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“Crystal” clear? Lysophospholipid receptor structure insights and controversies

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Abstract

Lysophospholipids (LPLs), particularly sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), are bioactive lipid modulators of cellular homeostasis and pathology. The discovery and characterization of five S1P- and six LPA-specific G protein-coupled receptors (GPCRs), S1P_{1–5} and LPA_{1–6}, has expanded their known involvement in all mammalian physiological systems. Resolution of the S1P₁, LPA₁, and LPA₆ crystal structures has fueled the growing interest in these receptors and their ligands as targets for pharmacological manipulation. In this review, we have attempted to provide an integrated overview of the three crystallized LPL GPCRs with biochemical and physiological structure-function data. Finally, we provide a novel discussion of how chaperones for LPLs may be considered when extrapolating crystallographic and computational data towards understanding actual biological interactions and phenotypes.

Keywords

apolipoprotein M; autotaxin; GPCR; lysophospholipid; lysophosphatidic acid; sphingosine 1-phosphate

Small but mighty: lysophospholipids

“Lipids,” broadly defined as fatty acids and their derivatives, encompass an enormous and growing number of molecules (over 43,000 unique structures in the LIPID MAPS database (<http://www.lipidmaps.org/data/structure/index.php>)) and range from simple saturated fatty acids to highly branched with varying hydrophilic head groups and **carbon chain saturation (e.g. 18:0)** (see Glossary). Lipids constitute at least 40% of the plasma membrane, and whereas the primary purpose of many lipids is structural, other lipids are primary and secondary messenger molecules and are referred to as “bioactive lipids” [1]. Two of the simplest bioactive lipids are **lysophospholipids** (LPLs, see Glossary), lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) [2, 3]. Although their

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parent molecules and biosynthetic pathways are quite different, LPA and S1P share similar basic structural characteristics (Figure 1A) [2–5].

The known physiological roles of S1P and LPA are diverse, affecting activity of every mammalian organ system [2, 3, 6]. The best-characterized roles for S1P include requirements in angiogenesis and vascular function, and regulation of lymphocyte egress from lymphoid organs, whereas regulation of neural cell development and migration are best characterized for LPA [5–9]. Almost every cell type expresses or can express receptors for both S1P and LPA at various stages of development and *in vitro* studies and *in vivo* models of receptor knockouts have demonstrated the importance of S1P and LPA receptors. The vast number of roles for S1PRs and LPARs have been comprehensively reviewed elsewhere [8–10].

All in the family-LPL receptors

While cell-specific effects of exogenous LPA and the S1P precursor sphingosine have been reported since the 1960s, they were attributed to changes in cell membranes, diffusion to act as second messengers, or calcium chelation [11–13]. The late 1980s to early 1990s brought the idea that simple LPLs may also affect cell activity via specific receptors, particularly **GPCRs** [14–18]. Evidence continued to build that GPCRs were responsible for effects of LPA and S1P and this concept was confirmed with the cloning of *Ventricular Zone Gene-1* (*VZG1*; historically, *EDG2* (*Endothelial Differentiation Gene- 2*) and currently *LPAR1*), that encodes for LPA receptor 1 (LPA₁) [19]. Its identification as the first LPL receptor allowed for **deorphanization** of multiple previously cloned genes, including the first S1P receptors [20–22].

There are now six *bona fide* LPA receptors, LPA_{1–6}, and five S1P receptors, S1P_{1–5} (Figure 1B) [23]. All five S1P receptors and the first three LPA receptors belong to the EDG receptor family, part of the rhodopsin GPCR family alpha subclass [23]. LPA_{4–6} are part of the rhodopsin delta subclass and are more closely related to the **P2Y purinergic receptors (P2YR)**, sharing only 25% or less amino acid (AA) homology with LPA_{1–3} while bearing almost 50% homology with certain P2YR [23–25]. The evolutionary significance of this functional convergence is unknown. Whereas mRNA for S1P₁ and LPA₁ are relatively ubiquitously expressed, mRNA for the other receptors is far more temporally and spatially restricted [6, 23]. While this restriction may have to do with generation of specific LPLs in different tissues, the consequences of tissue-specific LPL species utilization are largely unexplored.

The crystallized LPL receptors: S1P₁, LPA₁, and LPÄ₆

What special attributes would be required of such receptors in order for them to demonstrate substrate specificity, especially while embedded in membranes consisting of precursor molecules (Box 1)? The 2012 report of the **antagonist**-bound human S1P₁ crystal structure (Protein Data Bank ID (PDB):3v2w, 3v2y) was not only the first LPL GPCR crystallized, but also the first lipid GPCR crystallized (Figure 2) [26]. Driving selection of this receptor as the first lipid receptor to be targeted for crystallization efforts was the growing interest in

its potential for pharmacological modulation. The first FDA- approved LPL receptor modulator was FTY720/fingolimod/Gilenya, an immunomodulator approved in 2010 as the first oral treatment for **multiple sclerosis** (MS) [3, 27]. FTY720 is a **prodrug** phosphorylated to the active molecule FTY720- phosphate, which binds 4 of the 5 S1PR (S1P_{1, 3-5}) [27, 28]. Effectiveness of FTY720 spurred generation of other S1PR subtype-specific modulators for the treatment of MS, inflammatory bowel disease, psoriasis, and systemic lupus erythematosus (National Institutes of Health U.S. National Library of Medicine: <https://www.clinicaltrials.gov/>) [28]. The next LPL receptor crystallized was human LPA1 (PDB: 4Z34, 4Z35, 4Z36) in 2015, allowing comparisons to S1P1 and supporting analyses of LPAR-specific **agonists** or antagonists, which are in development for the treatment of pulmonary fibrosis and systemic sclerosis [3, 27, 29].

A third crystallized LPL receptor, LPA₆, is a non-EDG LPL receptor (PDB: 5XSZ) and of the three receptor crystal structures, it is the only non-human receptor (zebrafish) and receptor crystalized without a ligand, compared to antagonists that were used to stabilize both S1P₁ (ML-056) and LPA₁ (ONO-3080573, ONO-9780307, ONO-9910539) [26, 29, 30]. Despite ligand homology, the raw AA sequence of LPA₄₋₆ offers little in the way of determining agonist specificity because of their low AA sequence homology to LPA₁ and S1P₁ [23, 25, 31, 32]. Conversely, since structures of the nucleotide ligands for P2YR bear no resemblance to LPA other than containing a phosphate moiety, attempts to extrapolate from the P2Y₁ and P2Y₁₂ structures yielded no more information on the biology of the non-EDG LPA receptors than already existed from cell-based assays [25]. Resolution of the LPA₆ structure was regarded as a major advance in hopes of providing information about structure-function for the P2Y-related LPA receptors, since they were and remain something of an enigma.

Conserved motifs involved in ligand recognition, activation, signal termination, and internalization by rhodopsin family GPCRs are well documented from detailed mutational studies and crystal structures (Figure 3, Key Figure) [33–36]. Transduction of ligand-induced responses by GPCRs follows a series of standard structural alterations from outside to inside the cell [34, 37, 38]. Conserved AA motifs coordinate interaction of ligand with the receptor N-terminus and extracellular (EC) loops, then further contact between ligand and transmembrane (TM) residues leads to shifting and reorganization of the TM helices. This also shifts the C-terminus and intracellular (IC) loops to active conformations that elicit specific G α proteins, G protein-coupled receptor kinases (GRKs), and/or **arrestin** recruitment and subsequent signal transduction or, in the case of antagonists, prevent binding of agonist and those same signaling cascade movements, maintaining an inactive conformation [37–38]. The N-terminus, EC loops, and specific residues of the TM helices form the **orthosteric binding pocket** for the native ligand(s) [37, 40]. Many excellent reviews of GPCRs discuss steps from ligand binding through activation or inhibition [33, 34, 37, 40, 41]. Here, we present these features moving through the receptor structures from IC/TM to EC domains.

Cytoplasmic domains in LPL GPCRs

The contribution of IC (cytoplasmic) regions of the receptor to LPL signaling is the most difficult to determine from existing crystal structures as structural modifications were required to increase thermal stabilization and allow crystallization [31, 34, 42]. For S1P₁ and LPA₆, the bacteriophage T4 lysozyme (T4L) was inserted into IC loop 3 (ICL3, replacing S232-K44 (S1P₁) or V227-K233 (LPA₆) [26, 30]. Crystallizable LPA₁ was generated with insertion of an apocytochrome C (bRIL) protein replacing ICL3 residues R233-R247, with further addition of engineered disulfide bonds via computationally directed mutations D204C and V282C [29]. These modifications obfuscate cytoplasmic domain interactions of the crystal structures under antagonist or no ligand occupancy, and leave unassessed the structural details of agonist-bound receptors [41–43].

TM7 NPxxY GPCR recruitment

Moving into the transmembrane region, we come to the **NPxxY motif**, located on TM7 (Figure 3), an activation switch allowing interaction with G proteins [34, 43]. The highly conserved P^{7.50} (**BW nomenclature**; <http://www.gpcrdb.org/>) residue exhibits torsional changes that result in movement by Y^{7.53}. In the antagonist-bound/inactive state, TM7 NPxxY interacts with residues on TM3 for S1P₁ and LPA₁, similar to β_2 AR and P2Y₁ [26, 29, 44]. **Molecular dynamics** (MD) studies of S1P₁ indicate that S1P binding induces shifts in NPxxY residues, particularly Y311^{6.53} and P308^{7.50}, allowing increased TM7 interactions with TM2 (TM2 F83^{2.42}-L102^{2.61}; TM7 A293^{7.34}-T312^{7.54}) while decreasing interactions with TM3 and concomitant breakage of the TM3:TM6 ionic lock [45–47]. A report investigating the effect of S1P₁ mutations identified by the National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project (NHLBI GO ESP) found a mutation of G305^{7.47} to cysteine exhibited normal signaling but resulted in severely reduced receptor internalization [48]. The authors speculated that the location of the glycine to cysteine mutation upstream of the NPxxY motif could restrict flexibility of the TM7 helix and/or provide a novel disulfide bonding partner for one of four other TM cysteine residues. Thus, changes close to NPxxY may also alter the ability of the receptor to be activated or deactivated.

Earlier studies demonstrated that recombinant LPA₁ discriminated between myristoyl (14:0) and palmitoyl (16:0) LPA, recognizing the longer chain, but stearoyl (18:0), oleoyl (18:1), linoleoyl (18:2), or arachidonoyl (20:4) LPA stimulation all resulted in activation [49]. MD studies of the LPA₁ NPxxY response to various agonists yielded insight into how LPA species induce differential activation [50]. 14:0 LPA did not result in active NPxxY conformations, whereas 18:1 and 20:4 both drove NPxxY conformation to the active state and increased torsional changes of P308^{7.50}.

Motion of the NPxxY motif may allow H₂O molecules to enter the heptahelical bundle, indicative of full receptor activation status [51]. S1P₁ MD suggested that an empty receptor fluctuating between active and inactive conformations was more likely to have H₂O molecules in contact with Y311^{7.53} in the active conformation, and S1P binding induced H₂O flux to NPxxY [45, 46]. Alternatively, strong agonists of LPA₁, 18:1 and 20:4 LPA,

allowed H₂O influx from the extracellular side of the receptor, whereas weak agonist 16:0 and antagonist allowed only discontinuous flux [50]. Indicative of its divergence from the EDG family and rhodopsin alpha class, both human and the crystallized zebrafish LPA₆ have a DPxxY motif [30]. Replacement of the N^{7.49} with a negatively charged D^{7.49} residue could alter receptor affinity for LPA species or kinetics of the agonist-induced TM7 inward shift.

TM6 “toggle switch” CWxP motif

Another rotameric switch residue is W^{6.48} within the **CWxP motif** located at the bottom of the binding pocket, which plays a role in propagating TM6 outward shift upon full agonist binding [34, 43]. While S1P₁, LPA₁, and LPA₆ all have C^{6.47} and P^{6.50}, LPA₆ has a tryptophan to phenylalanine substitution at the conserved site (Figure 3) [26, 29, 30]. MD studies indicate the S1P acyl chain induces W269^{6.48} rotation, affecting movement of other binding pocket residues, specifically R120^{3.28}, E121^{3.29}, and M124^{3.32}, and mutation of W269^{6.48} to leucine reduced receptor activation [26, 45, 46]. R120P^{3.28} mutation was seen in the NHLBI GO ESP study and results in failure to activate receptor signaling and internalization [48].

The crystal structure of LPA₁ showed no interaction of antagonists with W271^{6.48} MD studies indicate this residue shifts in response to agonist, increasing binding pocket volume to accommodate ligands with different conformations and head groups [29]. LPA₁ R^{3.28} is also critical for 18:1 LPA-induced activity, thus alterations to W271^{6.48} could alter interactions with this residue in a fashion similar to S1P₁ R120^{3.28} [50, 52]. Although the role of this motif has not yet been examined in LPA₆, all of the non-EDG LPA receptors have the alternative CFxP motif and other receptors, including GPR35 and GPR55, also have this conservative substitution, although P2Y₁ has Y^{6.48} [30, 53].

TM3 D/ERY/F motif

The **D/ERY/F motif** at the TM3 cytoplasmic end is considered key to regulating receptor active conformation, allowing interactions with G proteins [33, 37, 41, 43, 54]. This occurs through an intrahelical salt bridge between the D/E and R residues and interhelical interactions with residues on TM5 after ligand binding. This motif shows variation amongst all three of the crystallized LPL receptors: E141^{3.49}R142^{3.50}Y143^{3.51} (S1P₁), E145^{3.49}R146^{3.50}H147^{3.51} (LPA₁), and D113^{3.49}R114^{3.50}F115^{3.51} (LPA₆) (Figure 3) [26, 29, 30]. The S1P₁ crystal structure does not show these interactions, but the importance of the ERY motif has been demonstrated by mutagenesis assays showing its necessity for receptor internalization [55, 56]. The LPA₁ structure has a salt bridge at a distance of 8.7 Å [29, 54]. MD indicated the ERH maintains LPA₁ in a locked conformation by forming an intrahelical lock with L256^{6.33} [29, 50, 54]. There is little information about the role of H^{3.51}, but mutational analyses of the lamprey gonadotropin-releasing hormone receptor (GnRH) wild-type H^{3.51} indicated involvement in maintaining the receptor at the cell surface [57]. LPA₆ has a more conventional DRF motif. Mutation of DRF in the chemokine receptor CXCR6 to a canonical DRY leads to altered Gα protein coupling, which already occurs in a cell-type specific fashion [58, 59]. This may explain some difficulties encountered when

attempting to measure activation of LPA₆ using traditional methodologies and cell lines [25, 32].

Ligand binding pockets

The orthosteric ligand-binding pocket differs from allosteric sites, although binding by allosteric modulators can dramatically affect orthosteric ligand binding [37, 40, 60]. For the sake of clarity, we will only be referring to the orthosteric site when using the term “binding pocket.”

An ideal scenario for these analyses involves crystal structures of S1P₁, LPA₁, and LPA₆ resolved with endogenous activating ligands in their respective binding pockets. Unfortunately, few GPCRs have been crystallized with agonists, meaning MD modeling must be based on inactive receptor conformations. Residues involved in LPL binding and specificity have been of great interest since the first S1P and LPA receptors were discovered (Figure 3). Crystal structures have provided results strikingly similar to those of earlier mutagenesis and computer modeling studies. An early study found that changing only one residue altered ligand preference by S1P₁ (E121^{3,29}) or LPA₁ (Q125^{3,29}) [61]. Both crystal structures and multiple modeling studies identified 3.29 as interacting with S1P or LPA polar head groups [26, 29, 47, 62]. In agreement with its crystal structure, the presence of V^{3,29} in both human and zebrafish LPA₆ indicates 3.29 does not play a role in binding or selectivity for this receptor [30].

S1P₁ binding pocket

Numerous S1P₁ MD analyses have been performed, many of which have highlighted the same binding residues as earlier mutational studies and human SNP analysis (Figure 3). Because the receptor was crystallized with an antagonist, modifications were necessary to fit S1P into the receptor. F125^{3,33}, F265^{6,44}, and F273^{6,52} required the largest shifts to accommodate the longer carbon chain of endogenous S1P and fit it into the hydrophobic cluster between TM3, TM5, and TM6 [26, 45, 63, 64]. W269^{6,48}, also in this cluster and involved in tail recognition, was identified earlier as important to receptor activation [65].

R120^{3,28} and E121^{3,29} were the first S1P₁ residues found to be required for S1P binding and activity and later studies based on the crystal structure confirmed roles in head group recognition [45, 62, 64]. N101^{2,60} was also identified early as important for recognition of the hydroxyl headgroup and found in models based on the crystal structure [45, 62]. Residues found by MD to interact with the phosphate headgroup include Y29 and K34, and S105^{2,64} may interact with the amine [26, 45, 47]. Residues involved in ligand recognition but not receptor activation include L128^{3,36} and F210^{5,47}, both identified in early studies and later MD [45, 63–65]. L276^{6,55} and V301^{7,42} may also form part of the binding pocket [63, 64].

LPA₁ binding pocket

A 2008 computational analysis of LPA₁ binding focused on the glycerolphosphate of LPA 18:1 and differential effects of mutations in conserved residues of LPA₁₋₃ [4] [66]. Several residues identified were later confirmed in more complex MD contemporary with crystal

structure publication. R124^{3.28}, Q125^{3.29}, and Y294^{7.36} affected head group binding, and mutation of 3.28 and 3.29 to alanine led to loss of receptor activity [50, 66]. Y34 and K39 are highly conserved in EDG LPA receptors and were found in the crystal and computational analyses to bind the head group [29, 50]. One group determined that K39 also affected LPA₁ affinity for AGP (alkylglycerol-3-phosphate 18:1), despite it lacking only the acyl carbonyl oxygen, implicating it in ligand discrimination (Figure 1) [50].

Other residues identified in the crystal structure as modifiers of LPA₁ binding pocket dimensions compared to S1P₁ were N129^{3.33} and W210^{5.43} [29]. G274^{6.51} is L272^{6.51} in S1P₁ and may provide van der Waals interactions [26]. LPA₁ may also be an endogenous cannabinoid metabolite receptor, and tryptophan substitution at 210^{5.43} versus S1P₁ C206^{5.43}, in combination with the conserved W271^{6.48}, may further expand the binding pocket to allow binding of ligands such as 2-ALPA (2-arachidonyl phosphatidic acid), found to be an agonist in functional studies (Figure 1) [29].

LPA₆ binding pocket

An *in silico* study of LPA₆ wild-type and mutant binding was conducted to address the potential role of known mutations in humans with autosomal recessive woolly hair/hypotrichosis; however, the group used LPA 18:1, not the higher affinity ligand 18:2 [25, 32, 67]. Complicating direct comparisons, zebrafish LPA₆ was used for crystallization, did not have ligand bound, and had amino terminus truncations [30]. Thus, determination of binding pocket residues was completely dependent on computer models and mutant analyses. Regardless, equivalents of several residues identified in human LPA₆ were found to affect binding in the model based on crystallized LPA₆. hR73^{2.60}/zR83^{2.60} bound the phosphate headgroup, and hM88^{5.42}/zM98^{5.42} participated in acyl chain binding [30]. Other binding residues found mutated in the human study and conserved in the crystal structure included N248Y^{6.54} and L277P^{7.38} [67].

Mutational analyses of binding residues identified by modeling LPA₆ with 18:2 LPA found the phosphate-binding K26^{1.31}, R83^{2.60}, R267^{6.62}, and R281^{7.32} displayed decreased activation when mutated to alanine or glutamine [30]. Residues V195^{5.39} and I198^{5.42} were important for acyl chain docking, and mutation resulted in loss of activity or decreased binding, respectively. Binding pocket depth appeared to be unimportant for acyl chain preference, since mutant residues at the bottom (L115^{3.41}, L153^{5.42}, V201^{5.45}) did not affect receptor activation [30]. Instead, binding pocket shape and size formed by TM3, TM4, and TM5 was more important, possibly for space to bend acyl chains of various ligands. This TM3–5 pocket may be common to all of the non-EDG and P2Y receptors [30].

Ligand delivery-what about the lipid chaperones?

Lipids are largely characterized by their hydrophobicity. Thus, in order to circulate in plasma, S1P and LPA must be bound to carrier proteins acting as lipid chaperones, protecting them from degradative enzymes and the aqueous environment [6, 68–70]. Chaperones also alter lipid functionality with regard to receptor activation. This has been characterized in far greater detail for S1P and its chaperones than for LPA carriers (Box 2).

GPCR crystallization has the potential to reveal unanticipated structures or interactions, as was the case when the S1P₁ crystal structure revealed an N-terminal “helical cap” appearing to prevent ligand delivery from the extracellular space, implying ligand must diffuse from within the lipid bilayer [26, 29]. The LPA₆ crystal structure suggests a unique autocrine activation pathway involving similar ligand diffusion access after biosynthesis of LPA 18:2 by hydrolysis of phosphatidic acid by membrane-associate phospholipase A1 α (PA-PLA1 α) [8, 71]. The N-terminus of LPA₁ is less structured, permitting extracellular ligand delivery [29, 30]. It was posited that this LPA₁ structural feature made sense, because of “the biological existence...of albumin-bound LPA” [29]. Indeed, LPA is found in plasma bound to albumin at ~10–50nM [8] [72]. However, ~30% of S1P in plasma is also albumin-bound, with most of the remaining ~70% bound specifically to apolipoprotein M (ApoM) tethered to high-density lipoprotein (HDL) particles at ~350–700nM [68]. The ApoM-HDL-bound S1P fraction not only initiates different signaling and internalization kinetics, but opposing biological outcomes in diverse cell types, possibly mediated by biased agonism (Box 2) [71, 73].

Apolipoprotein M (ApoM)

Whereas both LPA and S1P are bound to albumin, a majority of plasma S1P is carried by ApoM (Figure 4A) [68]. S1P preferentially transfers from albumin to HDL since dissociation from albumin has a low energetic barrier [75]. MD with the S1P-bound ApoM crystal structure (PDB: 2YG2) indicate that removal of S1P from the ApoM binding pocket requires a surprisingly large 65–75 kJ/mol **potential of mean force (PMF)** [76]. The proposed release mechanism involves breaking strong polar interactions with the S1P headgroup while the hydrocarbon tail of S1P moves out of the hydrophobic region until the headgroup is free and the tail follows. Double mutation of “gatekeeper” residues R98/R116 only reduced the required PMF to ~50kJ/mol, making it unclear how removal of S1P from ApoM to the cell membrane would be energetically favored. The authors stated “a specific mechanism decreasing the energetic barrier is essential for transfer of S1P to the S1P receptor.” [76]

Another MD study attempted to clarify the pathway for ligand diffusion into S1P₁; however, the antagonist used for crystallization, ML-056, was pre-inserted into the membrane before modeling migration into the binding pocket [76]. After binding to the outside of the receptor, the ML-056 polar head was attracted to a “**membrane vestibule**.” Accommodation of ligand binding required partial unfolding of the N-terminal helix and movement away from the receptor core [77]. **B-factor** representation of S1P₁ illustrates that the top of TM1 and the connecting loop to the N-terminal helix are the most flexible parts of the structure (Figure 4C). Since ApoM-S1P retains S1P₁ at the membrane and maintains prolonged signaling, this suggests that ApoM may interact with S1P₁ in such a manner as to deliver directly the ligand into the receptor [74, 75]. These models are further complicated by structural data that cannot assess antagonist “activation” since antagonists, by definition, do not activate receptors. Nevertheless, these proposed modes of S1P₁ receptor access are not mutually exclusive.

Autotaxin (ATX)

Autotaxin (ATX) is an ectonucleotide/phosphodiesterase secreted as an active lysophospholipase D (lysoPLD) that both synthesizes LPA (primarily from lysophosphatidylcholine (LPC)) and releases it through a separate exit tunnel (Figure 4B) [8, 69, 70, 71, 78]. The structure and function of ATX support a mechanism whereby it can deliver LPA directly to LPARs. The N-terminal somatomedin B-like (SMB)1 domain alters affinity of ATX for LPA, whereas the SMB2 domain allows direct interactions of ATX with cell surface β integrins [70, 71]. This specificity for cell surface molecules indicates that ATX delivery of LPA species could impart receptor selectivity compared to albumin, the concentration of which can also alter LPA binding to specific receptors [79–80]. The different delivery and activation mechanisms exhibited by ATX and albumin, combined with B factor analysis of LPA₁ demonstrating dramatically increased flexibility of the EC regions (Figure 4C), therefore support the potential for biased agonism of LPARs via direct delivery of LPA, but requires experimental validation.

Concluding remarks

The first successful crystallization of a lipid GPCR, S1P₁, and subsequent crystallization of two more LPL receptors, LPA₁ and LPA₆, have helped fuel the dramatically increased interest in LPL biology. These structures are representatives of a receptor family that shares structurally similar ligands and yet, in some cases, low AA sequence homology. We have compared the key structural features of GPCR ligand binding and subsequent activation across these three resolved structures. While these structures may provide a framework from which novel orthosteric and allosteric modulators can be derived, ligand-specific effects on receptor conformation resulting in recruitment of specific G α subunits or β -arrestin have yet to be deciphered and we still lack a structure of a LPL GPCR complexed with agonist or G proteins. Additionally, these structures have raised more questions than answers as to how LPL ligands are delivered to their receptors and how the EDG versus non-EDG family receptors recognize the same ligands, or how within the EDG family, they do not. Addressing these and other challenges (see Outstanding Questions) will impact the field of LPLs and the larger worlds of bioactive lipids and GPCR signaling towards understanding their biological roles, disease relevance, and identifying new therapeutic approaches.

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Glossary

Agonist

A molecule that binds the orthosteric binding site and activates a GPCR by stabilizing it in an active conformation. Full agonists induce maximal activation of the receptor. LPL receptors differ in their ability to be fully activated by LPL (their agonists) with varying hydroxyl carbon chain length and head groups.

Allosteric site

A site distinct from the orthosteric binding pocket, at which binding of a molecule alters receptor conformation, modulating its activity.

Antagonist

A molecule that binds the orthosteric (competitive) or allosteric (noncompetitive) site, blocking activation of the GPCR by stabilizing the inactive conformation, preventing coupling with and activation of G proteins or β arrestins and their respective downstream signaling pathways.

Arrestin

A group of four proteins (two visual arrestins, β -arrestin 1, and β -arrestin 2) that are recruited to phosphorylated GPCR C terminal tails. Arrestins can negatively regulate G protein signaling or initiate their own unique signaling cascades.

B factor

Also referred to as the temperature factor, B factor refers to the uncertainty of an atom's position in a crystal structure and is a measure of thermal motion. A region of the protein that can adopt multiple conformations in space will have a higher B factor than regions with less flexibility and motion. Because it is a measure of uncertainty, structures of lower resolution will tend to have higher B factors.

Bar code

In a GPCR, a specific sequence of phosphorylated residues recognized by arrestin proteins to initiate biased agonist signaling. P represents a phospho-serine or phospho-threonine and $\times$ can be any amino acid except proline in the second xx instance of either the PxxPxxP/E/D (short bar code) or PxxPxxP/E/D (long bar code). The final residue can also be either aspartate or glutamate.

BW nomenclature

Ballesteros-Weinstein generic numbering scheme for GPCR residues. The format of BW, "TM helix.XX", gives the TM helix number followed by "XX" as the residue number according to its distance from the most conserved residue of each TM helix, which is designated as "50". For example, the BW number 7. 50 indicates a residue on TM7 and is the most conserved residue at position 50, whereas residues 7.49 and 7.51 are the residues on either side of the conserved residue. The GPCRdb (<http://www.gpcrdb.org/>) has recently updated the numbering scheme based on structural alignments, designating residues that create a bulge in the structure with the same number as the preceding residue, appending a "1" to the residue number, e.g., both human and zebrafish LPA₆ have G^{5.461}. BW labels in this review are updated BW nomenclature derived from PDB alignments on GPCRdb. Thus, some of the residue labels differ from those given in original reports.

Carbon chain saturation (lipid nomenclature)

Saturated hydrocarbon carbon chains have no double bonds, whereas the presence of at least one double bond makes a carbon chain unsaturated. Saturation or unsaturation of the carbon chain is indicated by the C:D notation, where the number in the C position is the number of carbon atoms in the chain and D is the number of double bonds, or unsaturations. Thus, 18:1 S1P or LPA have an 18-carbon chain backbone with a single double bond. A detailed

explanation of IUPAC-IUB hydrocarbon chain and lipid nomenclature can be found here <http://www.sbcs.qmul.ac.uk/iupac/lipid/>

CWxP motif

An amino acid motif of cysteine (C), tryptophan (W), any single amino acid (x), and proline (P).

Deorphanization

A process whereby a GPCR with unknown ligand specificity-an “orphan”- is matched to a natural, physiological ligand to establish functional identity.

D/ERY/F motif

The most conserved form of this amino acid motif is DRY (aspartate (D), arginine (R), tyrosine (Y)). Other forms are not as common but not rare, including ERY (glutamate (E) substituted for D), seen in S1P₁, or DRF (phenylalanine (F) substituted for Y), seen in LPA₆. The ERH (E substituted for D and histamine (H) substituted for Y) seen in LPA₁ is far less common.

G protein-coupled receptor (GPCR)

A superfamily of heptahelical transmembrane (TM) receptors sharing a common structure and conserved activation motifs. GPCRs transduce extracellular cues to intracellular signals by changing their conformation to recruit heterotrimeric G proteins, G protein-coupled receptor kinases (GRK), or arrestins (e.g. visual or β arrestins).

Lysophospholipids (LPL)

Subgroup of lipids that includes S1P and LPA. The characterization as lysolipids stems from the loss of acyl chains from their parent compounds, but can also refer to early observations that some variants caused cell lysis. Both S1P and LPA have simple sphingoid or glycerol backbones, respectively, connecting phosphate head groups and saturated or unsaturated carbon chains, usually from C14 to C22, although shorter or longer chains can be found in some tissues.

Membrane vestibule

A site on the exterior (lipid-facing) portion of the GPCR to which ligands can bind before conformational changes allow ligands to move into the orthosteric binding pocket.

Molecular dynamics studies (MD)

An *in silico* method for integrating known structural and physical data and applying energetic restrictions to generate an atomistic model of the interactions between receptors and other molecules, such as ligands, the lipid membrane, signaling molecules, or other receptors.

Multiple sclerosis (MS)

A chronic disease of the central nervous system (CNS), the causes of which are unknown, but likely involve a combination of factors involving the immune, nervous, and vascular systems. The development and progression of MS require activation of the immune system

to attack cells and components of the CNS, which requires inflammatory activation of the vascular system.

NPxxY motif

An amino acid motif of asparagine (N), proline (P), any two amino acids (xx), and tyrosine (Y).

Orthosteric ligand binding pocket

A three-dimensional structure of TM helices, EC loops, and N-terminal residues where endogenous ligand(s) bind to propagate interactions by conserved motifs and other subtle, ligand- and receptor-specific events (e.g. H₂O ions or lipid interactions) leading to activation and signaling activity.

P2Y purinergic receptor (P2YR)

A family of GPCRs originally found to be stimulated by purines, but now including receptors stimulated by a variety of nucleotides, including purines (ATP and ADP) and pyrimidines (UDP and UTP).

Potential of mean force (PMF)

A profile of the average amount of free energy (energy available to perform work) required to move a molecule along a specific path (reaction coordinates (RC)). MD analysis calculates changes in free energy associated with the probability of each possible conformational state, giving the average (mean) force on the substrate as its interactions change with different atoms along the RC. In the case of the ApoM-S1P PMF calculations, the steered MD analyses were performed using the umbrella sampling method followed by WHAM (weighted histogram analysis method). The umbrella method samples different potential changes in free energy between the substrate (S1P) and the chaperone (ApoM) along the RC, including sampling of energetically unfavorable states while biasing the impact of potential reactions to a biophysically relevant range. Application of WHAM to umbrella sampling data allows combining these biased samples to obtain an unbiased PMF.

Prodrug

A biologically inactive molecule that is altered by *in vivo* metabolism to a chemically distinct, biologically active form.

Root mean square deviation (RMSD)

A quantitative measure of the similarity between two superimposed atomic coordinates. For any structural model, a root mean square (RMS) value is determined for each atom, which gives the range of values obtained from the crystal structure for the coordinates in space of that particular atom. After optimal superimposition of two or more crystal structures, RMSD is calculated to determine the distance between all of the atoms of the reference structure (in this review, the structure for LPA₁) versus the inquiry structures (S1P₁ or LPA₆). When both S1P₁ and LPA₆ were superimposed to LPA₁, RMSDs were the smallest, meaning the LPA₁ crystal structure was the most similar to both of the other receptors.

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Box1:**Lipids and crystals**

Much like the study of lipid biology, the study of GPCR biology has been historically difficult, despite their pharmacological importance. GPCRs are collectively the largest class of human membrane proteins [33]. Of the 475 drugs approved by the FDA, those that target GPCRs account for over 30%, and eleven S1P or LPA receptor modulating compounds are approved or in trials worldwide (<https://clinicaltrials.gov/>) [3, 28, 40, 60]. Lipid GPCRs, which recognize bioactive lipids including eicosanoids (e.g., prostaglandins and leukotrienes), cannabinoids, and LPLs, constitute 30% of nonolfactory GPCRs [60]. However, issues inherent in the study of their ligands compound the difficulty in their study. Lipids are difficult to isolate and quantitate, some are highly unstable, others are incredibly “sticky,” and yet others have structural similarities to membrane components that seemingly defy the ability of a GPCR to differentiate between specific ligands and the membrane in which it is embedded [1, 13, 81].

GPCR crystal structures provide a basis for molecular modeling and structure-function that cannot be gained from knowledge of the AA sequence alone [41, 44]. However, a major caveat when examining and utilizing these structures is that they are the equivalent of taking a snapshot at a wedding: they capture a single moment in time under specific and contrived circumstances. How that couple came together, experiences the next days or years, separation or bonding with other partners, is not based on that single moment in time. So, too, should scientists be wary of overinterpretation of crystal structure “snapshots” of GPCRs that are usually bound to an antagonist (less commonly with an agonist), disregarding how that snapshot fits into the normal physiological circumstances of the GPCR [33, 43].

The crystal structures of LPL GPCRs have led to novel hypotheses related to ligand entry into the receptor and accommodation of ligands within the binding pocket. It is proposed that ligands for S1P₁ and LPA₆ first enter the lipid bilayer and then laterally transfer between the transmembrane helices, contrasting with extracellular delivery of ligands to LPA₁ [26, 29, 30]. The implications of these different delivery systems have not been addressed in their full biological context, despite being one of the most critical aspects of their GPCR biology. The second hypothesis relates to accommodation of related lipid ligands, wherein a “baggy” binding pocket of LPA₁ predicted accommodation of other ligands, which was shown functionally for phosphorylated endocannabinoids that both exist endogenously and arise through biochemically distinct pathways [29]. Comparisons between the crystal structure of another lipid GPCR, human cannabinoid receptor CB₁, and S1P₁ and LPA₁ indicate that the ability of LPA₁ to be promiscuously activated by both LPA species and endocannabinoids may be due to this extended binding pocket in combination with the less restrictive structure of the N-terminal helix [26, 29, 82]. While CB₁ has a large 7 Å outward shift of TM1 to potentially accommodate long lipid tails of agonists, its N-terminal helix rests deeper within the binding pocket compared to the LPLR, positioning hydrophobic residues closer to the ligands and forcing polar residues

outward [82]. Alternatively, S1P₁ does not have such an accommodating binding pocket, resulting in more restrictive agonist binding [26, 82].

Box 2:**LPL receptor biased agonism**

Signaling cascades initiated by ligands binding to GPCRs can be transduced by either G proteins or alternative pathways served by arrestins, which also modulate receptor internalization [39]. When an agonist stabilizes a conformation that preferentially recruits arrestins to initiate divergent signaling cascades, it is considered “biased” [37]. With regard to the LPL receptor family, the ability of S1P carried by HDL to affect differential outcomes compared to albumin is gaining appreciation [68, 73–75]. Recent studies have demonstrated that S1P bound to ApoM-HDL can elicit opposing outcomes to albumin-S1P in multiple cell types, such as bone marrow lymphocyte progenitors or human umbilical vein endothelial cells (HUVEC) [73, 74]. This occurs in an apparent biased agonist manner, since knockdown of β -arrestin 2 in HUVEC abrogates vasoprotective and anti-inflammatory functions of S1P₁ signaling by ApoM-S1 P [74].

Recent crystallization of rhodopsin with visual arrestin and the vasopressin 2 receptor with β -arrestin led to generation of the postulated biased agonist “bar code” [83]. These bar codes, consisting of phosphorylated serine or threonine residues in the GPCR C-terminal tails that are necessary for high affinity arrestin recruitment and interaction. Although not resolved in the LPL receptor structures, these biased agonism signals are worthy of mention because of their potential importance to understanding receptor signaling mechanisms. S1P₁ contains three full short bar codes (PxPxxP/E/D) and fourteen partial bar codes, whereas LPA₁ contains three full long (PxxPxxP/E/D) and eight partial bar codes, and LPA₆ contains no full bar codes and eleven partial bar codes [83]. Full bar codes were required for high-affinity arrestin binding, but partial bar codes also resulted in arrestin recruitment and their phosphorylation might determine different arrestin docking positions. This report suggests that the biased agonism observed in S1P₁ signaling could also occur in some LPAR.

This also begs the question, “How do the S1P or LPA chaperones interact with the receptors to modify their conformation and transduce biased signals?” Much has been made of the S1P₁ crystal structure N-terminal “cap” and the proposed ligand delivery by diffusion from within the membrane. Such a proposition does not fully take into account the physiological context of ligand delivery. MD studies indicating the N-terminal cap must move to allow ligand receptor entry further supports the concept that ApoM-bound S1P induces specific receptor conformations resulting in β -arrestin recruitment and signaling [76]. It will be interesting to see how future studies resolve the conflict between the different ligand delivery mechanisms suggested by the crystal structure versus *in vitro* and *in vivo* experimental data.

Highlights

- As major regulators of mammalian physiology, G protein-coupled receptors (GPCR) for the structurally related lysophospholipids (LPLs) sphingosine 1-phosphate (S1P), lysophosphatidic acid (LPA), as well as other LPLs, are providing new insights into fundamental biology and genuine therapeutics.
- Despite their structural similarities, GPCRs for S1P and LPA do not show physiological promiscuity, indicating molecular selectivity. In an effort to clarify structural mechanisms underlying ligand recognition and discrimination, the LPL receptors S1P₁, LPA₁, and most recently, LPA₆, have been crystallized, generating hypotheses regarding differential ligand delivery and accommodation.
- Novel secondary and tertiary structures revealed by the three crystal structures support emerging evidence suggesting S1P, and possibly LPA, can act as biased agonists when bound to specific protein chaperones.

Outstanding Questions

1. What are the structures of agonist-bound LPL GPCRs with or without bound heterotrimeric G proteins, revealed by standard and newer technologies, like cryoelectron microscopy (cryo-EM)?
2. Would crystallization of an empty receptor better inform studies of lipid chaperones and ligand preference?
3. Does LPL receptor conformation affect which chaperone delivers ligands?
4. How does ligand delivery by different chaperones affect receptor conformation?
5. If receptors for the lipid chaperone are required for efficient delivery of ApoM-S1 P to S1 PR, do these directly interact with the S1 PR?
6. Do lipid chaperone receptors vary for different cell types?

A		B					
ligands		PDB ID number(s)	rhodopsin receptor subclass/family	protein name	human gene	mouse gene	historic names
18:1 S1P	3v2w, 3v2y(h)	alpha/EDG	S1P ₁	<i>S1PR1</i>	<i>S1pr1</i>	<i>EDG1</i>
			alpha/EDG	S1P ₂	<i>S1PR2</i>	<i>S1pr2</i>	<i>EDG5, GPCR13</i>
			alpha/EDG	S1P ₃	<i>S1PR3</i>	<i>S1pr3</i>	<i>EDG3</i>
			alpha/EDG	S1P ₄	<i>S1PR4</i>	<i>S1pr4</i>	<i>EDG6</i>
			alpha/EDG	S1P ₅	<i>S1PR5</i>	<i>S1pr5</i>	<i>EDG8</i>
18:1 LPA (1-acyl)		4Z34(h), 4Z35(h), 4Z36(h)	alpha/EDG	LPA ₁	<i>LPAR1</i>	<i>Lpar1</i>	<i>VZG1, EDG2, GPCR26^a</i>
18:1 LPA (2-acyl)			alpha/EDG	LPA ₂	<i>LPAR2</i>	<i>Lpar2</i>	<i>EDG4</i>
18:2 LPA (1-acyl)			alpha/EDG	LPA ₃	<i>LPAR3</i>	<i>Lpar3</i>	<i>EDG7</i>
2-ALPA			delta/P2Y	LPA ₄	<i>LPAR4</i>	<i>Lpar4</i>	<i>P2Y9, GPR23</i>
18:1 AGP			delta/P2Y	LPA ₅	<i>LPAR5</i>	<i>Lpar5</i>	<i>GPR92</i>
		5XSZ(d)	delta/P2Y	LPA ₆	<i>LPAR6</i>	<i>Lpar6</i>	<i>P2Y5, GPR87</i>

Figure 1. LPL receptor ligands, proteins, human and mouse genes, and historic names.

(A) Structures of the major S1PR and LPAR ligands referred to in the text are shown on the left of their respective receptor families. Lipid nomenclature (e.g., 18:1) is described in greater detail in the glossary. (B) Protein Data Bank (PDB) accession numbers for crystal structures used in figures in this text are shown in bold and species of receptor crystallized (human, *Homo sapiens* (h), or zebrafish, *Danio rerio* (d)) is given in parenthesis, followed by rhodopsin receptor subclass and family (EDG versus P2Y), current human and mouse gene names, and major historic names for each LPL receptor. ^a*GPCR26* should not be confused with *GPR26*, which is incorrectly given as a synonym for *LPAR1* in NCBI, Ensembl, and other databases. The mouse listings for *Lpar1* correctly list only *Gpcr26*. 2-ALPA, 2-arachidonylphosphatidic acid; AGP, alkylglycerol 3-phosphate; dhS1P, dihydrosphingosine 1-phosphate (also referred to as sphinganine 1-phosphate); LPA, lysophosphatidic acid.

Figure 2. S1P₁, LPA₁, and LPA₆ crystal structure comparisons and topologies.

S1P₁ and LPA₆ were first aligned to LPA₁, which gave the lowest **RMSD** (S1P₁:LPA₁ = 0.986 Å, LPA₆:LPA₁ = 3.691 Å). Superimposed crystal structures of S1P₁:LPA₁, LPA₁:LPA₆, and S1P₁:LPA₆ are shown from the plane of the membrane (A), looking down from the N-termini and extracellular space (B), and looking up from the C-termini and intracellular space (C). S1P₁ is shown in magenta, LPA₁ in blue, and LPA₆ in green. H, helix; N-term, N-terminus; TM, transmembrane helix

Figure 3. Overview of regions involved in LPL GPCR ligand binding and signaling. S1P₁ (magenta) and LPA₆ (green) are superimposed with LPA₁ (blue), with conserved GPCR regions amplified. Individual orthosteric ligand binding pockets are shown with co-crystallized ligands in dark grey. GPCRdb sequence alignments for human (h) and *Danio rerio* (d, LPA₆ only) with corresponding updated BW nomenclature are given for each region with conserved regions boxed in blue and ligand binding residues shaded in gray. H, helix; N-term, N-terminus; RMSD, root mean square deviation; TM, transmembrane helix

Figure 4. B factor coloring suggests ligand delivery mechanisms.

Crystal structure residues of ligand chaperones ApoM (A; PDB: 2YG2) and autotaxin (ATX, B; PDB: 3NKP) and LPL receptors (C) and are colored according to their B factors. Because of intrinsic differences in the properties of GPCRs versus soluble proteins, the scale for the receptors is 40–140 Å² and for chaperones, 1–60 Å². ApoM ligand S1P and ATX ligand LPA are shown as space-filling molecules and residues for ligand binding and retention are shown. The high degree of flexibility in the ATX SMB1 and SMB2 domains required for their proposed activities in ligand binding affinity and cell surface integrin interaction, respectively, are evident in their extremely high B factors, versus the relative rigidity of the ApoM ligand binding pocket.