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

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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 LDL 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) receptor (Krieger et al., 1985), decay-accelerating factor (Reddy et al., 1989), the Epstein-Barr virus envelope protein (Krieger et al., 1989), and glycoporphin (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-
normal posttranslational processing of the receptor in the Golgi (Kingsley et al., 1986a). At least in the case of IdIC 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., 1986a; Reddy and Krieger, 1989).

IdIB and IdIC 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., 1986a). 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 GM1), 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., 1986c), 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., 1986a). 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 luminal environment (pH, ion concentrations); or (e) some combination of these.

In the current work, we isolated a novel human cDNA (LDLCC) that corrects all of the pleiotropic defects in IdIC cells, and we also isolated an LDL homologue from Caenorhabditis elegans. We have examined the expression of the LDL gene and its protein product (LDLp), and the intracellular distribution of LDLp, in wild-type CHO and mutant IdIC and IdIB cells. IdLCp 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 LDL cDNAs suggests that IdLCp 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/LDLCC 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[251] (Amer sham Corp., Arlington Heights, IL); [35S]P4C1PCT, [35S]methionine, and [35S]ATP (New England Nuclear, Boston, MA); fluorescein-conjugated goat anti-rabbit (FGAR) and goat anti-mouse (FGAM) IgGs (Cappel Re- search 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/RJH, Lenexa, KA). Newborn calf lipoprotein-deficient serum, LDL, and [125I]-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. Compac tin was a gift of A. Endo (Tokyo Nudo University, Japan). Antibodies used for immunofluorescence localization experiments include a polyclonal antisera against Golgi mannosidase II (Moremen and Tsouster, 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 humified 5% CO2-95% air incubator unless specified otherwise. Wild-type CHO cells, IdLC (clone 475) and IdIB (clones 11 and WGA2) mutant CHO cells, and the transfectant LETB-144 were obtained as previously described (Krieger et al., 1981; Kingsley and Krieger, 1984; Kingsley et al., 1986a,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). IdLC transfecants 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 LDLC, and 40 µM compactin). Compac tin, 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 IdIC Cells

IdLC cells were transfected with calcium phosphate precipitates of human genomic DNA essentially as described by Graham and Van der Eb (1973). In brief, IdIC 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 Heps-buffered saline containing a calcium phosphate precipitate of genomic DNA from human A431 carcinoma cells (20 µg/dish) and 5 µl of [35S]transferase 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 Heps 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 were then set into 2100-mm dishes (4 × 106 cells/dish) in MeLoCo selection medium (medium F) containing 250 µg/ml G418, to isolate primary receptor-positive LDL endocytosis transfectants of IdIC cells (1° LETC cells). Five independent 1° LETC colonies were isolated from a total of 2 × 106 cells subjected to selection. Seven independent secondary LETC cells were then isolated from 2 × 106 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 the place of the A431 DNA. Finally, seven tertiary LETC (3° LETC) colonies were isolated from 6 × 106 cells after a third round of cotransfection/selection, using a 2° LETC colony (2° LETC-15) as the source of genomic DNA.

Cloning Human LDLCC cDNA

A 3.5-kb EcoRI DNA fragment was detected in the 2° LETC and 3° LETC colonies, using Southern blot analysis by BLURRI, a human Ali repeat element, as a probe (Jelinek et al., 1988). The BLURRI probe was then used to clone the 3.5-kbp fragment from a XZAPIII (Stratagene Inc., La Jolla, CA) library of EcoRI-digested, size-selected DNA from 2° LETC cells (colony V5). A 600-bp SacI-HindIII restriction fragment from the 3.5-kbp EcoRI clone, which did not contain the Ali repeat element, was then used as a probe to isolate candidate LDLCC 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 described by Ashkenazi 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 λ ZapII. Plaques (5 × 10^3) from the random-primer 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 IdlC clones from the oligo d(T) 12-18 primer library. cDNA clones with putative cDNA inserts bearing cDNA clones 1–16 were excised from the λ ZapII 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 +1 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 AGCTAGCT AGCTAGCT AGCTAGCT AGCTAGCT AGCTAGCT AGCTAGCTAGCT T3'. 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 IdlC Cells

The full-length cDNA insert from clone 2 was inserted into the plasmid pBluescript (Stratagene) and from the oligo d(T) 12-18 primer library, pBluescript constructs bearing cDNA clones 1–16 were excised from the λ ZapII 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 (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 +1 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 AGCTAGCT AGCTAGCT AGCTAGCT AGCTAGCT AGCTAGCT AGCTAGCTAGCT T3'. 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).

Cloning of a C. elegans Homologue of LDLC

The cDNA was cloned from C. elegans cDNA library in λ ZapII (Stratagene), prepared by R. Barstead, Washington University (St. Louis, MO). Six cDNA clones were isolated from 500,000 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-IdlCp Antipeptide Antibodies

Peptides Npep (EKSRMNLPKGPDTLC) and Cpep (CAELVAAAKDQAT) were synthesized containing the predicted NH2-terminal and COOH-terminal sequences of the IdlC protein, with terminal cysteines added to permit crosslinking to carrier proteins. Npep and Cpep were coupled to keyhole limpet hemocyanin (Sigma) in 20 mM sodium hydroxide at an molar ratio of 1:1, mixed with m-maleimido-benzenedicarboxylic 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 SlurpolyLink 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 soybean trypsin inhibitor, 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/ml) and transferred electrochemically 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 W containing 0.05% [vol/vol] NP-40, and 0.1% [wt/vol] SDS), and two times in buffer W, incubated with 125I-protein A (2–10 μCi/μg) in buffer W for 1–2 h, washed twice with buffer W, and three times with PBS. Antibody binding was visualized by autoradiography. Control samples for Fig. 5 and 8 A were probed with anti-tubulin antisera (not shown).

Immunofluorescence Microscopy

On day 0, cells were plated (200 cells/mm2) in medium C onto 12-mm square glass coverslips. On day 2, the coverslips were washed in cPBS (PBS with 0.5 mM MgCl2 and 0.9 mM CaCl2) at 37°C, fixed for 30 min at room temperature with 3% [vol/vol] formaldehyde in PBS, which was premixed to 37°C, and quenched for 10 min at room temperature. In PBS containing 0.1% Triton X-100 and 0.2% SDS. The samples 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 paraffin, 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, 1:1000 or 1:2000 dilution; FGAM, 1:500 dilution; or TRHAM, 1.5 μg/ml) for 45–60 min, rinsed four times in PBS and one time in H2O, 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., 1986a). The LDLp 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 125I-LDL (10 μg protein/ml, 400 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 125I-LDL degraded/105 cells. Protein concentrations were determined by the method of Lowry et al. (1951).

Metabolic labeling of cells, immunoprecipitation of LDL receptors with an affinity-purified 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-
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ment. Therefore, we used the same phenotype in the transfected cells, and that the human gene was probably responsible for the correction of the mutant phenotype with the restoration both of LDL receptor activity (as 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 pLDLC-1, but not with the empty vector, restored LDL receptor activity to levels as high as 160% of wild type (not shown). Analysis of other independent transfectants showed that pLDLC-1 restored receptor activity to levels as high as 160% of wild type (not shown). Therefore, human LDL 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 $\sim$125 kD precursor (p) which

### 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., 1986a). Due to the altered structures of cell surface glycoconjugates in ldlC cells (Kingsley et al., 1986a), 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 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 (1°-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 these clones, which comprises the entire predicted coding sequence (see below), was inserted into the vector pRe/CMV to generate the expression vector pLDLC-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., 1986a). To determine if pLDLC-1 could correct these mutant phenotypes, we isolated ldlC cells stably transfected with pLDLC-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 pRe/CMV lacking the cDNA insert. Table 1 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 pLDLC-1, but not with the empty vector, restored LDL receptor activity to 61% of wild-type levels. Analysis of other independent transfectants showed that pLDLC-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 $\sim$125 kD precursor (p) which

### Table 1. LDL Receptor Activities and Lectin Sensitivities of ldlC Transfectants

| Cells     | LDL receptor activity* | WGA | ConA | PHA | Ricin | Phenotype |
|-----------|------------------------|-----|------|-----|-------|-----------|
| 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 LD₅₀, 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., 1986a). 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 pLDLC-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 pLDLC-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 nucleotide and predicted ldlCp protein sequences. The

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 [35S]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 B-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 32P-labeled fragment of plasmid pLDLC-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 x 15 min at room temperature in 300 mM phosphate buffer (pH 7.0); 2 x 15 min at 60° in 300 mM phosphate buffer, 5% SDS, 5 mg/ml BSA, 1 mM EDTA; and 2 x 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.
The predicted protein product (ldlCp) of the 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 adenines of the LDLC cDNA are likely to represent the start of a poly(A) tail, as they follow a candidate polyadenylation signal (AATAAA) 13 bases.

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 adenines 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.

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 luminal 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 myristylation sites, nucleotide binding sites, heptad repeats, etc.

In addition, we have not detected any other common sequence motifs or predicted secondary or tertiary structural elements, such as isoprenylation sequences, amino terminal myristylation sites, nucleotide binding sites, heptad repeats, etc.
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. Goscochea, 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 IdlCp 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 TATA 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 IdlCp 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 IdlCp sequence lacks other notable structural features such as transmembrane domains or signal sequences. Overall, the conservation in the human and nematode IdlCp sequences suggests that the LDLC genes encode proteins which mediate important, highly conserved functions.

Preparation and Characterization of Anti-IdlCp Antibodies

Based on the abnormalities in medial and trans Golgi-associated glycoconjugate synthesis in IdlCp cells, we inferred that cytosolic IdlCp might physically associate with the Golgi apparatus. To determine the subcellular distribution of IdlCp by immunofluorescence microscopy, rabbit polyclonal antibodies were prepared using synthetic peptides which represent the amino (Npep)- and carboxy (Cpep)-termini of human IdlCp, 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-kDa protein, whose apparent mass is similar to the 83-kDa 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 IdlCp (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 IdlCp in CHO cell lysates (Fig. 5, lanes 7–9). The ~76-kD protein was not detected in lysates from IdlCp cells (Fig. 5, lanes 10 and 11), but was seen in IdlCp/LDLC lysates (lanes 12 and 13). (Replicate lanes of CHO, IdlC, and IdlCp/LDLC lysates, stained with anti-tubulin antisera, 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 IdlCp cells (Fig. 2). As was the case for HeLa cell lysates, anti-Cpep bound to smaller, unidentified species from CHO and IdlCp cells. Taken together, these data establish that the ~76-kD protein, which is the major specific antigen of anti-Cpep, is IdlCp and they suggest that at least a portion of the COOH terminus of IdlCp is conserved among several mammalian species.

Immunolocalization of IdlCp Protein

Immunofluorescence microscopy with affinity purified anti-Cpep was used to determine the distribution of IdlCp 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 IdlCp cells but present in transfected IdlCp/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 IdlCp. 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 IdlCp was characteristic of the distribution of the Golgi apparatus in CHO cells (Kao and Draper, 1992; Guo et al., 1994). For example, Fig. 6 b (top right) 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-derivated transport vesicles (Duden et al., 1991; Serafini et
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 Idc/P moa amino acid sequences. Vertical bars indicate identities, double and single dots indicate strong and weak similarities.

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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 125I-Protein A. The "ldlCp" (large arrow) indicates the position of the various mammalian ldlCp's, as described in Results.

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; Fujivara et al., 1988; Donaldson et al., 1990; Lippincott-Schwartz et al., 1989, 1990; Orci et al., 1991). BFA interferes with the assembly of the coatamer 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 distribution 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-
Aberrant Distribution of IdlCp in ldlB Cells Indicates Golgi Localization Is Required for IdlCp Function

The BFA-dependent reversible localization of IdlCp to the Golgi suggested that, as with β-COP, Golgi localization may be required for the effects of IdlCp on Golgi function. This suggestion was supported by studies of IdlCp'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 Kriger, 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., 1986a). 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 IdlCp. We therefore examined the expression of the endogenous LDLC gene and the localization of IdlCp 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., 1986a).

Northern blot analysis (not shown) and immunoblot analysis (Fig. 8 A) established that there were essentially wild-type levels of both LDLC mRNA and IdlCp 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 IdlCp. Fig. 8 B shows the immunofluorescence localization of IdlCp (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), IdlCp apparently did not localize to the Golgi apparatus in ldlB-11 cells (d). Instead, a uniform punctate background in IdlCp staining was seen, suggesting that IdlCp was distributed throughout the cytoplasm of ldlB-11 cells. These results were confirmed by examining an independently derived clone of ldlB cells (WGAr-2, Kingsley et al., 1986a) (not shown). In addition, the normal Golgi distribution of IdlCp 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 IdlC 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 IdlCp localization to the Golgi and raise the possibility that the distinct mutant phenotypes of ldlB cells are primarily due to abnormal localization of IdlCp.

Discussion

Three distinguishing characteristics of IdlC cells are their (a)
Figure 8. Immunoblotting (A) and immunofluorescence localization (B) of IdICp, β-COP and mannosidase II in CHO, IdIB, 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 125I-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., 1986a). Essentially identical defects are found in a genetically distinct class of CHO mutants, IdIB cells. All of these abnormalities arise from pleiotropic defects in multiple medial and trans Golgi-associated processes (Kingsley et al., 1986a). 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 intraluminal environment of the Golgi apparatus (Kingsley et al., 1986a).

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

The predicted sequence of IdICp is novel, lacking significant similarity to other known proteins. A portion of the IdICp 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 IdICp 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 IdICp 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 IdICp.

The predicted sequence of IdICp has no major common structural motifs such as GTP binding sites, transmembrane domains, or an ER translocation signal sequence. This suggests that IdICp is a cytoplasmic protein. Nevertheless, immunofluorescence studies indicated that IdICp 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 IdICp with the Golgi appears to be analogous to that of several other peripheral Golgi proteins, including p200 (Narula et al., 1992), the coatamer (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 coatamer 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 IdICp may undergo similar cycling between the cytoplasm and the Golgi membranes. The relative amounts of Golgi-associated and cytoplasmic IdICp and the affinity of IdICp for Golgi membranes have not yet been determined. The reversible nature of IdICp 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).
The mechanism by which IdlB 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 IdlC cells. Nevertheless, IdlBp 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 IdlCp, 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 IdlCp, and how these functions contribute to the normal activity of the Golgi apparatus.

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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.). 353:632-634.
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. Vasilie, 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. Tollefsen, 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.
Deveraux, J., P. Haabert, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395.
Dinis, 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.
Fujiiwa, 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 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. Vasilie, and M. Krieger. 1994. Disruptions in Golgi structure and membrane traffic in a conditional lethal mammalian cell mutant are corrected.
Kawai, S., and M. Nishizawa. 1984. New procedure for DNA transfection with 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. Detaining, et al. 1980. Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. USA. 77: 1398–1402.

Kao, C. T., 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 polyion and dimethyl sulfoxide. Mol. Cell. Biol. 4:1172–1174.

Kingsley, D., 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. 1986c. Three types of low density lipoprotein receptor-deficient mutant have pleiotropic effects in the synthesis of N-linked, O-linked, and lipid-linked carbohydrate chains. J. Cell Biol. 102:1576–1585.

Kingsley, D., D. Sege, K. F. Kozarsky, and M. Krieger. 1986b. DNA-Mediated Transfer of a Human Gene Required for Low-Density Lipoprotein Receptor Expression and for Multiple Golgi Processing Pathways. Mol. Biol. Cell. 6:273-2773.

Kozak, M. 1989. The scanning model for translation: an update. J. Mol. Biol. 213:409-415.

Krieger, M. 1986. Isolation of somatic cell mutants with defects in the endocytosis of low-density lipoprotein. Methods Enzymol. 129:227–237.

Krieger, M., J. G. Martin, M. Segal, and D. Kingsley. 1983. Amylophore B secretion from Chinese hamster cells mutants defective in the receptor-mediated endocytosis of low density lipoprotein. J. Cell. Biol. 100:167-184.

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.

Lemahn, 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 exon encoding transmembrane and cytoplasmic domains. Science (Wash. DC). 227:46-48.

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. Rosebroch, 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. McInerny, 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. Ferretti, 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 a cell surface expression and stability of decay-accelerating factor, a glycosphospholipid-anchored membrane protein. J. Biol. Chem. 264: 17329–17336.

Remaley, T. A., M. Igorski, N. Wu, L. Litzky, S. R. Burger, J. S. Moore, M. Matsuda, and S. Spitalni. 1991. Expression of human glycoporphin 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, R. K., and B. 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 2-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.

Takatsuka, A., and G. Tamura. 1983. 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. Agranit. Biol. Chem. 49:899-902.

Traub, L. M., J. A. Ostrowski, 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.