Absence of N-glycosylation at asparagine 43 in human lipoprotein lipase induces its accumulation in the rough endoplasmic reticulum and alters this cellular compartment

Roser Buscà,* Miguel Angel Pujana,* Philippe Pognonec,† Johan Auwerx,§ Samir S. Deeb, Manuel Reina,* and Senén Vilaró[1,*]**

Unit of Cellular Biology,* Departments of Biochemistry and Physiology, University of Barcelona, Avda. Diagonal, 645, 08028 Barcelona, Spain; Centre de Biochimie,† Université de Nice, Parc Valrose, 06108 Nice, Cédex 2, France; Laboratoire de Biologie des Régulations chez les Eukaryotes,§ Department d'Athérosclerose, Institut Pasteur, Rue Calmette, 59019 Lille, France; and Department of Genetics and Medicine,** University of Washington, Seattle, WA 98195

Abstract Lipoprotein lipase (LPL) is the enzyme responsible for the hydrolysis of plasma triglycerides from apolipoprotein C-II-containing lipoproteins at the capillary endothelium and it is synthesized in parenchymal cells of several tissues. Intracellular LPL processing is a major aspect of LPL regulation. The present study aims to determine the intracellular accumulation site of the LPL that is not glycosylated at Asn43. Human LPL (hLPL) cDNA was mutated by site-directed mutagenesis. An Ala residue was substituted for Asn at position 43 of the protein generating N43A hLPL. Wild type hLPL and the mutant hLPL were expressed in COS1 cells. Using immunofluorescence and immunoelectron microscopy we found that wild type hLPL in addition to being secreted into the medium was present in the rough endoplasmic reticulum (ER), Golgi compartments, and vesicles. Neither LPL activity nor protein was found in medium of cells expressing the mutant hLPL and all detectable protein was present exclusively in the ER identified with a specific antibody against the protein disulfide isomerase (PDI), an ER marker. In addition, the intracellular distribution of the ER of the cells that expressed the mutant protein was grossly altered. Treatment of COS1 cells with tunicamycin for 24 h had the same effect on wild type hLPL processing and endoplasmic reticulum distribution. Next, we investigated the influence of the accumulation of mutant hLPL on the intracellular transport of three other proteins that are N-glycosylated before reaching the plasma membrane: the related $B^{0,+}$ amino acid transporter (rBAT), the insulin-regulated glucose transporter (GLUT4), and the placental alkaline phosphatase (PLAP) protein. Coexpression of the mutant hLPL (but not wild type) caused the accumulation of rBAT and GLUT4 in the ER while PLAP reached the plasma membrane. Our findings demonstrate that glycosylation of Asn43 of human lipoprotein lipase in the endoplasmic reticulum is essential for its efflux from this compartment and that the retention of the non-glycosylated LPL induces morphological changes in the ER that could also affect its ability to modify the transport of other proteins.—**Buscà, R., M. A. Pujana, P. Pognonec, J. Auwerx, S. S. Deeb, M. Reina, and S. Vilaró.** Absence of N-glycosylation at asparagine 43 in human lipoprotein lipase induces its accumulation in the rough endoplasmic reticulum and alters this cellular compartment. *J. Lipid Res.* 1995. **36:** 939–951.

Supplementary key words intracellular processing • secretion • mutation • transfection • tunicamycin

Lipoprotein lipase (LPL) is an N-linked glycoprotein that is synthesized in a variety of extrahepatic tissues (1–3), including adipose tissue, heart, skeletal muscle, brain, and ovary (4, 5). After its synthesis by parenchymal cells, LPL is secreted and bound to heparan sulfate proteoglycans on the lumenal surface of the capillary endothelium (1, 2). At this functional site, LPL hydrolyzes the triglyceride core of chylomicrons and very low density lipoproteins. The monoglycerides and fatty acids thus liberated are used by subjacent tissues for storage or oxidation (6). The lipase can be released from the vascular endothelium and transported through the blood, bound to cholesterol-rich lipoproteins (7, 8), to the liver for degradation (9).

The response of LPL to nutritional or hormonal changes is a tissue-specific event that is often regulated at a posttranslational level (10–12). This regulation may require modifications to the LPL molecule that are essential for the expression of its catalytic activity. Among these,

Abbreviations: ER, rough endoplasmic reticulum; GLUT4, glucose transporter 4; GPI, glycosyl phosphatidylinositol; LPL, lipoprotein lipase; hLPL, human lipoprotein lipase; rBAT, related $B^{0,+}$ amino acid transporter; PDI, protein disulfide-isomerase; PLAP, placental alkaline phosphatase.

[1]To whom correspondence should be addressed.

asparagine-linked glycosylation (13) and dimerization (14) have been suggested as interrelated processes that confer catalytic activity to the enzyme (15). The role of other modifications, like the sulfation of one of the LPL oligosaccharide chains (16) is unknown at present.

Glycosylation of LPL is critical to normal LPL activity (13, 17–20) and may also be crucial to its normal intracellular processing. It initially involves the co-translational transfer of a lipid-linked oligosaccharide (Glc3-Man9-(GlcNAc)2) to specific asparagine residues in the nascent polypeptide. This early modification occurs in the ER (21). Studies by Olivecrona et al. (22) have shown that inactive LPL accumulates within the cell in tunicamycin-treated 3T3-L1 adipocytes and is not secreted to the medium. Later, Masuno et al. (18), using immunofluorescence assays, suggested that the unglycosylated lipase, in tunicamycin-treated brown adipocytes, might be retained in the ER. The human LPL amino acid sequence has two N-glycosylation sites: Asn-His-Ser (residues 43–45) and Asn-Lys-Thr (residues 359–361). Substitution of Asn43 by Ala completely abolishes LPL enzyme activity (13). Furthermore, using an ELISA assay for dimeric LPL, no significant amounts of mutant LPL were detected in the medium of transfected cells, suggesting that the loss of the N-linked glycosylation at Asn43 leads to the production of an inactive enzyme, which accumulates inside the cell (13). More detailed studies of the accumulation of non-glycosylated LPL are not available at present.

In order to elucidate the involvement of glycosylation in LPL secretion, we used site-directed mutagenesis to alter Asn43, the residue that is believed to be critical for intracellular processing (13). We show, using immunofluorescence and immunoelectron microscopy, that the substitution of Asn43 by Ala in human LPL results in *i)* retention of the mutant protein in the ER, similar to the retention of the non-mutated LPL induced by tunicamycin treatment, *ii)* altered intracellular distribution and structure of the ER, and *iii)* retention of some non-related proteins.

METHODS

Reagents

Muta-gene phagemid 'in vitro' mutagenesis kit was from (Bio-Rad Laboratories, Richmond, CA). The pCAGGS expressing vector was obtained from Miyazaki (23). The eukaryotic cells used were COS1 cells (ATCC CRL 1650). T4 DNA ligase, HhaI restriction enzyme, and the dideoxynucleotide sequencing kit (24) were from Pharmacia (Brussels, Belgium), Sequenase v.2.0 was from United States Biochemicals (Cleveland, OH). The synthetic oligonucleotide used in the mutagenesis was from Eurogentec (Seraing, Belgium). DMEM media, FBS, and trypsin-EDTA were from Whittaker (Walkersville, MD). Penicillin, streptomycin sulfate, glutamine, chloroquine, DEAE-Dextran, Tween 20, FITC-conjugated rabbit anti-chicken IgG, Hoechst 33342, glycine, bovine serum albumin (fraction V), polyvinylpyrrolidone (PVP), and methylcellulose were from Sigma (St. Louis, MO). Glycerol was from Panreac (Barcelona, Spain). Cellulosenitrat(e) BA85 from Schleicher and Schuell (Dassel, Germany) was used for blotting; the ECL detection system was from Amersham (Bucks, United Kingdom). The monoclonal antibody against bovine LPL (5D2) was from Oncogene (Uniondale, NY). The peroxidase-conjugated swine anti-mouse antibody, the FITC-conjugated goat anti-rabbit Ig, and the TRITC-conjugated goat anti-rabbit Ig were from Dakopatts (Glostrup, Denmark). The chicken affinity-purified polyclonal antibody against bovine LPL was obtained from Dr. Gunilla Bengtsson-Olivecrona (University of Umea, Sweden); the rabbit polyclonal antibody against rBAT protein was obtained by Ms. Conxi Mora and Dr. Manuel Palacin (University of Barcelona, Spain); the rabbit polyclonal antibody against PDI protein was from Dr. Gustavo Egea (University of Barcelona, Spain) (25); the rabbit polyclonal antibody against GLUT4 was obtained by Ms. Conxi Mora and Dr. Antonio Zorzano (University of Barcelona, Spain) (26); the rabbit polyclonal antibody against PLAP was provided by Dr. Ricardo Makiya (University of Umea, Sweden) (27); the FITC-conjugated and TRITC-conjugated sheep anti-mouse Ig were from Boehringer Mannheim (Mannheim, Germany) and the immunofluorescence mounting medium was from ICN Biomedicals Inc. (Costa Mesa, CA). Paraformaldehyde and glutaraldehyde fixatives and uranyl acetate were from Merck (Darmstadt, Germany); gelatin and sucrose were from Panreac; the polyclonal rabbit anti-chicken Ig was from Nordic (Tilburg, The Netherlands); and the protein A-gold 15 nm was purchased from Dr. Slot (University of Utrecht, The Netherlands).

LPL mutagenesis

The full-length human LPL (hLPL) clone was isolated by reverse transcription of RNA from THP-1 cells (ATCC TIB202) differentiated with phorbol esters and dexamethasone (28), followed by PCR amplification. The sequence was confirmed by the dideoxy chain termination method (24). Site-directed mutagenesis of the full-length human LPL cDNA cloned into the EcoRI site of PTZ18U vector was carried out according to the method of Kunkel, Roberts, and Zakour (29) using the site-directed mutagenesis kit from Bio-Rad. The oligonecleotide primer (5' CCTGTCATTTCGGCGCAC 3') used for mutagenesis contained the codon 43 substitution ATT (Asn)→GCG (Ala). This substitution created an HhaI restriction site. The mutant LPL clone was confirmed by digestion with HhaI and by sequencing. For expression, the wild type and mutant cDNAs were cloned into the EcoRI site of the

expression vector PCAGGS which contains the β-actin promoter and the SV40 replication origin.

Cell culture and transfection

COS1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics, and glutamine (2 mM). For immunofluorescence experiments, cells were cultured in six multi-well dishes containing glass coverslips. For electron microscopy experiments, cells were cultured in 10-cm plates containing 10 ml of medium. Cells at 80% of confluence, were transfected with 2.5 μg of DNA, by the DEAE-dextran method (30). All cells were examined 48 h after transfection. For cotransfection experiments, cells were incubated with DEAE-dextran solution containing 2.5 μg of each DNA (the hLPL DNA or N43A hLPL DNA) together with the rBAT gene cloned in pCMV-2 expressing vector or GLUT4 gene cloned in the pHβAPr-1 expressing vector (31) and the PLAP protein gene cloned in the pSVT7 expressing vector (32). The rest of the procedure was identical to that for single DNA transfection. When expressing the GLUT4 cDNA, cells at 47 h after transfection were treated with 1 μM insulin to induce GLUT4 translocation to the plasma membrane (26).

Western blotting and activity assay

The medium of transfected cells was removed from the plate and loaded onto an SDS-PAGE 10% acrylamide gel. The gel was blotted to nitrocellulose at 400 mA for 60 min using the Bio-Rad mini-protean apparatus. LPL was detected with a monoclonal antibody against bovine LPL (5D2) and a secondary peroxidase-conjugated anti-mouse antibody at a dilution of 1/2000. The blot was developed with the ECL system from Amersham. Lipoprotein lipase activity in medium was determined as described by Ramirez et al. (33). One milliunit of lipolytic activity represents release of 1 nmol fatty acid/min (33).

Immunofluorescence and immunoelecton microscopy

For immunofluorescence labeling (34), cells grown on glass coverslips were rinsed briefly in PBS, fixed with methanol (−20°C) for 2 min, washed twice in PBS, and processed. As primary antibodies, we used a monoclonal antibody anti-bovine LPL (5D2) and a polyclonal chicken antibody anti-bovine LPL (poly 66). For immunodetection of rBAT protein, we used a polyclonal antibody (35). To visualize the primary antibody, we used the following labeled secondary antibodies: FITC-conjugated sheep anti-mouse immunoglobulins, FITC-conjugated goat anti-rabbit immunoglobulins, FITC-conjugated rabbit anti-chicken immunoglobulins, TRITC-conjugated goat anti-rabbit immunoglobulins, and TRITC-conjugated goat anti-mouse immunoglobulins. All antibodies were diluted in PBS containing 0.5% of bovine serum albumin. Double immunofluorescence assays were performed by applying a mixture of mouse and chicken primary antibodies and a mixture of non-cross-reacting secondary antibodies. Primary antibodies were applied for 45 min at 37°C, followed by a 10-min wash in PBS, then 45-min incubation at 37°C with the secondary antibody followed by a final wash of 10 min in PBS. Finally, coverslips were labeled with the nuclear stain Hoechst 33342 diluted in PBS. The coverslips were mounted with immunofluorescence medium and viewed through a 40× or 100× objective using a Reichert Jung Polyvar II microscope equipped with epifluorescence illumination. Photographic exposures were made using Kodak Ektachrome 160 film and Kodak Tmax 400 film.

For electron microscopy, cells were washed in PBS and fixed on 10-cm plates with 2% paraformaldehyde–0.1% glutaraldehyde. Cell pellets were embedded in gelatin blocks and post-fixed overnight with 2% paraformaldehyde. Blocks were then cryoprotected for 10 h with PVP, mounted on sample carriers, and frozen in liquid nitrogen. Cryoultrasections were obtained in a Reichert-Jung ultramicrotome equipped with FC4 system for cryosectioning. Sections were retrieved with a copper loop containing 2.3 M sucrose in PBS and were transferred to a 100-mesh grid with carbon-coated Formvar film and placed on gelatin 2% until they were processed for immunodetection. For immunolabeling on ultrathin cryo-

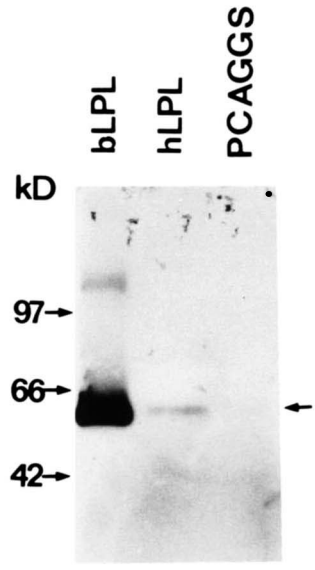

Fig. 1. Secretion of LPL by COS1 cells transfected with wild type human LPL cDNA. Media (15 μl) from cells transfected (48 h) with the wild type human LPL cDNA or the expression vector (PACGGS) were separated by SDS-PAGE, and blotted to a nitrocellulose filter. LPL was detected with the 5D2 antibody. In the first lane 0.5 μg of bovine LPL (bLPL) was applied. The molecular weight of standards is indicated on the left. The arrow on the right points to a single band immunodetected in medium from wild type transfected cells. Results are representative of two separate transfection experiments.

sections, we followed the procedure described by Slot et al. (36) with slight modifications. Grids were washed three times for 5 min with drops of 20 mM glycine in PBS and blocked with PBS-gly 1% BSA for 20 min. Incubation (30 min) with the primary chicken polyclonal antibody diluted in the blocking solution was performed, followed by three 5 min washes in PBS-gly. Incubation with a rabbit antibody against chicken immunoglobulins, used as a bridge, was then performed, followed by three 5 min washes in PBS-gly. The grids were incubated for 20 min in a solution of A-protein labeled with 15 nm gold particles. After three washes in PBS of 5 min each and six washes in double-distilled water of 2.5 min each, the sections were contrasted in 0.3% uranyl acetate in methyl cellulose for 10 min on ice. The grids were retrieved using a copper loop and the excess fluid was removed on a filter paper. Grids were examined at the Hitachi 600 AB electron microscope.

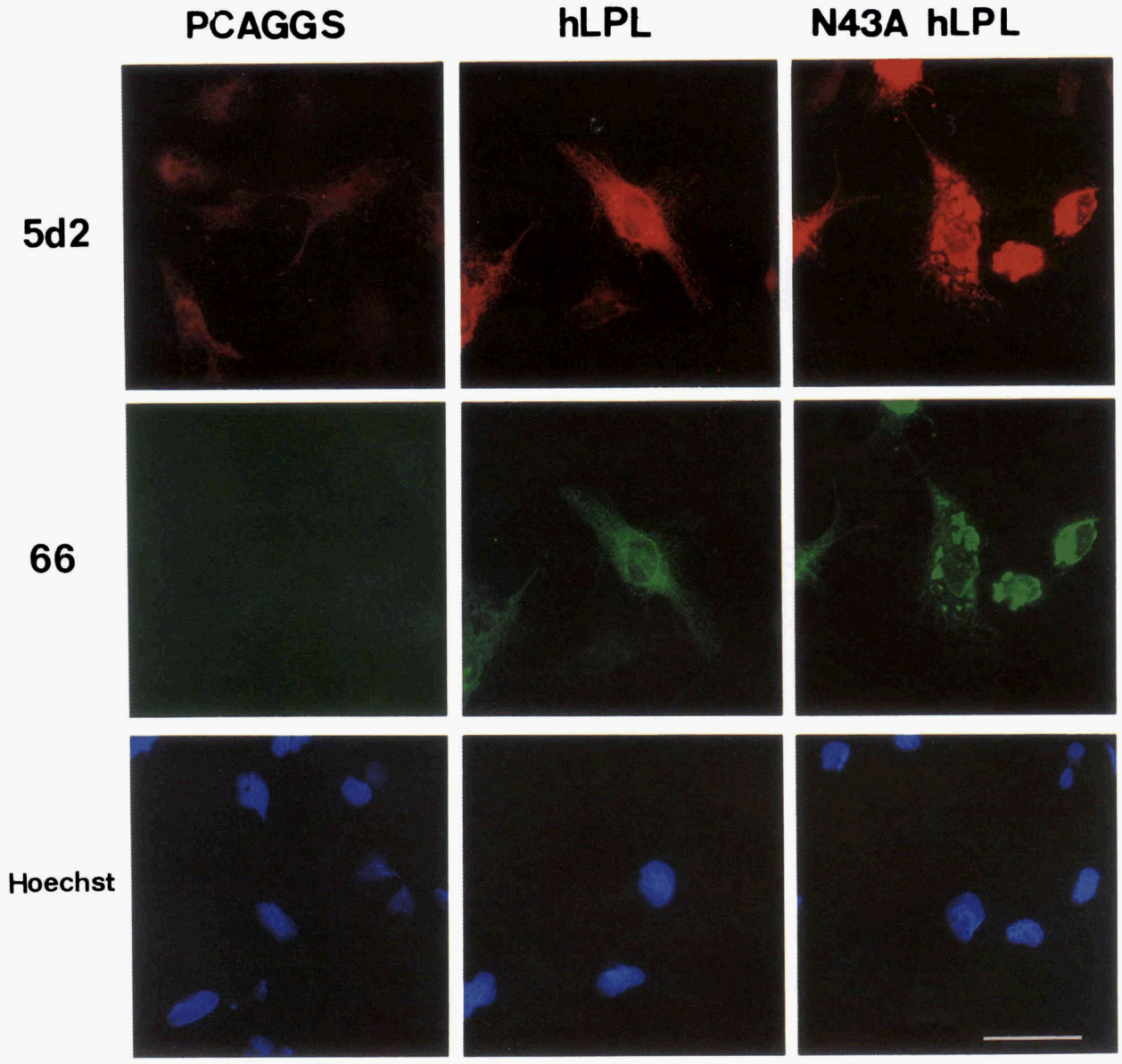

Fig. 2. LPL expression in hLPL and N43A hLPL transfected COS1 cells. COS1 cells were transfected only with the expression vector (PCAGGS), wild type LPL, or the mutant (N43A) cDNAs. Cells were fixed 48 h later and processed for immunofluorescence as indicated in Methods. Double immunofluorescence was performed using the mouse monoclonal (5D2) (row 1) and the affinity-purified chicken polyclonal anti-LPL (row 2) as primary antibodies, TRITC-conjugated sheep anti-mouse immunoglobulins and FITC-conjugated rabbit anti-chicken immunoglobulins as secondary antibodies. Nuclei were stained with Hoechst (row 3). Vertical panels show immunofluorescence obtained for each primary antibody and nuclear staining in the same field. Results are representative of at least three separate transfection experiments. Bar: 60 μm.

RESULTS

Intracellular distribution of hLPL and N43A hLPL

Semenkovich et al. (13) showed that Asn 43 is essential for both activity and secretion of LPL. In order to determine the intracellular location of mutant LPL, we transfected COS1 cells with cDNA constructs containing wild type or mutant LPL using the DEAE-dextran/chloroquine method. Cells transfected with wild type LPL produced 0.47 mU/ml of medium and 6.4 mU/mg protein in cell extracts 48 h after transfection. LPL activity was not detectable in cells transfected with the Asn 43 Ala mutant LPL cDNA clone. To confirm that the activity detected corresponded to human LPL and to check the specificity of the monoclonal antibody 5D2, we performed Western blot analysis of culture media of transfected COS1 cells (**Fig. 1**) 48 h after transfection. A single band with an apparent molecular mass of 58–60 kD (similar to bovine LPL) was detected in medium of cells transfected with wild type but not mutant LPL.

To determine the intracellular distribution of the LPL expressed, we performed double immunofluorescence studies using the monoclonal antibody 5D2, known to recognize human LPL (37), and chicken polyclonal antibodies against bovine LPL. As expected, when cells were transfected with the vector PACGGS only, no intracellular immunofluoresence was observed above background (**Fig. 2**). However, in cells transfected with either wild type or mutant LPL, intense intracellular staining was present 48 h after transfection, indicating that transfected cells efficiently expressed both the wild type and the mutated cDNAs (Fig. 2). The intracellular staining distribution obtained with each of the antibodies used was coincident, indicating that both antibodies could be used for immunostaining experiments. Approximately 20% of transfected cells showed intracellular staining.

Differences in the intracellular distribution pattern of wild type or mutant LPL in transfected expressing cells were observed by immunofluorescence (**Fig. 3**). Cells expressing the wild type enzyme showed intense fluorescent label surrounding the nucleus and from this perinuclear region a reticular network extended to the whole cytoplasm (Fig. 3A), which probably corresponded to the ER. Using double immunofluorescence with 5D2 to detect LPL and the 15C8 that recognizes an integral constitutive protein of *cis*-Golgi stacks (38), we observed that part of the LPL perinuclear staining corresponded to the Golgi complex (not shown). However, cells expressing the mutant protein only showed intense fluorescent spots, mostly in the perinuclear region (Fig. 3B), suggesting that mutant LPL accumulated within the transfected cells. We next studied the time course of LPL accumulation after transfection (not shown). Expression of wild type and mutant LPL was detected in a few cells 12 h after transfection. After 24 h the number of cells expressing the LPL increased substantially. At that time cells expressing wild type or mutant LPL showed the same pattern of immunofluorescence, consisting of a reticular network covering the whole cytoplasm. However, at 48 h after transfection the immunofluorescence pattern of cells expressing mutant LPL was very different from that of cells expressing the wild type enzyme, the first one characterized by intense perinuclear spots only. At longer periods (72–96 h) after transfection, the distribution pattern of mutant LPL did not change (not shown).

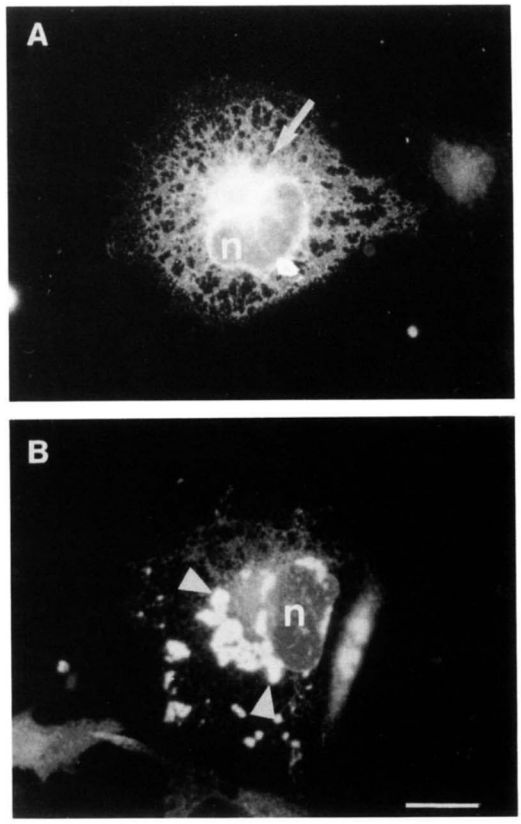

Fig. 3. hLPL and N43A hLPL transfected COS1 cells present different pattern of LPL distribution. High magnification photographs of cells expressing wild type (A) and mutant (N43A) LPL (B). Immunofluorescence was performed using the monoclonal 5D2 and FITC-conjugated sheep anti-mouse immunoglobulins. Cells expressing normal LPL (A) show intense fluorescence in a perinuclear area (arrow) and a reticular network extending over the cytoplasm. Cells expressing mutant LPL show fluorescent spots mostly surrounding the nucleus (n). Results are representative of at least ten separate experiments. Bar: 30 μm.

As immunofluorescence methods do not allow determination of intracellular distribution in detail (39), we performed immunoelectron microscopy to localize the accumulated mutant LPL. We used cryoelectron microscopy with the polyclonal chicken antibody against LPL which was stained by immunogold (**Fig. 4**). Using this approach, we found that most of the labeling in cells expressing wild type LPL was located in the ER, either surrounding the nucleus or widely distributed throughout

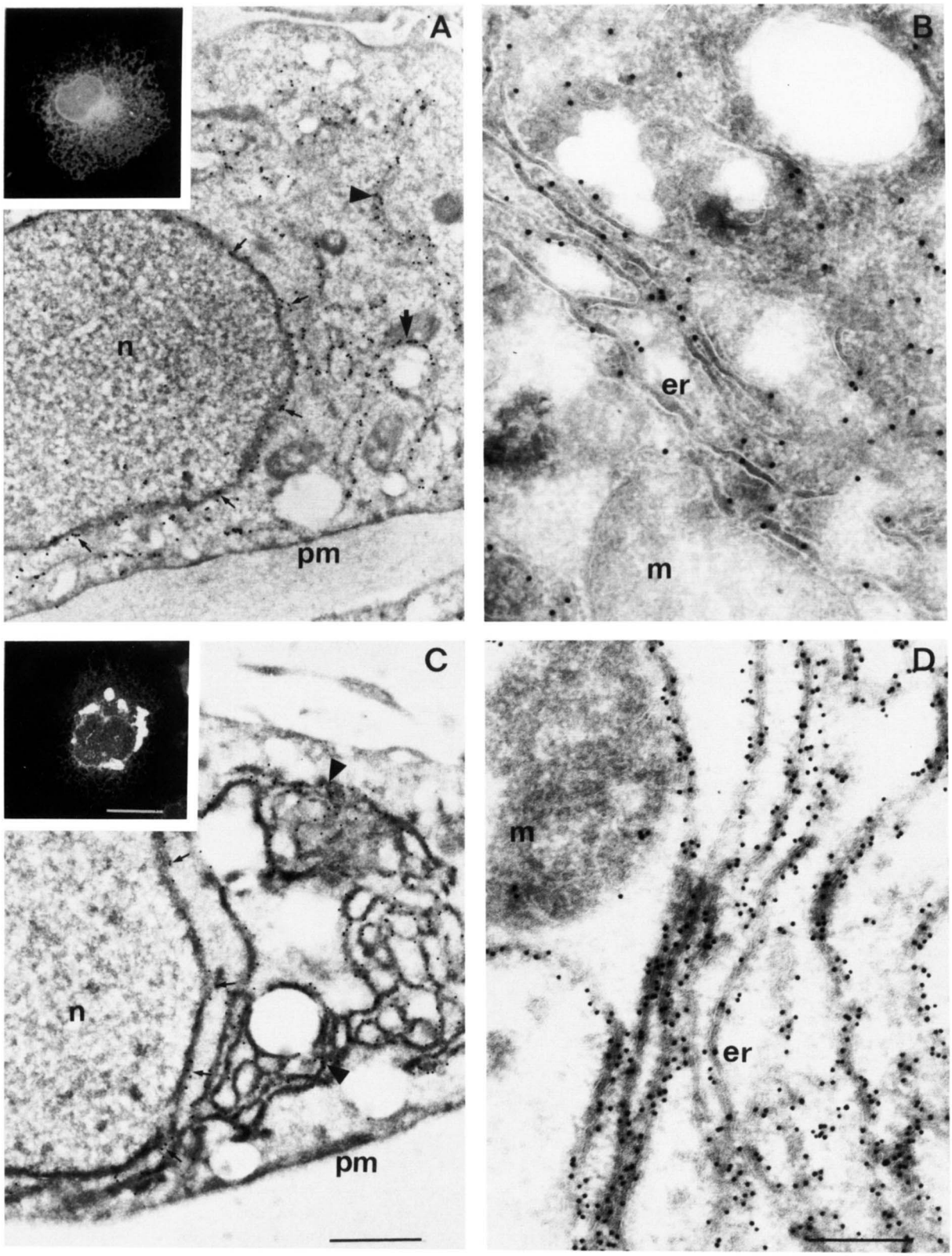

Fig. 4. Intracellular distribution of hLPL and N43A hLPL protein in transfected COS1 cells. COS1 cells were transfected with either wild type (A + B) or mutant (C + D) cDNAs. They were processed for immunoelectron microscopy (48 h post transfection) as indicated in Methods. Immunolabeling was performed using affinity-purified chicken anti-LPL, rabbit anti-chicken immunoglobulins, and protein A-gold (15 nm). Inserts show the immunofluorescence staining of wild type (A) and mutant LPL (C) transfected cells. er: endoplasmic reticulum; n: nucleus; m: mitochondria. Results are representative of three separate experiments. Bar for A and C: 1 μm. Bar for B and D: 300 nm.

the cytoplasm (Fig. 4A and B). In addition, some label was also detected inside vesicles that probably corresponded to Golgi or post-Golgi compartments. Most of the transfected cells appeared normal. However, a few appeared wrinkled, possibly as a consequence of the high expression level of the recombinant LPL (40). Cells expressing the mutant LPL showed a large number of gold particles distributed in membrane spaces around the nucleus and in cisternae, which correspond morphologically to the ER (Fig. 4C and D). However, the intracellular distribution of the ER in almost all of these transfected cells showed morphological alterations characterized by its bent appearance in perinuclear regions. Hence, the fluorescence spots that were detected by immunofluorescence corresponded to twisted ER (compare inset in Fig. 4C with the electron micrograph). To confirm that mutated LPL accumulated within the ER, we carried out double immunofluorescence using the anti-LPL monoclonal antibody 5D2 and a polyclonal antibody against PDI, a protein that is known to be located exclusively in the ER (25). As expected, the dense fluorescent spots observed in N43A hLPL-expressing cells were also stained with the anti-PDI antibody (**Fig. 5**), indicating that the intracellular site of accumulation of the mutant LPL is the ER.

Effects of tunicamycin on LPL distribution

Tunicamycin acts as a general inhibitor of N-glycosylation by blocking the synthesis of the dolichol-linked oligosaccharide (41) and it has been shown to inhibit both LPL activity and secretion (2). COS1 cells were transfected with wild type LPL cDNA and treated for a further 24 h with tunicamycin. Then they were fixed and processed either for immunofluorescence or for immunoelectron microscopy (**Fig. 6**). Highly fluorescent perinuclear

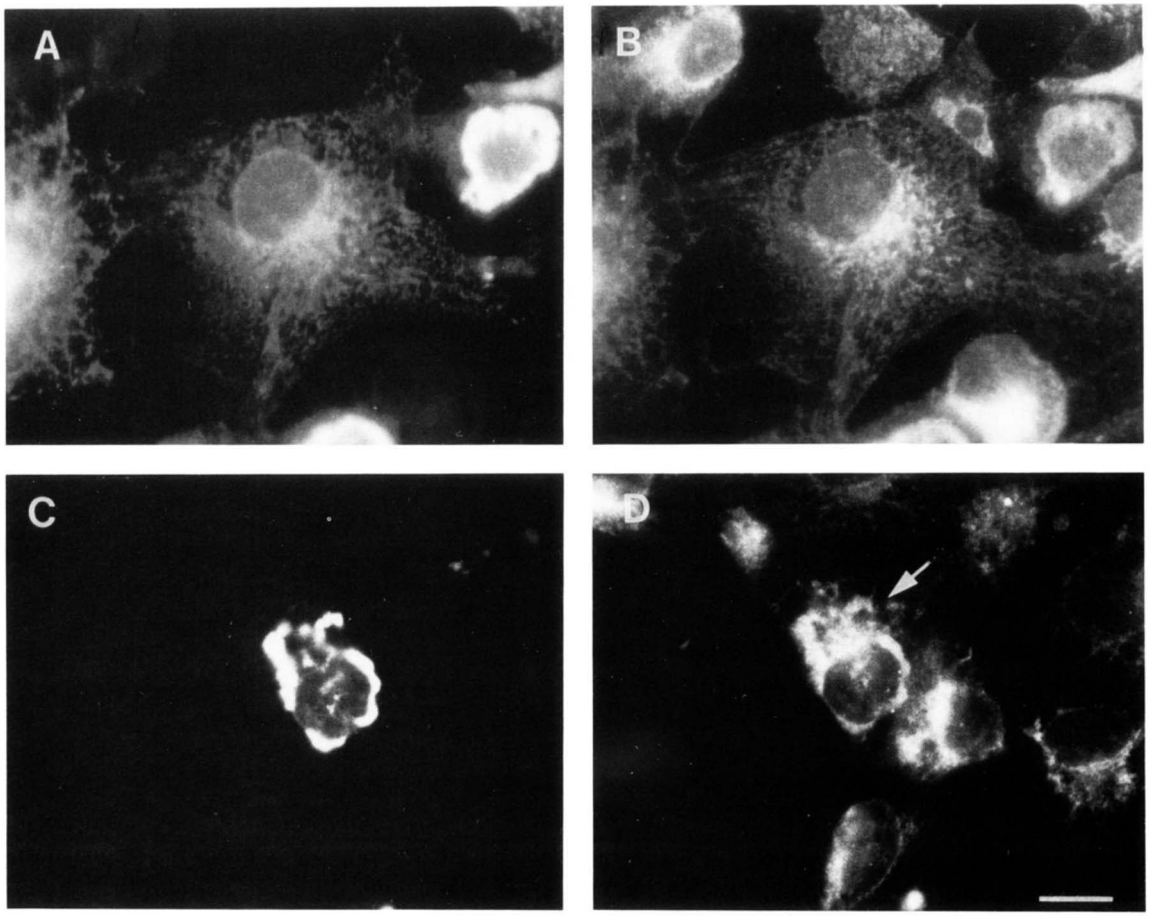

Fig. 5. Mutant N43A hLPL accumulates within the ER. COS1 cells were transfected with wild type hLPL (A and B) or N43A hLPL (C and D) and double immunofluorescence, using the monoclonal 5D2 antibody to detect LPL protein (A and C) and a rabbit polyclonal antibody to detect the PDI (B and D), was performed. Monoclonal antibodies were stained with FITC-conjugated sheet anti-mouse immunoglobulins and polyclonal antibodies with TRITC-conjugated goat anti-rabbit immunoglobulins. Note that the N43A hLPL accumulation sites are also stained with the anti-PDI antibody (arrow). Bar: 30 μm.

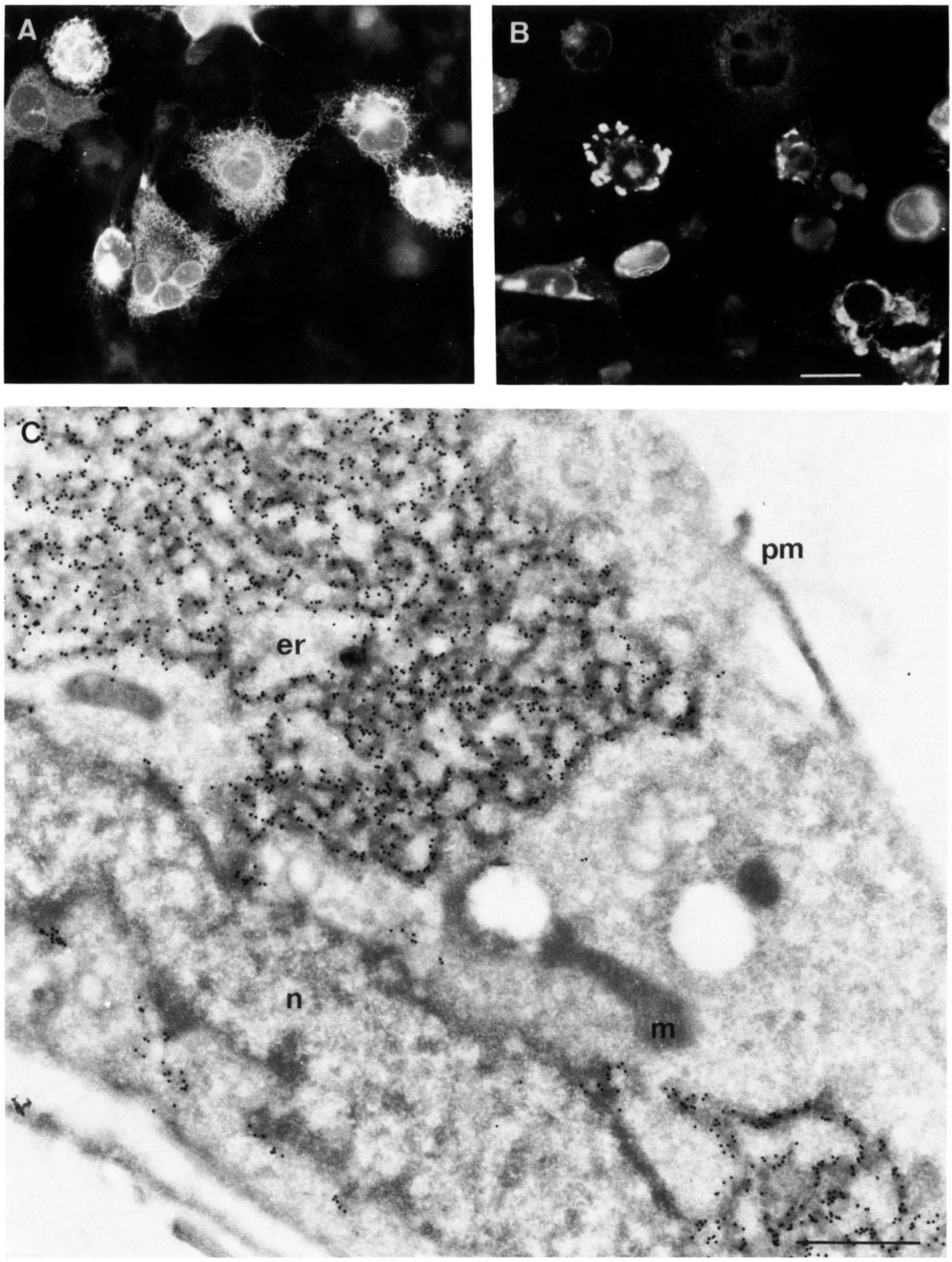

Fig. 6. Effects of tunicamycin in LPL distribution of COS1 transfected cells with wild type hLPL gene. A and B show the immunofluorescence pattern 48 h after transfection of COS1 cells non-treated (A) or treated for 24 with 5 μM of tunicamycin (B). Immunofluorescence was performed as in Fig. 3. Note the presence of fluorescent spots in tunicamycin-treated cells. C, immunogold staining of cryoultrasection from tunicamycin-treated COS1 cells expressing wild type LPL. Immunodetection was performed as in Fig. 4. er: endoplasmic reticulum; n: nucleus; m: mitochondria; pm: plasma membrane. Results are representative of two separate experiments. Bar for A and B: 30 μm, Bar for C: 500 nm.

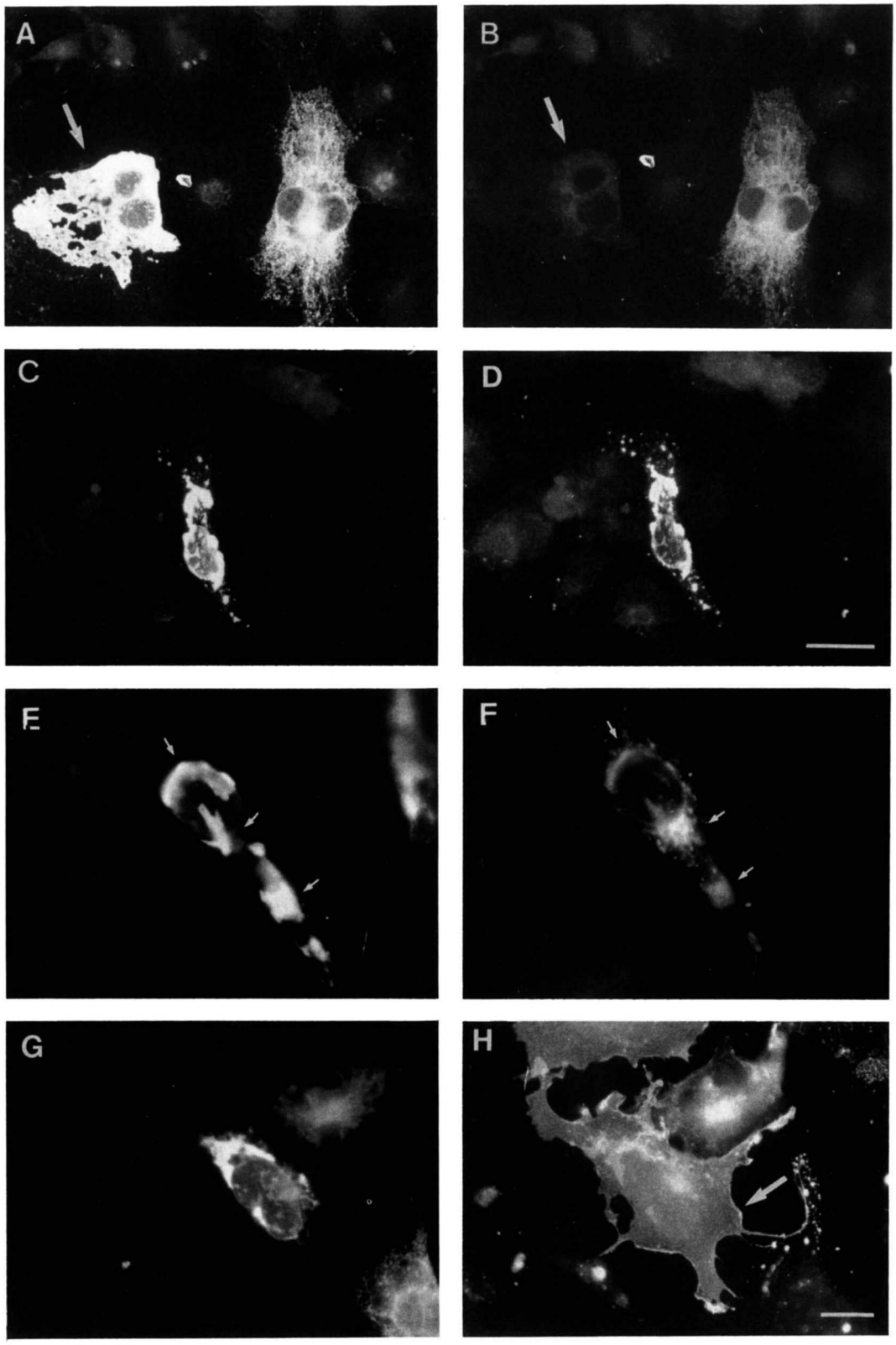

Fig. 7. Mutant N43A hLPL expression induces altered distribution of rBAT and GLUT4 but not PLAP protein. COS1 cells were cotransfected with wild type LPL and rBAT (A and B) or mutant N43A LPL and rBAT (C and D), with N43A hLPL and GLUT4 (E and F), and with N43A hLPL and PLAP (G and H). Double immunofluorescence was performed with mouse monoclonal 5D2 to detect LPL (A and C) and rabbit polyclonal anti-rBAT immunoglobulins (B and D), rabbit anti- GLUT4 immunoglobulins (F), and rabbit anti-PLAP immunoglobulins (H). As secondary antibodies FITC-conjugated sheep anti-mouse and TRITC-conjugated goat anti-rabbit were used. Arrows in A and B point to transfected cells expressing LPL but not rBAT, revealing the specificity of the antibodies used. Arrows in E and F indicate colocalization points, and the arrow in H points to labeling on the cell membrane. Results are representative of two separate experiments. Bars 30 μm.

spots were prominent in all transfected cells treated with tunicamycin (Fig. 5B). Immunoelectron microscopy revealed that most of the label was located in the ER, with a twisted appearance at the perinuclear region (Fig. 4). The effect of tunicamycin was not restricted to LPL-expressing cells, as non-transfected COS1 tunicamycin-treated cells also had the same ER alterations (not shown).

Cotransfection assays

We next investigated whether the structural modification of the ER induced by the expression of non-glycosylated LPL altered the processing of other cellular proteins. We cotransfected LPL together with the rBAT, GLUT4, and PLAP cDNAs. rBAT encodes a kidney transmembrane aminoacid transporter (42), GLUT4 encodes an transmembrane insulin-regulated glucose transporter (43) and PLAP encodes a placental alkaline phosphatase (a GPI-anchored plasma membrane protein) (44). The three proteins are N-glycosylated and transported to the plasma membrane. COS1 cells transfected with these three cDNAs, normally express the corresponding protein. The expression and distribution of LPL together with each of the three proteins was determined by double immunofluoresence (**Fig. 7**). When cells expressed the wild type LPL and rBAT, GLUT4 or PLAP, the label was broadly distributed (Fig. 7A and B show the distribution of rBAT and LPL in cells expressing the wild type LPL; for GLUT4 and PLAP this is not shown). However, coexpressed mutant LPL and rBAT or GLUT4 colocalized in a clear pattern of bright fluorescent perinuclear spots (Fig. 7C, D, E and F). Nevertheless, PLAP protein expressed in mutant LPL-expressing cells was clearly found in the plasma membrane (Fig. 7G and H), indicating that its processing was not affected by the ER accumulation of LPL. These findings suggest that the expression of the non-glycosylated LPL induces an alteration in the ER that may lead to accumulation of some proteins synthesized in this compartment while others are processed normally.

DISCUSSION

Previous studies have indicated that most LPL regulation occurs posttranslationally. The importance of N-linked glycosylation of LPL in the development of its activity has been demonstrated in several studies (for review, ref. 2). LPL glycosylation may be involved in the control of secretion of the protein because tunicamycin-treated 3T3-L1 adipocytes accumulate LPL within the cell (22). Tunicamycin-treated brown adipocytes were shown by immunofluorescence to retain LPL in the endoplasmic reticulum (18). As tunicamycin inhibits N-glycosylation of all proteins, it could, therefore, alter the accessory proteins that are needed for the correct processing of hLPL. In addition, it induces the synthesis of a number of proteins, such as BiP (45, 46), that bind misfolded proteins. These drawbacks, inherent in the use of a nonspecific inhibitor, do not occur using site-directed mutagenesis.

Semenkovich et al. (13) reported that Asn43 is essential for both enzymatic activity and secretion of the enzyme. In our study, both wild type human LPL and mutant (N43A) LPL cDNAs were expressed in COS1 cells and their intracellular distribution was studied by immunofluorescence and immunoelectron microscopy. Cells transfected with the wild type human LPL cDNA expressed high levels of active protein secreted into the medium. The intracellular distribution of wild type LPL in COS1 cells corresponded to what would be expected for a secretory protein, i.e., most of the staining was associated with the ER, and some was associated with Golgi or Golgi-derived vesicles. These findings indicate that transfected cells secrete the expressed LPL correctly. In contrast, the mutant LPL (Asn 43 Ala) protein accumulated within cells, indicating, as previously reported (13), that N-glycosylation is essential for proper intracellular transport and secretion. Immunofluorescence and immunoelectron microscopy showed that all the mutant LPL staining was associated with the ER, providing clear evidence of its retention in this biosynthetic compartment.

The retention of non-glycosylated LPL within the ER could be a consequence of its defective folding or oligomerization, or both. The intracellular site of LPL dimerization is still unknown (47), but there is evidence that most proteins oligomerize in the ER (25) before transit to the Golgi complex. Mutant, incompletely assembled, or aberrant glycosylated proteins are retained within the ER by complexing with proteins such as BiP (heavy chain-binding protein) (48). Furthermore, without the addition of N-linked sugars, numerous proteins fail to fold correctly (49). The bulky polar N-linked oligosaccharides added cotranslationally have three potential functions in the folding process: they may ensure the correct local positioning of the peptide segments to which they are bound; they may prevent the binding of chaperones to specific sites; and, because of their hydrophilic nature, they may render the folding intermediates more soluble, thus preventing their irreversible aggregation. N-glycosylation at Asn43 may thus be a critical step for LPL folding or oligomerization, so that its absence leads to the retention of LPL within the ER by binding to BiP or other ER folding proteins.

A relevant finding in the current study is the altered arrangement and structure of the ER observed in cells expressing mutant LPL. In addition, coexpression of the mutant LPL and rBAT or GLUT4, transmembrane N-glycosylated proteins (36, 44), results in the retention of

these proteins in the ER, although intracellular transport of the PLAP protein (a GPI-linked plasma membrane protein) is not affected. This differential effect can be the consequence of distinct intracellular processing pathways that are followed by transmembrane and GPI-linked plasma membrane proteins before reaching their final destination (50). In mice with the genetic defect of combined lipase deficiency (cld/cld), neither LPL nor hepatic lipase is processed beyond the high mannose stage and they are retained within a normal ER (51). However, the processing and secretion of adipsin, an N-linked glycoprotein (52), is not affected. The altered ER distribution found in this study could be the result of the blocked cotranslational transfer of the dolichol-linked oligosaccharide to the nascent polypeptide, the first step in N-glycosylation (21).

The altered distribution and structure of the ER resulting from either tunicamycin treatment or expression of mutant LPL is very similar to that observed after treatment with DTT (dithiothreitol) (49). When reducing agents like DTT or oxidizing agents like diamide are added to cells, the redox conditions of the ER change and the folding, maturation, and transport of proteins are affected (53, 54). DTT, for example, partially reduces and fully oxidizes monomers of viral membrane glycoproteins within the ER. DTT prevents protein folding in the ER by inhibiting the formation of disulfide bonds. This leads to transport-incompetent proteins and to swelling of the ER compartment in Chinese hamster ovary cells (49). Unfortunately, there is no evidence available to suggest that the absence of N-glycosylation in LPL could have any effect on the formation of disulfide bonds. Although several missense mutations have been reported in human LPL deficiency (55), until now, no patient with the Asn 43 Ala substitution has been found. Although highly speculative, it is possible that a human mutation at this N-linked glycosylation site could lead to the blocking of the normal intracellular processing of other proteins in mutant LPL expressing cells.

In summary, our findings demonstrate that core glycosylation of lipoprotein lipase in the ER is essential to its efflux from this compartment. In addition, we have provided evidence that retention of non-glycosylated LPL in the ER induces a morphological change in the ER and the accumulation of other non-related proteins.

We thank Dr. Gunilla Olivecrona for providing the bovine LPL and affinity-purified chicken anti-LPL immunoglobulins, Ms. Conxi Mora and Dr. Manuel Palacin for the rBAT clone and anti-rBAT immunoglobulins and for their stimulating comments; Dr. Paul Pilch and Dr. Antonio Zorzano for providing the GLUT4 clone and antibody, Dr. Ricardo Makiya for the PLAP clone and antibody, Dr. Egea for the anti-PDI antibody; Dr. Diego Haro for the pCMV-2 vector, and Dr. Jun. Ichi Miyazaki who provided the PCAGGS vector. We also thank all the members of LPL group at the Unit of Cell Biology for their critical support and especially David Bellido for his help in cryoultramicrotomy specimen preparation. We are grateful to David García, and Susanna Castel for their expert technical assistance, to Josep Julve for helping in LPL activity assays, and to Robin Rycroft for his expert editorial help. This work was supported by the Comision Interministerial de Ciencia y Tecnologia (grant SAF-92-0897), the Fondo de Investigaciones Sanitarias from Ministerio de Sanidad (grant 93/0423E), NIH Grant HL47151, the ARCOL, the International Life Sciences Institute (ILSI), and the European Community (BIOMED PL-921243).

Manuscript received 28 April 1994 and in revised form 28 September 1994.

REFERENCES

1. Olivecrona, T., and G. Bengtsson-Olivecrona. 1993. Lipoprotein lipase and hepatic lipase. *Curr. Opin. Lipidol.* **4:** 187–196.
2. Braun, J. E., and D. L. Severson. 1992. Regulation of the synthesis, processing and translocation of lipoprotein lipase. *Biochem. J.* **287:** 337–347.
3. Auwerx, J., P. Leroy, and K. Schoonjans. 1992. Lipoprotein lipase: recent contributions from molecular biology. *Crit. Rev. Clin. Lab. Sci.* **29:** 243–268.
4. Camps, L., M. Reina, M. Llobera, S. Vilaró, and T. Olivecrona. 1990. Lipoprotein lipase: cellular origin and functional distribution. *Am. J. Physiol.* **258:** C673–681.
5. Camps, L., M. Reina, M. Llobera, G. Bengtsson-Olivecrona, T. Olivecrona, and S. Vilaró. 1991. Lipoprotein lipase in lungs, spleen, and liver: synthesis and distribution. *J. Lipid Res.* **32:** 1877–1888.
6. Garfinkel, A. S., and M. C. Schotz. 1987. Lipoprotein lipase. *In* Plasma Lipoproteins A. M. Gotto, Jr., editor. Elsevier Science Publishers B. V., Amsterdam, New York, Oxford. 335–357.
7. Saxena, U., L. D. Witte, and I. J. Goldberg. 1989. Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. *J. Biol. Chem.* **264:** 4349–4355.
8. Vilella, E., J. Joven, M. Fernández, S. Vilaró, J. D. Brunzell, T. Olivecrona, and G. Bengtsson-Olivecrona. 1993. Lipoprotein lipase in human plasma is mainly inactive and associated with cholesterol-rich lipoproteins. *J. Lipid Res.* **34:** 1555–1564.
9. Vilaró, S., M. Llobera, G. Bengtsson-Olivecrona, and T. Olivecrona. 1988. Lipoprotein lipase uptake by the liver: localization, turnover and metabolic role. *Am. J. Physiol.* **254:** G711–G722.
10. Semenkovich, C. F., M. Wims, L. Noe, J. Etienne, and L. Chan. 1989. Insulin regulation of lipoprotein lipase activity in 3T3-L1 adipocytes is mediated at posttranscriptional and postranslational levels. *J. Biol. Chem.* **264:** 9030–9038.
11. Doolitte, M. H., O. Ben-Zeev, J. Elovson, D. Martin, and T. G. Kirchgessner. 1990. The response of lipoprotein lipase to feeding and fasting. Evidence for posttranslational regulation. *J. Biol. Chem.* **265:** 4570–4577.
12. Cisar, L. A., A. J. Hoogewerf, M. Arpp, C. A. Rapport, and A. Bensadoun. 1989. Secretion and degradation of lipoprotein lipase in cultured adipocytes. Binding of lipoprotein lipase to membrane heparan sulfate proteoglycans is necessary for degradation. *J. Biol. Chem.* **264:** 1767–1774.
13. Semenkovich, C. F., C. C. Luo, M. K. Nakanishi, S-H. Chen, L. C. Smith, and L. Chan. 1990. In vitro expression and site-specific mutagenesis of the cloned human lipoprotein lipase gene. *J. Biol. Chem.* **265:** 5429–5433.

14. Osborne, J. C., G. Bengtsson-Olivecrona, and N. S. Lee. 1985. Studies on activation of lipoprotein lipase. Role of the dimer to monomer dissociation. *Biochemistry.* **24:** 5606–5611.
15. Ailhaud, G. 1990. Cellular and secreted lipoprotein lipase revisited. *Clin. Biochem.* **23:** 343–347.
16. Hoogewerf, A. J., and A. Bensadoun. 1991. Occurrence of sulfate in an asparagine-linked oligosaccharide of chicken adipose lipoprotein lipase. *J. Biol. Chem.* **266:** 1048–1057.
17. Ben-Zeev, O., M. Doolittle, R. C. Davis, J. Elovson, and M. C. Schotz. 1992. Maturation of lipoprotein lipase. Expression of full catalytic activity requires glucose trimming but not translocation to the *cis*-Golgi compartment. *J. Biol. Chem.* **267:** 6219–6277.
18. Masuno, H., C. J. Schultz, J-W. Park, E. J. Blanchette-Mackie, C. Mateo, and R. O. Scow. 1991. Glycosylation, activity and secretion of lipoprotein lipase in cultured brown adipocytes of newborn mice. *Biochem. J.* **277:** 801–809.
19. Simsolo, R. B., J. M. Ong, and P. A. Kern. 1992. Characterization of lipoprotein lipase activity, secretion, and degradation at different sites of post-translational processing in primary cultures of rat adipocytes. *J. Lipid Res.* **33:** 1777–1784.
20. Carroll, B., O. Ben-Zeev, M. H. Doolittle, and D. L. Severson. 1992. Activation of lipoprotein lipase in cardiac myocytes by glycosylation requires trimming of glucose residues in the endoplasmic reticulum. *Biochem. J.* **285:** 693–696.
21. Hirschberg, C. B., and M. D. Snider. 1987. Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Annu. Rev. Biochem.* **56:** 63–87.
22. Olivecrona, T., S. S. Chernick, G. Bengtsson-Olivecrona, M. Garrison, and R. O. Scow. 1987. Synthesis and secretion of lipoprotein lipase in 3T3-L1 adipocytes: demonstration of inactive forms of lipase in cells. *J. Biol. Chem.* **262:** 10748–10759.
23. Miyazaki, J. I., K. Takaki, F. Tashiro, A. Tominega, K. Takatsu, and K. Yamamura. 1989. Expression vector system based on the chicken-actin promotor directs efficient production of interleukin-5. *Gene.* **79:** 269–277.
24. Sanger, F., S. Niklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
25. Hurtley, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5:** 277–307.
26. Gumà, A., C. Mora, T. Santalucía, F. Viñals, X. Testar, M. Palacín, and A. Zorzano. 1992. System A transport activity is stimulated in skeletal muscle in response to diabetes. *FEBS Lett.* **310:** 51–54.
27. Onishi, H. R., J. S. Tkacz, and J. O. Lampen. 1979. Glycoprotein nature of yeast alkaline phosphatase. *J. Biol. Chem.* **254:** 11943–11952.
28. Domin, W. S., A. Chait, and S. S. Deeb. 1991. Transcriptional activation of the lipoprotein lipase gene in macrophages by dexamethasone. *Biochemistry.* **30:** 2570–2574.
29. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154:** 367–382.
30. Kriegler, M. 1990. Gene Transfer and Expression: a Laboratory Manual. M. Stockton Press, New York. 99–100.
31. Gunning, P., J. Leavitt, J. Moscat, S-Y. Ng, and L. Kedes. 1987. A human β-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA.* **84:** 4831–4835.
32. Millán, J. L. 1986. Molecular cloning and sequence analysis of human alkaline phosphatase. *J. Biol. Chem.* **261:** 3112–3115.
33. Ramirez, I., A. J. Kryski, O. Ben-Zeev, M. C. Schotz, and D. L. Severson. 1985. Characterization of triacylglycerol hydrolase activities in isolated myocardial cells. *Biochem. J.* **232:** 229–236.
34. Alcalde, J., P. Bonay, A. Roa, S. Vilaro, and I. Sandoval. 1992. Assembly and disassembly of the Golgi complex: two processes arranged in a *cis* to *trans* direction. *J. Cell Biol.* **116:** 69–83.
35. Furriols, M., J. Chillarón, C. Mora, A. Castelló, J. Bertran, M. Camps, X. Testar, S. Vilaró, A. Zorzano, and M. Palacín. 1993. rBAT, related to L-cystine transport, is localized to the microvilli of proximal straight tubules, and its expression is regulated in kidney by development. *J. Biol. Chem.* **268:** 27060–27068.
36. Slot, J. W., H. J. Geuze, S. Gigengack, G. E. Lienhard, and D. E. James. 1991. Immunolocalization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* **113:** 123–135.
37. Babirak, S. P., P-H. Iverius, W. Y. Fujimoto, and J. D. Brunzell. 1989. Detection and characterization of the heterozygous state for lipoprotein lipase deficiency. *Atherosclerosis.* **9:** 326–334.
38. Yuan, L., L. G. Barriocanal, J. S. Bonifacino, and J. V. Sandoval. 1987. Two integral membrane proteins located in the *cis*-middle and *trans*-part of the Golgi system acquire sialylated N-linked carbohydrates and display different turnover and sensitivity to cAMP-dependent phosphorylation. *J. Cell Biol.* **105:** 215–227.
39. Griffiths, G., R. G. Parton, J. Lucocq, B. V. Deurs, D. Brown, J. W. Slot, and H. J. Geuze. 1993. The immunofluorescent era of membrane traffic. *Trends Cell Biol.* **3:** 214–219.
40. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene.* **108:** 193–200.
41. Elbein, A. D. 1987. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.* **56:** 497–534.
42. Bertran, J., A. Werner, J. Chillarón, V. Nunes, J. Biber, X. Testar, A. Zorzano, X. Estivill, H. Murer, and M. Palacin. 1993. Expression cloning of a human renal cDNA that induces high affinity transport of L-cystine shared with dibasic amino acids in *Xenopus* oocytes. *J. Biol. Chem.* **268:** 1482–14849.
43. Kotliar, N., and P. Pilch. 1992. Expression of the glucose transport isoform GLUT4 is insufficient to confer insulin-regulatable hexose uptake to cultured muscle cells. *Mol. Endocrinol.* **6:** 337–345.
44. McComb, R., and S. Posen, editors. 1979. Alkaline Phosphatase. Plenum, London, New York.
45. Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* **102:** 1558–1566.
46. Bole, D. G., R. Dowin, M. Doriaux, and J. D. Jamieson. 1989. Immunocytochemical localization of BiP to the rough endoplasmic reticulum: evidence for protein sorting by selective retention. *J. Histochem. Cytochem.* **37:** 1817–1823.
47. Peterson, J., W. Y. Fujimoto, and J. D. Brunzell. 1992. Human lipoprotein lipase: relationship of activity, heparin

affinity, and conformation as studied with monoclonal antibodies. *J. Lipid Res.* **33:** 1165–1170.
48. Gaut, J. R., and L. M. Hendershot. 1993. The modification and assembly of proteins in the endoplasmic reticulum. *Curr. Opin. Cell Biol.* **5:** 589–595.
49. Helenius, A., T. Marquardt, and I. Braakman. 1992. The endoplasmic reticulum as a protein folding compartment. *Trends Cell Biol.* **2:** 227–231.
50. Brown, D. A., and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* **68:** 533–544.
51. Masuno, H., E. J. Blanchette-Mackie, S. S. Chernick, and R. O. Scow. 1990. Synthesis of inactive, nonsecretable high mannose lipoprotein lipase by cultured brown adipose adipocytes of combined lipase processing and secretion. *J. Biol. Chem.* **265:** 1628–1638.
52. Davis, R. C., O. Ben-Zeev, D. Martin, and M. H. Doolittle. 1990. Combined lipase deficiency in the mouse. Evidence of the impaired lipase processing and secretion. *J. Biol. Chem.* **265:** 17960–17966.
53. Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein. 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. *Cell.* **60:** 781–790.
54. Braakman, I., J. Helenius, and A. Helenius. 1992. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO J.* **11:** 1717–1722.
55. Lalouel, J-M., D. E. Wilson, and P-H. Iverius. 1992. Lipoprotein lipase and hepatic lipase: molecular and genetic aspects. *Curr. Opin. Lipidol.* **3:** 86–95.