LDLC Encodes a Peripheral Golgi Protein Required for Normal Golgi Function

by

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B.S., Yale University (1987)

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Abstract.

ldlB and ldlC are two previously isolated classes of CHO cell mutant, with LDL receptor deficiencies and broad glycosylation defects. These mutant cells exhibit a broad set of defects in processing reactions within the Golgi apparatus, affecting virtually all N- and O-linked glycoproteins, and glycolipids. The LDL receptor deficiency in ldlB and ldlC cells is a secondary consequence of the Golgi processing defects. I have conducted a molecular analysis of ldlC mutant cells, to determine how a single defect can affect multiple reactions within the Golgi. I have cloned by complementation a human cDNA (*LDLC*) that fully corrects the mutant phenotypes when transfected into ldlC cells. The *LDLC* cDNA did not correct the apparently identical mutant phenotypes in ldlB cells. By Northern blot analysis, the endogenous *LDLC* mRNA was clearly expressed in wild-type CHO cells but not in ldlC mutant cells, suggesting that the *LDLC* cDNA is a normal human counterpart to the defective gene of ldlC cells. The *LDLC* cDNA was shown by sequence analysis to encode a protein (ldlCp) that was not similar in sequence to other known proteins. ldlCp has a predicted size of 83 kilodaltons, and contains no common structural motifs such as transmembrane domains or translocation signal sequences. Site-directed mutations introduced into the *LDLC* cDNA indicated that an amino-terminal portion of ldlCp may be dispensable for function. A cDNA related to the human *LDLC* cDNA was cloned from *Caenorhabditis elegans*, to extend the *LDLC* sequence analysis. It encodes a distant homolog of ldlCp, indicating that ldlCp mediates conserved cellular functions. This cDNA was physically mapped within the *Caenorhabditis elegans* genome, and several genetic loci were identified as *LDLC* candidates. Antibodies were raised against ldlCp, and used for immunofluorescence localization studies. In wild-type CHO cells, ldlCp co-localized with Golgi markers. Localization was disrupted by short treatment with the drug brefeldin A, which dissociates known peripheral proteins such as beta-COP from the Golgi. In ldlB cells, ldlCp was expressed at normal levels but was not localized to the Golgi. Together, these results demonstrate that ldlCp is a peripheral Golgi protein that requires the putative *LDLB* gene for Golgi localization.

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Contents.

Figures and Tables.

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Scafe, C., C. Martin, M. Nonet, S. Podos, S. Okamura, and R. A. Young. 1990. Conditional mutations occur predominantly in highly conserved residues of RNA polymerase II subunits. *Mol. Cell. Biol.* 10:1270-1275.

Abstracts:

Podos, S. D., E. Vasile, P. Reddy, J. Ashkenas, L. Hobbie, A. S. Fisher, S. I. Lee, A. Flint, and M. Krieger. CHO mutants with ER- and Golgi-associated defects. Poster at 1993 Gordon Conference, Molecular Membrane Biology.

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I also wish to thank here my immediate colleagues in this work. I didn't overlap with Pranhitha Reddy, but she deserves much credit for welcoming the *LDLC* cloning project into this world and for bringing it well into adolescence. Tremendous thanks are due to John Ashkenas, for guiding me into the ldlC project, working side-by-side with me in the early months, and contributing ideas throughout the course of this work. Thanks are also due to labmates Frank Traynor and Shangzhe Xu, who helped transform unsequenced DNA into sequenced DNA. My rabbits may disagree, but Alison Lux deserves thanks for her help in generating the polyclonal antibodies described within.

Dedication.

This thesis is dedicated to my friends and colleagues who made this work both doable and worth doing.

To Monty Krieger, for all the reasons described in the Acknowledgments, and for indescribable enthusiasm and support.

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Most of all, to my wife Judy Chevalier, for endless love, support, humor, encouragement, understanding, and patience. I couldn't have done it without you, and don't worry, this will be my last advanced degree.

Foreword.

It has long been apparent that eukaryotic cells are organized by internal membranes into a number of compartments, with distinct compositions and functions. The Golgi apparatus presents a striking case, as its layered cisternae somehow retain their morphologies and biochemical identities despite extensive bi-directional flow of their membranes and contents. Much has been learned in the past fifteen years about the functions of this organelle. Many protein and lipid processing steps within the Golgi have been characterized, and the distributions of enzymes within the Golgi cisternae have been described. Furthermore, much of the machinery which mediates membrane transport has been characterized through biochemical and genetic means. In fact, molecular dissection of Golgi membrane processes has been one of the great successes of recent years. However, many fundamental questions about Golgi structure and function remain.

The work presented in this thesis is rooted in a somatic cell genetics analysis of membrane activities. The strengths and the weaknesses of this approach are evident in the analysis described within. The greatest strength of the approach is still the obvious one, that requirements within living cells can be revealed which may be lost in an *in vitro* reconstitution. The *LDLC* gene was identified via an analysis of Chinese hamster ovary (CHO) cell mutants with LDL receptor deficiencies. Despite evidence that *LDLC* serves important and ancient roles in Golgi function, thus far it has been overlooked by other methods. The flip side of this very feature is that the net cast by the genetics approach is so wide that not all catches are readily traced to their sources. Again, *LDLC* is illustrative. Many of its properties have been uncovered in experiments presented here, which together point to an interesting role in membrane biology, yet its basic activity remains unsolved. Thus a limitation of the somatic cell genetics approach is the difficulty in interpreting and extending results, novel as they may be.

The one facile conclusion from all of this is that different phrasing of questions will often lead to different answers, and that no single approach can ever suffice. The utility of the somatic cell approach may be at its greatest for systems with architecture and logic as complex as found in the secretory system. A complete description may depend upon subtleties that are not available in reconstitutions. Furthermore, such features may not be shared with yeast, although at their cores the yeast and mammalian secretory systems are remarkably alike. I will not be surprised if the utility of somatic cell approach ultimately proves to be one of the important themes of the *LDLC* story, alongside its biological significance.

Chapter 1.

Introduction.

ldlB and ldlC.

The experiments in this thesis were conducted as an extension of the long-term efforts by the Krieger laboratory towards a somatic cell genetic description of LDL receptor activity. This work has aimed to characterize cellular requirements of receptor-mediated endocytosis, using the LDL receptor as a selectable reporter system (Krieger, 1985). Four distinct selections and screens have yielded numerous Chinese hamster ovary (CHO)[1] cell mutants, with defects in LDL receptor activity (Krieger, 1983; Krieger et al., 1981,1983,1985; Malmstrom and Krieger, 1991; Hobbie et al., 1994). These mutants define nine complementation groups, ldlA through ldlI (Kingsley and Krieger, 1984; Malmstrom and Krieger, 1991; Hobbie et al., 1994). ldlA mutants are defective in the structural gene for LDL receptor (Kingsley and Krieger, 1984; Kozarsky et al., 1986). These are analogous to cells from humans who are homozygous for familial hypercholesterolemia (FH) (Brown and Goldstein, 1986). Mutants in the remaining eight ldl complementation groups are defective in "supporting" genes. These mutants lack secondary general activities, and are not limited to the LDL receptor.

The initial motivation for this work was the characterization of endocytic processes, such as entry into coated pits, endosomal ligand/receptor uncoupling, and receptor recycling (Krieger, 1985). However, the LDL receptor-deficient mutants ldlB through ldlI have redirected the focus towards protein maturation in the secretory pathway (Kingsley et al., 1986a; Kingsley et al., 1986c; Hobbie et al., 1994; Guo et al., 1994; Podos et al, 1994). The broad context of the work on the ldlB and ldlC mutants, and thus the focus of this introduction, is the Golgi apparatus. The particular slant of this introduction rests upon features of ldlB and ldlC mutants, and of the *LDLC* gene and ldlCp protein.

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[1]*Abbreviations used in this thesis*: ARF, ADP-ribosylation factor; BFA, brefeldin A; CHO, Chinese hamser ovary; ConA, concanavalin A; COP, coatomer protein; endoH, endoglycosidase H; EST, expressed sequence tag; FGAM, fluorescein-conjugated goat anti-mouse IgGs; FGAR, fluorescein-conjugated goat anti-rabbit IgGs; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; LDL, low density lipoprotein; LETC, LDL endocytosis transfectants of ldlC cells; N-linked, asparagine-linked; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; O-linked, serine- or threonine-linked; PHA, phytohemagglutinin; TRHAM, Texas red-conjugated horse anti-mouse IgG; VSV, vesicular stomatitis virus; WGA, wheat germ agglutinin; WT, wild-type; YAC, yeast artificial chromosome.

ldlB, ldlC, and ldlD mutants all exhibit defective N- and O-linked glycosylation of the LDL receptor (Kingsley et al., 1986a). The underlying glycosylation machinery is affected, as N- and O-linked carbohydrate modeling is altered on other proteins and also on lipids (Kingsley et al., 1986a). The ldlD defects have been traced to the lack of a UDP-galactose/UDP-N-acetylgalactosamine 4-epimerase (Kingsley et al., 1986c). This enzyme generates the glycosylation substrates UDP-galactose and UDP-N-acetylgalactosamine (UDP-GalNAc), which are required for galactose and N-acetylgalactosamine (GalNAc) addition to carbohydrate moieties. GalNAc is a component of O-linked glycoconjugates, and is the most proximal sugar in the O-linked sugar chains on the LDL receptor. Galactose is found in N-linked, O-linked, and lipid-linked moieties, where it is added during terminal glycosylation in the Golgi. The deficiencies of these two sugars in ldlD cells can be reversed by the addition of exogenous galactose and N-acetylgalactosamine. Growth of ldlD cells in the presence of galactose but not GalNAc provides the only method available for specifically blocking O-linked glycosylation *in vivo*. Therefore, ldlD cells have been used for a variety of studies involving O-linkages. These cells have been used to study the kinetics of O-linked glycosylation of the LDL receptor (Kozarsky et al., 1988), and to measure the recycling of surface glycoproteins to the Golgi (Huang and Snider, 1993). ldlD cells have also been used to study the importance of O-linked glycosylation to the maturation and stability of a variety of proteins (Matzuk et al., 1987; Kozarsky et al., 1988; Reddy et al., 1989; Remaley, 1991).

ldlB and ldlC cells have virtually identical mutant phenotypes, which share major features with the ldlD defects. All three were first isolated during the same mutant selection (Krieger et al., 1981). All have broad defects in N- and O-linked protein glycosylation and in glycolipid maturation (Kingsley et al., 1986a). The LDL receptor is unstable in these glycosylation backgrounds. This instability is likely due to O-linked processing defects, even though a domain with most of the O-linked chains is dispensable (Davis et al., 1986; Reddy and Krieger, 1989). This LDL receptor instability is not a common feature among glycosylation mutants, many of which have been isolated because of resistance to lectin toxicity (Kingsley et al., 1986a; Stanley et al., 1985a). For example the major glycosylation pathways are affected in Lec8 mutant cells by the failure to import UDP-galactose into the Golgi (Deutscher and Hirschberg, 1986). Yet, Lec8 cells exhibit no severe LDL receptor phenotype (Kingsley et al., 1986a). In part by analogy to ldlD cells, and in part by a process of elimination, the LDL receptor deficiency in ldlB and ldlC cells may indicate a deficiency in GalNAc addition.

Despite the similarities to ldlD, the ldlB and ldlC mutant phenotypes appear distinct in fundamental ways. The breadth of the ldlB and ldlC mutant phenotypes has stymied efforts to explain them as straightforward glycosylation defects. In particular, the N-linked defects in these cells appear to have no overlap with the O-linked defects described above. The N-linked defects are first detected in the *medial* Golgi cisternae. Subsets of LDL receptor and of vesicular stomatitis virus (VSV) G proteins do not acquire the usual resistance to endoglycosidase H digestion, which normally indicates trimming by

mannosidase II in the *medial* Golgi cisternae. However, the N-linked structures in the *medial* Golgi are composed only of N-acetylglucosamine (GlcNAc) and mannose, neither of which are components of the O-linked chains. Thus, no single glycosyltransferase or nucleotide sugar transport activity yet described can account for these disparate mutant phenotypes. Furthermore, the salvage pathways that rescue ldlD cells do not restore ldlB and ldlC, indicating that nucleotide sugar synthesis is not at fault in ldlB and ldlC cells.

It is important to note that the ldlB and ldlC mutations lie within single genes, despite the complexity of their mutant phenotypes. The single ldlC isolate and the several independent ldlB mutants all share the mutant phenotypes, and spontaneous ldlB mutants arise in a non-mutagenized background at a frequency of 7×10^{-8} (Kingsley et al., 1986a). Full phenotypic revertants of ldlC arise spontaneously at a frequency of 1.2×10^{-6} (Reddy and Krieger, 1989), and both ldlB and ldlC cells can be corrected by DNA transfer (Kingsley et al., 1986b; Podos et al., 1994 and chapter 2 of this thesis). Thus, the multiple defects of ldlB and ldlC cells can be ascribed to single mutations.

Consideration of the ldlB and ldlC mutant phenotypes has led to the suggestion that these mutations affect general properties of the Golgi environment, such as ionic or protein compositions and distributions (Kingsley et al., 1986a). If this proposition proves correct, it will carry broad implications for the activities and organization of the eukaryotic organelles. To fully consider this proposition I will review some major features of the Golgi apparatus, before returning briefly to ldlB and ldlC. As many aspects of Golgi function have been extensively reviewed in the literature, my view will be selective and personal.

Glycosylation in the Golgi.

A complete description of glycosylation reactions in the Golgi is clearly beyond the scope of this thesis. However, I will draw some major principles. Protein and lipid glycoconjugates of all forms are built by sequential reactions within the secretory organelles. The N-linked glycosylation pathway in particular has been extensively reviewed (e.g., Kornfeld and Kornfeld, 1985). N-linked carbohydrates are branched structures which are first added as prefabricated cores within the ER. These cores are synthesized on dolichol lipids in the ER membrane, and are transferred *en bloc* to nascent proteins. These carbohydrates are then remodeled into mature forms, by combinations of trimming and addition reactions within the ER and Golgi. The O-linked and lipid-linked chains tend to be less complex than N-linked chains, and are built directly on their substrates with no lipid-linked intermediate (Carraway and Hull, 1989). These pathways are less clearly understood than the N-linked glycosylation pathway. The O-linked carbohydrates on LDL receptors have been examined in A431 cells. These oligosaccharides consist of galactose and GalNAc, with one or two terminal sialic acid residues (Cummings et al., 1983). The major glycolipid in CHO cells is GM_3 (sialyl-galactosyl-glucosylceramide) (Yogeeswaran et al., 1974). Lastly, it should not be forgotten that

glycosaminoglycans and proteoglycans are also assembled within the secretory pathway.

Oligosaccharide complexity is a product of the innate specificities of the glycysoltransferase and glycosidase enzymes, and also of their spatial segregation across the secretory pathway. Many glycosidase and glycosyltransferase activities have been identified biochemically and by cloning (Paulson and Colley, 1989). Many have also been localized to particular secretory compartments, such as by density fractionation (e.g., Dunphy and Rothman, 1983; Goldberg and Kornfeld, 1983) or by immuno-electron microscopy (e.g., Roth and Berger, 1982; Dunphy et al., 1985). The overriding conclusion from these experiments is that Golgi organization is reflected in the organization of glycoconjugate assembly. Spatial distribution of these enzymes therefore must be an important determinant of temporal order within glycosylation pathways. A recent double immunolocalization experiment suggests that the distributions of two glycosyltransferases can differ yet have substantial overlap (Nilsson et al., 1993). Thus the distributions of glycosylation enzymes within the Golgi need not be discrete. This observation likely derives from the mechanisms by which resident Golgi proteins are localized, to be considered later in this chapter.

In all glycosylation pathways, carbohydrate building blocks are presented in the form of nucleotide sugar substrates. These are synthesized by nucleotidylyl transferases in the cytoplasm (or in the nucleus, in the case of CMP-sialic acid). They are then transported into the Golgi cisternae by specific translocators (Hirschberg and Snider, 1987). These translocases have been defined primarily as transport activities within purified Golgi vesicles. Studies with radiolabeled substrates have established that the nucleotide sugars are imported intact, with obligate export of the corresponding nucleoside monophosphates (NMPs). As most nucleotide sugars liberate nucleoside diphosphates (NDPs) rather than NMPs, as for example UDP is liberated from UDP-galactose, the corresponding NMPs must therefore be generated by hydrolysis. Nucleoside diphosphatase (NDPase) activities in the Golgi are proposed to generate the NMPs, and thus to support antiporter activity (Abeijon et al., 1993; Berninsone, et al., 1994; Milla et al., 1992). Reconstitution and enrichment efforts have been commenced for Golgi transporters, but have not yet resulted in the purification of single transporter activities (Milla et al., 1992). Evidence suggests that each nucleotide sugar may utilize its own specific transporter. For example the Lec8 mutant is defective in the translocation of UDP-galactose but not of UDP-GalNAc or UDP-GlcNAc into Golgi vesicles (Deutscher and Hirschberg, 1986).

The functions of protein glycosylation are not easily generalizable. Wild-type processing is clearly not essential for viability of cells in culture, as shown by the existence of many distinct glycosylation mutants. However, glycosylation can contribute to processes as diverse as protein folding in the endoplasmic reticulum, mature glycoprotein conformation and stability, and cellular adhesion during morphogenesis or inflammatory responses (see for example Helenius, 1994; Jentoft, 1990; Brandley et al., 1990; Opdenakker et al., 1993). Cell

differentiation is often accompanied by changes in surface carbohydrates, and expression of glycosylation enzymes can be regulated according to the differentiation status of cultured cells (Datti and Dennis, 1993). N-linked glycosylation can influence the folding of nascent proteins in the endoplasmic reticulum, whether directly or through chaperones which respond to cycles of glucose addition and trimming (Helenius, 1994). A recent report of sequences related to sugar-binding plant lectins, both in the VIP36 protein in the Golgi and in the ERGIC-53 protein in the intermediate compartment between the ER and Golgi, suggests that these proteins may bind carbohydrates to regulate the transport or sorting of glycoproteins and glycolipids (Fiedler and Simons, 1994). The common theme in all of these processes is that glycosyl moieties present on glycoproteins contribute to the structure and function of proteins. In general, it appears that glycosylation expands the repertoire of conformations and surface properties available to proteins and lipids. Thus if biological events can be reduced to intermolecular recognition, then the overall importance of oligosaccharides to cellular processes must be acknowledged.

Organization and the dynamic Golgi.

The glycosylation events of the previous paragraphs, although compartmentalized along the secretory pathway, are conducted within a context of extensive membrane exchange. Membrane transport among the secretory organelles has been described with remarkable molecular and biochemical precision over the past decade, with advances continuing at dramatic pace. This work has benefited from a confluence of biochemical and genetic experiments, the former most extensively in mammalian cells and the latter primarily in yeast (Rothman and Orci, 1992; Pryer et al., 1992). Supporting the premise of transport by vesicular carriers, both approaches have allowed the description of the discrete steps of vesicle formation, targeting, and fusion, which can be individually dissected (Balch et al., 1984a, b; Beckers et al., 1990; Kaiser and Schekman, 1990; Rexach and Schekman, 1991; Ostermann et al., 1993). *In vitro* transport assays, particularly the intra-Golgi assay developed by Balch and Rothman and colleagues, have led to complete fractionation of the cytosolic proteins and also a description of energetic requirements, both for formation and for consumption of transport vesicles. Furthermore, a biochemical description of ER to Golgi transport in yeast is underway, using the collection of secretion mutants to great effect (Pryer et al., 1992; Barlowe et al., 1994). Many of the soluble transport proteins are conserved among yeast and mammals, as revealed by sequence similarity or even functional interchangeability; these include budding factors such as the coatomer proteins (Waters et al., 1991; Hosobuchi et al., 1992), and fusion proteins such as N-ethylmaleimide sensitive factor (NSF) and the soluble NSF attachment proteins (SNAPs) (Wilson et al., 1989). The biochemical reconstitution of the integral membrane activities is thus far less advanced. However, the availability of purified soluble factors has allowed affinity purification of a group of integral membrane constituents designated SNAP receptors or SNAREs (Söllner et al., 1993a). The diversity of SNARE isoforms has suggested that these proteins may contribute to the fidelity of vesicle targeting (Söllner et al., 1993b; Calakos

et al., 1994). Remarkably, the SNAREs share sequence similarity with a collection of proteins purified from synaptic vesicle membranes, as well as with yeast gene products (Bennet and Scheller, 1993). Thus, components of transport are conserved among membrane trafficking events throughout the cell (Pelham, 1991). Although each transport event is specific in terms of donor and target membrane identities, mechanisms are probably conserved.

A recurring theme in membrane transport is regulation by GTPase cycles. Vesicle coating and uncoating by Golgi coatomer and clathrin are both regulated by the small GTPase ADP-ribosylation factors (ARFs) (Robinson and Kreis, 1992; Donaldson et al., 1992; Stamnes and Rothman, 1993; Traub et al., 1993). Similarly, transport vesicles bound from the ER to the Golgi are coated with a newly recognized complex, called COPII, which is assembled and disassembled according to the GTP hydrolysis cycle of the small GTPase Sar1p (Barlowe et al., 1993a; 1994). The activity of Sar1p was first recognized in yeast, but has since been shown to be a universal factor for ER to Golgi transport (Kuge et al., 1994). Additional GTPases involved in transport including small GTPases such as Ypt1p and the rab proteins, and also trimeric G proteins. GTP hydrolysis cycles may allow for the precise regulation over the timing and extent of membrane fission events. It should be noted that GTPases are inherently regulable, as each cycle has several steps which can be controlled, and thus GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GNEFs) are likely to be involved in transport events (Yoshihisa et al., 1993; Barlowe and Schekman, 1993b).

In addition to constitutive flow of membranes, the dynamic nature of the Golgi is shown most dramatically by its ability to disassemble and accurately reassemble. The Golgi apparatus vesiculates during mammalian mitosis, and afterwards the fragments reassemble into the classical cisternae. When reconstituted *in vitro*, this process requires the coatomer complex (Misteli and Warren, 1994). Thus the normal flow of Golgi traffic and the regulated breakdown of the Golgi cisternae are mediated by common proteins. Although its visibly striking organization might suggest otherwise, the capacity to fragment is inherent in the normal activities of the Golgi. Extensive analysis with the drug brefeldin A (BFA) has led to the same conclusions (Klausner et al., 1992). Treatment with BFA causes the rapid disassembly of the Golgi apparatus into tubules and vesicles, by preventing GDP release from ARF and thereby blocking coatomer protein assembly onto budding vesicles (Takatsuki and Tamura, 1985, Fujiwara et al., 1988; Helms and Rothman, 1992; Donaldson et al., 1992). Therefore disruption of a biochemical step of normal membrane transport induces the dramatic reorganization of the Golgi compartments. By this action, BFA has played a significant role in uncovering the roles of coatomer and ARF proteins in membrane transport. Other proteins initially identified as membrane transport factors are capable of triggering a similarly dramatic Golgi disassembly. Ectopic expression of mutant rab or ARF constructs in which the GTPase hydrolysis is blocked also results in the dispersion of the Golgi stacks into small fragments (Dascher and Balch, 1994; B. Wilson et al., 1994). These observations reinforce the notion that the

capacity of the Golgi to disassemble is intimately connected to its normal interphase activities.

The somatic cell genetics analysis in the Krieger laboratory has established a further link between Golgi integrity and the control of membrane transport. ldlF mutants display temperature-sensitive defects in global protein secretion, in which the Golgi disassembles at the restrictive temperature (Hobbie et al., 1994; Guo et al., 1994). A cDNA cloned for its ability to fully correct the ldlF defects encodes the coatomer protein ε-COP (Guo et al., 1994). Therefore, the physiological membrane transport machinery is involved in a non-physiological membrane rearrangement. It remains to be seen whether the ldlF mutation lies in the ε-COP gene, or whether ε-COP overexpression confers an extragenic suppression. Either way ldlF is likely to prove useful to the study of membrane transport in the secretory system, such as for the study of coatomer complex assembly or stability, or for the identification of additional proteins which interact with coatomer proteins.

Reconciliation:

The two pictures of the Golgi apparatus presented above are in striking contrast. On the one hand, orderly arrangements of compartments and glycosylation activities are maintained. On the other hand, continual membrane flux and the capacity for sudden collapse are inherent. Therefore, it is clear that transport events must be tightly regulated, temporally and spatially, to maintain the balance of transport and the overall organization of the membranes. In addition to maintaining a balanced flux of lipids, mechanisms are required to maintain the steady state distributions of resident Golgi proteins. GTPase cycles are likely to be involved in the control of vesicle budding and fusion, but the input signals are unknown. Further characterization of the GTPase cycles is warranted, with particular interest in regulatory input such as GAP proteins and other accessory factors. GTPases may also help regulate the composition of transported membranes. Additional retention or retrieval mechanisms are likely to contribute to the localization of resident Golgi proteins. A variety of distinct mechanisms have been proposed for localization of Golgi proteins, including homotypic interactions, sensitivities of transmembrane domains to lipid composition, and interactions of cytoplasmic domains with specialized machinery (Weisz et al., 1993; Bretscher and Munro, 1993; Pelham and Munro, 1993; Nothwehr et al., 1993). Further analysis will be required to determine the relative contributions of these and other mechanisms to the localization of glycosylation enzymes within the Golgi.

ldlB and ldlC in Context.

The presumptive *LDLB* and *LDLC* genes influence multiple Golgi activities. Their activities must therefore be considered within the context of the discussion above. Therefore in the final chapter of this thesis (Chapter 6:

Discussion) I will attempt to place the experimental results and their implications in their larger context.

Chapter 2.

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

This chapter presents a series of experiments which focus on the function of the *LDLC* gene and its protein product ldlCp. This chapter was written by me and Dr. Krieger, as a manuscript for publication. I wrote all the original drafts, and Dr. Krieger and I edited all sections together. It has been published in the Journal of Cell Biology. The only modification made to this chapter for presentation here is that the references have been combined with references for other chapters, and are presented later within the thesis. Also, the figures and table have been renumbered with the prefix "2." to reflect the place of this chapter within the thesis.

The experiments described in this chapter start with the cloning of the human *LDLC* cDNA. This work was initiated by Dr. Pranhitha Reddy, then a post-doctoral fellow in the laboratory. Dr. Reddy performed the serial transfections of human genomic DNA through the ldlC mutant, to generate the primary, secondary, and tertiary LETC (LDL Endocytosis Transfectants of ldlC) cells. She also identified the common band within the LETC cells by hybridization to a human *Alu* repetitive element, isolated the corresponding EcoRI restriction fragment, and prepared a preliminary restriction map of this genomic clone. Working with Dr. John Ashkenas, then a graduate student in the laboratory, I refined the restriction map and prepared further probes with which to identify transcription units. I then identified the *LDLC* transcript, prepared the HeLa cDNA libraries, cloned the corresponding cDNA, and performed all subsequent experiments with the *LDLC* gene, *LDLC* mRNA, and ldlCp protein as described.

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

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Running title: ldlCp and Golgi Function

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Abstract.

Two genetically distinct classes of low density lipoprotein (LDL) receptor-deficient Chinese hamster ovary (CHO) cell mutants, ldlB and ldlC, exhibit nearly identical pleiotropic defects in multiple *medial* and *trans* Golgi-associated processes (Kingsley et al., 1986a). 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 homolog 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.

Introduction.

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 (Rothman and Orci, 1992; Hirschberg and Snider, 1987; Kornfeld and Kornfeld, 1985), 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 Chinese hamster ovary (CHO) cells with defects in LDL receptor activity (Krieger, 1983; Krieger et al., 1981,1983,1985; 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 abnormal post-translational processing of the receptor in the Golgi (Kingsley et al., 1986a). 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., 1986a; Reddy and Krieger, 1989).

ldlB and ldlC cells 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

[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.

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, 1985a; 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 post-translational 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* homolog 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 Laboratories, Grand Island, NY); $Na^{125}I$ (Amersham, 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 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 immunofluorescent 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., 1986a; Kingsley et al., 1986b) 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% (v/v) (medium B) or 10% (v/v) (medium C) FBS. Human HeLa and murine NIH 3T3 cells were obtained from P. Sharp and F. Solomon, M.I.T.. 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% (v/v) 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% (v/v) newborn calf lipoprotein-deficient serum) supplemented with MeLoCo (250 µM mevalonate, 2.5 µg protein/ml low density lipoprotein (LDL), and 40 µM compactin). Compactin, an inhibitor of HMG-CoA reductase, prevents cholesterol synthesis by inhibiting all mevalonate synthesis, with the supplemental 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 hr 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 ($4x10^6$ 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 $2 X 10^8$ cells subjected to selection. Seven independent secondary LETC (2° LETC) colonies were then isolated from $2 X 10^8$ cells by a second round of the co-transfection/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 $6 X 10^8$ cells after a third round of co-transfection/selection, using a 2° LETC colony (2° LETC-I5) as the source of genomic DNA.

Cloning human *LDLC* cDNA.

A 3.5 kbp 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, 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, Piscataway, NJ) as previously described (Ashkenas et al., 1993). The cDNAs were ligated to EcoRI/NotI adapters (Pharmacia), size-selected (>1kbp) by sedimentation through 5-20% KOAc gradients, and packaged into EcoRI-digested λZAPII. Plaques ($5 X 10^5$) 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, 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' - CCCTCATCTT CTCAAGCTTT ACCTTCTAAC TTCTGCACCA CCAGAAATTA AATTGATGGG CTTTTAAAAT AAATTGGTTA

CCAATAATTT CC - 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* homolog of *LDLC*.

The *C. elegans* cDNA fragment CEESW90 (GenBank #T01892) was identified as a potential *LDLC* homolog (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. Six cDNA clones were isolated from 500,000 λZAP plaques, each clone being approximately 2.0 kbp. One clone was sequenced on both strands, and its sequence (EMBL accession no. 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 figure 4B).

Preparation of polyclonal anti-ldlCp antipeptide antibodies.

Peptides Npep (EKSRMNLPKGPDTLC) and Cpep (CAELVAAAKDQATAEQP) were synthesized containing the predicted N-terminal and C-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) pre-activated with m-maleimidobenzoic acid N-hydroxysuccinimide ester (Sigma), and these complexes were used to prepare polyclonal antibodies in New Zealand white rabbits. Pre-immune and immune IgGs were isolated on Protein A Sepharose (Pharmacia) columns and are designated pre-immune IgG, anti-Npep, and anti-Cpep. For some experiments, anti-Cpep was affinity-purified on a Cpep-agarose column prepared by coupling approximately 6 mg of Cpep to a 2 ml SulfoLink column (Pierce, 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 2x sample buffer with protease inhibitors (final concentrations: 60 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) 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% polyacrylamide SDS 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% (w/v) 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 3 times with buffer X (buffer W containing 0.05% (v/v) NP-40, and 0.1% (w/v) SDS) and 2 times in buffer W, incubated with ^{125}I-protein A (2-10 μCi/μg) in buffer W for 1-2 h, washed 2 times with buffer X and 3 times with PBS. Antibody binding was visualized by autoradiography. Control samples for Figures 2.5 and 2.8A 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°, fixed for 30 min at room temperature with 3.7% (v/v) formaldehyde in cPBS which was prewarmed to 37°, and quenched for 10 min at room temperature in 50 mM NH_4Cl. Cells were then permeabilized for 10 minutes 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, pre-blocked for 30 min at 37°C face-down 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 minutes 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 4 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 minutes, 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 Figure 2.7) 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 40X and 100X 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 LD_{10} values presented represent estimates of the lectin concentrations which result in the killing of approximately 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 (Krieger, 1983; Goldstein et al., 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 hr 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-C-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) or Harlow and Lane (1988), respectively. Southern blot analyses were performed using Zetabind nylon filters (CUNO, 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 Methods for details). In brief, human genomic DNA was transfected into ldlC cells, and LDL receptor-positive revertants which exhibited normal glycogonjugate 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, 1985a; 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/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 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 sixteen overlapping human cDNA clones from two HeLa cell cDNA libraries (see *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 1) dramatically reduced LDL receptor activity, 2) abnormal post-translational processing (glycosylation) of LDL receptors and their consequent instability, and 3) global defects in cell surface glycoconjugates (Kingsley et al., 1986a). 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 2.1 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 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.

Figure 2.1 shows the post-translational 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 was rapidly converted to a ~155 kD mature form ("m") (Figure 2.1, upper panel). 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 (Figure 2.1, upper panel, 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 (Figure 2.1, middle panel 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 (Figure 2.1, bottom panel), LDL receptor post-translational 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 post-translational 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 2.1 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 homolog 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 (Figure 2.2, upper panel). The human *LDLC* probe recognized a single mRNA band of approximately 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 homologs. 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 (Figure 2.2, bottom panel). 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.

Table 2.1. LDL Receptor Activities and Lectin Sensitivities of ldlC Transfectants.

Cells	LDL Receptor Activity* (ng/5 hr/mg)	Lectin Sensitivities (LD_{10})¥ WGA (μg/ml)	ConA (μg/ml)	PHA (μg/ml)	Ricin (ng/ml)	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 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 approximately 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). The lectins are abbreviated as follows: WGA, wheat germ agglutinin; ConA, concanavalin A; and PHA, phytohemagglutinin.

Figure 2.1 (facing page).

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 minutes, 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 Methods. The immunoprecipitates were reduced with β-mercaptoethanol, and analyzed by 6% polyacrylamide SDS 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.

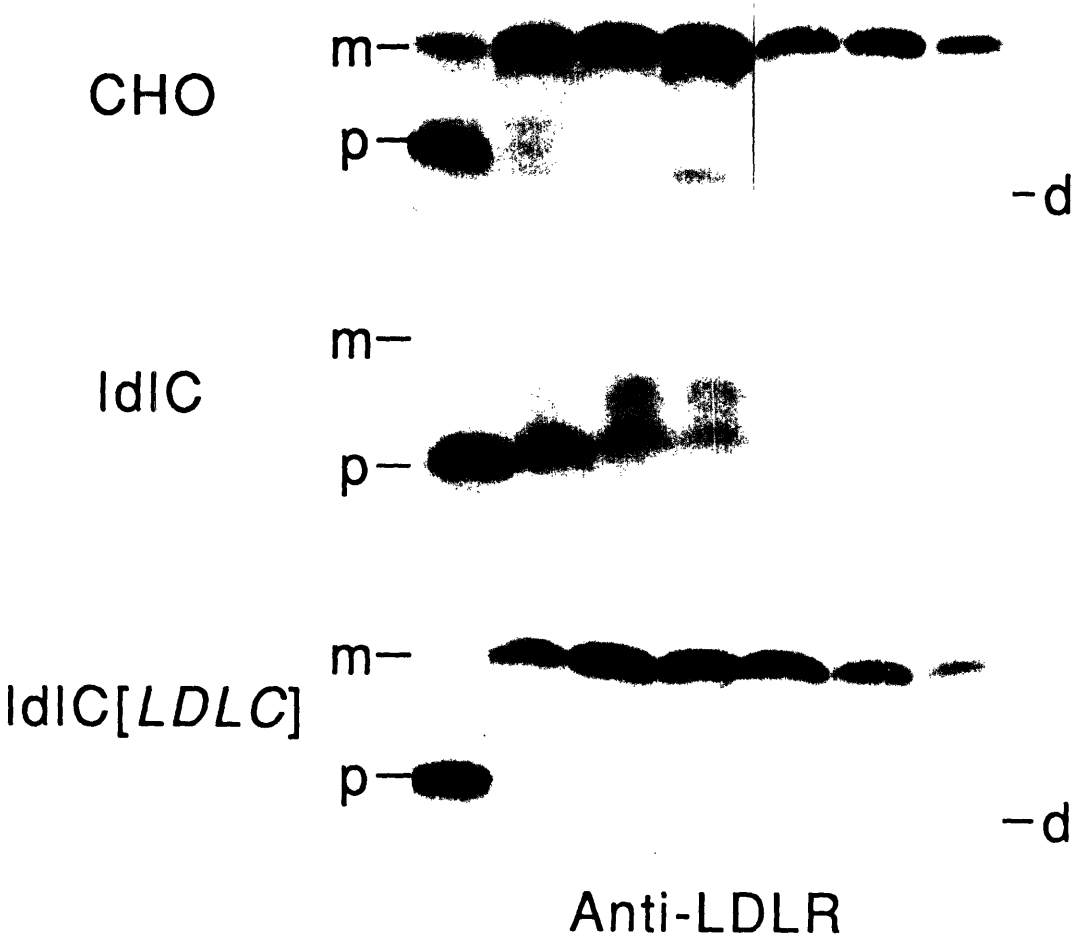
Figure 2.2 (immediately following figure 2.1).

Northern Blot Analysis of *LDLC* mRNA.

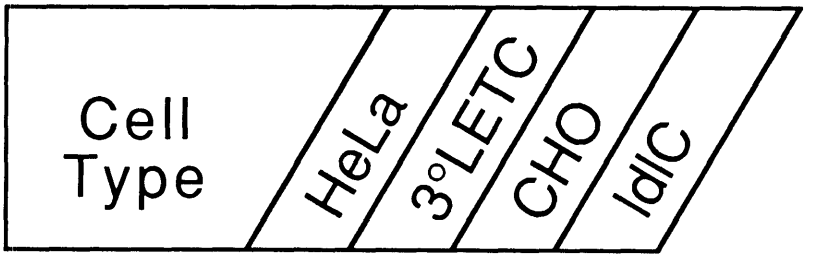
Poly(A)$^+$ RNAs from the indicated cells were prepared and subjected to Northern blot analysis as described in Materials and Methods. Upper panel: The filter was probed with a ^{32}P-labeled fragment of plasmid p*LDLC*-1 that contained the full open reading frame. The hybridization and washing conditions 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: 2X15 min at room temperature in 300 mM phosphate buffer (pH 7.0); 2X15 min at 60° in 300 mM phosphate buffer, 5% SDS, 5 mg/ml BSA, 1 mM EDTA; and 2X15 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. Lower panel: The same filter was stripped and reanalyzed using a portion of β-tubulin cDNA as a probe.

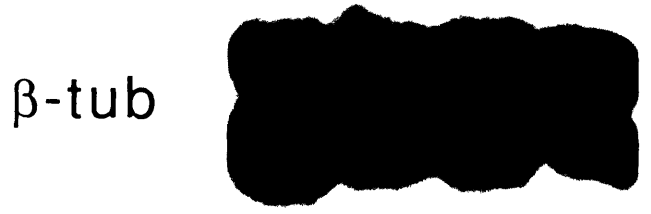
Synthesis and Processing of LDL Receptors in *LDLC* Transfectants

Pulse (h)	0.5						
Chase (h)	0	0.5	1	2	4	8	24



Northern Blot Analysis of *LDLC* mRNA





Results (continued).

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. Figure 2.3 presents the *LDLC* nucleotide and predicted ldlCp protein sequences. The sequence surrounding the putative initiator methionine (amino acid #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 7 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 Figure 2.3).

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.

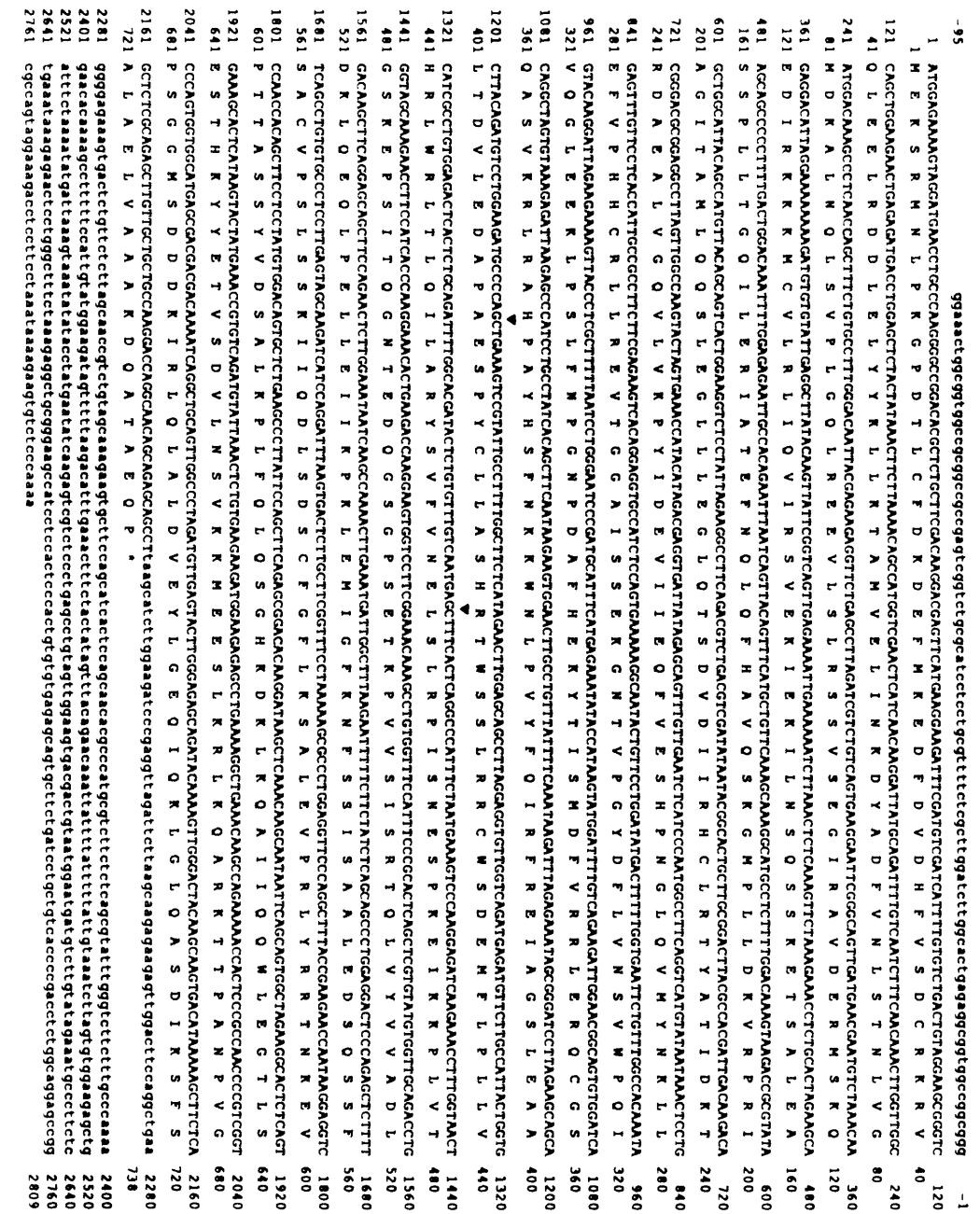
We have found no notable sequence similarities between *LDLC* and known genes reported in databases such as GenBank and EMBL. However, the *LDLC* cDNA sequence was significantly similar to three expressed sequence tags (ESTs), cDNA fragments which were cloned and sequenced at random. Two of the ESTs 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). 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* homolog 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.

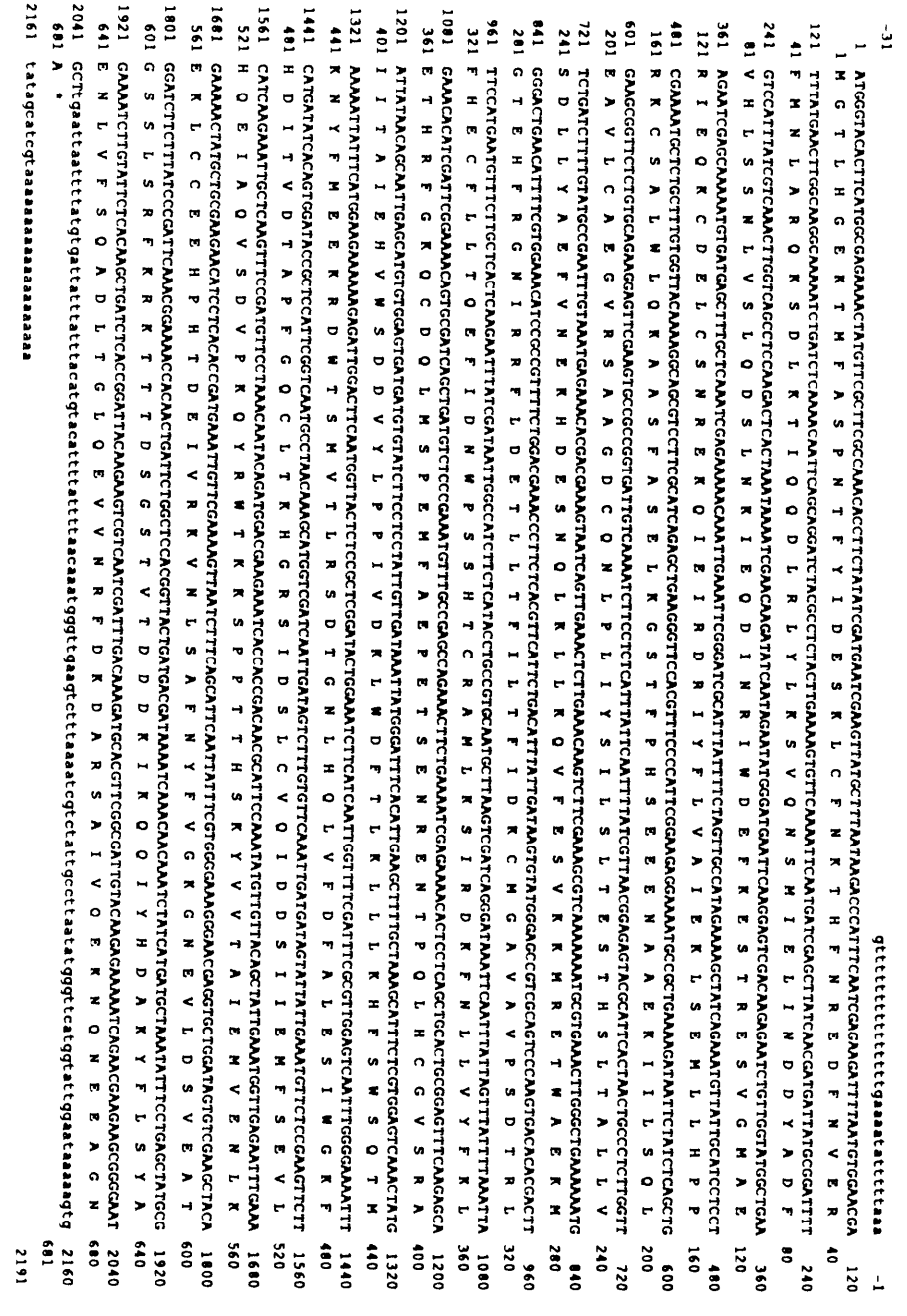
Furthermore, the predicted amino acid sequence within this region is 49% identical and 70% similar to the human ldlCp sequence. Therefore, the gene represented by this EST was a good candidate for an invertebrate homolog of the *LDLC* gene. To characterize the putative homolog, we used this EST to isolate six *C. elegans* cDNA clones. Each was approximately 2.0 kbp long, and they all had similar restriction maps. One clone was sequenced fully on both strands (see Figure 2.4A). Its 2222 base sequence includes an open reading frame of 681 codons from the first methionine (Figure 2.4A). 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 homologs are 26% identical and 53% similar when aligned as in Figure 2.4B. 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 Figure 2.4A 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.

Figure 2.3. Nucleotide (upper line) and predicted amino acid (lower line) sequences of human *LDLC* cDNA.



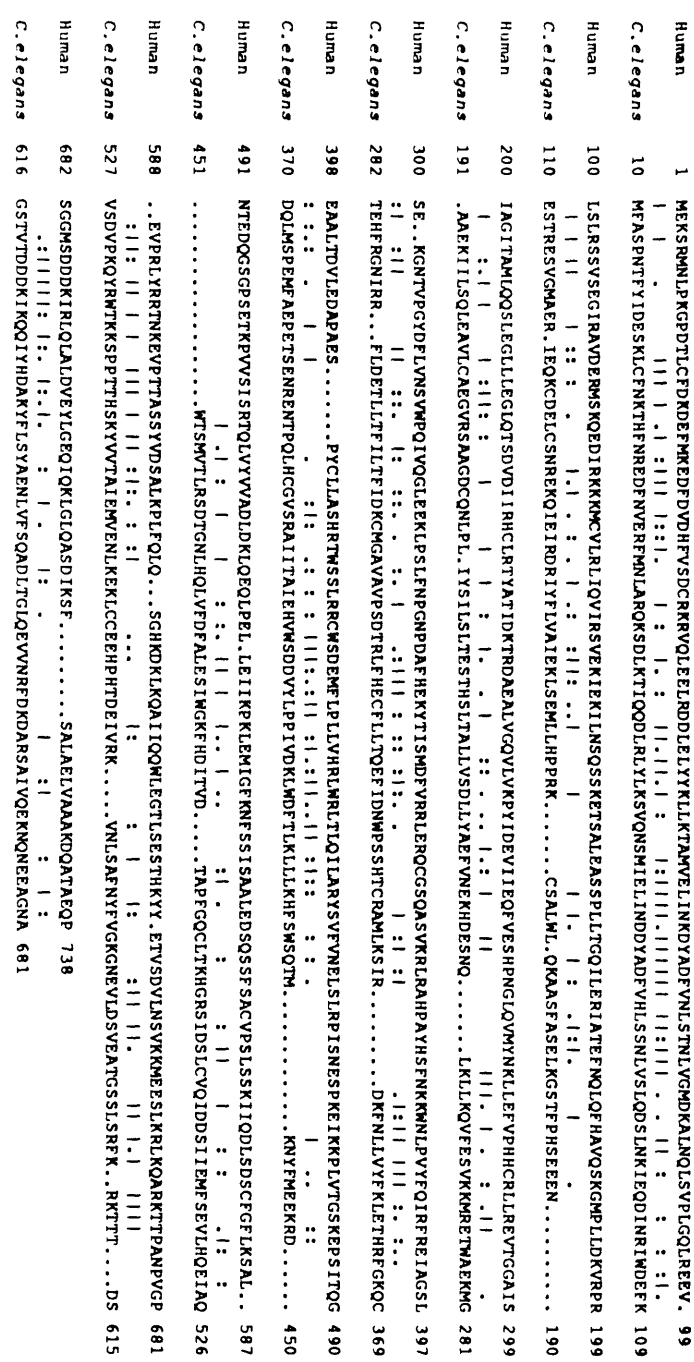
The human *LDLC* cDNA was cloned and sequenced and the sequence was analyzed as described in Methods. The nucleotide sequence is numbered so that the presumptive initiator codon starts at base 1. The arrowheads designate the positions of two introns. These were identified by sequencing a portion of the 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.

Figure 2.4. 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*).



Continuation and legend on facing page.

Figure 2.4 (continued).



(*A*) The *C. elegans LDLC* cDNA was cloned and sequenced as described in 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.

Results (continued).

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 approximately 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. Figure 2.5 compares the immunoblotting patterns of pre-immune IgG (p) and anti-Cpep (C), measured in the absence (-) or presence (+) of an excess of the Cpep peptide. Purified anti-Cpep, but not pre-immune IgG, bound to an ~76 kD protein in both human HeLa cell and murine 3T3 cell lysates (anti-Cpep, lanes 2 and 5; pre-immune IgG, lanes 1 and 4). This binding was competed by excess Cpep, suggesting that it may correspond to ldlCp (lanes 3 and 6). Anti-Cpep, but not preimmune IgG, also recognized two smaller species in the HeLa cell lysates (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 (lanes 7-9) The ~76 kD protein was not detected in lysates from ldlC cells (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 (Figure 2.2). 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 C-terminus of ldlCp is conserved among several mammalian species.

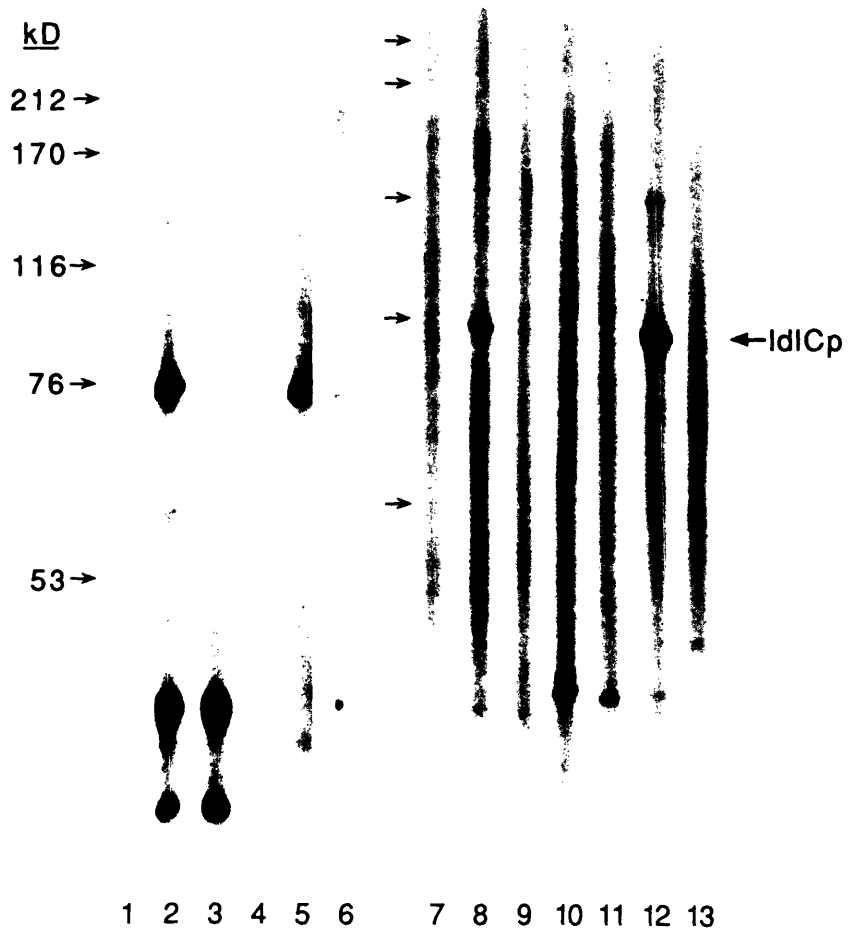
Figure 2.5 (facing page).

Immunoblot Analysis of IdlCp.

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 "IdlCp" (large arrow) indicates the position of the various mammalian IdlCp's, as described in Results.

Immunoblot Analysis of IdlC Protein

Cell Type	HeLa			3T3			CHO		IdlC	IdlC [*LDLC*]		
IgG : pre-immune (p) or anti-Cpep (C)	p	C		p	C		p	C	C	C		
Cpep Competition	-	-	+	-	-	+	-	-	+	-	+	-	+



Results (continued).

Immunolocalization of ldlC protein.

Immunofluorescence microscopy with affinity purified anti-Cpep was used to determine the distribution of ldlCp within wild-type CHO cells. Figure 2.6a (top left panel) 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 ten-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 pre-immune 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, Figure 2.6 (top row) also shows the staining of CHO cells with antibodies against two Golgi-associated proteins: β-COP (panel b) and mannosidase II (panel 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; Waters et al., 1991; Serafini 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 co-localized (panels 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; Lippincott-Schwartz et al., 1989,1990; Donaldson et al., 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).

Figure 2.6 shows ldlCp's redistribution following BFA treatment (left panels), compared with those of β-COP (center panels) and mannosidase II (right panels). After 2 minutes of BFA treatment (second row), perinuclear ldlCp was reduced but still evident, and the cytoplasmic staining increased (panel d). After 5 minutes (third row), only small remnants of perinuclear staining were observed (panel 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 minutes and dispersed after 5 minutes (panels e and h). In contrast, mannosidase II staining was largely unchanged after 2 minutes (panel f). After 5 minutes it had transformed into a more contiguous pattern which included some fiber-like projections (panel i), as previously described (Lippincott-Schwartz et al., 1990). Thus, after 5 minutes 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 minutes 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 minutes (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. Figure 2.7 shows that the distributions of β-COP (center panels) and mannosidase II (right panels) were essentially identical in all three types of cells, regardless of the presence or absence of ldlCp (left panels). 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 2.6 (following page).

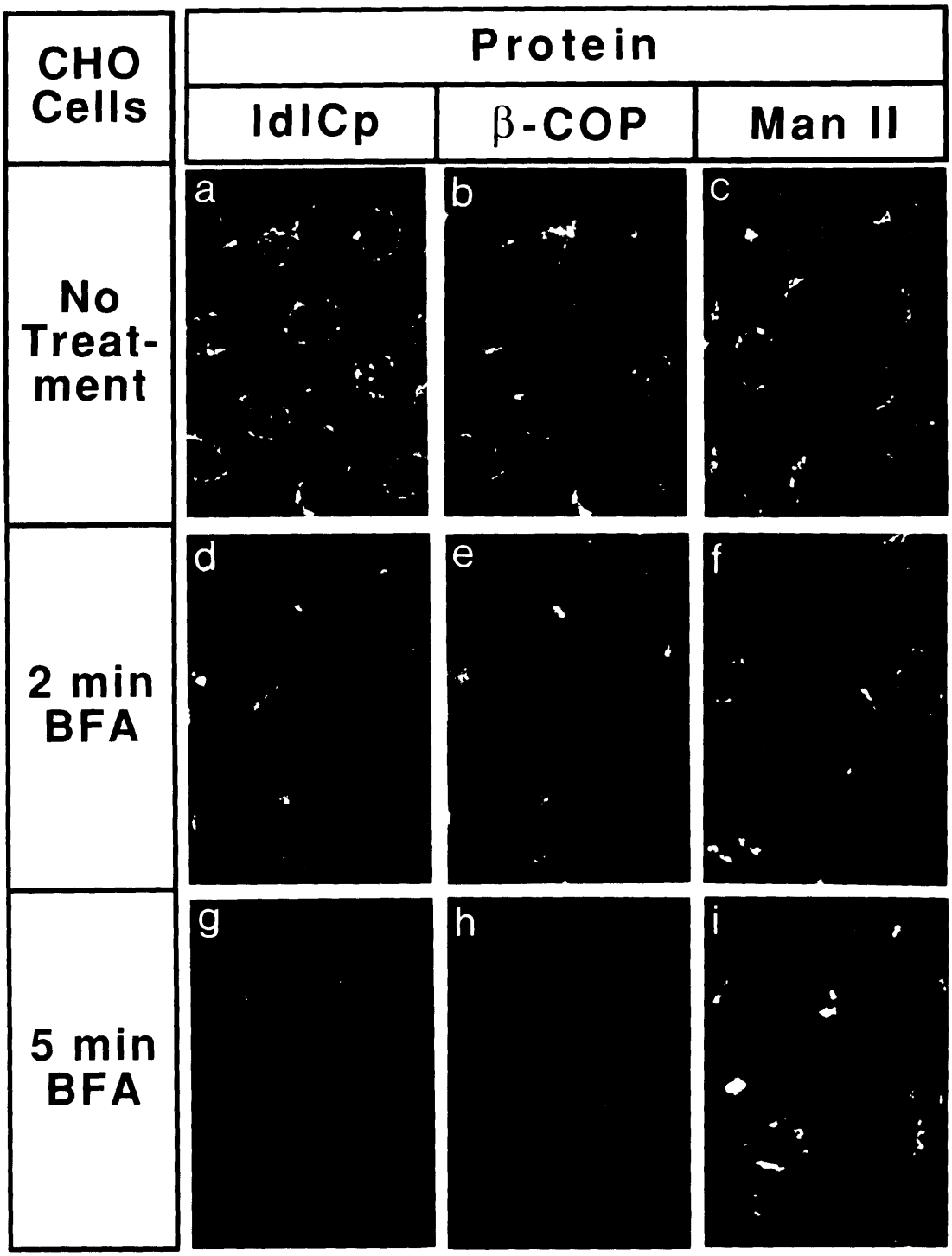
Immunofluorescence localization of ldlCp, β-COP,
and mannosidase II in CHO cells: effects of brefeldin A (BFA).

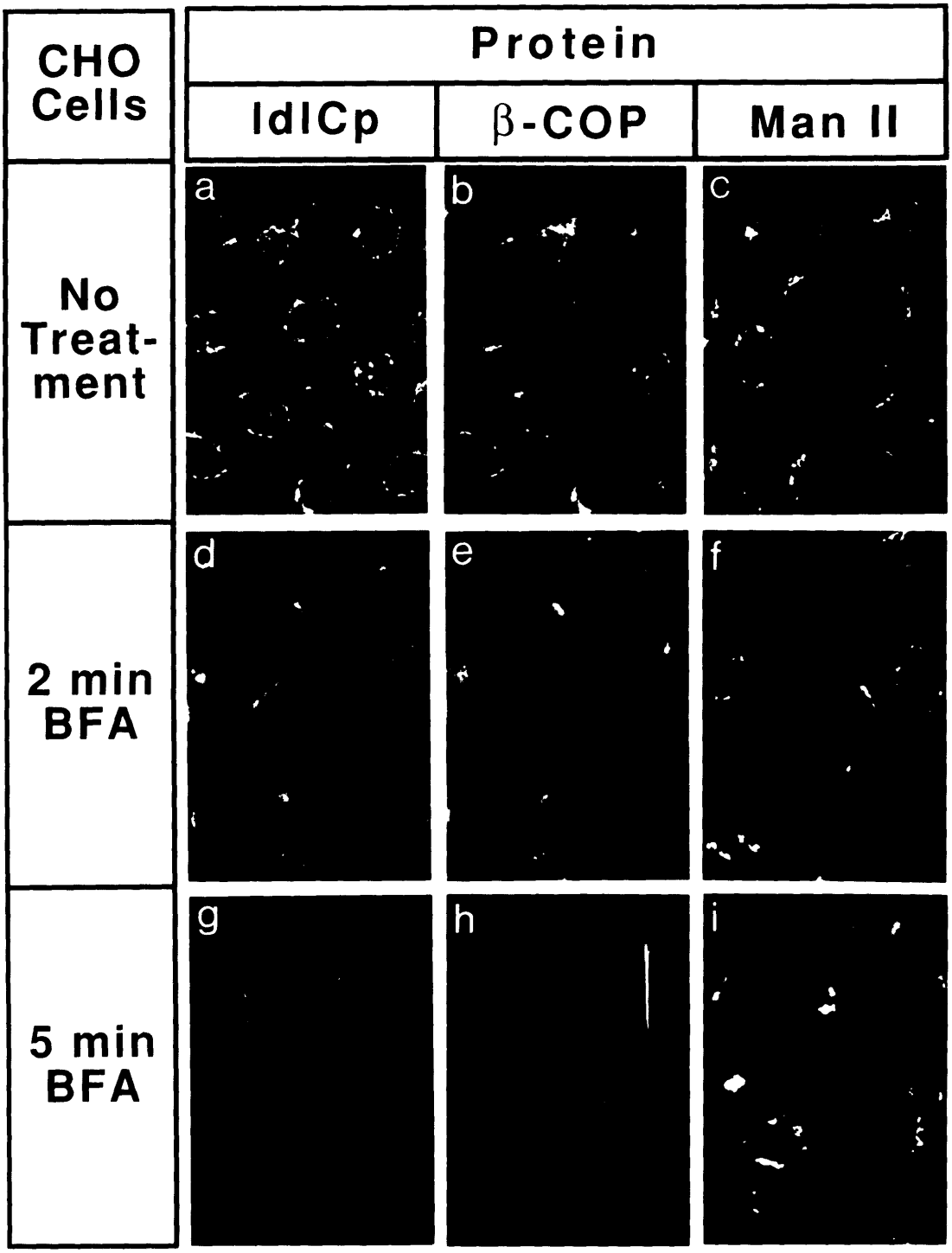
CHO cells were grown on glass cover slips as described in Methods. Prior to fixation and immunostaining, the cells were treated as follows: No additions (panels a, b and c); or incubation with 5 μg/ml BFA for two (panels d, e and f) or five (panels g, h and i) minutes. Cells were immunostained with peptide affinity-purified anti-Cpep (panels a, d and g), anti-β-COP monoclonal antibody M3A5 (panels b, e and h), and anti-mannosidase II (panels c, f and i) as described in Methods. Specimens were simultaneously double stained with the anti-Cpep and anti-β-COP antibodies, and the corresponding identical fields are shown.

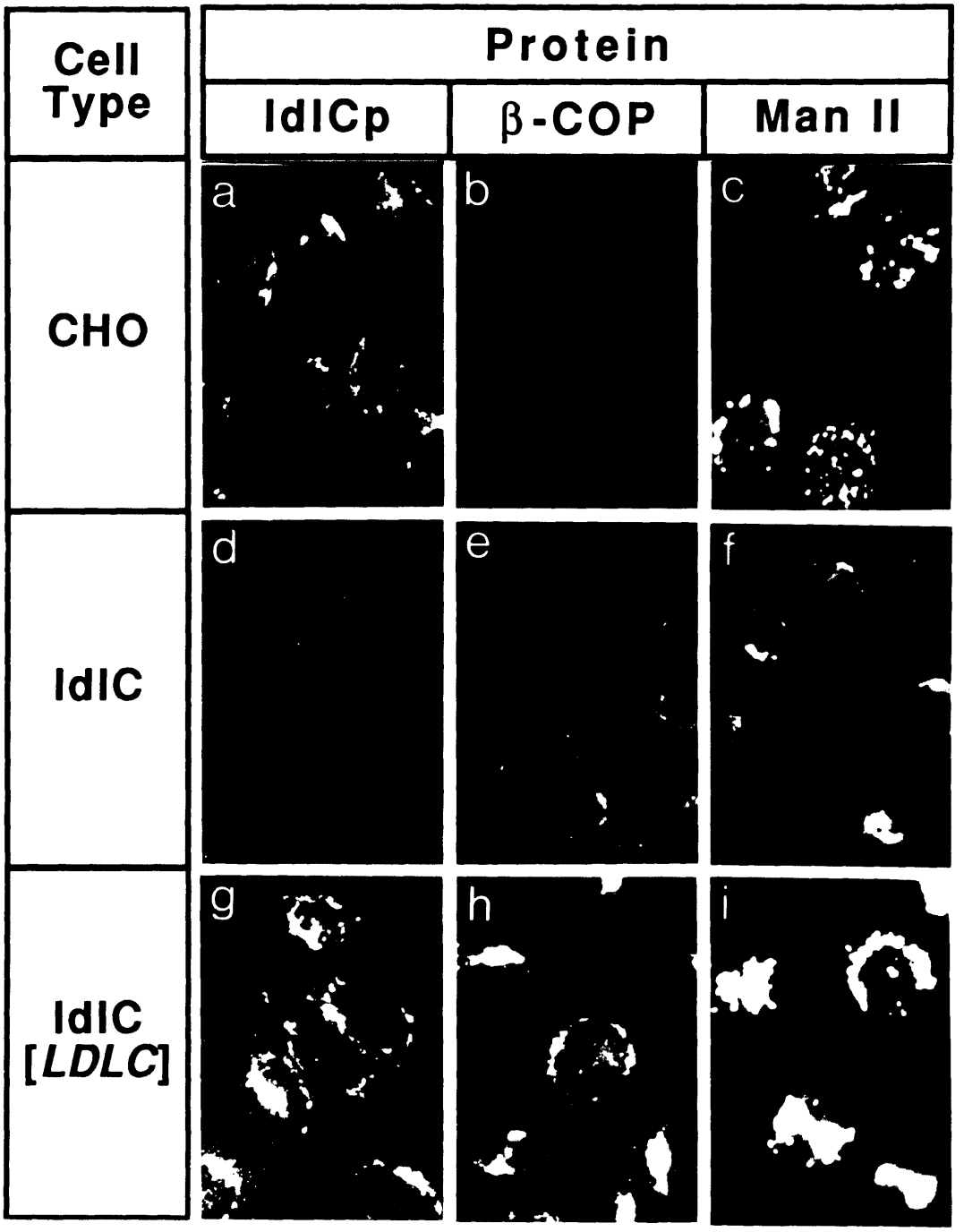
Figure 2.7 (immediately following figure 2.6).

Immunofluorescence 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 (panels a, d and g), anti-β-COP monoclonal antibody M3A5 (panels b, e and h), and anti-mannosidase II (panels c, f and i) as described in Methods.


CHO Cells
Protein
IdlCp
β-COP
Man II
No Treat-ment
2 min BFA
5 min BFA
a
b
c
d
e
f
g
h
i


CHO Cells
Protein
IdlCp
β-COP
Man II
No Treat-ment
2 min BFA
5 min BFA
a
b
c
d
e
f
g
h
i


Cell Type
Protein
IdlCp
β-COP
Man II
CHO
IdlC
IdlC [LDLC]
a
b
c
d
e
f
g
h
i

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 post-translational 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 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., 1986b).

Northern blot analysis (not shown) and immunoblot analysis (Figure 2.8A) 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. Figure 2.8B shows the immunofluorescence localization of ldlCp (left panels), β-COP (middle panels) and mannosidase II (right panels) 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 (*panel a*), ldlCp apparently did not localize to the Golgi apparatus in ldlB-11 cells (panel d). Instead, a uniform punctate background of 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., 1986a) (not shown). In addition, the normal Golgi distribution of ldlCp was restored in 2° LETB-144 cells (panel g). In both ldlB-11 and 2° LETB-144 cells, there were essentially wild-type distributions of β-COP (center column, panels b, e, and h) and mannosidase II (right column, panels 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.

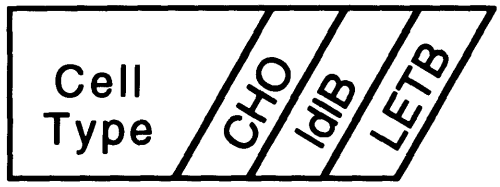
Figure 2.8 (facing page).

Immunoblotting (A) and immunofluorescence localization (B) of IdlCp, β-COP, and mannosidase II in CHO, IdlB, and LETB cells.

Panel 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.

Panel B: The indicated cells were grown on coverslips and immunostained with affinity purified anti-Cpep (panels a, d and g), anti-β-COP monoclonal antibody M3A5 (panels b, e and h), and anti-mannosidase II (panels c, f and i) as described in Methods.

A



1 2 3

B

Cell Type	Protein		
	IdlCp	β-COP	Man II
CHO	a	b	c
IdlB	d	e	f
LETB	g	h	i

Discussion.

Three distinguishing characteristics of ldlC cells are their 1) dramatically reduced LDL receptor activity, 2) abnormal post-translational processing (glycosylation) of LDL receptors, resulting in receptor instability, and 3) 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, ldlB 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 intralumenal environment of the Golgi apparatus (Kingsley et al., 1986a).

In the current study, we cloned a human *LDLC* cDNA which corrects the mutant phenotypes in 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 homolog 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 expressed sequence tag (EST) cDNA fragment from the nematode *Caenorhabditis 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 homologs 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.

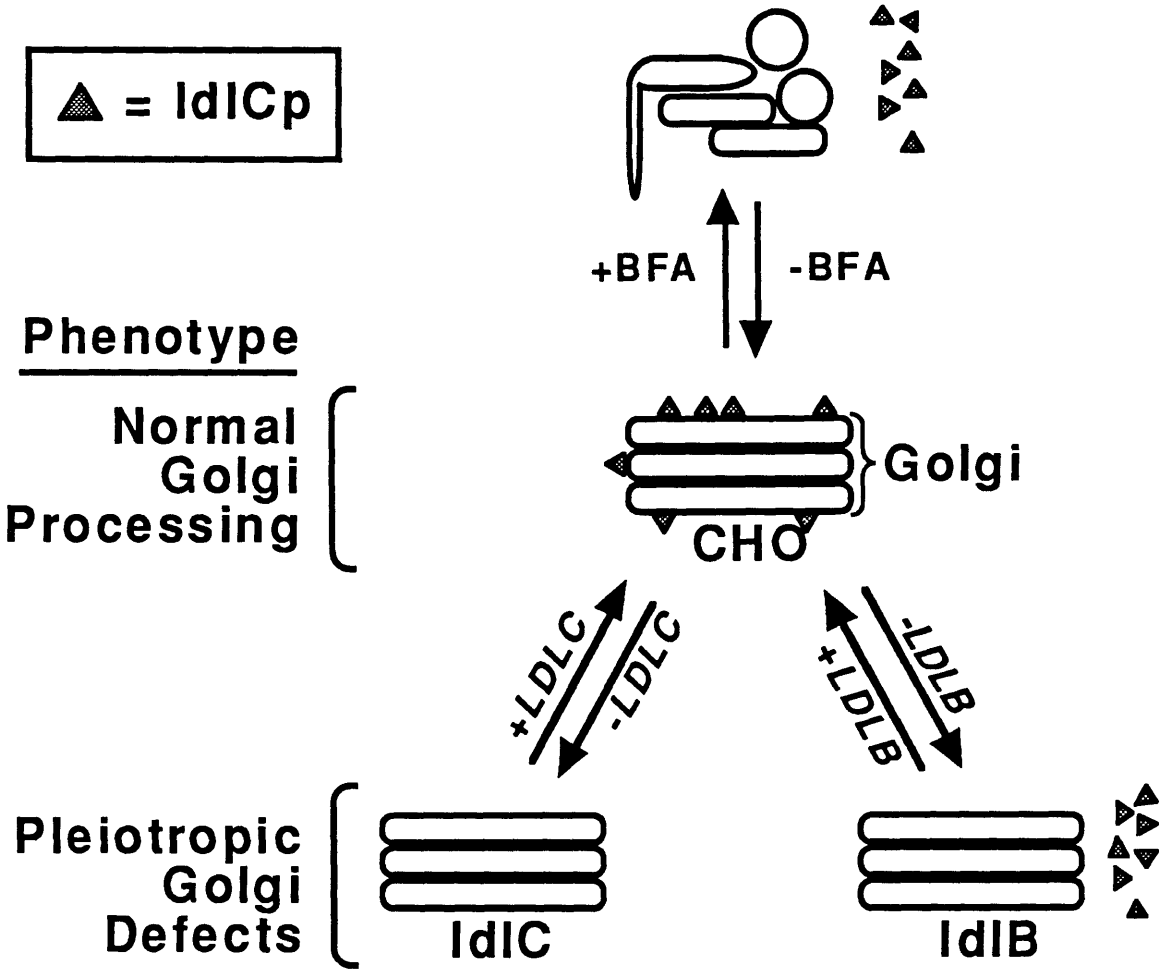
The predicted sequence of ldlCp has no major common structural motifs such as GTP binding sites, transmembrane domains, or an ER translocation signal sequence. This suggests that ldlCp is a cytoplasmic protein. Nevertheless, immunofluorescence studies indicated that ldlCp may be associated with the cytoplasmic face of the Golgi, as it co-localized with Golgi markers and was rapidly redistributed from the Golgi by the drug brefeldin A (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 (see, for example, Klausner et al., 1992; Donaldson et al., 1992; Helms and Rothman, 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).

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., 1986a), 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 ldlCp or to the loss of functional ldlBp, normal Golgi processing reactions are disrupted (see Figure 2.9). 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.

Figure 2.9. Model of the effects of brefeldin A treatment and ldlC and ldlB mutations on ldlCp and Golgi function.



ldlCp (triangles) is a brefeldin 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).

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References.

[The references from this manuscript have been incorporated into the overall reference section, at end of thesis.]

Chapter 3.

Bioactivity of the *LDLC* cDNA in ldlC Cells.

Abstract.

In chapter 2, the human *LDLC* cDNA was cloned and its activity in ldlC cells was established by transfection. The current chapter extends this line of experimentation. Specifically, several variant *LDLC* expression plasmids were constructed, and new experimental strategies were developed to assess their relative bioactivities when transfected into ldlC cells. These expression plasmids incorporated the following variables: *LDLC* was inserted in the antisense as well as sense orientation; frameshift and nonsense mutations were incorporated into the *LDLC* cDNA; and, vector sequences were altered independent of the *LDLC* insert. The following conclusions are drawn: 1) The *LDLC* cDNA reproducibly restores both the glycosylation and the LDL receptor phenotypes in ldlC cells; 2) The *LDLC* cDNA is effective in the antisense direction in pRc/CMV, likely due to the presence of a cryptic promoter activity within this construct. Furthermore, the suggestion is raised that first 60 amino acids of the human ldlCp may be dispensable for its activity. The latter prospect is considered further, as this region of ldlCp is well conserved between the human and nematode sequences.

Introduction.

In chapter 2, I presented the isolation of the human *LDLC* cDNA based upon its capacity to correct the mutant phenotypes of IdlC cells. The cloning strategy relied upon the physical linkage of the *LDLC* gene to an *Alu* repetitive element within the LETC genomes. This strategy carried the inherent possibility that linked but irrelevant genes could be cloned in place of *LDLC*, on the basis of their linkage to the same *Alu* repetitive element. Therefore, a demonstration of biological activity was required to confirm the identity of the cloned *LDLC* cDNA. For such a demonstration, the *LDLC* cDNA was placed into mammalian expression vectors and transfected into IdlC mutant cells. LDL receptor and glycosylation phenotypes were examined in these transfected cells, and compared to parental IdlC mutant cells and to wild-type cells.

This chapter presents an elaboration of the *LDLC* transfection experiments first described in chapter 2. In the experiments presented here, *LDLC* cDNA expression plasmids were transfected into IdlC mutant cells, and various methods were implemented to determine their relative efficacies at restoring normal LDL receptor and glycosylation phenotypes to IdlC cells. As in chapter 2, transfection with the human *LDLC* cDNA corrected the full set of mutant phenotypes in IdlC cells. In this chapter, however, several variants of the *LDLC* expression plasmid were also shown to retain activity. These variants included the antisense *LDLC* expression construct in pRc/CMV, and several *LDLC* cDNA mutants bearing site-directed alterations. The results of these experiments are discussed, with consideration of the advantages and limitations of the transfection assay as a means of determining *LDLC* activities.

Materials and Methods.

Plasmids.

The following expression constructs were used in these experiments:

Construct	cDNA Insert*	Orientation	Host Vector¥
pSP21 (=p*LDLC*-1)	+	sense	pRc/CMV
pSP22	+	antisense	pRc/CMV
pSP38	"frameshift 60"	sense	pRc/CMV
pSP40	"frameshift 190"	sense	pRc/CMV
pSP41	"frameshift 190"	antisense	pRc/CMV
pSP43	+	sense	Δ pRc/CMV
pSP44	+	antisense	Δ pRc/CMV
pSP45	"nonsense"	sense	pRc/CMV
pSP47	+	sense	pcDNAI neo
pSP48	+	antisense	pcDNAI neo

*The cDNA inserts are defined and were constructed as follows. All were confirmed by sequence analysis.

+ HeLa *LDLC* cDNA, as described in the Materials and Methods section of Chapter 2.

"frameshift 60" Derivative of HeLa *LDLC* cDNA, with 4 bp duplication of positions 179 through 182. Prepared from *LDLC* by digestion with NcoI, filling in of the 4 base 5' overhangs, and ligation of the blunt DNA ends.

"frameshift 190" Derivative of HeLa *LDLC* cDNA, with 4 bp deletion of positions 568 through 571. Prepared from *LDLC* cDNA by digestion with SphI, digestion of the four base 3' overhangs, and ligation of the blunt DNA ends.

"nonsense" Derivative of HeLa *LDLC* cDNA, with stop codons in place of glutamate 21 and lysine 57. Stop codons were introduced by PCR amplification of the 5' portion of the cDNA, using an antisense primer in which lysine 57 was replaced with a nonsense codon (AAA --> TAA). Subsequent sequence analysis revealed that the stop codon at amino acid 21 (GAG --> TAG) was introduced fortuitously during the PCR amplification.

¥The expression vectors are defined as follows:

pRc/CMV	Commercially available (Invitrogen, San Diego, CA). The cDNA insert is transcribed from the cytomegalovirus (CMV) promoter. The bacterial neomycin resistance gene is transcribed from the simian virus 40 (SV40) early promoter.
Δ pRc/CMV	A variant of pRc/CMV which lacks the CMV promoter. Created by excision of the internal SpeI fragment from pRc/CMV. The excised fragment contains most of the promoter, including nuclear protein binding sites and the TATA box. This vector is not expected to transcribe any cDNA inserts.
pcDNAI neo	Commercially available (Invitrogen). The cDNA insert is transcribed from the cytomegalovirus (CMV) promoter, as in pRc/CMV. The bacterial neomycin resistance gene is transcribed from the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter.

Transfections.

Transfections were performed by the polybrene method, as described in chapter 2. Transfectant colonies were selected either in 250 μg/ml G418, or in 0.25 ng/ml ricin, in medium E as defined in chapter 2. G418 selects for the expression of the neomycin resistance gene in the expression vectors, and ricin selects for *LDLC* activity in ldlC cells. The designs of the particular transfection experiments and selections are presented in the Results section.

Other methods.

^{125}I-LDL degradation assays and LDL receptor immunoprecipitation assays were performed as described in chapter 2. MeLoCo growth assays were performed on transfectants by setting 50,000 cells onto 100 mm dishes, and subjecting to selection in MeLoCo (mevalonate/LDL/compactin) medium, which was prepared as described in chapter 2. MeLoCo medium selects for cells with sufficient levels of LDL receptor activity.

Results.

LDLC corrects multiple defects in ldlC cells.

The first experiments to be presented in this chapter utilized the expression constructs pSP21 and pSP22, which bear the *LDLC* cDNA in the pRc/CMV vector in the sense and antisense orientations respectively. pSP21 is identical to the plasmid p*LDLC*-1 described in chapter 2. For the first experiment, pSP21 and pSP22 were transfected with polybrene into two and one independent dishes respectively of ldlC cells, and colonies were obtained in G418 selection. Between four and ten colonies were mechanically picked from each transfection dish. ^{125}I-LDL degradation activity was assayed in cells from each of these colonies, to determine whether cDNA expression corrected the ldlC mutant phenotypes. The ^{125}I-LDL degradation values were determined during one or both of two degradation experiments, which are presented in table 3.1 as degradations *a* and *b*. The LDL receptor activity in the ldlC standard was 12% of the wild-type level in degradation *a*, consistent with previous results. The higher apparent activity in ldlC cells in degradation *b* (33% wild-type) was due in part to the unusually low activity measured in wild-type CHO cells. Of the eleven pSP21 transfectants assayed in these two experiments, at least seven demonstrated at least partially restored levels of LDL receptor activity. Therefore, transfection with pSP21 restored to normal this phenotype of ldlC cells in colonies from two independent transfection dishes. Of the three colonies transfected with pSP22, one (ldlC[pSP22]-4) demonstrated a partial restoration of LDL receptor activity. This surprising observation suggested that the antisense construct might confer *LDLC* activity upon ldlC cells.

The colonies from this transfection were further analyzed, to determine whether *LDLC* transfection restored the underlying glycosylation phenotypes. LDL receptor protein processing was examined by immunoprecipitation analysis after labeling with ^{35}S-methionine for five hours. Immunoprecipitates from selected colonies were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography (not shown, but similar to figures 2.1 and 3.1). Some transfectant colonies with pSP21 and with pSP22 showed LDL receptors of wild-type mobility. Therefore, the *LDLC* cDNA in either orientation restored the LDL receptor glycosylation of ldlC cells at least partially to normal.

The experiments above established that the *LDLC* cDNA could correct the mutant phenotypes of ldlC cells. These experiments, however, raised several questions about the activity of the *LDLC* cDNA. In particular, the activity of the antisense construct pSP22 remained to be established, and if reproducible its mechanism remained to be explained. Furthermore, the distribution of activities measured in the transfectant colonies suggested that ldlC cells harbor a sharp threshold for *LDLC* activity, above which the phenotypes are restored to normal. Thus transfectants exhibit either mutant phenotypes or wild-type phenotypes, depending upon the activity of the *LDLC* cDNA incorporated upon transfection. To address these issues, experiments were conducted to measure the activities of the *LDLC* constructs within larger sample sizes of transfectants. The sense construct (pSP21), antisense

construct (pSP22), and empty vector (pRc/CMV) were each transfected into four independent dishes of ldlC cells. For each plasmid, the four dishes were designated A, B, C, and D. Transfectant colonies were selected in G418 as before, but were then collected *en masse* from each transfection dish and assayed as populations. Approximately 100 G418-resistant colonies that arose from each transfection dish were collected, and ^{125}I-LDL degradation activities were determined for each population (table 3.1). ldlC control cells in this experiment were measured at 11% of wild-type levels. Transfection with the sense construct (pSP21) elevated the ^{125}I-LDL degradation activity to between 35% and 60% (average = 49%, σ = 9%), and transfection with the antisense construct (pSP22) elevated the ^{125}I-LDL degradation activity to between 23% and 62% (average = 37%, σ = 15%). In contrast, the empty vector (pRc/CMV) did not elevate the ^{125}I-LDL degradation activity in ldlC cells (average = 8%, σ = 1%). Therefore the *LDLC* cDNA, in either orientation with respect to the CMV promoter, significantly elevated the LDL receptor activity of at least a subset of transfected ldlC cells. These results were confirmed by a MeLoCo growth assay, performed as described in Materials and Methods. In this assay, substantial numbers of cells within the populations transfected with pSP21 or with pSP22, but not with pRc/CMV bearing no cDNA insert, survived MeLoCo selection, which requires sufficient LDL receptor activity for survival (results not shown).

The transfection experiment above established that the *LDLC* cDNA has the capacity to correct the LDL receptor deficiency in ldlC cells. To extend this observation, I examined the glycosylation phenotypes of the twelve transfected populations; specifically, I analyzed the processing of the LDL receptor glycoproteins. Cell populations were labeled with ^{35}S-methionine for five hours, and LDL receptors were immunoprecipitated and examined by SDS polyacrylamide gel electrophoresis and autoradiography (Figure 3.1). The visible forms of the LDL receptor in this experiment corresponded to the mature protein in wild-type CHO cells ("m", = mature glycoprotein; lane 1), or to the partially processed unstable forms typical of ldlC mutant cells ("i" = intermediate mobility; lane 2). These mobilities were consistent with previous results (for example, see figure 2.1). LDL receptor mobilities within the pSP21 and pSP22 transfectant populations included both the mutant ("i") and the wild-type ("m") forms (lanes 3 through 6, and lanes 7 through 10), whereas only the mutant form was seen after transfection with pRc/CMV (lanes 11 through 14). These results indicate that each population transfected with *LDLC* included cells in which the glycosylation of the LDL receptor was restored to normal.

This set of experiments confirmed that the *LDLC* cDNA can correct reproducibly the LDL receptor deficiency and glycosylation defects in a subset of transfected ldlC cells. Furthermore, the *LDLC* cDNA retained its activity regardless of its orientation respective to the CMV promoter. The distribution of LDL receptor isoforms in the transfected populations reinforced the notion that correction of the mutant phenotypes in ldlC cells requires a minimal threshold of *LDLC* activity, as the mobility of the LDL receptor glycoprotein predominantly resembled either the wild-type or the mutant form.

Table 3.1. LDL receptor activities of sense and antisense *LDLC* transfectants.

Cell Type (Individual Colonies)*	LDL Receptor Activity,¥ (ng/5 hr/mg)			
	Degradation *a*		Degradation *b*	
CHO	2782	(100%)	1747	(100%)
ldlC	343	(12%)	578	(33%)
ldlC[pSP21]-1	2714	(98%)	1447	(83%)
ldlC[pSP21]-2	--		2182	(130%)
ldlC[pSP21]-4	284	(10%)	--	
ldlC[pSP21]-5	--		2096	(120%)
ldlC[pSP21]-6	3648	(130%)	3295	(190%)
ldlC[pSP21]-7	--		2270	(130%)
ldlC[pSP21]-8	384	(14%)	1662	(95%)
ldlC[pSP21]-14	--		478	(27%)
ldlC[pSP21]-15	--		381	(22%)
ldlC[pSP21]-16	--		2862	(160%)
ldlC[pSP21]-17	--		2552	(150%)
ldlC[pSP22]-2	488	(18%)	--	
ldlC[pSP22]-3	--		588	(34%)
ldlC[pSP22]-4	--		1027	(59%)

*ldlC cells were transfected with either pSP21 (human *LDLC* cDNA in pRc/CMV, sense orientation) or pSP22 (human *LDLC* in pRc/CMV, antisense orientation), 10 µg DNA per dish. After 2 days of recovery, cells were transferred to selection dishes. After 13 days in 250 µg/ml G418 in medium E, as defined in chapter 2, between 4 and 10 individual colonies were mechanically collected. These colonies are indicated above by name and number.

¥LDL receptor activity was determined in two experiments, using an ^{125}I-LDL degradation assay as described in Methods. Values represent ng ^{125}I-LDL protein degraded per mg cell protein in 5 h, and are also presented as percentages of the activity of wild-type CHO cells.

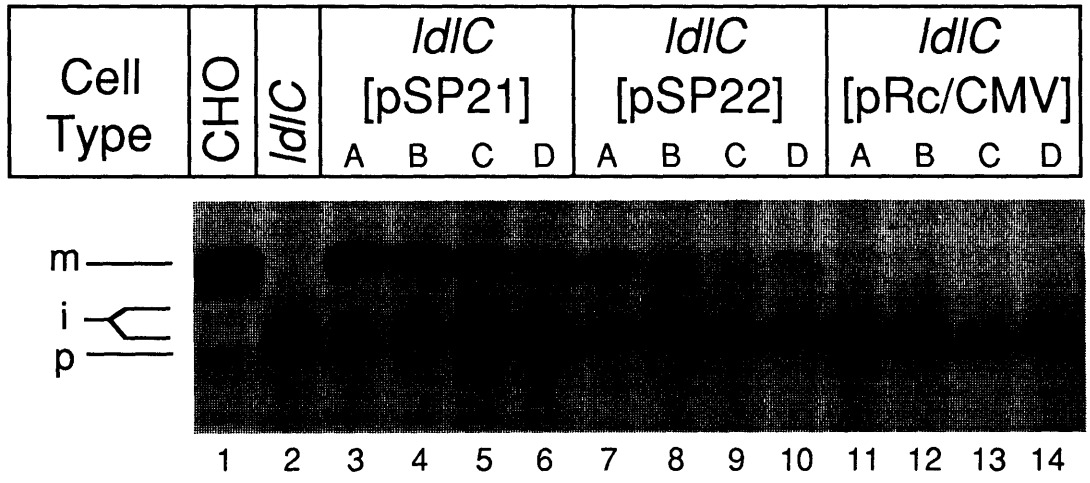
Table 3.2. Transfected populations of ldlC cells: LDL receptor activities.

Cell Type*	LDL Receptor Activity¥ (ng/5 hr/mg)	
CHO	1145	(100%)
ldlC	122	(11%)
ldlC[pSP21]-A	625	(55%)
ldlC[pSP21]-B	687	(60%)
ldlC[pSP21]-C	403	(35%)
ldlC[pSP21]-D	549	(48%)
ldlC[pSP22]-A	706	(62%)
ldlC[pSP22]-B	415	(36%)
ldlC[pSP22]-C	262	(23%)
ldlC[pSP22]-D	334	(29%)
ldlC[pRc/CMV]-A	104	(9%)
ldlC[pRc/CMV]-B	94	(8%)
ldlC[pRc/CMV]-C	94	(8%)
ldlC[pRc/CMV]-D	77	(7%)

*ldlC cells were transfected with pSP21, pSP22, and pRc/CMV, each on four independent transfection dishes A through D (10 µg DNA per dish). After two days of recovery, cells from each transfection dish were transferred to G418 selection dishes. After thirteen days, cells were collected as follows: three G418-resistant colonies were mechanically picked from each transfection dish and set aside, and the remaining ~100 colonies were trypsinized and collected as mixed populations from each plate. All twelve mixed populations are indicated in this table. As a note of interest, the ldlC[*LDLC*] colony that was used throughout chapter 2 and Podos et al. (1994) arose during this transfection.

¥LDL receptor activity was determined in the indicated lines and populations, using the ^{125}I-LDL degradation assay. Values represent ng ^{125}I-LDL protein degraded per mg cell protein in 5 h, and are also presented as percentages of the activity of wild-type CHO cells.

Figure 3.1. Processing of LDL receptors in ldlC cells transfected with sense and antisense *LDLC* constructs.



LDL receptor immunoprecipitation assays were performed on transfectant populations, comprising mixtures of approximately 100 G418-resistant colonies per dish as described in Results. Transfected DNAs were pSP21 (*LDLC*, sense), pSP22 (*LDLC*, antisense), and pRc/CMV (empty vector). A, B, C, and D signify the four independently transfected populations within each set, as described in the text and in table 3.2. Cells were labeled with ^{35}S-methionine (80 μCi/ml) in methionine-free medium E for 5 hours. The cells were then lysed and the lysates subjected to immunoprecipitation with an anti-LDL receptor antibody as in chapter 2. Immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. The mobilities of the mature ("m", 155 kD) and precursor ("p", 125 kD) forms of the LDL receptors in wild-type CHO cells are indicated, as is the mobility range of the receptor in ldlC cells, which is of intermediate size and is therefore marked "i".

Results (continued).

Activities of variant *LDLC* expression constructs:

As demonstrated above, the antisense *LDLC* expression construct pSP22 corrected the mutant phenotypes of ldlC cells, at an efficiency that was only somewhat less than that of the sense construct pSP21. To address the mechanism of this activity, I introduced frameshift mutations at two sites within the *LDLC* cDNA. These mutations were designed to discriminate between activity by ldlCp protein, and activity by *LDLC* cDNA such as by direct protein binding. Specifically, the latter should be immune to most local mutations whereas the former should be sensitive to frameshift mutations. Preparation of the frameshift constructs is described in Materials and Methods. These plasmids along with pSP21 and pSP22 were transfected into ldlC cells, to generate G418 resistant populations. LDL receptor activities were measured for each transfected population (table 3.3), as in table 3.2. Transfection with the empty vector or with an *LDLC* frameshift mutant at amino acid 190 (pSP40 and pSP41) did not increase the LDL degradation activity above background levels, suggesting that the *LDLC* cDNA acts by conventional mechanisms. Surprisingly, however, the *LDLC* frameshift mutant at amino acid 60 (pSP38) retained *LDLC* activity, elevating LDL receptor activity from 12% in untransfected cells to 19% in the transfected population. This effect was less than that mediated by the wild-type construct pSP21 (36%) but was comparable to that of the antisense construct pSP22 (19%). A MeLoCo growth assay confirmed that pSP38 restored LDL receptor activity to a subset of transfected ldlC cells, and that pSP40 and pSP41 did not (not shown). Furthermore, an immunoprecipitation experiment, performed as in figure 3.1, showed that pSP38 transfection restored normal processing to a substantial subset of LDL receptors (not shown). Therefore, the frameshift at amino acid 60 did not abolish the capacity of the *LDLC* cDNA to restore LDL receptor activity and normal LDL receptor processing to ldlC cells.

To confirm the activity of the *LDLC* frameshift plasmid pSP38, a new transfection assay was developed to more accurately indicate the relative efficacies of the *LDLC* expression constructs. In the previous assay, I allowed colonies to grow in G418 selection for ten to thirteen days before harvesting and analyzing the transfected populations. Thus the contribution of each colony to a population depended in part upon its rate of proliferation during the selection period. The new assay was designed to minimize this effect. In this assay, colonies were selected directly for *LDLC* activity, and were simply fixed and counted. Selection was achieved with the toxic lectin ricin, which was previously shown to selectively kill ldlC mutant cells. The numbers of colonies that arose under ricin selection were taken to represent the efficacies of the *LDLC* expression plasmids at restoring normal glycosylation to ldlC cells. Cells on control plates were selected in G418, to indicate the efficiencies of transfection. Table 3.4 presents the results from several such transfection experiments; each row presents a single plasmid, and shows results from one to three independent transfection dishes. The results in the first three rows demonstrate that pSP21 and pSP22 conferred ricin resistance to ldlC cells, and pRc/CMV did not, consistent with the previous experiments. Furthermore,

pSP21 activity was more penetrant than pSP22, indicating that *LDLC* expression from pSP21 was higher than from pSP22. The fourth row of table 3.4 shows that transfection of the "frameshift 60" *LDLC* plasmid pSP38 conferred *LDLC* activity, less efficiently than wild-type *LDLC* cDNA plasmid pSP21 but more efficiently than the antisense construct pSP22. This experiment therefore confirmed that transfection with the "frameshift 60" *LDLC* mutant can correct the defects of ldlC cells.

The activity of the "frameshift 60" plasmid could be explained by a translational start introduced into the ldlCp reading frame by the frameshift mutation, as will be considered in the Discussion section of this chapter. To eliminate this variable, I prepared a mutant *LDLC* cDNA with stop codons in place of amino acids 21 and 57 (designated the "nonsense" mutant). This cDNA was placed into pRc/CMV to create pSP45, which was transfected in ldlC cells. Table 3.4 shows that pSP45 conferred *LDLC* activity upon ldlC cells, at an efficiency similar to the wild-type *LDLC* antisense construct pSP22. The activity of both the "frameshift 60" and the "nonsense" mutants suggests that the first 60 amino acids of ldlCp may not be essential for *LDLC* activity in ldlC cells. This issue will be discussed further in the Discussion of this chapter.

The activity of the site-directed *LDLC* mutants leaves unanswered the question of how the antisense construct confers *LDLC* activity. The most likely mechanism is a bi-directional transcription of the *LDLC* cDNA which would allow synthesis of the sense strand from the antisense construct. Such transcription could occur independently of the CMV promoter, such as from a cryptic antisense promoter at the 3' end of the cDNA. To test this hypothesis, I prepared *LDLC* plasmids with altered vector sequences. In the first two of these constructs (pSP43 and pSP44), the cDNA was incorporated into a pRc/CMV variant (Δ pRc/CMV) from which the essentially complete CMV promoter was removed. These plasmids were transfected into ldlC cells, and the direct ricin selection assay was performed. Table 3.4 shows that the *LDLC* cDNA was active in either orientation in Δ pRc/CMV (pSP43 and pSP44). Therefore the *LDLC* cDNA did not require the CMV promoter for its activity, and was active regardless of its orientation. pRc/CMV contains a second promoter, the SV40 promoter, which transcribes the neomycin resistance gene responsible for G418 resistance. To determine the importance of the SV40 promoter to the activities of the various *LDLC* expression plasmids, the *LDLC* cDNA was incorporated into the expression vector pcDNAI neo, in the sense (pSP47) and antisense (pSP48) orientations. The pcDNAI neo vector is similar to pRc/CMV, but the neomycin resistance gene is transcribed from a different promoter. The final six rows of table 3.4 describe the activities of pSP47, pSP48, and pcDNAI neo; the first three rows and the last three rows were transfected on two separate days. These three plasmids generated fewer G418-resistant colonies than did the pRc/CMV constructs, most likely due to differences in the promoters that transcribe the neomycin resistance gene, but possibly due to real differences in transfection efficiency. However, comparisons among these three pcDNAI neo constructs are valid. The sense *LDLC* construct (pSP47) was fully active on all five transfection plates. In contrast, the antisense construct (pSP48) was completely inactive on two transfection plates on one day and

barely active on three transfection plates on the other day. Overall, the sense construct pSP47 was twenty times more active on average than the antisense construct pSP48, as indicated by the penetrance of ricin resistance normalized to the penetrance of ricin resistance. Therefore, the potency of the antisense construct of *LDLC* cDNA was restricted to the pRc/CMV vector, and can be considered the result of aberrant transcription in this particular vector.

Table 3.3. LDL receptor activities of ldlC transfectant populations.

Cell Type*	LDL Receptor Activity¥ (ng/5 hr/mg)	
CHO	2,348	(100%)
ldlC	284	(12%)
ldlC[pSP21]	851	(36%)
ldlC[pSP22]	437	(19%)
ldlC[pSP38]	446	(19%)
ldlC[pSP40]	173	(7%)
ldlC[pSP41]	220	(9%)
ldlC[pRc/CMV]	244	(10%)

*ldlC cells were transfected with the indicated plasmids (10 μg each). After three days of recovery, cells were transferred to G418 selection dishes. After twelve days in G418 selection, approximately 100 colonies were obtained. Three colonies were picked from each transfection dish and set aside, and the remaining ~100 colonies were collected as mixed populations. These populations were expanded and assayed for LDL receptor activity.

¥LDL receptor activity was determined using the ^{125}I-LDL degradation assay. Values represent ng ^{125}I-LDL protein degraded per mg cell protein in 5 h.

Table 3.4: Direct selection of transfectant colonies.

Construct*	Ricin-resistant Colonies¥	G418-Resistant Colonies§
pSP21	17,17	99,86
pSP22	4,5	91,115
pRc/CMV	0	79
pSP38	14,9	104,90
pSP45	4,9	62,141
pSP43	18,16	88,119
pSP44	3,11	62,81
pSP47	18,28	46,52
pSP48	0,0	49,38
pcDNAI neo	0	24
pSP47	21,18,35	53,41,81
pSP48	1,0,4	53,48,45
pcDNAI neo	0,0	34,46

*The constructs are described in Materials and Methods. The DNAs were transfected in three separate experiments, indicated in table by horizontal dividing lines.

¥IdlC cells were transfected with the indicated plasmids (5μg each), by the polybrene method. After three days, cells from each transfection dish were collected as pooled populations. Half of each population was plated into 0.25 ng/ml ricin selection in medium E (defined in chapter 2). The remaining half of each population was plated into G418 selection and processed as described below. After 9-12 days, ricin-resistant colonies were fixed and counted, and the colony counts are presented above. Multiple numbers within single lines indicate independent transfection dishes.

§Transfected populations were generated as described above, and half of each population was plated into 250 μg/ml G418 in medium E. After 9-12 days, G418-resistant colonies were fixed and counted, and the colony counts are presented above.

Discussion.

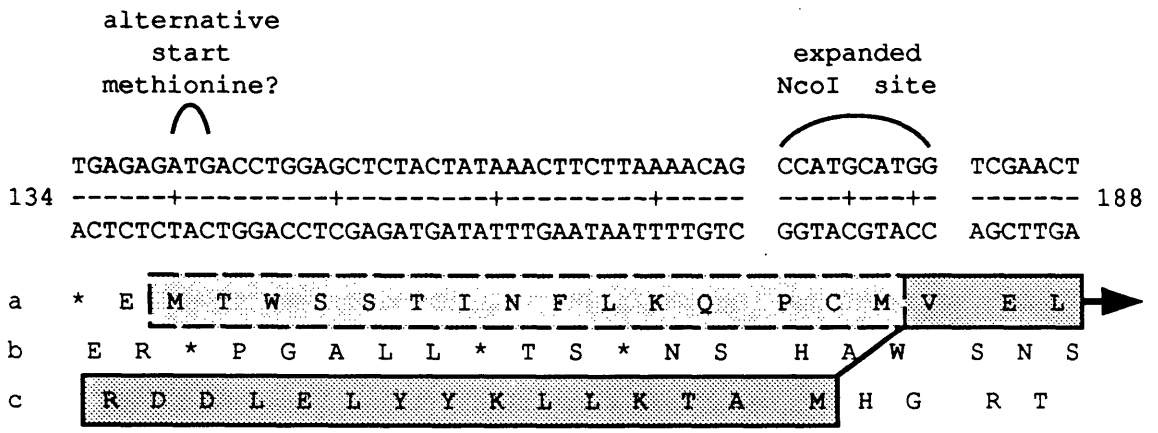
The experiments presented in this chapter demonstrated that the *LDLC* cDNA can reproducibly correct the mutant phenotypes of ldlC cells. However, the cDNA retained up to half of its apparent activity when in the antisense orientation in pRc/CMV (pSP22). Experimental perturbation of the pRc/CMV vector established that the activity of the antisense construct was independent of the CMV promoter. The most likely source for *LDLC* activity in the antisense construct was the SV40 promoter, which lies upstream of the neomycin resistance gene in pRc/CMV. This element could have directed transcription of the antisense strand, perhaps by enhancing cryptic promoter sites within the 3' untranslated region of the *LDLC* cDNA. Such a process could have been aided by the rearrangements that transfected DNAs are known to undergo. Thus, the same antisense construct might prove inactive if transfected by more benign means such as electroporation. There have been other reports of inadvertent activity of antisense constructs (for example, D. Housman, personal communication). The apparently high levels of antisense activity in this particular system must be considered in context. The experimental assays in this chapter measured the modification of ldlC mutant phenotypes, and did not directly measure ldlCp activity. If a tight threshold were in effect for *LDLC* activity in ldlC cells, then the apparent gap between the sense and antisense activities could be greatly narrowed. Thus, a great difference in productive *LDLC* transcription could be translated into a much more moderate difference in activity. This possibility can be tested readily by measuring the abundance of the sense and antisense transcripts in transfected cells. Furthermore, the number of colonies that are restored to wild-type is relatively small relative to the total number of G418-resistant transfectants. Therefore, the overall distribution of *LDLC* expression from the various constructs remains unknown. The assays simply distinguish insufficient *LDLC* activity from sufficient *LDLC* activity. As a final note, the presence of antisense mRNA could directly inhibit the activity of the sense mRNA. Thus a determination of ldlCp protein levels in sense and antisense transfectants could also be informative towards an effort to correlate *LDLC* expression with activity.

Further experiments presented in this chapter demonstrated that either a frameshift mutation or a pair of nonsense mutations within the first 60 amino acids of ldlCp failed to destroy the activity of the *LDLC* cDNA. A cursory sequence analysis of the "frameshift 60" and the "nonsense" mutations suggests possible mechanisms for their activities. Figure 3.2 shows the nucleic acid and protein sequences surrounding the lesions of these two mutants. In both cases, alternative translational start sites can be found which could bypass the mutations. Both alternative start sites are surrounded by appropriate sequences for translational initiation, although they are a distance from the 5' end of the cDNA. A difficulty with this explanation is that both alternative translations would remove the first 60 amino acids from ldlCp. As shown in chapter 2 and Podos et al. (1994), these 60 amino acids are predicted to be 33% identical to the corresponding region of the *C. elegans* ldlCp. Full interpretation of these observations will await description of the sequence requirements for human and nematode ldlCp function. Furthermore, it remains

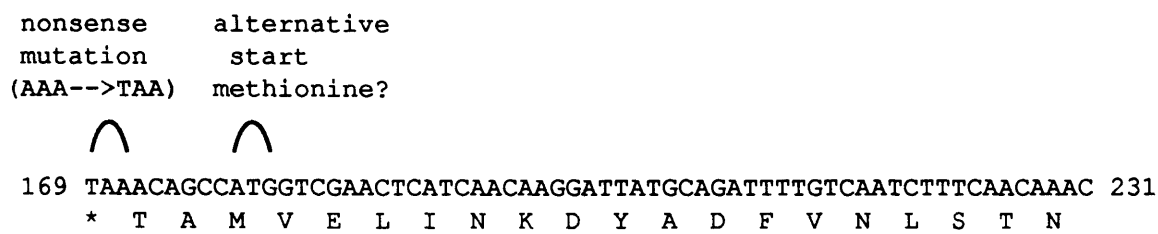
to be established that these first 60 amino acids are not expressed in ldlC cells. The ldlC lesion was induced by point mutagenesis, yet resulted in the loss of most *LDLC* mRNA. The molecular nature of the lesion is unknown, and may effect either the synthesis or the turnover of the mature *LDLC* mRNA. For example, other mRNAs have been shown to be sensitive to degradation when regions that normally are protected during translation become exposed by nonsense mutations (Peltz and Jacobson, 1992). If the *LDLC* mRNA were truncated by such means and thus not detectable as a uniform mRNA species in ldlC cells, then the amino-terminal portion of ldlCp could be synthesized in ldlC cells. It should be noted that the polyclonal antibody directed against the amino-terminus of the human ldlCp (anti-Npep, described in chapter 2) did not cross-react with the native hamster ldlCp, and therefore did not afford a direct search for amino terminal ldlCp fragments in ldlC cells. Thus, a transfected *LDLC* cDNA with an incomplete amino terminus might synthesize a product that could complement the endogenous amino terminus of ldlCp. Such intragenic complementation would closely mirror the α-complementation that has been described for bacterial β-galactosidase (Sambrook et al., 1989). Determination of the true relevance of the ldlCp amino terminus will therefore require the identification of the molecular lesion in ldlC mutant cells.

Figure 3.2: Analysis of sequence of "frameshift 60" and "nonsense" *LDLC* constructions.

A "frameshift 60" mutation.



B "nonsense mutation.



Nucleotide and protein sequences are presented for: A) the "frameshift 60" mutation, and B) the "nonsense" mutation. For the "frameshift 60" mutation, the "expanded NcoI site" is the restriction site which was modified to create the mutation. The ldlCp reading frame encoded by the normal *LDLC* cDNA is boxed. The dashed lines define an alternative reading frame, as discussed in the text. For the "nonsense" mutation, the mutation at codon 57 and a possible alternative start site are shown. The DNA sequence is numbered according to the system defined in chapter 2. The "alternative start methionine?"s in both A) and B) are surrounded by the appropriate start sequences defined by Kozak, despite their distance from the 5' end of the *LDLC* mRNA (1989).

Chapter 4.

Transfection of *LDLC* cDNA in ldlB cells.

Abstract.

The ability of the human *LDLC* cDNA to correct the LDL receptor deficiency in ldlB cells was assessed. The *LDLC* cDNA in the expression vector pRc/CMV (expression plasmid pSP21=p*LDLC*-1) was transfected into ldlB-11 mutant cells. Colonies expressing the neomycin resistance gene encoded within pRc/CMV were selected by treatment with G418, and these transfected colonies were collected as pooled populations. Two independent populations were collected after transfection with pSP21, and two after transfection with the parental expression vector pRc/CMV. The LDL receptor activity in each G418-resistant population was determined, by two separate assays. These experiments indicated that the human *LDLC* cDNA did not restore LDL receptor activity in ldlB-11 cells. Implications of these results are discussed.

Introduction.

The mutant phenotypes of ldlB cells are virtually identical to those of ldlC cells (Kingsley et al., 1986a). Both were isolated in screens for mutant CHO cells with deficiencies in LDL receptor activity (Krieger et al., 1981). The LDL receptor deficiencies in both cells are due to similar glycosylation defects, which affect N- and O- linked glycoprotein and glycolipid processing (Kingsley et al., 1986a). The ldlB and ldlC phenotypes have been indistinguishable by various methods, such as by analysis of glycoprotein mobility before and after digestion with glycosidases, or by comparing sensitivities of the mutants to a panel of toxic plant lectins. The parallels between ldlB and ldlC cells have led to the suggestion that the presumptive *LDLB* and *LDLC* genes are mechanistically related, i.e. that they contribute to common cellular functions. Thus an examination of the relationship between ldlB and ldlC mutant cells may shed light on the mechanisms by which the ldlCp protein influences Golgi functions.

Previous genetic experiments have indicated that the ldlB and ldlC loci are genetically distinct. In particular, ldlB and ldlC mutants define different complementation groups, as determined by cellular fusion experiments (Kingsley and Krieger, 1984). Hybrids between ldlB and ldlC mutants exhibit essentially wild-type levels of LDL receptor activity, and also exhibit normal glycoprotein processing. However, these results do not eliminate the possibility that the ldlB and ldlC mutants could complement intragenically. Specifically, ldlB and ldlC mutant phenotypes could both result from lesions within a single gene, but in distinct regions that can function separately.

As an initial effort to probe the genetic relationship between ldlB and ldlC cells, I transfected the human *LDLC* cDNA into ldlB-11 cells. As described in chapters 2 and 3, the *LDLC* cDNA corrects the mutant phenotypes in ldlC cells, and appears to be the normal counterpart to the gene which is defective in ldlC mutant cells. In the current chapter, the *LDLC* cDNA did not correct the LDL receptor deficiency when transfected into ldlB cells, and therefore did not correct the underlying glycoprotein processing defects. The genetic relationship between *LDLC* and the presumptive *LDLB* gene is discussed.

Materials and Methods.

The expression construct pSP21 (also designated p*LDLC*-1) bears the *LDLC* cDNA in the sense orientation within the expression vector pRc/CMV, as described in chapters 2 and 3. pSP21 and pRc/CMV were each transfected by the polybrene method into cells of the ldlB-11 isolate, as described in chapter 2. Transfectant colonies were selected by growth for eleven days in 250 μg/ml G418 in medium E. Approximately two or three hundred colonies arose from each transfection dish; the colonies from each transfection dish were trypsinized *en masse*, and the LDL receptor activity was assessed in each independently transfected population.

^{125}I-LDL degradation assays were conducted on each transfected population, to quantitatively determine the levels of LDL receptor activity averaged over each cell mixture. MeLoCo growth assays were also conducted, to provide an estimate of the percentage of transfectants in each population with LDL receptor levels sufficient for survival under MeLoCo selection. These assays are described in chapters 2 and 3, respectively.

Results.

Table 4.1 presents the ^{125}I-LDL degradation of pooled G418-resistant colonies from these transfection dishes. ldlB-11 cells transfected either with *LDLC* cDNA (pSP21) or with the empty vector (pRc/CMV) exhibited LDL receptor activities at levels between 4% and 6% of wild-type receptor activity, indicative of the ldlB mutant phenotype. Wild-type levels in this experiment are represented by LETB-144 and LETB-193 cells (Kingsley et al., 1986b). The LETB cells are derivatives of ldlB-11, in which the LDL receptor and glycosylation phenotypes were restored to normal by transfection with human DNA. The LETB cells were generated by the same methods as were the secondary LETC cells (Chapter 2 and Podos et al., 1994). Previous results have established that the LDL receptor-dependent ^{125}I-LDL degradation by LETB cells is comparable to that of wild-type CHO cells (Kingsley et al., 1986b). Therefore, the results in table 4.1 indicate that the *LDLC* cDNA does not correct the activity of ldlB cells.

These results were confirmed by a mevalonate/LDL/compactin (MeLoCo) growth assay. Briefly, 50,000 cells from each transfected population were grown under MeLoCo selection, to select for transfectants with normal levels of LDL receptor activity. This selection did not support the growth of ldlB cells after transfection with either pSP21 or pRc/CMV (not shown). In contrast, parallel control plates on which 1,000 LETB cells were set against a background of 50,000 ldlB cells resulted in the appearance of several hundred colonies. Therefore, expression of *LDLC* cDNA did not correct the LDL receptor deficiency of ldlB-11 cells, in even the small subset of transfectants which can be detected by this assay.

Table 4.1. LDL receptor activities and lectin sensitivities of ldlC transfectants.

Cell Type*	LDL Receptor Activity¥ (ng/5 hr/mg)	
ldlB-11	204	(7%)
LETB-144	2,741	(100%)
LETB-193	2,991	(109%)
ldlB[pSP21]-A	119	(4%)
ldlB[pSP21]-B	159	(6%)
ldlB[pRc/CMV]-A	140	(5%)
ldlB[pRc/CMV]-B	125	(5%)

*ldlB-11 is an isolate of the ldlB complementation group, defined in Kingsley and Krieger (1984), with mutant phenotypes that are identical to those of ldlC cells. LETB-144 and LETB-193 cells are DNA-mediated revertants of ldlB-11 cells, that were generated by transfection with human genomic DNA (Kingsley et al., 1986b). These LETB cells are secondary transfectants, equivalent to the secondary LETC cells described in chapter 2. The two ldlB[pSP21] populations and the two ldlB[pRc/CMV] populations were generated by transfection and selection in G418, as described in Materials and Methods.

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

Discussion.

The experiments described in this chapter provided the first direct demonstration that expression of the *LDLC* cDNA does not correct the mutant phenotypes of IdlB cells. Previous results had demonstrated that IdlB and IdlC mutant phenotypes were restored by somatic cell fusion, indicating that the IdlB and IdlC lesions defined two distinct loci. However, allelic complementation remained a possibility. The results presented in this chapter make this premise unlikely.

Transfection and assay conditions in this chapter were identical to those used for the transfection of *LDLC* cDNA into IdlC cells (as in chapters 2 and 3). Furthermore, comparable numbers of G418 colonies arose from transfection of IdlB and IdlC cells in these experiments, establishing that the IdlB cells were transfected with pRc/CMV at the same efficiency as were IdlC cells. Thus it is presumed that the transfected *LDLC* cDNA was transcribed at rates comparable to those which support *LDLC* activity in transfected IdlC cells. Thus the inability of the *LDLC* cDNA to restore LDL receptor activity to IdlB cells appears to be a valid result. Of course, it still remains possible that a hidden difference between IdlB-11 and IdlC-475 cells renders the IdlB-11 cells less susceptible to the activity of the *LDLC* cDNA, for example different levels of transcription initiation from the CMV promoter within pRc/CMV. It also remains possible that greater expression of the transfected *LDLC* cDNA, such as by stimulation with butyrate or by co-amplification with the dihydrofolate reductase gene, could correct the Golgi-based processing defects in IdlB cells.

The results presented here are supported by later experiments, which were presented previously (chapter 2 and Podos et al., 1994) Specifically, IdlB-11 cells have been shown to express normal levels of both the *LDLC* mRNA and the IdlCp protein. However, IdlCp in IdlB-11 cells is not localized to the Golgi. Taken together, these results support the conclusion that the IdlB and IdlC lesions are in distinct loci. The presumptive *LDLB* gene and IdlBp protein are therefore proposed to support IdlCp activity, either by directly modifying it or by collaborating with it in some way at its active position on the Golgi.

Chapter 5.

Map position of *LDLC* homolog in *Caenorhabditis elegans* genome.

Abstract.

Chapter 2 and Podos et al. (1994) report the cloning and the sequence analysis of a cDNA from *Caenorhabditis elegans*, with significant sequence similarity to the human *LDLC* cDNA. The current chapter presents the localization of the *C. elegans LDLC* cDNA within a physical map of the *C. elegans* genome. A DNA blot, containing a gridded pattern of overlapping yeast artificial chromosome (YAC) clones that span much of the *C. elegans* genome, was probed with a fragment of the *C. elegans LDLC* gene. A single YAC clone was identified that hybridized to this probe. Genetic interpretations of this physical mapping are discussed.

Introduction.

The cloning of the *C. elegans LDLC* cDNA arose as an outgrowth of my analysis of the sequence of the human *LDLC* and ldlCp. This nematode cDNA remains the only other gene with recognized similarity to human *LDLC*.

The immediate motivation for the isolation of the *C. elegans* homolog was to facilitate the analysis of the human *LDLC* sequence. The sequences of the human *LDLC* cDNA and of its predicted protein product show no significant similarity to other known genes within the common sequence databases. Using the Whitaker College Computing Facility at M.I.T., I have searched nucleic acid and protein databases such as GenBank and EMBL with the programs FASTA (University of Wisconsin) and BLAST (National Center for Biotechnology Information), and have not uncovered any matches of note. Furthermore, I submitted the *LDLC* and ldlCp sequences for comparison to the confidential dataset in the European Yeast Sequencing Project, which includes sequences from chromosomes II, IV, VII, X, XI, XIV, and XV of the yeast *Saccharomyces cerevisiae* (as of July 15, 1994). The search was conducted by I. Becker and H. W. Mewes (Martinsried Institute for Protein Sequences, Max Planck Institute for Biochemistry, Martinsried, Germany), who uncovered no significant homologies to yeast sequences. It is worth noting that ldlCp comparisons were made to DNA databases as well as to proteins databases. This is an essential point, as the protein databases are limited to sequences which have been recognized as coding regions. The large quantities of data arising from the various genome sequencing projects certainly include very many unrecognized loci, which are present in DNA databases but excluded from protein databases. Such coding regions become accessible to a protein query only if the DNA databases are translated in all reading frames and the translations then searched for similarity to the protein query sequence.

For the purpose of the ldlCp sequence alignment presented in chapter 2, any distant homolog would have been equally useful as the *C. elegans* clone. As discussed in chapter 2, this work has allowed the identification of domains and residues which may be important for ldlCp function. The alignment shows that the terminal regions are more highly conserved than the central portion. These terminal regions therefore are more likely to mediate interactions with other conserved components (perhaps an ldlBp protein?). Furthermore, the alignment can be applied in future work, to facilitate the identification of other related genes. The existence of the homolog in *C. elegans* indicates that ldlCp may serve conserved functions in eukaryotic cell biology, although the function of the nematode ldlCp in Golgi activities has not been established. It stands as a likely proposition that *LDLC* homologs may also reside within genomes of more distantly related eukaryotes, such as the yeast *Saccharomyces cerevisiae*. A genetic dissection of *LDLC* function in *S. cerevisiae* may identify genes which interact with ldlCp in the execution of its functions. Furthermore, such work may suggest possible conserved functions for ldlCp. This is not a trivial point, for the glycosylation pathways in yeast differ drastically from the mammalian pathways. In particular, O-linked glycosylation involves an entirely different set of glycoconjugates from those found on mammalian glycoproteins.

If a series of *LDLC* mutants results in a broad set of glycosylation defects in yeast, reminiscent of the ldlB and ldlC mutant phenotypes, this would suggest that ldlCp and the putative *LDLB* gene product may serve broad functions in regulating the activities or organization of the Golgi compartments.

The identification of the *LDLC* homolog in *C. elegans* introduces the possibility of a genetic dissection of the role of *LDLC* in a multicellular context. *C. elegans* has proven to be a powerful system for the genetic description of diverse processes such as cell fate decisions, signal transduction, neurodevelopment, and apoptosis (e.g., Ferguson et al., 1987; Estevez et al., 1993; Hengartner and Horvitz, 1994; Thomas, 1994). Numerous mutants have been collected and mapped genetically; these include general classes such as the uncoordinated (*unc*) and lethal (*let*) mutants, and mutants (*lin*) with alterations in the specification of cell lineage. The *C. elegans* genome has also been mapped physically; cosmids and yeast artificial chromosomes (YACs) carrying contiguous regions of *C. elegans* DNA have been isolated and an ordered map has been constructed. Many of the genetic markers have been cloned and mapped physically onto the YAC and cosmid maps, and some physical markers have also been mapped genetically. Therefore, one can now move readily among the genetic and physical maps, for a more thorough description of the function of a gene.

This chapter presents a preliminary analysis of the *C. elegans* cDNA in the context of the ongoing genome projects (for example see Coulson et al., 1991; R. Wilson et al., 1994). I have physically mapped the *LDLC* cDNA within the *C. elegans* genome, by probing a blot displaying physically ordered YACS which cover most of the genome. The correlation between this physical map and a genetic map are discussed.

Materials and Methods.

A filter bearing the YAC polytene grid, designated "poly 1", was provided by the laboratory of H. R. Horvitz, M. I. T.. The filter was constructed as described in Coulson et al. (1991). The "poly 1" blot displays 958 different YAC clones that together compose a physical map spanning greater than 80% of the *C. elegans* genome. DNA from these mapped YAC clones was fixed onto the filter in a pre-arranged pattern that allows one to determine the identities of YAC clones that hybridize to a particular probe.

The *LDLC* probe was prepared by PCR amplification of *C. elegans* chromosomal DNA, with the oligonucleotide primers ATGGGTACACTTCATGGCGA and CGATTCTTTCAGCCATACCAAC. The product of this amplification corresponds to positions 1 through 367 of the *C. elegans LDLC* cDNA, as numbered in chapter 2 and in Podos et al. (1994). The probe was approximately 460 bp long, and included two introns of approximately 50 bp as revealed by restriction mapping and sequence analysis. This same genomic probe was used to isolate the *C. elegans LDLC* cDNA clones, in chapter 2 and Podos et al. (1994). Hybridization to the YAC blot was conducted at high stringency, under standard conditions as defined in chapter 2.

Computer analysis of the physical and genetic maps was performed in the Horvitz laboratory, using the database ACEDB (A *C. elegans* Database) assembled by R. Durbin (M. R. C., U. K.) and J. Thierry-Mieg (C. N. R. S, France). As a matter of note, the map location of *LDLC* is to be entered into the ACEDB database in the near future.

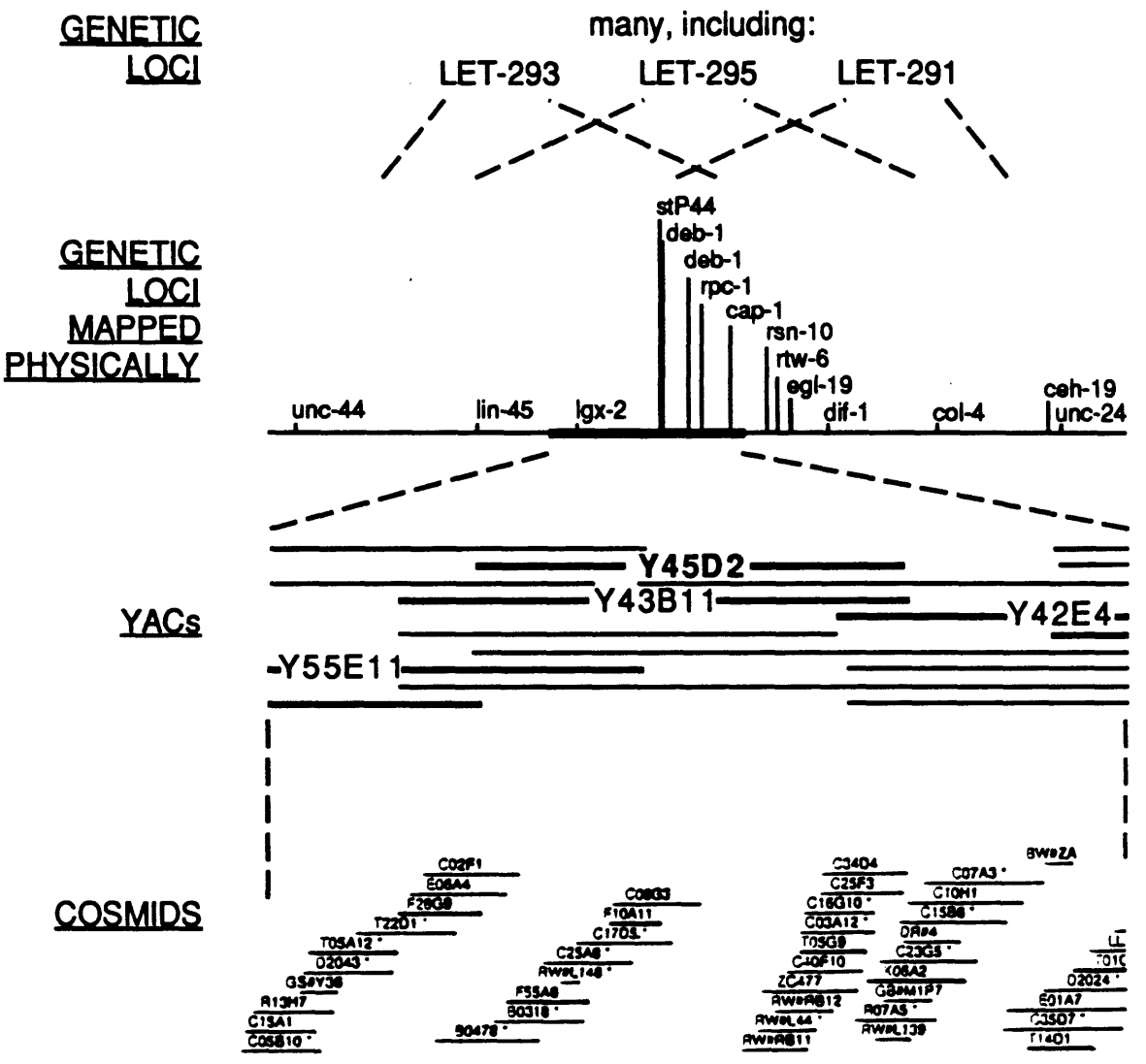
Results.

The "poly 1" polytene filter, displaying YAC clones bearing *C. elegans* genomic DNA, was probed with the *C. elegans LDLC* probe at high stringency by standard methods. The radiolabeled probe hybridized to a single spot on the "poly 1" filter (not shown). This position appeared to correspond to a YAC clone with the designation Y45D2, which is derived from *C. elegans* chromosome IV. Alignment of the positive spot with the known grid pattern was accomplished with the aid of alignments taken from past probings of the same filter within the Horvitz laboratory, as the overall grid pattern was not evident in the background of my autoradiograph.

Figure 5.1 presents the genetic and physical map of the region of chromosome IV surrounding the YAC Y45D2, as reported in the database ACEDB. The genetic markers in the top two rows correspond to loci that have been identified by mutational analysis and recombinant mapping. Classical markers such as these make up the core of the genetic map of the *C. elegans* chromosomes. The mutations indicated in the first row of figure 5.1 have not been defined molecularly, and have been mapped to this region by genetic crosses with other mutations. The markers in the second row of figure 5.1 are genetic loci that have been cloned and placed onto the physical map by mapping methods similar to those used in this chapter for the *LDLC* gene. The best candidates for loci that may correspond to the *LDLC* gene therefore reside among the genes not yet cloned, such as those in the first row.

The third row of figure 5.1 presents overlapping YAC clones, which provide the framework for the physical map of the *C. elegans* genome. The YACs shown here include the clone Y45D2, which hybridized to the *LDLC* probe in the experiment described above. The YACs that are represented by bold lines have been placed onto polytene grids such as the poly 1 filter. Also shown in this figure are ordered cosmid clones (fourth row), that have been correlated with the YAC map and therefore elevate the resolution available for physical mapping. Not shown are numerous molecular markers of smaller size, such as restriction fragment length polymorphisms (RFLPs), PCR tags, and cDNA clones such as the expressed sequence tags (not shown in figure), that have been placed on the physical map by methods similar to those used for *LDLC* in this chapter. The vertical and diagonal dashed lines between the various rows in this figure show the known correspondences between the physical and the genetic maps of the *C. elegans* genome. It is these correspondences that may ultimately allow the identification of a genetic locus that corresponds to *LDLC*, based upon the physical map location of *LDLC*. This is considered further in the Discussion below.

Figure 5.1. Partial genetic and physical map of *C. elegans* chromosome IV, in the vicinity of the *LDLC* gene.



Genetic and physical map of the region of *C. elegans* chromosome IV surrounding the YAC clone Y45D2, which has an approximate size of 230 kilobase pairs. The various types of landmarks that are shown above are discussed within the text of the Results section. The *LDLC* gene has been mapped to the YAC Y45D2 and therefore may correspond to one of the genetic loci in this vicinity, such as the lethal mutations *let-293*, *let-295*, and *let-291* in the upper row of this figure, or other mutations described in the text but not shown.

Discussion.

This chapter takes advantage of the new intersection between genetic and physical mapping tools. Molecular methods were used to place the *LDLC* gene onto the physical map of the *C. elegans* chromosomes, and the physical map was examined in the vicinity of the *LDLC* gene. Numerous genetic loci have been identified in this region. Of particular interest are genes that confer mutant phenotypes yet that have not been characterized molecularly, as these are the most likely candidates for the *C. elegans LDLC* gene. Figure 5.1 indicates three such genes, defined by the lethal mutations *let-293*, *let-295*, and *let-291*. The cellular bases for the lethality of these mutations are not known. These three loci have been placed onto the map by meiotic recombination between the markers *unc-44* and *unc-24*, which form the endpoints of the genetic data presented in figure 5.1 (mapping data reported to the ACEDB database by D. L. Riddle). The *let-294*, *let-297*, *let-292*, *let-296*, *let-289*, and *let-290* mutations have been similarly mapped to the region between *unc-44* and *unc-24*, and have been omitted from figure 5.1 only for simplicity (reported by D. L. Riddle). Numerous other mutations have also been placed within the vicinity, by meiotic mapping against more distant markers. These loci include *bli-6*, *egl-19*, *emb-26*, *emb-31*, *lag-1*, *lin-33*, *mec-17*, *mor-2*, *sog-3*, and *unc-8*. For simplicity these additional loci have not been included in figure 5.1. There is presently no reason to favor the likelihood of any one of these genes as corresponding to the native *LDLC* gene. However, all are implicated by reason of proximity within the genome.

The data presented in this chapter will ultimately require independent confirmation, such as by identification of a *C. elegans* cosmid that hybridizes to the *LDLC* gene. The YAC map within figure 5.1 highlights the need for independent confirmation. The *LDLC* cDNA hybridized to Y45D2 but not to Y43B11, yet both are present on the "poly 1" filter. Yet, the former YAC is shown in figure 5.1 to be fully included within the latter YAC. This inconsistency is entirely plausible, as *LDLC* sequences could have been lost during the construction of Y43B11. Alternatively, however, the physical map within the ACEDB database could be assembled incorrectly.

The experiment described within this chapter is clearly of a preliminary nature. However, it provides a launching point for potentially informative future experiments. To test whether any of the known genetic loci correspond to the *C. elegans LDLC* gene, mutant animals can be obtained and tested for DNA rearrangements, decreased transcription, or even sequence mutations within the *LDLC* gene. Furthermore, *LDLC* DNA can be cloned from each of these mutant lines and tested for activity such as in ldlC mutant cells (the activity of the wild-type *C. elegans LDLC* cDNA must first be established). Such experiments could lead to the identification of *LDLC* mutants from among known mutant animals. Additionally, homozygotes from deficiency strains can be examined for the loss of the *LDLC* gene, and the *LDLC* gene within insertional mutant animals can be examined for transposon integrations. Lastly, the effects of ectopic *LDLC* expression on the viability, development, and behaviors can be analyzed in transgenic animals. Therefore, the isolation of an *LDLC* cDNA in *C.*

elegans provides potential entrypoints for a new line of experimentation involving *LDLC* function *in situ*. There are no obvious expectations for possible phenotypes of an *in situ LDLC* mutation. If the broad alterations in glycosylation that are seen in ldlC mutant cells are reproduced in the nematode, then one might predict that a *C. elegans* mutant would suffer from gross defects in early morphogenetic events due to alterations in cell adhesion and cell-cell recognition. Alternatively, of course, mutant phenotypes could be more restricted or even undetectable.

Chapter 6.

Discussion.

ldlC cells and the *LDLC* gene.

ldlB and ldlC cells are Chinese hamster ovary (CHO) cell mutants that were isolated because they display unusually low levels of active LDL receptor (Krieger et al., 1981). The underlying cause of the LDL receptor deficiency in both ldlB and ldlC cells has been traced to a broad set of defects in the protein and lipid glycosylation pathways of the Golgi apparatus (Kingsley et al., 1986a; Reddy and Krieger, 1989). The mutations in these cells affect global glycoprotein and glycolipid processing, altering the synthesis of N-linked and O-linked protein glycoconjugates and also of glycolipids (Kingsley et al., 1986a). Despite their complexity, the ldlB and ldlC mutant phenotypes are caused by single mutations (Kingsley et al., 1986a, b; Reddy and Krieger, 1989). The pleiotropic nature of the defects in the Golgi apparatus of these cell mutants has led to the suggestion that the ldlB and ldlC mutations may alter the membrane or lumenal compositions of the Golgi cisternae.

With the aim of determining how a single gene can influence seemingly independent glycosylation pathways in the Golgi apparatus, I have cloned a human cDNA that corrects the mutant phenotypes of ldlC cells (chapter 2). This cDNA is designated *LDLC* because of its ability to reproducibly correct by transfection the full set of ldlC mutant phenotypes. The mutant phenotypes that I have examined include the LDL receptor deficiency, defects in LDL receptor processing and stability, and defects in global glycosylation as detected by altered sensitivities to toxic lectin proteins. Northern blot analysis showed that the corresponding *LDLC* mRNA is barely expressed in ldlC mutants, relative to wild-type CHO cells. This result provided strong evidence that the *LDLC* cDNA is the normal counterpart to the gene whose defect accounts for the ldlC mutant phenotypes. Furthermore, this finding indicated that the ldlC defect may be the functional equivalent of a null mutation. Both of these conclusions will require further substantiation, particularly in the form of molecular analysis of the genetic lesion that accounts for the ldlC mutation. For the present it remains possible that the ldlC lesion lies in a separate gene that is required for expression of the *LDLC* mRNA and possibly other mRNAs. If the ldlC mutation does reside within the cloned *LDLC* gene, it remains plausible that the mutation did not generate a true functional null allele. Specifically, the *LDLC* gene in ldlC cells may direct the translation of low but functionally significant levels of protein product, or perhaps of a truncated protein that supplies an active portion of the *LDLC* gene product. This latter issue is discussed in chapter 3, with a brief discussion of mechanisms by which such a truncated protein might be produced and also of circumstances under which it may retain partial activity.

The latter caveats notwithstanding, the cloned *LDLC* activity appears to be required for multiple processing reactions in the Golgi apparatus. To shed light on the molecular functions of the human *LDLC* cDNA, its nucleotide

sequence was examined (chapter 2). An open reading frame within the cDNA sequence encodes a putative protein, ldlCp, with a predicted size of 83 kilodaltons. The sequence of ldlCp contains no major common structural motifs, such as putative transmembrane domains, sequences specifying lipid anchor attachment, or an ER translocation signal sequence. Thus the sequence of ldlCp is more notable for what it lacks than for what it contains. The lack of recognizable membrane insertion sequences suggested that ldlCp might affect multiple reactions within the Golgi cisternae from a cytoplasmic location. Furthermore, no significant sequence similarity was found between *LDLC* and known genes reported in public and private sequence databases. These databases included such resources as GenBank and EMBL (discussed in chapter 2), as well as unpublished sequences collected as part of the European Yeast Sequencing Project (chapter 5). Both nucleotide and protein sequences were searched. The only sequences within these databases that were similar to *LDLC* were contained within three cDNA fragments called expressed sequence tags (ESTs), two from human tissues and one from the nematode *Caenorhabditis elegans*. As the ESTs had been cloned and sequenced without regard to function, they could provide no immediate functional information (Adams et al., 1991).

An *LDLC* family?

The *C. elegans* EST described above represented the only known DNA that contained divergent homology with the human ldlCp, and thus presented a valuable resource to further the analysis of the ldlCp sequence. Accordingly, I isolated and sequenced the corresponding cDNA from a *C. elegans* cDNA library (chapter 2). The sequence of this cDNA includes an open reading frame that encodes an apparent homolog of the human ldlCp; the cDNA and its protein are simply designated the *C. elegans LDLC* and ldlCp. The similarity between the human and nematode *LDLC* sequences was scattered throughout their lengths, indicating that these cDNAs may encode functional as well as structural homologs. The strongest similarity was found in a stretch of 72 amino acids near the amino termini, within which the two sequences are 49% identical and 71% similar (as determined either by the algorithm of chapter 2, or by the BESTFIT program, Genetics Computer Group, University of Wisconsin), when aligned with no gaps. Comparison with the human ldlCp sequence suggests that I have isolated the entire coding region of the nematode *LDLC* cDNA, although more information may still remain unidentified at the 5' end of the coding region.

The similarity between the human and *C. elegans LDLC* sequences suggests the existence of a larger family of *LDLC* genes. The native functions of the *LDLC* gene in *C. elegans* have not been determined, nor has functional interchangeability been established such as by transfection into ldlC cells. However, I will offer the hypothesis that the human and nematode *LDLC* genes represent a larger family of *LDLC* genes, whose members perform related cellular functions in animals and possibly within other eukaryotes. Examination of *LDLC* family members from *C. elegans* and from other sources may therefore

prove informative about the functions of the mammalian ldlCp protein in Golgi processes. A genetic dissection of *LDLC* function in *C. elegans* presents one potential source of future contributions. Chapter 5 presents a first step in that direction. The *C. elegans LDLC* gene was mapped within the *C. elegans* genome, using recently developed methods for physical mapping (Coulson et al., 1991). In particular, the *LDLC* gene was determined to hybridize to one among an ordered set of 958 yeast artificial chromosome (YAC) clones, each bearing *C. elegans* genomic DNA. This result placed the *LDLC* gene with some precision onto the map of *C. elegans* chromosome IV. The physical location of *LDLC* suggests a number of previously isolated mutations that could represent lesions within the *C. elegans LDLC* gene. Furthermore, the physical map location can serve as a starting point for a directed effort at isolating new chromosomal *LDLC* mutants, for example in *trans* to a chromosomal deficiency. Admittedly, these are not trivial experiments. It is unclear what effects an *LDLC* mutation would have in a multicellular organism. Unlike ldlC cells in culture, cells in the context of a developing nematode, or mouse or human, are expected to be highly sensitive to changes in cell adhesion interactions. As cellular adhesion can depend upon carbohydrate recognition (e.g. Brandley et al., 1990) a mutant glycosylation phenotype might have wide-ranging effects on morphogenesis. However, a *C. elegans* glycosylation phenotype would be difficult to assess, given the relative lack of knowledge about normal glycosylation in *C. elegans*.

The hypothesis that the human and nematode ldlCp proteins represent a larger family raises the issue of multiple *LDLC* homologs even within mammalian cells, whether expressed or latent. For example like the rab and ras GTPases (Novick and Brennwald, 1993; Zerial and Stenmark, 1993), multiple ldlCp proteins might perform diverse cellular functions through a conserved biochemical mechanism. Northern blot analysis in chapter 2 did not reveal multiple mRNA species that hybridized to the *LDLC* cDNA, but at reduced stringency such species might be revealed. Alternatively, the alignment between the human and nematode ldlCp sequences can guide the design of degenerate primers and probes with which to isolate additional *LDLC* genes within genomic DNA or cDNA libraries.

The conservation between the human and *C. elegans* ldlCp sequences further suggests that even unicellular eukaryotes might rely upon *LDLC* genes for related cellular processes. For a directed analysis of the functions of ldlCp in cellular processes, the yeast *Saccharomyces cerevisiae* presents a highly promising experimental system. Once identified, an *LDLC* gene in *S. cerevisiae* can be readily manipulated within the yeast genome to allow for example the determination of the true null phenotype. Genes that interact functionally with *LDLC* can be identified, via mutations that modify *LDLC* mutant phenotypes. *S. cerevisiae* has been used extensively for molecular and genetic studies of membrane transport and of glycosylation within the Golgi (Pryer et al., 1992; Herscovics and Orlean, 1993). These pathways have already been described in significant detail, which would aid phenotypic analysis of new mutants. Large collections of yeast mutants with Golgi phenotypes are presently available to allow studies of genetic interactions with

LDLC mutations. Genetic interactions can be assessed with pre-existing yeast mutants such as *mnn9* and other *mnn* mutants that, like ldlC, have broad defects in outer chain glycosylation (Ballou, 1990; Herscovics and Orlean, 1993).

Interpretation of results involving any homologs to the human *LDLC* gene will require the acknowledgment that ldlCp functions may have diverged from their common origin, and therefore may be diverse. The absence of a direct biochemical assay for ldlCp function complicates matters. However, it seems likely that study of new *LDLC* genes, whether closely or distantly related to the known mammalian *LDLC* gene, will shed light on ldlCp function in unexpected ways.

Properties of ldlCp.

To provide for direct studies of the properties of the ldlCp protein, polyclonal antibodies were raised against the synthetic peptides Npep and Cpep that correspond to the amino and carboxy termini respectively of the human ldlCp (chapter 2). These antibodies, designated anti-Npep and anti-Cpep, both recognized the human ldlCp in immunoprecipitation and Western blotting experiments. Recognition by both anti-Npep and anti-Cpep antibodies indicated that the human ldlCp protein was expressed at full or nearly full length, despite an observed mobility in SDS polyacrylamide gels (76 kilodaltons) that was somewhat smaller than the predicted molecular weight (83 kilodaltons). The anti-Cpep antibody was found to cross-react with the murine and hamster ldlCp proteins, suggesting that the carboxy terminal decahepta-peptide sequence is at least partially conserved among the mammalian ldlCp species. This terminus therefore may contribute to ldlCp function, although related sequences were not observed in the ldlCp of *C. elegans*. The anti-Npep antibody did not cross-react with the hamster ldlCp protein, and could not be used to determine expression levels in CHO cells and in the ldlC mutant. As a cross-reacting antibody, however, the anti-Cpep antibody was used to assay cellular ldlCp expression levels. The endogenous hamster ldlCp was readily detected in wild-type CHO cells but not in ldlC mutant cells. These observations indicated that the complete ldlCp was not expressed in ldlC cells, which is consistent with the poor expression of the *LDLC* mRNA in ldlC cells. These results do not rule out the possible expression of truncated ldlCp in ldlC cells (as discussed in chapter 3). ldlCp expression in ldlC cells, as detected by the anti-Cpep antibody, was restored by transfection with the human *LDLC* cDNA.

The experiments described above established the validity of the anti-Cpep antibody as a reagent that specifically recognizes the ldlCp protein. This antibody therefore was used to determine the subcellular distribution of ldlCp within wild-type cells (chapter 2). The apparent lack of sequences specifying entry of ldlCp into the secretory pathway was striking, given the Golgi-based mutant phenotypes of ldlC cells. To address how ldlCp could exert its effects while not in contact with the Golgi lumenal spaces, I performed immunolocalization experiments in CHO cells with the anti-Cpep antibodies

against ldlCp. The central conclusion from these experiments was that ldlCp associates peripherally with the Golgi membranes. ldlCp staining was localized in a distinctive perinuclear distribution that closely resembled the distributions of the known Golgi markers mannosidase II and β-COP, and ldlCp co-localized with β-COP in double-staining immunofluorescence experiments. These results suggested that ldlCp localizes to the Golgi membranes, although other structures can be found in the same vicinity of the cell (Griffiths et al., 1993). Further evidence of Golgi association was provided by treatment with the drug brefeldin A, which causes the dissociation of β-COP and other peripheral Golgi proteins from the Golgi membranes (Donaldson et al., 1990; Klausner et al., 1992). Brefeldin A caused the rapid diffusion of the ldlCp staining pattern, thereby uncoupling the localization of ldlCp with that of the integral Golgi membrane protein mannosidase II. This dissociation of ldlCp staining from the Golgi membranes was qualitatively similar to the behavior of β-COP, a Golgi coat protein whose brefeldin A-sensitive association with Golgi membranes is well established (Donaldson et al., 1990, 1992; Orci et al., 1991; Helms and Rothman, 1992). The kinetics of dissociation of ldlCp and β-COP were not readily compared, as immunofluorescence staining is not easily quantitated. The conclusion that ldlCp associates with the Golgi will ultimately require further substantiation, such as by immunoelectron microscopy or by a demonstration *in vitro* of ldlCp association with Golgi membranes. Significant association *in vitro* of cytoplasmic ldlCp with isolated Golgi membranes has not yet been achieved (D. Finazzi and R. Klausner, unpublished results).

An examination of ldlCp localization in ldlB mutant cells has suggested that the localization of ldlCp to the Golgi apparatus may be essential for its activity (chapter 2). Previous experiments indicated that the ldlB and ldlC defects lie in separable activities, despite their identical mutant phenotypes (Kingsley and Krieger, 1984; Kingsley et al., 1986a). Furthermore, transfection with the *LDLC* cDNA did not correct the mutant defects in ldlB cells, and ldlB cells express normal levels of both the *LDLC* mRNA and the ldlCp protein (chapters 2 and 3). Thus, the mutant block in ldlB cells does not lie upstream of ldlCp expression. Despite its apparently normal expression, ldlCp was not localized to the Golgi apparatus in ldlB cells. Rather, ldlCp staining was seen in a diffuse pattern that suggested a cytoplasmic distribution. These results established that the presumptive *LDLB* gene is required for the peripheral localization of ldlCp to the Golgi apparatus. Thus we are allowed the beginnings of a genetic pathway, in which the *LDLB* gene product (ldlBp?) lies downstream of ldlCp synthesis but upstream of ldlCp localization to the Golgi. Numerous models can be offered to explain how the *LDLB* gene directs the localization of ldlCp to the Golgi. The presumptive *LDLB* gene might encode an equal partner with ldlCp, such as a co-subunit in a complex, or perhaps a receptor for ldlCp on the Golgi membranes. Alternatively, ldlBp might catalytically modify ldlCp or its target site without directly populating the bound state. Whatever its *modus operandi*, the *LDLB* gene appears to be intimately connected to ldlCp in its contributions to normal Golgi functions. A biochemical and molecular analysis of the presumptive *LDLB* gene and gene product will be essential to a full explanation of ldlCp localization. The *LDLB* gene can be cloned by complementation of the ldlB mutant phenotype, and its products can

be analyzed much as the *LDLC* gene has been analyzed. The presence of an activity in the human genome that can correct the defects of ldlB mutant cells has already been demonstrated by transfection experiments (Kingsley et al., 1986b). Analysis of the *LDLB* gene and gene products will likely provide important insights into the immediate activities of ldlCp in its role in Golgi functions.

ldlCp and Golgi function.

One major implication of the ldlCp immunolocalization studies described above is that ldlCp must influence the lumenal Golgi reactions from across the Golgi membranes. How it does so remains unknown. Observations described in chapter 2 open the possibility that ldlCp can influence the structure or organization of the Golgi apparatus. Immunofluorescence localization experiments revealed a notable decrease in the intensity of Golgi staining in ldlC mutant cells, as detected by antibodies against either the integral membrane protein mannosidase II or the peripheral protein β-COP. The perinuclear distributions of these proteins appeared approximately normal, correcting for differences in staining intensity and in cell morphology. These decreased intensities were coupled to the mutant phenotypes, as the intensities returned to or exceeded normal levels upon transfection with the human *LDLC* cDNA. Staining intensities of mannosidase II and β-COP were similarly decreased in ldlB mutant cells, and staining levels again were elevated with the DNA-mediated restoration of phenotype. The diminution of lumenal mannosidase II staining in ldlB and ldlC mutant cells might be ascribed to altered carbohydrate epitopes, but β-COP is not a glycoprotein and its decreased staining cannot be so ascribed. Therefore, it appears that the *LDLB* and *LDLC* gene products may contribute in some way to the structure or organization of the Golgi apparatus. It must be noted that the effects of the ldlB and ldlC mutations on Golgi organization are relatively subtle, as the mutant cells are fully viable and the Golgi in these cells is not subject to catastrophic loss. It is not currently known whether the ldlB and ldlC mutations alter the subcellular distributions of β-COP and possibly of mannosidase II, thereby diminishing their perinuclear concentrations, or whether the total cellular concentrations of these proteins are reduced. Either effect might be consistent with the ldlB and ldlC mutant phenotypes, as even subtle alterations in the expression of glycosylation enzymes or nucleotide-sugar transport proteins within the Golgi might result in pleiotropic glycosylation defects similar to those of ldlB and ldlC mutant cells. Before such models can be considered seriously, the metabolic fates and subcellular distributions of a number of glycosylation enzymes would need to be examined.

Further analysis of the Golgi membranes and lumenal spaces in ldlB and ldlC mutant cells may also contribute to an explication of ldlCp function. For example, the ionic compositions of the mutant Golgi compartments could be aberrant, such as by altered localization or activity of a proton or calcium ATPase pump. The endosomal system provides a precedent for ionic control of secretory functions, as somatic cell mutants with defects in pH regulation have

been shown to have pleiotropic defects both in endocytosis and in the secretory pathway (e.g. Robbins et al., 1984). Another precedent is provided by the calcium system in yeast. The *PMR1* gene encodes a calcium ATPase pump which has been localized on or near the Golgi membranes. *pmr1* mutants exhibit defective outer chain glycosylation, and thus bear resemblance to IdlB and IdlC cells (Antebi and Fink, 1992). Although the properties of IdlCp are inconsistent with function as a membrane pore, it could regulate such a pump indirectly or directly.

A biochemical dissection of the products of Golgi processing steps could also prove informative. Specifically, the compositions and structures of carbohydrates on glycoproteins and glycolipids can be assessed. Such analyses may reveal particular structures that are over- or under-represented in the mutant cells. The defects in these structures have been inferred by the effects of probes such as glycosidases on glycoprotein mobilities, but have not been determined directly. A large set of lectin proteins can be used as fairly specific probes for particular structures within carbohydrate moieties, and as discussed throughout this thesis can be used as cell toxins to select for or against glycosylation mutants (Wu et al., 1988; Stanley, 1985b). As structural probes, however, lectins are more informative when applied to isolated carbohydrates rather to whole cells. For example, the toxicity of ricin has been used as an indicator of cellular glycosylation state throughout this thesis and elsewhere, yet ricin can also be used to select for cells with defects in endocytosis (Sandvig and van Deurs, 1994). The heterogeneity of the carbohydrate products is likely to complicate the interpretation of an analysis of the glycosylation products (Kingsley et al., 1986a).

In addition to sugar analysis, biochemical assays for Golgi functions could be developed, with the hope that an IdlC mutant phenotype could be replicated *in vitro*. The well studied intra-Golgi transport assay might be suitable, as it measures the combined activities of membrane transport and glycosylation. Although the IdlB and IdlC mutant cells display no gross defect in membrane transport as measured by the transport of LDL receptor proteins to the cell surface, it remains possible that these cells express defects within the transport machinery. In principle, the native function of IdlCp could be to modulate the fidelity of vesicle targeting within the Golgi without affecting the overall amount of transport through the Golgi. If this were the case, then a loss of IdlCp activity could result in pleiotropic alterations in glycosylation without reducing the net amount of protein transport through the Golgi. If the IdlB and IdlC mutant defects can be replicated *in vitro*, then the defective process could be dissected, regulatory and energetic requirements determined, and complementing activities purified.

Clues to IdlCp function are likely to reside in the nature of its presumed association with the Golgi. The apparent peripheral association of IdlCp is wholly consistent with the sequence of IdlCp, as this type of membrane association does not require entry into or across the Golgi membranes. Other peripheral Golgi proteins include components of the coatomer complex, and GTPases such as rab6, ARF, the trimeric G protein subunit $G_i\alpha$-3, which all have

been characterized as effectors or regulators of secretory transport processes in the Golgi (Zerial and Stenmark, 1993; Bomsel and Mostov, 1992). Numerous other peripheral Golgi proteins have also been identified but not yet characterized at the level of sequence, including a 115 kilodalton protein that is required *in vitro* for Golgi membrane transport (Waters et al., 1992), and proteins of 54, 86, 200 and 230 kilodaltons with unknown functions (Chicheportiche et al., 1984; Kooy et al., 1992; Narula et al., 1992). The mechanisms by which these proteins are localized to the Golgi may be sufficiently diverse to defy generalizations. This may be the case for the human ldlCp, as it lacks apparent signals for fatty acylation or isoprenylation and therefore may depend strictly upon interactions with other Golgi proteins. The sequences within ldlCp that are required for Golgi localization can be identified. Such analysis has been done for such peripheral Golgi proteins as $G_{i}\alpha$-3, the oxysterol binding protein OSBP (Ridgway et al., 1992) and the Golgi-associated isoform of glutamic acid decarboxylase, GAD_{65}, the enzyme that synthesizes the neurotransmitter γ-amino butyric acid (de Almeida, 1994; Shi et al., 1994; Solimena et al., 1994). The brefeldin A sensitivity of ldlCp can be examined further. It remains unknown whether this brefeldin A sensitivity indicates a functional interaction with ARF and other transport machinery, or is merely an indirect consequence of ARF dissociation. It does suggest that the normal localization of ldlCp to the Golgi may be regulated by cycles of GTP hydrolysis. Biochemical experiments can be conducted to identify proteins that contact ldlCp, either at the Golgi apparatus or in the cytoplasm. Proteins in contact ldlCp might be identified by co-immunoprecipitation experiments, perhaps with the aid of covalent cross-linkers, or by molecular methods such as two-hybrid cloning.

Final Words.

The preceding paragraphs demonstrate the facility with which models can be offered to explain the mutant phenotypes of the ldlB and ldlC mutants. Ultimately, I expect that the roles of the *LDLB* and *LDLC* gene products will be plainly understood in the context of other components of the Golgi apparatus. In the meantime, the interesting and interconnected properties of ldlCp and of the presumptive *LDLB* gene should provide more than sufficient motivation for future studies of the interactions of these gene products with each other and with other cellular proteins, and of their roles in normal Golgi functions.

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