

Published in final edited form as:

Biochim Biophys Acta. 2013 January ; 1831(1): 2–12. doi:10.1016/j.bbaliip.2012.09.002.

Integrating the puzzle pieces: The current atomistic picture of phospholipids–G protein coupled receptor interactions★

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Abstract

A compelling question of how phospholipids interact with their target receptors has been of interest since the first receptor-mediated effects were reported. The recent report of a crystal structure for the SIP₁ receptor in complex with an antagonist phospholipid provides interesting perspective on the insights that had previously been gained through structure–activity studies of the phospholipids, as well as modeling and mutagenesis studies of the receptors. This review integrates these varied lines of investigation in the context of their various contributions to our current understanding of phospholipid–receptor interactions. This article is part of a Special Issue entitled *Advances in Lysophospholipid Research*.

Keywords

SIP; LPA; GPCR structure; Structure–activity relationships

1. Introduction

Twenty years has passed since the late eighties, when the debate began whether lysophospholipids elicit their wide-ranging biological actions through receptors or by detergent-like actions. The first crystal structure of a lipid receptor, the sphingosine-1-phosphate type 1 receptor has now been solved. Twenty years only! Considering that the concept of cholinergic or adrenergic receptors preceded atomic resolution structures of these biomolecules by almost a hundred years, lysophospholipid receptor biology and structural characterization have outpaced the development of any other receptor field. In the early nineties, lysophosphatidic acid (LPA) was humorously called lytic precipitating artifact, while the low cellular level of sphingosine-1-phosphate made some investigators think that it was a trace lipid that could even be a purification artifact. Today, we all know that research has provided solid answers to these early dilemmas. There are five SIP and six LPA plasma membrane receptors that have been firmly established and several others await further characterization. The dilemma whether lysophospholipids work via plasma membrane GPCR or intracellular targets is settled and several cell surface and intracellular targets have been identified.

★This article is part of a Special Issue entitled *Advances in Lysophospholipid Research*.

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The interest in lysophospholipids is also fueled by the potential development of drugs that harness the biology of these lipids. The field has received a big impetus from the FDA approval of FTY720/Gilenya as first-line treatment for multiple sclerosis and the granting of orphan drug status to an LPA₁ receptor antagonist, AM152 (now BMS-986202), for the treatment of idiopathic pulmonary fibrosis. This is just the beginning. Inhibitors of sphingosine kinases, autotaxin, and receptor antagonists are in the drug development pipeline.

In the present review we provide a selective review of the discovery process that provides the foundation for developing agonist pharmacons targeting lysophospholipid receptors. Our groups started tackling the problem of ligand recognition of lysophospholipid receptors in 1998 and we have been gratified last year when the crystal structure of S1P₁ was unveiled confirming the two key residues R3.28 and E3.29 interacting with the polar headgroup of the ligand which we have predicted by computational modeling a decade earlier. This review begins with some of the indirect reflections obtained about the interactions between phospholipids and their receptors through investigations of the structure–activity relationships of phospholipid analogs, proceeds through more direct structural insights, and highlights some of the protein–protein interactions modulated by receptor activation.

1.1. S1P receptor family agonist SAR trends

Several key findings led to an explosive increase in the investigation of structure–activity relationships for members of the S1P receptor family. First among these was the linkage of the immunomodulatory action of FTY720 (fingolimod) with S1P receptor agonism after *in vivo* phosphorylation [1,2]. Since FTY720 was already in the human clinical trial pipeline [3], its connection to S1P receptors stimulated preparation and testing of a wide variety of phospholipid analogs and subsequently non-lipid and more drug-like molecules. Later discovery that the long-lasting removal of S1P₁ from the cell surface occurs after stimulation by FTY720-phosphate (functional antagonism [4]) and is responsible for lymphocyte trafficking effects [5] stimulated consideration of antagonists, as well as agonists. Non-lipid molecules with S1P receptor activity appeared first in the patent literature (reviewed in [6]) and subsequently in peer-reviewed journals. These non-lipid molecules have not often been linked with specific binding sites through mutagenesis studies or confirmation of strictly competitive binding modes. In contrast, the wealth of SAR data generated using S1P analogs and a subset of the non-lipid agonists that have been investigated in the contexts of both wild type and mutated receptors provide valuable indirect information about phospholipid–receptor interactions.

The SAR data for S1P analogs and select non-lipid agonists can be analyzed based on an arbitrary division of these analogs into three pieces, a polar headgroup, a linker, and a tail, as shown for S1P in Fig. 1. Using this framework, S1P has a phosphate headgroup, a 2-aminopropanol linker, and a fifteen carbon tail with a trans double bond near the linker region.

Headgroup changes have been investigated by several research groups in the context of different linker and tail combinations. In one of these studies, 2-amino propane linkers and para-substituted nonyl phenyl methylene tails were used to investigate a range of headgroups including phosphate, phosphonate, alpha-hydroxyphosphonate, sulfonate and carboxylate [7]. Among these headgroups, the carboxylate and sulfonate were poorly tolerated at S1P₁, S1P₃, S1P₄ and S1P₅ (S1P₂ data not reported) with the smallest potency shift relative to the phosphate headgroup observed for the methylene carboxylate analog at S1P₁ (100-fold difference). Replacement of phosphate with methylene phosphonate reduced potency at all receptors tested. Selectivity for S1P₁, however, was enhanced due to a more limited 10-fold potency difference at this receptor relative to the 50 to 400-fold potency

differences at S1P₃₋₅. The inclusion of a hydroxyl group on the alpha carbon of the phosphonate, in contrast, leads to improved potency at S1P₁ relative to the phosphate headgroup, while still showing potency reductions of 5 to 85-fold at the other receptors tested. A second study utilized 2-amino ethane as the linker, and connected the para-substituted nonyl phenyl methylene tail directly to the amino group as the context in which to investigate headgroup preferences [8]. The range of headgroups tested included phosphonate, phosphinate, carboxylate, and both alpha and beta fluorine or hydroxyl-substituted carboxylates. The analog in this series with the methyl phosphonate headgroup showed the best potency at all receptor subtypes. As in the previous series, the methylene carboxylate was best tolerated by the S1P₁ receptor (40-fold worse than methylene phosphonate), but all substitutions of hydroxyl groups or fluorine atoms alpha or beta to the carboxylate group further decreased potency [8] in contrast to the increased potency observed for similar substitutions alpha to a phosphonate headgroup [7]. Two different publications investigated S1P receptor preferences between phosphate and thiophosphate head groups using the three linker–tail combinations shown in Fig. 2 [9,10]. S1P_{1,2,5} showed a preference for phosphate over thiophosphate regardless of linker–tail combination, with the single exception that the two headgroups in the context of scaffold B were equipotent at the S1P₁ receptor. The S1P₃ receptor also preferred the phosphate headgroup in the context of all scaffolds except B, although only partial agonism at S1P₃ was observed for both analogs. The S1P₄ receptor also showed a context-sensitive headgroup preference, in which the phosphate headgroup was preferred except for scaffold C when R = OH, although partial agonism was observed for this pair of analogs. The trends in all these studies suggest that anionic headgroups with charges delocalized over three atoms are better able to activate all S1P receptors than anionic headgroups with reduced overall charge, or charge shared only by two atoms as in the carboxylate headgroup. Highly delocalized negative charge is not an absolute requirement, however, as highly potent non-lipid molecules with carboxylate headgroups [11–18] as well as uncharged trifluoromethyl [19,20] and other headgroups [21–25] combined with a variety of aromatic and heteroaromatic linker/tail combinations have been reported. The headgroup pocket also seems to be limited in size, as the sulfur atom in the thiophosphate is substantially larger than the corresponding oxygen atom in a phosphate group. These SAR findings are consistent with prior identification of multiple cationic ion-pairing partners by modeling-driven mutagenesis studies [26,27].

The S1P receptors tolerate a broad selection of linkers, including saturated and unsaturated hydrocarbons with a variety of functional groups [1,7,8,10,28], secondary amines connecting the linker to the tail [8,29], amides [10,30–33], and a selection of aromatic heterocycles [9,34–36]. The single site mutation of the conserved glutamate residue at position 3.29 to glutamine (E3.29Q) is sufficient to confer tolerance of glycerol as a linker [37,38], though the wild type receptors require an ionizable nitrogen in all of these linker types. As with the preferences noted for highly delocalized negative charges in the headgroup, this requirement is also not absolute when combined with substantial changes in the headgroup and tail [39]. In this review, we will focus on one of these SAR studies, as it provides interesting insights into differences in phospholipid recognition in the S1P receptor family. Fig. 3 shows a subset of conformationally-constrained molecules used to define the agonist linker shape preferred by the various S1P receptors [29]. The unconstrained compound, D, showed potencies of 3.7, 580, 3.6, 140, and 13 nM at S1P₁₋₅, respectively. Compound E, with a sharp bend induced by the pyrrolidine ring, showed poorer potencies at all receptors than did compound D. However, the S1P₄ receptor showed only a 6.2-fold increase in IC₅₀ in the radioligand binding assay relative to more than 20-fold for S1P₁, S1P₃, and S1P₅. Binding was not detected at S1P₂. This finding matches well with comparative modeling of all five S1P receptors which concluded that the S1P₄ receptor binding pocket requires ligands to bend sharply near the ammonium functional group [40]. Two isomers of compound F (cis and trans geometries of substituents on the piperidine ring)

highlight additional geometry differences in the S1P receptor binding pockets. S1P₁ and S1P₄ showed several fold better affinity for the trans isomer over the cis isomer. S1P₂ and S1P₅ showed several fold better affinity for the cis over the trans isomer. Both isomers showed similar affinities for S1P₃. The cis isomer can adopt more extended conformations, in contrast to the trans isomer which bends at the ring. As noted previously, the S1P₄ receptor showed the most pronounced bend in the binding pocket in modeling studies. These data suggest that S1P₁ can also accommodate mildly bent shapes, but that S1P₂ and S1P₄ prefer more linear ligand geometries. The ability of S1P₃ to recognize both bent and linear shapes demonstrates why achieving S1P₁-selective agonists that lack the bradycardia side effect mediated by S1P₃ action has been achieved by large jumps in structural space from the flexible phospholipid agonist analogs of S1P [11,13,17–19,21,22,25,36,41,42].

SAR studies focused on the hydrophobic tail have defined the preferred length, tolerance for aromatic insertion, and preferred position of aromatic insertions in several headgroup-linker combinations. The linkage of FTY720 with S1P receptors provided the first evidence that the S1P₂ receptor does not tolerate aromatic ring insertions immediately adjacent to 2-aminopropanediol linker [1]. This finding extended to most analogs with amide linkers [30–32] as well as secondary amine linkers [8,36]. Interestingly, the aromatic substitution pattern (ortho versus meta versus para) plays an important role in ligand function, with meta-substituted aromatic rings positioned adjacent to the linker showing antagonist activity at S1P₁ and S1P₃ [31]. This functional impact is discussed further in the context of the S1P₁ crystal structure. Aromatic ring position has a tremendous impact on receptor activation profiles, with S1P₂ activity evident for analogs in which the aromatic ring is placed at or near the end of the tail region, S1P₃/S1P₄ potency showing an optimum when the aromatic ring appears near the middle of the hydrophobic tail, and S1P₁/S1P₅ showing about a ten-fold preference for the aromatic ring to be adjacent to the linker [8]. An atomistic explanation for the anti-S1P₂ selectivity of the analogs with aromatic rings adjacent to the linker was difficult to find, as it stems not from any single amino acid substitution, but from differences in the amino acid sequence in the first intracellular loop, located quite distant from the ligand binding pocket [43]. Extensive molecular dynamics simulations indicate that the differences in the intracellular loop sequence impact the mobility of the hydrophobic tail in a manner dependent on the presence or absence of an aromatic ring. The anti-S1P₂ selectivity is therefore due to binding entropy differences, rather than differences in binding enthalpy. Chain length preferences have been investigated using saturated linkers [8], amine linkers [8], and amide linkers [32]. These studies all suggest that the majority of S1P receptors are optimized for lengths similar to S1P (18 heavy atoms extending from the phosphate headgroup to the end of the tail) although the S1P₅ receptor shows the best retention of potency with shorter chain lengths. A preference for lengths similar to that of S1P was also identified for the S1P₄ receptor E3.29Q mutant using LPA species of varying chain lengths, with a preference for the 14-carbon tail and a four-atom glycerol linker [38].

1.2. LPA receptor family agonist SAR trends

Important biological roles for LPA were discovered much earlier than those for S1P, with several hundred publications related to LPA appearing in the literature prior to 1985, and only about ten related to S1P in the same time period. The structure of LPA additionally facilitates SAR explorations due to the natural occurrence of multiple LPA species that differ in the fatty acid linked to the sn-1 position of the glycerol linker (Fig. 2 shows LPA with a 14:0 fatty acid). SAR studies of phospholipid analogs of LPA therefore began earlier and have already been thoroughly reviewed, although most of these studies were limited to examination of the EDG-family LPA receptors, LPA₁, LPA₂, and LPA₃ [44,45]. These reviews note that changes in the phosphate headgroup are, in general, poorly tolerated. Only alpha-hydroxy substituted phosphates and thiophosphates had been incorporated into active

EDG LPA receptor agonists. These headgroups have opposing selectivities among LPA receptors, with the alpha-hydroxy substituted phosphonates activating LPA₁ and LPA₂, and the thiophosphate activating LPA₃. A more recently published set of alpha-fluorophosphonate analogs also show selectivity among the EDG LPA receptors [46], but with greater similarity to the LPA₃ selectivity of the thiophosphate headgroup; than to the LPA₁/LPA₂ selectivity of the more structurally similar alpha-hydroxyphosphonates. Although the alpha-fluoro phosphonate and thiophosphate analogs of LPA proved to be selective agonists, alpha-fluoro phosphonate and thiophosphonate analogs of cyclic phosphatidic acid generally showed antagonist activity at LPA₁ and LPA₃ [47,48]. The unsubstituted phosphonate analogs of cyclic phosphatidic acid (carba-cyclic phosphatidic acids) showed no activity at LPA₁₋₄, but proved to be partial agonists of LPA₅ with E_{max} values of 58 and 71% for the two regioisomers [49]. A final set of phosphonates in which methylene phosphonate is attached to the sn-3 oxygen of glycerol (methylene-extended) proved to be full to partial agonists of LPA₁, LPA₂ and LPA₄, but antagonists of LPA₃ [50]. The strict limitations on the headgroup were further validated for the EDG LPA receptors and extended to the non-EDG LPA₄ receptor with the finding that two uncharged biomimetics of the phosphate group, trifluoromethanesulfonamide (–NHSO₂CF₃) and trifluoromethanesulfonamidoxy (–ONHSO₂CF₃) replacements for phosphate did not produce LPA analogs capable of activating LPA₁₋₄ [51]. These recent additions to the headgroup SAR for the LPA receptors highlight the sensitivity of these receptors to even small changes in headgroup structure, size, and charge.

LPA analog linker SAR has been previously reviewed [44,45]. In summary, LPA receptors have a relatively high tolerance for glycerol with varying numbers and positions of tails and neutral ethanolamines linked into amide functional groups to attach the tail in the linker region. Interestingly, the LPA₂ receptor can be activated by a variety of linker-free fatty alcohol phosphates, many of which show weak antagonism of LPA₁ and LPA₃ [52,53]. The linker, therefore, plays an important functional role in the EDG LPA receptors.

More recently published SAR data has focused largely on the non-EDG LPA receptors, and changes in the hydrophobic tails. The LPA₅ receptor is unique in showing a preference for the hydrophobic tails connected to the linker by an ether rather than ester functional group (alkyl-glycerol phosphates, AGP), although AGP is tolerated at the EDG LPA receptors and equipotent with LPA at LPA₄ [49,54]. The LPA₆ receptor is analogous to the LPA₃ receptor in its preference for the hydrophobic tail placement at the central sn-2 position of glycerol, rather than the terminal sn-1 position [55]. This finding is in contrast with results for LPA₁ and LPA₂, in which little preference between the regioisomers was observed [55,56]. Many short-chain phosphatidic acids with two tails had previously been shown to act as antagonists at members of the EDG LPA receptor subfamily, but display partial agonist activity at LPA₅ [54,57].

1.3. Inferred similarities and differences between LPA and S1P recognition

The SAR trends for the S1P and LPA receptor families suggest both similarities and differences in phospholipid recognition. SAR trends all demonstrate a relatively limited tolerance for changes to the phosphate headgroup relative to the natural ligands. Mutagenesis studies done in multiple LPA [49,58,59] and S1P [26,27] receptors as well as the recently published crystallographic structure of the S1P₁ receptor [60] allow this limited tolerance to be attributed to the presence of multiple cationic amino acids in close proximity to each other that must be balanced by the distributed negative charge of a headgroup quite similar in size, charge, and charge distribution to a phosphate group. A substantial contribution to phospholipid selectivity between the LPA and S1P receptor families centers on selective recognition of a cationic ammonium group in the linker region of active S1P receptor agonists that is absent in LPA receptor agonists. Mutagenesis studies have

demonstrated that S1P receptors select for ammonium-containing phospholipids using an anionic glutamate residue in the third transmembrane domain that is absent in all LPA receptors [37,38].

1.4. An overview of the structural puzzle pieces

1.4.1. Crystal structure of S1P₁—The year 2012 marked the first published and publically available crystal structures of a phospholipid GPCR, the S1P₁ receptor in complex with a phospholipid antagonist, ML056 (ML056 structure in Fig. 4) [60]. The crystallized construct is another in a series of GPCR structures that have proven amenable to crystallization after replacement of the third intracellular loop (IL3) with T4 lysozyme, as pioneered in the solution of the structure of the β -2 adrenergic receptor [61]. In the S1P₁ receptor, IL3 residues 232–244 were replaced by T4-lysozyme, and therefore are not represented in the structure. Additionally missing due to poorly defined electron density are either 15 or 16 N-terminal amino acids (16 in 3V2W and 15 in 3V2Y), as well as residues 40–46 leading into the first transmembrane segment (3V2W only). The reduced number of missing amino acids in the 3V2Y structure is due to the application of enhanced data assembly methods in processing the same diffraction data. An N-acetylglucosamine molecule is associated with the extracellular surface of the receptor, and one crystallographic water molecule forms bridging interactions between the phosphate headgroup of ML056 and residues in the amino terminus and TM7. Fig. 5 shows these features of the S1P₁ crystal structure (PDB ID: 3V2Y [62]).

The S1P₁ structures provide atomistic experimental confirmation of several phospholipid interactions previously predicted by modeling studies and supported by mutagenesis experiments. In particular, the 3.28R/3.29E motif in the third transmembrane segment conserved in S1P₁₋₅ was observed in the crystal structure to form ion-pairing interactions with the phosphate (R3.28) and ammonium (E3.29) functional groups of ML056. These interactions are directly analogous to the observed interactions with the phosphate and ammonium groups of S1P (structure in Fig. 4) in modeling studies [26,27,37–40,63–66]. These interactions result in phosphate group placement within the transmembrane helical bundle at a depth analogous to the membrane:water interface surrounding the receptor. Additionally, the hydrophobic tail of the bound phospholipid extends deeper into the transmembrane helical bundle, rather than extending upward toward the extracellular loops of the receptor.

The crystal structure does demonstrate substantial differences in phospholipid:receptor interactions relative to prior modeling studies, which may be attributed either to differences in binding modes between the antagonist crystallized and the agonists investigated in modeling studies, or may indicate errors in the modeling and interpretation of mutagenesis results. These two differences include the hydrophobic tail position and the identity of the second ion-pairing partner for the phosphate head group of the bound phospholipid. Fig. 6 highlights these differences in a side-by-side comparison of the crystallographic complex between S1P₁ and ML056 and our modeled complex between S1P₁ and S1P.

The hydrophobic tail of ML056 in the crystal structure [60] makes a sharp bend toward TM5, passing underneath F125(3.33) and ending near C206(5.44), T207(5.45), F210(5.48) and L276(6.55). In contrast, the hydrophobic tail of S1P in the model complexes reported by ourselves [67] and others [39,65,66] extends in a more linear fashion into the TM bundle from the polar headgroup, ending near V6.40 and L3.43 [67] or near L6.41 and L3.43 [66]. Both mutagenesis [65,67] and structure–activity relationship (SAR) results suggest that differences in the hydrophobic tail position may reflect real differences that differentiate agonists from antagonists. In particular, the C5.44D mutant of S1P₁ displayed normal responses to S1P [67], which would not be expected if the hydrophobic tail of S1P occupied

the same location as that of ML056 as that would represent an entropically favorable interaction type that should lead to decreased responsiveness to S1P. S1P was also minimally affected by F3.33Y and F3.33A mutations [65], even though F3.33 makes van der Waals contact with five atoms in the hydrophobic tail of ML056, which would be lost upon mutation to alanine and would be expected to show impaired activity if similar interactions with S1P occur. Interestingly, F3.33A mutants were poorly activated by agonists with aromatic moieties, indicating that this residue differentially contacts ligands of different types [65]. Another residue showing a variable role in ligand binding as a function of ligand structure was identified by the Deng et al. [66]. These studies demonstrated that mutations of L6.55F in S1P₁ and F6.55L in S1P₃ had little impact on S1P binding, but reversed the S1P₁/S1P₃ selectivity of a non-lipid agonist, compound A (structure in Fig. 4). In addition to these mutations to residues that do interact with ML056 in the crystallographic complex, mutations at sites that do not interact with ML056 also support different binding modes for S1P and ML056. In particular, the V6.40L mutant of S1P₁ showed no response to S1P (although mutations to smaller amino acids were tolerated) [67], suggesting that the increased size of leucine compared to valine blocked a part of the pocket occupied by the hydrophobic tail of S1P. Interestingly, the V6.40L mutant showed reduced response to SEW2871 (E_{\max} at 3 μM = 34.8% and structure in Fig. 4) [67], indicating that the receptor is not grossly misfolded, and a slightly shorter agonist retains some ability to activate the receptor in the presence of the larger sidechain at position 6.40. In contrast, V6.40 is very distant (>17 Å) from any atoms of ML056 in the crystal structure. Finally, SAR data has demonstrated that longer tails on meta-substituted compounds similar in structure to ML056 as well as their para-substituted analogs act as agonists, rather than antagonists [31]. These data in aggregate suggest that the short hydrophobic subpocket angling toward TM5 is likely to allow binding, but does not allow bound ligands to stabilize the active conformation of the receptor. In contrast, the longer and more linearly arranged subpocket that would be occupied by longer analogs and para-substituted analogs must be occupied in order to observe receptor activation. The crystal structure itself clearly includes not only the hydrophobic pocket occupied by the tail of ML056, but also another hydrophobic subpocket similar to the more linear arrangement observed for the S1P hydrophobic tail in modeling studies [68]. Thus the differences in hydrophobic tail location are likely to represent differences that can be used in the rational optimization of agonists versus antagonists.

A second substantial phospholipid interaction difference between the S1P₁ crystal structure and previous modeling studies is the identity of the second ion-pairing partner for the phosphate group. ML056 interacts with K34 from the N-terminal helical cap that covers the bound phospholipid in the crystal structure. In contrast, modeling and mutagenesis studies in our group identified R7.34 in TM7 as the second ion-pairing partner for the bound phosphate group of S1P [26,63], although other modeling studies did not suggest any additional ion-pairing partner [39,64–66]. It is worth mentioning that the S1P₁ model used in all of these modeling studies did not include N-terminal residues, and therefore could not consider K34 as a potential ion-pairing partner. In support of our model-derived hypothesis of a direct role for R7.34 in phospholipid binding, the R7.34A mutant of S1P₁ showed no ability to bind 1 μM S1P in a radioligand binding assay, and showed no response to 1 μM S1P in a [³⁵S]GTP γ S binding assay. Yet the sidechain of R7.34 is located almost 12 Å from the phosphate group of ML056 in the crystal structure. The apparent discrepancy between these data can be attributed either to a ligand-specific and differing role for R7.34, potentially dependent on whether ligands act as agonists or antagonists. An alternative explanation is that R7.34 plays a crucial role in ligand recognition without making direct contact in the final complex. This latter explanation is intriguing due to the location of R7.34 in the crystallographic structure, where the cationic sidechain sits at the top of a gap between TM1 and TM7, suggested to serve as the phospholipid entry and exit pathway between the binding pocket and the surrounding membrane [60]. It is therefore possible that R7.34 is

responsible for recruiting phospholipid binding partners from the surrounding membrane, and that its replacement with a non-polar amino acid results in no recruitment event, even if the final complex stability would be unchanged.

Several additional features of published models are interesting to review in the context of the S1P₁ crystal structure with ML056. A hydrogen bond between Y98(2.57) was indicated in one modeling study [64], but the role of this residue was suggested to stabilize the polar portion of the binding pocket through a direct interaction with R3.28 in a different modeling study [65]. The latter study experimentally demonstrated that the Y2.57F mutation showed wild-type responses to S1P and other agonists, in contrast to the Y2.57A mutant, which showed impaired responses to all agonists tested with the exception of FTY720-phosphate. This finding seemed to rule out a hydrogen bond between Y2.57 and agonists. The crystal structure [60] demonstrates neither role for Y2.57, which is positioned 9 Å below the sidechain of R3.28 but does form a hydrophobic interaction with V301(7.43) that may contribute to stabilizing the shape of the hydrophobic part of the binding pocket, and makes van der Waals contact with the aromatic ring of ML046 (<4 Å).

1.4.2. NMR structure of first extracellular loop of S1P₄—The first atomic-resolution experimental structure that shed light on GPCR–phospholipid interactions was reported five years prior to the report on the S1P₁ crystal structure, but was limited to describing the structure of only the first extracellular loop (EL1) of the S1P₄ receptor [69]. This study utilized NMR spectroscopy to characterize the structure of a synthetic peptide which included the sequence of the S1P₄ EL1 in between two segments of an antiparallel coiled-coil as a folding constraint. The synthetic peptide retained the R3.28/E3.29 motif that had been demonstrated by mutagenesis to be required for phospholipid binding and activation in S1P₄ [27]. The structure of S1P₄ EL1 includes a short 3¹⁰ helical segment for residues R109, T110, and F111 at the N-terminal end of the loop. These residues correspond to residues T108, T109, and Y110 in the S1P₁ crystal structure, which also form a helical secondary structure (Fig. 7). Notably, ligand titration studies using O-phosphoethanolamine (a mimic of the S1P headgroup) but not N-acylethanolamine (a mimic of the ceramide headgroup) showed chemical shift changes localized to E3.29 and residues in the flexible C-terminal end of the loop (residues 109–116).

1.5. Extending the structural puzzle pieces to new GPCR

The crystallographic structure of S1P₁ represents the first experimental structure of a phospholipid receptor, an important representative of a poorly-characterized segment of GPCR sequence space. S1P₁ shares little sequence identity with the more than ten other class A GPCR that have been crystallized (<22%). Several subsets of the class A GPCR that have been documented to respond to a variety of lipid ligands have much higher sequence homology with S1P₁ than with any other currently available class A GPCR crystal structure, including the remaining S1P receptors (S1P_{2–5}), the EDG LPA receptors (LPA_{1–3}), the cannabinoid receptors (CB1–2), and the GPR3/6/12 cluster (alignment shown in Fig. 8). Comparison of the NMR structure of a peptide mimetic of EL1 from the S1P₄ receptor to the crystallographic structure of S1P₁ shows that a short helical segment in this loop (residues GATT in the S1P₁ EL1 and GART in the S1P₄ EL1) is conserved in two different lipid receptors. It is useful, therefore, to compare sequence features in a larger set of aligned lipid receptors that correspond with interesting structural features in the S1P₁ structure in order to suggest which structural features are likely to be observed in other receptors.

1.5.1. Disulfide bonding pattern analogies—The S1P₁ receptor crystal structure exhibits a unique disulfide bonding pattern among the currently available class A GPCR crystal structures. This pattern involves two intraloop disulfide bonds, one involving the two

cysteine residues in the second extracellular loop (EL2) and the other involving the two cysteine residues in EL3. Fig. 8 shows that the disulfide-bonded cysteine residues in EL2 of S1P₁ are strictly conserved in these lipid receptors, although S1P₄, LPA₁₋₃, and CB2 have an additional cysteine. Fig. 8 also shows that the disulfide-bonded cysteine residues in EL3 of S1P₁ show much more limited conservation only in S1P_{1-3,5} and LPA₁₋₃. Several of these lipid receptors completely lack cysteine residues in EL3, particularly S1P₄, GPR3/6/12, and the cannabinoid receptors. An additional cysteine residue is present in EL3 of LPA₁. These comparisons suggest that most of the lipid receptors will share the intraloop disulfide bond found in EL2 of S1P₁, with the possible exception of receptors exhibiting an additional cysteine residue in that loop. However, the majority of the lipid receptors cannot be assumed to exhibit the same disulfide bond in EL3 that was observed in S1P₁, and further studies will be needed to identify the role disulfide bonds play in determining structural differences among the extracellular regions of these lipid receptors.

1.5.2. N-terminal helical cap analogies—The S1P₁ crystal structure exhibits a unique extracellular architecture relative to the other crystallized class A GPCR [60,68]. This architecture includes a helical cap formed from residues 23–32 in the N-terminus. The N-terminal helix includes asparagine 30, which is N-glycosylated in the crystallized construct. Fig. 8 demonstrates that N-glycosylation is not possible in the GPR3/6/12 subset of lipid receptors, due to the absence of asparagine or glutamine as found in the remaining receptors. Tyrosine 29 in the N-terminal helix forms a hydrogen bond with W117 at the C-terminal end of EL1. This pair of residues is conserved in the entire EDG family (S1P₁₋₅ and LPA₁₋₃). Isoleucine 25 of the N-terminal helix sits in a hydrophobic pocket formed by P114 at the C-terminal end of EL1 and L197 near the C-terminal end of EL2. Isoleucine 25 corresponds to branched hydrophobic sidechains in the EDG receptors, with the exception of the linear hydrophobic methionine residue found in LPA₃. The EL2 interaction site is highly conserved in the EDG receptors, with the only substitution of isoleucine for leucine appearing in LPA₃. The EL1 interaction site is less well-conserved, with the proline residue appearing in the S1P receptors and other hydrophobic residues appearing in the LPA receptors. Given these conserved interaction types, it is likely that the entire set of S1P and LPA receptors may exhibit a similar N-terminal helical cap. It is unlikely that this structural feature generalizes into the cannabinoid or GPR3/6/12 subsets.

1.6. C-terminal motifs form a signaling hub

Lysophospholipid GPCR engage heterotrimeric G-proteins and activate canonical downstream signal transduction pathways. These pathways have been well characterized in many of the original papers describing these receptors as well as in reviews. One less discussed aspect of signaling by many of the lysophospholipid receptors is not coupled to the activation of G proteins but is mediated by the ligand-dependent assembly of macromolecular signaling complexes via docking sites encoded by distinct sequence motifs in their C-termini (Fig. 9). Thus far, there are two such motifs identified and characterized primarily among LPA-specific lipid receptors. The C-termini of LPA₁, LPA₂ and LPA₅ contain a –SxL/V motif, where x can be any amino acid, which is a docking site for the PSD95/Dlg/ZO-1 (PDZ) family proteins. It was first reported by Yamada and colleagues that PDZ-RhoGEF and leukemia-associated Rho GEF (LARG), two guanine nucleotide exchange factors (GEF) of the small molecular weight G protein RhoA, physically interact with the PDZ motif of LPA₁ and LPA₂ [70]. PDZ-RhoGEF and LARG contain PDZ domains as well as regulators of G protein signaling (RGS) domains. The PDZ domain mediates their binding to LPA₁ and LPA₂, whereas the RGS domain binds to the G₁₂ or G₁₃ α subunits. We demonstrated that a PDZ motif-dependent macromolecular complex of the cystic fibrosis transmembrane regulator (CFTR), the Na⁺/H⁺ exchange regulatory factor 2 (NHERF2), and the LPA₂ receptor subtype is essential for LPA-dependent inhibition of

cholera toxin-induced diarrhea. Through this interaction, LPA₂-dependent Gi-mediated inhibition of adenylyl cyclase reduces Cl⁻ secretion and attenuates the symptoms of secretory diarrhea in vivo. Zhang et al. [71] screened a library of PDZ proteins for interaction with the LPA₂ C-terminus and identified multiple potential interacting proteins including NHERF1, NHERF2, MAGI-2, MAGI-3 and neurabin. Singh et al. provided further functional evidence for the functional regulation of CFTR localization and activity by PDZ-dependent interactions involving LPA₂ and either NHERF1 or NHERF2, respectively [72]. MAGI-3 binding to LPA₂ regulates LPA-activated ERK1/2 and RhoA activation [71]. Interestingly, if both proteins are expressed in the same cell there is a dynamic competition between MAGI-3 and NHERF2 binding to the PDZ motif of the LPA₂ C-terminus, for example in intestinal epithelial cells. In such a case MAGI-3 binding attenuates NF- κ B and c-Jun terminal kinase activation mediated by LPA₂ [73]. Among the P2Y family LPA receptors, LPA₅ has a PDZ motif that has been shown to physically interact with the second PDZ domain of NHERF2 and regulate the Na⁺/H⁺ exchanger 3 that mediates fluid absorption in the intestine [74]. Altogether, the type 1 PDZ binding motif in the C-termini of LPA₁, LPA₂, and LPA₅ is a functionally important docking site for several proteins with PDZ domains, which modulate the activation of canonical G protein-coupled signaling.

Yeast two hybrid experiments conducted with the C-terminus of LPA₂ have identified Siva-1, a non-PDZ protein, and also the thyroid receptor interacting protein 6 (TRIP6) as high-affinity binding partners [75]. Siva-1 is an early response gene regulated by p53 and E2F transcription factors. Siva-1 is rapidly expressed following DNA damage and makes a complex with the antiapoptotic protein Bcl-X_L [76,77]. However, upon ligand activation of LPA₂, Siva-1 forms a macromolecular complex with this receptor and the complex becomes poly-ubiquitinated and degraded in the proteasome. Siva-1 recognizes the -C₃₁₁xxC- motif in the LPA₂ C-terminus, which is a half zinc finger motif [78]. Double zinc finger structures promote protein-protein interactions [79] and Siva-1 has its own zinc finger motif. TRIP6 belongs to the Lin-11, Isl-1 and Mec-3 (LIM) family of proteins that contain zinc finger motifs. LIM proteins can shuttle between focal adhesions and the nucleus and control gene expression [80,81]. TRIP6 also contains a PDZ motif in its C-terminus and can interact with NHERF2. The zinc finger and PDZ motifs present in TRIP6 are the foundation of a ternary macromolecular complex formed between LPA₂-TRIP6-NHERF2 that is required for the antiapoptotic action mediated by this receptor subtype [78]. The physical interaction between LPA₂ and TRIP6 is required for LPA-induced activation of NF κ B that involves the physical interaction of TRIP6 with the p65 subunit of NF κ B [82]. Furthermore, this complex augments the activation of the ERK1/2 kinases. Thus the potent antiapoptotic action of LPA depends on the depletion of the apoptotic mediator Siva-1 as well as activation of the ERK1/2 and NF κ B prosurvival pathways all of which involve macromolecular complexes assembled at the C-terminus of LPA₂. Although here we focused on macromolecular complexes of the LPA-specific lipid receptors, there is emerging evidence that the SIP₁ and SIP₂ receptors also interact with the P-Rex1 and LARG PDZ proteins, which are involved in migratory and differentiation responses [83,84].

2. Summary

The coupled progress made over the last twenty years in the areas of phospholipid biology and biomolecular target structural biology has led to an impressive understanding of how phospholipid interactions with their biomolecular targets lead to downstream biological effects. Even in the face of the rapid progress in the field, however, there still remains ample room for future research focused on a wide variety of topics. Compelling unanswered questions remain in areas ranging from the structural differences within receptor subfamilies that can be exploited in the development of receptor-specific therapeutics to detailed

understanding of the protein–protein interactions that participate in the complex cascade of interactions that lead to downstream systemic responses. Several groups have effectively applied modeling to discover sources of receptor selectivity. The first of these was our identification of position E/Q3.29 as the source of selectivity between S1P and LPA [37,38]. More applicable to the drug discovery, however, are the findings of Deng et al., and Schurer et al., that position L/F6.55 contributes to S1P₁/S1P₃ selectivity among the non-lipid agonists [39,66]. However, care must be taken not to ignore the fact that binding free energy differences include both enthalpic differences and entropic differences. Consideration of docked complexes or even crystallographic complex structures and specific interactions within those complexes can provide insights into the enthalpic differences between different complexes, but do not aid in identification of entropic differences that can dramatically influence specificity. We recently undertook an extensive study to define the source of the FTY720-phosphate selectivity against the S1P₂ receptor [43]. An extensive set of first site mutations and then S1P₁/S1P₂ receptor chimeras surprisingly identified intracellular loop 1 as providing this selectivity. Modeling studies that considered the dynamic nature of the receptor ligand complexes demonstrated that entropic penalties due to limited mobility of the hydrophobic tail occurred in the inactive receptor–ligand pairings. Thus modeling studies can provide pathways to understand both the enthalpic and entropic contributions to ligand selectivity.

Acknowledgments

The work was supported by grants to AP from the National Heart, Lung, and Blood Institute, HL084003 and to GT from the National Cancer Institute, CA092160, National Institute of Allergy Medical Countermeasures Radiological and Nuclear Threats Program AI80405 and by the Harriet Van Vleet Endowment for Basic Oncology Research.

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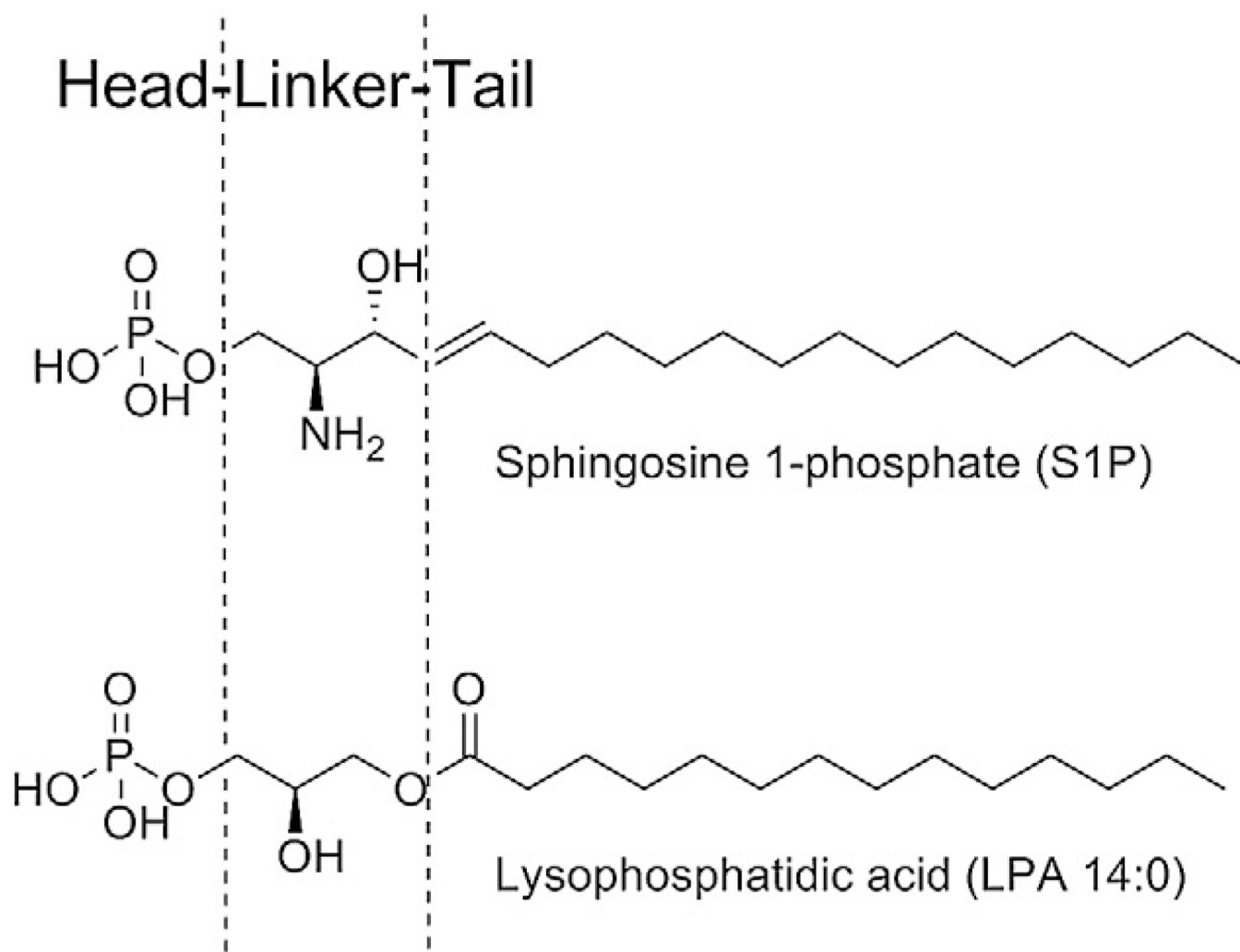


Fig. 1. Arbitrary division of S1P and LPA phospholipid analogs into headgroup, linker region, and tail for SAR analysis.

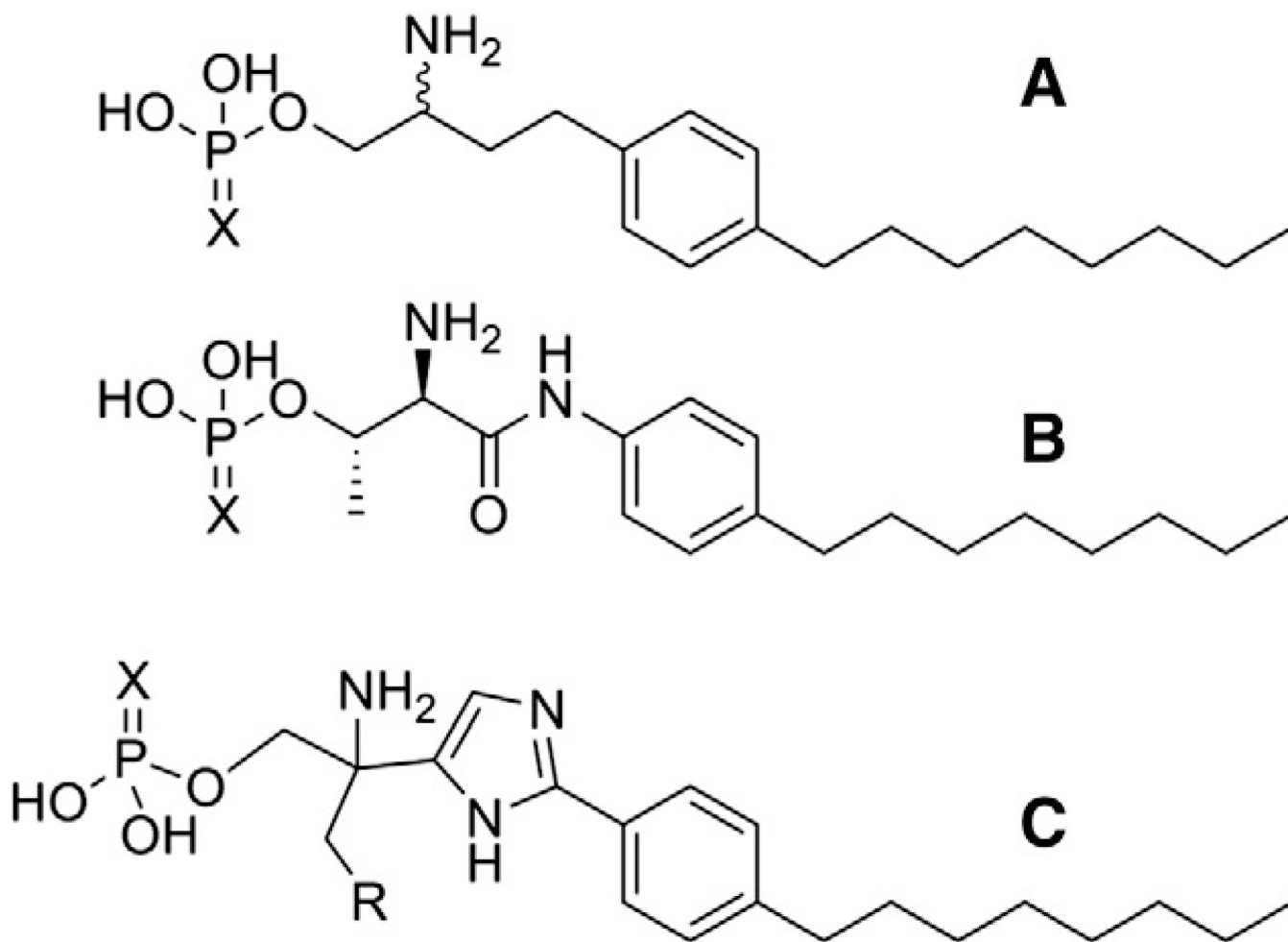


Fig. 2. Linker-tail combinations used to investigate S1P receptor preferences between phosphate (X = O) and thiophosphate (X = S) headgroups. R = H or OH.

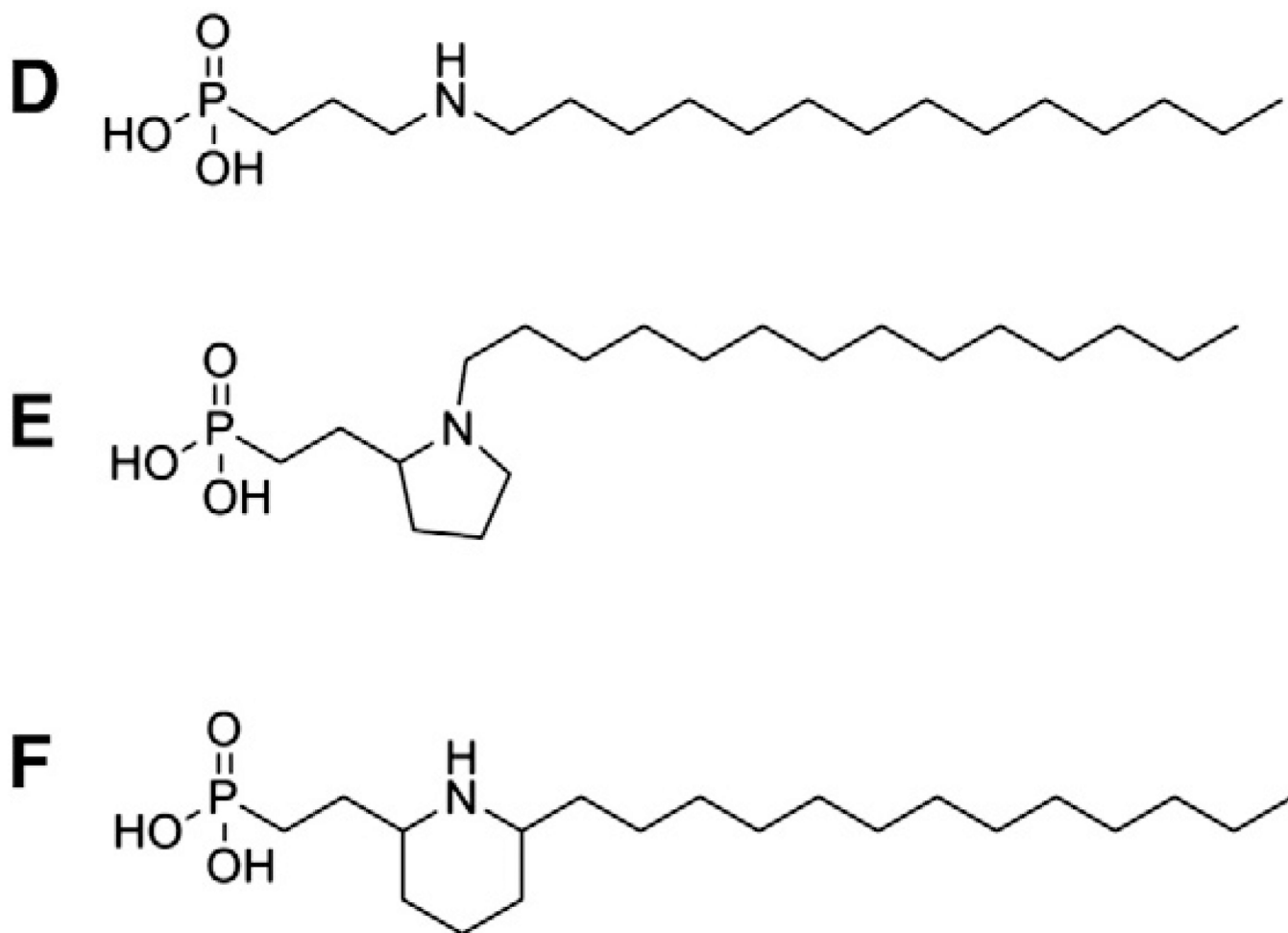


Fig. 3. Examples of conformationally-constrained linkers used to define S1P receptor binding pocket shapes.

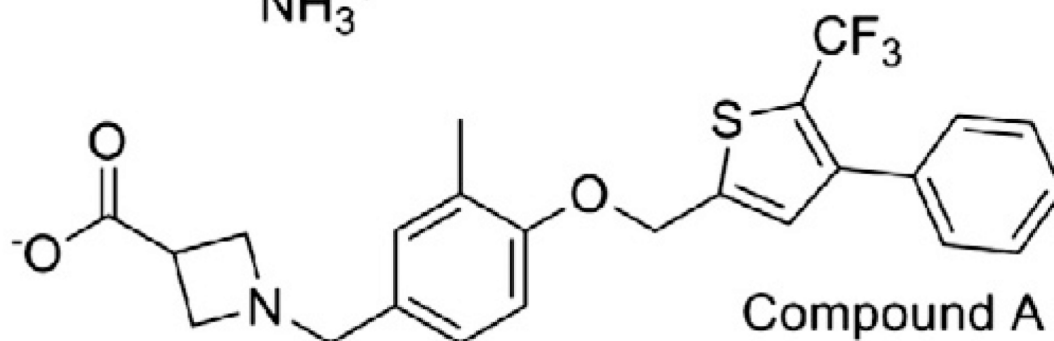
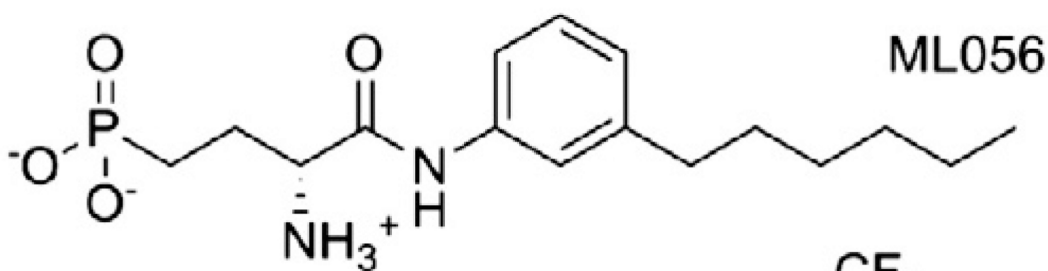
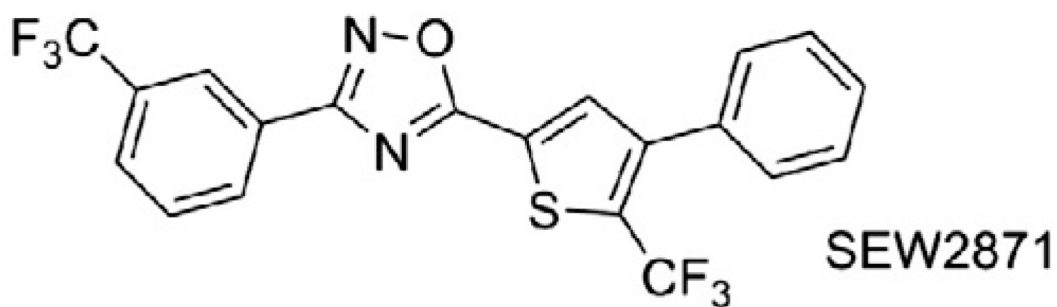
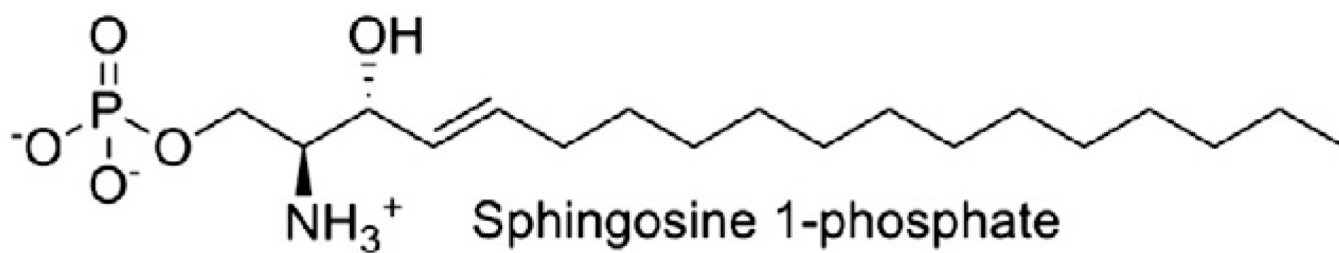


Fig. 4.
Structures of S1P, SEW2871, ML056, and compound A.

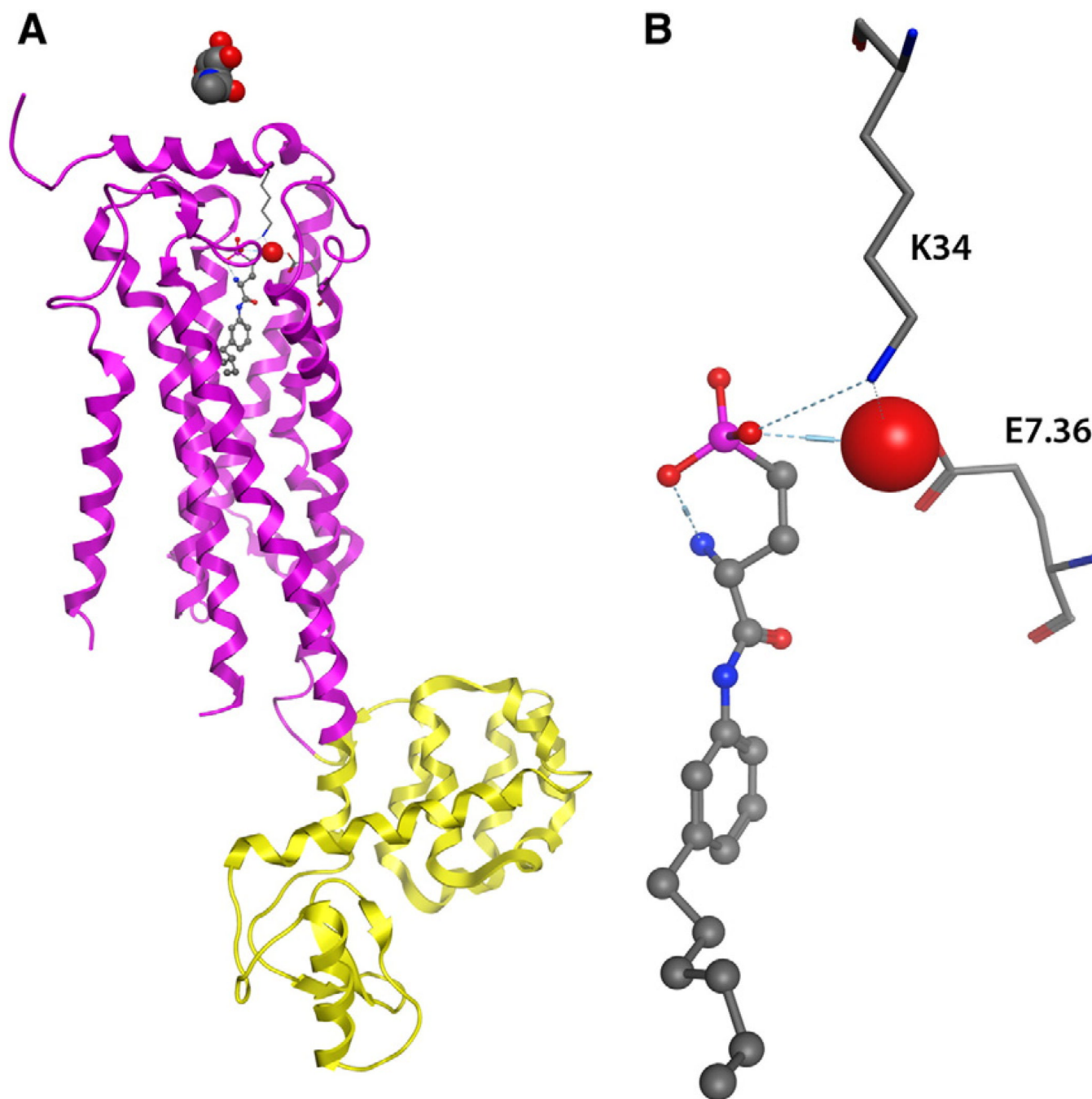


Fig. 5. S1P₁ crystal structure. Panel A: Protein structure rendered in ribbons with residues from S1P₁ colored magenta and those from T4 lysozyme colored yellow. N-acetylglucosamine and the water mediating ligand-receptor interactions are shown as spacefilling models. ML056 is shown using ball & stick and select receptor residues are shown using sticks. Panel B: Close up of water-mediated ligand-receptor interactions.

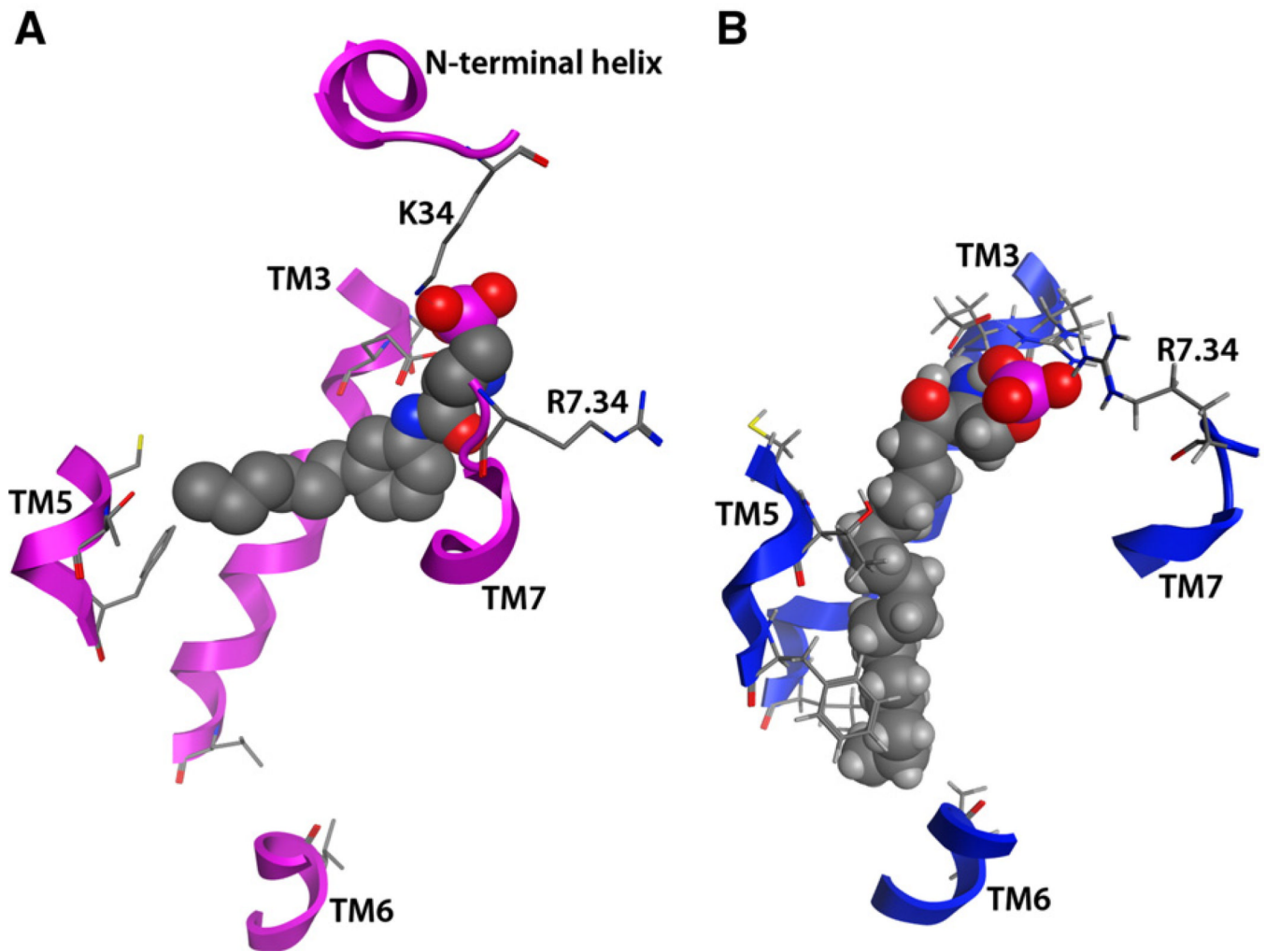


Fig. 6. Phospholipid interaction comparison between the S1P₁ crystallographic complex with ML056 (Panel A) and an S1P₁ modeled complex with S1P (Panel B). Portions of TM3, TM5–7, and the N-terminal helix are shown for reference, and other protein segments are hidden to allow clear depiction of ligand–receptor interactions.

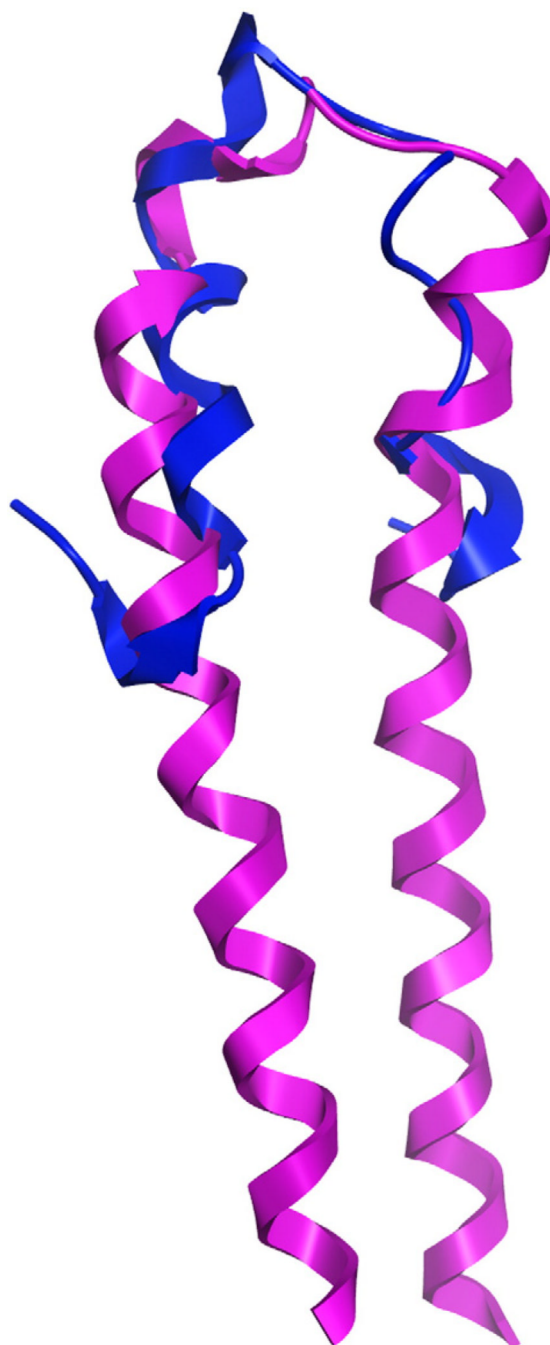


Fig. 7. NMR solution structure of synthetic S1P₄ EL1 peptide (PDB ID: 2DCO, blue ribbon) superposed on the crystallographic structure of S1P₁ (PDB ID: 3V2Y, magenta ribbon, TM2–3 segment only).

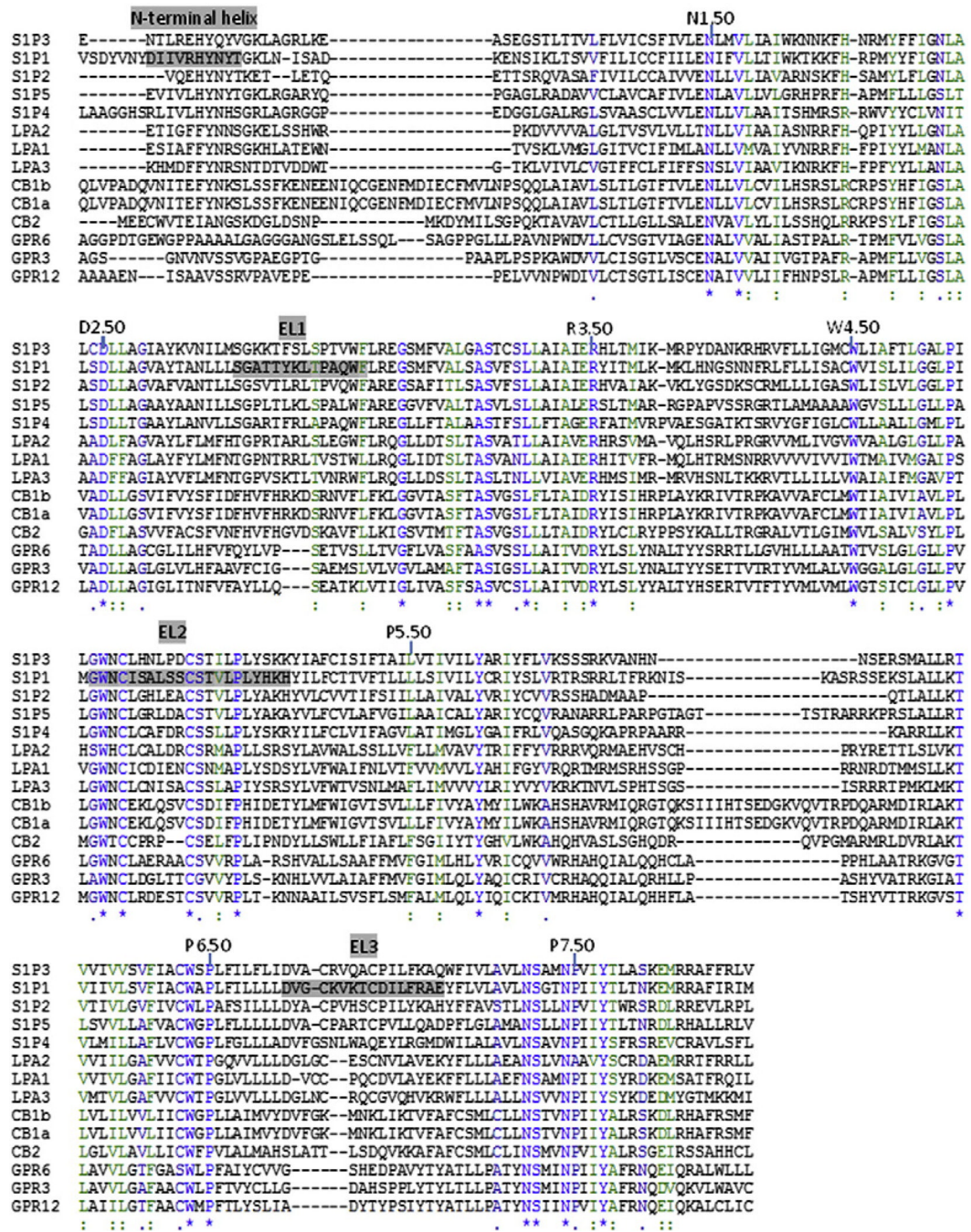


Fig. 8. Alignment of human lipid receptor sequences. Strictly conserved residues are shown in bright blue (marked with * under the alignment). Residues with strong or weak conservation within groups of receptors are colored green (marked with :) and dark blue (marked with .), respectively. Alignment is truncated at both N-terminal and C-terminal ends to exclude residues corresponding to unresolved segments of the S1P₁ crystal structure (prior to residue 16 and after residue 326 of S1P₁).

	Helix 7	C - terminus
	
	Human LPA ₁ -YRDKE MSTAF RQILC CQRSE NPTGP TESSD RSASS LNHTI LAGVH SNDHS VV	
EDG	Human LPA ₃ -YKDED MYGTM KKMIC CFSQE NPERR PSRIP STVLS RSDTG SQYIE DSISQ GAVCN STS	
	Conserved ---*-- *--*- ----* *----- ----*-----	
	Human LPA ₂ -CRDAE MRRTF RRLLC CA CLR QSTRE SVHYT SSAQG GASTR IMPLE NGHPL MD STL	
P2Y	Human LPA ₄ -ESFQK SFYIN AHIRM ESLFK TETPL TTKPS LPAIQ EEVSD QTTNN GGELM LESTF	
	Human LPA ₅ -RARTS ATNGT RAALA QSERS AVTTD ATRPD AASQG LLRPS DSHSL SFTQC PQD SAL	
	Human LPA ₆ -YYFTS DTIQN SIKMK NWSVR RSDFR FSEVH GAENF IQHNL QTLKS KIFDN ESAA	
	 Half Zn-finger motif	 PDZ recognition motifs
Sequence motif	Binding partners	Signaling links
-CxxC- half Zn-finger	Siva-1 TRIP6	Bcl-XL, Mdm2, p53 c-Src, PTPL1, ERK1/2, AKT, NFκB
-SxLV PDZ	NHERF1 NHERF2 MAGI-2 MAGI-3 Neurabin PDZ-RhoGEF LARG	CFTR CFTR, PLC-β3, AKT, ERK1/2 ? NHERF2, ERK1/2 ? RhoA RhoA

Fig. 9.
C-terminal docking motifs for signaling complexes formed by LPA receptors. See text for details.