methods

Characterization of low density lipoprotein receptor ligand interactions by fluorescence resonance energy transfer

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Abstract **The low density lipoprotein receptor (LDLR) is the prototype of a family of cell surface receptors involved in a wide range of biological processes. A soluble low density lipoprotein receptor (sLDLR) and a tryptophan (Trp)-deficient variant human apolipoprotein E3 (apoE3) N-terminal domain (NT) were used in binding studies. The sole cysteine in apoE3-NT was covalently modified with an extrinsic fluorescence probe, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-napthyl)ethylenediamine (AEDANS), and the protein was complexed with lipid. Incubation of sLDLR with AEDANS-Trp-null apoE3-NT dimyristoylphosphatidylcholine (DMPC) disks, but not lipid-free AEDANS-apoE, induced an enhancement in AEDANS fluorescence emission intensity (excitation, 280 nm) consistent with intermolecular energy transfer from excited Trp in sLDLR to receptor-bound apoE. Ligand binding to sLDLR required calcium and was saturable. In competition binding assays, unlabeled apoE3-NT DMPC inhibited AEDANS-apoE DMPC binding to sLDLR more effectively than low density lipoprotein. Fluorescence changes in this system reflected pH-dependent ligand binding and release from sLDLR consistent with models derived from the X-ray crystal structure of the receptor at endosomal pH. Intermolecular energy transfer from excited Trp in LDLR family members to fluorescently tagged ligands represents a sensitive and convenient assay for the characterization of the myriad molecular interactions ascribed to this family of receptor.**—Yamamoto, T., J. Lamoureux, and R. O. Ryan. **Characterization of low density lipoprotein receptor ligand interactions by fluorescence resonance energy transfer.** ***J. Lipid Res.*** **2006.** 47: **1091–1096.**

Supplementary key words fluorescence spectroscopy • apolipoprotein E • apolipoprotein B • assay • lipid • ligand binding

Since the discovery of the low density lipoprotein receptor (LDLR), a large number of homologous receptors have been identified and/or characterized (1, 2). All members of this ancient receptor family, of which LDLR is the prototype, possess a modular organization minimally composed of a short intracellular domain, a single membrane-spanning sequence, epidermal growth factor (EGF) precursor homology segments, and a series of complement-like, ligand binding, LDL-A repeats (2). Despite their similarities, family members manifest distinct ligand binding preferences and functional properties. Indeed, ligand binding among LDLR family members is now recognized to affect cellular functions ranging from lipoprotein endocytosis to cell migration, pericellular proteolysis, signal transduction, and synaptic plasticity (2, 3).

Classical assays of LDLR family members involve radiolabeled ligand binding to receptors present on the surface of cultured cells (4). Others assays, including surface plasmon resonance, ligand blotting, and solid phase microtiter plate systems, have also been used to characterize binding (5). These methods involve multiple steps and, in general, are not well-suited for detailed molecular characterization studies. Here, we report a fluorescence-based solution assay system that is applicable to all members of this expanding receptor family. Binding is detected by intermolecular fluorescence resonance energy transfer between excited tryptophan (Trp) residues in a soluble low density lipoprotein receptor (sLDLR) and an extrinsic fluorophore covalently bound to a Trp-deficient protein ligand. Using this assay, apolipoprotein E (apoE) interaction with LDLR shows saturability, a requirement for calcium and ligand lipid association as well as competition by unlabeled ligand and pH-dependent ligand release. Given the growing size of this receptor family and its documented ligand diversity, adaptation of this method to other ligands may reveal new insights.

MATERIALS AND METHODS

sLDLR expression and isolation

The nucleotide sequence encoding human LDLR residues 1–699 (a soluble LDLR fragment lacking the transmembrane and

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Abbreviations: AEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-napthyl)ethylenediamine; apoE, apolipoprotein E; DMPC, dimyristoylphosphatidylcholine; EGF, epidermal growth factor; LDLR, low density lipoprotein receptor; sLDLR, soluble low density lipoprotein receptor; Trp, tryptophan.

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intracellular modules) plus a 20 residue signal peptide and a C-terminal histidine tag was amplified, digested, and cloned into the pBluescript (Stratagene) plasmid at vector-encoded *Kpn*I and *Not*I restriction sites. The sLDLR sequence was subcloned into pCEP4 vector (Invitrogen), and the plasmid construct was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). Cells were cultured in DMEM plus 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). Stably transfected cells were obtained by culturing in medium supplemented with hygromycin (200 μg/ml). sLDLR was isolated from conditioned medium by a combination of Ni^{2+} chelation chromatography and heparin-Sepharose chromatography. Wild-type human apoE3 N-terminal domain (NT; residues 1–183) and a Trp-null variant (6) were expressed in *Escherichia coli* BL21 cells as described (7). The protein was labeled at cysteine 112 with *N*-(iodoacetyl)-*N*′-(5-sulfo-1-napthyl)ethylenediamine (AEDANS) as described (8), and labeling efficiency was determined spectrophotometrically. Human LDL, isolated by ultracentrifugation, was obtained from Intracel (Frederick, MD).

Receptor ligand incubations

ApoE3-NT dimyristoylphosphatidylcholine (DMPC) complexes were prepared as described elsewhere (9). Various apoE3-NT ligands (1 μg of protein, unless specified otherwise) were incubated with sLDLR (2 μg) in 20 mM Tris, pH 7.2, 2 mM $CaCl_2$, and 90 mM NaCl (final sample volume, 300 μl) at 25°C for 1 h before spectroscopy.

Spectroscopy

Fluorescence measurements were obtained on a Perkin-Elmer LS 50B luminescence spectrometer equipped with a thermostatted cell holder. Samples were excited at 280 nm, and emission was monitored from 300 to 550 nm (5.0 nm slit width).

Analytical procedures

Protein concentrations were determined by the BCA assay (Pierce Chemical Co.) using BSA as a standard. SDS-PAGE was performed on 4–20% acrylamide gradient slabs at 30 mA constant current and stained with Coomassie blue. For immunoblotting, protein samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with a rabbit polyclonal 6x His-Tag antibody (Abcam, Inc.) followed by detection with horseradish peroxidase-linked secondary antibody.

RESULTS

Component characterization and assay design

Isolated recombinant sLDLR comprising N-terminal residues 1–699 gives rise to a single band on SDS-PAGE that migrates faster under nonreducing than reducing conditions (**Fig. 1A**), a known property of correctly folded cysteine-rich, LDL-A repeats present in all members of this receptor family (5). A fluorescence emission spectrum (excitation, 280 nm) of AEDANS-labeled Trp-null apoE3-NT DMPC disks verified the lack of Trp in this protein and revealed low fluorescence emission intensity around 470 nm attributable to the direct excitation of AEDANS

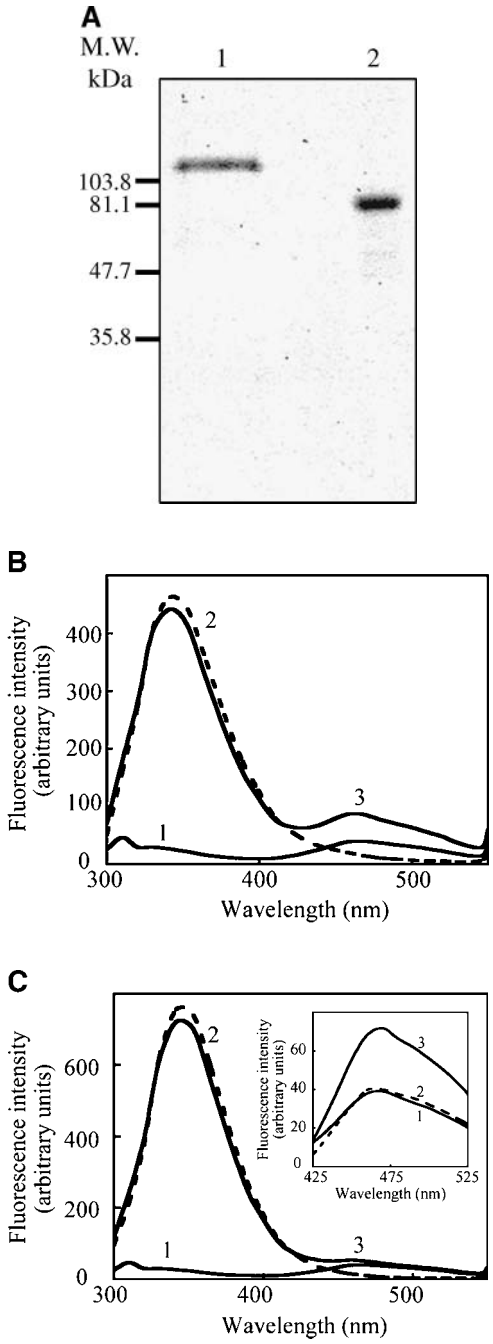

Fig. 1. Characterization of soluble low density lipoprotein receptor (sLDLR) and *N*-(iodoacetyl)-*N*′-(5-sulfo-1-napthyl)ethylenediamine-apolipoprotein E (AEDANS-apoE). A: SDS-PAGE of sLDLR under reducing (lane 1) and nonreducing (lane 2) conditions. M.W., molecular mass. B: Fluorescence emission spectra of AEDANS-tryptophan (Trp)-null apoE3-N-terminal domain (NT) dimyristoylphosphatidylcholine (DMPC; 1 μg) (curve 1), sLDLR (2 μg) (curve 2), and sLDLR plus AEDANS-Trp-null apoE3-NT DMPC (curve 3). C: Fluorescence emission spectra of AEDANS-Trp-null apoE3-NT DMPC (1 μg) (curve 1), BSA (5 μg) (curve 2), and BSA plus AEDANS-Trp-null apoE3-NT DMPC (curve 3). In B and C, samples (300 μl final volume) were excited at 280 nm. C inset: Background-subtracted fluorescence emission spectra of AEDANS-Trp-null apoE3-NT DMPC (1 μg) (curve 1), panel C spectrum 3 minus spectrum 2 (curve 2), and panel B spectrum 3 minus spectrum 2 (curve c).

at 280 nm (Fig. 1B). Consistent with the known properties of this fluorophore (6), excitation of the sample at 336 nm elicited strong fluorescence emission centered at 470 nm (data not shown). By the same token, excitation of sLDLR at 280 nm results in strong fluorescence emission around 340 nm, with negligible fluorescence emission at 470 nm. Energy transfer between Trp and AEDANS occurs when an excited Trp donor and AEDANS acceptor reside within 100 Å of each other, with the efficiency of energy transfer being dependent on the inverse sixth power of the distance between the chromophores (10). In our system, we hypothesize that the AEDANS-apoE interaction with LDL-A repeats in sLDLR will result in the localization of AEDANS moieties at a distance wherein energy transfer will occur. Consistent with this, incubation of AEDANS-Trp-null apoE3-NT DMPC with sLDLR and excitation of the sample at 280 nm results in a reproducible enhancement in AEDANS fluorescence emission intensity. To determine whether AEDANS fluorescence emission enhancement observed upon incubation with sLDLR is attributable to a specific binding interaction between ligand and receptor, AEDANS-Trp-null apoE3-NT DMPC was incubated with BSA (Fig. 1C). The presence of excess albumin relative to AEDANS-apoE had no effect on AEDANS fluorescence emission intensity, indicating that this unrelated Trp-containing protein is unable to serve as an energy donor to AEDANS-Trp-null apoE3-NT DMPC in this system. The inset in Fig. 1C shows background-subtracted fluorescence emission spectra obtained when AEDANS-Trp-null apoE3-NT DMPC is incubated alone (spectrum 1), with 5 μg of BSA (spectrum 2), and with 2 μg of sLDLR (spectrum 3).

Effect of ligand concentration and EDTA on receptor binding

In addition to binding specificity, a hallmark of ligand interactions with LDLR family members is saturability. When increasing amounts of AEDANS-Trp-null apoE3-NT DMPC were incubated with a fixed amount of sLDLR, background-subtracted AEDANS fluorescence emission intensity increased in a concentration-dependent manner and reached a plateau, after which no further fluorescence emission enhancement was observed (**Fig. 2A**). By contrast, when lipid-free AEDANS-Trp-null apoE3-NT was used, sLDLR had little effect on AEDANS fluorescence emission intensity. This result is consistent with data from classical LDLR binding assays that revealed that lipid-free apoE3-NT does not serve as a ligand for LDLR yet has receptor recognition properties upon association with lipid (11). Likewise, LDL-A repeats are known to require calcium for correct folding and ligand binding (12). When sLDLR was preincubated with EDTA before incubation with AEDANS-Trp-null apoE3-NT DMPC, an EDTA concentration-dependent diminution in sLDLR-induced AEDANS fluorescence emission intensity enhancement was noted (Fig. 2B). A similar trend was seen with EGTA, although the effect was less dramatic (data not shown).

Effect of competitor ligands on sLDLR-dependent AEDANS-apoE3 fluorescence intensity enhancement

To further evaluate the specificity of AEDANS-Trp-null apoE3-NT DMPC interaction with sLDLR, competition binding experiments were conducted (**Fig. 3**). Introduction of unlabeled Trp-null apoE3-NT DMPC resulted in a concentration-dependent decline in sLDLR-induced AEDANS-Trp-null apoE3-NT DMPC fluorescence emission intensity. When wild-type apoE3-NT was used as the competitor ligand, a similar result was obtained, illustrating that competition experiments may be performed with Trp-containing ligands. Likewise, human LDL induced a concentration-dependent decrease in AEDANS-apoE3 fluorescence emission intensity (Fig. 3). In keeping with the results of classical cell-based receptor binding assays, LDL was a less effective competitor than apoE (13).

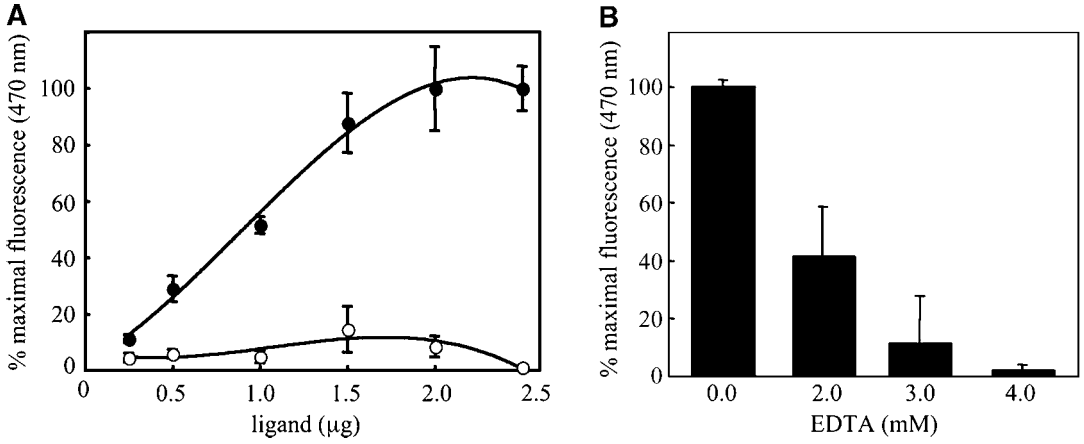

Fig. 2. Factors affecting sLDLR-induced AEDANS-apoE3 fluorescence emission intensity. A: Two micrograms of sLDLR was incubated with increasing amounts of AEDANS-Trp-null apoE3-NT DMPC (closed circles) or lipid-free AEDANS-Trp-null apoE3-NT (open circles). Fluorescence emission intensity values reported were obtained after subtraction of fluorescence emission intensity of ligand alone at each concentration. B: One microgram of AEDANS-Trp-null apoE3-NT DMPC and 2 μg of sLDLR were incubated in the presence of the indicated amounts of EDTA. Samples were excited at 280 nm, and fluorescence emission intensity was determined at 470 nm. Values reported are averages ± SD (n = 3).

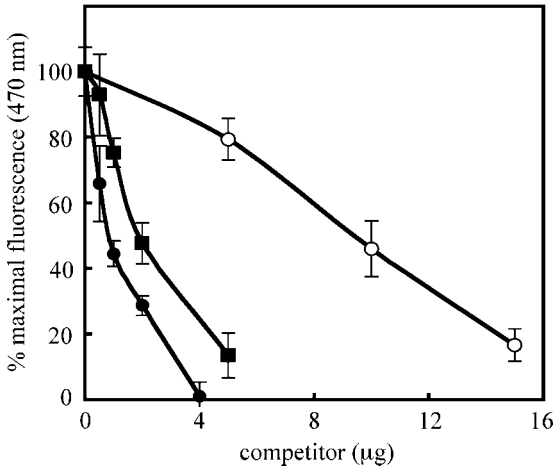

Fig. 3. Effect of unlabeled apoE3 and LDL on sLDLR-induced enhancement of AEDANS-apoE3 fluorescence emission intensity. One microgram of AEDANS-Trp-null apoE3-NT DMPC and 2 μg of sLDLR were incubated in the presence of increasing concentrations of unlabeled Trp-null apoE3-NT DMPC (closed circles), wild-type apoE3-NT (closed squares), or human LDL (open circles). Samples (300 μl final volume) were excited at 280 nm, and fluorescence emission intensity was determined at 470 nm. Values reported are averages ± SD (n = 3).

Studies of pH-dependent ligand release

Rudenko et al. (14, 15) reported the X-ray crystal structure of a large extracellular portion of human LDLR at endosomal pH. The structure revealed the organization of six of the seven LDL-A repeats in LDLR as well as an intact EGF precursor homology region with its β-propeller segment and three EGF repeats. A striking finding of this structure is that the β-propeller domain makes close contact with LDL-A repeats 4 and 5. Because the structure was determined at pH 5.3 and LDL-A repeats 4 and 5 are critical for ligand interaction (16, 17), these data provide a structural rationale for how LDLR releases bound ligand within the endosome. By acting as a cryptic, pH-dependent, intramolecular alternative substrate, the β-propeller domain may be capable of displacing bound ligand. To test this, AEDANS-Trp-null apoE3-NT DMPC was incubated with sLDLR at pH 7.2 or 5.3 (**Fig. 4A**). In control incubations, no change in AEDANS emission intensity occurred as a function of pH in the absence of sLDLR. At pH 7.2, but not pH 5.3, sLDLR induced the expected enhancement in AEDANS fluorescence emission intensity, consistent with ligand interaction. Further study revealed that bound ligand is rapidly released from sLDLR upon shifting the pH from 7.2 to 5.3 (Fig. 4B). For comparison, competition-based displacement of receptor-bound AEDANS-apoE by unlabeled apoE3-NT DMPC was slower, as judged by time-dependent changes in AEDANS fluorescence emission intensity.

DISCUSSION

The LDLR family of proteins functions in fundamental cellular processes through interaction with an array of oftentimes unrelated ligands (1, 2). Since the discovery by Brown and Goldstein that LDLR is a key regulator of plasma cholesterol homeostasis (18, 19), members of this receptor family have been implicated in numerous additional processes, including the regulation of proteases and their inhibitors, protein reabsorption from glomerular filtrate, neuronal migration, signal transduction, and intracellular sorting (1). LDLR family members have been

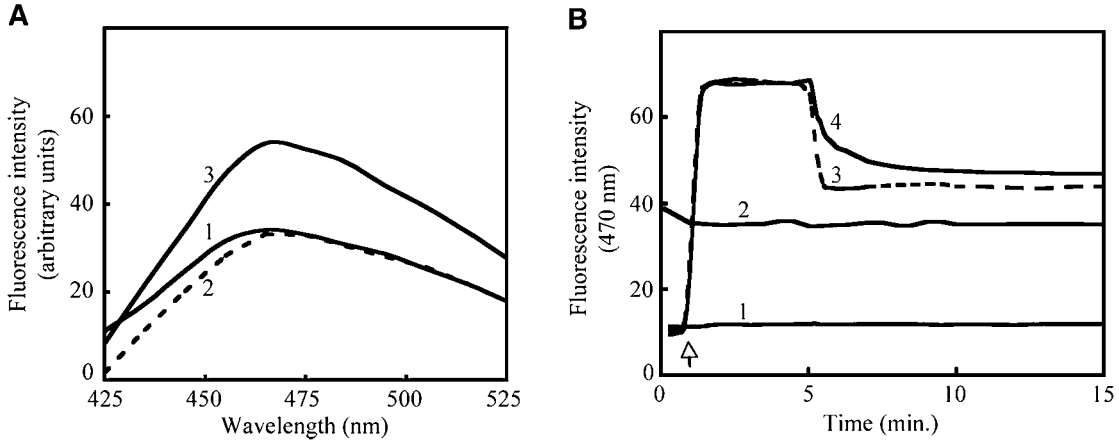

Fig. 4. Effect of solution pH on apoE-sLDLR interactions. A: Fluorescence emission spectra of 1 μg of AEDANS-Trp-null apoE3-NT DMPC alone (pH 7.2) (curve 1), 1 μg of AEDANS-Trp-null apoE3-NT DMPC plus 2 μg of sLDLR (pH 5.3) (curve 2), and 1 μg of AEDANS-Trp-null apoE3-NT DMPC plus 2 μg of sLDLR (pH 7.2) (curve 3). Samples were excited at 280 nm, and spectra were obtained from 425–525 nm. B: Time-dependent changes in AEDANS-apoE fluorescence emission. Trace 1, 2 μg of sLDLR alone; trace 2, 1 μg of AEDANS-Trp-null apoE3-NT DMPC alone; trace 3, sLDLR plus AEDANS-Trp-null apoE3-NT DMPC at pH 7.2 (arrow) and after shifting the pH to 5.3 (5 min time point); trace 4, 2 μg of sLDLR and 1 μg of AEDANS-Trp-null apoE3-NT DMPC at pH 7.2 (arrow) and after the introduction of 4.0 μg of unlabeled Trp-null apoE3-NT DMPC (5 min time point). Samples were excited at 280 nm, and emission was monitored at 470 nm as a function of time.

identified and/or characterized in nematodes, insects, birds, and mammals and manifest a complex, oftentimes overlapping ligand specificity that is likely related to distinct biological processes. Whereas LDLR interacts with two major ligands, apoB-100 and apoE, the LDLR-related protein has >20 ligands (2). The ability to characterize ligand receptor recognition sites, binding overlap, ligand stoichiometry, and binding kinetics would be facilitated by a solution assay that permits real-time monitoring.

In contrast to other assays of ligand binding to LDLR family members, our method is performed in solution under conditions that can be easily manipulated. Furthermore, the amount of material required is low, yet sensitivity is high. Although we demonstrated that unrelated Trp-containing proteins do not serve as energy donors to AEDANS-apoE in this system, it remains to be determined precisely which Trp residues in sLDLR contribute to the AEDANS fluorescence emission enhancement observed upon binding. Of seven LDL-A repeats in LDLR, repeats 1, 2, 5, and 7 contain one Trp, whereas repeat 4 has two. In addition, the remainder of the sLDLR contains nine Trp residues. Although these latter Trps do not participate in ligand binding, it is conceivable that they could contribute to energy transfer to receptor-bound AEDANS-apoE. Site-directed mutagenesis studies designed to eliminate existing Trps or place new Trps in specific locations should yield insights into the ligand recognition properties of this receptor as well as other members of this receptor family.

The ligand used in these studies is known to interact with at least six members of the LDLR family (2). The independently folded NT domain of apoE bears the amino acid residues responsible for recognition by the LDLR (20). In the absence of lipid, the NT domain is organized as an antiparallel up-and-down bundle of four elongated amphipathic α-helices (21). Features of this protein that facilitate its use in this system include the presence of a single cysteine residue in helix 3 of the four helix bundle. Cysteine-112 is accessible to sulfhydryl-reactive reagents, and efficient AEDANS labeling is readily achieved. Wild-type apoE3-NT possesses four Trp residues that are located in the region of helix 1. Given the location of the receptor recognition sequence in apoE in helix 4 (20), replacement of Trp residues in helix 1 by phenylalanine and/or covalent modification of the cysteine-112 side chain would not be expected to have a major effect on LDLR interactions. Features of apoE3-NT conformation have been used to validate our LDLR binding assay. For example, it is recognized that the lipid-free helix bundle conformation of apoE-NT does not bind LDLR (11). To achieve a conformation that is recognized by LDLR, the NT domain undergoes a conformational change that is dependent upon interaction with lipid. In keeping with this, we found that binding of AEDANS-apoE3-NT DMPC to sLDLR is inhibited by unlabeled apoE3-NT DMPC but not by unlabeled lipid-free apoE3-NT. Because there is negligible overlap between Trp emission (excitation, 280 nm) and AEDANS emission, Trp-containing competitor ligands can be used in this system, as illustrated by the use of wild-type apoE3-NT DMPC and LDL as competitor ligands. This finding is important because it permits the design of competition binding studies using candidate ligands that naturally possess Trp.

Another advantage of our system is the ability to monitor receptor ligand interaction in real time. For example, we demonstrated that pH-dependent ligand release occurs within seconds after a change in pH from 7.2 to 5.3. On the other hand, displacement of bound ligand by unlabeled competitor occurs more slowly. Indeed, this system is fully amenable to the determination of association and dissociation kinetic parameters, with the additional potential to investigate the effect of mutations in LDL-A repeats or elsewhere in LDLR on ligand binding and/or release. As the size of this receptor family and the importance of the biological processes involved continue to expand, the availability of a convenient, reliable, and adaptable solution binding assay will see broad application.

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