Immunocytochemical Localization of Mutant Low Density Lipoprotein Receptors that Fail to Reach the Golgi Complex

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Abstract. In the low density lipoprotein (LDL) receptor system, blocks in intracellular movement of a cell surface receptor result from naturally occurring mutations. These mutations occur in patients with familial hypercholesterolemia. One class of mutant LDL receptor genes (class 2 mutations) produces a receptor that is synthesized and glycosylated in the endoplasmic reticulum (ER) but does not reach the cell surface. These receptors contain serine/threonine-linked (O-linked) carbohydrate chains with core N-acetylgalactosamine residues and asparagine-linked (N-linked) carbohydrate chains of the high mannose type that are only partially trimmed. To determine the site of blockage in transport, we used electron microscope immunohistochemistry to compare the intracellular location of LDL receptors in normal human fibroblasts with their location in class 2 mutant fibroblasts. In normal cells, LDL receptors were located in coated pits, coated vesicles, endosomes, multivesicular bodies, and portions of the Golgi complex. In contrast, the mutant receptors in class 2 cells were almost entirely confined to rough ER and irregular extensions of the rough ER. Metabolic labeling studies with [³H]glucosamine confirmed that these mutant receptors contain core O-linked sugars, suggesting that the enzymes that attach these residues are located in the rough ER or the transitional zone of the ER. These studies establish that naturally occurring mutations in cell surface receptors can cause the receptors to remain trapped in the ER, thereby preventing their normal function and producing a genetic disease.

RECENT studies in a variety of systems have disclosed that animal cells have a mechanism by which they can prevent the movement to the cell surface of improperly folded secretory or membrane proteins. The failure of such transport has been observed with mutant viral lipid envelope proteins (Gething et al., 1986), chimeric fusions between secretory and membrane-bound proteins (Rizzolo et al., 1985), and with naturally occurring human mutations in the genes for α-1-antitrypsin (Hercz et al., 1978) and the low density lipoprotein (LDL)[1] receptor (Tolleshaug et al., 1983; Yamamoto et al., 1986).

Biochemical studies suggest that these abnormal proteins are usually blocked in movement from the endoplasmic reticulum (ER) to the Golgi complex. The site within the ER at which this block occurs has been studied in detail at the morphologic level in only one case—that of a chimeric protein between growth hormone and the influenza hemaggluti-

1. *Abbreviations used in this paper:* ABC, avidin–biotin complex; Con A, concanavalin A, DAB, 3,3′-diaminobenzidine tetrahydrochloride; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; GalNAc, N-acetylgalactosamine; HRP, horseradish peroxidase; LDL, low density lipoprotein.

nin (Rizzolo et al., 1985). In cells that expressed extremely high levels of this fusion protein, the protein accumulated in an abnormal expanded compartment of smooth-surfaced membranes that was felt to represent a hypertrophied transitional zone between the ER and the Golgi complex. Whether this same organelle represents the site of blockage in the movement of naturally occurring mutant proteins is not known.

In the current studies, we have sought to answer this question by examination of cultured fibroblasts from patients with mutations in the gene for the LDL receptor. The LDL receptor is a 160-kD transmembrane glycoprotein that moves continually from one organelle to another (Goldstein et al., 1985). In the steady state, most of the receptors are located in coated pits on the cell surface. These receptors continually enter the cell when coated pits invaginate to form coated vesicles. LDL that is bound to this receptor is delivered to acidic endosomes where it dissociates. The unoccupied receptor returns to the cell surface where it binds another LDL and initiates another round of internalization. After it is deposited in the endosome, the LDL is transported to the lysosome where it is degraded. During its 20-h life span each LDL

receptor mediates >100 rounds of internalization (Brown et al., 1983).

Before it can embark upon this recycling pathway, the LDL receptor must first travel to the cell surface from its site of synthesis in the rough ER. Its progress along this path can be followed biochemically by monitoring the apparent molecular mass of the receptor on SDS–polyacrylamide gels (Tolleshaug et al., 1983). Newly synthesized LDL receptors show an apparent molecular mass of 120 kD on SDS–polyacrylamide gels. These receptor precursors contain several immature high mannose N-linked oligosaccharide chains and many O-linked N-acetlygalactosamine (GalNAc) residues. The majority of the O-linked GalNAc residues are clustered in a serine- and threonine-rich region of 48 amino acids just outside the plasma membrane (Cummings et al., 1983; Davis et al., 1986). Within 30 min after synthesis, the receptor is transported from the ER to the Golgi complex where the high mannose N-linked oligosaccharide chains are converted to a complex form, and where each of the O-linked core GalNAc sugars is modified by addition of galactose and sialic acid residues. As a result of these changes, the apparent molecular mass of the receptor increases to 160 kD, an increase that is attributable mainly to anomalous migration on SDS gels resulting from the elongation of the clustered O-linked sugar chains (Cummings et al., 1983; Davis et al., 1986). The mature 160-kD LDL receptor is rapidly transported to the cell surface where it immediately clusters in coated pits and begins to recycle whether or not LDL is available (Goldstein et al., 1985).

The molecular signals that regulate the intracellular traffic of the LDL receptor are being elucidated through the study of naturally occurring human mutant fibroblasts that synthesize receptors that are defective in intracellular movement. These mutations occur in individuals with a common genetic disease called familial hypercholesterolemia (FH). One class of mutant LDL receptor genes (class 2 mutations) produce 120-kD LDL receptor precursors that cannot be processed to the mature 160-kD form (Goldstein et al., 1985). The 120-kD precursor never reaches the cell surface, as revealed by its resistance to digestion with extracellular proteases (Tolleshaug et al., 1983; Cummings et al., 1983). The N-linked sugars remain in the endoglycosidase H–sensitive form, suggesting that the receptors have not been transported to the Golgi stacks. In one of these patients, the trapped precursor was shown to contain core GalNAc residues attached to serine and threonine (Cummings et al., 1983). This latter modification is catalyzed by a polypeptide, GalNAc transferase, that is thought, on the basis of cell fractionation studies, to be located in the Golgi complex (Hanover et al., 1982; Abeijon and Hirschberg, 1987). This apparent inconsistency in the location of the class 2 mutant LDL receptors and the enzymes that modify them can only be resolved by ultrastructural studies.

The molecular basis for two of the class 2 mutations have been disclosed through molecular cloning (Yamamoto et al., 1986). One of these occurs in a strain of rabbits designated WHHL rabbits and the other occurs in an FH patient designated FH 563. In both instances there is a small in-frame deletion that removes several amino acids from a region of the receptor that is rich in disulfide-bonded cysteine residues. We have suggested that the failure of transport results from improper folding caused by the deletions with consequent

failure to form normal disulfide bonds. Aberrant folding has been implicated as a cause of slow movement from ER to Golgi complex in mutants of viral lipid envelope proteins (Gething et al., 1986).

In the current studies we have used several techniques of histochemical visualization to compare the intracellular location of the LDL receptor in normal human fibroblasts with that in fibroblasts from three FH individuals with class 2 mutations. Our results indicate that in normal cells in the steady state the LDL receptor is located primarily in coated pits on the cell surface and in endosomes; very little receptor is found in the ER. In the class 2 mutant cells the receptor is located exclusively in the ER and in tubular extensions of the ER that are believed to correspond to transition elements between this organelle and the Golgi complex.

Materials and Methods

Reagents

We obtained BSA (No. A-7638), cycloheximide, 3,3'-diaminobenzidine tetrahydrochloride (DAB), Hepes, hydrogen peroxide, saponin, Trizma (hydroxymethyl aminomethane) base, and Trizma hydrochloride from Sigma Chemical Co. (St. Louis, MO). Paraformaldehyde, tetrachloroauric acid (No. 50800), and 2,4,6-trinitrophenol were purchased from Fluka (Hauppauge, NY). Lysine monohydrochloride and sodium metaperiodate were obtained from Fisher Scientific Co. (Plano, TX); glutaraldehyde, osmium tetroxide, propylene oxide, and Spurr's low viscosity embedding medium from Electron Microscopy Sciences (Fort Washington, PA). Rabbit immunoglobulin Vectastain avidin–biotin complex (ABC) kit (No. PK4001) containing affinity-purified biotinylated goat anti-rabbit IgG, avidin DH (reagent A), and biotinylated horseradish peroxidase (HRP) H (reagent B) was purchased from Vector Laboratories, Inc. (Burlingame, CA). Affinity-purified sheep anti-rabbit IgG conjugated to HRP (No. BI-2407) was obtained from Bio-Sys (Compiegne, France). A polyclonal antibody directed against the human LDL receptor was prepared by immunizing a New Zealand white rabbit with purified bovine receptor as described (Russell et al., 1984). mAbs IgG-C7 and IgG-2001 were prepared as described (Beisiegel et al., 1982). We obtained [^{35}S]methionine (1,100 Ci/mmol) and D-[1,6-^{3}H]glucosamine (30–60 Ci/mmol) from New England Nuclear (Boston, MA); D-[6-^{3}H]glucosamine (20–40 Ci/mmol) from Amersham Corp. (Arlington Heights, IL). DME and PBS (No. 310-4190) were from Grand Island Biological Co. (Grand Island, NY); and ITS Premix (containing insulin, transferrin, and selenium) was from Collaborative Research Inc. (Bedford, MA). Human LDL (density, 1.019–1.063 g/ml) and lipoprotein-deficient serum (density, >1.215 g/ml) were isolated from the plasma of healthy donors by ultracentrifugation (Goldstein et al., 1983). LDL was radiolabeled with ^{125}I as described (Goldstein et al., 1983).

Pronase was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and concanavalin A (Con A)–Sepharose was obtained from Pharmacia P-L Biochemicals (Milwaukee, WI). N,N'-Diacetylchitobiose, N,N',N'',-triacetylchitobiose, N,N',N'',N'''-tetraacetylchitotetraose, and N,N',N'',N''',N''''-pentaacetylchitopentaose were prepared from chitin (Sigma Chemical Co.) by the procedure of Rupley (1964). The reduced forms of these oligosaccharides were prepared by reduction with NaB^{3}H$_4$. N-[^{3}H]Acetylglucosaminitol and N-[^{14}C]acetylgalactosaminitol were prepared by NaBH$_4$ reduction of the appropriate radiolabeled reducing sugars and were purified after reduction by passage over a column of mixed bed ion exchange resin in water. The disaccharide standard [^{3}H]Gal-β1,4GlcNAc was prepared enzymatically from GlcNAc (Sigma Chemical Co.) using bovine galactosyltransferase (UDP-galactose/D-glucose-4-galactosyl-transferase, Sigma Chemical Co.) and UDP-[6-^{3}H]galactose (Amano et al., 1988). The reduced form of this disaccharide was prepared by reduction with NaBH$_4$. The standard Galβ1,3-[^{3}H]GalNAcitol was prepared from glycoproteins derived from [^{3}H]glucosamine-labeled adult schistosomes (Nyame et al., 1987).

Cell Culture

Human fibroblasts were derived from skin biopsies obtained from normal subjects and subjects with FH. Cells were grown in monolayers and set up for experiments according to a standard format (Goldstein et al., 1983). On

day 0, 2 × 10⁴ cells were seeded into each Petri dish (60 × 15 mm) containing 3 ml DME supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (vol/vol) FCS. Fresh medium of the same composition was added on day 3. On day 5 of cell growth, each monolayer received 2 ml of medium supplemented with penicillin, streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium, and 10% (vol/vol) human lipoprotein-deficient serum. All experiments except those in Fig. 5 were performed on day 7 after a 48-h incubation in lipoprotein-deficient serum. The experiments in Fig. 5 were performed on day 6 after a 24-h incubation in lipoprotein-deficient serum.

Immunochemical Analysis of LDL Receptors

On day 6 of cell growth, monolayers of fibroblasts were pulse-labeled with [³⁵S]methionine or [³H]glucosamine, and the isotopically labeled LDL receptors were immunoprecipitated with antireceptor mAb IgG-C7 and analyzed by electrophoresis on 7% SDS–polyacrylamide gels, followed by autoradiography (Tolleshaug et al., 1983). Apparent molecular masses of the radiolabeled proteins were calculated by positions of migration of the following standards as determined by Coomassie Blue staining: myosin (200 kD), β-galactosidase (116 kD), phosphorylase G (97 kD), and BSA (68 kD).

Analysis of Radiolabeled Glycopeptides and Oligosaccharides Released from LDL Receptors

The following procedures were performed as described by Cummings et al. (1983) and David et al. (1986): labeling of LDL receptors in intact fibroblasts with [³H]glucosamine, preparation and separation of ³H-labeled glycopeptides, treatment of glycopeptides with mild alkaline borohydride, and paper chromatography of ³H-oligosaccharides released from glycopeptides.

Immunocytochemical Localization of LDL Receptors

ABC Immunoperoxidase Method. On day 7 of cell growth, fibroblast monolayers were fixed for 1 h at room temperature with a fixative that contained 3% (wt/vol) paraformaldehyde and 0.069% (wt/vol) trinitrophenol in buffer A (100 mM Hepes, 4 mM CaCl₂, 2 mM KCl, and 4 mM MgCl₂ at pH 7.4) followed by three washes (2 ml each) with buffer B (50 mM Hepes, 150 mM NaCl, 4 mM CaCl₂, 2 mM KCl, and 4 mM MgCl₂ at pH 7.4). Monolayers were then incubated in buffer C (buffer B containing 0.54% [wt/vol] NH₄Cl, 0.2% [wt/vol] BSA, and 0.06% [vol/vol] saponin) for 30 min at room temperature followed by three washes with buffer D (buffer B plus 0.2% BSA and 0.01% saponin). Permeabilized cells were incubated with either a nonimmune IgG or a polyclonal anti–LDL receptor IgG (0.1 mg/ml in buffer D) for 1 h at 37°C. IgG binding sites were localized using the Vectastain ABC kit with the following modifications: (a) a 1-h incubation at 37°C with biotinylated, goat anti-rabbit IgG (diluted 1:222 [vol/vol] in buffer D); and (b) a 1-h incubation at 37°C with ABC reagent (1 part [vol/vol] avidin DX [reagent A] mixed with 1 part [vol/vol] biotinylated HRP [reagent B] in buffer D and preincubated 60 min at room temperature). Between each incubation, monolayers were washed three times for 10 min each at room temperature with buffer D. After the last wash, dishes were rinsed three times, 5 min each, with buffer B and fixed with 2.5% (vol/vol) glutaraldehyde and 5% (wt/vol) sucrose in buffer B overnight at 4°C. Dishes were washed three times, 10 min each, with buffer B followed by a brief wash with 100 mM Tris-HCl (pH 7.2). To detect peroxidase activity, cells were incubated with 0.1% (wt/vol) DAB in 100 mM Tris-HCl, pH 6.0, for 15 min at room temperature. This incubation medium was replaced with fresh 0.1% DAB in 100 mM Tris-HCl (pH 6.0) containing 0.01% (vol/vol) H₂O₂, and the cells were incubated another 10 min. At the end of the reaction, dishes were washed six times, 5 min each, with 100 mM Tris-HCl (pH 7.2) at room temperature.

Anti-IgG Immunoperoxidase Method. Monolayers subjected to the indicated treatments were fixed immediately for 45 min at 4°C in fixative that contained 2% paraformaldehyde, 37 mM sodium phosphate buffer, 75 mM lysine monohydrochloride, and 10 mM sodium metaperiodate at pH 6.2. Cells were then washed with PBS and processed for indirect immunoperoxidase staining by the method of Louvard et al. (1982) using 0.2 mg/ml of a polyclonal anti–LDL receptor antibody and a 1:75 dilution of sheep anti-rabbit IgG conjugated to HRP. Peroxidase reaction product was visualized by incubating cells in 0.2% DAB and 0.01% hydrogen peroxide in 50 mM Tris-HCl (pH 6.5) for 10 min at room temperature. Cells were then washed and processed for electron microscopy as described (Anderson et al., 1981).

Preparation of [¹²⁵I]LDL–Gold

Colloidal gold particles were prepared by the reduction of tetrachloroauric acid with trisodium citrate (Frens, 1973). [¹²⁵I]LDL (1.7 × 10⁶ cpm/ng protein; 5 mg/ml) was dialyzed against 50 mM sodium EDTA at pH 6 at 4°C for 2 h. The dialyzed [¹²⁵I]LDL was conjugated with colloidal gold at pH 6, according to the method of Horisberger (1979), by mixing 5 ml of colloidal gold with 42 μl of [¹²⁵I]LDL (1 mg/ml) and incubating the mixture for 30 min at room temperature. To remove [¹²⁵I]LDL–gold from unbound LDL, the mixture was centrifuged at 17,400 g in polyethelene glycol-20–coated polysulfone tubes (Oak Ridge type) for 30 min at 4°C. The supernatant fraction was discarded, and the loose pellet was carefully collected in 0.5 ml of PBS and dialyzed 1 h at 4°C against PBS. The protein concentration of [¹²⁵I]LDL in the [¹²⁵I]LDL–gold preparation was determined by scintillation counting in a gamma counter.

Electron Microscopy

Cells were postfixed with 2% (wt/vol) OsO₄ in 100 mM sodium cacodylate (pH 7.2) containing 4.5% sucrose for 90 min at room temperature. Dishes were washed several times with distilled water and incubated with 0.05% (wt/vol) uranyl acetate for 10 min at room temperature. Cells were then dehydrated through graded ethanol and were separated from the dishes using propylene oxide (Anderson et al., 1981). After three rinses with propylene oxide, the cells were infiltrated with and embedded in Spurr's low viscosity embedding medium at 75°C for 18 h. Sections were cut on an ultramicrotome (MT2 B, Sorvall Instruments Div., Newton, CT) and examined with an electron microscope (100 CX, JEOL USA, Peabody, MA) without any further staining.

Results

Distribution of Intracellular LDL Receptors in Normal Fibroblasts

The total number of LDL receptors in normal human fibroblasts under maximal induction is <70,000 per cell (Goldstein et al., 1983). In mutant fibroblasts the number of receptors is much lower. This low level of expression is at the limits of detection by electron microscope immunohistochemistry. To localize this small number of receptors, we used a complex amplification technique (the ABC procedure) in which fixed cells are permeabilized with saponin and then incubated with a rabbit anti-receptor IgG, followed by a biotinylated goat anti-rabbit IgG, and then a mixture of avidin and biotinylated HRP. Because of the light fixation that is initially used and the many washes that are necessary, some of the cytoplasmic detail is not preserved in the micrographs. Moreover, in order to clearly demonstrate the peroxidase reaction product, the photographs must be printed with maximal contrast, which also tends to make the unstained areas seem washed out in appearance. The only alternative procedure would involve the use of antibody–gold complexes on thin sections of cells, which is a less sensitive technique than immunoperoxidase. Unfortunately, we have been unable to visualize a low number of LDL receptors with the gold technique. Although the ABC procedure sacrifices cellular detail, the visualization was sufficient to allow definite conclusions to be drawn, as illustrated below.

To analyze the distribution of the class 2 mutant LDL receptors, we first had to study the steady-state distribution of intracellular receptors in normal cells. Although the surface distribution of these receptors has been studied extensively (Anderson et al., 1977, 1982), the distribution of intracellular LDL receptors has not been visualized previously by electron microscopy. Normal human fibroblasts incubated in the absence of LDL were fixed, permeabilized with saponin,
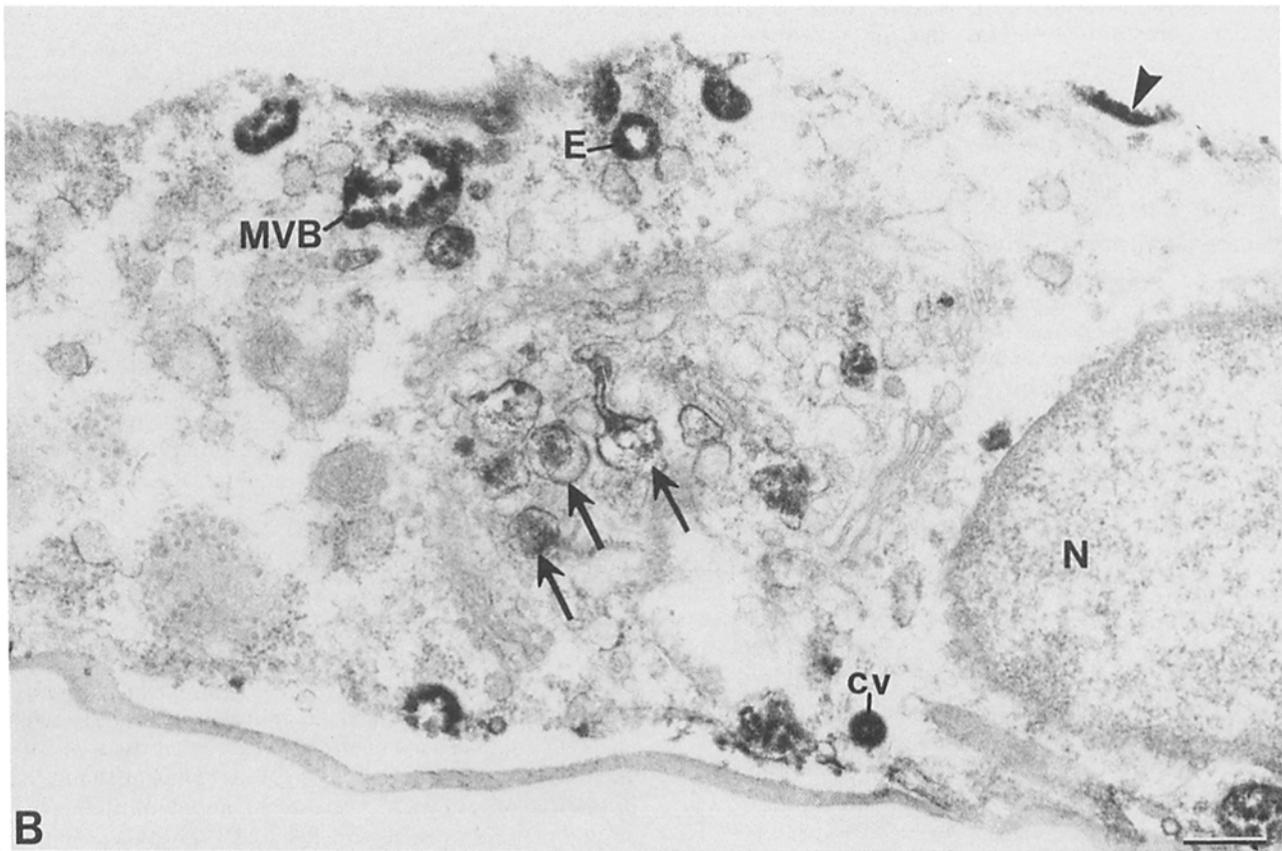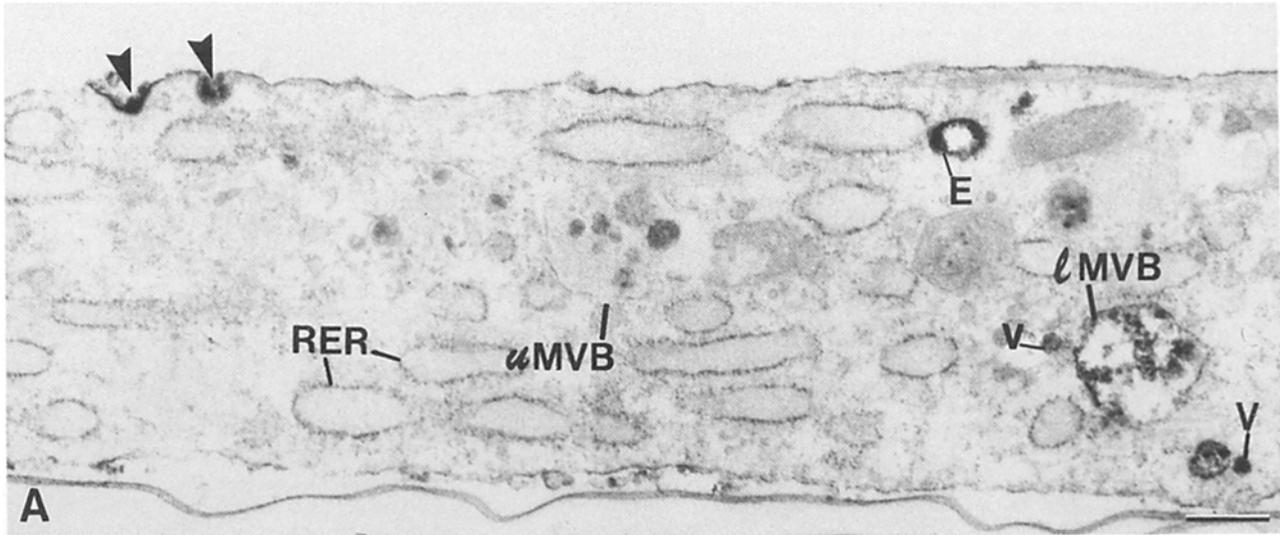
Figure 1. Distribution of anti–LDL receptor IgG binding sites in normal human fibroblasts. Monolayers of normal human fibroblasts were fixed and processed to localize intracellular LDL receptors using the ABC immunoperoxidase procedure. (*A*) A low power electron micrograph showing the distribution of anti–LDL receptor IgG binding sites as detected by the presence of electron dense HRP reaction product. *Arrowheads*, coated pit; *E*, endosomes; *uMVB*, lightly labeled multivesicular body; *ℓ MVB*, labeled multivesicular body; *RER*, rough ER; *V*, small vesicles. (*B*) Low power electron micrograph showing the distribution of anti–LDL receptor IgG binding sites. *Arrowhead*, coated pit; *arrows*, Golgi vesicles and cisternae; *E*, endosomes; *cv*, coated vesicle; *MVB*, multivesicular body; *N*, nucleus. Bar, 0.4 μm.

and processed for immunoperoxidase localization of LDL receptors by the ABC method using a polyclonal anti–LDL receptor IgG. Dark HRP reaction product due to anti–LDL receptor IgG binding was present in coated pits (*arrowhead*, Fig. 1, *A* and *B*), coated vesicles (*cv*, Fig. 1 *B*), endosomes (*E*, Fig. 1, *A* and *B*), small vesicles (*v*, Fig. 1 *A*), multivesicular bodies (*ℓ MVB* and *MVB*, Fig. 1, *A* and *B*, respectively), and vesicles and cisternae of the Golgi complex (*arrows*, Fig. 1 *B*). Reaction product was not evident in the rough ER (*RER*, Fig. 1 *A*) or the nuclear envelope (Fig. 1 *B*), and some
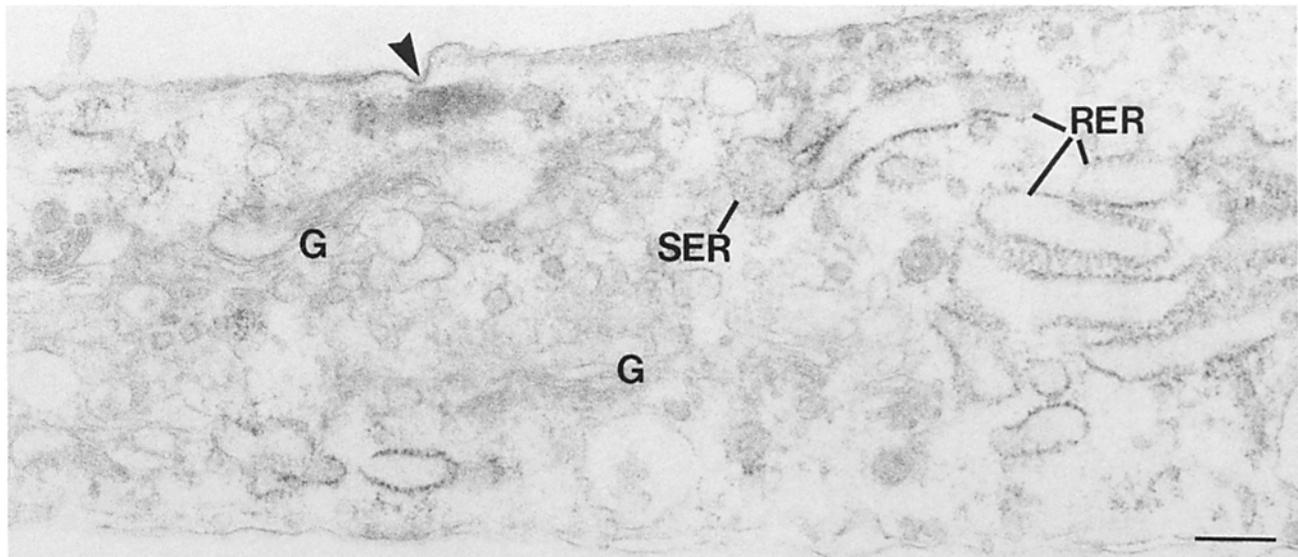
Figure 2. Distribution of anti–LDL receptor IgG binding sites in mutant human fibroblasts that do not synthesize LDL receptors (class 1 mutation). Monolayers of fibroblasts from FH 549 were fixed and processed to localize LDL receptors by the ABC procedure. *Arrowhead,* coated pit; *RER,* rough ER; *G,* Golgi complex; *SER,* irregular extensions of the RER. Bar, 0.4 μm.

multivesicular bodies had a reduced amount of reaction product (*uMVB,* Fig. 1 *A*). As a control for the specificity of the antibodies used in these experiments, we studied mutant fibroblasts that do not synthesize any immunoreactive LDL receptors (class 1 mutation). No HRP reaction product was detected in any intracellular compartment of these cells (Fig. 2). Moreover, when normal fibroblasts were processed using a nonimmune primary antibody, HRP reaction product was not detected (data not shown).

The receptor-positive membranes in the endocytic pathway were further defined by localizing receptors in cells that had been incubated with LDL–gold to mark the sites of LDL internalization and accumulation (Fig. 3, *A* and *B*). LDL–gold particles and immunodetectable LDL receptors were colocalized in coated pits (*arrowhead,* Fig. 3 *A*), nascent coated vesicles (*arrowhead,* Fig. 3 *B*), uncoated endocytic vesicles (*E,* Fig. 3, *A* and *B*), and some multivesicular bodies (*MVB,* Fig. 3 *A*). Other multivesicular bodies contained LDL–gold but little HRP reaction product (*MVB,* Fig. 3 *C*). Surrounding the multivesicular bodies were numerous small vesicles that did not contain LDL–gold but were heavily labeled with HRP reaction product (*arrows,* Fig. 3, *C* and *D*). Some of these receptor-positive vesicles appeared to be in the process of budding from the multivesicular body membrane as evidenced by their location at the ends of membranous stalks (*arrowhead,* Fig. 3, *C* and *D*).

LDL Receptors in Golgi Complex Are Newly Synthesized

The receptors that were seen in the Golgi complex (Fig. 1 *B*) could represent receptors that are recycling through this compartment en route to the cell surface or they could represent newly synthesized receptors. To distinguish between these possibilities, we incubated fibroblasts in the presence of cycloheximide for 4 h to prevent receptor synthesis (Brown et al., 1981). This treatment does not affect the internaliza-

tion or recycling of already synthesized receptors (Brown et al., 1981), but it should deplete newly synthesized receptors from the secretory pathway. The cycloheximide-treated cells showed a marked depletion of receptors in the Golgi complex (Fig. 4, compare *B* with *A*). This treatment did not affect the distribution of receptors in coated pits (*arrowheads,* Fig. 4, *A* and *B*), coated vesicles, or endocytic vesicles. We therefore conclude that the receptors in the Golgi complex were newly synthesized, rather than recycling, receptors. Consistent with this interpretation was our failure to observe internalized LDL–gold in any portion of the Golgi complex (data not shown).

Biochemical Analysis of 120-kD Receptor in Fibroblasts with Class 2 Mutation

Fibroblasts from three unrelated FH homozygotes with class 2 mutations were studied. The defect in LDL receptor processing was demonstrated by incubating the cells in the presence of [^{35}S]methionine for 60 min and then transferring them to medium that contained an excess of unlabeled methionine for 2 h. The LDL receptor was immunoprecipitated from detergent-solubilized cells and analyzed by SDS-PAGE and autoradiography (Fig. 5). After a 60-min pulse, the normal cells showed about equal amounts of the 120-kD precursor and 160-kD mature receptor (Fig. 5, lane *2*). After 2 h of chase, virtually all of the precursor was converted to the mature form (lane *3*). In cells from two of the class 2 mutants, FH 429 and FH 261, no detectable amount of precursor was converted to the mature form (lanes *6* and *9*). In cells from one class 2 mutant, FH 563, a small amount of precursor (~5% by densitometry of the gels) was processed to the mature form (lane *12*). These results are similar to previous results with the class 2 mutant cells (Tolleshaug et al., 1983; Yamamoto et al., 1986).

The glycosylation of the 120-kD receptor precursors from normal fibroblasts and fibroblasts from two of the class 2 mu-
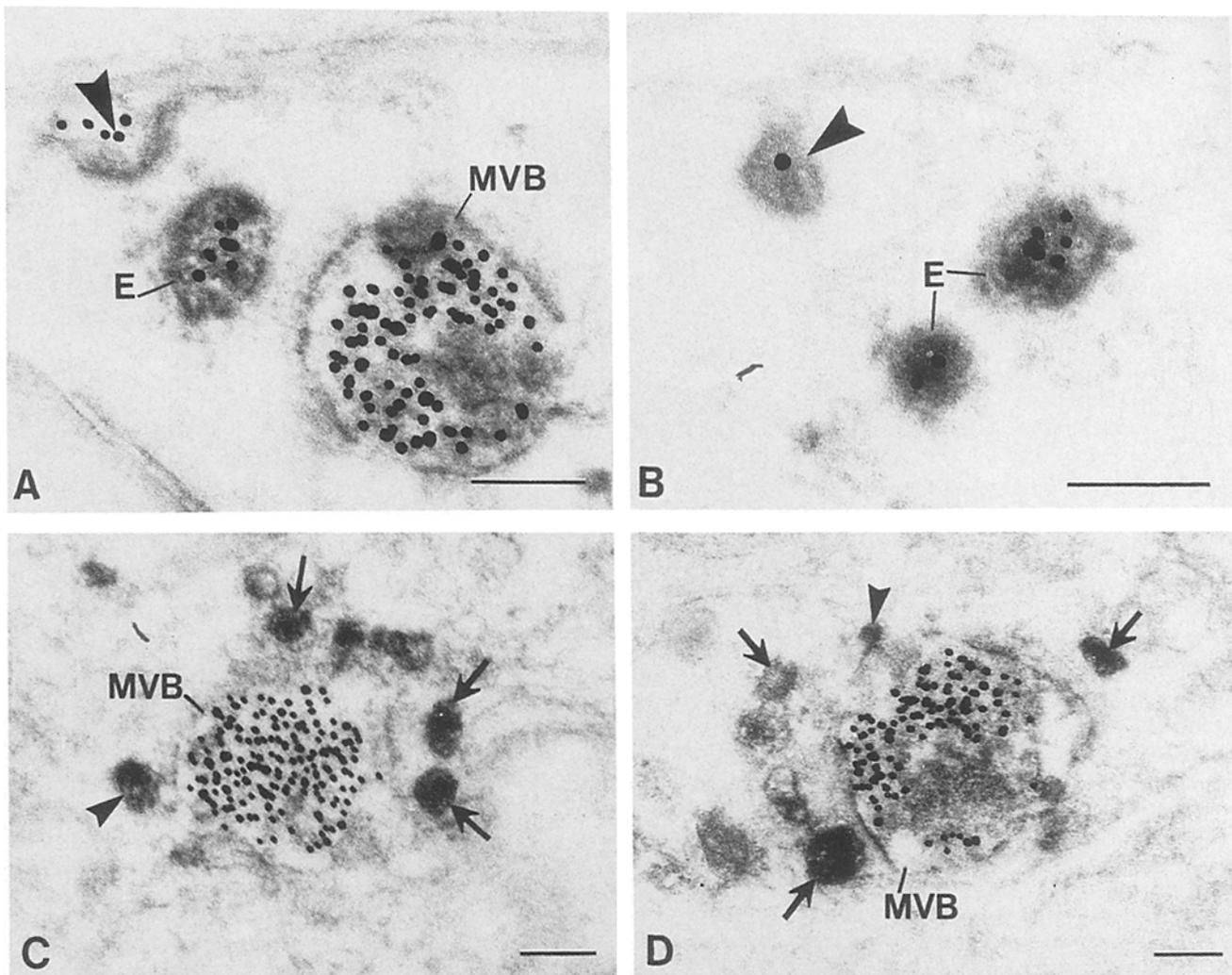
Figure 3. Colocalization of LDL–gold and anti–LDL receptor IgG binding sites in normal human fibroblasts. Monolayers of normal human fibroblasts were incubated for 30 min at 37°C with 20 μg protein/ml of LDL–gold. At the end of the incubation, the cells were washed with PBS, fixed, and processed for the localization of LDL receptors using the anti-IgG immunoperoxidase procedure. (*A* and *B*) Both LDL–gold and anti–LDL receptor IgG binding in coated pit (*arrowheads*), endosomes (*E*), and multivesicular bodies (*MVB*). (*C* and *D*) Multivesicular bodies (*MVB*) that contained LDL–gold and anti–LDL receptor IgG binding sites. *Arrows*, receptor-positive vesicles surrounding multivesicular bodies; *arrowheads*, receptor-positive evaginations from multivesicular body membrane. Bar, 0.2 μm.

tants, FH 429 and FH 563, were analyzed by metabolic radiolabeling (Cummings et al., 1983). The cells were incubated in medium containing [³H]glucosamine, and the LDL receptors were immunoprecipitated and subjected to SDS-PAGE and fluorography as described in Materials and Methods. [³H]Glucosamine was incorporated into the 120-kD receptor precursor of all three cell strains (data not shown).

The observation that the LDL receptor precursor in normal fibroblasts is glycosylated is consistent with the results of our previous study in which the receptor precursor was found to contain both O-linked chains and high mannose-type Asn-linked sugar chains (Cummings et al., 1983). In that study we found that a large amount of [³H]glucosamine-derived material was contained in O-linked sugar chains that did not bind to Con A-Sepharose. Con A-Sepharose does not bind glycopeptides containing O-linked sugar chains. It also does not bind certain complex-type tri- and tetraantennary and bisected biantennary asparagine-linked sugar chains (Cummings and Kornfeld, 1984).

To determine whether the [³H]glucosamine in the mutant 120-kD receptors was in O-linked sugar chains, the 120-kD band was excised from the SDS gel and digested with pronase. The resulting ³H-labeled glycopeptides were analyzed by previously described techniques (Cummings et al., 1983; Davis et al., 1986). The glycopeptides were first fractionated by chromatography on Con A-Sepharose (Fig. 6). Greater than 95% of the radioactivity from the normal precursor (Fig. 6 *A*) and the FH 563 precursor (Fig. 6 *C*) and 85% of that from FH 429 precursor (Fig. 6 *B*) did not bind to Con A-Sepharose. The remainder of the radioactivity from the FH 429 receptor required 100 mM α-methyl-mannoside for elution, a behavior that is typical for high mannose-type asparagine-linked sugar chains (Cummings et al., 1983).

The [³H]glucosamine-labeled glycopeptides that did not bind to Con A-Sepharose were treated with mild base borohydride and then analyzed by descending paper chromatography. This treatment releases O-linked sugars from serine and threonine residues in peptides via a β-elimination reac-
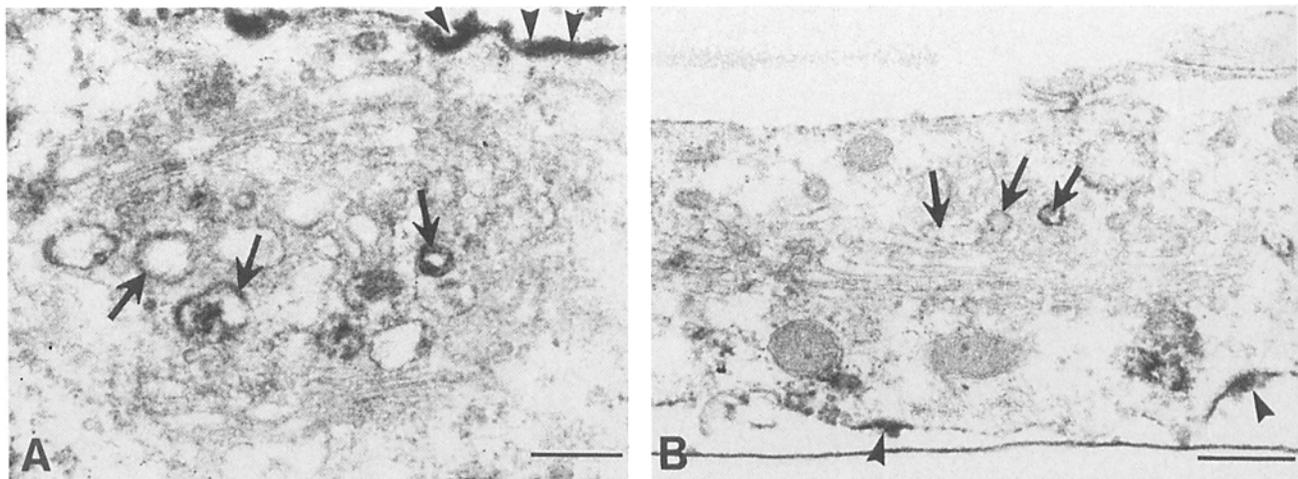
Figure 4. Distribution of anti–LDL receptor IgG binding sites in the Golgi complex of normal human fibroblasts that were incubated in the absence (*A*) or presence (*B*) of cycloheximide. Monolayers of normal human fibroblasts were incubated in the absence (*A*) or presence (*B*) of 200 μM cyclohexiniide for 4 h at 37°C. At the end of the incubation, the cells were fixed and processed for localization of LDL receptors using the ABC immunoperoxidase procedure as described. (*A*) *Arrows*, Golgi cisternae and vesicles that contain anti–LDL receptor IgG binding sites; *arrowheads*, coated pits. (*B*) *Arrows*, Golgi cisternae and vesicles; *arrowheads*, coated pits. Bar, 0.4 μm.

tion (Carlson, 1968; Baenziger and Kornfeld, 1974). Glycopeptides from the two mutant cell strains yielded two major radiolabeled species that had chromatographic mobilities similar to tri- and tetrasaccharides (Fig. 7, *A* and *B*). These results are similar to those obtained previously with the LDL receptor precursor from normal fibroblasts (Cummings et al., 1983) and indicate that the receptor precursors accumulating in the FH 429 and FH 563 cells contain O-linked sugar chains.

Intracellular Localization of the 120-kD LDL Receptor in Fibroblasts with Class 2 Mutation

The distribution of LDL receptors in the class 2 mutant cells was analyzed by electron microscopy using the ABC immunoperoxidase procedure. Only faint traces of LDL receptor reaction product were detectable on the cell surface, in coated pits (*arrowhead*, Fig. 8, *A* and *C*), or in recognizable vesicles in the endocytic pathway (*MVB*, Fig. 8 *B*). Instead, most of the reaction product was present in elements of the

rough ER (*RER*, Fig. 8, *A*, *C*, and *D*). The highest concentration of reaction product was in membrane extensions of the rough ER (*SER*, Fig. 8, *A* and *C*) that appeared to be devoid of ribosomes. In addition, irregularly shaped vesicles (*arrows*, Fig. 8, *A* and *B*) that contained reaction product were scattered throughout the cell. Each of the three FH cell strains that we studied — FH 429, FH 261, and FH 563 — had the same LDL receptor distribution (Fig. 8, *A*, *B*, and *C*, respectively) except that FH 563 cells had a somewhat larger amount of HRP reaction product in coated pits (data not shown). This finding is consistent with the small degree of receptor processing that occurs in this mutant (Fig. 5).

The irregularly shaped vesicles that contained LDL receptors appeared to represent extensions of the rough ER where the connection with rough ER was in a different plane of section. To make certain that these vesicles were not part of the endocytic pathway, we incubated class 2 mutant cells in the presence of colloidal gold to delineate endocytic vesicles that participated in bulk-phase uptake and then processed the cells for receptor localization. As seen in Fig. 8 *D*, the vesi-

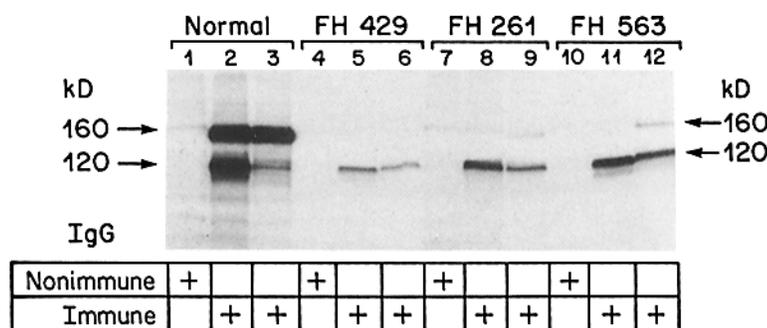|	Normal			FH 429			FH 261			FH 563		
	1	2	3	4	5	6	7	8	9	10	11	12
Nonimmune	+			+			+			+		
Immune		+	+		+	+		+	+		+	+

Figure 5. Biosynthesis and processing of LDL receptors in fibroblasts from three different FH homozygotes with the class 2 mutation. On day 6 after incubation for 24 h in lipoprotein-deficient serum, cells from the indicated subject were pulse labeled for 60 min with 110 μCi/ml [³⁵S]methionine in methionine-free medium and either not chased (lanes *2, 5, 8,* and *11*) or chased for 2 h in medium containing 200 μM unlabeled methionine (lanes *1, 3, 4, 6, 7, 9, 10,* and *12*). Detergent-solubilized cell extracts were prepared and subjected to immunoprecipitation with either anti–LDL receptor mAb IgG-C7 (*Immune*) or an irrelevant mAb IgG-2001 (*Nonimmune*) as indicated. The immunoprecipitates were processed for SDS-PAGE and autoradiography. The gel was exposed to x-ray film for 18 h. The positions of migration of the receptor precursor (120 kD) and the mature receptor (160 kD) are indicated.
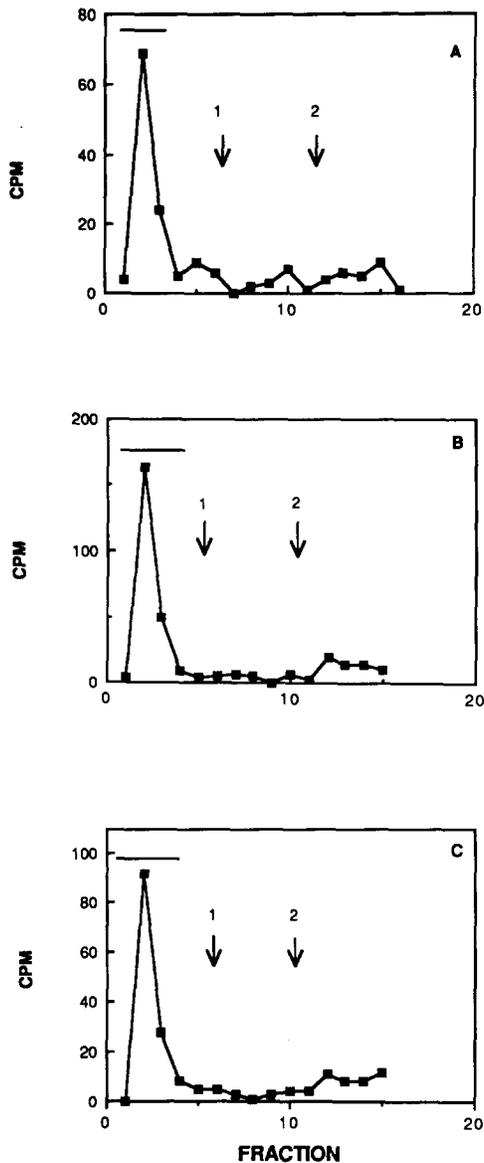
Figure 7. Descending paper chromatograms of [³H]glucosamine-labeled oligosaccharides released by NaOH/NaBH₄ from the class 2 mutant receptors. The [³H]glucosamine-labeled glycopeptides from the mutant receptors (*A*, FH 429; *B*, FH 563) were fractionated on Con A-Sepharose as described in Fig. 6. The glycopeptides not bound by the lectin were treated with NaOH/NaBH₄, and the released radioactivity was analyzed by descending paper chromatography as described in Materials and Methods. The migration of standards is indicated by the arrowheads and corresponds to the following: *1*, N,N′,N″,N‴,N‴′-[³H]pentaacetylchitopentaitol; *2*, N,N′,N″,N‴[³H]tetraacetylchitotetraitol; *3*, N,N′,N″[³H]triacetylchitotriitol; *4*, [³H]Galβ1,4GlcNacitol; *5*, N,N′[³H]diacetylchitobiitol; *6*, Calβ1,3[³H]GalNAcitol; *7*, [³H]GlcNAcitol; and *8*, [¹⁴C]GalNAcitol.

Figure 6. Chromatography on Con A-Sepharose of radiolabeled glycopeptides from the 120-kD LDL receptor precursor isolated from normal and two different FH homozygotes with the class 2 mutation. On day 6 after incubation for 24 h in lipoprotein-deficient serum, 10 dishes (60 mm) from a normal subject (*A*) and 35 dishes (60 mm) from an FH subject (*B*, FH 429 mutant; *C*, FH 563 mutant) were washed with PBS, preincubated for 1 h at 37°C in glucose-free DME supplemented with 10% lipoprotein-deficient serum, and then pulse labeled for either 20 min (*A*) or 2.5 h (*B* and *C*) with 200 μCi/ml of [³H]glucosamine. Detergent-solubilized cell extracts were prepared and immunoprecipitated with anti-LDL receptor mAb. The immunoprecipitates were subjected to electrophoresis in 7% SDS-polyacrylamide gels. LDL receptors were located by fluorography, excised from the gel, and digested with Pronase. The released [³H]glycopeptides from the antireceptor immunoprecipitates were chromatographed on Con A-Sepharose as described in Materials and Methods. As denoted by the arrows, 10 mM α-methyl glucoside (arrow *1*) or 100 mM β-methyl mannoside (arrow *2*) were applied to elute glycopeptides bound by the lectin. Portions of each fraction were removed and counted for radioactivity. Horizontal lines represent the fractions of the run-through material that were pooled for further analysis in Fig. 7.
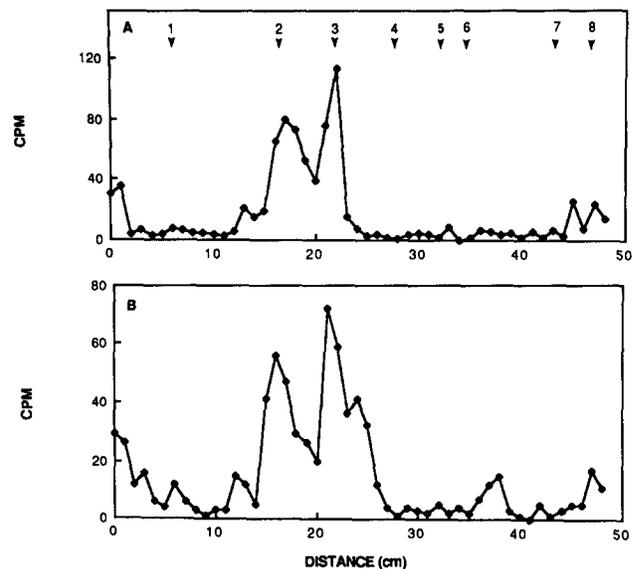
cles that contained gold particles did not contain LDL receptor reaction product (Fig. 8 *D*, *inset*).

We also analyzed the Golgi complex of the class 2 mutant cells to determine if any receptor became localized to this compartment. In all mutant cells examined, there was very little receptor in this compartment as compared with normal cells (Fig. 9, compare *A* with *B*).

Discussion

The current results establish the following new points about the intracellular distribution of the LDL receptor in normal human fibroblasts: (*a*) internalized LDL receptors can be visualized, together with their ligand, in irregularly shaped multivesicular bodies that resemble the structures previously defined as endosomes (Helenius et al., 1983), receptosomes (Pastan and Willingham, 1981), or compartment of uncoupling of receptor and ligand (Geuze et al., 1983); (*b*) within the multivesicular bodies the LDL receptors appear clustered in regions that have the appearance of budding vesicles, which may represent the morphologic counterpart of previously postulated recycling vesicles (Brown et al., 1983); and (*c*) some receptors were associated with the Golgi complex, but these appear to be newly synthesized receptors, since they disappear within 4 h after inhibition of receptor synthesis with cycloheximide.

The above pathway for recycling of the LDL receptor in

fibroblasts is similar to the previously described recycling pathways for the asialoglycoprotein receptor, the mannose-6-phosphate receptor, and the polymeric IgA receptor in hepatocytes (Geuze et al., 1983, 1984), and for the mannose-6-phosphate receptor in fibroblasts (Brown et al., 1986). Previous studies of the fibroblast endosome have revealed it to be a morphologically complex structure consisting of a central vacuole with many radiating tubular extensions (Marsh et al., 1986). The LDL receptor-rich vesicles that we observed in association with multivesicular bodies most likely correspond to cross-sectional profiles of these tubular extensions. These data suggest that once the LDL receptor reaches the endosome, it migrates into these tubules. These tubules give rise to vesicles that carry the receptor back to the cell surface.

In fibroblasts from FH patients previously identified as having the class 2 mutant phenotype at the LDL receptor locus, the receptor was found almost exclusively in the rough ER and in tubulo-vesicular structures that appeared to be smooth-surfaced extensions of the ER, although we cannot be sure that the peroxidase reaction product did not obscure ribosomes present on this membrane. We found no more than trace amounts of receptors in the Golgi complex, on the cell surface, in coated pits, or in endocytic vesicles (the latter defined as those structures that take up colloidal gold particles).

The LDL receptors produced by the class 2 mutant cells remain sensitive to endoglycosidase H (Tolleshaug et al., 1983), a finding that is consistent with the morphologic evidence that these receptors never reach the Golgi complex. However, these receptors also contain O-linked sugars (Figs. 6 and 7). The polypeptide, GalNAc transferase, that attaches the initial component of O-linked sugars to serine or threonine residues in rat liver and cultured human choriocarcinoma cells has been reported to be located in the Golgi complex, as determined by cell fractionation (Hanover et al., 1982; Abeijon and Hirschberg, 1987). The current ultrastructural data suggest that these core sugars are added to the LDL receptor in the ER of human fibroblasts. We cannot rule out the remote possibility that the class 2 mutant receptors move transiently to the Golgi complex where they undergo O-linked glycosylation, but not removal of mannose residues, and then return to the ER so rapidly that their presence is undetectable in the Golgi complex in the steady state. It is also possible that there are cell type-specific differences in the subcellular localization of these enzymes.

The composition of the O-linked sugars on the normal LDL receptor precursor and on the class 2 mutant precursors remains to be determined. In the current studies, the carbohydrates released from the mutant precursors had mobilities comparable to tri- and tetrasaccharides on paper chromatography (Fig. 7). Unfortunately, the small amounts of label incorporated made further characterization impossible. This question may be answered in the future by overexpressing normal and class 2 mutant receptors after transfection of the respective cDNAs into cultured cells.

In the mutant FH cells the density of HRP reaction product appeared to be greatest in the irregular extensions of the ER, which suggests that these extensions were the sites at which the movement of the receptor out of the ER was blocked. This portion of the ER is morphologically similar to the transitional zone of the ER that has been implicated in the trans-

port of secretory proteins to the Golgi apparatus (Palade, 1975). This ER in class 2 mutant fibroblasts has the same morphology as in fibroblasts that do not synthesize an LDL receptor (compare SER in Fig. 8 A with SER in Fig. 2), indicating that it was not hypertrophied in the mutant cells producing an abnormal LDL receptor. This observation is in contrast to the hypertrophied transitional elements seen in cells that were producing high levels of the growth hormone–influenza hemagglutinin fusion protein (Rizzolo et al., 1985). This difference was probably related to the fact that mutant fibroblasts produce only small amounts of LDL receptors, which can apparently accumulate within this region of the ER without causing it to expand.

Considered together, the available data suggest that the ER contains some mechanism for distinguishing between native and denatured proteins and that only native protein structures can migrate to the Golgi complex. Further studies should reveal the nature of the mechanism that makes this distinction.

The mutations in the LDL receptor that occur in FH are the only naturally occurring mutations in a cell surface receptor that have been elucidated to date. It is likely that similar mutations exist in genes for other receptors and that these mutations will produce disease by mechanisms similar to those identified for the LDL receptor. Unfortunately, cell surface receptors are generally present in only trace amounts within cells, thus making morphological studies of naturally occurring mutant receptors much more difficult than that of model experimental systems, such as viral envelope proteins. Despite these technical difficulties, with highly sensitive immunocytochemical methods it is possible to localize mutant receptors in intracellular organelles, and the current results establish that a major site of blockage in the transport of mutant cell surface receptors lies in the ER. It is likely that other mutant receptors will show a similar defect.

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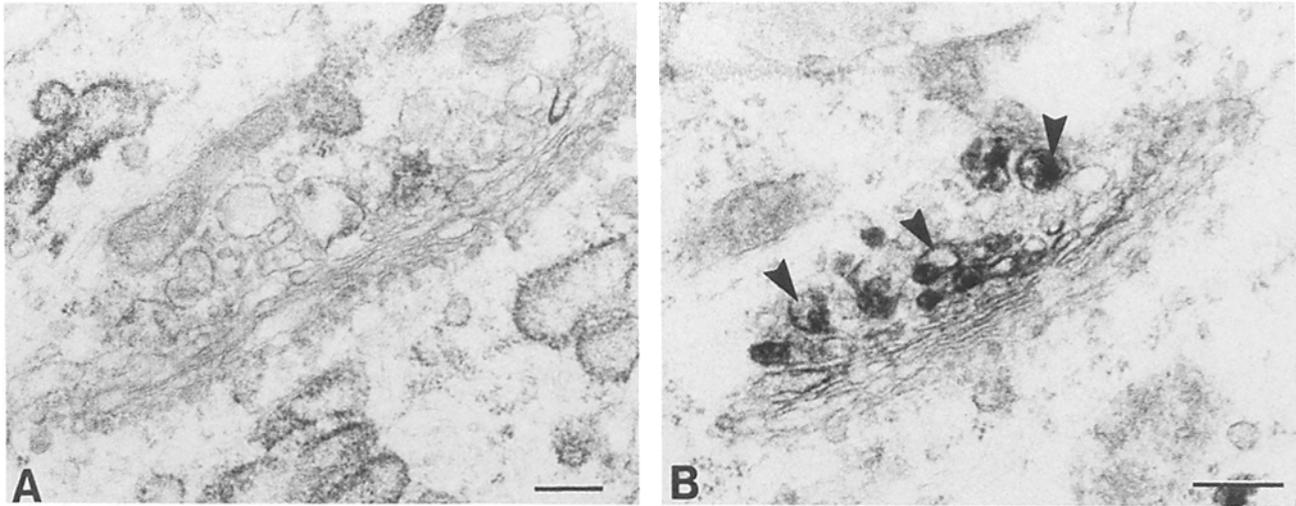Brown, M. S., R. G. W. Anderson, S. K. Basu, and J. L. Goldstein. 1981.
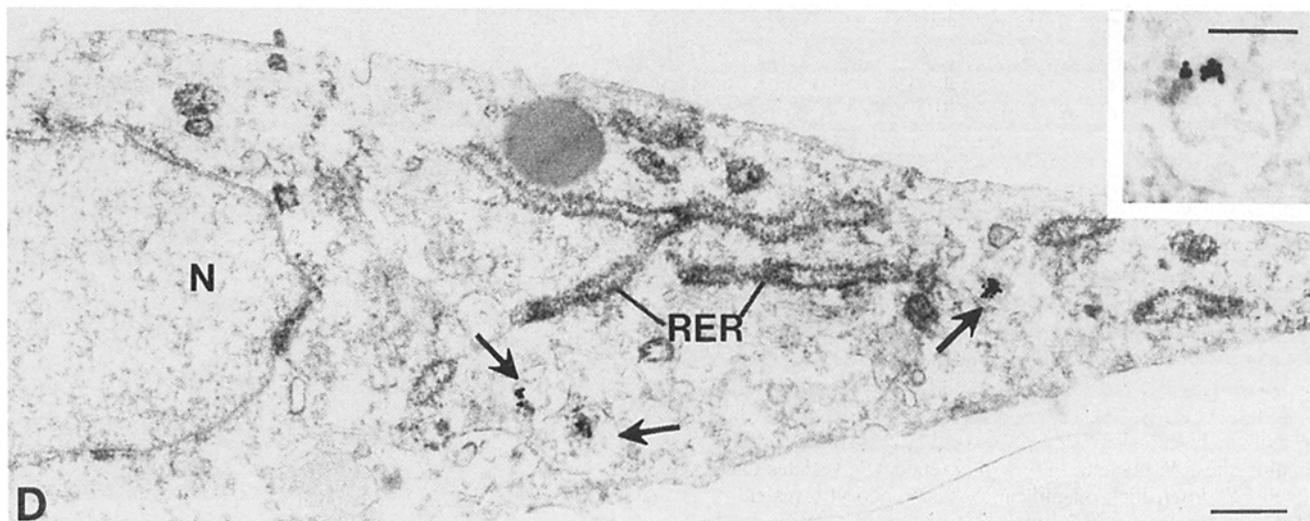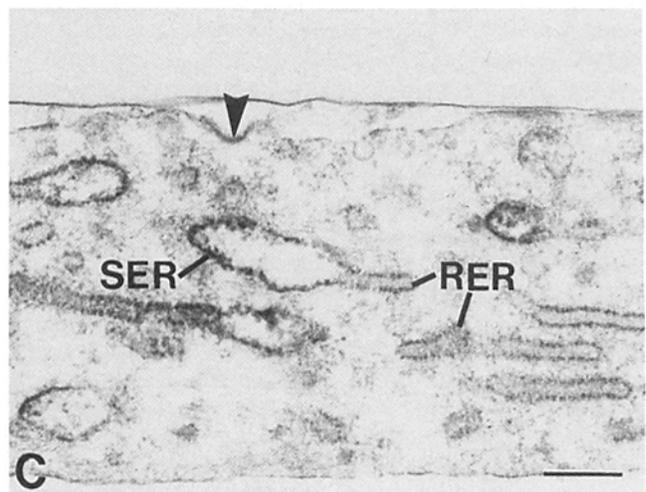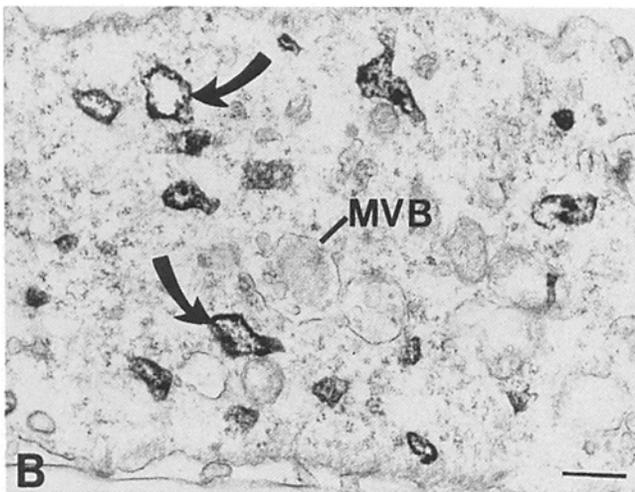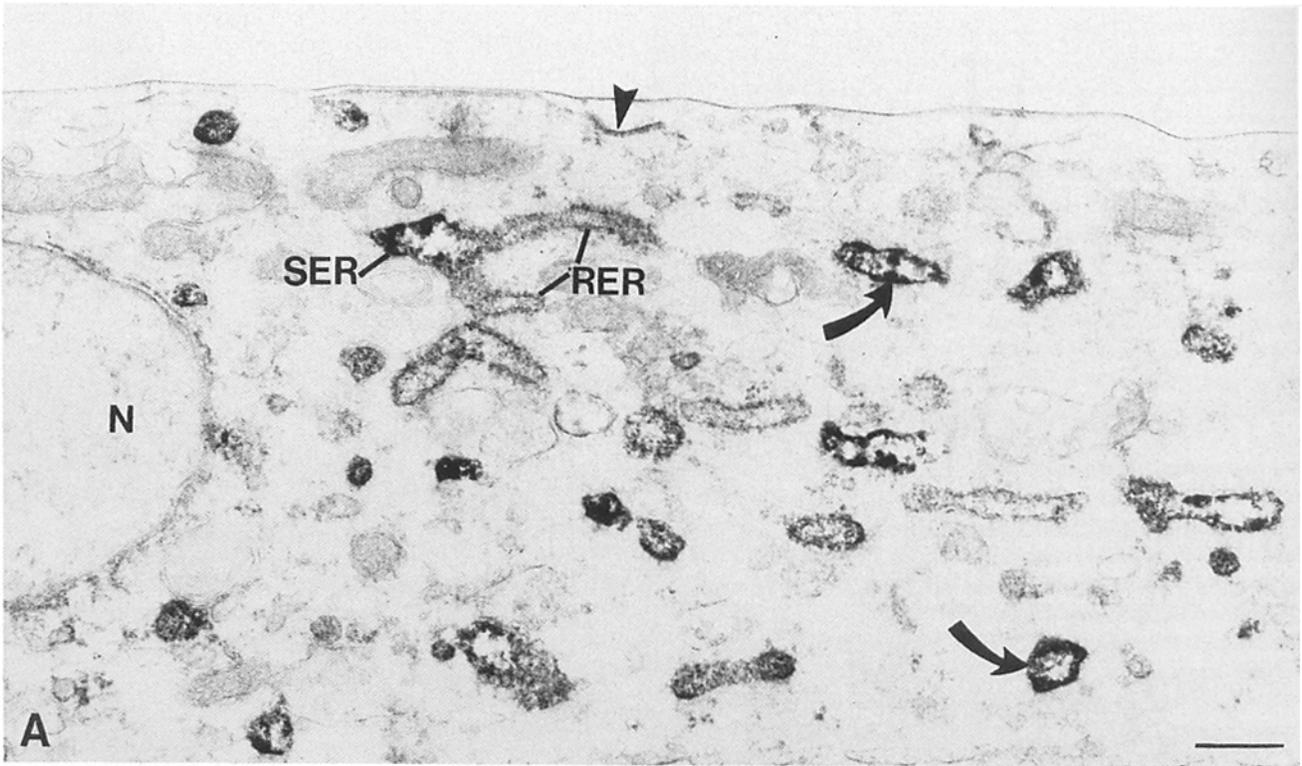
Figure 9. Distribution of anti-LDL binding sites in the Golgi complex of normal (*B*) and class 2 mutant human fibroblasts (*A*). Monolayers of normal (*B*) and FH 429 (*A*) fibroblasts were fixed and processed to localize LDL receptors by the ABC immunoperoxidase procedure. *Arrowheads*, Golgi vesicles and cisternae. Bar, 0.3 μm.

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Figure 8. Distribution of anti-LDL receptor IgG binding sites in class 2 mutant fibroblasts. Monolayers of fibroblasts from three different FH homozygotes with the class 2 mutation—FH 429 (*A*), FH 261 (*B*), FH 563 (*C*)—were fixed and processed for the localization of LDL receptors using the ABC immunoperoxidase procedure. A second set of FH 429 cells was incubated for 45 min at 37°C with a 1:40 dilution of colloidal gold particles before processing for localization of LDL receptors (*D*). (*A–C*) *Arrowheads*, coated pits; *RER*, rough ER; *SER*, irregular extensions of the rough ER; *MVB*, multivesicular bodies; *arrows*, irregularly shaped vesicles that contain anti-LDL receptor IgG binding sites; *N*, nucleus, (*D*) *Arrows*, endocytic vesicles that contain colloidal gold but not anti-LDL receptor IgG binding sites; *RER*, rough ER; *inset*, high magnification of an endocytic vesicle that contains colloidal gold but not anti-LDL receptor binding sites. Bars: (*A–D*) 0.4 μm; (*inset*) 0.2 μm.