Localizing the N-terminal Domain of the Low Density Lipoprotein Receptor*

The low density lipoprotein (LDL) receptor binds cholesterol-rich lipoproteins for receptor-mediated endocytosis and plays a key role in the regulation of cholesterol metabolism. At the N terminus, the LDL receptor has modular cysteine-rich repeats in both the ligand binding domain and the epidermal growth factor (EGF) precursor homology domain. Each repeat contains six disulfide-bonded cysteine residues, and this structural motif has also been found in many other proteins. The bovine LDL receptor has been purified and reconstituted into egg yolk phosphatidylecholine vesicle bilayers. Using gel electrophoresis and cryoelectron microscopy (cryoEM), the ability of the reconstituted LDL receptor to bind its ligand LDL has been demonstrated. After reduction of the disulfide-bonds in the N-terminal domain of the receptor, the reduced LDL receptor was visualized using cryoEM; reduced LDL receptors showed images with a diffuse density region at the distal end of the extracellular domain. Gold labeling of the reduced cysteine residues was achieved with monomaleimido-Nanogold, and the bound Nanogold was visualized in cryoEM images of the reduced, gold-labeled receptor. Multiple gold particles were observed in the diffuse density region at the distal end of the receptor. Thus, the location of the ligand binding domain of the LDL receptor has been determined, and a model is suggested for the arrangement of the seven cysteine-rich repeats of the ligand binding domain and two EGF-like cysteine-rich repeats of the EGF precursor homology domain.

The low density lipoprotein (LDL) receptor binds cholesterol-rich lipoproteins for receptor-mediated endocytosis and plays a key role in the regulation of cholesterol metabolism. This 115-kDa receptor has a mosaic structure containing five distinguishable domains based originally on the sequence of the human LDL receptor (1); the sequences of LDL receptor from other species also suggest the same domain organization (2–7). These domains, beginning at the N terminus are (i) ligand binding domain, (ii) epidermal growth factor (EGF) precursor homology domain, (iii) O-linked sugar domain, (iv) transmembrane domain, and (v) cytoplasmic domain. The ligand binding domain has the characteristic 7 cysteine-rich repeats consisting of a highly homologous 40-residue amino acid sequence. A study using mutated receptors found that different combinations of the cysteine-rich repeats was responsible for binding to LDL or \( \beta \)-migrating very low density lipoprotein (\( \beta \)-VLDL), both ligands for the LDL receptor (8). Mutations deleting one of the third to the seventh repeats in the ligand binding domain of the LDL receptor resulted in a marked reduction in LDL binding. \( \beta \)-VLDL binding to the LDL receptor was decreased only by deletion of the fifth cysteine-rich repeat in the ligand binding domain. LDL contains a single high molecular mass (550 kDa) protein, apoB\(_{100}\) (9), whereas \( \beta \)-VLDL has multiple copies of the smaller (33 kDa) apoE plus one molecule of apoB\(_{100}\) or apoB\(_{48}\) (10). It is thought that the receptor binding domain of apoB\(_{100}\) of LDL may require several of the cysteine-rich repeat modules for efficient binding, whereas the presence of multiple small apoE copies in \( \beta \)-VLDL may allow it to contact with other cysteine-rich repeats of the LDL receptor even when a repeat is deleted.

The EGF precursor homology domain has three cysteine-rich “growth factor-like” repeats, two at its N terminus (adjacent to the ligand binding domain) and one at its C terminus (next to the O-glycosylation domain). Deletion analysis in this region of the LDL receptor suggested that the EGF precursor homology domain is required for both efficient binding of LDL and for the acid-dependent conformational change that allows the LDL receptor to release its bound ligand after acidification of the endocytic vesicle (11). However, the LDL receptor with these deletions was able to bind \( \beta \)-VLDL and to internalize it at a normal rate even though the receptor-recycling rate was slowed. Similar growth factor-like repeats have been identified in many proteins; cell surface receptors, viral proteins, factor IX and X of the blood clotting system, growth factors, and developmental proteins of lower eukaryotes (12–18). Structural studies of the growth factor-like repeats in different proteins have provided convincing evidence that these cysteine-rich repeat modules are engaged in protein-protein interactions (19).

Recently, the atomic resolution structures of the 40-residue cysteine-rich repeats of both the ligand binding and EGF precursor homology domains of the LDL receptor have been determined by NMR spectroscopy and x-ray crystallography. These include the structures of cysteine-rich repeats 1, 2, and 5 in the ligand binding domain (20–22) and the structures of EGF-like modules corresponding to the EGF-like repeats in the EGF precursor homology domain (19, 23, 24). The crystal structure of the fifth cysteine-rich repeat in the ligand binding domain revealed a hydrophobic ligand binding surface with a well coordinated calcium-binding site (22). The disulfide bond linkages were between Cys(I)-Cys(III), Cys(II)-Cys(V), and Cys(IV)-

Received for publication, March 27, 2000, and in revised form, June 30, 2000
Published, JBC Papers in Press, July 10, 2000, DOI 10.1074/jbc.M002582200
Cys(VI), an identical pattern to that reported for the first and the second repeats (20, 21). The structure of repeat 5 explained why calcium ion is essential for a proper folding and disulfide bond formation of this repeat; furthermore, a sensible rationale for the calcium requirement in ligand binding (25) was proposed (26). The structure of a concatamer of repeats 1 and 2 suggests that little or no intermolecular interactions occur between the two modules (27), and a recent study of the concatamer of repeats 5 and 6 again indicated that each module is structurally independent of the other (28).

The human EGF is a 53-residue peptide hormone that has a sequence similarity with an EGF-like cysteine-rich repeat in the EGF precursor homology domain of the LDL receptor. The human EGF contains three disulfide-bond cross-links between Cys(I)-Cys(III), Cys(II)-Cys(IV), and Cys(V)-Cys(VI) in a conserved order for disulfide-bonds in the EGF-like modules (29). The structure of the EGF-like module in blood clotting factor X suggested that the calcium-binding site maintains the conformation of the N-terminal region and mediates protein-protein interactions (19).

In the accompanying paper (31), stick-like, bent stick-like, and Y-shaped images of frozen, hydrated reconstituted LDL receptors have been observed using cryoelectron microscopy (cryoEM). In this paper, we use reduction of the disulfide linkages, maleimide-Nanogold labeling of the now available cysteine residues, and cryoEM to locate the cysteine-rich ligand binding domain.

**EXPERIMENTAL PROCEDURES**

**Purification and Reconstitution**—Purification of the LDL receptor from bovine adrenal cortices and reconstitution into egg yolk phosphatidylcholine (EYPC) vesicles followed the same procedures as reported previously (31).

**LDL Binding**—Human LDL was purified by sequential ultracentrifugation from the plasma of normal donors (32). LDL between the density 1.025 and 1.050 g/ml was used for the ligand binding assays. The LDL protein concentration was determined (33) using bovine serum albumin solution as a standard. To test LDL binding to the detergent-solubilized receptor, three samples, with LDL to LDL receptor molar ratios of 3:1, 6:1, and 12:1, were incubated for 1 h at room temperature then applied to native gel electrophoresis. Samples were run on 7.5% homogeneous gel under non-reducing conditions using modified Laemmli buffer system with no SDS (34). The gel was transferred to a nitrocellulose membrane electrophoretically and blotted with monoclonal antibodies, IgG-C7 and IgG-4A4, against the N and C termini, respectively.

**Reduction of Disulfide Bonds and Nanogold Labeling**—2-Mercaptoethanol chloride (MEA) was used to reduce disulfide bonds in the LDL receptor. MEA was added to produce final concentrations of 0, 5, 15, and 30 mM in the reconstituted LDL receptor solution at concentrations of 0.1 mg/ml LDL receptor and 1 mg/ml EYPC. Each solution was incubated for 1 h at room temperature then transferred into the microdialyzer wells and dialyzed against ~1 liter of dialysis buffer (50 mM HEPES, 2 mM CaCl$_2$, pH 6.15) overnight to remove excess reducing agent. Monomaleimido-Nanogold was solubilized with 20 µl of dimethyl sulfoxide or isopropanol, then with 180 µl of deionized water. Immediately after solubilization, 10 µl of the solution was added to the reduced LDL receptor solution and incubated at 4°C for approximately 18 h. Unbound gold particles were removed by microdialysis against 2 liters of dialysis buffer at 4°C for 48 h. Four 7.5% homogeneous gels were run simultaneously with identical samples under non-reducing conditions. Each gel was applied to LI silver stain, homogeneous gels were run simultaneously with identical samples under non-reducing conditions. Each gel was applied to LI silver stain, and Western blot with both N-terminal and C-terminal antibodies.

**Cryoelectron Microscopy**—The specimens for cryoEM were prepared and visualized as described previously (31).

**RESULTS**

**LDL Binding**—The LDL binding assay using detergent-solubilized LDL receptor was performed using native gel electrophoresis before cryoEM visualization of LDL-bound reconstituted receptors. The LDL receptor showed two bands for each lane in the native gel, probably indicating monomer and dimer forms of the receptor under these conditions (Fig. 1). The N-terminal IgG-C7 and the C-terminal IgG-4A4 antibodies detected both monomer and dimer bands in the presence and the absence of calcium ions. In the presence of calcium, the bands blotted with the N-terminal antibody showed less of both the monomeric and dimeric LDL receptors in the presence of LDL, as the bound LDL-receptor complex remained on the top of the gel. The intensity of the band corresponding to the dimer form decreased proportionally as the LDL:LDL receptor ratio was increased in the presence of calcium ions. A similar result, where the intensity of the dimeric LDL receptor decreased at higher LDL:LDL receptor ratios, was observed on the Western blot detected by the C-terminal antibody. Although there was less monomer form in the presence of LDL, the intensity of the monomer band appeared to be independent of the LDL concentration. In the absence of calcium ions, the LDL:LDL receptor ratio did not seem to affect the intensity of the LDL receptor bands corresponding to either the monomeric or dimeric form. This in turn suggests, as expected, that little or no LDL binding occurred in the absence of calcium.

The EYPC-reconstituted LDL receptor incubated with the purified human LDL was visualized by cryoEM. The LDL:LDL receptor molar ratio was adjusted to approximately 1/15 for optimal visualization by cryoEM. The resulting cryoEM images showed clearly that in the presence of calcium LDL particles bound to the outer surface of the LDL receptor-containing vesicles (Fig. 2B). Occasionally, images showed stick-like density linking the vesicle membrane to the bound LDL; this presumably represents part of the reconstituted LDL receptor (31). In the absence of calcium, the LDL receptors reconstituted into vesicles showed no interaction with LDL, even though the vesicles showed evidence of LDL receptor molecules extending from the bilayer surface (data not shown). Control EYPC vesicles lacking the LDL receptor, following incubation with LDL under the same conditions described above, did not show any significant interactions with the LDL particles (Fig. 2A). Again, these cryoEM images provide direct visual evidence that the reconstituted LDL receptor is functional and, in the presence of calcium, can bind its ligand, LDL.

**Reduction of Disulfide Bonds and Nanogold Labeling of the Reconstituted LDL Receptor**—To localize the ligand binding domain of the LDL receptor, reduction of the disulfide bonds by MEA was examined. The cysteine sulfhydryl groups in this domain could be labeled by monomaleimido-Nanogold if they were made accessible by reduction of disulfide bonds. The mild reducing reagent, MEA, was selected for the reductive cleavage of the disulfide cross-links. In preliminary studies using MEA...
concentrations up to 70 mM, the maximal concentration of MEA for optimal reduction of the LDL receptor was determined to 30 mM (data not shown).

The LDL receptor reconstituted into EYPC vesicles was incubated with MEA at final concentrations of 0, 5, 15, and 30 mM. The reaction mixtures were dialyzed to remove extra reducing agent present in the solutions. Aliquots of these samples were taken to prepare cryoEM specimens of reduced unlabeled LDL receptor. The remaining samples were incubated with Nanogold then dialyzed again to eliminate unbound Nanogold particles. The reduced Nanogold-labeled receptor was analyzed by SDS-PAGE to confirm the labeling. The LDL receptor was detected as a dark band in all lanes of silver stained gel for each of the reducing agent concentrations tested (Fig. 3A). There was no significant difference between lanes other than that the Nanogold-containing samples showed stronger bands due to the presence of gold. Detecting gold-labeled receptor after reduction with LI silver stain demonstrated the presence of gold-containing bands in all lanes for Nanogold-containing samples (Fig. 3B). The higher the concentration of reducing agent, the weaker the observed receptor band was in Western blots using the two antibodies against the N and C termini (Fig. 3C). However, both antibodies could detect some LDL receptor even after reduction with 30 mM MEA.

CryoElectron Microscopy—Images of the reconstituted LDL receptor after incubating with reducing agent were obtained. After reduction with 5 mM MEA, there seemed to be no change in the observed images compared with control, unreduced LDL receptor (data not shown). In contrast, the LDL receptor images at reducing agent concentrations of 15 and 30 mM showed a large diffuse density region at the end of the stick-like projections from the vesicle surface, although each image was unique (Fig. 4). The additional diffuse density that appeared at the distal end of the LDL receptor was attributed to the effect of the reducing agent. Presumably, disulfide reduction produces an altered, more extended receptor structure, particularly in the cysteine-rich ligand binding domain and is responsible for the observed diffuse density at the distal end of the receptor.

For the reduced and Nanogold-labeled LDL receptors, cryoEM images were recorded at two different defocus values, −1.7 μm, optimal for visualization of protein, and −0.5 μm, optimal for gold. These images of the reduced, gold-labeled LDL receptor also showed additional density at the end of the extracellular domain (Fig. 5). The receptors visualized for optimization of Nanogold displayed multiple gold particles in the diffuse density region. The number of bound Nanogold particles, their arrangement, and the receptor image were different for each imaged receptor. Presumably, this variability resulted from individual variations in the number of disulfide bonds.
reduced in the N-terminal domain of each receptor. The reduced Nanogold-labeled receptors confirmed that the distal region, showing diffuse density, is the N-terminal cysteine-rich domain.

**DISCUSSION**

The functionality of the LDL receptor was confirmed by binding studies to its major ligand, LDL. It has been shown that human LDL binds to the bovine LDL receptor as well as the human LDL receptor (35), and for the region that can be compared (the C-terminal ~25%), their amino acid sequences exhibit very high homology (36). This similarity of the human and bovine LDL receptors validates our use of the human LDL for binding studies with the bovine LDL receptor. The detergent-solubilized receptor and the reconstituted receptor showed LDL binding using native gel electrophoresis (Fig. 1) and cryoEM (Fig. 2). The existence of some dimer form seen in the native gels supports the earlier report of receptor dimerization (37). The dimer band of the LDL receptor shown by Western blot analysis using the N-terminal antibody seemed to correspond (the C-terminal ~25%) for optimal visualization of gold. For the panels 1–6 at ~1.7-μm defocus, the arrow points to the reduced, Nanogold-labeled LDL receptor.

![CryoEM images of the reduced, Nanogold-labeled LDL receptor preserved in vitreous ice. Micrographs were recorded as defocus pairs, −1.7 μm defocus (number 1–6) for optimal visualization of protein and −0.5 μm defocus (1′–6′) for optimal visualization of gold. For the panels 1–6 at ~1.7-μm defocus, the arrow points to the reduced, Nanogold-labeled LDL receptor.](image)

Fig. 5.

Images obtained by cryoEM showed bound LDL particles at the surface of LDL receptor-containing vesicles. Many individual LDL particles were bound to the outside surface of the receptor-containing vesicles, and in some images, the stick-like extracellular domain of the LDL receptor was visible, extending from the membrane surface to the bound LDL particle. Further studies using detergent-solubilized LDL receptor (or expressed LDL receptor domains) may eventually be used to map the location on LDL where the receptor binds; a similar approach has been used to detect antibody binding sites on the surface of LDL (38).

The successful application of monomaleimido-Nanogold labeling methods in our structural studies of the LDL receptor (31) encouraged us to use additional cysteine residues for further labeling, particularly those of the cysteine-rich N-terminal region. The reduction of these disulfide bonds was performed carefully so as not to perturb the whole structure of the LDL receptor. The LDL receptor bands that were detected in LI silver stain, silver stain, and Western blot analyses after MEA disulfide reduction were normal in appearance. Images of the reduced LDL receptor obtained by cryoEM showed more diffuse density at the end of the extracellular domain. Some images of the reduced LDL receptor still had visible stick-like or Y-shaped density as the previously reported images of the reconstituted receptor (31). The distal end of both the stick-like receptor and the Y-shaped receptor images showed additional diffuse density.

CryoEM of reduced, Nanogold-labeled LDL receptor localized the N-terminal cysteine-rich repeats to the distal end of the observed receptor images. There were significant differences in the number of Nanogold particles bound in this distal region, presumably reflecting differences in the number of accessible free cysteine residues and their Nanogold labeling. It seems reasonable to conclude that this distal domain of the imaged LDL receptor contains the seven cysteine-rich repeats of the ligand binding domain plus the two N-terminal cysteine-rich repeats of the EGF precursor homology domain.

Studies of repeat motifs common to the human LDL receptor, human EGF, and other proteins have provided structural information relevant to our studies of the bovine LDL receptor. The seven cysteine-rich repeats in the ligand binding domain of the human LDL receptor sequence are connected to each other with 4-residue linker sequences, with the exception of a 12-residue sequence connecting the fourth and the fifth repeats. Two EGF-like cysteine-rich repeats of the EGF precursor homology domain closely follow the seventh repeat of the ligand binding domain. This proximity in the sequence and the proposed function of the EGF-like module in protein-protein interactions suggests that both EGF-like repeats are located near the ligand binding domain at the N terminus of the LDL receptor. The spacer sequence of the EGF precursor homology domain occupies about 70% of this 400-amino acid domain and connects the second and the third cysteine-rich EGF-like repeats. The relatively short (58 amino acids) O-linked sugar domain has been suggested to function as a strut, extending from the membrane surface (36), and it seems reasonable that the major part of the EGF precursor homology domain essentially extends this strut to the cysteine-rich domain.

In Fig. 6 we suggest possible arrangements, particularly for the cysteine-rich repeat modules of both the ligand binding domain and the EGF precursor homology domain, consistent with the observed cryoEM images of both unlabeled and gold-labeled LDL receptor. Two issues led us to the proposed (2 + 5)-type and (4 + 3)-type arrangements of the 7 cysteine-rich repeat modules of the ligand binding domain. First, for the (2 + 5) type, binding of LDL was severely affected by mutations in repeats 3 to 7, which might imply that the first and the second repeats were located away from the binding site for the LDL (see Fig. 6A). Second, for the (4 + 3) type, the presence of a longer linker sequence between repeats 4 and 5 suggests that a turn may occur after the fourth repeat in the ligand binding domain (see Fig. 6B). Since the biological function of the repeated EGF-like modules in protein-protein interaction is significantly calcium-dependent, it has been suggested that an adjacent pair of this module could act as a functional unit (39). Therefore, the two EGF-like repeats were positioned as a pair...
in the model (see Fig. 6, A and B). Finally, we have suggested possible arrangements for dimeric assemblies of the LDL receptor based on these (2 + 5) and (4 + 3) structural motifs (Fig. 6, right).

In summary, the LDL receptor is a single chain membrane receptor that requires detergents or lipids for solubilization. This characteristic of the LDL receptor has made it difficult to investigate its detailed structure by x-ray crystallography. Our cryoEM approach has provided low resolution images of the full-length bovine LDL receptor (31), and in this study we have used the gold labeling approach to determine the location of the cysteine-rich ligand binding domain. However, more detailed structural information must await improvements in specimen preparation for cryoEM and the crystallization of progressively larger extracellular domains of the LDL receptor (40, 41).

Acknowledgments—We thank Drs. David Atkinson, Esther Bullitt, Kumkum Saxena, and Christine Woldin for helpful advice. Technical assistance was provided by Ann Tercyak, Cheryl England, Michael Gigliotti, Cynthia Curry, and Donald Gantz. Also, we thank Drs. Michael S. Brown and Joseph L. Goldstein (University of Texas-Southwestern Medical Center, Dallas, TX) for providing us with the monoclonal antibody IgG-4A4.

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FIG. 6. Schematic representation of the LDL receptor and its N-terminal cysteine-rich repeat domains. The (2 + 5) and (4 + 3) models represent different arrangements of the 7 cysteine-rich repeats (numbered 1–7) of the ligand binding domain of the LDL receptor.
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