A Single Amino Acid Determines Lysophospholipid Specificity of the S1P1 (EDG1) and LPA1 (EDG2) Phospholipid Growth Factor Receptors*

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The phospholipid growth factors sphingosine-1-phosphate (S1P) and lysosphatidic acid (LPA) are ligands for the related G protein-coupled receptors S1P/EDG1 and LPA/EDG2, respectively. We have developed a model of LPA1 that predicts interactions between three polar residues and LPA. One of these, glutamine 125, which is conserved in the LPA receptor subfamily (LPA1/EDG2, LPA/EDG4, and LPA/EDG7), hydrogen bonds with the LPA hydroxyl group. Our previous S1P1 study identified that the corresponding glutamate residue, conserved in all S1P receptors, ion pairs with the S1P ammonium. These two results predict that this residue might influence ligand recognition and specificity. Characterization of glutamate/glutamine interchange point mutants of S1P1 and LPA, validated this prediction as the presence of glutamate was required for S1P recognition, whereas LPA recognition was possible with either glutamine or glutamate. The most likely explanation for this dual specificity behavior is a shift in the equilibrium between the acid and conjugate base forms of glutamic acid due to other amino acids surrounding that position in LPA1, producing a mixture of receptors including those having an anionic glutamate that recognize S1P and others with a neutral glutamic acid that recognize LPA. Thus, computational modeling of these receptors provided valuable information necessary for understanding the molecular pharmacology of these receptors.

Lysosphatidic acid (LPA)1 and sphingosine-1-phosphate (SIP, see Fig. 1A) are members of the phospholipid growth factor family (for reviews, see Refs. 1–3). The responses elicited by phospholipid growth factors are pleiotropic and include the enhancement of cell survival, induction of cell proliferation, regulation of the actin-based cytoskeleton affecting cell shape, adherence, and chemotaxis, and the activation of Cl− and Ca2+ ion conductances. LPA has been implicated in a number of disease and injury states, due to elevated levels of LPA in fluids surrounding the tissues involved, including corneal injury, lung disease, atherosclerosis, ovarian cancer, and wound healing. The eight receptors in the endothelial differentiation gene (EDG) family encode G protein-coupled receptors activated by the phospholipid growth factors LPA and S1P (4, 5). The EDG family is subdivided into two clusters based on ligand selectivity. S1P1, S1P2, and S1P5 receptors (formerly EDG1/3/5) are specifically activated by S1P (4), whereas LPA1, LPA2, and LPA4 receptors (formerly EDG2/4/7) are specifically activated by LPA (5).

Members of the S1P receptor subfamily display 40–50% sequence identity to each other and 30–35% identity to the members of the LPA receptor subfamily (5, 6). These homologies and a distant relatedness to the cannabinoid receptors (7, 8) suggest that the LPA- and S1P-specific subfamilies may have evolved from a common ancestral lipid receptor through the evolutionary development of distinct ligand binding pockets. If so, ligand selectivity should be determined by a limited number of conserved and/or related amino acids. We have previously shown that three residues, Arg120, Glu121, and Arg292, are all required for S1P recognition by S1P1/EDG1 (9). One of these residues, Arg120 in S1P1/EDG1, which is predicted to ion pair with the phosphate moiety of S1P, is a conserved arginine in the entire EDG family. The model further predicted that Arg292 interacts with the phosphate and Glu121 interacts with the amine of S1P. Alanine substitution for any of these three amino acids in S1P1/EDG1 caused loss of binding and activation by S1P (9). However, recognition of LPA and discrimination between S1P and LPA by the highly related EDG family G protein-coupled receptors have not yet been determined.

In this study, we have developed a theoretical model of the LPA/EDG2 receptor alone and in complex with LPA based on the previously described and experimentally validated S1P/EDG1 receptor model. Computational analysis of the ligand binding interactions has predicted that a single amino acid, Glu121 in S1P1/EDG1, which corresponds to Gin125 in LPA1/EDG2, influences the specificity for S1P or LPA. Experimental characterization of site-directed mutants confirms that the glutamate-to-glutamine replacement is sufficient to change the specificity of S1P1 from S1P to LPA, whereas the glutamine-to-glutamate replacement in LPA1 resulted in recognition of both S1P and LPA.

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1 The abbreviations used are: LPA, lysosphatidic acid; S1P, sphingosine-1-phosphate; EDG, endothelial differentiation gene; GTPγS, guanosine 5'-3-O-(thio)triphosphate.
EXPERIMENTAL PROCEDURES

**Computational Studies**—To facilitate comparison between aligned positions in different receptors with sequential residue positions, the numbering system introduced first by Ballesteros and Weinstein will be used (10). In this numbering system, residues are given two-part numbers. The first part of the number indicates the transmembrane helical domain in which the residue occurs. The second part of the number is the position in that domain relative to a residue most conserved throughout the G protein-coupled receptor family, which is given the number 50. Thus Arg^{217} in S1P and Arg^{218} in LPA1 are both numbered 3.28, indicating their position in the third transmembrane helical domain relative to the conserved arginine in the (E/D/R/Y) motif which is numbered 3.50.

A model of LPA1 (GenBank™ accession number U80811) was developed previously and was refined to optimize the interhelical hydrogen bonding network. Subsequent homology modeling utilized default conditions to generate a preliminary model that was then manually refined to optimize the interhelical hydrogen bonding network. cis-amide bonds present in the loop regions were converted to the trans conformation by manual rotation followed by optimization of two amino acids on either side of the amide bond to a root mean square gradient of 0.1 kcal/mol Å using the AMBER94 forcefield (11). After these manual refinements, the receptor model was optimized using the AMBER94 forcefield to a root mean square gradient of 0.1 kcal/molÅ. C18:1 LPA was docked into the LPA1 model, and the complexes were evaluated based on electrostatic interactions between the receptor and ligand complex with the best electrostatic interactions selected from the docking procedure was further refined using molecular dynamics simulations under constant volume at 300 K. Molecular dynamics simulations utilized a 1-ns time step with 30 ps of equilibration prior to the 100-ps data collection phase. The final snapshot from the simulation was subsequently minimized to a root mean square gradient of 0.01 kcal/molÅ using the AMBER94 forcefield.

**Site-directed Mutagenesis**—The N-terminal FLAG epitope-tagged S1P and LPA1 receptor constructs (GenBank™ accession numbers AF233365 and U80811, respectively) were provided by Drs. Timothy Hla and Songzhu An, respectively. Site-specific mutations were generated using the ExSite™ mutagenesis kit (Stratagene, La Jolla, CA). S1P-3.29Q and S1P-3.29A were generated by replacement of GAA in codon 121 with GAC and GCA, respectively. LPA1-3.29E and LPA1-3.29A were generated by substitution of CAG in codon 125 with GAA and GCA, respectively. At the same time a silent mutation was introduced that provided a BamHI restriction site in these constructs but did not affect coding for glycine and serine in codons 122 and 123. For LPA1-3.29E and LPA1-3.29A, a silent mutation was introduced adding a StuI restriction site between codons 125 and 127. Clones were verified by sequencing of the inserts.

**Cell Culture, Transfection, and Western Blot**—RH7777 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified minimal essential medium containing 10% fetal bovine serum (Summit Biotechnology, Manassas, VA) were maintained in Dulbecco’s modified minimal essential medium for an additional 6 h. The cells were homogenized by 30-s sonication in 20 mM HEPES (pH 7.4) buffer containing 50 mM NaCl, 1 mM EDTA, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, and protease inhibitor mixture (Sigma-Aldrich). Nuclei and cell debris were removed by centrifugation at 2000 × g for 5 min at 4 °C. The supernatant was centrifuged at 40,000 × g for 30 min at 4 °C. The membrane pellet was resuspended in 20 mM HEPES (pH 7.4) containing 100 mM NaCl, 10 mM glucose, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5 mM EGTA, protease/phosphatase inhibitor mixture (Sigma-Aldrich), and 5 mM fatty acid-free bovine serum albumin (binding buffer) and stored at −80 °C. Membrane protein concentration was measured by Micro BCA assay kit (Pierce). A 40-μg aliquot of membrane protein was used to perform the binding experiments. Incubations were performed at 4 °C for different times (10–120 min) using a constant 30 μM concentration of [35S]-labeled C18:1 LPA (Avanti Polar Lipids) or using different concentrations (0.3–500 nM) of [35S]-labeled C18:1 LPA while incubating a constant 30 min. Membrane-bound radioactivity was separated by filtration on GF/B filters (Whatman) using a Brandel harvester (Gaithersburg, MD). The filter was washed first with 1 ml of ice-cold Ca<sup>2+</sup>-free phosphate-buffered saline containing 10 μM bovine serum albumin and two more times with phosphate-buffered saline alone. Nonspecific LPA binding was determined in the presence of 100 μM cold C18:1 LPA (Avanti Polar Lipids). The band radioactivity was measured by liquid scintillation counting. S.E. was computed on the basis of triplicate samples. Every binding experiment was repeated using membrane samples isolated at least three times.

**Receptor Activation Assays**—Functional assays were performed in RH7777 cells transiently transfected with the constructs by measuring S1P- and LPA-activated [35S]GTP<sub>S</sub> binding as described previously (9). Briefly, membranes were isolated and stored as described for the LPA binding assays. A 20-μg aliquot of membrane protein was incubated in 1.0 ml of 50 mM HEPES (pH 7.5) assay buffer containing 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 μM GDP, 2 mM dithiothreitol, and 0.1 mM [35S]GTP<sub>S</sub> (119 Ci/mmol; Amersham Biosciences, Inc.) with different concentrations of S1P or LPA for 30 min at 30 °C. Membrane-bound radioactivity was then separated by filtration through a Whatman GF/B glass filter and quantitated by liquid scintillation counting. Samples were run in triplicate, and the activation assay was performed using membranes isolated from at least three transfections.

**RESULTS**

**Computational Modeling of LPA1/EDG2**

The eight known members of the EDG family were aligned based on amino acid sequence using the MOE program (Fig. 1B). The LPA1 model was developed by homology to the previously described and experimentally validated S1P1 model (9). The first 35 and the last 33 amino acid residues were deleted from the LPA1 amino acid sequence because there was no corresponding S1P<sub>1</sub> template structure available for these regions. Manual adjustments removed gaps in the transmembrane domains of the aligned sequences prior to homology modeling. Polarity-conserved positions (14) were identified and examined for interhelical hydrogen bonding interactions. Where geometrically possible, side chains were rotated manually to improve hydrogen bonding among these residues. These interactions, present in the high-resolution crystal structure of rhodopsin (15), are important for maintaining helical packing. The LPA1 model included all of the hydrogen bonding networks among the following residues: Asp<sup>1.50</sup>-Glu<sup>2.50</sup>-His<sup>7.49</sup>, Ser<sup>2.39</sup>-Ser<sup>2.46</sup> Leu<sup>2.42</sup> Asn<sup>3.45</sup> Asp<sup>3.42</sup> Ala<sup>6.43</sup> Asn<sup>7.45</sup> corresponding to hydrogen bonding interactions originally developed in the S1P<sub>1</sub> model and also observed in the crystal structure of rhodopsin (15). The entire model was then minimized to a gradient of 0.1 kcal/molÅ. This relatively high gradient prevented collapse of the binding pocket due to an absence of ligand or water molecules during the minimization.

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Three-dimensional Model of LPA₁/EDG2 in Complex with LPA

The LPA₁ model was docked with LPA using the MOE program. A complex in which LPA was positioned within the helical bundle with the highest electrostatic score was chosen for further evaluation. This complex resembles the experimentally validated S1P/H₁₈₅₂₈ S₁P₁ complex. However, in LPA₁ amino acid side chains were found to protrude further into the binding pocket than in the S₁P₁ model. These side chains were manually rotated to decrease steric interactions. Geometry optimization with the use of distance restraints between the phosphate of LPA and the nearest cationic amino acids was used to optimize ion-pairing interactions involving the phosphate due to experimental evidence underlining the requirement of the phosphate in receptor binding (16). The resulting complex was then minimized to a root mean square gradient of 0.001 kcal/mol by a multistep process that first constrained the backbone atoms while allowing amino acid side chains to minimize followed by minimization of the entire complex. The resulting complex (Fig. 2) shows considerable similarity to the model of the S₁P₁ complex with regard to the region of the receptor occupied by the ligand.

The model of the LPA₁-LPA complex derived from computational docking studies indicates several key interactions between polar amino acids in LPA₁ and the polar head group of LPA (Fig. 2B). In particular, Arg₃.₂₈ and Lys₇.₃₆ form ion pairs with the phosphate group of LPA. Examination of the aligned amino acid sequences of the entire EDG family showed two intriguing features. First, the position 3.₂₈ is a conserved arginine in every EDG receptor (Fig. 1B). Second, position 7.₃₆ is a positively charged amino acid in the LPA-binding EDG subfamily. The model also indicates that the sn-2 hydroxyl of LPA accepts a hydrogen bond from the amide of Gln₃.₂₉ in LPA₁. The oxygen of the side chain carbonyl in Gln₃.₂₉ also accepts a hydrogen bond from the phosphate hydrogen (Fig. 2B).

Comparison of the interactions in the S₁P₁ complex (9) with those in the LPA₁ complex (Fig. 2B) suggests that ligand specificity might be determined by the naturally occurring single amino acid difference at position 3.₂₉. In S₁P₁, Glu₃.₂₉ is positioned within 2.₅ Å of the positively charged ammonium group of the sphingosine backbone and forms an ion pair with the ammonium group of S₁P. Computational modeling of the S₁P₁ receptor in complex with LPA, a glycerophospholipid that lacks an ammonium group, indicates that it is unable to ion pair with Glu₃.₂₉, leaving the carboxylate of Glu₃.₂₉ within 5 Å of the anionic phosphate of LPA with no counterion to mitigate the repulsive interaction between the two. This hypothesis is much more focused than could be obtained by simple primary sequence comparisons of the S₁P₁ and LPA receptors (Fig. 1B).

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![Fig. 1. Structures of phospholipid growth factors and their receptors. A, structures of S₁P and LPA. B, alignment of amino acid sequences of the S₁P and LPA receptor families. Helical reference numbers used in the text are marked above the relevant position. Positions enclosed in boxes have conserved residues or residue types in either the S₁P or LPA receptor subfamily differing in the other subfamily.](http://www.jbc.org/)

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The subfamily is altered in the other. The addition of three-dimensional information, such as the homology models we have developed or simpler snake models demonstrating positions of residues relative to the extracellular surface, reduce this number. The three-dimensional computational model described herein thus enabled us to formulate two specific hypotheses. First, Gln3.29 is required for LPA binding to LPA1. Second, position 3.29 is the single amino acid residue that accounts for the LPA and S1P specificity of LPA1 and S1P1, respectively. To test these two hypotheses we generated point mutations in both receptors by converting Glu3.29 to Gln in S1P1 or mutating Gln3.29 to Glu in LPA1. To establish that these polar interactions are essential for ligand binding, two additional alanine replacement mutants, S1P1-3.29A and LPA1-3.29A, were generated. The predicted influence of position 3.29 on ligand specificity was evaluated by characterizing the S1P1 and LPA1 receptor constructs having Glu, Gln, and Ala at position 3.29 transiently transfected into LPA and S1P receptor-negative RH7777 cells (17).

Experimental Validation of Theoretically Predicted Ligand Specificity

Construct Expression and Localization—To verify that the point mutants were expressed in comparable amounts, cell lysates were analyzed for receptor expression by Western blot using the N-terminal FLAG epitope present in every construct (Fig. 3). The level of expression was similar for every mutant and wild type receptor. To verify proper targeting of the mutant receptors to the plasma membrane, immunocytochemical staining for the FLAG epitope was done in serum-starved cells (Fig. 4). Laser confocal microscopy showed that every receptor construct was predominantly targeted to the cell surface as seen for the wild type receptors.

Recognition of S1P—Cells expressing the two receptor constructs having glutamate at position 3.29, namely wild type S1P1 and LPA1-3.29E, both showed significantly higher specific [32P]S1P binding as compared with cells that were transfected with the empty pcDNA3 vector. Fitting a one-site model to the [32P]S1P binding data for these two constructs (Table I) gave Kd values of 36 ± 2 and 79 ± 12 nM, respectively. Likewise, dose-response curves for S1P-induced [35S]GTPγS binding (Fig. 5) provided EC50 values for these constructs of 1.7 ± 0.7 and 453 ± 95 nM (Table I). As expected from the radioligand binding
and receptor activation results, the S1P₁ and LPA₁-3.29E receptors also internalized with a similar time course when incubated with 100 nM S1P for 15 min (Fig. 4).

Cells expressing receptor constructs having either glutamine or alanine at position 3.29 did not show a statistically significant increase in [32P]LPA binding over cells transfected with empty vector (LPA₁ data shown in Fig. 6, B and F) at a 30 nM concentration, which is near the $K_D$ value of S1P₁. The dose-response curves for S1P-induced [35S]GTPγS binding showed no dose-dependent activation by S1P for either the two alanine mutants, S1P₁-3.29A (Fig. 5A) and LPA₁-3.29A (Fig. 5B), or the two receptors with glutamine at position 3.29, wild type LPA₁ (Fig. 5B) and S1P₁-3.29Q (Fig. 5A). None of the receptors lacking glutamate at position 3.29 internalized when incubated with 100 nM S1P for 15 min (Fig. 4).

**LPA Binding Assay**—LPA binding has traditionally been difficult to measure due to unfavorably high nonspecific binding presumed to be due to its partitioning in the bilayer and its rapid breakdown by endogenous phosphatases and phospholipases. The assay developed for these studies utilizes vanadate to inhibit endogenous phosphatases (18) to prevent the breakdown of LPA. Validation of the assay for RH7777 cells transfected with vector, S1P₁, and LPA₁ is presented in Fig. 6. LPA binding is dependent on transfection with LPA₁, as membranes isolated from vector-transfected cells show neither concentration-dependent (Fig. 6A) nor time-dependent (Fig. 6C) LPA binding. Membranes isolated from LPA₁-transfected cells, however, show establishment of a steady state (equilibrium binding) after 30 min (Fig. 6C) with a strongly concentration-dependent specific binding that provides a $K_D$ of 27 ± 3 nM and $B_{max}$ of 1.56 ± 0.05 fmol/μg of membrane protein by nonlinear regression ($r = 0.997$, Fig. 6D). For comparative purposes, vector-transfected (Fig. 6B) as well as LPA₁-transfected cells show neither time-dependent (Fig. 6E) nor concentration-dependent (Fig. 6F) S1P binding.

**Recognition of LPA**—In contrast to the recognition of S1P, LPA recognition was detectable in membranes isolated from cells expressing the receptor constructs having glutamine rather than glutamate at position 3.29, namely wild type LPA₁ and S1P₁-3.29Q. These constructs both showed significantly higher specific [32P]LPA binding than membranes isolated from cells that were transfected with the empty vector. Unexpectedly, the LPA₁-3.29E construct also showed significantly greater specific [32P]LPA binding than the empty vector control. Nonlinear regression fits of the [32P]LPA binding data to a one-site model for wild type LPA₁, S1P₁-3.29Q, and LPA₁-3.29E provided apparent $K_D$ values of 27 ± 3, 139 ± 32, and 32 ± 3 nM, respectively (Table I). Scatchard plots were constructed in every case to validate the use of the one-site binding model. Likewise, dose-response curves for LPA-activated [32P]GTPγS binding (Fig. 7) provided EC₅₀ values of 2.6 ± 0.6, 5.8 ± 3.7, and 11.0 ± 2.9 nM (Table I) for these same constructs. In agreement with the ligand binding and receptor activation results, these three constructs also internalized after a 15-min incubation with 100 nM LPA (Fig. 4).

The two receptors having alanine at position 3.29 as well as wild type S1P₁ did not respond to LPA in these assays. These did not show significantly increased binding of [32P]LPA (data shown for S1P₁ in Fig. 6F), did not demonstrate LPA-induced [32P]GTPγS binding (Fig. 7), and did not internalize in response to a 15-min exposure to 100 nM LPA (Fig. 4).

**DISCUSSION**

Theoretical models of LPA₁ and its complex with C18:1 LPA have been developed by homology to a validated model of S1P₁. The LPA₁ complex model predicts that three critical polar residues interact with LPA, Arg³.28 and Gln³.29 in helix 3 and Lys⁷.36 in helix 7. Position 3.28 is a conserved arginine throughout the S1P and LPA receptor families, although some changes in its position may be tolerated. The third position in the LPA₁ model predicts that the charged residue Arg⁷.27 is facing inward but two hydrogen bonds from the phosphate group of LPA. The computational models of S1P₁ and LPA₁ predict that these two positively charged residues ion pair with the phosphate groups in S1P and LPA, respectively. Furthermore, a positively charged residue occurs near position 7.34 in most members of the LPA and S1P receptor subfamilies (Fig. 1B). In the S1P₅ sequence we speculate that the corresponding positively charged residue is Arg⁷.37, which is seven positions earlier than position 7.34. This shift should orient it facing inward but two turns higher, making it a part of the third extracellular loop rather than the seventh transmembrane α-helix. Thus a catalytic residue in helix 7 may be required throughout the S1P and LPA receptor families, although some changes in its position may be tolerated. The third position in the LPA₁ model predicted to interact with LPA is Gln³.29. A position conserved in LPA₁, LPA₁, and LPA₅, thus in every LPA-specific EDG receptor. Characterization of the LPA₁-3.29A mutant confirmed that this residue is required for LPA binding and ligand activation by LPA (Table I and Fig. 7). The model predicts that Gln³.29 donates a hydrogen bond to the hydroxyl group and accepts a hydrogen bond from the phosphate of LPA. Position 3.29 is a
Binding constants were derived from nonlinear regression analysis of radioligand binding assays with S1P and LPA, fitting a one-site model to the data. Scatchard plots were constructed to visualize the validity of the one-site model. Binding and activation parameters and their confidence intervals were calculated with the Kaleidagraph software (v3.51, Synergy Software).

**Table I**  
Properties of S1P, LPA, and their mutants

| Specific binding | Receptor activation |
|------------------|---------------------|
|                  | Apparent $K_D$      | $B_{max}^{a}$ | $EC_{50}$ | Internalization |
|                  | $nm$                | $nm$          | $nm$      |                |
| S1P              | 36 ± 2              | None          | 1.7 ± 0.7 | None           |
| LPA              | 79 ± 12             | 32 ± 3        | 453 ± 95  | Yes            |
| LPA,3.29E        | None$^b$            | 6.31 ± 0.36   | 5.5 ± 3.7 | No             |
| S1P,3.29Q        | None$^b$            | None          | None      | No             |
| S1P,3.29A        | None$^b$            | None          | None      | No             |
| LPA,3.29A        | None$^b$            | None          | None      | No             |  

$^a$ $B_{max}$ units are fmol/$1 \times 10^6$ cells for S1P binding and fmol/$\mu g$ of membrane protein for LPA binding.

$^b$ Denotes $K_D$ values that were greater than 10 $\mu m$, the limit that could be meaningfully derived from the radioligand binding assay.

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Asn7.49 instead (25). Mutation of Lys 3.28 and Ser3.31 simultaneously resulted in a loss of affinity for the classical cannabinoid receptors CB1 and CB2. The overall amino acid sequence homology between LPA1/LPA2/LPA3 (EDG2/4/7) or S1P/S1P1/S1P2/S1P3/S1P4/S1P5 (EDG1/3/5/6/8) and CB2 is 21.7 and 22.6%, respectively. Mutagenesis studies of the CB1 receptors have found that a positive charge at position 3.28(192), occupied by lysine in the wild type receptor, is critical for the binding of some agonists (23, 24). The importance of charge at position 3.28 is consistent with its interaction with the phosphate group of LPA and positions 3.28 and 7.36 in the LPA1 receptor are consistent with the inability of hydrogen phosphate and methyl phosphate derivatives of LPA to induce Ca$^{2+}$ mobilization in human A431 cells (16). Likewise, a cyclic phosphate derivative of dioxa-zaphosphocane with a phosphorus-oxide bond induces plateau aggregation, while a similar dioxa-zaphosphocane with a phosphorus-hydrogen bond does not (19). Second, the oxygen of the LPA hydroxyl group is predicted to accept a hydrogen bond from Gln$^{3.29}$ (25). This result is consistent with the similar abilities of 1-O-hexadecyl-sn-glycero-3-phosphate and 1-O-hexadecyl-2-O-methyl-sn-glycero-3-phosphate to induce Ca$^{2+}$ mobilization in human A431 cells (16). Likewise, several N-acyl aminoethanol phosphatidic acids are able to induce plateau aggregation (20) and mobilize calcium in MDA MB-231 cells (21). The oxygen of the amide group in the N-acyl aminoethanol phosphatidic acid analogs is geometrically able to occupy a position corresponding to the hydroxyl oxygen of LPA and thus serve as the analogous hydrogen bond acceptor. Finally, the hydrocarbon chain of LPA occupies a hydrophobic binding pocket in the model. Although the dispersive van der Waals interactions between this hydrocarbon chain and the receptor are not individually as strong as the polar interactions of the lipid head group, they do contribute to the overall affinity of LPA for the receptor. This observation is consistent with the observed inability of C12:0 LPA and significantly reduced ability of C14:0 LPA to induce Ca$^{2+}$ mobilization in Si9 cells transfected with the LPA1/EDG2 receptor (22).

Receptors in the EDG family are most closely related to the cannabinoid receptors CB1 and CB2. The overall amino acid sequence homology between LPA1/LPA2/LPA3 (EDG2/4/7) or S1P/S1P1/S1P2/S1P3/S1P4/S1P5 (EDG1/3/5/6/8) and CB2 is 21.7 and 22.6%, respectively. Mutagenesis studies of the CB1 receptors have found that a positive charge at position 3.28(192), occupied by lysine in the wild type receptor, is critical for the binding of some agonists (23, 24). The importance of charge at position 3.28 is consistent with its interaction with the phosphate group of LPA in the model complex with LPA1. Mutation of the same residue in the CB2 receptor to alanine, however, failed to influence the binding profiles of the same agonists (25). Molecular modeling studies reported by the same researchers located a binding pocket for classical cannabinoids in the CB2 receptor involving residues Ser$^{3.31}$, Thr$^{3.35}$, and Asn$^{7.49}$ instead (25). Mutation of Lys$^{3.28}$ and Ser$^{3.31}$ simultaneously resulted in a loss of affinity for the classical cannabinoids and validated the model. Thus the role of position 3.38 in agonist binding is not conserved among the cannabinoid receptors in contrast with our expectation that it is important throughout the EDG receptor family. Only experimental characterization of replacement mutations at position 3.28 of other...
S1P and LPA receptors will verify this expectation, which is beyond the scope of the present study.

The predicted ligand binding residues derived from the previously validated S1P1 and the present LPA1 models lead to a prediction of how the EDG receptors discriminate between S1P and LPA. Namely it is the glutamine residue that determines the specificity for LPA, whereas the glutamate residue determines specificity for S1P. To test our hypothesis we have generated the S1P1-3.29Q and the LPA1-3.29E mutants. Both mutant receptors were expressed and targeted to the plasma membrane. In agreement with the predictions of the computational models, the S1P1-3.29Q mutant lost the ability to bind S1P and acquired the ability to bind LPA with an apparent $K_D$ of 139 ± 11 nM. This value is slightly higher than that of the wild type S1P1 receptor. The S1P1-3.29Q receptor not only bound LPA with a high affinity but also was activated by LPA as indicated by the 5.8 ± 3.7 nM $EC_{50}$, which is comparable to the 2.6 ± 0.6 nM $EC_{50}$ of the wild type LPA1 receptor. We also obtained confirmatory evidence from ligand-induced receptor internalization experiments for the ligand specificity observed in radioligand- and ligand-activated [35S]GTPγS binding experiments. These data, together with the inability of the S1P1-3.29A mutant to bind S1P or LPA, support the hypothesis that an ionic interaction between the negatively charged glutamic acid stabilizes the binding of S1P in the binding pocket of the receptor, whereas a neutral polar glutamine interacts favorably with LPA.

Experimental characterization of the LPA1-3.29E mutant provides only partial support to this hypothesis. This latter mutant has become capable of binding S1P with an apparent $K_D$ of 79 ± 12 nM that is 2 times higher than that of the wild type S1P1 receptor. This receptor mutant was also activated in a dose-dependent manner by S1P with an apparent $EC_{50}$ value of 453 ± 95 nM and showed S1P-induced internalization (Fig. 4). In contrast, the wild type LPA1 showed no concentration-dependent high affinity specific binding of S1P. While these properties clearly establish that the mutant acquired S1P recognition and activation, at the same time it retained recognition of its natural ligand, LPA. The apparent $K_D$ value of the LPA1-3.29E mutant for LPA was 32 ± 11 nM with an $EC_{50}$ of 11.0 ± 2.9 nM, which are approximately equivalent to and 4 times less, respectively, than those of wild type LPA1.

The properties of the LPA1-3.29E mutant suggest that the glutamate residue is able to interact to some degree with the hydroxyl group of LPA, perhaps through hydrogen bonding. Such an interaction could occur in two different ways. First, the glutamate might occur in the protonated, neutral form in a significant fraction of the receptors thus being capable of donating a hydrogen bond in the same fashion as a glutamine residue. The reduced LPA binding $B_{\text{max}}$ value for this construct (0.74 fmol/µg) relative to LPA1 (1.56 fmol/µg) and the reduced S1P binding $B_{\text{max}}$ value (6.3 fmol/1 × 10⁶ cells) relative to S1P1 (11.7 fmol/1 × 10⁶ cells) support the possibility that some of the receptors expressed are recognizing S1P and others are recog-
for the interaction of LPA and S1P also agrees with the experimental observations of Lee et al. (12), who reported that S1P1 could bind LPA with a 20-fold lower affinity than its physiological ligand, S1P.

In conclusion, our work demonstrates the applicability of computational modeling to correctly predict the influence of a single amino acid on ligand specificity, thus supporting the utility of a model-driven approach to gene function. This application of molecular modeling to understand the molecular basis for ligand selectivity between receptor subfamilies sets the stage to use the molecular models to design ligand derivatives with selectivity within a receptor subfamily.

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Fig. 7. LPA-induced receptor activation. Ligand-induced [35S]GTPγS binding was measured using 20 μg of membrane protein in the presence and absence of ligand. A, LPA-induced [35S]GTPγS binding for LPA1 and its mutants. Filled circles, squares, and triangles represent wild type LPA1, LPA1-3.29E, and LPA1-3.29A, respectively. B, activation assays for ligand-induced [35S]GTPγS binding to S1P1 and its mutants. Filled circles, squares, and triangles represent wild type S1P1, S1P1-3.29Q, and S1P1-3.29A, respectively. See Table I for the EC50 values. Data represent the mean ± S.E. of three independent determinations.
A Single Amino Acid Determines Lysophospholipid Specificity of the S1P₁ (EDG1) and LPA₁ (EDG2) Phospholipid Growth Factor Receptors
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