

Calcium induces a conformational change in the ligand binding domain of the low density lipoprotein receptor

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Abstract We previously have shown that LDL-R³⁵⁴, a truncated low density lipoprotein (LDL) receptor, is a calcium binding protein. LDL-R³⁵⁴ is composed of the ligand binding domain and repeat A of the EGF precursor homology domain of the full-length human LDL receptor. We also found that Ca²⁺ was required for the interaction between LDL-R³⁵⁴ and its ligand, LDL (Dirlam et al. 1996. *Protein Expr. Purif.* **8**: 489–500). In the current study, calcium-induced changes in the structure and function of LDL-R³⁵⁴ were examined. When calcium bound to LDL-R³⁵⁴, its apparent size increased, as determined by native and SDS-gel electrophoresis. The calcium-saturated form of LDL-R³⁵⁴ was more resistant to trypsin proteolysis than the calcium-depleted form. In the presence of calcium, the disulfide bonds in the truncated receptor were stabilized, rendering them more resistant to reduction by dithiothreitol. Calcium binding affinities were measured by monitoring increased tryptophan fluorescence intensities. LDL-R³⁵⁴ bound Ca²⁺ with high affinity (EC₅₀ = 60 nM at pH 7.4) and specificity, as 400 μM Mg²⁺ did not compete for calcium binding. The affinity of LDL-R³⁵⁴ for calcium decreased when the pH was lowered. These results suggest that calcium induces a conformational change in the ligand binding domain of the LDL receptor and that a receptor conformer capable of binding ligand should be stabilized at physiological extracellular Ca²⁺ concentration and pH. Drops in pH may regulate LDL receptor function by altering the amount of calcium bound to the receptor.—**Dirlam-Schatz, K. A., and A. D. Attie.** Calcium induces a conformational change in the ligand binding domain of the low density lipoprotein receptor. *J. Lipid Res.* 1998. **39**: 402–411.

Supplementary key words disulfide bonds • divalent cations • epidermal growth factor-like repeats • protein folding • protein structure

The low density lipoprotein (LDL) receptor (-R) is an 839-amino acid integral membrane glycoprotein that mediates cholesterol homeostasis by removing apolipoprotein (apo) B- and apoE-containing lipoproteins from the circulation (1). Calcium is required for this interaction between receptor and ligand (2), but the exact role this cation plays in ligand binding is not fully known.

The LDL receptor contains two types of repeats that would be predicted to bind calcium, the ligand binding repeats and the epidermal growth factor (EGF)-like repeats. Seven imperfect cysteine-rich ligand binding repeats that share homology with the human complement component, C9, constitute the ligand binding domain (3, 4). The first indication that the LDL receptor is a calcium binding protein was based on antibody binding studies: recognition by monoclonal antibodies directed against repeat 1 required the presence of calcium (5). The next domain of the LDL receptor, the EGF precursor homology domain, also contains three cysteine-rich EGF-like repeats, A, B, and C (3, 6). EGF-like repeats have been shown to bind calcium in a variety of proteins, such as fibrillin (7, 8), factors IX (9) and X (10), Protein C (11), and Protein S (12).

The calcium binding properties of individual repeats from the ligand binding domain of the LDL receptor have been more extensively studied than those from the EGF precursor homology domain (13–15). Recent studies of repeat 5 revealed that a cluster of acidic amino acids, which is found in the carboxy-terminus of each ligand binding repeat, forms a calcium binding site, and calcium binding is required for proper protein folding (15, 16). When repeat 5 is allowed to refold in the presence of calcium, a single isomer

Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; apo, apolipoprotein; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; BSA, bovine serum albumin; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EC₅₀, effective concentration at 50% response; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; kDa, kilodalton, LDL, low density lipoprotein; LDL-R low density lipoprotein receptor; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; *Sf21*, *Spodoptera frugiperda* insect cells.

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containing one disulfide bonding pattern is formed (15). In contrast, if disulfide exchange is allowed to proceed in the absence of calcium, multiple isomers are formed.

Calcium binding also affects the structure of certain recombinant proteins composed of single repeats from the ligand binding domain. Repeat 5 binds calcium with an affinity in the nm range and adopts a more folded structure upon Ca^{2+} addition, as determined by NMR (15). Similarly, the calcium-bound form of repeat 2 is more ordered than the calcium-free form (14). In contrast, addition of calcium to repeat 1 does not result in any significant change in its conformation, as determined by CD and NMR (13). These results may help to explain the fact that in the full-length receptor, repeat 1 is not directly involved in ligand binding, whereas repeats 5 and 2 are (5, 17).

The ligand binding domain of the LDL receptor contains 42 cysteines, all of which are involved in disulfide bonds (18–20). This highly cross-linked protein would be expected to form a very stable and rigid structure. However, calcium is still required for the association of ligand with the LDL receptor (2). Therefore, we explored the possibility that calcium induces additional structural changes in the entire ligand binding domain of the LDL receptor that stabilize this protein. These studies were made possible due to the availability of a truncated LDL receptor (LDL-R³⁵⁴) that contains repeats 1–7 and repeat A from the ligand binding domain and the EGF precursor homology domain of the full-length human LDL receptor, respectively (21). LDL-R³⁵⁴ binds LDL with high affinity in a calcium-dependent manner (21). LDL-R³⁵⁴ also binds calcium, but only if the disulfide bonds are intact (21). In the present study, we show that LDL-R³⁵⁴ undergoes a conformational change upon binding calcium and is stabilized in a more ordered, solvent-inaccessible structure.

MATERIALS AND METHODS

Expression and purification of LDL-R³⁵⁴

Spodoptera frugiperda (SF21) cells were infected with AcNPV-LDL-R³⁵⁴, and LDL-R³⁵⁴-containing media were produced as described previously (21). Media were filtered through a 0.22-micron syringe filter and subjected to anion exchange chromatography [DEAE Sepharose Fast Flow, HR 5/10 column (Pharmacia)]. A 20 mm sodium phosphate buffer (pH 6.5) containing 2 mm benzamidine was used to equilibrate and wash the column. The column was then eluted with a NaCl gradient at a flow rate of 1 ml per min. The use of this

buffer system allowed the separation of LDL-R³⁵⁴ monomers from multimers. Column fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (22) on 10% gels under non-reducing conditions and staining with Coomassie brilliant blue to determine the location of LDL-R³⁵⁴ monomers. Fractions containing predominantly monomers were pooled for future experiments. Protein concentrations were determined by the Micro BCA protein assay (Pierce), using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis and immunoblot analysis

LDL-R³⁵⁴-containing fractions were exchanged into 10 mm Tris-HCl (pH 7.4) (TRIS Ultra Pure, Schwarz/Mann Biotech [ICN]), 60 mm KCl with a Centriprep 30 concentrator (Amicon). Purified receptor was incubated with 10 mm CaCl_2 or 10 mm EDTA for 15 min. For native (non-denaturing) PAGE, 4× native gel sample buffer (40% sucrose, 0.05% bromophenol blue) was added and samples were electrophoresed on 4–15% Tris-Glycine Ready Gels (Bio-Rad) in a buffer containing 90 mm Tris, 80 mm boric acid, 3 mm azide (pH 8.3). Bovine serum albumin (4 μg) and ovalbumin (4 μg) were run for molecular weight comparison. For SDS-PAGE, 6 × SDS sample buffer [50 mm Tris-HCl (pH 6.8), 1.0% SDS, 0.1% bromophenol blue, 1.0% glycerol, 1 mm urea: final concentrations] was added and samples were electrophoresed on the 4–15% gradient gels in a buffer containing 25 mm Tris, 192 mm glycine, 0.1% SDS (pH 8.3). Kaleidoscope prestained standards (Bio-Rad) were used for molecular weight comparison. Resolved proteins were detected by Coomassie brilliant blue staining. Immunoblot analysis (23) was performed with the anti-human LDL-R monoclonal antibody IgG-C7 (5, 24) or IgG-HL1 (25) and an alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (Hyclone). All nitrocellulose blots were developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 175 $\mu\text{g}/\text{ml}$) and nitro blue tetrazolium (NBT, 350 $\mu\text{g}/\text{ml}$).

⁴⁵Calcium blots

Proteins subjected to native or SDS-gel electrophoresis were transferred to nitrocellulose, and the resultant blots were soaked in 10 mm HEPES (pH 7.4), 60 mm KCl, 5 mm MgCl_2 for 1 h with three buffer changes (26). The membranes were then incubated in the HEPES buffer containing 1 $\mu\text{Ci}/\text{ml}$ ⁴⁵Ca for 15 min at room temperature, followed by a 5-min wash with distilled water. Membranes were allowed to air dry, after which they were exposed to Kodak XAR 5 film over-

night. For ^{45}Ca blots done at varying pHs, buffers are indicated in the fluorescence studies section of Materials and Methods and contained 1 mM as opposed to 5 mM MgCl_2 .

Trypsin proteolysis

LDL-R³⁵⁴ (10 mM HEPES (pH 7.4), 60 mM KCl) was incubated in 2 mM CaCl_2 or 2 mM EDTA for 30 min at room temperature. An aliquot was removed, and 6× SDS sample buffer plus either 2 mM CaCl_2 or 2 mM EDTA were added, making the final concentration of both CaCl_2 and EDTA in each sample 2 mM, as EDTA has been shown to increase the mobility of the receptor during electrophoresis. This sample represents the zero time point. Trypsin (~1% weight/weight) was added to the protein, and samples were incubated at 37°C for the indicated times (10, 60, and 120 min). At each time point, an aliquot was removed, and sample buffer plus calcium/EDTA were added. The samples were immediately boiled for 5 min to inactivate the trypsin. Half of the samples contained β-mercaptoethanol (final concentration ~1.6%), while the other half contained no reducing agent. Protein samples were subjected to SDS-PAGE on 12% gels. The dye front was not allowed to run off the bottom of the gels. Gels were stained with Coomassie blue. Alternatively, proteins were electroblotted onto nitrocellulose for immunoblot analysis with IgG-C7 and IgG-HL1 or ^{45}Ca blot analysis.

Fluorescence studies

Purified LDL-R³⁵⁴ was buffer exchanged into 10 mM Tris (pH 8.5 or 7.4), 60 mM KCl or 10 mM MES (pH 6.5 or 5.5), 60 mM KCl. To a 100-μl aliquot of protein, 20 μl of a 6× calcium/EDTA solution was added, and the sample was incubated for at least 30 min at room temperature. The final concentration of EDTA was 100 μM and the concentrations of added calcium required to give the indicated free (unchelated) Ca^{2+} concentrations were determined with the Chelator program (Theo Schoenmakers, Department of Animal Physiology, Netherlands). Spectra were acquired on a SLM 8000 C Spectrofluorometer. Samples were excited at 280 nm, and emission was monitored from 300–400 nm at 1 nm increments. The band pass was 8 nm for excitation and 16 nm for emission. Fluorescence intensity measurements are reported as the ratio of channel A, the fluorescence of the sample cell, to channel B, the fluorescence of the reference cell containing rhodamine B. Non-linear curve-fitting was performed with the program DeltaGraph Pro 3.5 (DeltaPoint, Inc.) to determine the amount of free calcium required to result in a 50% response (EC_{50}).

DTT reduction of disulfide bonds and DTNB quantitation of free sulfhydryls in LDL-R³⁵⁴

Purified LDL-R³⁵⁴ (10 mM HEPES (pH 7.4), 60 mM KCl) was incubated with 5 mM CaCl_2 or 5 mM EDTA for 1 h at room temperature. Increasing concentrations of dithiothreitol (DTT) were added to the truncated receptor protein. As a control to ensure the efficient removal of DTT in later steps, the same concentration of DTT was added to buffer alone, and these samples were processed in the same manner as the protein-containing samples. After a 1 h incubation at room temperature in the presence of DTT, aliquots were removed and subjected to SDS-PAGE. Proteins were either stained with Coomassie blue or electroblotted onto nitrocellulose for ^{45}Ca blot analysis.

DEAE Sepharose (75 μl in 10 mM HEPES (pH 7.4), 60 mM KCl) was added to the remaining sample, and all tubes were rotated end over end for 1.5 h at room temperature. Samples were spun in a microcentrifuge (2000 g, 4 min) to pellet the Sepharose resin that has now bound the truncated receptor protein. Supernatants were removed and buffer (3 × 1 ml 10 mM HEPES (pH 7.4), 60 mM KCl) was added to wash away residual DTT, which would interfere with the assay. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB, ~33 μM) (27) in 50 mM acetate (pH 4.8) containing 1 M NaCl was added to the resin, allowing the elution of LDL-R³⁵⁴ from the DEAE Sepharose and reaction of DTNB with free sulfhydryls. This reaction was allowed to proceed at room temperature for 1 h after which the resin was removed from the samples, and the absorbance (412 nm) of the supernatant was measured. All washes were saved, and the protein concentration of each was determined, using the Coomassie Plus reagent (Pierce), to confirm that binding of LDL-R³⁵⁴ to the DEAE Sepharose was quantitative and that protein was not lost during the wash steps.

RESULTS

Electrophoretic mobility of calcium-saturated and calcium-depleted LDL-R³⁵⁴

To determine whether calcium binding affects the truncated receptor's mobility, purified LDL-R³⁵⁴ was subjected to native (non-denaturing) PAGE in the presence (10 mM CaCl_2) or absence (10 mM EDTA) of calcium. When calcium was bound to the truncated receptor, its electrophoretic mobility was decreased relative to the calcium-depleted receptor (Fig. 1). LDL-R³⁵⁴ was also subjected to non-reducing SDS-PAGE in the presence or absence of calcium. The apparent molecular

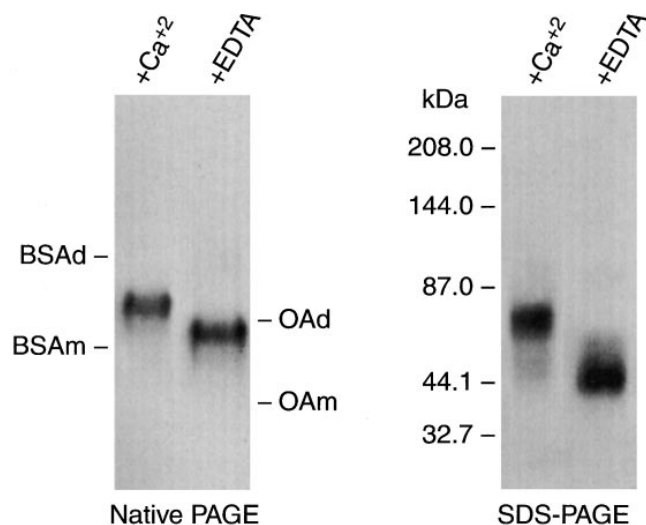


Fig. 1. Electrophoretic mobility of LDL-R³⁵⁴ in the presence and absence of calcium, as determined by ⁴⁵Ca blot analysis. Purified LDL-R³⁵⁴ (2 μ g, 10 mM Tris, 60 mM KCl, pH 7.4) was incubated with 10 mM CaCl₂ or 10 mM EDTA for 15 min. Samples were then subjected to native (non-denaturing) or SDS-polyacrylamide gel electrophoresis on 4–15% gradient gels. The receptor was transferred to nitrocellulose and ⁴⁵Ca binding activity was determined in a HEPES buffer (pH 7.4) at room temperature. The resultant blots were exposed to film for 14.5 h. (BSA, bovine serum albumin; OA, ovalbumin, m, monomer; d, dimer)

mass of the calcium-saturated receptor (~65 kDa) was greater than that of the calcium-depleted form (~44 kDa). The fact that calcium-bound LDL-R³⁵⁴ had a larger apparent molecular mass than calcium-free LDL-R³⁵⁴, as determined by both native and SDS-PAGE, suggested that the shape and/or the charge of LDL-R³⁵⁴ changed upon binding Ca²⁺.

Trypsin proteolysis of LDL-R³⁵⁴

As proteins fold, they become less susceptible to protease digestion. To determine whether calcium affects the folding state of the LDL receptor ligand binding domain, LDL-R³⁵⁴ was subjected to trypsin proteolysis in the presence or absence of calcium, and the resulting digestion products were analyzed. The truncated receptor was more resistant to digestion by trypsin when treated with 2 mM CaCl₂ than when treated with 2 mM EDTA (**Fig. 2**). In the Ca²⁺-treated samples, the intact receptor (~60 kDa) was still detectable after a 120-min incubation, whereas in the EDTA-treated ones, full-length LDL-R³⁵⁴ was no longer present after 60 min (**Fig. 2A**). In the presence of calcium, two major bands were present on the gel. One band was the intact receptor (~60 kDa), and the other was a digestion product (~31 kDa). There were more digestion products in EDTA-treated than in Ca²⁺-treated samples.

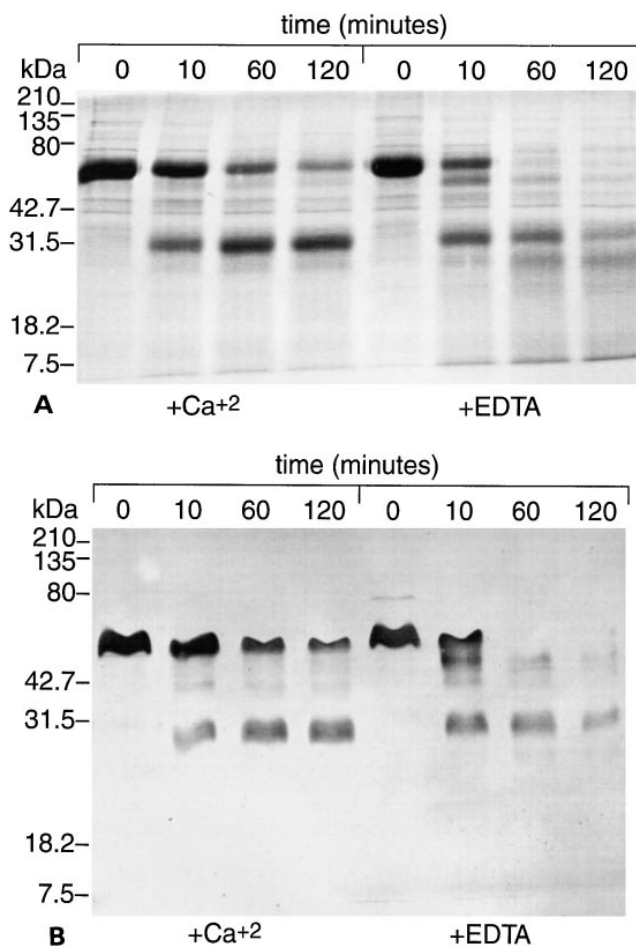


Fig. 2. Trypsin proteolysis of LDL-R³⁵⁴. Purified LDL-R³⁵⁴ was incubated with 2 mM CaCl₂ or 2 mM EDTA and then digested with trypsin (1% weight/weight) for the indicated periods of time. Panel A shows proteins (8.8 μ g) subjected to reducing SDS-PAGE and Coomassie blue staining. Panel B is the immunoblot analysis with IgG-HL1 of proteins (2.2 μ g) subjected to reducing SDS-PAGE

Immunoblot and ⁴⁵Ca blot analyses of the trypsin-digestion products were carried out to determine where the truncated receptor was cleaved. We used antibodies recognizing repeat 1 (IgG-C7) or the linker between repeats 4 and 5 (IgG-HL1). The major digestion product of 31 kDa did not react with IgG-C7 (data not shown) but was recognized by IgG-HL1, suggesting that it still contained the region between repeats 4 and 5, but not repeat 1 (**Fig. 2B**). This 31-kDa fragment also bound calcium on ⁴⁵Ca blot (data not shown).

There are many predicted trypsin cleavage sites in the truncated receptor; however, LDL-R³⁵⁴ appeared relatively trypsin-resistant. To determine whether this was due to a compact structure maintained by the disulfide bonds, the truncated receptor was treated with 1%

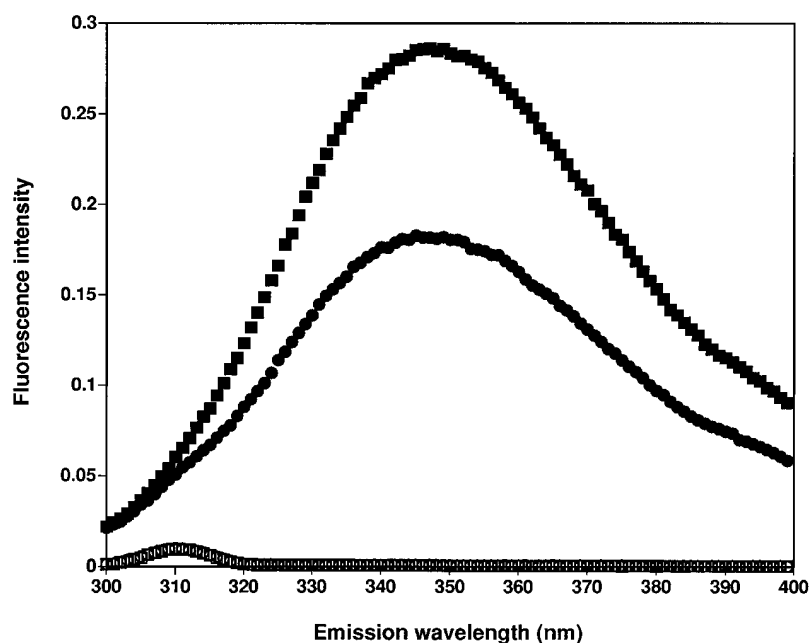


Fig. 3. Changes in intrinsic tryptophan fluorescence upon calcium addition. The fluorescence emission of tryptophan residues in LDL-R³⁵⁴ was measured from 300 to 400 nm at 1-nm increments after excitation at 280 nm. Fluorescence intensity measurements are expressed in arbitrary units as the ratio of channel A (sample channel) to channel B (reference channel). Each sample contained 7.4 μg of protein in 10 mm Tris, 60 mm KCl, pH 7.4. (Squares, 100 μM free Ca^{2+} ; circles, 100 μM EDTA; closed symbols, 7.4 μg LDL-R³⁵⁴; open symbols, buffer alone [no protein]).

β -mercaptoethanol prior to incubation with trypsin. Reduction of LDL-R³⁵⁴ made it very susceptible to trypsin proteolysis; it was completely digested within 10 min (data not shown).

Intrinsic tryptophan fluorescence

Changes in tyrosine or tryptophan fluorescence are commonly measured to monitor changes in protein conformation (28). LDL-R³⁵⁴ contains six tryptophans, and the fluorescence of these was monitored in the presence and absence of calcium. Purified LDL-R³⁵⁴ was incubated with 100 μM EDTA or 100 μM EDTA plus the required amount of CaCl_2 to yield 100 μM free (unchelated) Ca^{2+} . When the truncated receptor bound calcium, the fluorescence intensity, expressed in arbitrary units as the ratio of channel A (sample cell) to channel B (reference cell), increased from 0.18 to 0.29 (Fig. 3). Calcium binding reached equilibrium within 15 min (data not shown). Addition of excess EDTA to calcium-bound LDL-R³⁵⁴ resulted in a decrease in fluorescence intensity back to baseline (100 μM EDTA value), indicating that calcium binding was reversible (data not shown). Magnesium at 50 μM ($A/B = 0.28$) or 400 μM ($A/B = 0.27$), the physiological concentration in blood, did not compete for the binding of 0.1 μM calcium to the receptor ($A/B = 0.26$), suggesting that calcium binding was specific.

Susceptibility of disulfide bonds to reduction by DTT

The aforementioned results suggested that calcium induced a conformational change in the ligand bind-

ing domain of the LDL receptor. As intact disulfide bonds are required to maintain the calcium binding sites in LDL-R³⁵⁴ (21), the stability of the disulfide bonds in the presence and absence of calcium was determined. Purified LDL-R³⁵⁴ was incubated with 5 mm CaCl_2 or 5 mm EDTA and increasing concentrations of DTT. After SDS-PAGE, proteins were stained with Coomassie blue (Fig. 4A) or electroblotted onto nitrocellulose for ⁴⁵Ca blot analysis (Fig. 4B). LDL-R³⁵⁴ cannot bind calcium when the disulfide bonds are reduced (21). Thus, ⁴⁵Ca binding to LDL-R³⁵⁴ monitors the extent of reduction of the disulfide bonds by DTT. ⁴⁵Ca binding was essentially lost upon addition of 0.5 mm DTT to the calcium-treated receptor. EDTA-treated LDL-R³⁵⁴ was even more susceptible to reduction and lost the ability to bind ⁴⁵Ca after incubation with only 0.1 mm DTT.

The number of free sulfhydryls generated by DTT reduction of LDL-R³⁵⁴ was determined by reaction of the reduced receptor with DTNB. In the presence of calcium, addition of more than 1 mm DTT was required to reduce 50% of the disulfide bonds in LDL-R³⁵⁴ (Fig. 5). In the absence of calcium, half of the disulfides were already reduced upon addition of only 0.5 mm DTT. Calcium binding to LDL-R³⁵⁴ appeared to stabilize its disulfide bonds, making them more resistant to reduction by DTT. When LDL-R³⁵⁴ was incubated with DTNB without first treating it with DTT (0 mm DTT) there was no reaction, indicating that all the cysteines in LDL-R³⁵⁴ were oxidized. Protein-free controls did not react with DTNB, indicating that excess DTT was

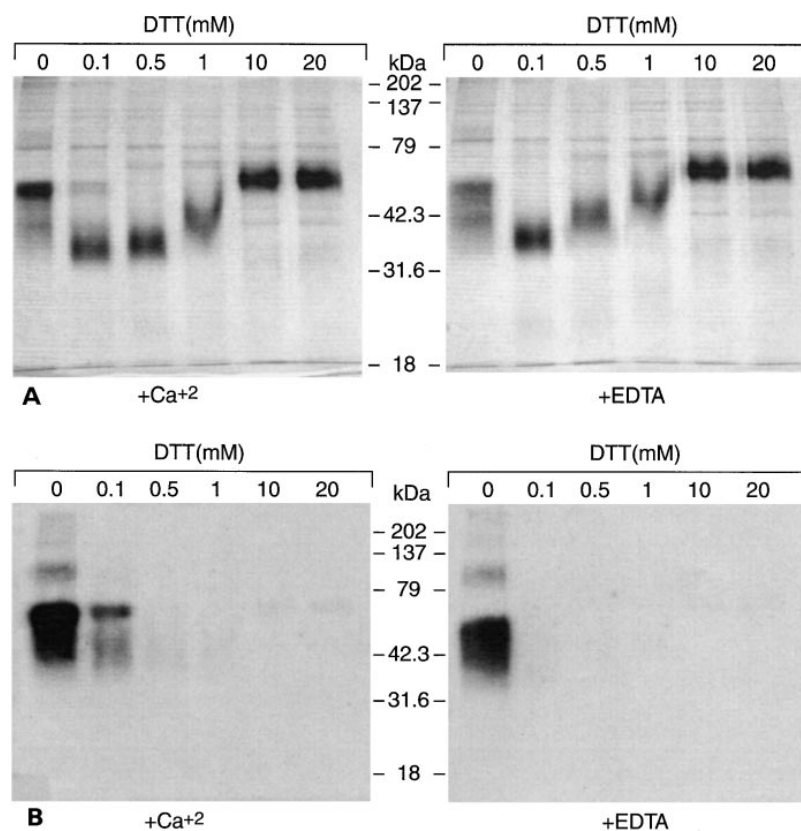


Fig. 4. Disulfide bond stability in calcium-saturated and calcium-depleted LDL-R³⁵⁴. Purified LDL-R³⁵⁴ was incubated with 5 mM CaCl₂ or 5 mM EDTA. The indicated concentrations of DTT were added to the truncated receptor, and a 1-h incubation at room temperature followed. Samples were subjected to SDS-PAGE on 10% gels. Panel A shows the Coomassie blue-stained gel. Panel B shows the autoradiograph from the ⁴⁵Ca blot analysis performed in a HEPES buffer, pH 7.4. The blots were exposed to film for 17 h. Each lane contains 3.8 μg of total protein.

efficiently removed from the samples prior to reaction with DTNB and did not interfere with the assay.

pH-dependent calcium binding

LDL dissociates from the LDL receptor in the endosome when the pH drops below 6.5 (1). To explore the possibility that pH-dependent ligand release might occur through pH-dependent Ca²⁺ release, we measured the binding affinity of Ca²⁺ as a function of pH. Purified LDL-R³⁵⁴ was incubated with increasing concentrations of free Ca²⁺ at pH 8.5, 7.4, 6.5, and 5.5 at 25°C and the fluorescence emission at 346 nm was monitored (**Fig. 6A**). The affinity of the truncated receptor for calcium decreased approximately 10-fold for each unit decrease in pH below 7.4 (**Fig. 6B**). A decrease in calcium binding was also observed as the pH was lowered in the ⁴⁵Ca blot analysis of LDL-R³⁵⁴ used in the fluorescence studies (**Fig. 6C**).

DISCUSSION

The LDL receptor is an unusually stable protein. Receptor-mediated endocytosis leads to the formation of an endosome where the receptor is exposed to an

acidic environment. In the endosome, the ligand dissociates from the receptor and the receptor recycles to the plasma membrane where it regains full ligand binding capability (1). In vitro, the LDL receptor can be subjected to electrophoresis in the presence of denaturants and still binds to LDL (29). A large part of the receptor's stability can be attributed to the presence of 21 intra-repeat disulfide bonds in the ligand binding domain (18, 19, 30), as reduction of the LDL receptor results in loss of ligand binding (29). However, even with intact disulfide bonds, calcium is still required to promote LDL receptor–ligand interactions (2).

In the current study, we used multiple approaches to show that calcium binding alters the structure of LDL-R³⁵⁴ and imparts additional stability to the ligand binding domain of the receptor. Changes in receptor structure were first monitored by gel electrophoresis. Under both non-denaturing and denaturing conditions, the calcium-saturated form of LDL-R³⁵⁴ had a larger apparent molecular mass compared to the calcium-depleted form. This suggests that calcium causes the receptor to adopt a more extended structure. This calcium-induced conformational change in LDL-R³⁵⁴ also appears to be a transition to a more native, folded structure, as reflected in the truncated receptor's ability to bind ligand (21).

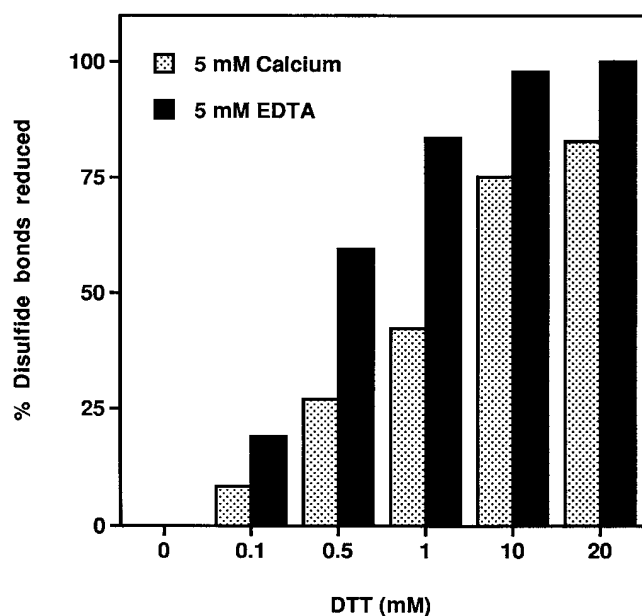


Fig. 5. Quantitation of free sulfhydryls in DTT-treated LDL-R³⁵⁴. Purified LDL-R³⁵⁴ was treated with 5 mM CaCl₂ or 5 mM EDTA and incubated in the indicated concentrations of DTT for 1 h at room temperature. LDL-R³⁵⁴ was separated from excess DTT by precipitation with DEAE-Sephacel. LDL-R³⁵⁴ was eluted from the resin and reacted with 33 μ M DTNB. The absorbance of the samples was read at 412 nm. The percentage of disulfide bonds reduced in each sample was calculated using the 5 mM EDTA, 20 mM DTT sample as the 100% value.

The extent of digestion of proteins by proteolytic enzymes is a function of the number of solvent-exposed cleavage sites. Folded proteins are therefore relatively resistant to proteolytic digestion. LDL-R³⁵⁴ was most resistant to proteolysis when the disulfide bonds were intact and calcium was bound. The removal of calcium from LDL-R³⁵⁴ exposed additional trypsin cleavage sites, suggesting that the conformation of LDL-R³⁵⁴ had changed to one that was less folded, more flexible, and more solvent-accessible.

The susceptibility of disulfide bonds to reduction by DTT is also a probe for the mature native state of a protein (31). It required a higher DTT concentration to reduce the disulfide bonds in LDL-R³⁵⁴ when it was bound to calcium. This result suggests that calcium locks in a conformation in which the disulfide bonds are more buried, making them more inaccessible to solvent and therefore less likely to be reduced.

The more ordered structure and increased stability of Ca²⁺-bound LDL-R³⁵⁴ is in accord with the more ordered structures seen in the NMR studies of Daly et al. (14) and Blacklow and Kim (15). Once the native disulfide bonds have been formed in a recombinant protein containing only repeat 2 or in one containing repeat 5,

calcium addition results in a conformational change to a form that is more characteristic of a folded protein, as determined by the enhancement of amide dispersion resonances and NOESY cross-peaks in the ¹H NMR spectra (14, 15).

Calcium binding resulted in increased tryptophan fluorescence intensities in LDL-R³⁵⁴, which further suggests that the truncated receptor protein underwent a conformational change upon binding calcium. By monitoring the fluorescence emission at 346 nm we were able to determine the amount of free Ca²⁺ required to produce 50% of the maximal increase (EC₅₀) in LDL-R³⁵⁴ fluorescence, a measure of *n* calcium binding to receptor. The EC₅₀ was 60 nM at pH 7.4, 25°C. If all the calcium-binding sites in LDL-R³⁵⁴ bind calcium independently and with equal affinity, then this value is in close agreement with the 70 nM equilibrium dissociation constant reported for repeat 5 binding to calcium (15). The fact that the receptor bound to calcium with an affinity in the nM range predicts that at physiological calcium concentrations (2 mM), the LDL receptor would exist predominantly in the calcium-bound conformer.

We showed that calcium binding to LDL-R³⁵⁴ was specific. Magnesium, the only other divalent cation present in blood at significant levels (0.4 mM), did not compete for calcium binding. This is consistent with the previously reported observation that 2 mM magnesium is unable to promote ligand binding to the full-length receptor (2).

Calcium binding to LDL-R³⁵⁴ was pH-dependent. Therefore, pH might serve as a regulator of LDL receptor conformation by altering the amount of calcium bound to the receptor. LDL dissociates from the receptor in the endosome when the pH drops below 6.5, due to an influx of hydrogen ions. The LDL receptor is then recycled back to the cell surface where it initiates a new cycle of ligand binding (1). A decrease in pH might facilitate the release of bound ligand in the endosome as a consequence of the dissociation of calcium from the receptor. However, when the calcium-depleted LDL receptor is recycled back to the cell surface, it would rapidly bind calcium, preparing it for another round of ligand binding. pH-dependent calcium release could occur in both the ligand binding repeats and the EGF-like repeats. The amount of calcium released would depend on the affinity of the repeats for calcium and the Ca²⁺ concentration in the endosome. In other proteins containing EGF-like repeats, the calcium binding affinities of the repeats have been reported to be in the micromolar range (9, 32). Therefore, calcium may dissociate from the lower affinity EGF-like repeats before the ligand binding repeats. A pH-dependent conformational change in EGF-like repeats A and B has been proposed to mediate acid-dependent ligand release (33).

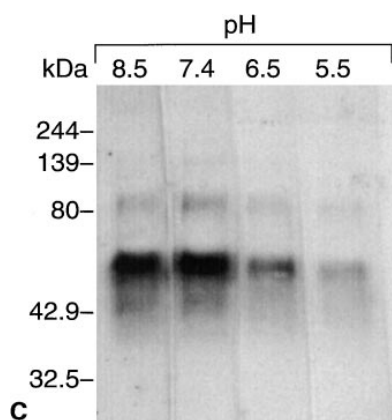
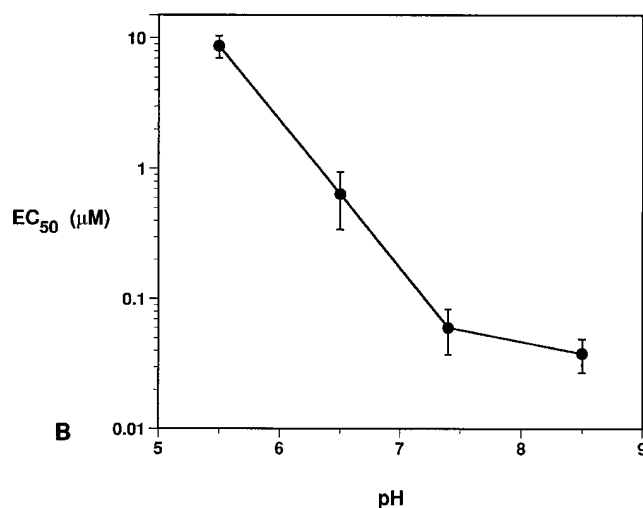
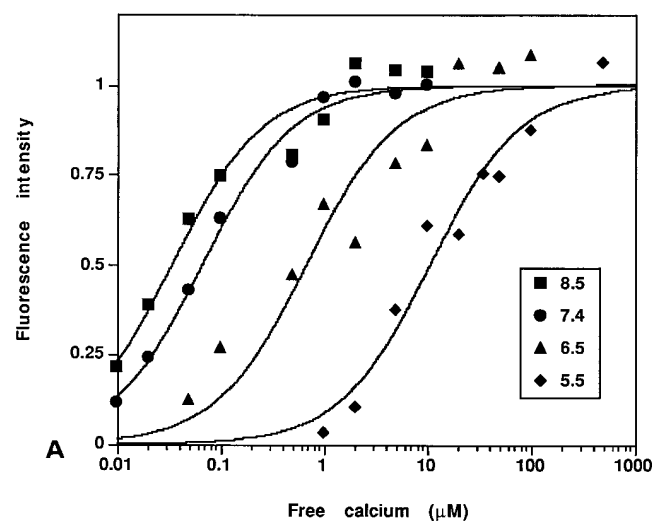


Fig. 6. pH dependence of calcium binding to the truncated receptor. LDL-R³⁵⁴ at pH 8.5, 7.4, 6.5, and 5.5, in the buffers indicated under Materials and Methods, was incubated with increasing concentrations of free Ca²⁺. The fluorescence emission of the tryptophans was monitored at 346 nm after excitation at 280 nm. In each case, the maximum value of the fluorescence intensity (ratio of channel A to channel B) was normalized to approximately one. Representative binding curves are shown in panel A. Calcium binding curves, including the ones shown in Fig. 6A, were fit with the program DeltaGraph Pro 3.5. The resulting EC₅₀ values (amount of free calcium required to produce 50% of the maximal response), which are the mean of 4 independent experiments \pm standard deviation, were plotted as a function of pH, and the graph is shown in panel B. LDL-R³⁵⁴ was also subjected to non-reducing SDS-PAGE and ⁴⁵Ca blot analysis at the indicated pHs. The autoradiograph is shown in panel C. Each lane contains 1.6 μ g LDL-R³⁵⁴.

The idea that calcium binding to a cysteine-rich domain can stabilize a particular protein conformation has precedence (9, 32, 34–38). There appear to be many parallels between the LDL receptor and fibrillin in their structural and functional relationship with calcium. Calcium maintains the interdomain structure of EGF-like repeats in fibrillin, the protein defective in Marfan syndrome (8, 39). Incubation of fibrillin microfibrils with EDTA alters their morphology and causes the collapse of the interbead domains (40). In the presence of calcium, fibrillin becomes stabilized and is more resistant to proteolytic degradation (41). Calcium also affects the shape of fibrillin, causing it to become more extended and rigid (42). Additionally, introduction of a Marfan mutation (Arg₁₁₃₇ \rightarrow Pro) into a calcium-binding EGF-like repeat of fibrillin results in a protein that is unable to fold correctly (43). Certain mutations in the LDL receptor that cause familial hypercholesterolemia also alter key calcium-binding sites that are required for proper receptor folding, as recently proposed (15, 16).

In summary, we show that calcium induces a conformational change in the ligand binding domain of the LDL receptor and maintains the cysteine-rich regions in a more folded, native state. Without calcium, even though the structure of the receptor is stabilized by multiple disulfide bonds, the LDL receptor still does not attain the proper conformation for ligand binding (21, 44). Consequently, only when the receptor is exposed to calcium at physiological pH, as it is when it resides on the cell surface, is it competent to bind ligand. **■**

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