The low density lipoprotein (LDL) receptor plays a key role in cholesterol homeostasis, mediating cellular uptake of lipoprotein particles by high affinity binding to its ligands, apolipoprotein (apo) B-100 and apoE. The ligand-binding domain of the LDL receptor contains 7 cysteine-rich repeats of approximately 40 amino acids; each repeat contains 6 cysteines, which form 3 intra-repeat disulfide bonds. As a first step toward determining the structure of the LDL receptor, both free and bound to its ligands, we produced in *Escherichia coli* a soluble fragment containing the ligand-binding domain (residues 1–292) as a thrombin-cleavable, heat-stable thioredoxin fusion. Modest amounts (5 mg/liter) of partially purified but inactive fragment were obtained after cell lysis, heat treatment, thrombin cleavage, and gel filtration under denaturing conditions. We were able to refold the receptor fragment to an active conformation with approximately 10% efficiency. The active fragment was isolated and purified with an LDL affinity column. The refolded receptor fragment was homogeneous, as determined by sodium dodecyl sulfate or non-denaturing polyacrylamide gel electrophoresis and isoelectric focusing. The purified fragment did not react with fluorescein-5-maleimide, indicating that all 42 cysteines were disulfide linked. In addition, the refolded fragment exhibited properties identical to those of the intact native receptor: Ca\(^{2+}\)-dependent binding and isoform-dependent apoE binding (apoE2 binding <5% of apoE3). Furthermore, antibodies to the fragment recognized native receptors and inhibited the binding of \(^{125}\)I-LDL to fibroblast LDL receptors. We conclude that we have produced a properly folded and fully active receptor fragment that can be used for further structural studies.

The low density lipoprotein (LDL)\(^1\) receptor plays a central role in lipid metabolism by mediating the binding and uptake of plasma lipoprotein particles that serve to transport cholesterol and other lipids throughout the body [1]. This binding is dependent upon a high affinity interaction between the LDL receptor and its protein ligands, apolipoprotein (apo) E and apoB-100, which are present on the surface of plasma lipoprotein particles. Mutations in the LDL receptor gene that decrease or eliminate ligand binding have been linked to a genetic disorder, familial hypercholesterolemia, which results in a significant elevation of plasma LDL cholesterol concentrations, severe atherosclerosis, and premature death [2, 3].

In recent years, a number of structurally related receptors, all of which mediate the binding and endocytosis of multiple ligands, have been identified. These receptors, which include the LDL receptor-related protein or \(\alpha_2\)-macroglobulin receptor [4, 5], the very low density lipoprotein receptor [6], the apoE receptor 2 [7], and the renal glycoprotein gp330 [8], constitute a superfamily. The cardinal structural feature of this superfamily is the presence of various numbers of cysteine-rich repeats approximately 40 amino acids in length. Each repeat contains 6 cysteines whose positions within the repeats are stringently conserved. The LDL receptor contains 7 such repeats, clustered at the amino terminus, which together constitute the ligand-binding domain of the receptor [9–11]. Each repeat also features a conserved cluster of negatively charged residues (Ser-Asp-Glu) located between the 5th and 6th cysteines in each repeat, which are required for binding of apoE and apoB-100 although the contributions of individual repeats appear to be quite variable [11]. These clusters are thought to interact with corresponding clusters of positively charged residues in the receptor binding regions of apoE and apoB-100 [12]. In the case of apoE, the receptor binding region lies in the vicinity of residues 136–150, a region enriched in positively charged residues [12, 13]. The x-ray structure of a fragment of apoE containing the receptor binding region is an anti-parallel four-helix bundle [14]. The receptor binding region lies on the face of the fourth helix in the bundle. The binding region in apoB-100 has been suggested to be located in the vicinity of residues 3359–3369, a sequence similar to the receptor binding region of apoE [15].

To understand the interaction of apoE with the LDL receptor at the molecular level, it will be necessary to determine the structure of the ligand-binding domain of the receptor and, ultimately, the structure of a complex of the receptor and apoE. Although the individual structures of the first 2 of the 7 ligand-binding repeats have been determined by nuclear magnetic resonance [16, 17], this information provides little insight into either the overall structure of the ligand-binding domain of the receptor or the details of its interaction with apoE. As a first step in delineating this interaction, we have expressed and purified from *Escherichia coli* the entire 292-amino acid ligand-binding domain of the human LDL receptor, including all 7

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\(^1\) The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein; PAGE, polyacrylamide gel electrophoresis; DMPC, dimyristoyl phosphatidylcholine.

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cysteine-rich repeats, with 42 cysteine residues that likely form 21 intra-repeat disulfide bonds. This fragment has been successfully refolded and reoxidized to yield a homogeneous and active preparation that displays all the properties and specificities of the native intact receptor.

**EXPERIMENTAL PROCEDURES**

**Vector Construction**—The plasmid pTZ1, containing the complete human LDL receptor sequence, was the kind gift of Dr. David Russell, University of Texas Southwest Medical Center. A fragment coding for residues 1–292 was amplified by reaction and ligated into pET32a (Novagen), a third expression vector. The resulting plasmid, pET32-292, was further modified by polymerase chain reaction mutagenesis to generate a thrombin-cleavage consensus sequence immediately upstream of residue 1 of the receptor. This plasmid, pET32NT-292, was used in all subsequent expression studies. Pfu polymerase (Stratagene) was used for all polymerase chain reactions. All constructs were verified by DNA sequencing (18).

**Expression and Purification**—For expression of the receptor fusion, pET32NT-292 was transformed into the T7 expression strain BL21(DE3) (Novagen). Fresh transformants or frozen glycerol stocks were used to inoculate a 500-ml overnight culture in LB broth containing 100 μg/ml ampicillin (Sigma). The overnight culture was pelleted (15 min at 4000 rpm in a Beckman J6B centrifuge) and resuspended in 500 ml of fresh medium. 50 ml of this suspension was used to inoculate each of six 1-liter cultures in 2800-ml Fernbach flasks. The cultures were grown at 37 °C until midlog phase (A500 of approximately 0.6) and harvested by centrifugation at 4000 rpm for 20 min. The pellets were resuspended in 1% of the original volume of lysis buffer (25 mM Tris, pH 7.5, 2.5 mM EDTA) and lysed by sonication with a Branson 450 sonicator, and centrifuged at 18,000 × g for 20 min to remove cellular debris. The cleared lysate was transferred to a 400-ml glass beaker and incubated at 80 °C water bath for 7 min with constant agitation to precipitate heat-labile proteins. The precipitate was then removed by centrifugation for 20 min at 18,000 × g. The receptor fusion was then cleaved by incubation with thrombin (Hemotologic Technologies) overnight at room temperature at a weight ratio of 1:100. Guanidine hydrochloride (ICN) and β-mercaptoethanol (Sigma) were added to the cleaved mixture in final concentrations of 7 M and 1%, respectively. The mixture was incubated for 4 h at 4 °C, loaded onto a Sepharcl S-300HR (Pharmacia Biotech Inc.) column (4.8 × 120 cm, 1 M guanidine hydrochloride) and eluted with 4 M guanidine hydrochloride. 100 mM Tris, pH 7.4, 1 mM EDTA, and 0.1% β-mercaptoethanol. Fractions containing the receptor fragment were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) and pooled. Western blot analysis and isoelectric focusing were performed as described previously (19, 20). Protein concentrations were determined by the method of Lowry et al. (21). The presence of free –SH groups was determined by SDS-PAGE after the addition of 0.5 M of 20 mM fluorescein-5-maleimido dibromide in dimethylformamide (Molecular Probes, Eugene, OR) to protein samples in sample buffer, containing 2% SDS (22). The mixture was incubated for 15 min at room temperature and electrophoresed, and modifications were detected by UV irradiation on a transilluminator. Refolding, Isolation, and Affinity Chromatography—The purified receptor fragment was dialyzed against 4 M guanidine at 4 °C under argon to remove the β-mercaptoethanol. The receptor was refolded and oxidized by dilution to ~60 μM in refolding buffer (20 mM glycyglycine, pH 9.0, 20 mM CaCl₂, 10 mM cysteamine (Sigma), 1 mM cysteine (Fluka)), incubated overnight at room temperature, and dialyzed against 20 mM glycyglycine, pH 9.0, and 20 mM CaCl₂. The active 292 receptor fragment was isolated by affinity chromatography using LDL-Sepharose. Freshly prepared human LDL was coupled to cyanogen bromide-activated Sepharose 4B (30 mg of LDL/g of Sepharose) according to the manufacturer's instructions. After dialysis, refolded LDL receptor preparations were incubated with the LDL-Sepharose, previously equilibrated with 20 mM glycyglycine, pH 9.0, and 20 mM CaCl₂ for 4 h at 4 °C. After the LDL-Sepharose was washed with equilibration buffer, the active receptor fragment was eluted with 2 M NaCl and 20 mM CaCl₂.

**Plate Assay for Binding Activity**—Plasma LDL and apoE isoforms were isolated as described previously (23–25). As a positive control for receptor binding activity, a preparation of bovine LDL receptor isolated from adrenal glands was used (26). For LDL binding assays, LDL (500 ng/well) in 150 mM NaCl, 20 mM sodium phosphate, pH 7.4 (phosphate-buffered saline) was incubated overnight at 4 °C in 96-well microtiter plates. For apoE binding assays, apoE4, apoE3, and apoE2 (100 ng/well) were incubated as described above for LDL. The refolded and purified receptor (1 mg/ml) was diluted (1:10,000), added to the wells, and detected as described above. Determinations were performed in triplicate. In parallel wells without added receptor, an anti-apoE antibody was used for detection to ensure that the microtiter wells were coated with comparable amounts of each apoE isoform.

**Preparation of Antibodies and Tissue Culture**—Antiserum to the affinity-purified receptor was prepared in pathogen-free rabbits. The IgG fraction was prepared on a protein A cartridge (Bio-Rad) according to the manufacturer's instructions. Human fibroblast binding assays were performed with [125I]-LDL as described previously (27). To determine the ability of receptor fragment antibodies to compete with LDL for LDL receptors on fibroblasts, increasing concentrations of receptor fragment IgG were incubated with the fibroblasts for 1 h at 4 °C before addition of [125I]-LDL. Bound receptor was detected by incubation with an anti-human LDL antibody (Amersham) and color development with o-phenylenediamine dihydrochloride (Sigma) according to the manufacturer's instructions.

For apoE binding assays, apoE4, apoE3, and apoE2 (100 ng/well) were incubated as described above for LDL. The refolded and purified receptor (1 mg/ml) was diluted (1:10,000), added to the wells, and detected as described above. Determinations were performed in triplicate. In parallel wells without added receptor, an anti-apoE antibody was used for detection to ensure that the microtiter wells were coated with comparable amounts of each apoE isoform.

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**RESULTS AND DISCUSSION**

**Expression and Purification of a Soluble Fragment of the LDL Receptor**—The multidomain structure of the human LDL receptor, including the ligand-binding domain with its 7 cysteine-rich repeats is shown in Fig. 1. Since we long-term goal is to determine the interaction of apoE with the LDL receptor, our strategy was to express and purify a soluble fragment of the receptor that includes this domain for structural studies: residues 1–292. Following very limited success with expression in the yeast Pichia pastoris, we focused on expression in E. coli encouraged by reports of the successful expression and refold-
ing of the individual repeats 1 and 2 (16, 17). Although a variety of expression vectors failed to express detectable amounts of the receptor fragment, we were successful using a thioredoxin fusion system (28). This T7-driven expression cassette produced a soluble 52-kDa fusion protein in modest amounts (Fig. 2).

The fusion construct, as originally designed, coded for an enterokinase cleavage site upstream of the receptor fragment; however, the fusion protein was insoluble at the low pH levels required for enterokinase activity. Therefore, a thrombin cleavage consensus sequence (Leu-Val-Pro-Arg-Gly-Ser) was introduced immediately upstream of residue 1 of the receptor fragment by polymerase chain reaction mutagenesis. With this construct, complete cleavage of the fusion protein was obtained at pH 7.5 (Fig. 2). However, in addition to the thrombin site that we inserted, the fusion contains an additional site 34 amino acids upstream, generating a product only slightly (~3.6 kDa) larger than the receptor fragment itself. Because the latter site was preferentially cleaved, it was critical to monitor the progress of the reaction by SDS-PAGE to ensure complete cleavage of both sites. Thioredoxin-containing fusion proteins often retain one or both of its unique properties, heat stability and preferential release during osmotic shock, both of which are useful for purification (29). Although the receptor fragment fusion protein was not preferentially released by osmotic shock (data not shown), it turned out to be strikingly stable to heat denaturation. The fusion protein remained soluble at 80 °C for as long as 15 min (Fig. 2), whereas the majority of contaminating bacterial proteins precipitated, allowing us to use heat treatment as a major purification step. Using this method, we typically obtain approximately 10 mg of partially purified fusion protein per liter of cells in culture.

The partially purified fusion protein was then cleaved with thrombin, and the released receptor fragment was separated from thioredoxin by gel filtration under denaturing conditions (Fig. 2). Even after extended incubation with thrombin, there were no apparent cleavages within the receptor fragment. The accuracy of cleavage was confirmed by amino-terminal protein sequencing (data not shown), which showed the expected two amino acid amino-terminal extension (Gly-Ser) generated by thrombin cleavage within its recognition sequence. Although there was little LDL receptor binding activity associated with the purified fragment at this point (Fig. 3), comparison of reduced and non-reduced lanes showed some disulfide bond formation (Fig. 2). At this step, we routinely obtain 5–6 mg of the semipurified receptor fragment per liter.

Refolding and Final Purification—Initially, we concentrated the pooled fractions containing the receptor fragment from the gel filtration column to approximately 5–10 mg/ml before refolding to ensure low guanidine concentrations in the diluted refolding mixture. However, it was discovered that concentration promoted the formation of high molecular weight, cross-linked aggregates (data not shown). After later experiments demonstrated that guanidine concentrations of ~1 M could be tolerated in the diluted refolding mixtures, pooled fractions were diluted directly to approximately 60 µg/ml. The final refolding conditions were arrived at by screening an extensive set of conditions for their ability to generate LDL receptor binding activity as assessed using an LDL enzyme-linked immunosorbent assay. A variety of factors, including pH, temperature, buffers and their concentrations, additives, and time, were explored. Although the majority of the conditions that were assayed resulted in some degree of binding activity, several factors proved critical in generating high levels of the correct receptor species. The presence of Ca\(^{2+}\) at a high concentration (20 mM) was absolutely essential, as were a relatively high pH (>8.0) and the addition of a thiol exchange system. The importance of Ca\(^{2+}\) in the folding of the LDL receptor and repeat 5 was recently pointed out (30) and may result from its ability to stabilize folding intermediates (31). The cysteamine/cystamine thiol exchange system was more efficient than the more widely utilized reduced/oxidized glutathione system. This may be due to the smaller relative size of cysteamine and cystamine, which might allow better access to the interior of the relatively compact repeat structure. It is also possible that the positively charged amino group on cysteamine is more effective in the highly negatively charged environment that characterizes the receptor repeats.

Another key step was the removal of both the thiol exchange system and residual guanidine by dialysis. This step proved to be absolutely essential in generating a fully folded receptor fragment. It is possible that removal of the thiol exchange system and guanidine facilitates a final oxidative refolding step in which a critical disulfide link (or links) is formed, leading directly to the fully folded state. As shown in Fig. 2, this dialysis step produces a narrow compact band above the broad heterogeneous mixture of bands, which likely contain a number of bands...
of misfolded intermediates. This compact band turns out to be the fully folded and an active receptor fragment.

The final step in the isolation was affinity chromatography. The refolded mixture was applied to an LDL-Sepharose column, and as shown in Fig. 2, only the thin compact band was bound. This refolded receptor fragment could be eluted with either a salt or a pH gradient. At this point, the receptor fragment is approximately 99% pure. We estimate that approximately 10% of the starting material is successfully refolded. Our overall yield is approximately 0.5 mg of purified and refolded receptor fragment per liter of cells.

At various stages of purification, the receptor binding activity of the receptor fragment was monitored with an LDL plate assay. Little, if any, activity is present in the 80 °C supernatant or the thrombin-digested mixture (Fig. 3). A low level of activity is present following the gel filtration step, indicating that some refolding and reoxidation occurs in 4 M guanidine and 0.1% β-mercaptoethanol or occurs after dialysis. It is possible that this activity represents the presence of a significant fraction of partially folded and hence weakly active species, rather than a small amount of fully active receptor fragment. Western blot experiments with monoclonal antibody C7 (32, 33), which recognizes only correctly folded repeat 1, demonstrated that the majority of the receptor was partially folded even at this early stage, whereas no species migrating in the position of the fully folded receptor fragment could be detected on SDS gels (data not shown). Refolding led to a 10-fold increase in total binding activity, followed by another 10-fold increase in activity after the affinity step (Fig. 3). Taking the differences in molecular mass into account, the 292 receptor fragment has approximately the same level of activity as the full-length bovine receptor, as assessed in the LDL plate assay, suggesting the fragment has full binding activity. However, this comparison must be viewed as a qualitative rather than a quantitative argument given the potential for differences in both intrinsic activity and antibody recognition between the two species.

Analysis of the Refolded 292 Receptor Fragment—To assure that a homogeneous, correctly folded, and fully active fragment was produced, the fragment was subjected to a variety of analyses based on the known properties of the intact native receptor, the results of which are presented below.

Although the refolded receptor fragment appeared to be homogeneous on non-reducing SDS gels, it was important to establish that it does, in fact, represent a single molecular species. As shown in Fig. 4, the refolded fragment migrated as a single species on a non-denaturing polyacrylamide gel, as well as on an isoelectric focusing gel, providing strong evidence that it comprises a structurally homogeneous population. As expected from the amino acid composition, the pI of the receptor fragment is acidic (pI ~4.0).

The disulfide connectivities of the 6 cysteine residues in the independently folded repeats 1, 2, and 5 are the same in each repeat: Cys-1–Cys-3, Cys-2–Cys-5, and Cys-4–Cys-6 (16, 17, 30). Thus, it is reasonable to expect that the same disulfide connectivity would occur in all 7 repeats in the intact native receptor and that all 42 cysteine residues would be disulfide bonded with each repeat containing 3 intra-repeat disulfide bonds. Therefore, if the receptor fragment is properly folded, it should contain no free cysteine residues. To determine if this was the case, we used fluorescein-5-maleimide, a fluorescent cysteine-specific reagent that reacts with free –SH groups. As shown in Fig. 5, the refolded receptor fragment when modified under denaturing conditions did not fluoresce, indicating that it contains no detectable free –SH groups. For comparison, apoE3, which contains a single cysteine residue, was easily detected when loaded in an equimolar amount, whereas apoE4, which contains no cysteines, did not fluoresce, as expected. This experiment provides strong evidence that all of the cysteines in the refolded receptor fragment are disulfide bonded.

Another characteristic of the native LDL receptor is that it exhibits a Ca2+ requirement for high affinity binding to LDL (33). To test whether the receptor fragment also exhibited Ca2+-dependent binding, the binding activity of the receptor fragment in the presence and absence of Ca2+ was examined. The standard binding buffer for the plate assay contains 2 mM CaCl2, and the LDL binding of the fragment in this buffer was defined as 100%. As shown in Fig. 6, binding was significantly reduced if Ca2+ was not included in the binding buffer. Although no Ca2+ was added, some is likely to be receptor-bound because Ca2+ is present at a relatively high concentration in the refolding and affinity isolation procedures. This residual binding was virtually eliminated by adding EGTA to the incubation mixture. Hence, like the native receptor, the receptor fragment shows highly Ca2+-dependent binding activity.

In addition to apoB-100 on LDL, apoE-containing lipoproteins are the other major ligand for the LDL receptor (34). To bind with high affinity to the LDL receptor, apoE must be complexed with lipid (35). This is typically done by reconstituting apoE into vesicles with dimyristoyl phosphatidylcholine.
(DMPC) to mimic its natural environment (35). Using apoE-DMPC complexes in the plate assay, we demonstrated the same Ca²⁺-dependent binding as was observed for LDL (data not shown). Interestingly, lipid-free apoE bound to the microtiter wells also bound with high affinity to the LDL receptor fragment. Thus, it appears that apoE binding to a plastic surface mimics binding to lipid with respect to the high affinity receptor interaction.

Another key characteristic of the native LDL receptor is its differential binding to the three common human apoE isoforms, apoE2, apoE3, and apoE4. These isoforms are distinguished by cysteine and arginine differences at positions 112 and 158; apoE3 contains cysteine and arginine at these positions, respectively, whereas apoE2 contains cysteine and apoE4 contains arginine at both positions (36, 37). Whereas apoE3 and apoE4 bind to the LDL receptor with essentially the same affinity, apoE2 displays defective binding (<1% of that of apoE3 or apoE4) (38). The defective binding of apoE2 is an underlying cause of type III hyperlipoproteinemia, a lipoprotein disorder associated with elevated plasma lipid levels and premature heart disease (39).

If the receptor fragment is correctly folded and fully active, it should exhibit differential binding to the apoE isoforms. This expectation was tested with either apoE-DMPC complexes or lipid-free apoE in a plate assay. The results with both forms of apoE were essentially identical; the data for the lipid-free apoE isoforms are presented in Fig. 7. As shown, the amounts of receptor fragment bound to apoE3 and apoE4 were identical, whereas binding to apoE2 was less than 5% of that of apoE3 and apoE4 (comparable amounts of each isoform were bound to the wells). These results clearly demonstrate that the isoform specificities of the intact LDL receptor are mirrored by the receptor fragment.

As a further test of the authenticity of the receptor fragment, we reasoned that an antibody raised to the refolded receptor fragment should recognize the native receptor. As determined by Western blot analysis, an anti-receptor fragment antibody bound to LDL receptors from rat liver and bovine adrenal membranes, whereas it did not bind to misfolded or non-folded receptor fragments, i.e. fusion protein or S-300 fraction (data not shown). In addition, the antibodies completely inhibited the binding of human LDL to LDL receptors on human fibroblasts (Fig. 8), demonstrating that the antibodies recognized the native human receptor.

In summary, the evidence presented above strongly supports the conclusion that the binding activity of the LDL receptor fragment is biologically relevant and that the receptor fragment has been successfully refolded to its native conformation. With this correctly folded receptor fragment available, we can now begin to address structural questions, such as the overall topology of the 7 cysteine-rich repeats, the role of calcium in ligand interaction, the number of repeats involved in apoE interaction, and how the Arg-150 conformational change in apoE2 reduces receptor binding activity. In addition, with the methods developed here, production and refolding of the ligand-binding domains of other members of the LDL receptor superfamily are potentially feasible.

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