A Two-Module Region of the Low-Density Lipoprotein Receptor Sufficient for Formation of Complexes with Apolipoprotein E Ligands†

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ABSTRACT: The low-density lipoprotein (LDL) receptor transports two different classes of cholesterol-carrying lipoprotein particles into cells: LDL particles, which contain a single copy of apolipoprotein B-100 (apoB-100), and β-migrating very low-density lipoprotein (β-VLDL) particles, which contain multiple copies of apolipoprotein E (apoE). The ligand-binding domain of the receptor lies at its amino-terminal end within seven adjacent LDL-A repeats (LA1−LA7). Although prior work clearly establishes that LA5 is required for high-affinity binding of particles containing apolipoprotein E (apoE), the number of ligand-binding repeats sufficient to bind apoE ligands has not yet been determined. Similarly, uncertainty exists as to whether a single lipid-activated apoE receptor-binding site within a particle is capable of binding to the LDLR with high affinity or whether more than one is required. Here, we establish that the LA4−5 two-repeat pair is sufficient to bind apoE-containing ligands, on the basis of binding studies performed with a series of LDLR-derived "minireceptors" containing up to four repeats. Using single chain multimers of the apoE receptor-binding domain (N-apoE), we also show that more than one receptor-binding site in its lipid-activated conformation is required to bind to the LDLR with high affinity. Thus, in addition to inducing a conformational change in the structure of N-apoE, lipid association enhances the affinity of apoE for the LDLR in part by creating a multivalent ligand.

The human low-density lipoprotein receptor (LDLR)[1] is the patriarch of a family of cell-surface receptors that combine LDL-A (LA), YWTD, and EGF-like modules in different arrangements to perform a wide variety of biological functions. Proteins of the LDLR family not only mediate cellular uptake of ligands ranging from lipoprotein particles to protease-inhibitor complexes but also participate in the communication of biological signals across membranes in a diverse array of signal transduction cascades (*1*−*3*).

The primary role of the LDLR is to remove cholesterol-laden lipoprotein particles from the circulation (*4*, *5*). The receptor exhibits high-affinity binding both to LDL particles (*4*), which contain a single copy of apolipoprotein B-100 (apoB-100), and to β-VLDL particles (*6*), which contain multiple copies of apolipoprotein E (apoE). Other receptors of the LDL receptor family most closely related to the LDLR, including the VLDL receptor (VLDLR), apolipoprotein E receptor 2 (apoER2), the LDL receptor-related protein (LRP-1), and megalin (LRP-2), also bind apolipoprotein E-containing ligands with high affinity (*7*−*11*).

The ligand-binding domain of the LDLR lies within the seven adjacent LDL-A repeats (LA1−LA7) at the amino-terminal end of the receptor (Figure 1A) (*12*, *13*). Previous studies have shown that deletion of any single module from LA3 through LA7 abrogates binding of LDL, whereas only deletion of LA5 prevents high-affinity binding of β-VLDL. Systematic mutation of conserved residues within each repeat yields similar findings in such loss of binding studies (*13*).

The 1.7 Å crystal structure of LA5 (*14*), as well as the X-ray structure of the entire LDLR ectodomain (*15*) and structures of other LA modules and module pairs (*16*−*22*), shows that the ligand-binding modules of the LDLR share a similar tertiary fold. Each module is organized around a single bound calcium ion tightly coordinated by four highly conserved acidic residues at the C-terminal end of the module (*14*). The structure is also held together by three disulfide bonds, which stabilize the module in conjunction with the bound calcium ion (*23*). The ion binding site explains the observed calcium requirement for proper folding and disulfide bond formation in LDL-A modules (*14*, *23*−*26*) and provides a rationale for the known calcium requirement in lipoprotein binding (*27*).

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[1] Abbreviations: apoB, apolipoprotein B; apoE, apolipoprotein E; apoER2, apolipoprotein E receptor 2; β-VLDL, β-migrating very low-density lipoprotein; DMPC, dimyristoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGFP, epidermal growth factor precursor; GST, glutathione S-transferase; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; LA, low-density lipoprotein receptor type-A repeat; LBD, ligand binding domain; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LRP, low-density lipoprotein receptor-related protein; N-apoE, apoE N-terminal domain; RAP, receptor-associated protein; TBST, Tris-buffered saline with Tween 20; TEV, tobacco etch virus; VLDLR, very low-density lipoprotein receptor; YWTD, Tyr-Trp-Thr-Asp containing repeat; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Ni-NTA, nickel nitrilotriacetic acid.

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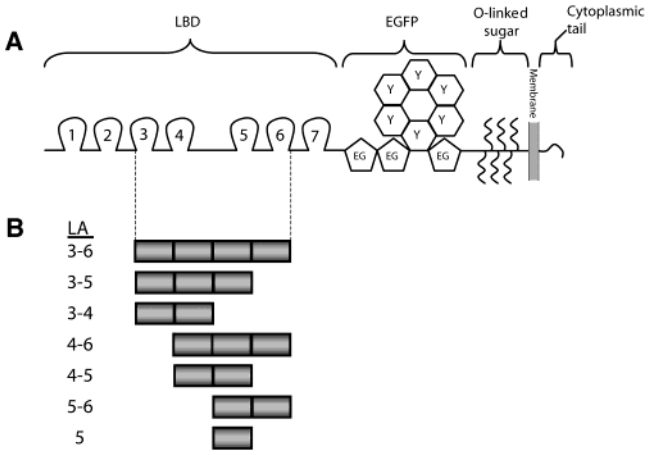

FIGURE 1: (A) Modular organization of the LDLR and (B) schematic of LA-repeat minireceptors used in this study: LBD, ligand binding domain; EGFP, epidermal growth factor precursor; EG, epidermal growth factor-like module; Y, repeat containing a YWTD motif.

Of the two apolipoprotein ligands, apoE, an exchangeable apolipoprotein of only 299 residues (*28*), has been more amenable to detailed structural characterization than apoB-100, a large, nonexchangeable apolipoprotein of 4536 residues (*29*). Structural and biochemical studies of apoE have identified two distinct domains (*30*, *31*), an N-terminal domain (N-apoE) that adopts a four-helix bundle structure in the apo state (*32*, *33*) and a ~10 kDa C-terminal lipophilic region (*30*, *31*). Mutagenesis, antibody blocking, and chemical modification studies all implicate a region rich in basic amino acids on the fourth helix as the primary site involved in receptor binding (*34*−*36*). Remarkably, N-apoE binds to the LDLR with high affinity only after complexation with lipid, and there is a large body of evidence that N-apoE (*37*−*42*) and structurally related apolipoproteins (*43*, *44*) undergo a conformational opening upon lipid association. A region rich in basic residues within apoB has also been implicated in receptor binding, although apoB exhibits little overall sequence similarity to apoE (*45*).

Although prior work clearly establishes that LA5 is required for high-affinity binding of particles containing apoE, the number of ligand-binding repeats sufficient to bind apoE ligands has not yet been determined. Similarly, with regard to the ligand, there are conflicting observations regarding whether one copy of lipid-activated N-apoE within a particle is capable of binding to the LDLR with high affinity or whether more than one copy is required (*46*−*48*). Here, we determine how many LA repeats are required for binding of apoE-containing ligands on the basis of binding studies performed with a series of LDLR-derived "minireceptors" containing up to four repeats (Figure 1B) and address the role of lipid in activation of N-apoE by using single-chain N-apoE multimers as LDLR ligands.

EXPERIMENTAL PROCEDURES

Subcloning of LDLR Minireceptors for Expression. LDLR minireceptors (Figure 1) were introduced into GST-fusion expression vectors downstream of GST by recombinational cloning. The residues included in each minireceptor are as follows (numbers in parentheses refer to residue position in the mature LDLR): LA3−6 (K86−N251), LA3−5 (K86−A211), LA3−4 (K86−G171), LA4−6, (V124−N251). LA4−5 (V124−A211), LA5−6 (D172−N251), and LA5 (D172−A211). LDLR inserts, flanked by an N-terminal tobacco etch (TEV) protease cleavage site (ENLTFQG) and either with or without a C-terminal c-Myc epitope tag (preceded by a three-residue glycine linker when present, i.e., *GGG*EQKLISEEDL), were amplified from a derivative of pLDLR2 (*49*) by nested PCR. Site-specific recombination sites were also included at the 5′ and 3′ termini to enable recombinational cloning first into the donor vector, pDONR201 (Gateway Cloning System, Life Technologies), and then into the expression vector, pDEST15, after verification of the identity of the inserts by DNA sequencing. The resulting expression constructs synthesize the various minireceptors as GST-{TEV-site}-LA chimeric proteins with or without a C-terminal myc epitope tag. Because TEV cleavage of the LA4−5 and LA4−6 constructs was inefficient, a six-residue AGASGG spacer was ultimately placed between the TEV cleavage site and the N-terminal residue of LA4 by site-directed mutagenesis to enable efficient cleavage.

Minireceptor Expression, Refolding, and Purification. Protein expression was induced by the addition of 0.5 mM IPTG during log phase growth (A_{595} of 0.6−0.8) of *E. coli* BL21(DE3) cells harboring the desired vector. Bacteria were harvested by centrifugation and lysed by probe sonication in Buffer A (50 mM Tris buffer pH 8.0, containing 150 mM NaCl, 20% (w/v) sucrose, and 1 mM EDTA). The cell debris was removed by centrifugation at 12 000*g* for 30 min at 4 °C, and the expressed fusion protein remaining in the supernatant was then captured onto Glutathione Sepharose beads (Pharmacia). After two washes with TBS (Tris-buffered saline; 150 mM NaCl, 20 mM Tris, pH 8.0), the fusion protein was cleaved with TEV protease in TBS supplemented with 0.5 mM EDTA and 1 mM DTT for 1 h at 20 °C to release the desired minireceptor from the beads. The supernatant was separated from the beads by centrifugation, and TEV protease was then removed from solution by capture onto Ni-NTA beads (Qiagen) via its hexahistidine tag, followed by filtration through fritted glass. Each minireceptor was refolded under conditions permitting disulfide exchange by exhaustive dialysis (72 h with daily buffer changes) at 4 °C against 50 mM Tris buffer, pH 8, containing 10 mM $CaCl_2$, 2 mM L-cysteine, and 0.5 mM L-cystine. The extent of disulfide bond formation was monitored by analytical reversed-phase HPLC using a Vydac C-18 column (The Separations Group, Hesperia, CA). For each minireceptor, the predominant disulfide isomer was purified to apparent homogeneity by reversed-phase HPLC on a preparative-scale Vydac C-18 column (The Separations Group, Hesperia, CA) using a shallow acetonitrile gradient (0.1%/min). The fractions containing the desired protein (confirmed in each case by MALDI-TOF mass spectrometry) were combined and lyophilized for storage at −80 °C. Protein concentration was determined by measurement of protein absorbance in 20 mM sodium phosphate buffer, pH 6.5, containing 6 M guanidine hydrochloride, using a molar extinction coefficient calculated as described (*50*).

Subcloning and Site-Directed Mutagenesis of N-apoE and Its Single-Chain Multimeric Variants. DNA encoding N-apoE (residues 1−191) with an N-terminal hexahistidine tag was subcloned into a derivative of pET 24 (Novagen).

The K143A, K146A variant was created by site-directed mutagenesis with a Quickchange mutagenesis kit (Stratagene). Single-chain multimers were assembled by first introducing a linker sequence, which contained an Nhe I restriction site between residue R191 and the TAA stop codon, by PCR and then inserting additional copies of N-apoE on a cassette spanned by Spe I and Bam HI restriction sites. When assembled, each single-chain multimer encoded a hexahistidine tag, followed by two to four copies of N-apoE separated by a GGGASG six-residue spacer and terminated with the sequence GGGAS.

Expression of the various N-apoE polypeptides was induced in BL21(DE3) cells, and cells were lysed in Buffer A as above. The desired N-apoE variant was captured onto Ni-NTA agarose beads (Qiagen) which were washed twice in TBS buffer (pH 8.0) containing 20 mM imidazole, and the protein was eluted from the beads with 250 mM imidazole in TBS. After Ni-NTA affinity purification, N-apoE variants were purified to apparent homogeneity by gel-filtration chromatography on a Superdex 75 column (Pharmacia). Only fractions containing monomeric protein, as judged by the elution volume, were pooled and stored in aliquots at −80 °C. Protein concentration was determined by absorbance as above.

Discoidal complexes of the different N-apoE proteins were prepared by bath sonication with dimyristoylphosphatidylcholine (DMPC) as described (*51*). Certain disk preparations were further purified away from uncomplexed N-apoE by pooling high molecular weight fractions by passage over a Superdex 300 gel filtration column (Pharmacia); these fractions were concentrated by ultrafiltration (Vivascience). In all cases, 95% or more of the N-apoE was incorporated into disks as judged by native gel electrophoresis (Phast gel, Pharmacia), which was also used to estimate the size of discoidal complexes by comparison with molecular weight standards.

RAP Expression and Purification. Briefly, a clone for expression of human RAP as a GST fusion in bacteria was kindly provided by Dr. Guojun Bu (*52*). After induced expression and cell lysis, RAP was purified by affinity chromatography on glutathione−agarose beads, released from GST and the beads by cleavage with thrombin, and purified to apparent homogeneity on SDS−PAGE using size-exclusion chromatography in a final step.

Isothermal Titration Calorimetry (ITC). Calcium titrations were performed at pH 5.6 in Chelex-treated MES buffer at 25 °C using a MicroCal Inc. VP-ITC calorimeter (Northampton, MA) in a 1.3 mL reaction vessel. A $CaCl_2$ stock solution (0.5−1 mM) was added in 4−6 μL increments to various minireceptors present at concentrations from 30 to 120 μM, depending on the protein. Calorimetry data were analyzed with the program Origin 5.0 (OriginLab Corp., Northampton, MA).

Immunoprecipitation Binding Assay. Myc-tagged minireceptors, purified by reversed-phase HPLC in their calcium-free form, were used as the starting point for these experiments. Each minireceptor was reconstituted in TBS binding buffer containing 0.1% Tween 20 and either 2 mM $CaCl_2$ or 2 mM EDTA and gently rocked for 1 h at 4 °C in the presence of equimolar amounts (based on the number of N-apoE molecules) of N-apoE·DMPC. Complexes were then recovered by incubation (1 h at 4 °C) with protein G agarose (Sigma) beads either loaded with (Figures 4 and 6) or covalently coupled to (Figures 5 and 6) an anti-c-Myc monoclonal antibody. The beads were pelleted by centrifugation, washed twice with incubation buffer, and then resuspended in 30 μL of sample loading buffer for SDS−PAGE. After boiling for 10 min, bound proteins were separated by SDS−PAGE on a 15% gel and were analyzed by Western blot using a polyclonal anti-apoE antibody conjugated to horseradish peroxidase (HRP; Biodesign Intl., Kennebunk, ME). Signal was visualized with a chemiluminescent detection method (Pierce, SuperSignal West Femto). To assess the amount of c-Myc tagged protein in each sample, gels were either run in duplicate and subjected to Western blotting with an HRP-conjugated anti-myc antibody or acid-stripped and reprobed with HRP-conjugated anti-myc (Figure 6). The RAP concentration and the untagged competitor concentration used for competition binding studies ranged from 1.7 to 35 μM, which corresponds to 1−20 molar equivalents when compared with the amount of input N-apoE ligand molecules.

RESULTS

LDLR Minireceptors Containing up to Four LA Repeats Refold Efficiently to a Predominant Disulfide Isomer. Because the LDLR minireceptors are overexpressed recombinantly in bacteria, they must be refolded under conditions that allow for formation of native disulfide bonds. The buffer used to promote folding of LA modules must permit disulfide exchange and must also contain calcium, because coordination of calcium by a conserved cluster of acidic residues at the C-terminal end of each module stabilizes the module in conjunction with its three native disulfide bonds (*14*). Redox refolding in the presence of calcium has been used successfully to fold individual modules, module pairs, and even a fragment of the LDLR encompassing all seven LA modules of the ligand-binding domain in active form (*53*).

In the studies reported here, all minireceptors were refolded in the presence of calcium under conditions that permit disulfide exchange. Minireceptors containing as many as four LA repeats fold to a single preferred disulfide isomer, as judged by reversed-phase HPLC analysis of the product distribution (Figure 2, bottom trace). When this refolded sample is incubated under conditions permitting disulfide exchange in the presence of the calcium chelator EDTA, a broad distribution of nonnative disulfide-bonded products is observed (Figure 2, middle trace). Reduction of either sample with dithiothreitol overnight yields a single peak with a significantly later retention time (Figure 2, top trace), consistent with previous studies of individual LA modules and module pairs (*23*, *26*).

Stoichiometry of Calcium Binding by Various Minireceptors Deduced Using Isothermal Titration Calorimetry Confirms Refolding to the Native State. All LA repeats studied thus far have a single calcium coordination site required for maintenance of the structural integrity of the folded module. Therefore, to verify that the LA repeats present in each protein were all properly folded, we also tested whether the stoichiometry of calcium binding per folded module was ∼1:1 in our refolded minireceptors by isothermal titration calorimetry (Figure 3). Titration of LA5-myc shows saturation of a single site for calcium binding with an approximate

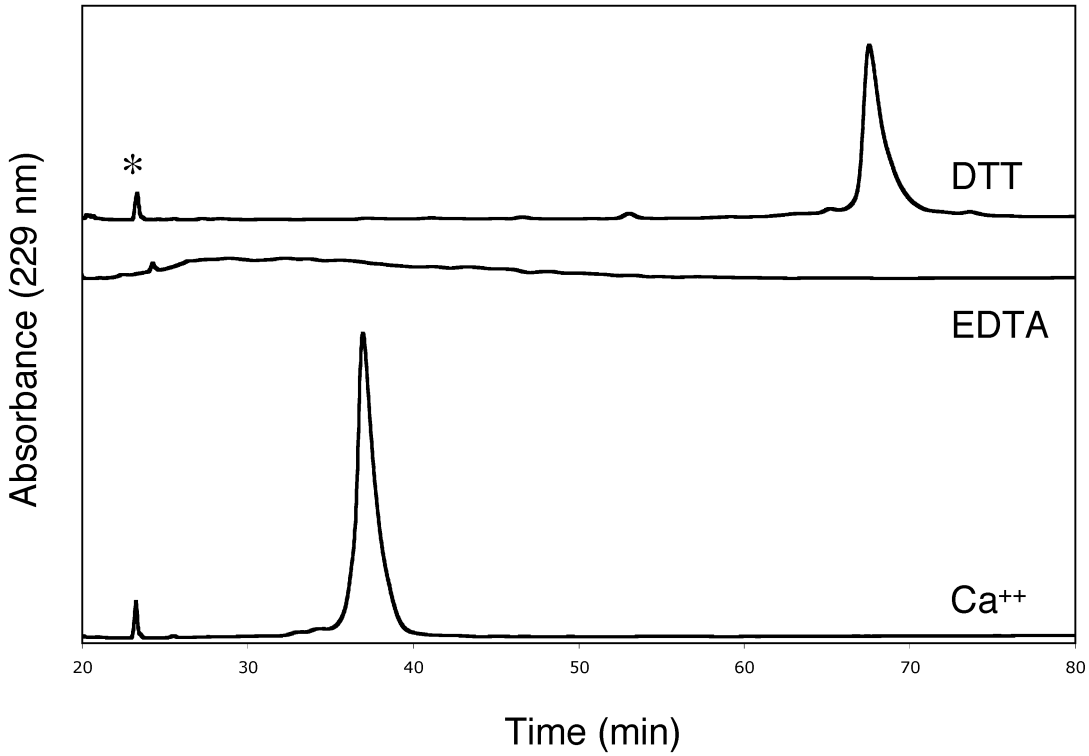

FIGURE 2: HPLC chromatogram of products formed after incubation of LA3−6-c-Myc under different conditions: (bottom trace) chromatogram after refolding in a redox buffer containing 10 mM $CaCl_2$ (see methods); (middle trace) chromatogram after refolding in redox buffer with 10 mM EDTA added in place of $CaCl_2$; (top trace) chromatogram after reduction with 50 mM DTT. The asterisk at an elution time of approximately 23 min denotes a column-derived impurity.

K_d of 7.7×10^{-7} M at pH 5.6 (Figure 3A,B). This value is fully consistent with the K_d for calcium of 40 nM measured by fluorescence at neutral pH (*23*, *26*) and with the 25-fold reduction in K_d observed by Gettins and colleagues upon shifting the pH from 7.4 to 5.0 (*22*; see also ref *54*). In contrast, titration of LA3−6 only exhibits saturation after addition of four calcium ions per polypeptide, as predicted for a minireceptor spanning four modules with one calcium-binding site each (Figure 3C,D). Similar ITC results were obtained for each minireceptor tested.

LA3−6 Binds Native DMPC-Complexed N-apoE but not the K143A, K146A N-apoE Double Mutant. To detect formation of complexes between different minireceptors and N-apoE·DMPC, we developed an immunoprecipitation assay using purified recombinant minireceptors bearing a C-terminal myc epitope tag. Complexes were first captured onto beads loaded with an anti-myc antibody. Bound N-apoE·DMPC was then liberated by denaturation in boiling SDS loading buffer and detected by Western blot using an HRP-conjugated anti-apoE polyclonal antibody. The immunoprecipitation data confirm that the LA3−6 minireceptor binds to N-apoE·DMPC and requires calcium for binding (Figure 4A). The binding of N-apoE·DMPC to the LA3−6 minireceptor is specific because no binding is detected with the N-apoE K143A, K146A variant, which has point mutations of two key basic residues known to be important for binding to the native LDLR (*36*, *55*) and because binding is blocked by addition of the receptor-associated protein (RAP), which is known to compete with apoE-containing lipoproteins (*56*) for binding to the LDLR (Figure 5).

LA4−5 Constitutes the Minimum-Length Minireceptor Sufficient To Coimmunoprecipitate N-apoE·DMPC. We next screened all of the recombinant minireceptors (Figure 1B) in the immunoprecipitation assay to identify the smallest minireceptor that specifically recognized N-apoE·DMPC. LA3−6, LA3−5, LA4−6, and LA4−5 all bound N-apoE·DMPC, and in each case, calcium was required for binding (Figure 4B). In contrast, LA3−4, LA5−6, and LA5 fail to bind N-apoE·DMPC, even in the presence of calcium (Figure 4B). In addition, the same group of minireceptors, LA3−6, LA3−5, LA4−6, and LA4−5, effectively compete in solution for binding of N-apoE·DMPC to LA3−6, whereas the others do not (Figure 5). Taken together, these results clearly demonstrate that LA4−5 is the minimal fragment capable of specific, high-affinity binding to N-apoE·DMPC.

Binding of Lipid-Activated N-apoE to LA3−6 Requires Contact with More Than One Intact Receptor Binding Site. To investigate how lipid activates N-apoE for binding to LA3−6, we constructed single-chain multimers containing from two to four copies of N-apoE separated from one another by a short linker sequence (GGGASG). Following sonication with DMPC and incubation at 24 °C, >95% of each multimeric N-apoE variant is incorporated into high molecular weight DMPC complexes of approximately equivalent size (∼600 kDa), as judged by native gel electrophoresis, and each multimer exhibits calcium-dependent binding to LA3−6 only after complexation with lipid (data not shown).

To test whether the lipid-induced conformational rearrangement of a single active copy of N-apoE is sufficient to confer high-affinity binding to LA3−6, we created altered forms of the single-chain N-apoE trimer in which one or two of the three receptor binding sites were inactivated by introducing the K143A/K146A pair of point mutations.

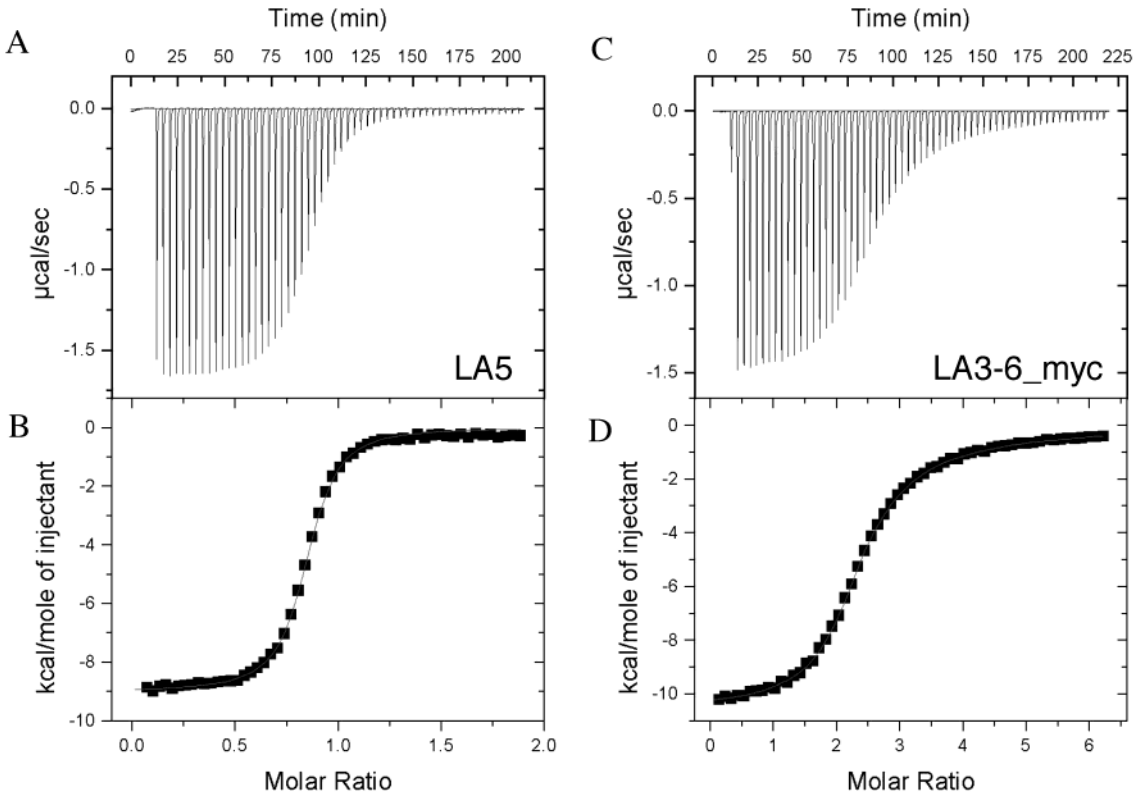

FIGURE 3: Representative calorimetric titrations for the binding of Ca^{2+} to different minireceptors. Panels A and B show titration of LA5-c-myc. A 0.9 mM stock solution of $CaCl_2$ was added in 5 μL increments to a 110 μM solution of LA5-c-Myc in 20 mM MES buffer, pH 5.6. Panel A shows the raw data; panel B shows the binding isotherm fitted to a single-site model. Panels C and D show titration of LA3−6. A 1 mM stock solution of $CaCl_2$ was added in 4 μL increments to a 30 μM solution of LA3−6 in 20 mM MES buffer, pH 5.6. Panel C shows the raw data; panel D shows the binding curve fitted to a four-site model.

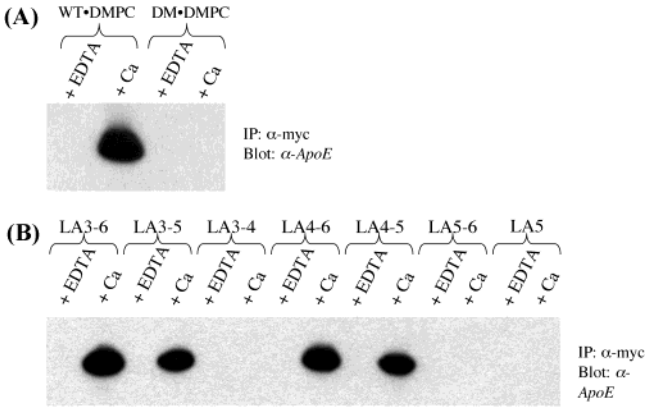

FIGURE 4: (A) LA3−6-c-Myc binds to native N-apoE•DMPC (WT) but not to K143A, K146A mutant N-apoE•DMPC (DM). Complexes were immunoprecipitated with anti-myc monoclonal antibody in the presence of either 2 mM EDTA or 2 mM $CaCl_2$ and analyzed by Western blot with an anti-apoE polyclonal antibody. Panel B shows binding of different c-Myc-tagged minireceptors to N-apoE•DMPC. Complexes were immunoprecipitated with anti-myc monoclonal antibody in the presence of either 2 mM EDTA or 2 mM $CaCl_2$, and analyzed by Western blot with an anti-apoE polyclonal antibody.

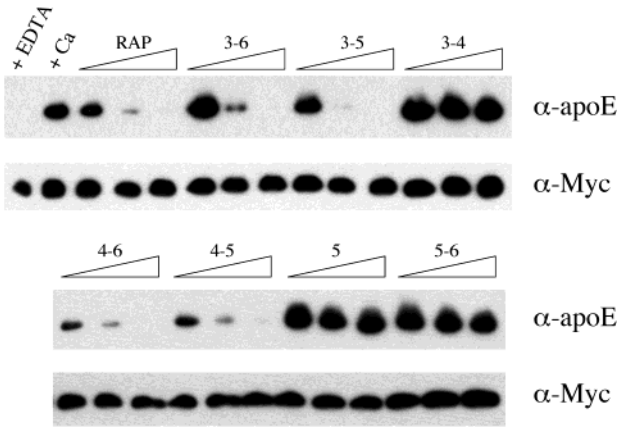

FIGURE 5: Competition binding assay. Complexes between N-apoE•DMPC and myc-tagged LA3−6 minireceptors were immunoprecipitated with anti-myc monoclonal antibody in the presence of various untagged competitors and $CaCl_2$ (2 mM), except as indicated (EDTA, 2 mM). Competitors were included at stoichiometries of 1, 5, or 20 molar equivalents with respect to input N-apoE molecules. The upper panels show the Western blot analyzed with an anti-apoE polyclonal antibody. The bottom panels show duplicate blots analyzed by Western blot with an anti-myc monoclonal antibody. RAP denotes receptor-associated protein.

Whereas binding to LA3−6 is detected with multichain N-apoE constructs containing two (DWW) or three (WWW) intact receptor binding sites, binding is not detected when only a single intact binding site (DWD) is present, suggesting that lipid association alone is not sufficient to convert a single copy of N-apoE into a high-affinity LDLR ligand (Figure 6).

DISCUSSION

The primary function of the LDL receptor is to transport lipoprotein particles into cells. The LA modules at the N-terminal end of the LDL receptor harbor the binding sites for both LDL particles and apoE-containing ligands. LA modules found clustered in groups of 2−11 repeats also

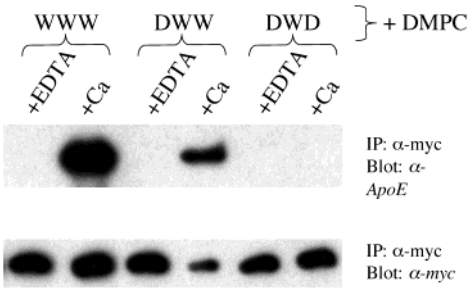

FIGURE 6: Binding of LA3−6-c-Myc to lipid-associated N-apoE trimers harboring either one, two, or three intact receptor-binding sites. Complexes were immunoprecipitated with anti-myc monoclonal antibody in the presence of either 2 mM EDTA or 2 mM $CaCl_2$. The upper panel shows the Western blot analyzed with an anti-apoE polyclonal antibody; the bottom panel shows the same blot probed with an anti-myc monoclonal antibody after stripping with acid. W denotes wild-type N-apoE; D denotes the double mutant (K143,146A) N-apoE; DMPC denotes dimyristoylphosphatidylcholine.

confer apoE binding activity upon close relatives of the LDLR, including the VLDLR, apoER2, LRP-1, and LRP-2.

In the work reported here, we deduce that the LA4−5 two-repeat pair is sufficient to bind apoE-containing ligands, on the basis of binding studies performed with a series of LDLR-derived "minireceptors" containing up to four repeats (Figures 4 and 5). Though LA5 is necessary for association with both apoE- and apoB-100-containing ligands (*13*), it alone is not sufficient to mediate high-affinity binding.

The minimal requirement of two LA modules for formation of stable complexes between the LDLR and N-apoE is similar to that observed in the characterization of complexes between LRP-1 and two of its ligands, the receptor-associated protein (RAP) and α-1-macroglobulin (*57*−*59*). Any of several repeat pairs from the second cluster of LA modules in LRP-1 bind to the receptor-associated protein RAP (*57*), and both the repeat 3−4 and repeat 4−5 pairs from the same cluster exhibit sufficiently strong affinity for α-1-macroglobulin to enable isolation of a complex by size exclusion chromatography (*58*). Despite this similarity, though, the requirement for formation of complexes between the LDLR and N-apoE is more stringent; complexes of N-apoE·DMPC with the LA4−5 module pair are readily detected, whereas complexes with the LA3−4, LA5−6, or LA6−7 pairs are not (Figure 5 and data not shown).

One possible explanation for the observed binding specificity is that the linker separating modules 4 and 5 is much longer than the linker separating all other module pairs: a 12-residue linker separates the terminal cysteines of modules 4 and 5, whereas only four residues separate the other repeat pairs. Thus, the 12-residue spacer may enable repeats 4 and 5 to be positioned far enough away from each other to contact different N-apoE subunits in an effectively bivalent arrangement (see below), whereas the shorter four-residue linkers between the other module pairs may not be as permissive.

Remarkably, repeats LA4 and LA5 also correspond precisely to the two modules that form direct intramolecular contacts with the YWTD β-propeller domain in the X-ray structure of the LDLR ectodomain determined at endosomal pH (*15*). The finding that the same central repeats confer high-affinity binding of apoE-containing ligands lends further credence to the proposal that at endosomal pH the YWTD propeller domain either directly displaces the ligand from the LA modules or prevents rebinding of ligands after release by acting as an intramolecular ligand.

There is substantial evidence that lipid is required for high-affinity binding of apoE to the LDLR. Two possible mechanisms, which are not mutually exclusive, have been proposed to explain how lipid enhances the affinity of apoE for the receptor. The first supposes that association with lipid triggers a conformational change in apoE that permits binding to the receptor, while the second argues that lipid association creates a multivalent ligand for the receptor.

On one hand, biophysical studies of the isolated N-terminal domain clearly show that the four-helix bundle rearranges when incorporated into complexes with lipid (*37*−*42*). In addition, the N-terminal domain of apoE becomes a high-affinity ligand for the LDLR when incorporated into complexes with the phospholipid dimyristoylphosphatidylcholine (DMPC), and early studies of particles bearing varying molar ratios of active and inactivated forms of apoE did not appear to support a multivalent ligand model (*46*). On the other hand, a linear tandem repeat of residues 141−155 of apoE (*47*, *48*) inhibits uptake of LDL particles by the LDLR. The latter finding suggests (1) that the peptide binds directly to the receptor and (2) that a multivalent ligand may be all that is required for apoE to bind to the LDLR. Thus, whether lipid activation of apoE occurs because (i) the N-terminal domain of native apoE undergoes a conformational change upon complexation with lipid, (ii) lipid serves to create a multivalent ligand, or (iii) a combination of these two events takes place remained unresolved.

To address the role of multivalency, we prepared three different N-apoE linear trimers: the first has three intact receptor-binding sites (WWW), the second has two adjacent intact receptor-binding sites (DWW), and the third only has the central receptor-binding site intact (DWD). The data show that the WWW and DWW polypeptides are competent for receptor binding, whereas the DWD form is not. These findings indicate that a single local receptor-binding site in its lipid-activated conformation is not sufficient to bind to the LDLR with high affinity and supports the conclusion that multivalency (at least two nearby intact receptor binding epitopes) is required for receptor binding. This interpretation also reconciles the observations that, on one hand, lipid induces a conformational change in N-apoE and, on the other hand, multiple copies of either N-apoE or peptides encompassing the receptor-binding region are required to promote receptor binding; in addition to inducing a conformational change in the structure of N-apoE, lipid association enhances the affinity of apoE for the LDLR in part by creating a multivalent ligand. Note that this interpretation does not require that only one receptor trimer be loaded per disk; if disks containing two or more DWD trimers do form, the distance between the nearest intact receptor binding sites would be too great to be spanned by the LA3−6 receptor fragment.

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