cld and *lec23* are disparate mutations that affect maturation of lipoprotein lipase in the endoplasmic reticulum

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Abstract The mutations *cld* (combined lipase deficiency) and *lec23* disrupt in a similar manner the expression of lipoprotein lipase (LPL). Whereas *cld* affects an unknown gene, *lec23* abolishes the activity of α-glucosidase I, an enzyme essential for proper folding and assembly of nascent glycoproteins. The hypothesis that *cld*, like *lec23*, affects the folding/assembly of nascent LPL was confirmed by showing that in cell lines homozygous for these mutations (Cld and Lec23, respectively), the majority of LPL was inactive, displayed heterogeneous aggregation, and had a decreased affinity for heparin. While inactive LPL was retained in the ER, a small amount of LPL that had attained a native conformation was transported through the Golgi and secreted. Thus, Cld and Lec23 cells recognized and retained the majority of LPL as misfolded, maintaining the standard of quality control. Examination of candidate factors affecting protein maturation, such as glucose addition and trimming, proteins involved in lectin chaperone cycling, and other abundant ER chaperones, revealed that calnexin levels were dramatically reduced in livers from *cld/cld* mice; this finding was also confirmed in Cld cells. We conclude that *cld* may affect components in the ER, such as calnexin, that play a role in protein maturation. Whether the reduced calnexin levels per se contribute to the LPL deficiency awaits confirmation.—Briquet-Laugier, V., O. Ben-Zeev, A. White, and M. H. Doolittle. ***cld* and *lec23* are disparate mutations that affect maturation of lipoprotein lipase in the endoplasmic reticulum. *J. Lipid Res.* 1999.** 40: **2044–2058.**

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Lipoprotein lipase (LPL), the major enzyme hydrolyzing the triglyceride core of chylomicrons and very low density lipoproteins, is synthesized and secreted by the parenchymal cells of extrahepatic tissues and by neonatal liver cells. In these tissues, LPL is anchored to the capillary endothelium via heparan sulfate proteoglycans, and functions in liberating free fatty acids for storage or oxidation (1, 2).

Glycosylation at the conserved *N*-terminal site in LPL has been shown to be crucial for the formation of active enzyme (3). Similar to other glycoproteins (4), core oligosaccharide chains, consisting of the 14-saccharide unit $Glc_3Man_9GlcNAc_2$, are added co-translationally in the endoplasmic reticulum (ER). Early on, these core high mannose chains undergo a series of trimming reactions that involve removal of the terminal glucose by glucosidase I, followed by successive hydrolysis of the inner glucoses by glucosidase II. Inhibition of these trimming reactions by specific glucosidase inhibitors (castanospermine, deoxynojirimycin) severely inhibits catalytic activity of LPL, and most of the inactive enzyme is retained intracellularly (5–8).

Addition and removal of the outer glucose residues from core glycans is a prerequisite for entry of nascent glycoproteins into a folding cycle involving lectin chaperones. Specifically, these chaperones (e.g., calnexin, calreticulin) recognize and bind the innermost glucose residue of nascent glycoproteins, after trimming of the two distal glucose residues by glucosidase I and II (9–12). Removal of the final glucose residue by glucosidase II liberates nascent proteins from their lectin anchors. If the released proteins are still improperly folded, binding is resumed after reglucosylation of the protein by UDP-glucose:glycoprotein glucosyltransferase (UGGT) (13). Thus, cycles of re- and de-glucosylation permit repeated access of nascent glycoproteins to the chaperones (14). The bound glycoproteins may interact with ERp57, a protein that associates with calnexin/calreticulin (15–17), that may promote

Abbreviations: Cld, cell line derived from *cld/cld* mice; Cs, castanospermine; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERGIC, ER-to-Golgi intermediate compartment; endo H, endoglycosidase H; Het, cell line derived from +/*cld* mice; Lec23, glucosidase I deficient CHO cell line; LPL, lipoprotein lipase; LPL_{TR}, lipoprotein lipase tandem repeat construct; PL, pancreatic lipase; Pro5, control CHO cell line; UGGT, UDP-glucose:glycoprotein glucosyltransferase; BFA, brefeldin A.

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folding through its protein–disulfide isomerase activity (18) and translgutamination activity (19). Upon attaining a native conformation, proteins released from the chaperones by glucosidase II are no longer recognized by UGGT, and thus exit from the cycle and continue along the secretory pathway. The components of the folding cycle (e.g., lectin chaperones, ERp57, glucosidase II, and UGGT) comprise a functional complex within the ER referred to as the "calnexin cycle". If any of these components are absent or non-functional, the calnexin cycle is compromised. Thus, in cell lines deficient in glucosidase I (Lec23) or glucosidase II (Pha^R2.7) activity, association of nascent glycoproteins with calnexin is virtually nonexistent (20).

While initial glycosylation events are crucial in LPL maturation, a naturally occurring mutation in the mouse, *cld* (combined lipase deficiency), also affects lipase activity posttranslationally, although the mechanism is unknown (reviewed in ref. 21). Located on chromosome 17, this recessive mutation virtually abolishes LPL and hepatic lipase activity, resulting in severe chylomicronemia and death 2–3 days after birth (21–23). Mice homozygous for the mutation synthesize normal levels of lipase protein that is inactive and retained intracellularly (24–26). The mutation does not affect the lipase coding sequence, as *cld* does not co-localize with the lipase structural genes (27, 28). Rather, it has been proposed that *cld* might interfere with early glycan modification events, or with the course of lipase folding carried out in conjuncture with these events (21).

In this study, we attempt to elucidate the underlying basis of the lipase deficiency caused by the *cld* mutation. To accomplish this aim, a cell line derived from *cld*/*cld* mice (Cld) was established and compared with the Lec23 cell line deficient in glucosidase I activity (29). This allowed a side-by-side comparison of the effect of the two mutations on lipase activity, as well as on lipase structural characteristics such as assembly state and heparin affinity. Based on these criteria, the majority of LPL failed to attain a native conformation, supporting the hypothesis of improper folding/assembly as the underlying defect of both mutations. We then tested possible candidate processes in the ER that could explain lipase misfolding, including ER-to-Golgi transport, glucose addition and removal, and components of the calnexin cycle and other ER chaperones. Of these, only calnexin levels were found to be substantially decreased in microsomal-enriched fractions from either *cld*/*cld* livers or the Cld fibroblast cell line. Whether reduced calnexin levels in Cld cells contribute to the lipase deficiency awaits further experimentation. However, as *cld* reduces calnexin levels, and *lec23* affects binding of glycoproteins to calnexin, it is tempting to speculate that both mutations may impair maturation of LPL by limiting its access to the calnexin cycle.

EXPERIMENTAL PROCEDURES

Materials

Ionomycin and brefeldin A were purchased from Calbiochem and Epicentre Technologies, respectively. Castanospermine (Cs), deoxynojirimycin, and endoglycosidase H (endo H) were from Genzyme. Endoglycosidase D (endo D), collagenase A, and insulin were from Boehringer Mannheim Biochemicals. Jack bean α-mannosidase was from Oxford GlycoSystems. Heparin-Sepharose was from Pharmacia. Biotinylated rabbit anti-chicken IgG, PVDF blotting membranes, and SuperSignal® chemiluminescent substrate were from Pierce. Hyperfilm-ECL photographic film was from Amersham. Antibodies against calnexin, calreticulin, BiP, GRP94 and PDI were purchased from Affinity Bioreagents, Inc. or from Stressgen Biotechnologies Corp. The antibody against ERp57 was a kind gift from M. Kito, Kyoto University.

Cell lines

Lec23, the mutant Chinese hamster ovary (CHO) cell line deficient in α-glucosidase I activity (29), was a generous gift from Pamela Stanley. Pro5, used as a control cell line for Lec23, was obtained from the American Type Culture Collection (ATCC).

Fibroblast cell lines were derived from skin explants removed from *cld*/*cld* and unaffected (+/?) neonatal mice. After washing in sterile PBS, 10–20 of these skin explants (2–3 mm in size) were placed with the dermal side down onto a collagen-coated 60-mm dish containing 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), penicillin–streptomycin, sodium pyruvate, glutamine, and non-essential amino acids. After 3 days, the volume of growth medium was increased to 3 ml, and non-adherent explants were removed. The medium was changed weekly until a substantial outgrowth of cells was observed. At this time adherent skin explants were removed, and when the outgrowing cells reached 50% confluency the cells were passaged. The cultures were maintained in the same growth medium except for lowering serum supplementation to 10% FBS. After about 20 passages, the primary cultures were overgrown by foci of spontaneously immortalized fibroblasts. At this time, FBS was replaced by 10% calf serum and the cell lines were passaged at split ratios of 1:8. Cells maintained for over 200 passages retained normal growth characteristics and morphology. Immortalized fibroblasts derived from either unaffected or *cld*/*cld* mice did not express detectable lipase activity.

To estimate chromosome number per cell, Het and Cld cells were arrested in metaphase using 0.1 mm colcemid, swollen in hypotonic solution (0.04 M KCl, 0.025 M sodium citrate), and fixed in ice-cold acetic methanol (30). Chromosome number was determined in about 50 metaphase-arrested cells, by counting chromosomes under a phase-contrast microscope. As expected for immortalized cells, both cell lines exhibited heteroploidy, predominately in the subtetraploid range; in some cells aneuploidy was also evident. Because of the abnormal chromosomal counts and the possibility of genetic deletions, both cell lines were genotyped for the microsatellite markers *D17Au126*, *D17Au57*, and *D17Au100* that are linked to *cld* on chromosome 17 (31). All three markers were present in both unaffected and affected cell lines, indicating that chromosome 17 in the region of *cld* was intact. In addition, the polymorphic nature of these markers was used to genotype the cells lines: the cell lines derived from normal and affected mice were found to be heterozygous and homozygous, respectively, for the region of chromosome 17 containing the *cld* mutation. Based on these genotypes, the cell lines are referred to as Het and Cld.

cDNA expression constructs

Human cDNA for LPL (32) and PL (33) were subcloned into pRc/RSV and pcDNA1/Neo expression vectors driven by enhancer/promoter sequences from the Rous sarcoma virus long terminal repeat and the human cytomegalovirus, respectively. Both vectors contain a neomycin resistance gene used for selec-

tion of G418-resistant stable cell lines. The LPL tandem repeat (LPL_{TR}) expression construct (a generous gift from Howard Wong) contains in succession the sequence coding for the LPL signal peptide, residues 1–448 (the complete LPL sequence), an 8 amino acid linker (GSIEGRLE), LPL residues 1–448, and 20 bases of the LPL 3′ untranslated region cloned into the pcDNA3 expression vector (34).

Transfection

Transient transfection was carried out by electroporation (35). Briefly, 2–5 × 10^7 cells were exposed to a single voltage pulse (0.33 V, 960 μF) in the presence of 40 μg plasmid DNA, comprised of 30 μg of lipase DNA mixed with 10 μg of a β-galactosidase reporter construct (pCH110). After electroporation, the cell suspensions were distributed into 3 × 60 mm tissue culture plates. The medium was changed after 4 h, and the cells were harvested after reaching confluency, within 24–48 h.

To obtain stably transfected cell lines, cells were transfected using co-precipitates of $CaPO_4$ and plasmid DNA (pRc/RSV constructs for Het/Cld cell lines; pcDNA1/Neo constructs for Pro5/Lec23 cell line) according to manufacturer's instruction (Invitrogen). Cells expressing resistance to antibiotics were selected at a concentration of 400–800 μg/ml G418 (Gibco). Individual antibiotic-resistant colonies were then screened for lipase activity, and the highest expressing clones were retained and expanded for subsequent studies.

Treatment and harvesting of cells

For most experiments, cells were subcultured into 60-mm plates containing 3 ml medium. Treatment of the cells with heparin (10 U/ml), with cytoskeleton-perturbing agents (3 μm ionomycin or 5 μg/ml BFA), or with glucosidase inhibitors (1 mm Cs or 2 mm deoxynojirimycin) was carried out by removing the culture medium, replacing with 2 ml fresh medium containing the pertinent reagent, and incubating the plates for an additional 4.5–5 h. After the experiment, media were recovered and the cell layers were washed twice with phosphate-buffered saline (PBS). The cells were scraped from the plate in 1 ml PBS, centrifuged for 5 min at 4°C, and the pellets were stored at −80°C until use.

Density gradient centrifugation

A detailed description of the sucrose gradient centrifugation protocol has been reported elsewhere (36). Briefly, gradients of 5–20% sucrose (11.7 ml) were prepared in LPL lysis buffer (50 mm $NH_4OH \cdot HCl$ buffer, pH 8.1, containing 10 U/ml heparin and 0.2% deoxycholate). Gradients were overlaid with a sample of cell lysate (approx. 2 mg cellular protein) containing two internal molecular size markers: 35 units glucose-6-phosphate dehydrogenase (G-6-PDH, 114 kDa) and 15 units malic dehydrogenase (MDH, 74 kDa). Each sedimentation experiment included a separate gradient containing additional molecular size markers: 200 μg cytochrome C (12 kDa), 200 μg ovalbumin (45 kDa), and 400 μg catalase (240 kDa) in LPL lysis buffer. Gradients were centrifuged at 4°C in a Beckman SW 41 Ti rotor for 22 h at 200,000 *g*, and fractionated into 26 aliquots of 480 μl, starting from the top; fractions were stored at −80°C until assayed.

Heparin-sepharose chromatography

One ml heparin-Sepharose (Pharmacia Biotech.), suspended and washed in distilled water, was packed into 1 × 10 cm columns and equilibrated with column buffer (10 mm Tris-HCl, pH 7.3, containing 0.1% Triton X-100). Samples subjected to chromatography were cell lysates obtained from five or six 100-mm plates or 8 ml of medium from Cld cells incubated overnight with 10 U/ml heparin; Triton X-100 was added to the medium prior to chromatography (final concentration, 0.1%). The column was washed with 10 bed-volumes of column buffer, and LPL elution was carried out in two stages: addition of 0.75 m NaCl, followed by 2 m NaCl, in the column buffer. The eluate was collected in 0.6-ml fractions. To preserve LPL activity, heparin (10 U/ml) was added to all eluted fractions and samples were immediately frozen at −80°C until assayed.

LPL western blotting

The detailed protocol of LPL immunodetection by Western blotting has been described (37). Briefly, LPL immunoprecipitates, generally representing 10 mu activity, were dissociated from Staph A pellets using 30 μl 0.5% SDS in 50 mm sodium phosphate buffer, pH 5.75. For samples subjected to digestion by endoglycosidase (endo) H (Boehringer), 2.5 mu of endo H were added and the sample was incubated overnight at 37°C. When the amount of NaCl in the samples varied (e.g., in heparin-Sepharose eluates), the salt concentration of all samples was adjusted to equivalence. After PAGE and transfer of the proteins onto polyvinylidene difluoride (PVDF) membranes, blots were submerged for 1 h in blocking buffer (2% casein hydrolysate in 50 mm Tris-HCl, pH 7.5, 0.1% Tween-20) and incubated overnight with chicken anti-bovine milk LPL (0.3–0.4 μg/ml in 10 mm sodium phosphate, pH 7.2, containing 0.15 m NaCl and 0.1% Triton X-100 (PBS-T)). After washing, the membrane was incubated for 30 min with biotinylated rabbit anti-chicken IgG (1:10,000 in PBS-T), washed again, and incubated for 10 min with horseradish peroxidase (HRP)–streptavidin (1:5,000) in PBS-T. After a last series of washes, the blots were then developed by chemiluminescence and exposed to photographic film. Densitometric scanning of lipase bands obtained by chemiluminescence was carried out with the aid of an AMBIS Radioanalytic Imaging System. Integration of the scanned bands was performed using the QuantProbe™ Software, version 4.31.

Jack bean mannosidase digestion of LPL

LPL isolated by immunoprecipitation was incubated overnight at 37°C with jack bean mannosidase (50 mU) in a 60 μl volume containing 100 mm sodium acetate, pH 5.0, 2% Triton X-100, and 0.25% SDS. At the end of incubation, 40 μl of a glycerol/SDS stock (20% glycerol, 5% SDS, 0.01% bromophenol blue) was added prior to Western blotting.

Preparation of microsomes

Liver microsomes from unaffected and *cld*/*cld* neonatal mice were isolated as previously described (38). For microsome-enriched fractions from Cld and Het cells, two 100-mm plates of each cell line were harvested (0.8 ml/plate) in 20 mm HEPES buffer, pH 7.5, containing 0.25 m sucrose, 1 mm EDTA, and protease inhibitors (leupeptin and pepstatin, 10 μm). Cell suspensions were homogenized in a 2-ml Dounce homogenizer with 10 strokes using a loose-fitting pestle followed by 12 strokes using a tight-fitting pestle. The homogenates were subjected to a 10-min centrifugation at 2,500 *g*, followed by a 20 min centrifugation of the supernatant at 12,000 *g*. The microsomal-enriched fractions were stored at −80°C; prior to use, they were resuspended in by sonication for 6 sec at 1 gram-force in 20 mm HEPES buffer, pH 7.5, containing 0.2% deoxycholate (sodium salt) and protease inhibitors (leupeptin and pepstatin, 1 μg/ml).

Enzyme assays

Glucosidase II was assayed as described (39). The incubation mixture contained, in a total volume of 50 μl, 20 mm HEPES buffer, pH 7.5, 10 mm *p* nitrophenyl α-d-glucopyranoside, and sample. As a control for the specificity of the reaction, 2 mm deoxynojirimycin or castanospermine was included in similar assay mixtures. After incubating for 60 min at 37°C, the reaction

was stopped by addition of 50 μl of 2 M Tris base, and the absorbance was monitored at 405 nm.

UGGT was assayed based on Trombetta, Bosch, and Parodi (40), using as substrate either native or denatured thyroglobulin; the latter was obtained by dialyzing the protein (20 mg/ml) for 8 h in 10 mM Tris-HCl, pH 8.0, containing 8 M urea, followed by dialysis in the same buffer without urea for 24 h with a change of buffer after 4 h. For the UGGT assay, the incubation mixture contained in a final volume of 100 μl: 20 mM Tris-HCl, pH 8.0, 300 μg native or denatured thyroglobulin, 10 mM $CaCl_2$, 0.6% Triton X-100, 5 μM UDP-[^{14}C]Glc, 40 μM deoxynojirimycin, and 50-100 μg microsomal protein. Reactions were carried out at 37°C for 40 min, and stopped by addition of 1 ml of 10% trichloroacetic acid (TCA). The mixtures were placed in a 60°C water bath for 10 min, allowed to cool, and poured onto glass filters (2.4 cm). The filters were washed with 10% TCA, dried by rinsing with 95% ethanol and acetone, and placed into scintillation vials containing 1 ml Solvable:H_2O 1:1. After overnight incubation at room temperature, scintillation fluid was added and radioactivity was measured.

For all lipase assays, cell lysates were prepared by sonication in 50 mM NH_4OH-HCl buffer, pH 8.1, containing 10 U/ml heparin and either 0.2% deoxycholate or 0.1% Triton X-100. LPL was assayed using a triolein substrate prepared by polytron emulsification (41). PL was assayed using an emulsion of triolein prepared by sonication (42) with the following modifications: a mixture of 0.5 μg colipase (Boehringer), 0.8 μmol tauro-deoxycholic acid (Sigma), 0.2 μmol $CaCl_2$ was prepared in a final volume of 50 μl water; enzyme source and ammonium buffer (50 mM ammonium-HCl, pH 8.0) were then added to complete the volume to 100 μl, and the reaction was initiated by addition of substrate (100 μl).

Other assays

Cellular protein was assayed using the bicinchoninic acid (BCA) reagent (Pierce). β-Galactosidase activity was determined by standard methods (43).

Renaturation studies

The source of LPL, LPL_{TR}, and PL used for denaturation/renaturation experiments was medium from Pro5 cells obtained after transient transfection with the respective lipase cDNA. Guanidine HCl (GuHCl) was added to 0.5 ml medium to a final concentration of 1.5 M; a similar volume of H_2O was added to a second 0.5 ml aliquot as control, and both samples were incubated on ice for 45 min. After incubation, only the control samples of LPL, LPL_{TR}, and PL were found to contain lipase activity. Both control and GuHCl-denatured samples were then dialyzed overnight in 4 liter of 50 mM ammonium-HCl buffer, pH 8.0, containing 0.2% deoxycholic acid (sodium salt) and 10 U/ml heparin, and reassayed for lipase activity. Whereas removal of the denaturant in the LPL, LPL_{TR} samples was not accompanied by detectable lipase activity, the PL sample regained 90% activity relative to its untreated control.

RESULTS

Lipoprotein lipase is structurally malformed in Cld and Lec23 cells

As the majority of LPL in tissues of *cld*/*cld* mice is inactive and exhibits an immature glycan structure distinctive of glycoproteins in the ER (24, 26), we determined whether these characteristics were present in fibroblast cell lines carrying the *cld* mutation. In Het and Cld cells, which are heterozygous and homozygous for *cld* (see Experimental Procedures), LPL expression was determined after transfection with a human LPL cDNA expression construct. Lipase activity in Cld cells and medium was reduced over 90% relative to Het cells (**Fig. 1A**, left panel). When LPL from equal amounts of cellular protein was immunoprecipitated (representing 10 and 0.7 mu of activity for Het and Cld, respectively), similar amounts of enzyme mass were detected (middle panel); this was not seen in the medium, where levels of LPL mass were proportional to the activity (see below). Thus, in Cld cells, intracellular activity per unit LPL mass was dramatically reduced, while secreted activity per unit LPL mass remained unaffected. As assessed by differential glycan processing using endoglycosidase (endo) H digestion, the subcellular localization of LPL also differed between normal and mutant cells (Fig. 1A, right panel). In Het cells, LPL immunoprecipitates treated with endo H exhibited three forms: one resistant (57 kDa), one partially resistant (55 kDa), and the third completely sensitive (52 kDa) to endo H digestion. In the resistant forms, either both glycan chains of LPL (57 kDa) or one of the chains (55 kDa) are of the complex type, indicating *medial*/*trans*-Golgi processing. Conversely, the 52 kDa form, with an exclusive endo H-sensitive high-mannose structure, is located in the ER/*cis*-Golgi compartments. In contrast to Het cells, LPL in Cld cells was virtually all endo H sensitive (Fig. 1A, lane 4), suggesting its residence in the ER.

The reduced activity per unit LPL mass in Cld cells and its location in the ER implied that the structure of the lipase may be aberrant. Thus, we compared the effect of *cld* with that of *lec23*, a mutation causing glucosidase I deficiency that is known to disrupt protein folding and assembly within the ER. Lec23 cells, along with control CHO cells that exhibit normal levels of glucosidase I activity (Pro5), were stably transfected with human LPL cDNA. As shown in Fig. 1B, the overall pattern of LPL expression in Pro5/Lec23 was very similar to that observed in Het/Cld cell lines. Lipase activity in Lec23 cells and medium was reduced 70–80% relative to Pro5 cells (left panel), although intracellular enzyme mass per mg cellular protein was similar (middle panel). Like Cld cells, levels of LPL mass in the medium of Lec23 cells were proportional to the activity (see below). LPL glycan chains in Lec23 cells were also entirely endo H sensitive (lane 4). Thus, like *cld*, the *lec23* mutation resulted in a decreased intracellular activity per unit LPL mass that was accompanied by the apparent retention of the enzyme in the ER/*cis*-Golgi. However, *lec23* and *cld* are not identical mutations. This was apparent from the delayed electrophoretic migration rate of LPL in Lec23 cells, due to lack of glucose trimming, that was not observed in Cld cells (Figs. 1A and B, compare lane 2 to the respective control in lane 1).

The atypical patterns of LPL expression in the mutant cell lines could be explained if the lipase was structurally malformed. To establish whether this was the case, several approaches were used. The first approach was to examine two physical properties of LPL, native size and heparin affin-

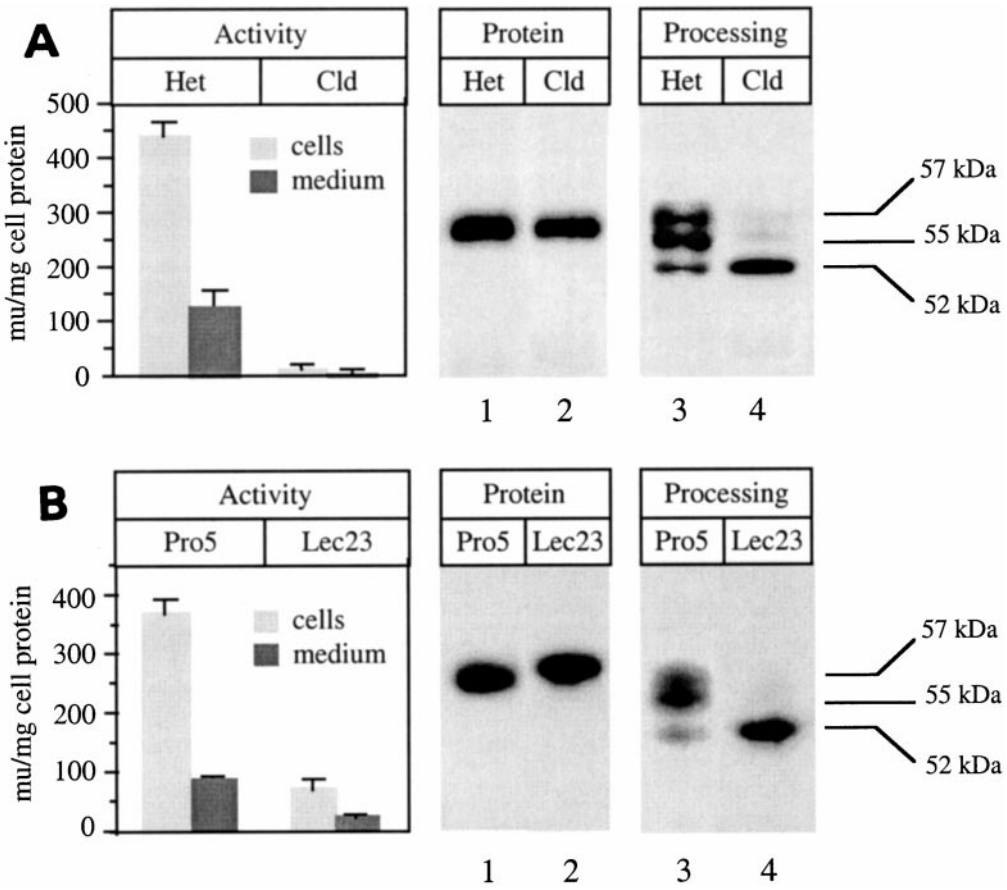

Fig. 1. LPL expression in unaffected and mutant cell lines. A: LPL expression in Het and Cld cells. Left panel, LPL activity in cells and medium, normalized to mg cell protein. Middle panel, LPL mass derived from immunoprecipitation of equal amounts of lysate protein in Het and Cld cells, representing 10 and 0.7 mu activity, respectively. Right panel, oligosaccharide processing of intracellular LPL. Samples identical to those shown in the middle panel were subjected to endo H digestion prior to Western blot analysis. B: LPL expression in Pro5 and Lec23 cells. The data are represented as described in *A*, except that LPL mass was determined from equal amounts of lysate protein in Pro5 and Lec23 cells representing 10 and 4 mu activity, respectively. Note the delayed migration rate of LPL from Lec23 cells, resulting from untrimmed glucose residues.

ity, as both are sensitive to changes in three-dimensional conformation. LPL size was determined by examining the sedimentation profile of LPL from normal and mutant cell lysates following density gradient centrifugation. As shown in the histogram portions of **Fig. 2**, active LPL in control and mutant cell lines sedimented exclusively as a dimer, the peak fraction (#12) corresponding to a molecular mass of 110 kDa. In contrast, the distribution of LPL mass, as determined by Western blotting (shown below the histograms), differed significantly between the mutant and the normal cells. In Het and Pro5 cells, the distributions of LPL mass and activity were superimposable and Gaussian in form; only a small amount of protein was detected at the bottom of the gradient (fraction 26), indicative of some aggregated protein. In contrast, in mutant cells, the protein was clearly skewed towards higher molecular weight complexes (fractions 14–25), and a significant amount migrated to the bottom of the gradient suggesting that it was highly aggregated. The aggregated state of LPL in the mutant cells is indicative of proteins that inappropriately expose hydrophobic regions due to misfolding or denaturation (44, 45).

Heparin affinity is another physical property of LPL that is dependent on the structural integrity of the lipase molecule: active, dimeric LPL binds to heparin with high affinity, and dissociates from the ligand only at high ionic strength (>1 m NaCl); LPL inactivation, which reflects alterations in folding and stability, is associated with a reduction in heparin affinity. Thus, the LPL elution profile from heparin-Sepharose was compared in mutant and control cells. A two-step elution protocol, consisting of 0.75 m NaCl followed by 2.0 m NaCl, was used to elute LPL with low- and high-affinity for heparin, respectively. As shown in **Fig. 3** (top), in all four cell lines LPL activity appeared only in the 2.0 m NaCl eluate, although the activity in Cld and Lec23 was dramatically reduced. Analysis of LPL mass (Fig. 3, bottom) revealed that in control cells most of the protein was associated with the 2.0 m NaCl eluate (fractions 14–15). In contrast, all detectable mass in Cld cells, and a significant proportion of the mass in Lec23, eluted at 0.75 m

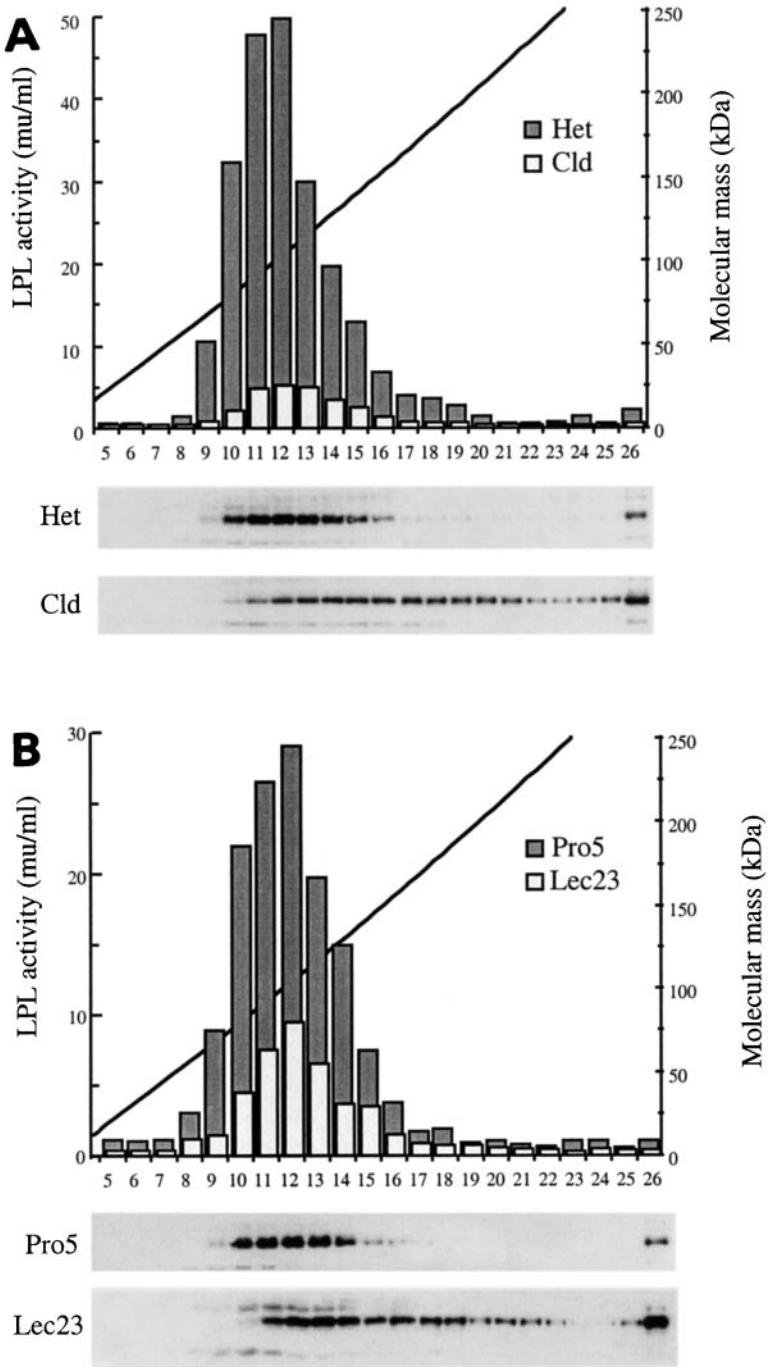

Fig. 2. Sedimentation profile of LPL activity and mass in unaffected and mutant cells. Lysates obtained from two 100-mm plates from each cell line were combined and subjected to rate-zonal centrifugation through a 5–20% sucrose gradient. Fractions obtained were assayed for LPL activity and sucrose concentration and subjected to LPL Western blotting. Molecular mass was estimated using internal and external standards, which were used to construct a standard curve (background line). Based on this curve, the human LPL dimer (estimated to be 107,500 Da) is expected to sediment in fraction 12. A: LPL sedimentation profile in Het and Cld cells. B: LPL sedimentation profile in Pro5 and Lec23 cells. Note the similar pattern of heterogeneous aggregation displayed by Cld and Lec23 cells, in contrast to the expected dimer distribution in Het and Pro5 cells.

NaCl (fractions 2–5). Thus, as evidenced by decreased heparin affinity and a highly aggregated state, inactive LPL in the mutant cells appears to be structurally malformed.

A second, more indirect, approach was also used to verify the aberrant physical nature of inactive LPL in the mutant cells. This approach was based on the cell's ability to recognize misfolded or unassembled secretory proteins by quality control mechanisms operating in the ER (9, 46, 47). Thus, if inactive LPL is structurally malformed in the mutant cells, then this fraction should be retained in the ER; conversely, any LPL that exits the ER should possess a native structure exhibiting full catalytic activity.

In both mutant and control cells, low-affinity LPL was exclusively endo H sensitive, suggesting retention of inactive LPL in the ER (**Fig. 4**, lanes 1, 4, 7, 10). Only high-affinity LPL exhibited endo H-resistant forms, although a discernible amount of high mannose LPL was also present, particularly in the mutants (lanes 2, 5, 8, 11). In controls, the majority of high-affinity LPL was localized to Golgi/post-Golgi compartments, as evidenced by the similar degree of processing compared to secreted LPL (lanes 3, 9). However, a small fraction of high-affinity LPL was endo H sensitive (lane 8), indicating that LPL can assume a native conformation in the ER/*cis*-Golgi.

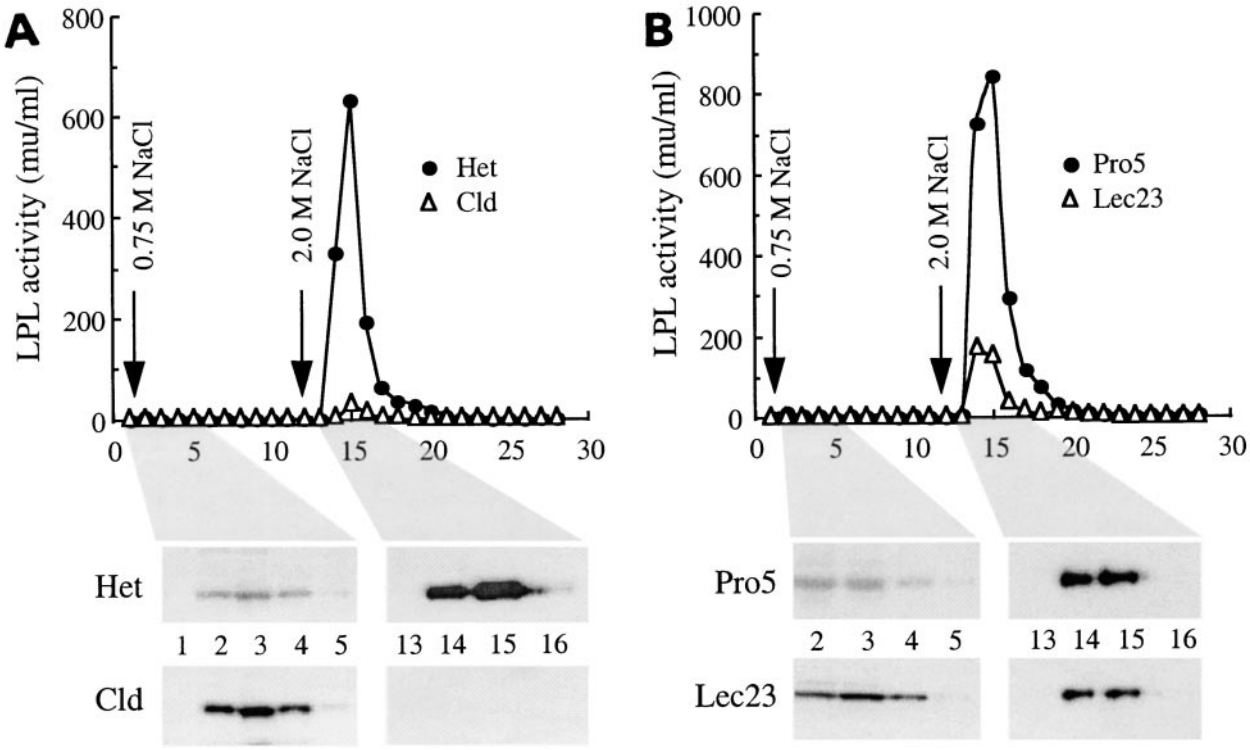

Fig. 3. Heparin affinity of LPL from unaffected and mutant cells. Cell lysates were subjected to heparin-Sepharose chromatography. Bound LPL was sequentially eluted with 0.75 M NaCl (fractions 1–12) and 2.0 M NaCl (fractions 13–28). The graphs illustrate the elution profile of LPL activity (mu/ml). The bottom figures show LPL mass detected by Western blot analysis of equal volumes from fractions 2–5 (low-affinity LPL) and 13–16 (high-affinity LPL). A: Elution profile of Het and Cld cells. B: Elution profile of Pro5 and Lec23 cells. Although LPL protein was not seen in the high-affinity fractions (lanes 13–16), it can be visualized after concentration (see Fig. 4, lane 5).

In Cld, a significant proportion of high-affinity LPL displayed complex glycan chains (lane 5), in contrast to inactive LPL in the ER (lane 4). The ability of Cld cells to carry out normal Golgi processing of LPL glycans was confirmed by concentrating the small amount of lipase secreted to the medium. Indeed, endo H digestion of this fraction indicated that LPL exhibited only endo H-resistant (complex) forms (lane 6), similar to LPL secreted

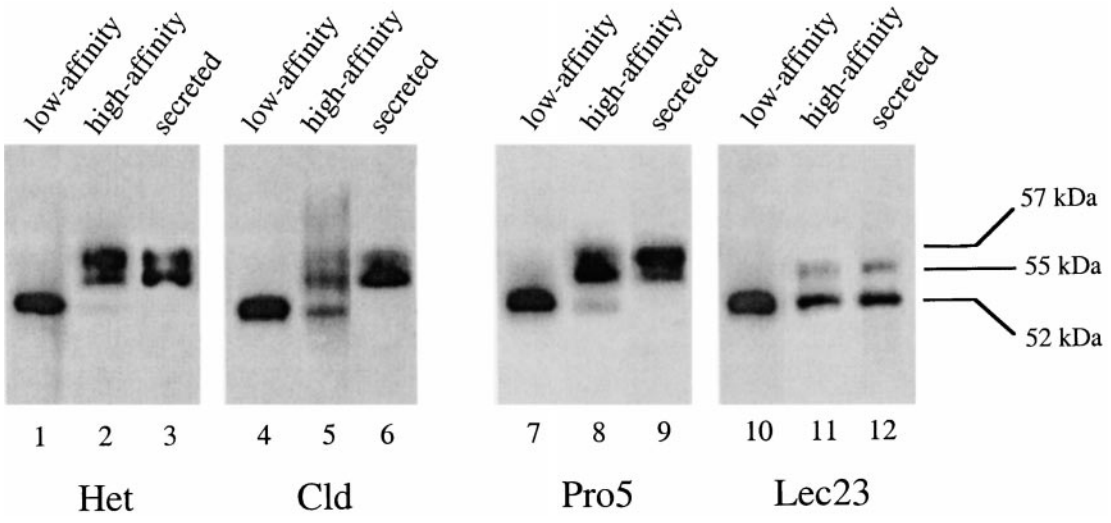

Fig. 4. Active and inactive forms of LPL are localized in different subcellular compartments. Intracellular LPL eluted from heparin-Sepharose at 0.75 M and 2.0 M NaCl (low- and high-affinity, respectively), as well as secreted LPL, was immunoprecipitated and subjected to endo H digestion. Sample volumes used for LPL immunoprecipitation were adjusted to obtain signals of comparable intensity, due to the different proportions of high- and low-affinity LPL mass in unaffected and mutant cells. Thus, the volumes of 0.75 M NaCl eluate, 2.0 M eluate, and medium, respectively, were: Het, 50 μl, 15 μl, and 20 μl; Cld, 75 μl, 250 μl, and 8 ml (the latter was concentrated by heparin-Sepharose chromatography prior to immunoprecipitation); Pro5, 100 μl, 10 μl, and 10 μl; Lec23, 50 μl, 30 μl, and 900 μl.

by Het and Pro5 cells (lanes 3, 9). In contrast to Cld, high-affinity LPL in Lec23 cells remained predominantly endo H sensitive (52 kDa), with only a minor component becoming partially endo H resistant (55 kDa; lane 11). Clearly, lack of glucosidase I activity disrupted the normal course of LPL Golgi processing. However, as a similar pattern was displayed by the small amount of LPL secreted by Lec23 cells (lane 12), we concluded that, despite lack of processing, most LPL in the high-affinity fraction was located in Golgi/post-Golgi compartments. Thus, in both Cld and Lec23 cells, the small proportion of LPL attaining a high-affinity, native conformation was normally secreted, emphasizing that these mutant cells recognize and retain the majority of LPL due to its misfolded structure.

To confirm that high-affinity LPL in the mutants was indeed comprised of only fully functional LPL molecules, LPL mass was compared in 2.0 m NaCl eluates from Lec23 and Pro5 cells containing identical amounts of activity (1, 3, and 5 mu; **Fig. 5A**, top). Densitometric scanning of the bands obtained by Western blotting revealed that activity per unit LPL mass was identical in the high-affinity eluates from Lec23 and Pro5 cells (Fig. 5A, bottom). Thus, despite incomplete glycan processing in Lec23 cells, acquisition of high heparin affinity was exclusively associated with formation of a native, active LPL structure. In Cld cells, the exceedingly small amount of high-affinity LPL prohibited a similar type of analysis. However, concentration of a large volume of medium derived from Cld cells followed by immunoprecipitation enabled us to compare activity per unit LPL mass in the medium of unaffected and mutant cells. Indeed, equal ratios of activity per unit LPL mass in the medium of Pro5, Lec23, and Cld confirmed that only functional LPL was secreted (Fig. 5B). In conclusion, neither *cld* nor *lec23* represent mutations that affect LPL secretion; rather, quality control mechanisms operating in both unaffected and mutant cells appear to limit secretion to only native enzyme, while retaining misfolded LPL in the ER.

In Cld and Lec23 cells, the failure of most LPL to assume its proper structure suggests that this lipase has polypeptide domains or subunits whose folding or assembly may require assistance by ER-mediated processes that are defective in the mutant cells. Accordingly, a protein able to fold in the absence of cellular factors should not be affected by either mutation. To test this hypothesis, the expression of pancreatic lipase (PL) was examined in the mutant cells, an enzyme structurally related to LPL but capable of renaturation to an enzymatically active state in vitro (see Experimental Procedures). Human PL cDNA, along with a β-galactosidase reporter gene, were co-transfected into the Het/Cld and Pro5/Lec23 cell lines. In both Cld and Lec23 cells, relative PL activity levels were found to be unaffected (**Table 1**). Activity levels in medium of Cld cells were also comparable to wild-type, although a 50% decrease was observed in medium of Lec23 (Table 1). While the reason for this decrease is unknown, the high levels of intracellular activity clearly indicated that PL expression was not affected by either mutation.

The specificity of *cld* and *lec23* for LPL expression indicated a common structural feature of this lipase that is not

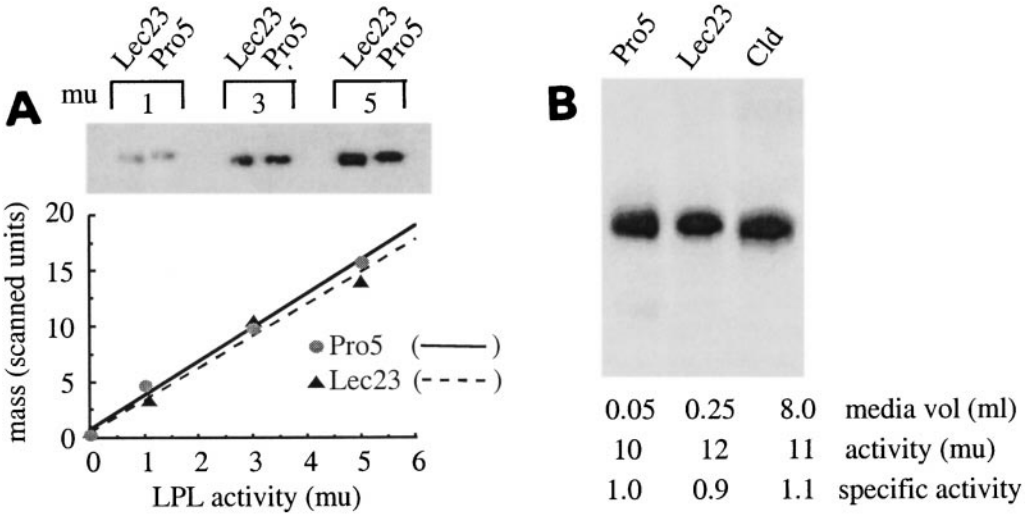

Fig. 5. Maintenance of quality control in unaffected and mutant cell lines. A: LPL specific activity in the high-affinity heparin-Sepharose fractions from Pro5 and Lec23 cells. Samples from the 2.0 m heparin-Sepharose eluates containing increasing activity levels (1, 3, 5 mu) were subjected to Western blotting (top figure), and relative mass was determined by densitometry. The corresponding regression lines of LPL mass versus activity (bottom figure) yielded slopes that were 30.6 and 28.8 for Pro5 and Lec23, respectively. Thus, LPL mass in the high-affinity fraction from control and mutant cells was equally active. B: LPL specific activity in medium from unaffected and mutant cells. Cells were incubated for 4 h in the presence of heparin (10 U/ml) to maximize the amount of LPL in the medium. LPL was immunoprecipitated from the volumes indicated under each lane. LPL in medium from Cld cells was concentrated by heparin-Sepharose chromatography prior to immunoprecipitation. LPL mass was calculated by densitometry; the relative activity/unit LPL mass obtained for Pro5 was arbitrarily set at 1.0.

TABLE 1. Effect of the cld and lec23 mutations on the expression of pancreatic lipase and the LPL tandem repeat molecule

Expression Construct	Relative LPL Activity			
	Het	Cld	Pro5	Lec23
PL				
Cells	8.11 ± 1.33	5.90 ± 4.33	31.7 ± 2.05	41.6 ± 8.70
Medium	96.6 ± 3.72	82.5 ± 4.36	95.9 ± 4.04	47.2 ± 5.05[a]
LPL_{TR}				
Cells	76.9 ± 9.80[b]	11.4 ± 12.1[a,b]	21.3 ± 1.95	3.16 ± 0.49[a]
Medium	90.6 ± 8.28	6.22 ± 1.46[a]	95.4 ± 12.7	5.39 ± 0.16[a]

Control and mutant cells were transfected either with a human PL or a LPL_{TR} expression construct: a β-galactosidase plasmid was co-transfected with each lipase to account for variations in transfection efficiency. LPL activity was assayed in cells and medium, and normalized to β-galactosidase (referred to as Relative LPL Activity). For LPL_{TR}, heparin (10 U/ml) was added to the medium 4 h prior to harvesting unless otherwise indicated. Values represent mean ± SD obtained from three plates.

[a] The decrease relative to control cells (Het, Pro5) was significant at $P < 0.01$.

[b] Values are from cells not incubated with heparin.

TABLE 2. Effect of BFA on activity per unit LPL mass

	No BFA	+BFA
Wild type	1.00 ± 0.25	1.22 ± 0.77
cld/*cld*	0.12 ± 0.1	0.3 ± 0.12[a]
Pro5	1.00 ± 0.4	1.01 ± 0.38
Lec23	0.37 ± 0.10	0.52 ± 0.05[a]

Activity per unit LPL mass was calculated as the ratio between the activity of the sample subjected to Western blotting and the mass determined by densitometric scanning. The ratio obtained for wild-type cells (no BFA) was designated as 1.00; all other values were expressed relative to this ratio. In all cases, values obtained from the mutants were significantly lower than those obtained for wild-type cells ($P < 0.05$).

[a] The change in LPL specific activity in BFA-treated cells relative to control was significant at $P < 0.02$.

shared by PL. A possible candidate was homodimer assembly, as only PL is functional as a monomer. To examine this possibility, we used a construct consisting of two human LPL monomers connected by a short polypeptide linker; this tandem repeat molecule (LPL_{TR}) does not oligomerize and expresses full enzymatic activity (34).

LPL_{TR} cDNA was transfected into wild-type and mutant cell lines, and LPL activity was determined relative to that of co-transfected β-galactosidase. As shown in Table 1, LPL_{TR} activity levels were reduced in Cld cells and medium by 85% and 93%, respectively. This clearly affected phenotype was nearly identical to the deficiency observed for native LPL. Similarly, LPL_{TR} expression levels in Lec23 cells and medium were decreased by 85% and 94%, respectively (Table 1), once again reflecting the phenotype exhibited by the native enzyme. Thus, LPL_{TR} is affected by the mutations even though the attainment of LPL_{TR} activity does not require subunit assembly in the ER.

Testing candidate processes in the ER that may be affected by the *cld* mutation

While Lec23 cells are deficient in glucosidase I activity (29), the nature of the *cld* defect awaits identification of the gene on chromosome 17. However, we examined some processes that have been proposed to be affected by *cld* as they are known or suspected to affect LPL expression posttranslationally. As LPL in *cld*/*cld* mice is located predominately in the ER, one of the proposed effects of *cld* was a block in LPL transport from the ER to more distal compartments, where its final maturation would occur (24–26, 48). This possibility has recently gained support in studies suggesting that treatment with brefeldin A (BFA), an agent causing resorption of post-ER components back into the ER (49), was associated with a gain in LPL activity in *cld*/*cld* adipocytes (50). However, when we treated *cld*/*cld* adipocytes and Lec23 cells with BFA in the presence of heparin, BFA induced only a small increase in activity per unit LPL mass (**Table 2**). This increase in activity per unit LPL mass reflects, at least in part, intracellular accumulation of active LPL that would normally be secreted. In fact, when BFA was replaced by ionomycin, an agent that induces indiscriminate transport of ER proteins to distal compartments but does not abolish secretion (51, 52), no increase in activity per unit LPL mass was observed in either Cld or Lec23 cells (data not shown).

Glycan processing of LPL was used to verify that BFA treatment had resulted in the resorption of Golgi components back to the ER. Analysis of LPL glycan processing in wild-type and *cld*/*cld* adipocytes treated with heparin alone (no BFA, **Fig. 6A**) indicated that LPL was predominately endo H-sensitive (52 kDa; lanes 2, 6) and thus localized in the ER/*cis*-Golgi. However, incubation with BFA (+ BFA) resulted in conversion of LPL to endo H-resistant forms (57 kDa; lanes 4, 8) due to retrieval of Golgi components, including oligosaccharide-processing enzymes, back to the ER. Similar results were obtained in Pro5 and Lec23 cells (Fig. 6B, lanes 2, 4, 6), except for incomplete processing of LPL in BFA-treated Lec23 cells (lane 8). This latter result was expected, as untrimmed LPL glycans in Lec23 cells are only poorly processed by Golgi enzymes (Fig. 4). However, a partially processed 55 kDa form was apparent even in Lec23 cells, indicating that Golgi retrieval had occurred (Fig. 6B, compare lanes 6, 8). Taken together, our results indicate that combined lipase deficiency is not the result of a blockage in transport of immature LPL from the ER to the Golgi.

As an alternative to deficient transport, it has been proposed that impaired lipase maturation in *cld*/*cld* mice could result from a defect in glycosylation (22, 25, 26, 48). The numerous reports stressing the importance of glucose trimming for lipase activity (5–8, 53) led to the proposal that *cld* may affect this initial glycan processing step (54, 55). Although in this study we showed that *cld* does not affect glucosidase I activity (see above), cognate factors involved in addition or complete removal of glucose residues from the glycan chains of nascent lipases could still be affected. For instance, it is possible that the core oligosaccharides added to nascent proteins in Cld cells contain no glucose residues at all, i.e., are of the type $Man_{7-9}GlcNAc_2$. This possibility was eliminated when Cld cells were incubated with or without castanospermine (Cs),

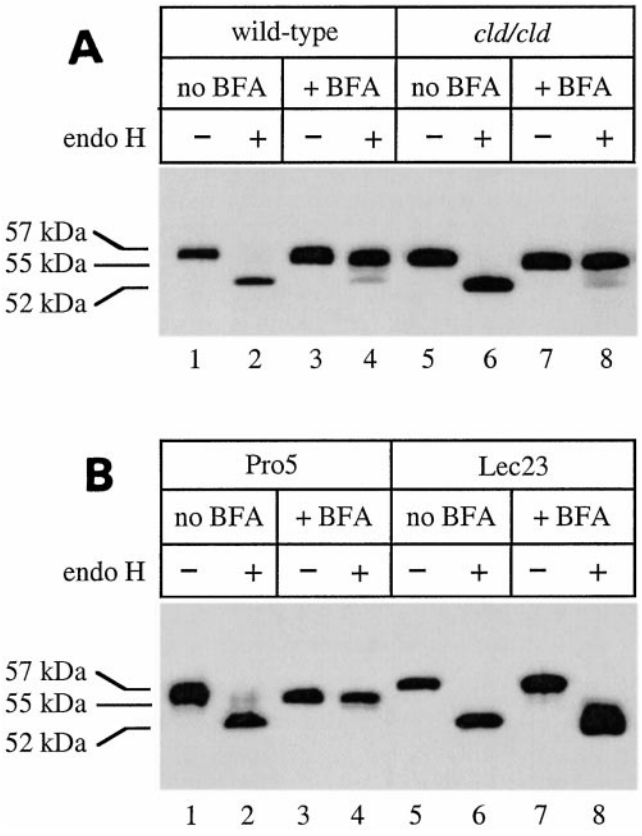

Fig. 6. Effect of brefeldin A on oligosaccharide processing of LPL from wild-type and mutant cells. Cells were incubated with medium containing 10 U/ml of heparin. After 30 min, the medium was changed and replaced with fresh medium containing 10 U/ml heparin in the absence (no BFA) or presence (+ BFA) of brefeldin A (5 μg/ml). The cells were harvested after additional 4.5 h. LPL immunoprecipitates from cell lysates were incubated with (+) or without (−) endo H prior to Western blot analysis. A: LPL exhibits Golgi-processed glycan chains in BFA-treated brown adipocytes isolated from wild-type and *cld*/*cld* littermates. B: Differential processing of LPL glycan chains in Pro5 versus Lec23 cells after treatment with BFA. LPL migrating at 55 and 57 kDa indicates the presence of one high-mannose and one complex (Golgi-processed) glycan chain, or two complex chains, respectively; the 52 kDa form contains exclusively high-mannose glycan chains.

a potent glucosidase I and II inhibitor (56). As shown in **Fig. 7**, the migration of LPL from Cs-treated Cld cells was delayed compared to that of LPL from untreated Cld cells, and closely resembled the migration of LPL from Lec23 cells (lanes 1–3). Thus, in the presence of Cs, core oligosaccharides in Cld cells contained the expected $Glc_3Man_9GlcNAc_2$ structure (compare diagrams *a* and *b*, top Fig. 7).

As LPL in Cld cells contained fully glucosylated glycan chains, the proper removal of these glucose residues was next investigated. The extent of glucose removal was assessed using jack bean α-mannosidase, an exomannosidase that distinguishes between oligosaccharides with incompletely or fully glucose-trimmed glycans (57, 58). As shown schematically in Fig. 7, the enzyme removes maximally five mannose residues from glucose-containing chains (diagram *d*), but has access to eight α-linked mannoses in unglucosylated chains (diagram *c*). Thus, the shift in mobility after mannose trimming of glucose-free glycans is expected to be larger than that of glycans still containing glucose(s); this shift, however, should not be as large as that produced by endo H digestion, when the entire glycan, except the innermost GlcNAc, is removed (diagram *e*). As shown in Fig. 7, the shift in mobility of mannosidase-digested LPL from Cld cells (lane 4) was indeed larger than the shift seen in LPL from Cs-treated Cld or from Lec23 cells (lanes 5, 6), but smaller than that caused by endo H digestion (lanes 7, 8). Thus, *cld* affects neither addition nor subsequent trimming of glucose residues from nascent oligosaccharide chains.

Finally, we examined whether *cld* affected key elements comprising the lectin chaperone cycle, including calnexin, calreticulin, ERp57, UGGT, and glucosidase II, as a defect in any of these components may affect lipase folding. UGGT and glucosidase II activities were measured in microsomal preparations of Het and Cld cells and were found to be unaffected by the mutation (data not shown). The levels of calnexin, calreticulin, and ERp57 were examined by Western blotting of liver microsomes isolated from wild-type and *cld*/*cld* mice. While calreticulin and ERp57 levels were not affected, a dramatic decrease (about 85%) was found in calnexin levels of *cld*/*cld* cells (**Fig. 8A**). The levels of three abundant ER chaperones not involved in the calnexin/calreticulin cycle, but playing essential roles in protein folding, were also compared. These were GRP94 and BiP (prominent members of the stress-70 and stress-90 protein family (59)) and PDI (catalyzing disulfide bond formation and isomerization (60)). As shown in Fig. 8A, the levels of these proteins were similar in microsomes of wild-type and *cld*/*cld* mice.

The reduced calnexin levels in liver microsomes from *cld*/*cld* mice was confirmed in ER-enriched fractions from the Cld cell line. Whereas GRP94 levels remained unaffected, calnexin levels were dramatically decreased relative to the Het control (Fig. 8B). Thus, calnexin is the only component of the lectin chaperone cycle, or among other abundant ER chaperones, that appears to be affected by the *cld* mutation.

DISCUSSION

We have demonstrated that the Cld fibroblast cell line exhibits patterns of LPL expression that are indistinguishable from those described in tissues (25, 26, 61) or primary brown adipocytes (24, 50) of *cld*/*cld* mice. The decreased LPL activity in the Cld cell line was associated with reduced activity/unit LPL mass and a high-mannose glycan structure, implying that most of the intracellular lipase was catalytically inactive and located in the ER (Fig. 1A). This suggested that *cld* may decrease the ability of the newly synthesized lipase to form a correct three-dimensional structure.

While the identity of the *cld* mutation is unknown, *lec23* is a well-characterized mutation that inhibits trimming of

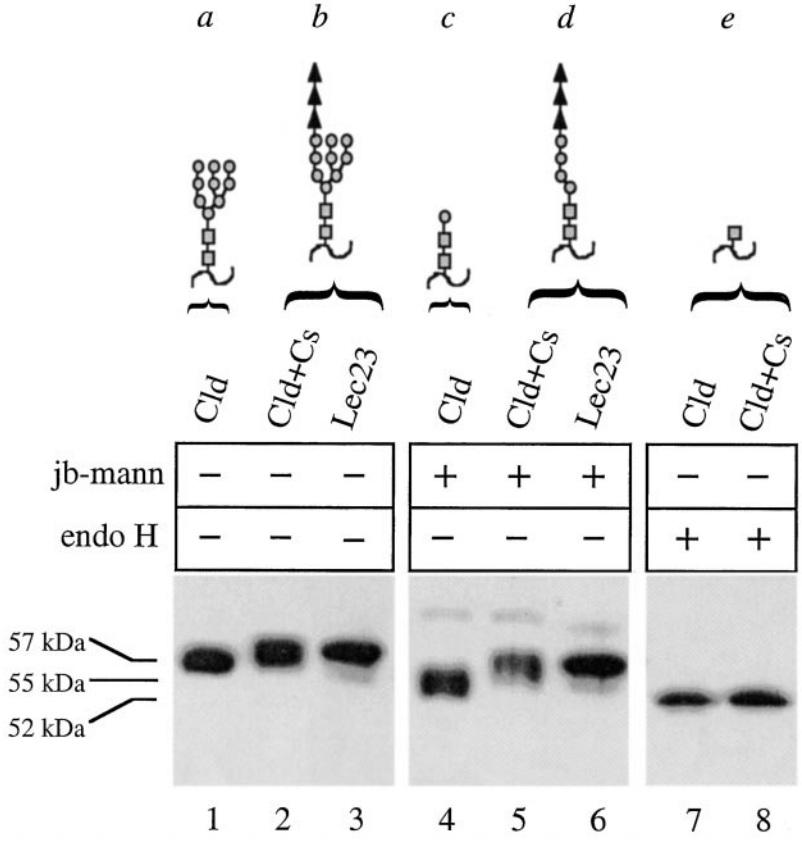

Fig. 7. The *cld* mutation does not affect addition or trimming of glucose residues on LPL oligosaccharides. Cells were grown to near-confluency in 60-mm dishes, and incubated for 4 h either with medium alone (Cld, Lec23) or with medium containing 1 mm castanospermine (Cld + Cs). LPL immunoprecipitated from these cells was incubated with (+) or without (−) jack bean mannosidase (jb-mann) or endo H prior to Western blot analysis. The top diagram is a schematic representation of the inferred oligosaccharide structure: ■, N-acetylglucosamine; ●, mannose; ▲, glucose.

the outer glucose residue from newly synthesized glycoproteins. This step is essential to the folding/assembly of nascent glycoproteins in the ER (62, 63). Indeed, *lec23* affected LPL expression in a manner remarkably similar to *cld*, including diminished activity per unit LPL mass and a high mannose structure suggestive of its localization in the ER. A notable difference was the delayed electrophoretic migration of LPL in Lec23, caused by the untrimmed glucose residues (Fig. 1), indicating that *cld* and *lec23* represent distinct mutations.

A significant fraction of LPL in Cld and Lec23 cells exhibited physical features of a misfolded protein, including sedimentation as large complexes that trailed out from the region of the gradient containing active, dimeric LPL (Fig. 2). This skewed distribution is characteristic of heterogeneous aggregation and, as observed for LPL, these aggregates can range in size from small oligomers to very large complexes (64). The inactive LPL in the mutant cells also displayed low affinity for heparin (Fig. 3). Indeed, a decrease in heparin affinity has been found to be highly correlated with factors that induce loss of LPL activity, such as heat denaturation, chaotropic agents, and several structural gene mutations (65–68). Although this decreased affinity has been generally interpreted as mere dissociation of LPL into monomers, all factors mentioned above affect protein organization, including tertiary and secondary structures. Thus, in the mutant cells, the large fraction of inactive LPL eluting from heparin-Sepharose at low ionic strength implies misfolding or improper assembly.

Our results demonstrating LPL aggregation and reduced heparin affinity are in full agreement with those reported by others, who used primary cells from *cld*/*cld* mice and chemically induced glucosidase inhibition of wild-type cells (8, 24, 50). In the present study, however, we extended the analysis to include the small fraction of LPL that, despite the deleterious effects of the mutations, had acquired enzymatic activity (Figs. 4, 5). This active fraction exhibited physical characteristics indistinguishable from LPL expressed in control cells, including Golgi-processing, high heparin affinity, and a normal activity per unit LPL mass. Thus, while severely impairing the ability of LPL to achieve an active conformation, neither *cld* nor *lec23* compromise the sorting function known as quality control. This cellular mechanism ensures that incompletely folded or misfolded proteins are retained in the ER, where they are eventually degraded (69, 70), while properly folded, functional proteins are permitted to continue unhindered through the secretory pathway (9, 46, 47).

While virtually all LPL was endo H-sensitive in Cld cells (Fig. 1), further analysis clearly showed that these cells were capable of Golgi glycan processing (Fig. 4), and thus indicated that the high mannose structure was due solely to retention of the enzyme in the ER. Lec23 cells, however, were unable to efficiently process LPL past the high-mannose stage. In these cells, the majority of active LPL remained in the high-mannose form, even after passing through the Golgi en route to secretion (Fig. 4). The appearance of a limited amount of the Golgi-processed 55 kDa form was most likely due to the activity of the Golgi endo-α-mannosidase, which provides an alternative pathway for

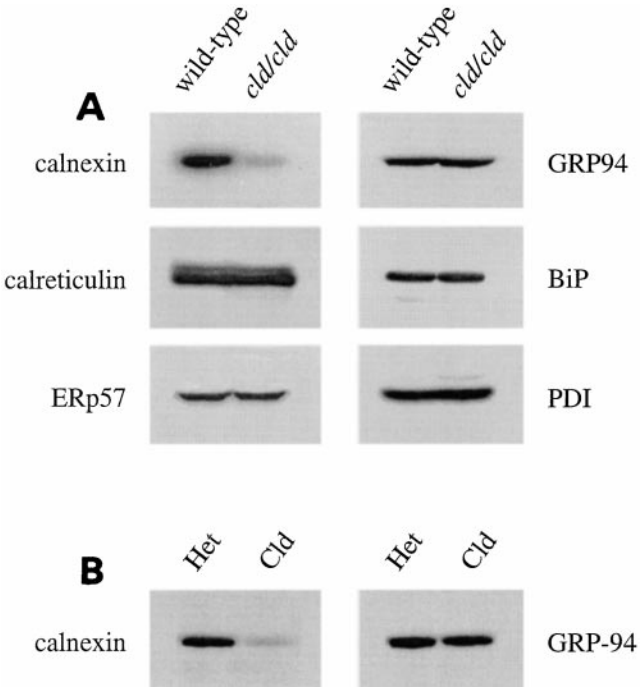

Fig. 8. Effect of the *cld* mutation on levels of ER chaperones. A: Calnexin, calreticulin, and ERp57 (members of the calnexin cycle, left panel) and GRP94, BiP, and PDI (abundant ER chaperones, right panel) were detected by Western blot analysis using microsome-enriched fractions (20 μg) from livers of wild-type and *cld*/*cld* mice. The diminished calnexin levels in *cld*/*cld* were confirmed using several preparations of microsomes from different groups of mice. B: Levels of calnexin were confirmed using ER-enriched fractions of Het and Cld cells (100 μg). As in liver microsomes, these fractions exhibited equivalent levels of GRP94.

glucose removal from untrimmed or incompletely trimmed glycoproteins ($Glc_{1-3}Man_9GlcNAc_2$) that exit from the ER. The enzyme removes en bloc the $Glc_{1-3}Man$ moiety, permitting subsequent addition of the normal array of complex sugars (71, 72). However, as tri- and diglucosylated species ($Glc_{2-3}Man_9GlcNAc_2$) are cleaved by the endo-mannosidase more slowly than $Glc_1Man_9GlcNAc_2$ (73), this may explain the relatively small amount of LPL in Lec23 cells that was processed to the 55 kDa form.

Unlike the deleterious nature of *cld* and *lec23* on LPL expression, PL activity was essentially unaffected (Table 1). The only difference was a 50% reduction in secreted PL activity observed in Lec23. The reason for this diminished secretion rate may be due to delayed or incorrect intracellular trafficking resulting from the inability of these cells to normally process the core oligomannosyl group (74). Regardless of the reduced secretion, however, the normal activity levels intracellularly clearly demonstrate that PL maturation remained unaffected. Although PL is a monomer, the LPL tandem-repeat molecule (LPL_{TR}) was fully affected by the *cld* and *lec23* mutations, ruling out the assembly of correctly folded monomers as the basis for the defect. Rather, as PL was found to renature in vitro, newly synthesized PL may "escape" the *cld* and *lec23* defects because of its inherent ability to fold productively, unassisted by factors affected by these mutations.

To begin to assess what factors may be affected by the *cld* mutation, we examined processes that have been proposed to be defective, including LPL transport from ER to Golgi, and glucose addition and removal. A defect in LPL transport was investigated using BFA to retrieve Golgi components back to the ER. BFA clearly dispersed Golgi components into the ER, evidenced both by processing of LPL to endo H-resistant forms, and by cessation of secretion resulting in LPL intracellular accumulation (Fig. 6). However, in BFA-treated mutant cells, we did not find an increase in LPL activity levels approaching wild-type values, as would be predicted if the inactive LPL in these cells was a nascent precursor awaiting transport to a post-ER compartment (Table 2). Indeed, a number of studies suggest that LPL maturation does not require transport to Golgi (5, 75–77). Thus, inactive LPL in the mutant cells does not appear to represent a sequestered nascent precursor, but rather the end-product of unproductive folding reactions occurring in the ER.

We also ruled out that *cld* might affect the glucosyltransferases responsible for transfer of glucose residues to the $Man_9GlcNAc_2$–pyrophosphoryldolichol substrate (78), or even earlier steps in the formation of the dolichol substrate, leading to truncated structures such as $Man_7GlcNAc_2$ and $Man_5GlcNAc_2$ (79, 80). Thus, it appeared that in Cld cells, normally glucosylated core oligosaccharides are added to nascent LPL (Fig. 7). Furthermore, subsequent removal of the glucose residues in these cells occurred to completion (Fig. 7), ruling out a defect in glucose trimming caused by *cld*. Thus, unlike *lec23* or chemical inhibition of glucose trimming, *cld* does not disrupt early steps in oligosaccharide synthesis or processing that might interfere with entry of LPL into the lectin chaperone cycle.

Finally, examination of the components of the calnexin cycle indicated a substantial decrease in calnexin levels in microsome preparations from livers of *cld*/*cld* mice and the Cld cell line (Fig. 8). The decrease was specific to calnexin, not only with regard to other members of the cycle, but also relative to three additional major ER chaperones, PDI, BiP, and GRP94, which were found unaffected. The unchanged levels of BiP and GRP94 in *cld*/*cld* cells suggest that misfolding may be restricted to a small number of proteins, as the levels of these chaperones increase substantially under conditions of general protein misfolding (81).

Although the effect of *cld* on calnexin levels appears specific, the mutation, located on chromosome 17 (22), does not colocalize with the calnexin gene on chromosome 11 (82). Thus, like the lipases, calnexin levels are affected in trans. Preliminary studies have indicated that the decreased calnexin levels are not due to reduced calnexin synthesis, but may result from missorting to non-ER compartments (e.g., the plasma membrane). Whether *cld* also results in calnexin dysfunction is not known.

It is tempting to speculate that the similar nature of the *lec23* and *cld* mutations on lipase expression indicate that reduced calnexin levels, and possibly impaired function, could contribute to the lipase deficiency in *cld*. However, as calnexin is present in all tissues and is found associated

with many nascent glycoproteins, reduced calnexin levels might be expected to have a general affect on the expression of many proteins, including the lipases. Nonetheless, in this regard, mutations in the ubiquitous ERGIC-53 protein, a lectin required for selective transport through the early secretory pathway (83), also had a surprisingly specific effect on the secretion of only two proteins. Thus, while plasma levels of the homologous coagulation factors V and VIII were extremely reduced (akin to combined lipase deficiency), the integrity of the ER and general ER-to-Golgi transport were not affected (84). In an analogous manner, while many proteins associate with calnexin, not all may require its presence for normal expression. For example, the expression and transport of the MHC class I complex is not affected in a calnexin-deficient cell line (85), although this protein associates with calnexin under normal circumstances (86, 87). Further investigation will be required to ascertain whether reduced calnexin levels contribute to the combined lipase deficiency in *cld*/*cld* mice, and whether other calnexin-associated proteins are similarly affected.

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