *trans* Golgi-associated processes (Kingsley, D., K. F. Kozarsky, M. Segal, and M. Krieger. 1986. *J. Cell Biol.* 102:1576–1585.). In these mutants, the synthesis of virtually all N- and O-linked glycoproteins and of the major lipid-linked oligosaccharides is abnormal. The abnormal glycosylation of LDL receptors in ldlB and ldlC cells results in their dramatically reduced stability and thus very low LDL receptor activity. We have cloned and sequenced a human cDNA (*LDLC*) which corrects the mutant phenotypes of ldlC, but not ldlB, cells. Unlike wild-type CHO or ldlB cells, ldlC cells had virtually no detectable endogenous *LDLC* mRNA, indicating that *LDLC* is likely to be the normal human homologue of the defective gene in ldlC cells. The predicted sequence of the human *LDLC* protein (ldlCp, ~83 kD) is not similar to that of any known proteins, and contains no major common structural motifs such as transmembrane domains or an ER translocation signal sequence. We have also determined the sequence of the *Caenorhabditis elegans* ldlCp by cDNA cloning and sequencing. Its similarity to that of human ldlCp suggests that ldlCp mediates a well-conserved cellular function. Immunofluorescence studies with anti-ldlCp antibodies in mammalian cells established that ldlCp is a peripheral Golgi protein whose association with the Golgi is brefeldin A sensitive. In ldlB cells, ldlCp was expressed at normal levels; however, it was not associated with the Golgi. Thus, a combination of somatic cell and molecular genetics has identified a previously unrecognized protein, ldlCp, which is required for multiple Golgi functions and whose peripheral association with the Golgi is both *LDLB* dependent and brefeldin A sensitive.

In eukaryotes, nascent secretory and integral membrane proteins, glycosaminoglycans, and glycolipids typically traverse the Golgi en route to their final destinations. Often, chemical modification of these molecules within the Golgi is essential for their stability or function. For example, mucin-type serine/threonine-linked (O-linked) oligosaccharides are known to protect from rapid proteolysis several cell surface proteins, including the low density lipoprotein (LDL)[1] receptor (Krieger et al., 1985), decay-accelerating factor (Reddy et al., 1989), the Epstein-Barr virus envelope protein (Krieger et al., 1989), and glycophorin (Remaley et al., 1991). Also, asparagine-linked (N-linked) glycosylation is required for normal folding, assembly, and intracellular transport of proteins such as the vesicular stomatitis virus G protein and the influenza virus hemagglutinin protein (Rose and Doms, 1988; Doms et al., 1993). Although previous biochemical and genetic analyses have uncovered a wealth of information about the molecular mechanisms underlying intracellular protein transport and processing in the Golgi (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Rothman and Orci, 1992), much remains to be learned about the structure and function of the Golgi.

To help define and analyze the gene products and functions required for normal Golgi activity, we have analyzed mutant CHO cells with defects in LDL receptor activity (Krieger et al., 1981, 1983, 1985; Krieger, 1983; Malmstrom and Krieger, 1991; Hobbie et al., 1994). These mutants define nine complementation groups, designated ldlA through ldlI (Kingsley and Krieger, 1984; Malmstrom and Krieger, 1991; Hobbie et al., 1994). The LDL receptor deficiency of mutants in two of these groups, ldlB and ldlC, is a consequence of dramatically decreased LDL receptor stability due to ab-

Address all correspondence to Monty Krieger, Room 68-483, Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139. Tel.: (617) 253-6793. Fax: (617) 258-6553.

Steven Podos' current address is Dept. of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637.

Pranhitha Reddy's current address is Immunex Corp., Seattle, WA 98101.

John Ashkenas' current address is Laboratory of Radiobiology and Environmental Health, University of California at San Francisco, San Francisco, CA 94143-0750.

1. *Abbreviations used in this paper*: BFA, brefeldin A; ConA, concanavalin A; EST, expressed sequence tag; FGAM, fluorescein-conjugated goat anti-mouse IgGs; FGAR, fluorescein-conjugated goat anti-rabbit IgGs; LDL, low density lipoprotein; LETC, LDL endocytosis transfectants of ldlC cells; PHA, phytohemagglutinin; TRHAM, Texas red-conjugated horse anti-mouse IgG; WGA, wheat germ agglutinin; WT, wild-type.

© The Rockefeller University Press, 0021-9525/94/11/679/13 $2.00

normal posttranslational processing of the receptor in the Golgi (Kingsley et al., 1986*a*). At least in the case of ldlC cells, this aberrant processing and the resulting instability do not prevent the initial appearance of the abnormal receptors on the cell surface and do not alter the receptors' ligand-binding and endocytic properties (Kingsley et al., 1986*a*; Reddy and Krieger, 1989).

ldlB and ldlC exhibit nearly identical pleiotropic defects in *medial* and *trans* Golgi-associated processes, which result in the abnormal synthesis of virtually all N-linked, O-linked and lipid-linked glycoconjugates (Kingsley et al., 1986*a*). The global nature of the glycosylation defects in these mutants was demonstrated both by examining the synthesis of several distinct molecules (LDL receptor, vesicular stomatitis virus G protein, the major surface glycolipid GM_3), and by establishing that the mutants exhibit abnormal sensitivities to a panel of toxic plant lectins. In contrast to many other glycosylation mutants (Stanley, 1985; Kingsley et al., 1986*c*), the diverse defects in these mutants cannot readily be explained by single deficiencies in the activities of either a glycosidase or a glycosyltransferase. Therefore, we have suggested that the genes defined by these mutants may affect the regulation, compartmentalization, or activity of several different Golgi enzymes or substrates (Kingsley et al., 1986*a*). The primary biochemical defects in these cells might cause Golgi disruptions by: (*a*) blocking the synthesis of small and/or macromolecular substrates or their access to Golgi enzymes; (*b*) blocking Golgi enzyme transport to or retention at the appropriate site; (*c*) preventing the posttranslational activation or stabilization of multiple Golgi enzymes; (*d*) disrupting the basic structure of the Golgi or its lumenal environment (pH, ion concentrations); or (*e*) some combination of these.

In the current work, we isolated a novel human cDNA (*LDLC*) that corrects all of the pleiotropic defects in ldlC cells, and we also isolated an *LDLC* homologue from *Caenorhabditis elegans*. We have examined the expression of the *LDLC* gene and its protein product (ldlCp), and the intracellular distribution of ldlCp, in wild-type CHO and mutant ldlC and ldlB cells. ldlCp is a peripheral Golgi protein whose association with the Golgi is dependent on the *LDLB* gene and sensitive to the drug brefeldin A. The high degree of similarity between the sequences of the human and *C. elegans LDLC* cDNAs suggests that ldlCp mediates a well-conserved cellular function. Thus, somatic cell genetic analysis of LDL receptor activity has defined a previously unrecognized gene which plays an important role in establishing or maintaining multiple Golgi functions. Additional molecular genetic and biochemical analysis of the *LDLB*/*LDLC* system should provide new insights into Golgi structure and function.

Materials and Methods

Materials

Reagents (and sources) were: methionine- and cysteine-free Ham's F12 medium (GIBCO BRL, Grand Island, NY); $Na^{125}I$ (Amersham Corp., Arlington Heights, IL); $\alpha[^{32}P]$dCTP, $[^{35}S]$methionine, and $[^{35}S]$dATP-α-S(>1000 Ci/mmol) (DuPont NEN, Boston, MA); fluorescein-conjugated goat anti-rabbit (FGAR) and goat anti-mouse (FGAM) IgGs (Cappel Research Reagents, Organon, Teknika, Durham, NC); Texas red-conjugated horse anti-mouse IgG (TRHAM) (Vector Laboratories, Burlingame, CA); and cell culture media and supplements (GIBCO BRL or Hazelton/JRH, Lenexa, KA). Newborn calf lipoprotein-deficient serum, LDL, and ^{125}I-LDL were prepared as previously described (Krieger, 1983). Lectins were purchased from Sigma Chemical Co., St. Louis, MO. Other reagents were obtained as previously described (Krieger, 1983) or were purchased from standard commercial suppliers. Compactin was a gift of A. Endo (Tokyo Nodo University, Japan). Antibodies used for immunofluorescence localization experiments include a polyclonal antiserum against Golgi mannosidase II (Moremen and Touster, 1985), and the anti-β-COP monoclonal antibody M3A5 (Allan and Kreis, 1986).

Cell Culture

All incubations with intact cells were performed at 37°C in a humidified 5% CO_2/95% air incubator unless specified otherwise. Wild-type CHO cells, ldlC (clone 475) and ldlB (clones 11 and WGA^r-2) mutant CHO cells, and the transfectant LETB-144 were obtained as previously described (Krieger et al., 1981; Kingsley and Krieger, 1984; Kingsley et al., 1986*a,b*) and were maintained in medium A (Ham's F12 containing glutamine [2 mM], penicillin [50 U/ml] and streptomycin [50 μg/ml]), supplemented with either 5% (vol/vol) (medium B) or 10% (vol/vol) (medium C) FBS. Human HeLa and murine NIH 3T3 cells were obtained from P. Sharp and F. Solomon, M. I. T. (Cambridge, MA). HeLa cells were maintained in medium B or C. 3T3 cells were maintained in medium D (Dulbecco's Modified Eagle Medium with glutamine, penicillin, streptomycin, and 5% [vol/vol] FBS). ldlC transfectants were maintained with or without 250 μg/ml G418 in either medium B or medium F, which is composed of medium E (medium A with 3% [vol/vol] newborn calf lipoprotein-deficient serum) supplemented with MeLoCo (250 μM mevalonate, 2.5 μg protein/ml LDL, and 40 μM compactin). Compactin, an inhibitor of HMG-CoA reductase, prevents cholesterol synthesis by inhibiting all mevalonate synthesis, with the supplemented mevalonate providing only enough precursor for nonsteroidal isoprenoid synthesis; thus, the LDL is the only source of cholesterol for cell growth (Goldstein et al., 1979; Krieger, 1986). Consequently, cells can grow in medium F containing MeLoCo only if they express essentially normal levels of functional LDL receptors.

Isolation of LDL Receptor-positive Genomic Transfectants from ldlC Cells

ldlC cells were transfected with calcium phosphate precipitates of human genomic DNA essentially as described by Graham and Van der Eb (1973). In brief, ldlC cells were plated on day 0 in medium B (500,000 cells/100-mm dish), and on day 2 the medium from each dish was replaced with 1.5 ml of Hepes-buffered saline containing a calcium phosphate precipitate of genomic DNA from human A431 carcinoma cells (20 μg/dish) and pSV2neo DNA (1 μg/dish). After 10 min, 10 ml of medium B were added. After a 5-h incubation, the DNA-calcium phosphate solution was removed and the cells in each dish were shocked with 2 ml of 15% glycerol in Hepes buffered saline for 3 min, washed twice in Ham's F12 medium, and incubated overnight in medium B. On day 3, the cells were refed with medium B and on day 4 harvested with trypsin/EDTA. Cells from each transfection dish were then reset into 2 100-mm dishes (4×10^6 cells/dish) in MeLoCo selection medium (medium F) containing 250 μg/ml G418, to isolate primary receptor-positive LDL endocytosis transfectants of ldlC cells (1° LETC cells). Five independent 1° LETC colonies were isolated from a total of 2×10^8 cells subjected to selection. Seven independent secondary LETC (2° LETC) colonies were then isolated from 2×10^8 cells by a second round of the cotransfection/selection procedure, except that genomic DNA isolated from one of the 1° LETC colonies (1° LETC-3C) was used in place of the A431 DNA. Finally, seven tertiary LETC (3° LETC) colonies were isolated from 6×10^8 cells after a third round of cotransfection/selection, using a 2° LETC colony (2° LETC-I5) as the source of genomic DNA.

Cloning Human LDLC cDNA

A 3.5-bp EcoRI DNA fragment was detected in the 2° LETC and 3° LETC colonies by Southern blot analysis, using BLUR11, a human *Alu* repeat element, as a probe (Jelinek et al., 1980). The BLUR11 probe was then used to clone the 3.5-kbp fragment from a λZAPII (Stratagene Inc., La Jolla, CA) library of EcoRI-digested, size-selected DNA from 2° LETC cells (colony V5). A 600-bp SacI-HincII restriction fragment from the 3.5-kbp EcoRI clone, which did not contain the *Alu* repeat element, was then used as a probe to isolate candidate *LDLC* cDNAs from two cDNA libraries.

These libraries were prepared from human HeLa cell poly(A)$^+$ RNA, synthesized both from random hexamer primers (Amersham) and from oligo d(T) 12-18 primers (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) as previously described (Ashkenas et al., 1993). The cDNAs were ligated to EcoRI/NotI adaptors (Pharmacia), size-selected (>1 kbp) by sedimentation through 5-20% KOAc gradients, and packaged into EcoRI-digested λZAPII. Plaques (5 × 10^5) from the random-primed library were transferred to nitrocellulose for hybridization. Eight positive clones (1-8) were identified, and cDNA from these clones was then used to isolate eight additional clones (9-16) from the oligo d(T) primed library. pBluescript constructs bearing cDNA clones 1-16 were excised from the λZAPII clones according to the manufacturer's instructions. Partial or complete sequences on one or both strands from all 16 clones were determined using internal and pBluescript primers with Sequenase 2 (United States Biochemical Corp., Cleveland, OH), and were assembled into a single consensus sequence (EMBL accession number Z34975) with Staden DNA and protein analysis software (Cambridge, UK; see e.g., Staden, 1990). Clone 2, which encompasses the complete protein coding sequence (bases 1-2214), was fully sequenced on both strands. Clone 2 starts at base −15 and continues through base 2780. The sequence of the 3′ most 92 bases in clone 2 does not match the consensus sequence derived from the other clones. This divergent sequence is: 5′-AGCTAGCTAG CTAGCTAGCT AGCTAGCTAG CTAGCTAGCT AGCTAGCTAG CTAGCTAGCT AGCTAGCTAG CTAGCTAGCT AGCTAGCTAG CT-3′. Surveys of sequence databases and analysis of protein sequence motifs were performed using the programs FASTA and MOTIFS (with PROSITE database, version 10.2, from Amos Bairoch, Geneva, Switzerland) from the Sequence Analysis Software Package from the Genetics Computer Group at the University of Wisconsin (versions through 7.3) (Devereux et al., 1984), and BLAST from NCBI (Altschul et al., 1990).

Transfection of LDLC cDNA into ldlC Cells

The full-length cDNA insert from clone 2 was inserted into the plasmid pRc/CMV (Invitrogen, San Diego, CA) to generate the expression construct p*LDLC*-1. p*LDLC*-1 DNA was transfected into ldlC cells using polybrene (Kawai and Nishizawa, 1984). Transfected cells were isolated by selection in medium B containing 250 μg/ml G418. In some cases, transfectants were isolated by incubation in MeLoCo selection medium F with G418, to select directly for LDL receptor-positive transfectants. One colony transfected with p*LDLC*-1, designated ldlC[*LDLC*], was used for all cDNA transfectant experiments presented here, and all results were verified using independently isolated transfectants (not shown).

Cloning a C. elegans Homologue of LDLC

The *C. elegans* cDNA fragment CEESW90 (GenBank accession number T01892) was identified as a potential *LDLC* homologue (see Results). A probe comprising sequences from CEESW90 was generated by PCR amplification of *C. elegans* genomic DNA using the oligonucleotide primers ATGGGTACACTTCATGGCGA and CGATTCTTTCAGCCATACCAAC. This probe was used to screen a *C. elegans* cDNA library in λZAP (Stratagene), prepared by R. Barstead, Washington University (St. Louis, MO). Six cDNA clones were isolated from 500,000 λZAP plaques, each clone being ~2.0 kbp. One clone was sequenced on both strands, and its sequence (EMBL accession number Z34976) was analyzed as described above for the human *LDLC*. The sequence of its protein product was compared to that of the human protein using the BESTFIT program from the Genetics Computer Group, and the amino acid similarities described in Guo et al., 1994 (see Fig. 4 *B*).

Preparation of Polyclonal Anti-ldlCp Antipeptide Antibodies

Peptides Npep (EKSRMNLPKGPDTLC) and Cpep (CAELVAAAKDQATAEQP) were synthesized containing the predicted NH_2-terminal and COOH-terminal sequences of the ldlC protein, with terminal cysteines added to permit crosslinking to carrier proteins. Npep and Cpep were coupled to keyhole limpet hemocyanin (Sigma) preactivated with m-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma), and these complexes were used to prepare polyclonal antibodies in New Zealand white rabbits. Preimmune and immune IgGs were isolated on Protein A-Sepharose (Pharmacia) columns and are designated preimmune IgG, anti-Npep, and anti-Cpep. For some experiments, anti-Cpep was affinity-purified on a Cpep-agarose column prepared by coupling ~6 mg of Cpep to a 2 ml SulfoLink column (Pierce Chem. Co., Rockford, IL); anti-Cpep was isolated after adsorption to the column by washing the column with 100 mM Tris (pH 8.0), and eluting with 100 mM glycine (pH 2.5).

Immunoblot Analysis

Cells were grown to confluence in medium C in 150-mm dishes, washed and collected in PBS, lysed by addition of an equal volume of 2× sample buffer with protease inhibitors (final concentrations: 60 mM Tris [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.71 M β-mercaptoethanol, 1.5 μg/ml aprotinin, 2 μM leupeptin, 10 μg/ml tosyl arginine methyl ester, 0.5 mM phenylmethylsulfonyl fluoride), boiled, and passed repeatedly through a 25-gauge needle. Protein concentrations were determined after trichloroacetic acid precipitation by the Lowry method (Lowry, 1951), and samples were resolved by electrophoresis on 0.8-mm thick 8% SDS-polyacrylamide gels (70 μg protein/mm^2) and transferred electrophoretically to 0.22 μm nitrocellulose (Schleicher and Schuell, Keene, NH). Approximately 2-mm wide strips were cut, and nonspecific protein-binding sites were blocked by incubating the strips in buffer W (2% [wt/vol] hemoglobin in PBS) for at least 1 h at room temperature. The specimens were then incubated overnight with primary antibody (10 μg/ml for anti-Cpep) in buffer W, washed three times with buffer X (buffer W containing 0.05% [vol/vol] NP-40, and 0.1% [wt/vol] SDS), and two times in buffer W, incubated with ^{125}I-protein A (2-10 μCi/μg) in buffer W for 1-2 h, washed two times with buffer X, and three times with PBS. Antibody binding was visualized by autogradiography. Control samples for Fig. 5 and 8 *A* were probed with anti-tubulin antiserum (not shown).

Immunofluorescence Microscopy

On day 0, cells were plated (200 cells/mm^2) in medium C onto 12-mm square glass cover slips. On day 2, the cover slips were washed in cPBS (PBS with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$) at 37°C, fixed for 30 min at room temperature with 3.7% (vol/vol) formaldehyde in cPBS which was prewarmed to 37°C, and quenched for 10 min at room temperature in 50 mM NH_4Cl. Cells were then permeabilized for 10 min at room temperature in PBS containing 0.1% Triton X-100 and 0.02% SDS. The cover slips were then processed as follows: rinsed once quickly in PBS, preblocked for 30 min at 37°C facedown on a 25-μl droplet of blocking solution (PBS, 5% FBS, 0.1% Triton X-100, 0.02% SDS) on parafilm, rinsed once with PBS, incubated for 90-120 min at 37°C with 25 μl primary antibody diluted in blocking solution (affinity purified anti-Cpep, 3 μg/ml; anti-β-COP monoclonal antibody M3A5, 1:2 dilution; and anti-mannosidase II, 1:1000 dilution), rinsed four times in PBS, incubated with fluorescently labeled secondary antibody in blocking solution (FGAR, 1/1,000 or 1/2,000 dilution; FGAM, 1/500 dilution; or TRHAM, 1.5 μg/ml) for 45-60 min, rinsed four times in PBS and one time in H_2O, and mounted on Vinol gel (Air Products and Chemicals, Allentown, PA) with 1,4-diazabicyclo[2.2.2]octane (15 mg/ml) (Sigma). Double-staining experiments (e.g., see Fig. 7) were performed by simultaneous addition of two primary antibodies and then addition of the corresponding secondary antibodies. Control experiments (not shown) established that results from doubly stained samples were indistinguishable from those of singly stained samples. Cells were examined on a Zeiss axioplan microscope using 40× and 100× oil immersion objectives and fluorescein or rhodamine filter packages, and photographed with Kodak T-max 400 film.

Other Methods

Lectin sensitivity assays were performed as previously described (Kingsley et al., 1986*a*). The LD_{10} values presented represent estimates of the lectin concentrations which results in the killing of ~90% of the cells.

LDL receptor activity was determined using an ^{125}I-LDL (10 μg protein/ml, 490 cpm/ng protein) degradation assay as described previously (Goldstein et al., 1983; Krieger, 1983). The high affinity degradation values shown represent the differences between measurements made in the absence (duplicate determinations) and presence (single determinations) of excess unlabeled LDL (400 μg protein/ml) and are presented as ng of ^{125}I-LDL degraded in 5 h per mg of cell protein. Protein concentrations were determined by the method of Lowry et al. (1951).

Metabolic labeling of cells, immunoprecipitation of LDL receptors with an anti-COOH terminus anti-peptide antibody, electrophoresis, and autoradiography were performed as previously described (Kozarsky et al., 1986).

Unless otherwise indicated, recombinant DNA and immunological techniques were performed as described in Sambrook et al. (1989) and Har-

low and Lane (1988), respectively. Southern blot analyses were performed using Zetabind nylon filters (Cuno Inc., Meriden, CT) and poly(A)$^+$ RNA Northern blot analyses using GeneScreen filters (DuPont NEN, Boston, MA).

Results

Cloning of the Human LDLC cDNA

To clone the *LDLC* gene, we adapted the strategy pioneered by Shih and Weinberg (1982) for the cloning of the *ras* oncogene (see Materials and Methods for details). In brief, human genomic DNA was transfected into ldlC cells, and LDL receptor-positive revertants which exhibited normal glycoconjugate synthesis were isolated using a nutritional selection method (MeLoCo, described in Krieger, 1986). LDL receptor activity was determined using an LDL degradation assay, which measures the receptor-dependent internalization and lysosomal degradation of ^{125}I-LDL (Goldstein et al., 1983; Krieger, 1983). The global glycosylation defects in ldlC cells and their correction by transfection were detected using a lectin sensitivity assay (Stanley, 1985; Kingsley et al., 1986*a*). Due to the altered structures of cell surface glycoconjugates in ldlC cells (Kingsley et al., 1986*a*), these mutants, relative to wild-type CHO, are hypersensitive to the lectins concanavalin A (Con A) and ricin, and resistant to phytohemagglutinin (PHA) and wheat germ agglutinin (WGA). The transfectants from this first round of transfection/selection are designated primary (1°) LETC cells (LDL Endocytosis Transfectants of ldlC). Genomic DNA from one 1° LETC line was transfected into ldlC cells to generate LDL receptor-positive secondary (2°) LETC cells and an additional round of transfection and selection was used to isolate tertiary (3°) LETC cells (not shown).

The presence of human DNA in the LETC cells was assessed by Southern blotting, using either total human genomic DNA or a cloned fragment of human repetitive DNA (*Alu*) as the probe (not shown). In all secondary and tertiary transfectants examined, there was a correlation of the presence of a 3.5-kbp EcoRI human DNA-containing fragment with the restoration both of LDL receptor activity (^{125}I-LDL assay or growth in selective medium, see Materials and Methods) and of normal glycosylation (lectin sensitivity assay). This suggested that transfer of the human *LDLC* gene was probably responsible for the correction of the mutant phenotype in the transfected cells, and that the human *LDLC* gene was physically linked to the 3.5-kbp EcoRI fragment. Therefore, we used the *Alu* probe to clone this 3.5-kbp DNA fragment from a size-selected library of EcoRI-digested genomic DNA prepared from a 2° LETC colony.

A 600-bp *Alu* repeat-free SacI-HincII restriction fragment from this 3.5-kbp clone was then used as a probe for Northern blot analysis (not shown). Under high stringency hybridization conditions, the probe recognized a single 3.1–3.5-kb mRNA from both 3° LETC-B6 cells and human HeLa cells, but not from untransfected *ldlC* or wild-type CHO cells. Thus, this mRNA was likely to be the transcription product of the human gene that corrected the ldlC defects. We therefore used the SacI-HincII fragment as a probe to isolate 16 overlapping human cDNA clones from two HeLa cell cDNA libraries (see Materials and Methods). The cloned DNA is designated *LDLC* cDNA. One of the clones, which comprises the entire predicted coding sequence (see below), was inserted into the vector pRc/CMV to generate the expression vector p*LDLC*-1.

Human LDLC cDNA Corrects the Abnormal Phenotypes of ldlC Cells

Three distinguishing characteristics of ldlC cells are (*a*) dramatically reduced LDL receptor activity, (*b*) abnormal posttranslational processing (glycosylation) of LDL receptors and their consequent instability, and (*c*) global defects in cell surface glycoconjugates (Kingsley et al., 1986*a*). To determine if p*LDLC*-1 could correct these mutant phenotypes, we isolated ldlC cells stably transfected with p*LDLC*-1. One transfectant, designated ldlC[*LDLC*], was used in the experiments described below; all results were confirmed using independently generated transfectants (not shown). Control transfectants, designated ldlC[*control*] cells, were generated by transfection with the vector pRc/CMV lacking the cDNA insert. Table I shows the LDL receptor activities, determined using an ^{125}I-LDL degradation assay, of wild-type CHO, ldlC, ldlC[*LDLC*], and ldlC[*control*] cells. In the experiment shown, transfection of ldlC cells with p*LDLC*-1, but not with the empty vector, restored LDL receptor activity to 61% of wild-type levels. Analysis of other independent transfectants showed that p*LDLC*-1 restored receptor activity to levels as high as 160% of wild type (not shown). Therefore, human *LDLC* cDNA restored normal LDL receptor activity to ldlC cells.

Fig. 1 shows the posttranslational processing of LDL receptors, using a pulse/chase immunoprecipitation assay (Kozarsky et al., 1986). In wild-type CHO cells the LDL receptor was synthesized as a ~125 kD precursor (*p*) which

Table I. LDL Receptor Activities and Lectin Sensitivities of ldlC Transfectants

Cells	LDL receptor activity*	Lectin sensitivities (LD_{10})‡				Phenotype
		WGA	ConA	PHA	Ricin	
	ng/5 hr/mg	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	
CHO	1770	3	20	50	50	WT
ldlC	183	30	5	>300	0.1	Mutant
ldlC[*LDLC*]	1083	5	20	50	5	WT
ldlC[*control*]	225	30	3	>300	0.05	Mutant

* LDL receptor activity determined using an ^{125}I-LDL degradation assay as described in Materials and Methods. Values represent ng ^{125}I-LDL protein degraded per mg cell protein in 5 h.

‡ Values represent LD_{10}s, or the lectin concentrations sufficient to reduce cell density to 10% of that of untreated cells. Lectin sensitivity phenotypes are classified as WT (characteristic of wild-type CHO cells) or as Mutant (characteristic of ldlC cells).

was rapidly converted to a ∼155 kD mature form (*m*) (Fig. 1, *top*). Previous experiments have established that the precursor is an endoglycosidase H sensitive ER protein, that is processed to an endoglycosidase H resistant, sialylated, mature protein during transport through the Golgi apparatus to the cell surface (Tolleshaug et al., 1982; Cummings et al., 1983; Kozarsky et al., 1986). The shift in electrophoretic mobility between the precursor and mature forms is due to maturation of the numerous O-linked and several N-linked oligosaccharides on the receptor. The mature form of the receptor is stable, with a half-life of ∼16-20 h. The band of lower apparent mass (*d*) represents a previously described degraded form of the receptor (Fig. 1, *top*, and see Lehrman et al., 1985; Kozarsky et al., 1986). In contrast, the LDL receptor in ldlC cells was converted from an apparently normal precursor to a heterogeneous mixture of abnormally glycosylated intermediates, with significantly lower stability than that of the mature receptor in wild-type cells. (Fig. 1, *middle*, and see Kingsley et al., 1986*a*). These abnormally glycosylated LDL receptors are transported to the cell surface, where they can bind LDL with normal affinity and mediate endocytosis; their dramatically reduced stability is the primary cause of the reduction in receptor activity in ldlC cells (Kingsley et al., 1986; Reddy and Krieger, 1989). In ldlC[*LDLC*] cells (Fig. 1, *bottom*), LDL receptor posttranslational processing and stability were restored to those seen in wild-type cells, while processing and stability in ldlC-[*control*] cells remained essentially identical to those in untransfected ldlC cells (not shown). Therefore, the human *LDLC* cDNA corrected the abnormal posttranslational glycosylation and instability of LDL receptors in ldlC cells.

To determine if p*LDLC*-1 corrected the global abnormalities in the synthesis of N-linked, O-linked, and lipid-linked oligosaccharides in ldlC cells, we measured the lectin sensitivities of these transfected and untransfected cells. Table I shows that, indeed, ldlC[*LDLC*] cells as well as wild-type CHO cells exhibited the wild-type (WT) pattern of lectin sensitivities, while ldlC and ldlC[*control*] cells expressed the mutant phenotype (hypersensitivity to ConA and ricin, resistance to WGA and PHA). Thus, all three major mutant phenotypes of ldlC cells were corrected by transfection with the *LDLC* cDNA.

Expression of LDLC in Wild-type, Mutant, and Transfected Cells

Plasmid p*LDLC*-1 could encode the human homologue of the defective gene in ldlC cells, or an extragenic suppressor of this gene (e.g., see Rine, 1991; Reddy and Krieger, 1989). To address this issue, we examined by Northern blot analysis the expression of the endogenous *LDLC* gene in ldlC cells (Fig. 2, *top*). The human *LDLC* probe recognized a single mRNA band of ∼3.4 kb in human HeLa cells, in a 3° LETC colony, and in wild-type CHO Cells. The somewhat reduced intensity of the band in CHO cells relative to HeLa and 3° LETC cells was presumably due to imperfect sequence complementarity between the human and hamster homologues. Strikingly, this hamster *LDLC* mRNA was essentially undetectable in ldlC cells, although a longer exposure revealed a very faint signal (not shown). Examination of the same filter with a control tubulin probe indicated that comparable levels of mRNA were loaded for each of the samples (Fig. 2, *bottom*). The dramatically reduced levels of *LDLC* mRNA in ldlC cells relative to wild-type CHO cells reflects either decreased synthesis or increased degradation of the *LDLC* mRNA. Therefore, a mutation in the *LDLC* gene itself, or, perhaps less likely, in a gene which regulates *LDLC* mRNA expression, is responsible for the mutant phenotypes of ldlC cells.

Human LDLC cDNA Encodes a Novel Cytosolic Protein

Sequence analysis of the human *LDLC* cDNA clones defined a contiguous 2904-base pair sequence, containing an open reading frame of 738 codons. Fig. 3 presents the *LDLC* nucleotide and predicted ldlCp protein sequences. The se-

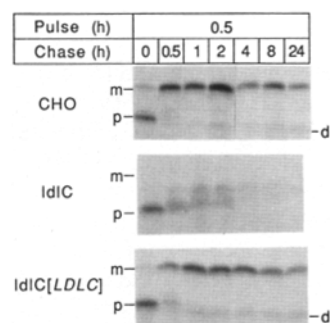

Figure 1. Synthesis and processing of LDL receptors in wild-type CHO cells, ldlC mutants and ldlC[*LDLC*] transfectants. On day 0, the indicated cells were plated in 6-well dishes (150,000 cells/well in medium E). On day 2, the cells were pulse-labeled with [^{35}S]methionine (180 μCi/ml) in methionine-free medium E for 30 min, washed once with Ham's F12 medium, and then chased for the indicated times in medium E supplemented with 1 mM unlabeled methionine. The cells were then lysed and the lysates subjected to immunoprecipitation with an anti-LDL receptor antibody as described in Materials and Methods. The immunoprecipitates were reduced with β-mercaptoethanol, and analyzed by 6% SDS-polyacrylamide gel electrophoresis and autoradiography as previously described (Kozarsky et al., 1986). The mobilities of the mature (*m*, 155 kD), precursor (*p*, 125 kD), and degraded (d, 118 kD) forms of the LDL receptors in wild-type CHO cells are indicated.

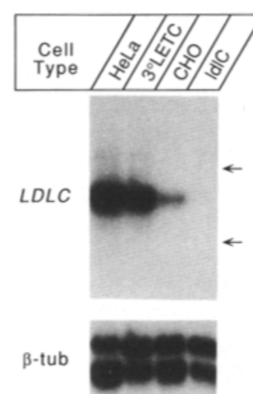

Figure 2. Northern blot analysis of *LDLC* mRNA. Poly(A)$^+$ RNAs from the indicated cells were prepared and subjected to Northern blot analysis as described in Materials and Methods. (*Top*) The filter was probed with a ^{32}P-labeled fragment of plasmid p*LDLC*-1 that contained the full open reading frame. The hybridization and washing conditions were chosen to permit hybridization of the human probe to hamster mRNA. Prehybridization and hybridization were carried out at 60° in 500 mM phosphate buffer (pH 7.0), 7% SDS, 1 mM EDTA, 10 mg/ml BSA, and 0.1 mg/ml sheared salmon sperm DNA. Washes were as follows: 2 × 15 min at room temperature in 300 mM phosphate buffer (pH 7.0); 2 × 15 min at 60° in 300 mM phosphate buffer, 5% SDS, 5 mg/ml BSA, 1 mM EDTA; and 2 × 15 min at 60° in 300 mM phosphate buffer, 1% SDS, and 1 mM EDTA. The arrows indicate the positions of the two major ribosomal RNA bands. (*Bottom*) The same filter was stripped and reanalyzed using a portion of β-tubulin cDNA as a probe.

```
-95        ggaaactggcggtggccgcggccgccgagtcggtctgcgcatcctcctgcgttttctcgcttggatcttggcactgagaggcggtggccggcggg   -1
   1 ATGGAGAAAAGTAGGATGAACCTGCCCAAGGGGCCGGACACGCTCTGCTTCGACAAGGACGAGTTCATGAAGGAAGATTTCGATGTCGATCATTTTGTGTCTGACTGTAGGAAGCGGGTC   120
   1 M E K S R M N L P K G P D T L C F D K D E F M K E D F D V D H F V S D C R K R V   40
 121 CAGCTGGAAGAACTGAGAGATGACCTGGAGCTCTACTATAAACTTCTTAAAACAGCCATGGTCGAACTCATCAACAAGGATTATGCAGATTTTGTCAATCTTTCAACAAACTTGGTTGGC   240
  41 Q L E E L R D D L E L Y Y K L L K T A M V E L I N K D Y A D F V N L S T N L V G   80
 241 ATGGACAAAGCCCTCAACCAGCTTTCTGTGCCTTTGGGACAATTACGAGAAGAGGTTCTGAGCCTTAGATCGTCTGTCAGTGAAGGAATTCGGGCAGTTGATGAACGAATGTCTAAACAA   360
  81 M D K A L N Q L S V P L G Q L R E E V L S L R S S V S E G I R A V D E R M S K Q   120
 361 GAGGACATTAGGAAAAAAAAGATGTGTGTATTGAGGCTTATACAAGTTATTCGGTCAGTTGAGAAAATTGAAAAAATCTTAAACTCTCAAAGTTCTAAAGAAACCTCTGCACTAGAAGCA   480
 121 E D I R K K K M C V L R L I Q V I R S V E K I E K I L N S Q S S K E T S A L E A   160
 481 AGCAGCCCCCTTTTGACTGGACAAATTTTGGAGAGAATTGCCACAGAATTTAATCAGTTACAGTTTCATGCTGTTCAAAGCAAAGGCATGCCTCTTTTGGACAAAGTAAGACCGCGTATA   600
 161 S S P L L T G Q I L E R I A T E F N Q L Q F H A V Q S K G M P L L D K V R P R I   200
 601 GCTGGCATTACAGCCATGTTACAGCAGTCACTGGAAGGTCTCCTATTAGAAGGCCTTCAGACGTCTGACGTCGATATAATACGGCACTGCTTGCGGACTTACGCCACGATTGACAAGACA   720
 201 A G I T A M L Q Q S L E G L L L E G L Q T S D V D I I R H C L R T Y A T I D K T   240
 721 CGGGACGCGGAGGCCTTAGTTGGCCAAGTACTAGTGAAACCATACATAGACGAGGTGATTATAGAGCAGTTTGTTGAATCTCATCCCAATGGCCTTCAGGTCATGTATAATAAACTCCTG   840
 241 R D A E A L V G Q V L V K P Y I D E V I I E Q F V E S H P N G L Q V M Y N K L L   280
 841 GAGTTTGTTCCTCACCATTGCCGCCTTCTTCGAGAAGTCACAGGAGGTGCCATCTCCAGTGAAAAAGGCAATACTGTTCCTGGATATGACTTTTTGGTGAATTCTGTTTGGCCACAAATA   960
 281 E F V P H H C R L L R E V T G G A I S S E K G N T V P G Y D F L V N S V W P Q I   320
 961 GTACAAGGATTAGAAGAAAAGTTACCCTCGCTTTTTAATCCTGGGAATCCCGATGCATTTCATGAGAAATATACCATAAGTATGGATTTTGTCAGAAGATTGGAACGGCAGTGTGGATCA   1080
 321 V Q G L E E K L P S L F N P G N P D A F H E K Y T I S M D F V R R L E R Q C G S   360
1081 CAGGCTAGTGTAAAGAGATTAAGAGCCCATCCTGCCTATCACAGCTTCAATAAGAAGTGGAACTTGCCTGTTTATTTCAAATAAGATTTAGAGAAATAGCGGGATCCTTAGAAGCAGCA   1200
 361 Q A S V K R L R A H P A Y H S F N K K W N L P V Y F Q I R F R E I A G S L E A A   400
                         ▼
1201 CTTACAGATGTCCTGGAAGATGCCCCAGCTGAAAGTCCGTATTGCCTTTTGGCTTCTCATAGAACTTGGAGCAGCCTTAGGAGGTGTTGGTCAGATGAGATGTTCTTGCCATTACTGGTG   1320
 401 L T D V L E D A P A E S P Y C L L A S H R T W S S L R R C W S D E M F L P L L V   440
                                                   ▼
1321 CATCGCCTGTGGAGACTCACTCTGCAGATTTTGGCACGATACTCTGTGTTTGTCAATGAGCTTTCACTCAGGCCCATTTCTAATGAAAGTCCCAAGGAGATCAAGAAACCTTTGGTAACT   1440
 441 H R L W R L T L Q I L A R Y S V F V N E L S L R P I S N E S P K E I K K P L V T   480
1441 GGTAGCAAAGAACCTTCCATCACCCAAGGAAACACTGAAGACCAAGGAAGTGGTCCTTCGGAAACAAAGCCTGTGGTTTCCATTTCCCGCACTCAGCTCGTGTATGTGGTTGCAGACCTG   1560
 481 G S K E P S I T Q G N T E D Q G S G P S E T K P V V S I S R T Q L V Y V V A D L   520
1561 GACAAGCTTCAGGAGCAGCTTCCAGAACTCTTGGAAATAATCAAGCCAAAACTTGAAATGATTGGCTTTAAGAATTTTTCTTCTATCTCAGCAGCCCTGGAGGACTCCCAGAGCTCTTTT   1680
 521 D K L Q E Q L P E L L E I I K P K L E M I G F K N F S S I S A A L E D S Q S S F   560
1681 TCAGCCTGTGTGCCCTCCTTGAGTAGCAAGATCATCCAGGATTTAAGTGACTCTTGCTTCGGTTTCCTAAAAAGCGCCCTGGAGGTTCCCAGGCTTTACCGAAGAACCAATAAGGAGGTC   1800
 561 S A C V P S L S S K I I Q D L S D S C F G F L K S A L E V P R L Y R R T N K E V   600
1801 CCAACCACAGCTTCCTCCTATGTGGACAGTGCTCTGAAGCCCTTATTCCAGCTTCAGAGCGGACACAAGGATAAGCTCAAACAAGCAATAATTCAGCAGTGGCTAGAAGGCACTCTCAGT   1920
 601 P T T A S S Y V D S A L K P L F Q L Q S G H K D K L K Q A I I Q Q W L E G T L S   640
1921 GAAAGCACTCATAAGTACTATGAAACCGTGTCAGATGTATTAAACTCTGTGAAGAAGATGGAAGAGAGCCTGAAAAGGCTGAAACAAGCCAGAAAAACCACTCCCGCCAACCCCGTCGGT   2040
 641 E S T H K Y Y E T V S D V L N S V K K M E E S L K R L K Q A R K T T P A N P V G   680
2041 CCCAGTGGTGGCATGAGCGACGACGACAAAATCAGGCTGCAGTTGGCCCTAGATGTTGAGTACTTGGGAGAGCAGATACAAAAGTTGGGACTACAAGCAAGTGACATAAAAAGCTTCTCA   2160
 681 P S G G M S D D D K I R L Q L A L D V E Y L G E Q I Q K L G L Q A S D I K S F S   720
2161 GCTCTCGCAGAGCTTGTTGCTGCTGCCAAGGACCAGGCAACAGCAGAGCAGCCTtaagcatcttggaagatcccgaggttagattcttaagcaagagaagagttggacttccaggctgaa   2280
 721 A L A E L V A A A K D Q A T A E Q P *   738
2281 ggggagaaagtgactctgttctcttagcaaccgtctgtagcaaagaagtgcttccagcatcactccagcaacacgcccatgcgtcttctctcagcgtatttgggtcttctttgcccaaaa   2400
2401 gaacacaaaagccttttccattgtatggaagatagttttaagacatttgaaactttctactatagtttacagaacaaattattttattttattgtaaatcttagtgtggaagagctg   2520
2521 atttctaaaatatgattaaagtaaatatatacctatgaatatcaagagtcgtctccctgagcctgtagttggaagtgacgactgtaatggaatgatgtcttgtatagaaatgcccttctc   2640
2641 tgaaataaagagaactcctgggctttctaaagaggctgcgggaagccatcctccactcccactgtgtgtgagagcagtgcttctgatcctgctgtcaccccgacctctggcaggagccgg   2760
2761 cgccagtaggaaagacctccttcctaaataaaagaagtgtctcccaaaa   2809
```

Figure 3. Nucleotide (*upper line*) and predicted amino acid (*lower line*) sequences of human *LDLC* cDNA. The human *LDLC* cDNA was cloned and sequenced and the sequence was analyzed as described in Materials and Methods. The nucleotide sequence is numbered so that the presumptive initiator codon starts at base 1. The arrowheads designate the positions of two introns. These were identified by sequencing a portion of the genomic probe which was used to clone the *LDLC* cDNA. The terminal four adenosines of the *LDLC* cDNA are likely to represent the start of a poly(A) tail, as they follow a candidate polyadenylation signal (AATAAA) by 13 bases.

quence surrounding the putative initiator methionine (amino acid no. 1) is consistent with the consensus sequence described by Kozak (1989). This ATG is preceded by a 95-bp 5′ untranslated region, which includes an in-frame stop codon seven triplets upstream of the start methionine. The 2214-bp open reading frame is followed by a 595-bp 3′ untranslated region. A 154-bp sequence within the SacI-HincII genomic fragment used to clone the cDNA was identical to the corresponding sequence in the cDNA (bases 1227–1380). This region of the genomic DNA was flanked at both ends by unrelated sequence, suggesting that this overlap defines a single exon within the *LDLC* gene (see arrowheads in Fig. 3).

The predicted protein product (ldlCp) of the human *LDLC* gene has a calculated mass of 83,207 D. Surveys of various DNA and protein sequence databases have revealed no similarities to any known genes or proteins. Furthermore, we have detected no signal sequences for translocation into the ER, and no candidate transmembrane domains. This suggests that the ldlCp is a novel, soluble protein which does not enter the secretory pathway and is probably a cytoplasmic protein. Thus, it appears that *LDLC* encodes a protein that influences lumenal Golgi reactions from the cytoplasm. In addition, we have not detected any other common sequence motifs or predicted secondary or tertiary structural elements, such as isoprenylation sequences, amino terminal *N*-myristylation sites, nucleotide binding sites, heptad repeats, etc.

We have found no notable sequence similarities between *LDLC* and known genes reported in databases such as GenBank and EMBL. However, the *LDLC* cDNA sequence was significantly similar to three expressed sequence tags (ESTs), cDNA fragments which were cloned and sequenced at random. Two of the ESTs were derived from human cDNA libraries (EST01264 from hippocampus, GenBank no. M79116, Adams et al., 1992; and EST clone HEB069 from heart atrium, GenBank no. Z25929, Genexpress, unpublished observations). These two ESTs are nearly identical to

the *LDLC* cDNA from bases 1805 through 2072 (99% identity) and from 1674 through 1875 (96% identity), respectively. The few mismatches are probably due either to polymorphisms or to sequence errors arising from the preliminary nature of EST sequences (Adams et al., 1991). The third EST (CEESW90; GenBank no. T01892, McCombie, W. R., J. M. Kelley, L. Aubin, M. Goscoechea, M. G. Fitzgerald, A. Wu, M. D. Adams, M. Dubnick, A. R. Kerlavage, J. C. Venter, and C. A. Fields, unpublished information) was obtained from the nematode *Caenorhabditis elegans*.

Cloning of an LDLC homologue from C. elegans

The *C. elegans* EST clone is 382 bases long, and includes a 203-bp region which is 60% identical to bases 40–242 of the human *LDLC* cDNA. Furthermore, the predicted amino acid sequence within this region is 49% identical and 70% similar to the human ldlCp sequence. Therefore, the gene represented by this EST was a good candidate for an invertebrate homologue of the *LDLC* gene. To characterize the putative homologue, we used this EST to isolate six *C. elegans* cDNA clones. Each was ~2.0 kbp long, and they all had similar restriction maps. One clone was sequenced fully on both strands (see Fig. 4 *A*). Its 2222 base sequence includes an open reading frame of 681 codons from the first methionine (Fig. 4 *A*). The sequence surrounding the putative initiator codon is consistent with the consensus sequence described by Kozak (1989). The reading frame is preceded by a putative 31-bp 5′ untranslated region which lacks in-frame stop codons; this 5′ untranslated region includes a T_{15} which may be an artifact of cDNA synthesis. The open reading frame is followed by a 148-bp 3′ untranslated region which includes a 20-bp polyadenylate tail. Throughout their lengths, the predicted protein sequences of the *C. elegans* (calculated mass of 78,565 D) and human ldlCp homologues are 26% identical and 53% similar when aligned as in Fig. 4 *B*. The first methionine in the human sequence best corresponds to the methionine at position 10 of the *C. elegans* sequence, raising the possibility that the first nine amino acids of the *C. elegans* sequence in Fig. 4 *A* may not be translated. These nine residues include a potential myristylation site. As with its human counterpart, the nematode ldlCp sequence lacks other notable structural features such as transmembrane domains or signal sequences. Overall, the conservation in the human and nematode ldlCp sequences suggests that the *LDLC* genes encode proteins which mediate important, highly conserved functions.

Preparation and Characterization of Anti-ldlCp Antibodies

Based on the abnormalities in *medial* and *trans* Golgi-associated glycoconjugate synthesis in ldlC cells, we inferred that cytosolic ldlCp might physically associate with the Golgi apparatus. To determine the subcellular distribution of ldlCp by immunofluorescence microscopy, rabbit polyclonal antibodies were prepared using synthetic peptides which represent the amino (Npep)- and carboxy (Cpep)-termini of human ldlCp, and are designated anti-Npep and anti-Cpep, respectively. Both immunoprecipitation and immunoblot analyses (not shown) established that anti-Npep and anti-Cpep antibodies bound to an ~76-kD protein which was present in HeLa cells (not shown). This binding was specifically blocked by an excess of soluble peptide, and this 76-kD protein, whose apparent mass is similar to the 83-kD predicted from the *LDLC* sequence, was not detected when either preimmune serum was used.

Anti-Cpep was affinity purified on a Cpep-agarose column, and its specificity was assessed by immunoblot analysis. Fig. 5 compares the immunoblotting patterns of preimmune IgG (*p*) and anti-Cpep (*C*), measured in the absence (−) or presence (+) of an excess of the Cpep peptide. Purified anti-Cpep, but not preimmune IgG, bound to an ~76-kD protein in both human HeLa cell and murine 3T3 cell lysates (anti-Cpep, Fig. 5, lanes *2* and *5*; preimmune IgG, lanes *1* and *4*). This binding was competed by excess Cpep, suggesting that it may correspond to ldlCp (Fig. 5, lanes *3* and *6*). Anti-Cpep, but not preimmune IgG, also recognized two smaller species in the HeLa cell lysates (Fig. 5, lanes *1* and *2*); however, this binding was not inhibited by excess Cpep (lane *3*). The identities of these smaller molecules and the significance of their recognition here are unknown. Anti-Cpep also specifically recognized the ~76-kD endogenous hamster ldlCp in CHO cell lysates (Fig. 5, lanes 7–9). The ~76-kD protein was not detected in lysates from ldlC cells (Fig. 5, lanes *10* and *11*), but was seen in ldlC[*LDLC*] lysates (lanes *12* and *13*). (Replicate lanes of CHO, ldlC, and ldlC[*LDLC*] lysates, stained with anti-tubulin antiserum, showed that these samples contained equivalent amounts of protein [not shown]). These results are consistent with the dramatically reduced levels of *LDLC* mRNA observed in ldlC cells (Fig. 2). As was the case for HeLa cell lysates, anti-Cpep bound to smaller, unidentified species from CHO and ldlC cells. Taken together, these data establish that the ~76-kD protein, which is the major specific antigen of anti-Cpep, is ldlCp and they suggest that at least a portion of the COOH terminus of ldlCp is conserved among several mammalian species.

Immunolocalization of ldlC Protein

Immunofluorescence microscopy with affinity purified anti-Cpep was used to determine the distribution of ldlCp within wild-type CHO cells. Fig. 6 *a* (*top left*) shows that the major anti-Cpep signal in CHO cells emanated from clearly defined, punctate, and sometimes annular, structures surrounding the nucleus. This perinuclear staining was absent from ldlC cells but present in transfected ldlC[*LDLC*] cells (see below), and was largely competed by a 10-fold molar excess of soluble Cpep (not shown). Thus, the perinuclear staining represents the localization of ldlCp. A fine, granular, yet otherwise uniform, background was often present. This background was resistant to Cpep competition, and was indistinguishable from the staining pattern observed with preimmune IgG or in controls in which the primary antibody was omitted (not shown).

The perinuclear distribution of ldlCp was characteristic of the distribution of the Golgi apparatus in CHO cells (Kao and Draper, 1992; Guo et al., 1994). For example, Fig. 6 (*top row*) also shows the staining of CHO cells with antibodies against two Golgi-associated proteins: β-COP (*b*) and mannosidase II (*c*). β-COP is a subunit of the Golgi coatomer complex, which associates reversibly with Golgi membranes and which is a major component of the protein coat on Golgi-derived transport vesicles (Duden et al., 1991; Serafini et

```
A

  -31                                                                                          gtttttttttttttttgaaaatatttttaaa    -1
    1  ATGGGTACACTTCATGGCGAGAAAACTATGTTCGCTTCGCCAAACACCTTCTATATCGATGAATCGAAGTTATGCTTTAATAAGACCCATTTCAATCGAGAAGATTTTAATGTGGAACGA   120
    1  M  G  T  L  H  G  E  K  T  M  F  A  S  P  N  T  F  Y  I  D  E  S  K  L  C  F  N  K  T  H  F  N  R  E  D  F  N  V  E  R    40
  121  TTTATGAACTTGGCAAGGCAAAAATCTGATCTCAAAACAATTCAGCAGGATCTACGCCTCTACTTGAAAAGTGTTCAAAATTCAATGATCGAGCTTATCAACGATGATTATGCGGATTTT   240
   41  F  M  N  L  A  R  Q  K  S  D  L  K  T  I  Q  Q  D  L  R  L  Y  L  K  S  V  Q  N  S  M  I  E  L  I  N  D  D  Y  A  D  F    80
  241  GTCCATTTATCGTCAAACTTGGTCAGCCTCCAAGACTCACTAAATAAAATCGAACAAGATATCAATAGAATATGGGATGAATTCAAGGAGTCGACAAGAGAATCTGTTGGTATGGCTGAA   360
   81  V  H  L  S  S  N  L  V  S  L  Q  D  S  L  N  K  I  E  Q  D  I  N  R  I  W  D  E  F  K  E  S  T  R  E  S  V  G  M  A  E   120
  361  AGAATCGAGCAAAAATGTGATGAGCTTTGCTCAAATCGAGAAAAACAAATTGAAATTCGGGATCGCATTTATTTTCTAGTTGCCATAGAAAAGCTATCAGAAATGTTATTGCATCCTCCT   480
  121  R  I  E  Q  K  C  D  E  L  C  S  N  R  E  K  Q  I  E  I  R  D  R  I  Y  F  L  V  A  I  E  K  L  S  E  M  L  L  H  P  P   160
  481  CGAAAATGCTCTGCTTTGTGGTTACAAAAGGCAGCGTCCTTCGCATCAGAGCTGAAGGGTTCCACGTTTCCCCATTCGGAAGAGGAAAATGCCGCTGAAAAGATAATTCTATCTCAGCTG   600
  161  R  K  C  S  A  L  W  L  Q  K  A  A  S  F  A  S  E  L  K  G  S  T  F  P  H  S  E  E  E  N  A  A  E  K  I  I  L  S  Q  L   200
  601  GAAGCGGTTCTCTGTGCAGAAGGAGTTCGAAGTGCCGCCGGTGATTGTCAAAATCTTCCTCTCATTTATTCAATTTTATCGTTAACGGAGAGTACGCATTCACTAACTGCCCTCTTGGTT   720
  201  E  A  V  L  C  A  E  G  V  R  S  A  A  G  D  C  Q  N  L  P  L  I  Y  S  I  L  S  L  T  E  S  T  H  S  L  T  A  L  L  V   240
  721  TCTGATCTTTTGTATGCCGAATTTGTAAATGAGAAACACGACGAAAGTAATCAGTTGAAACTCTTGAAACAAGTCTTCGAAAGCGTCAAAAAAATGCGTGAAACTTGGGCTGAAAAAATG   840
  241  S  D  L  L  Y  A  E  F  V  N  E  K  H  D  E  S  N  Q  L  K  L  L  K  Q  V  F  E  S  V  K  K  M  R  E  T  W  A  E  K  M   280
  841  GGGACTGAACATTTTCGTGGAAACATCCGCCGTTTTCTGGACGAAACCCTTCTCACGTTCATTCTGACATTTATTGATAAGTGTATGGGAGCCGTCGCAGTCCCAAGTGACACACGACTT   960
  281  G  T  E  H  F  R  G  N  I  R  R  F  L  D  E  T  L  L  T  F  I  L  T  F  I  D  K  C  M  G  A  V  A  V  P  S  D  T  R  L   320
  961  TTCCATGAATGTTTCTTGCTCACTCAAGAATTTATCGATAATTGGCCATCTTCTCATACCTGCCGTGCAATGCTTAAGTCGATCAGGGATAAATTCAATTTATTAGTTTATTTAAATTA  1080
  321  F  H  E  C  F  L  L  T  Q  E  F  I  D  N  W  P  S  S  H  T  C  R  A  M  L  K  S  I  R  D  K  F  N  L  L  V  Y  F  K  L   360
 1081  GAAACACATCGATTCGGAAAACAGTGCGATCAGCTGATGTCTCCCGAAATGTTTGCCGAGCCAGAAACTTCTGAAAATCGAGAAAACACTCCTCAGCTGCACTGCGGAGTTTCAAGAGCA  1200
  361  E  T  H  R  F  G  K  Q  C  D  Q  L  M  S  P  E  M  F  A  E  P  E  T  S  E  N  R  E  N  T  P  Q  L  H  C  G  V  S  R  A   400
 1201  ATTATAACAGCAATTGAGCATGTGTGGAGTGATGATGTGTATCTTCCTCCTATTGTTGATAAATTATGGGATTTCACATTGAAGCTTTTGCTAAAGCATTTCTCGTGGAGTCAAACTATG  1320
  401  I  I  T  A  I  E  H  V  W  S  D  D  V  Y  L  P  P  I  V  D  K  L  W  D  F  T  L  K  L  L  L  K  H  F  S  W  S  Q  T  M   440
 1321  AAAAATTATTTCATGGAAGAAAAAAGAGATTGGACTTCAATGGTTACTCTCCGCTCGGATACTGGAAATCTTCATCAATTGGTTTTCGATTTCGCGTTGGAGTCAATTTGGGGAAAATTT  1440
  441  K  N  Y  F  M  E  E  K  R  D  W  T  S  M  V  T  L  R  S  D  T  G  N  L  H  Q  L  V  F  D  F  A  L  E  S  I  W  G  K  F   480
 1441  CATGATATCACAGTGGATACCGCTCCATTCGGTCAATGCCTAACAAAGCATGGTCGATCAATTGATAGTCTTTGTGTTCAAATTGATGATAGTATTATTGAAATGTTCTCCGAAGTTCTT  1560
  481  H  D  I  T  V  D  T  A  P  F  G  Q  C  L  T  K  H  G  R  S  I  D  S  L  C  V  Q  I  D  D  S  I  I  E  M  F  S  E  V  L   520
 1561  CATCAAGAAATTGCTCAAGTTTCCGATGTTCCTAAACAATACAGATGGACGAAGAAATCACCACCGACAACGCATTCCAAATATGTTGTTACAGCTATTGAAATGGTTGAGAATTTGAAA  1680
  521  H  Q  E  I  A  Q  V  S  D  V  P  K  Q  Y  R  W  T  K  K  S  P  P  T  T  H  S  K  Y  V  V  T  A  I  E  M  V  E  N  L  K   560
 1681  GAAAAACTATGCTGCGAAGAACATCCTCACACCGATGAAATTGTTCGAAAAGTTAATCTTTCAGCATTCAATTATTTCGTGGGGAAAGGGAACGAGGTGCTGGATAGTGTCGAAGCTACA  1800
  561  E  K  L  C  C  E  E  H  P  H  T  D  E  I  V  R  K  V  N  L  S  A  F  N  Y  F  V  G  K  G  N  E  V  L  D  S  V  E  A  T   600
 1801  GGATCTTCTTTATCCCGATTCAAACGGAAAACCACAACTGATTCTGGCTCCACGGTTACTGATGACGATAAAATCAAACAACAAATCTATCATGATGCTAAATATTTCCTGAGCTATGCG  1920
  601  G  S  S  L  S  R  F  K  R  K  T  T  T  D  S  G  S  T  V  T  D  D  D  K  I  K  Q  Q  I  Y  H  D  A  K  Y  F  L  S  Y  A   640
 1921  GAAAATCTTGTATTCTCACAAGCTGATCTCACCGGATTACAAGAAGTCGTCAATCGATTTGACAAAGATGCACGTTCGGCGATTGTACAAGAGAAAAATCAGAACGAAGAAGCGGGGAAT  2040
  641  E  N  L  V  F  S  Q  A  D  L  T  G  L  Q  E  V  V  N  R  F  D  K  D  A  R  S  A  I  V  Q  E  K  N  Q  N  E  E  A  G  N   680
 2041  GCTtgaattaattttatgtgattatttatttacatgtacattttattttaacaaatgggttgaagtcttttaaaatcgtctattgccttaatatgggttcatggtattggaataaaaagtg  2160
  681  A  *                                                                                                                        681
 2161  tatagcatcgtaaaaaaaaaaaaaaaaaaaa                                                                                             2191
```

```
B

Human        1   MEKSRMNLPKGPDTLCFDKDEFMKEDFDVDHFVSDCRKRVQLEELRDDLELYYKLLKTAMVELINKDYADFVNLSTNLVGMDKALNQLSVPLGQLREEV. 99
C.elegans   10   MFASPNTFYIDESKLCFNKTHFNREDFNVERFMNLARQKSDLKTIQQDLRLYLKSVQNSMIELINDDYADFVHLSSNLVSLQDSLNKIEQDINRIWDEFK 109

Human      100   LSLRSSVSEGIRAVDERMSKQEDIRKKKMCVLRLIQVIRSVEKIEKILNSQSSKETSALEASSPLLTGQILERIATEFNQLQFHAVQSKGMPLLDKVRPR 199
C.elegans  110   ESTRESVGMAER.IEQKCDELCSNREKQIEIRDRIYFLVAIEKLSEMLLHPPRK.......CSALWL.QKAASFASELKGSTFPHSEEEN.......... 190

Human      200   IAGITAMLQQSLEGLLLEGLQTSDVDIIRHCLRTYATIDKTRDAEALVGQVLVKPYIDEVIIEQFVESHPNGLQVMYNKLLEFVPHHCRLLREVTGGAIS 299
C.elegans  191   .AAEKIILSQLEAVLCAEGVRSAAGDCQNLPL.IYSILSLTESTHSLTALLVSDLLYAEFVNEKHDESNQ.......LKLLKQVFESVKKMRETWAEKMG 281

Human      300   SE..KGNTVPGYDFLVNSVWPQIVQGLEEKLPSLFNPGNPDAFHEKYTISMDFVRRLERQCGSQASVKRLRAHPAYHSFNKKWNLPVYFQIRFREIAGSL 397
C.elegans  282   TEHFRGNIRR...FLDETLLTFILTFIDKCMGAVAVPSDTRLFHECFLLTQEFIDNWPSSHTCRAMLKSIR.........DKFNLLVYFKLETHRFGKQC 369

Human      398   EAALTDVLEDAPAES.......PYCLLASHRTWSSLRRCWSDEMFLPLLVHRLWRLTLQILARYSVFVNELSLRPISNESPKEIKKPLVTGSKEPSITQG 490
C.elegans  370   DQLMSPEMFAEPETSENRENTPQLHCGVSRAIITAIEHVWSDDVYLPPIVDKLWDFTLKLLLKHFSWSQTM.............KNYFMEEKRD...... 450

Human      491   NTEDQGSGPSETKPVVSISRTQLVYVVADLDKLQEQLPEL.LEIIKPKLEMIGFKNFSSISAALEDSQSSFSACVPSLSSKIIQDLSDSCFGFLKSAL.. 587
C.elegans  451   ..................WTSMVTLRSDTGNLHQLVFDFALESIWGKFHDITVD....TAPFGQCLTKHGRSIDSLCVQIDDSIIEMFSEVLHQEIAQ 526

Human      588   ..EVPRLYRRTNKEVPTTASSYVDSALKPLFQLQ...SGHKDKLKQAIIQQWLEGTLSESTHKYY.ETVSDVLNSVKKMEESLKRLKQARKTTPANPVGP 681
C.elegans  527   VSDVPKQYRWTKKSPPTTHSKYVVTAIEMVENLKEKLCCEEHPHTDEIVRK.....VNLSAFNYFVGKGNEVLDSVEATGSSLSRFK..RKTTT....DS 615

Human      682   SGGMSDDDKIRLQLALDVEYLGEQIQKLGLQASDIKSF.........SALAELVAAAKDQATAEQP 738
C.elegans  616   GSTVTDDDKIKQQIYHDAKYFLSYAENLVFSQADLTGLQEVVNRFDKDARSAIVQEKNQNEEAGNA 681
```

Figure 4. Nucleotide and predicted amino acid sequences of the *Caenorhabditis elegans LDLC* cDNA (*A*), and alignment of the protein sequences of the human and *C. elegans* homologs (*B*). (*A*) The *C. elegans LDLC* cDNA was cloned and sequenced as described in Materials and Methods. The presumptive initiator codon starts at base 1 (however, see text for further discussion). The 3′-terminal 20 adenosines are likely to represent the start of a poly(A) tail, as they follow a candidate polyadenylation signal (AATAAA) by 16 bases. (*B*) Alignment of the human and nematode ldlCp amino acid sequences. Vertical bars indicate identities, double and single dots indicate strong and weak similarities.

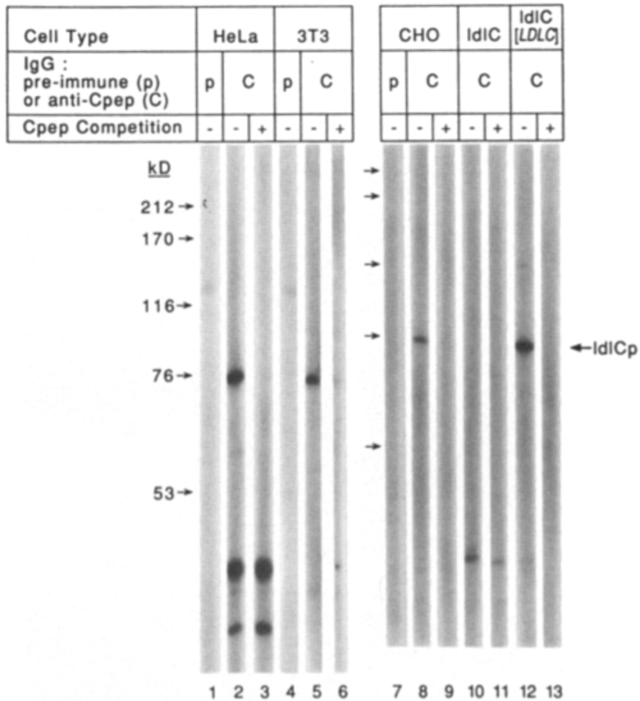

Figure 5. Immunoblot Analysis of ldlCp. The indicated cells were grown to confluence and lysed, and the lysates subjected to immunoblot analysis using either preimmune IgG (10 μg/ml, *p*) or anti-Cpep (10 μg/ml, *C*), the latter in the presence (+) or absence (−) of a 10-fold molar excess (2 μg/ml) of the Cpep peptide. Bound antibody was detected autoradiographically using ^{125}I-Protein A. The "ldlCp" (*large arrow*) indicates the position of the various mammalian ldlCp's, as described in Results.

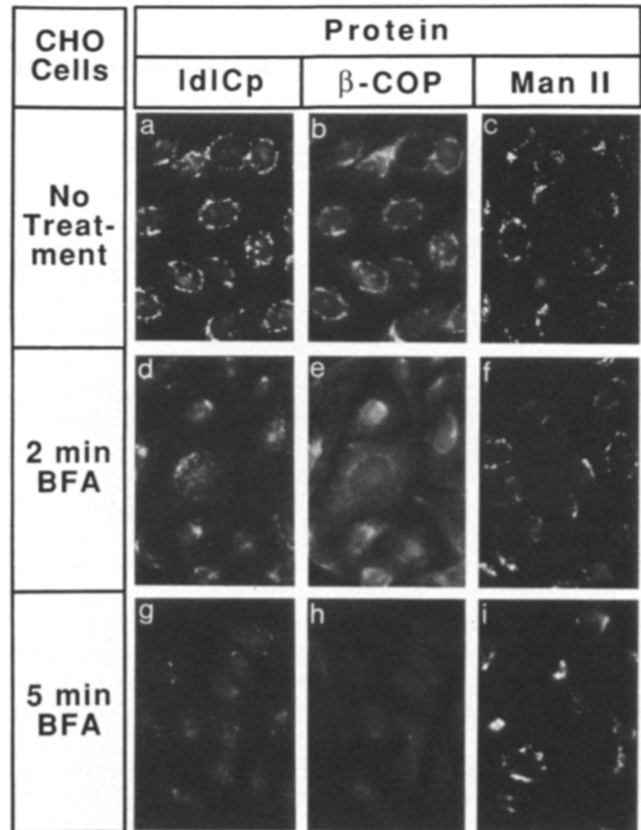

Figure 6. Immunofluorescence localization of ldlCp, β-COP and mannosidase II in CHO cells: effects of BFA. CHO cells were grown on glass cover slips as described in Materials and Methods. Before fixation and immunostaining, the cells were treated as follows: no additions (*a–c*) or incubation with 5 μg/ml BFA for two (*d–f*) or five (*g–i*) min. Cells were immunostained with peptide affinity-purified anti-Cpep (*a*, *d*, and *g*), anti-β-COP monoclonal antibody M3A5 (*b*, *e*, and *h*), and anti-mannosidase II (*c*, *f*, and *i*) as described in Materials and Methods. Specimens were simultaneously double stained with the anti-Cpep and anti-β-COP antibodies, and the corresponding identical fields are shown.

al., 1991; Waters et al., 1991; Ostermann et al., 1993). Mannosidase II is an integral membrane protein required for normal processing of N-linked oligosaccharide chains in the lumen of the Golgi apparatus (Moremen and Touster, 1985). The perinuclear immunofluorescence of ldlCp and β-COP colocalized (Fig. 6, *a* and *b* show essentially the same field from a doubly-stained sample), and their distributions clearly resembled that of mannosidase II. Thus, ldlCp appears to be a Golgi-associated protein in wild-type CHO cells. Similar results were obtained using 3T3 cells (not shown).

Effects of Brefeldin A on the Localization of ldlCp

Because the sequence of ldlCp suggested that it is a cytosolic protein, it appeared likely that ldlCp would associate peripherally, rather than integrally, with Golgi membranes. We therefore compared the behavior of ldlCp with those of the peripheral Golgi protein β-COP and the integral membrane protein mannosidase II, when the structure of the Golgi apparatus was disrupted with the drug brefeldin A (BFA) (Takatsuki and Tamura, 1985; Fujiwara et al., 1988; Donaldson et al., 1990; Lippincott-Schwartz et al., 1989, 1990; Orci et al., 1991). BFA interferes with the assembly of the coatomer complexes onto Golgi membranes resulting in the division of Golgi-associated proteins into at least two kinetically and morphologically distinguishable groups. β-COP and other peripherally associated coat proteins rapidly redistribute from the Golgi surface into the cytoplasm (Donaldson et al., 1990). Subsequently, the Golgi membranes and their integrally associated proteins, such as mannosidase II, more slowly fragment into tubules and vesicles, which then mix with the endoplasmic reticulum. The effects of BFA on the distributions of β-COP and mannosidase II are reversed after the drug is removed from the cells (Donaldson et al., 1990).

Fig. 6 shows ldlCp's redistribution following BFA treatment (*left column*), compared with those of β-COP (*center column*) and mannosidase II (*right column*). After 2 min of BFA treatment (Fig. 6, *second row*), perinuclear ldlCp was reduced but still evident, and the cytoplasmic staining increased (*d*). After 5 min (*third row*), only small remnants of perinuclear staining were observed (*g*). In this regard, the effects of BFA on the distribution of ldlCp resembled those on β-COP, which was reduced in intensity after 2 min and dispersed after 5 min (*e* and *h*). In contrast, mannosidase II staining was largely unchanged after 2 min (*f*). After 5 min it had transformed into a more contiguous pattern which included some fiber-like projections (*i*), as previously de-

scribed (Lippincott-Schwartz et al., 1990). Thus, after 5 min of BFA treatment, the staining of ldlCp and of β-COP were distinct from that of mannosidase II. The staining with all three antibodies was almost fully dispersed after 20 min of BFA treatment, and was restored to an essentially normal distribution after the BFA was removed and the cells were permitted to recover for 30 min (not shown). Taken together with the predicted sequence of ldlCp, these data strongly suggest that ldlCp is peripherally associated with the Golgi apparatus and its association appears similar to that of β-COP.

To determine if ldlCp was required to maintain the normal structure of the Golgi apparatus, we compared the distributions of β-COP and mannosidase II in CHO, ldlC, and ldlC[*LDLC*] cells. Fig. 7 shows that the distributions of β-COP (*center column*) and mannosidase II (*right column*) were essentially identical in all three types of cells, regardless of the presence or absence of ldlCp (*left column*). Thus, expression of ldlCp was not required for the formation of the Golgi. It should be noted that the intensities of the perinuclear staining of the Golgi markers varied among these cell types. In general, there was a tendency for somewhat reduced perinuclear β-COP and mannosidase II staining intensity in ldlC cells. Expression of the transfected human ldlCp in ldlC[*LDLC*] cells elevated the intensity of these two markers to wild-type, and often even greater than wild-type levels. The significance of these differences in staining intensities remains unclear, but may reflect a subtle role of ldlCp in regulating the structure or quantity of Golgi membranes.

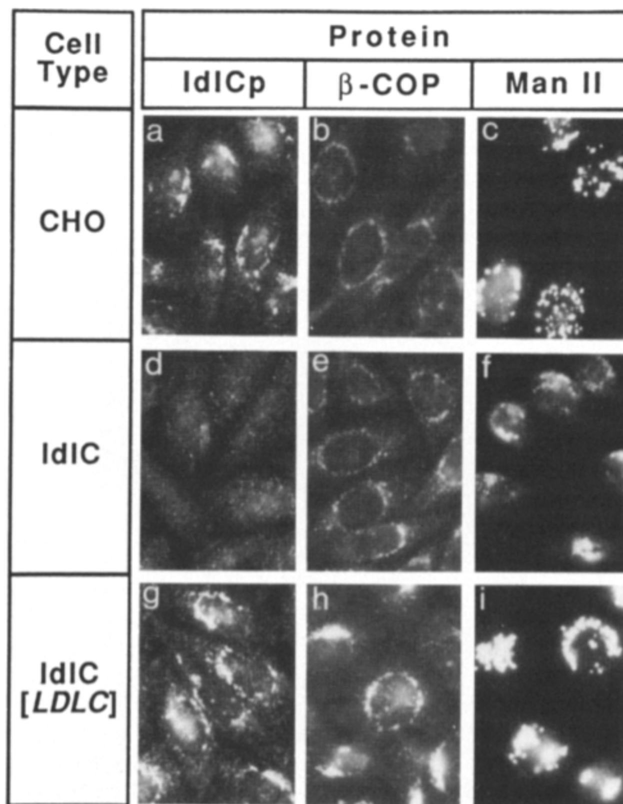

Figure 7. Immunofluroescence localization of ldlCp, β-COP and mannosidase II in CHO, ldlC and ldlC[*LDLC*] cells. The indicated cells were grown on coverslips and immunostained with peptide affinity-purified anti-Cpep (*a*, *d*, and *g*), anti-β-COP monoclonal antibody M3A5 (*b*, *e*, and *h*), and anti-mannosidase II (*c*, *f*, and *i*) as described in Materials and Methods.

Aberrant Distribution of ldlCp in ldlB Cells Indicates Golgi Localization Is Required for ldlCp Function

The BFA-dependent reversible localization of ldlCp to the Golgi suggested that, as with β-COP, Golgi localization may be required for the effects of ldlCp on Golgi function. This suggestion was supported by studies of ldlCp's distribution in another class of CHO cell mutant, ldlB. ldlC and ldlB cells are genetically distinct; they define discrete recessive complementation groups (Kingsley and Krieger, 1984), and transfection of the cloned *LDLC* cDNA into ldlB cells did not correct the pleiotropic defects of ldlB cells (not shown). Nevertheless, the mutant phenotypes of ldlB and ldlC cells are virtually indistinguishable: reduced LDL receptor activity, abnormal posttranslational processing and stability of LDL receptors, and global defects in cell surface glycoconjugates (Kingsley et al., 1986*a*). This raised the possibility that the *LDLB* gene could exert its effects on Golgi function by regulating the expression or function of the *LDLC* gene or of ldlCp. We therefore examined the expression of the endogenous *LDLC* gene and the localization of ldlCp in a clone of ldlB cells, designated ldlB-11, and in a secondary human genomic DNA transfectant of ldlB-11 cells, designated 2° LETB-144, in which the mutant phenotypes had reverted to wild type (Kingsley et al., 1986*b*).

Northern blot analysis (not shown) and immunoblot analysis (Fig. 8 *A*) established that there were essentially wild-type levels of both *LDLC* mRNA and ldlCp, in both ldlB-11 and 2° LETB-144 cells. Thus, *LDLB* gene function was not required for the synthesis or maintenance of normal steady-state levels of ldlCp. Fig. 8 *B* shows the immunofluorescence localization of ldlCp (*left column*), β-COP (*middle column*) and mannosidase II (*right column*) in wild-type CHO (*first row*), ldlB-11 (*second row*), and 2° LETB-144 cells (*third row*). In contrast to its typical Golgi localization in wild-type CHO cells (*a*), ldlCp apparently did not localize to the Golgi apparatus in ldlB-11 cells (*d*). Instead, a uniform punctate background in ldlCp staining was seen, suggesting that ldlCp was distributed throughout the cytoplasm of ldlB-11 cells. These results were confirmed by examining an independently derived clone of ldlB cells (WGA^r-2, Kingsley et al., 1986*a*) (not shown). In addition, the normal Golgi distribution of ldlCp was restored in 2° LETB-144 cells (*g*). In both ldlB-11 and 2° LETB-144 cells, there were essentially wild-type distributions of β-COP (*center column*, *b*, *e*, and *h*) and mannosidase II (*right column*, *c*, *f*, and *i*), indicating that the Golgi in these cells was essentially normal. As was the case for ldlC cells, there was a tendency for the intensity of immunofluorescence to be lower in the mutant than in wild-type or phenotypically reverted transfected cells; the significance of this observation is unclear. Taken together, these results establish that the *LDLB* gene is necessary for ldlCp localization to the Golgi and raise the possibility that the distinctive mutant phenotypes of ldlB cells are primarily due to abnormal localization of ldlCp.

Discussion

Three distinguishing characteristics of ldlC cells are their (*a*)

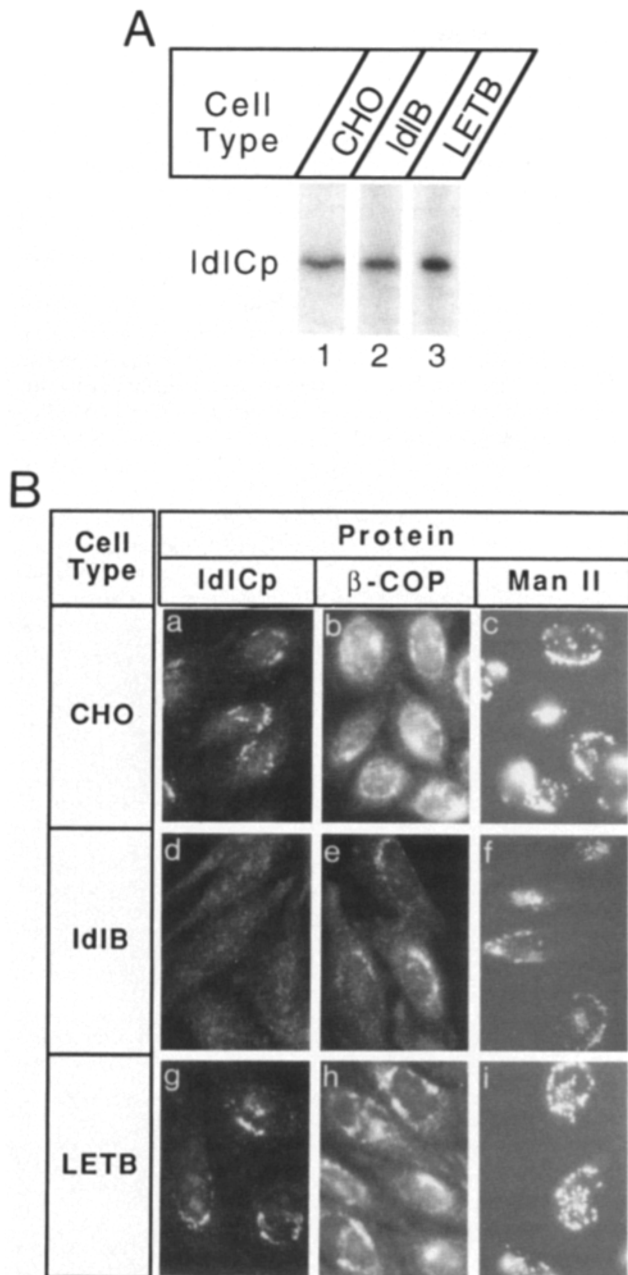

Figure 8. Immunoblotting (*A*) and immunofluorescence localization (*B*) of ldlCp, β-COP and mannosidase II in CHO, ldlB, and LETB cells. (*A*) The indicated cells were grown to confluence and lysed, and the lysates subjected to immunoblot analysis using anti-Cpep (10 μg/ml). Bound antibody was detected autoradiographically using ^{125}I-Protein A. (*B*) The indicated cells were grown on coverslips and immunostained with affinity purified anti-Cpep (*a*, *d*, and *g*), anti-β-COP monoclonal antibody M3A5 (*b*, *e*, and *h*), and anti-mannosidase II (*c*, *f*, and *i*) as described in Materials and Methods.

dramatically reduced LDL receptor activity, (*b*) abnormal posttranslational processing (glycosylation) of LDL receptors, resulting in receptor instability, and (*c*) global defects in cell surface glycoconjugates (N-linked, O-linked, and lipid-linked oligosaccharides) (Kingsley et al., 1986*a*). Essentially identical defects are found in a genetically distinct class of CHO mutants, ldlB cells. All of these abnormalities arise from pleiotropic defects in multiple *medial* and *trans* Golgi-associated processes (Kingsley et al., 1986*a*). The complex nature of these defects suggests that the *LDLB* and *LDLC* genes may be critically important for generating or maintaining the compartmental organization or the intralumenal environment of the Golgi apparatus (Kingsley et al., 1986*a*).

In the current study, we cloned a human *LDLC* cDNA which corrects the mutant phenotypes of ldlC, but not ldlB, cells. Unlike wild-type CHO or ldlB cells, ldlC cells had virtually no detectable endogenous *LDLC* mRNA, suggesting that *LDLC* is the normal human homologue of the defective gene in ldlC cells. Alternatively, the cloned *LDLC* gene may have acted as an extragenic suppressor of the defective gene in the ldlC cells. In either case, it appears that the gene which is defective in ldlC cells either directly or indirectly controls the expression of the *LDLC* mRNA and its protein product (ldlCp), and ldlCp apparently plays an important role in the normal functioning of the Golgi.

The predicted sequence of ldlCp is novel, lacking significant similarity to other known proteins. A portion of the ldlCp sequence was, however, highly similar to that of an EST cDNA fragment from the nematode *C. elegans*. We cloned and sequenced the *C. elegans* cDNA, and found a high degree of sequence similarity throughout the entire lengths of the mammalian and nematode sequences (26% identity, 53% similarity). This similarity suggests that ldlCp plays an ancient role in eukaryotic cell biology. The highly conserved portions of these sequences should facilitate the construction of probes which will permit the identification of ldlCp homologues from other species, possibly including the yeast *Saccharomyces cerevisiae*. Genetic studies in *C. elegans* and *S. cerevisiae* should help further define the functions of ldlCp.

The predicted sequence of ldlCp has no major common structural motifs such as GTP binding sites, transmembrane domains, or an ER translocation signal sequence. This suggests that ldlCp is a cytoplasmic protein. Nevertheless, immunofluorescence studies indicated that ldlCp may be associated with the cytoplasmic face of the Golgi, as it colocalized with Golgi markers and was rapidly redistributed from the Golgi by the drug BFA. Thus, the association of ldlCp with the Golgi appears to be analogous to that of several other peripheral Golgi proteins, including p200 (Narula et al., 1992), the coatomer (Donaldson et al., 1990; Orci et al., 1991), the small GTPase ADP-ribosylation factor (ARF) (Klausner et al., 1992), clathrin, and type I clathrin-associated proteins (Robinson and Kreis, 1992; Stamnes and Rothman, 1993; Traub et al., 1993), most of which have been implicated in intracellular membrane transport. Because ARF and coatomer proteins cycle on and off of Golgi membranes in a guanine nucleotide-dependent fashion (for example see Donaldson et al., 1992; Helms and Rothman, 1992; Klausner et al., 1992), it seems likely that ldlCp may undergo similar cycling between the cytoplasm and the Golgi membranes. The relative amounts of Golgi-associated and cytoplasmic ldlCp and the affinity of ldlCp for Golgi membranes have not yet been determined. The reversible nature of ldlCp association with the Golgi suggests that the association may be regulated. Regulated association of Golgi proteins has been implicated in the mitotic disassembly of the Golgi, as well as in normal trafficking during interphase (Rothman and Warren, 1994).

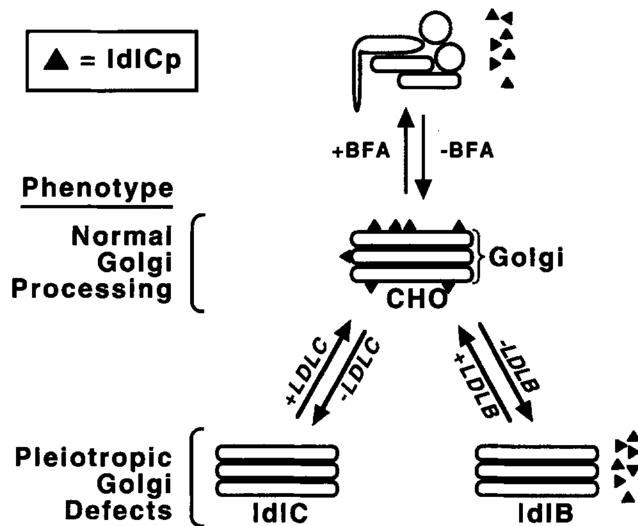

Figure 9. Model of the effects of BFA treatment and ldlC and ldlB mutations on ldlCp and Golgi function. ldlCp (*triangles*) is a *BFA*-sensitive peripheral Golgi protein (*top*) required for normal *medial*- and *trans*-Golgi processing reactions. Abnormal processing of glycoproteins and glycolipids in the lumen of the Golgi occurs when ldlCp is not associated with the Golgi, either because ldlCp is not synthesized (ldlC mutants, *lower left*) or because it cannot associate with the Golgi in the absence of normal *LDLB* gene function (ldlB mutants, *lower right*).

Analysis of ldlB mutants suggested that the association of ldlCp with the Golgi apparatus is required for its normal function. Essentially wild-type levels of ldlCp were present in ldlB cells; however, immunofluorescence microscopy indicated that the ldlCp was not localized to the Golgi complex in ldlB cells. A simple model, which accounts for the virtually identical phenotypes of ldlB and ldlC cells (Kingsley et al., 1986*a*), is that the product of the *LDLB* gene is required for the Golgi association of ldlCp and that this association is required for ldlCp function. When this association is prevented, due either to the absence of the ldlCp or to the loss of functional ldlBp, normal Golgi processing reactions are disrupted (See Fig. 9). ldlBp might serve as a Golgi receptor for ldlCp, a component of a heterooligomer with ldlCp, or a processing enzyme that renders ldlCp competent to bind to Golgi membranes. Further experiments will be required to determine how ldlBp influences the localization and activity of ldlCp, and what other roles the *LDLB* gene may play in normal Golgi functions.

The mechanism by which ldlCp influences lumenal Golgi processing reactions has not yet been established. At the resolution of the immunofluorescence microscopy described here, we observed no major defects in the ultrastructure of the Golgi in ldlC cells. Nevertheless, ldlCp might play a role in determining the compositions of the Golgi's membranes or lumenal spaces, including the amounts or types of proteins, lipids, carbohydrates, or ions present. Alterations in the localization or amounts of these components could interfere with multiple Golgi processing reactions. For example, the distributions of enzymes within the Golgi may depend on the distributions of lipids (Bretscher and Munro, 1993). It is also possible that the membrane association of ldlCp, which is BFA sensitive, is required for normal membrane trafficking through the Golgi. A defect in transport through one or more of the Golgi stacks might result in pleiotropic processing defects without grossly disrupting either the Golgi's ultrastructure or protein transport to the cell surface. Additional biochemical and genetic studies will be required to determine the functions of ldlCp, and how these functions contribute to the normal activity of the Golgi apparatus.

We thank Marsha Penman, Alison Lux, Frank Traynor, and Shangzhe Xu for excellent technical assistance; F. Solomon and R. Hynes for use of the fluorescence microscope in the Center for Cancer Research; R. Klausner and J. Donaldson for providing the anti-mannosidase II antibody and the anti-β-COP antibody (M3A5, originally from T. Kreis); and R. Horvitz, F. Solomon and the members of their laboratories for reagents and advice. Oligopeptides for immunizations were synthesized at the Biopolymers Laboratory of the Howard Hughes Medical Institute, Massachusetts Institute of Technology. We also thank F. Solomon, C. Kaiser, D. Housman, and members of our laboratory for helpful discussions.

This work was supported by National Institutes of Health grants HL41484, HL32459, and RR05734 (BRS Shared Instrumentation Grant). S. Podos was supported by a National Science Foundation predoctoral fellowship, and S. Podos and J. Ashkenas were supported by National Institutes of Health Institutional NRSA 5 T32 GM/HG07287. This work was performed during the tenure of a research fellowship to P. Reddy from the American Heart Association, Massachusetts Affiliate, Inc.

References

Adams, M. D., J. M. Kelley, J. D. Gocayne, M. Dubnick, M. H. Polymeropoulos, H. Xiao, C. R. Merril, A. Wu, B. Olde, R. F. Moreno, et al., 1991. Complementary DNA sequencing: expressed sequence tags and human genome project. *Science (Wash. DC).* 252:1651–1656.

Adams, M. D., M. Dubnick, A. R. Kerlavage, R. F. Moreno, J. M. Kelley, T. R. Utterback, J. W. Nagle, C. Fields, and J. C. Venter. 1992. Sequence identification of 2375 human brain genes. *Nature (Lond.).* 355:632–634.

Allan, V. J., and T. E. Kreis. 1986. A microtubule-binding protein associated with membranes of the Golgi apparatus. *J. Cell Biol.* 103:2229–2239.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. Lipman. 1990. A basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

Ashkenas, J., M. Penman, E. Vasile, S. Acton, M. Freeman, and M. Krieger. 1993. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J. Lipid Res.* 34:983–1000.

Bretscher, M. S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science (Wash. DC).* 261:1280–1281.

Cummings, R. D., S. Kornfeld, W. J. Schneider, K. K. Hobgood, H. Tolleshaug, M. S. Brown, and J. L. Goldstein. 1983. Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. *J. Biol. Chem.* 258:15261–15273.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* 12:387–395.

Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius. 1993. Folding and assembly of viral membrane proteins. *Virology.* 193:545–562.

Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *J. Cell Biol.* 111:2295–2306.

Donaldson, J. G., D. Finazzi, and R. D. Klausner. 1992. Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. *Nature (Lond.).* 360:350–352.

Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kreis. 1991. β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.

Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* 263:18545–18552.

Goldstein, J. L., A. S. Helgeson, and M. S. Brown. 1979. Inhibition of cholesterol synthesis with compactin renders growth of cultured cells dependent on the low density lipoprotein receptor. *J. Biol. Chem.* 254:5403–5409.

Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* 98:241–260.

Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456–467.

Guo, Q., E. Vasile, and M. Krieger. 1994. Disruptions in Golgi structure and membrane traffic in a conditional lethal mammalian cell mutant are corrected

by ε-COP. *J. Cell Biol.* 125:1213–1224.
Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 726 pp.
Helms, J. B., and J. E. Rothman. 1992. Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. *Nature (Lond.).* 360:352–354.
Hirschberg, C. B., and M. D. Snider. 1987. Topography of glycosylation in the rough endoplasmic reticulum and golgi apparatus. *Annu. Rev. Biochem.* 56:63–87.
Hobbie, L., A. S. Fisher, S. Lee, A. Flint, and M. Krieger. 1994. Isolation of three classes of conditional-lethal Chinese hamster ovary cell mutants with temperature-dependent defects in LDL receptor stability and intracellular membrane transport. *J. Biol. Chem.* 269:20958–20970.
Jelinek, W. R., T. P. Toomey, L. Leinwand, C. H. Duncan, P. A. Biro, P. V. Choudary, S. M. Weissman, C. M. Rubin, C. M. Houch, P. L. Deininger, et al. 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. *Proc. Natl. Acad. Sci. USA.* 77: 1398–1402.
Kao, C. Y., and Draper, R. K. 1992. Retention of secretory proteins in an intermediate compartment and disappearance of the Golgi complex in an End4 mutant of Chinese hamster ovary cells. *J. Cell Biol.* 117:701–715.
Kawai, S., and M. Nishizawa. 1984. New procedure for DNA transfection with polyction and dimethyl sulfoxide. *Mol. Cell. Biol.* 4:1172–1174.
Kingsley, D. M., and M. Krieger. 1984. Receptor-mediated endocytosis of low density lipoprotein: somatic cell mutants define multiple genes required for expression of surface-receptor activity. *Proc. Natl. Acad. Sci. USA.* 81: 5454–5458.
Kingsley, D., K. F. Kozarsky, M. Segal, and M. Krieger. 1986*a*. 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. *J. Cell Biol.* 102:1576–1585.
Kingsley, D., R. D. Sege, K. F. Kozarsky, and M. Krieger. 1986*b*. DNA-Mediated Transfer of a Human Gene Required for Low-Density Lipoprotein Receptor Expression and for Multiple Golgi Processing Pathways. *Mol. Cell. Biol.* 6:2734–2737.
Kingsley, D. M., K. F. Kozarsky, L. Hobbie, and M. Krieger. 1986*c*. Reversible defects in O-linked glycosylation and LDL receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell.* 44:749–759.
Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116:1071–80.
Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–634.
Kozarsky, K., H. A. Brush, and M. Krieger. 1986. Unusual forms of low density lipoproteins in hamster cell mutants with defects in the receptor structural gene. *J. Cell Biol*, 102:1567–1575.
Kozak, M. 1989. The scanning model for translation: an update. *J. Cell Biol.* 108:229–241.
Krieger, M. 1983. Complementation of mutations in the LDL pathway of receptor-mediated endocytosis by cocultivation of LDL receptor-defective hamster cell mutants. *Cell.* 33:413–422.
Krieger, M. 1986. Isolation of somatic cell mutants with defects in the endocytosis of low-density lipoprotein. *Methods Enzymol.* 129:227–237.
Krieger, M., M. S. Brown, and J. L. Goldstein. 1981. Isolation of Chinese hamster cell mutants defective in the receptor-mediated endocytosis of low density lipoprotein. *J. Mol. Biol.* 150:167–184.
Krieger, M., J. Martin, M. Segal, and D. Kingsley. 1983. Amphotericin B selection of mutant Chinese hamster cells with defects in the receptor-mediated endocytosis of low density lipoprotein and cholesterol biosynthesis. *Proc. Natl. Acad. Sci. USA.* 80:5607–5611.
Krieger, M., D. Kingsley, R. Sege, L. Hobbie, and K. Kozarsky. 1985. Genetic analysis of receptor-mediated endocytosis. *Trends Biochem. Sci.* 10: 447–452.
Krieger, M., P. Reddy, K. Kozarsky, D. Kingsley, L. Hobbie, and M. Penman. 1989. Analysis of the synthesis, intracellular sorting, and function of glycoproteins using a mammalian cell mutant with reversible glycosylation defects. *Methods Cell Biol.* 32:57–84.
Lehrman, M. A., W. J. Schneider, T. C. Sudhof, M. S. Brown, J. L. Goldstein, and D. W. Russell. 1985. Mutation in LDL receptor: Alu-Alu recombination deletes exons encoding transmembrane and cytoplasmic domains. *Science (Wash. DC).* 227:140–146.
Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from the Golgi to ER. *Cell.* 56:801–813.
Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.
Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.
Malmstrom, K., and M. Krieger. 1991. Use of radiation suicide to isolate constitutive and temperature-sensitive conditional Chinese hamster ovary cell mutants with defects in the endocytosis of low density lipoprotein. *J. Biol. Chem.* 266:24025–24030.
Moremen, K. W., and O. Touster. 1985. Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 Cells. *J. Biol. Chem.* 260: 6654–6662.
Narula, N., I. McMorrow, G. Plopper, J. Doherty, K. S. Matlin, B. Burke, and J. L. Stow. 1992. Identification of a 200-kD, brefeldin-sensitive protein on Golgi membranes. *J. Cell Biol.* 117:27–38.
Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell.* 64:1183–1195.
Ostermann, J., L. Orci, K. Tani, M. Amherdt, M. Ravazzola, Z. Elazar, and J. E. Rothman. 1993. Stepwise assembly of functionally active transport vesicles. *Cell.* 75:1015–1025.
Reddy, P., and M. Krieger. 1989. Isolation and characterization of an extragenic suppressor of the low-density lipoprotein receptor-deficient phenotype of a Chinese hamster ovary cell mutant. *Mol. Cell Biol.* 9:4799–4806.
Reddy, P., I. Caras, and M. Krieger. 1989. Effects of O-linked glycosylation on the cell surface expression and stability of decay-accelerating factor, a glycophospholipid-anchored membrane protein. *J. Biol. Chem.* 264: 17329–17336.
Remaley, A. T., M. Ugorski, N. Wu, L. Litzky, S. R. Burger, J. S. Moore, M. Fukuda, and S. Spitalnik. 1991. Expression of human glycophorin A in wild type and glycosylation-deficient Chinese hamster ovary cells. *J. Biol. Chem.* 266:24176–24183.
Rine, J. 1991. Gene overexpression in studies in Saccharomyces cerevisiae. *Methods Enzymol.* 194:239–251.
Robinson, M. S., and T. E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell.* 69:129–138.
Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257–288.
Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409–415.
Rothman, J. E., and G. Warren. 1994. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Current Biol.* 4: 220–233.
Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Serafini, T., G. Stenbeck, A. Brecht, F. Lottspeich, L. Orci. J. E. Rothman, and F. T. Wieland. 1991. A coat subunit of Golgi-derived non-clathrin-coated vesicles with homology to the clathrin-coated vesicle coat protein β-adaptin. *Nature (Lond.).* 349:215–220.
Shih, C., and R. A. Weinberg. 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. *Cell.* 29:161–169.
Staden, R. 1990. Searching for patterns in protein and nucleic acid sequences. *Methods Enzymol.* 183:193–211.
Stamnes, M. A., and J. E. Rothman. 1993. The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell.* 73:999–1005.
Stanley, P. 1985. Lectin-resistant glycosylation mutants. *In* Molecular Cell Genetics: The Chinese Hamster Cell. M. M. Gottesman, editor. John Wiley & Sons, Inc., New York. 745–772.
Tolleshaug, H., J. L. Goldstein, W. J. Schneider, and M. S. Brown. 1982. Posttranslational processing of the LDL receptor and its genetic disruption in familial hypercholesterolemia. *Cell.* 30:715–724.
Takatsuki, A., and G. Tamura. 1985. Brefeldin A, a specific inhibitor of intracellular translocation of vesicular stomatitis virus G protein: intracellular accumulation of high-mannose type G protein and inhibition of its cell surface expression. *Agric. Biol. Chem.* 49:899–902.
Traub, L. M., J. A. Ostrom, and S. Kornfeld. 1993. Biochemical dissection of AP-1 recruitment onto Golgi membranes. *J. Cell Biol.* 123:561–573.
Waters, M. G., T. Serafini, and J. E. Rothman. 1991. 'Coatomer': a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature (Lond.).* 349:248–251.