Subtype-specific Residues Involved in Ligand Activation of the Endothelial Differentiation Gene Family Lysophosphatidic Acid Receptors

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Lyso-phosphatic acid (LPA) is a ligand for three endothelial differentiation gene family G protein-coupled receptors, LPA1–3. LPA1–3. We performed computational modeling-guided mutagenesis of conserved residues in transmembrane domains 3, 4, 5, and 7 of LPA1–3 predicted to interact with the glycerophosphate motif of LPA C18:1. The mutants were expressed in RH7777 cells, and the efficacy (E_{max}) and potency (EC_{50}) of LPA-elicited Ca^{2+} transients were measured. Mutation to alanine of R3.28 universally decreased both the efficacy and potency in LPA1–3 and eliminated strong ionic interactions in the modeled LPA complexes. The alanine mutation at Q3.29 decreased modeled interactions and activation in LPA1 and LPA2 more than in LPA3. The mutation W4.64A had no effect on activation and modeled LPA interaction of LPA1 and LPA2 but reduced the activation and modeled interactions of LPA3. The R5.38A mutant of LPA2 and R5.38N mutant of LPA3 showed diminished activation by LPA; however, in LPA3, the D5.38A mutation did not, and mutation to arginine enhanced receptor activation. In LPA2, K7.36A decreased the potency of LPA; in LPA3, this same mutation increased the E_{max}. In LPA3, R7.36A had almost no effect on receptor activation; however, the mutation K7.35A increased the EC_{50} in response to LPA 10-fold. In LPA1–3, the mutation Q3.29E caused a modest increase in EC_{50} in response to LPA but caused the LPA receptors to become more responsive to sphingosine 1-phosphate (S1P). Surprisingly micromolar concentrations of S1P activated the wild type LPA2 and LPA3 receptors, indicating that S1P may function as a weak agonist of endothelial differentiation gene family LPA receptors.

Lyso-phosphatic acid (LPA) and sphingosine 1-phosphate (S1P) are structurally related lysosphospholipid growth factors that mediate a variety of cellular effects, including regulation of cellular proliferation, survival, migration, and morphology. S1P is structurally closely related to the purinoreceptor cluster of GPCRs (16–19). The EDG family of GPCRs includes eight closely related genes that show the conserved GPCR topology of an extracellular amino terminus followed by seven α-helical transmembrane domains (TMs) (15). Three of these genes (LPA1–3) are cellular receptors for LPA and share 55% overall homology in humans. The other five (S1P1–5) are cellular receptors for S1P and share 50% homology in humans. The two subclusters are 35% homologous with each other. The transmembrane domains of human LPA1–3, where ligand binding takes place show 81% homology with each other. LPA has also been shown to elicit cellular responses through binding to three non-EDG family GPCRs, p2y9/LPAq, GPR92/LPAq, and GPR87/LPAq, which are more closely related to the purinoreceptor cluster of GPCRs (16–19).

Modeling and mutagenesis studies of S1P receptors in the EDG family have demonstrated that conserved residues can play either conserved or non-conserved roles in different family members. A validated computational model of S1P1 was developed that successfully identified residues in TM3 and TM7 of S1P1 that participated in ligand binding. A critical role for residues R3.28, E3.29, and R7.34 of S1P1 in ligand binding and receptor activation was experimentally confirmed using a site-directed mutagenesis strategy (20). Later studies determined that in S1P1, the residues R3.28, E3.29, W4.64, and K5.38 were critical for ligand binding and receptor activation (21), whereas K5.38 was not essential in S1P1 (22). Based upon the high sequence homology within the EDG family, the experimentally validated S1P1 model was used as a template to map the ligand-binding pocket of the LPA-specific EDG receptors. Computa-

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Novel computational modeling predicted that the residues R3.28, Q3.29, R5.38, and K7.35 of LPA3 form critical interactions with the polar head group of LPA, and this was confirmed experimentally (23). These previous studies suggest a conserved and essential role for R3.28 in all receptors examined so far but a variable role for K5.38 in two family members.

Position 3.29, which is conserved as a glutamine in LPA-specific EDG receptors and a glutamate in S1P-specific EDG receptors, was computationally identified and experimentally validated as a key residue that determines receptor selectivity for LPA or S1P in the S1P1 and LPA1 receptor pair (24). The E3.29Q mutant of S1P1 responded to LPA rather than S1P; the reciprocal Q3.29E mutation in LPA1 showed diminished activation by LPA but was activated by S1P, indicating the involvement of additional residues in ligand recognition. Alanine mutation at this position diminished activation by either ligand in both receptors (24). Similarly the E3.29Q mutation in S1P1 conferred responsiveness to LPA but decreased responsiveness to S1P (25).

Most cell types express multiple EDG receptor subtypes (26). The role of the different LPA receptor subtypes in physiological and pathophysiological processes is often difficult to determine because of the lack of LPA receptor subtype-specific reagents. Subtype-specific agonists and antagonists could elucidate the role of the different LPA receptor subtypes in physiological and disease states as well as function as lead compounds in drug development. To aid in the development of subtype-specific reagents, it is important to identify differences between the EDG family LPA receptor subtypes in the ligand-binding pocket.

The fundamental assumption underlying homology modeling and comparative sequence analysis is that identical residues fulfill the same role in homologous proteins. Given the very high degree of sequence identity, especially in the transmembrane domains of the EDG receptors, one would hypothesize that the function of those residues validated to play a role in ligand recognition applies universally within the family. The variable importance of K5.38 in the S1P1 and S1P4 receptors, however, suggests that this assumption is not always accurate (21, 22). In the present study we carried out a comparative analysis of the conserved key residues experimentally validated to be involved in ligand recognition in one or another LPA– or S1P-specific EDG family receptor to find that many of these head group-interacting residues play different roles. We extended our analysis to include all three of the LPA-specific EDG receptors and generated mutations at sites that are computationally predicted to impact ligand recognition: R3.28, Q3.29, W4.64, D/R5.38, K7.35, and K/R7.36. We determined the effect of each mutation upon the potency ($EC_{50}$) and efficacy ($E_{max}$) of LPA relative to the activation elicited in the wild type receptors. We also evaluated the impact of some of these mutations on receptor activation by the related lipid mediator S1P. Experimental results were correlated to predictions based upon computational modeling of the wild type and mutant receptors docked with ligand. These studies reveal that major differences exist between the different LPA receptor subtypes in the functional utilization of several conserved residues in the predicted ligand-binding pocket. Only one residue when mutated to alanine identically impacted the three receptor subtypes; the mutation R3.28A universally reduced both the efficacy and potency in LPA1–3 and eliminated strong ionic interactions in the modeled LPA complexes. The different roles that conserved residues can play among the highly homologous members of the EDG family provide insight into nature’s diverse answers for high affinity molecular recognition and challenge the concept that automatically assigns identical function to homologous residues.

EXPERIMENTAL PROCEDURES

Reagents—All analogs of LPA and S1P were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were prepared before use as a 1 mM stock in PBS containing 1 mM charcoal- stripped bovine serum albumin (BSA). Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR). Anti-FLAG M2 monoclonal antibody was purchased from Sigma.

Residue Nomenclature—Amino acids in the TM sets were assigned index positions by the method of Ballesteros and Weinstein (27) based upon homology found in the seven helical TMs of GPCRs. Index positions are in the format X.YY where X refers to the number of the TM in which that residue is found and YY refers to the position within that TM relative to the most highly conserved residue in that TM throughout the GPCR superfamily, which is arbitrarily designated position 50 (27).

Computational Homology Modeling—Previously developed computational models of LPA1, LPA2, and LPA3 (23, 28) were used for mutation studies. LPA C18:1 was docked into each receptor with a −2 charge because previous quantum mechanical studies suggest that is appropriate for phospholipid binding sites with multiple cationic residues (22). Docking studies were done using Autodock 3.0 (29). Default docking parameters were used except for number of runs (15), energy evaluations ($9.0 \times 10^{10}$), generations (30,000), and local search iterations (3000). The complex with the greatest number of cationic interactions with the LPA phosphate group was chosen and subjected to molecular dynamics simulations using a 1-fs time step at 500 ps. The lowest energy structure from the simulation was geometry-optimized and used as the wild type receptor for mutation studies. Mutation studies were done as described previously (22). Mutant models were generated by amino acid side chain replacement. Each mutant was modeled with LPA bound. The models were refined using the MOE (Molecular Operating Environment) software (version 2004.03, Chemical Computing Group, Montreal, Canada). The models were subjected to molecular dynamics and geometry optimization. The MMFF94 force field (30) was used for all force field simulations. Default parameters for molecular dynamics simulations were used with the exception of the total simulation length, which was 1 ns. LPA was removed from each mutant receptor and docked back into the receptor using Autodock 3.0. The best LPA complex with each mutant was selected as the one with the most cationic interactions with the phosphate group.

Site-directed Mutagenesis—Amino-terminal FLAG epitope-tagged LPA1, LPA2, and LPA3 receptor constructs were subcloned into pcDNA3.1 vector (Invitrogen). Receptor constructs were mutated at residues computationally predicted to participate in ligand recognition using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). In some cases, a PCR-based site-directed mutagenesis strategy was used to generate the desired mutation as described previously (23). TOP10 competent
cells (Invitrogen) were transformed with the mutant constructs, and clones were verified by complete sequencing of the inserts.

Cell Culture and Transfection—LPA does not elicit Ca\textsuperscript{2+} transients in the parental McArT1 rat hepatoma 7777 (RH7777) cells (31) (ATCC, Manassas, VA). RH7777 cells and rat hepatoma HTCC4 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 10 μg/ml streptomycin, and 2 mM glutamine. RH7777 cells stably expressing LPA\textsubscript{1} and LPA\textsubscript{3} receptors have been characterized elsewhere (32). RH7777 cells stably expressing LPA\textsubscript{2} were a generous gift from Dr. Fumikazu Okajima (Gunma University, Gunma, Japan) and were characterized previously (33). Stable transfectants were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 10 μg/ml streptomycin, 2 mM glutamine, and 250 μg/ml G418. Transient transfections of RH7777 cells and HTCC4 cells were performed using Effectene transfection reagent (Qiagen, Valencia, CA).

Flow Cytometric Analysis—Expression of all receptor constructs on the cell surface was confirmed by flow cytometric analysis using indirect immunofluorescence staining with anti-FLAG M2 antibody. RH7777 cells were transfected with FLAG epitope-tagged LPA receptor constructs, replated after 16 h, and cultured for an additional 24 h. The culture medium was replaced with Krebs buffer (120 mM NaCl, 5 mM KCl, 0.62 mM MgSO\textsubscript{4}, 1.8 mM CaCl\textsubscript{2}, 6 mM glucose, 10 mM HEPES, pH 7.4) for 4 h before collection; cells were detached using HyQTrase Cell Detachment Solution (Hyclone Laboratories) and collected on ice. Cells were washed with PBS that contained 3% BSA and incubated with anti-FLAG M2 monoclonal antibody (1:200) in PBS containing 0.001% pluronic acid for 30 min and rinsed with Krebs buffer, and the Ca\textsuperscript{2+} response to LPA C18:1 or S1P was measured and reported in terms of maximal activation (E\textsubscript{max}) and efficacy (EC\textsubscript{50}) ± S.D.

Theoretical Models of LPA\textsubscript{1}, LPA\textsubscript{2}, and LPA\textsubscript{3}—Mutation Site Selection—In LPA\textsubscript{1–3}, we evaluated the effect of alanine mutation of residues in TM3, TM4, TM5, and TM7 that were computationally predicted to impact binding to LPA C18:1. The summary of the computationally predicted ionic ligand-receptor interactions in each receptor model. Mutation of other residues was applicable; the residue to be mutated does not occur or that mutation was not made in that receptor.

| Construct | LPA\textsubscript{1} | LPA\textsubscript{2} | LPA\textsubscript{3} |
|-----------|----------------|----------------|----------------|
| Wild type | 2              | 2              | 3              |
| R3.28A    | 0              | 0              | 0              |
| Q3.29E    | 1              | 2              | 0              |
| Q3.29A    | 0              | 0              | 3              |
| W4.64A    | 1              | 3              | 0              |
| D5.38A    | 2              | NA             | NA             |
| D5.38R    | 2              | NA             | NA             |
| R5.38A    | NA             | 1              | 0              |
| R5.38N    | NA             | NA             | 0              |
| K7.36A    | 1              | 2              | NA             |
| R7.36A    | NA             | NA             | 3              |
| K7.35A    | NA             | NA             | 0              |

RESULTS

Radioligand Binding Assay—HEK293T cells were plated in 24-well plates at 4 × 10\textsuperscript{5}/well and the following day transiently transfected with 0.4 μg of receptor constructs using Lipofectamine 2000 (Invitrogen). Two days later cells were washed with ice-cold binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl). Cells were then incubated in binding buffer containing 4 mg/ml fatty acid-free BSA and \textsuperscript{32}P]-S1P ranging from 10 nM to 1 μM in the presence or absence of 10 μM unlabeled S1P as a competitor on ice for 45 min. After washing twice with cold binding buffer containing 0.4 mg/ml BSA, cells were lysed in 0.5% SDS, and binding was quantified by scintillation counting. Triplicate samples were measured for each condition.

Receptor Internalization Assays—RH7777 cells were transiently transfected with FLAG-tagged LPA\textsubscript{2} using Effectene transfection reagent. Cells were serum-starved for 4 and then incubated with a 10 μM concentration of either LPA, S1P, ATP, or vehicle for 30 min at 37 °C before collection on ice and subsequent anti-FLAG flow cytometric analysis. The assay was repeated three times with similar results.

TABLE 1

The computationally predicted number of close charge-charge interactions between LPA receptor constructs and LPA

The number of interactions that occur over distances of less than 4.5 Å between the polar head group of LPA and nitrogen atoms of charged residues in TM3, TM5, and TM7 was calculated based upon computational models. NA, not applicable; the residue to be mutated does not occur or that mutation was not made in that receptor.
of amino-terminal FLAG epitope-tagged LPA1, LPA2, and LPA3 receptor constructs at residues in TM3, TM4, TM5, and TM7 that were computationally identified to surround the glycerophosphate portion of LPA C18:1 in the wild type LPA1–3 complexes. Additionally in LPA1, LPA2, and LPA3, Q3.29 was also mutated to glutamate, the residue occurring at this position throughout the S1P receptors in the EDG receptor family; previously this mutation was shown to change the ligand specificity of LPA1 from LPA to S1P (24).

Expression and surface targeting of the receptor constructs was confirmed by indirect immunofluorescence flow cytometric analysis using antibodies directed against the amino-terminal FLAG epitope present in the constructs (Table 2). All receptor constructs showed targeting to the cell surface when transiently transfected into RH7777 cells except for the R5.38A mutant of LPA3, which showed low (11.9% of cells) surface expression compared with the wild type receptor (55.2%). In LPA3, surface expression of R5.38A was not detected above background (6.2% compared with 5.0% for empty vector-transfected cells); the R5.38N mutant did show cell surface expression although somewhat less than the wild type receptor (13.2% versus 18.1% for wild type).

Variation in surface expression was also noted among several mutant constructs compared with the wild type receptors (Table 2). For LPA1 constructs, cells transfected with Q3.29A showed low (11.9% of cells) surface expression compared with cells transfected with wild type (42.1%) likely due to diminished cell surface targeting or stability of this construct. In the case of LPA2, surface expression levels of two mutants, Q3.29A (20.5%) and R5.38A (10.7%), were less than half of the level measured for the wild type receptor (55.2%). In LPA3, surface expression of R5.38A was not detected above background (6.2% compared with 5.0% for empty vector-transfected cells); the R5.38N mutant did show cell surface expression although somewhat less than the wild type receptor (13.2% versus 18.1% for wild type).

Because of the relatively high expression levels of the receptors in our transient transfection system, the variation in cell surface expression of the different constructs should have only minor effects on receptor activation as measured in our calcium mobilization assays. In particular the measure of potency (EC50) was not expected to vary much with receptor levels because this is a measurement that reflects the affinity of the ligand for the receptor and is the most informative measured parameter in our assays as far as indicating how mutation of a residue affects ligand recognition.

To assess the impact of LPA receptor surface expression levels on potency and efficacy of the measured LPA response, we transfected RH7777 cells with different ratios of FLAG-tagged LPA1 to vector (pcDNA3.1). Transfection of RH7777 cells with LPA1 diluted with different amounts of vector showed that the variations we observed in our wild type and mutant constructs should have no effect on potency and only a minor effect on efficacy (Table 3). The measured EC50 was not significantly affected by up to 20-fold dilution of the LPA1 plasmid; the measured E_max showed some decrease with dilution of receptor by vector but still retained 51% of E_max when diluted 20-fold with empty vector plasmid (Table 3).

**Effect of Point Mutations on Receptor Activation**—We evaluated the impact of each mutation on the potency (EC50) and efficacy (E_max) elicited by LPA as measured by Ca^{2+} mobilization in transiently transfected RH7777 cells. The effects of these

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**TABLE 2**

| Construct | Anti-FLAG-stained cells, percentage of total cells |
|-----------|-----------------------------------------------|
|           | LPA1 | LPA2 | LPA3 |
| Wild type | 42.1 | 55.2 | 18.1 |
| R3.28A    | 51.6 | 66.1 | 39.7 |
| Q3.29E    | 48.6 | 27.4 | 15.0 |
| Q3.29A    | 11.9 | 20.5 | 19.9 |
| W4.64A    | 43.8 | 47.6 | 17.6 |
| D5.38A    | 54.7 | NA   | NA   |
| D5.38R    | 53.0 | NA   | NA   |
| R5.38A    | NA   | 10.7 | 6.2  |
| R5.38N    | NA   | NA   | 13.2 |
| K7.36A    | 54.7 | 41.7 | NA   |
| R7.36A    | NA   | NA   | 38.3 |
| K7.35A    | NA   | NA   | 32.3 |

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**TABLE 3**

| LPA1:vector (percentage of LPA3) | EC50 ± S.D. (nM) | Relative E_max | Percentage of anti-FLAG-stained cells |
|---------------------------------|-----------------|---------------|-------------------------------------|
|                                 | nM  | %  |                                |                                   |
| 1:0 (100%)                      | 145 ± 29 | 100 | 34.5                               |
| 1:1 (50%)                       | 107 ± 37 | 95  | 24.3                               |
| 1:4 (20%)                       | 180 ± 77 | 72  | 20.9                               |
| 1:19 (5%)                       | 105 ± 46 | 51  | 12.0                               |
| 0:1 (0%)                        | No activation | No activation | 5.0                               |

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**Residues Involved in Ligand Activation of LPA Receptors**

Table 2 shows the effect of receptor expression levels on potency (EC50) and maximal response (E_max) to LPA. EC50 values (mean ± S.D.) and E_max values were determined in RH7777 cells transiently transfected with different ratios of FLAG-LPA1 to vector (pcDNA3.1). EC50 values are expressed relative to the maximal response for Ca^{2+} mobilization measured in cells transfected with 100% FLAG-LPA1 plasmid.
mutations on the pharmacological properties of the receptors are summarized in Table 4.

Characteristics of LPA₁, LPA₂, and LPA₃ Receptor Mutations at Strictly Conserved Sites—Three strictly conserved residues surround the glycerophosphate head group of LPA in the modeled LPA receptor complexes: R3.28, Q3.29 (Fig. 1, A–C), and W4.64 (Fig. 2, A–C). Alanine mutants at only one of these sites showed a universal effect across the three receptors. Modeled complexes of LPA with the R3.28A mutant of all three receptors showed a complete lack of close ionic interactions (Table 1).
Likewise the LPA-induced Ca\(^{2+}\) responses in the R3.28A mutants showed right-shifted dose-response curves with EC\(_{50}\) values >1000 nM and E\(_{\text{max}}\) values less than 80% at the highest concentrations tested at each receptor (Fig. 1, D–F, and Table 4).

Receptor-dependent effects were observed for mutations at positions Q3.29 and W4.64. Modeled complexes of LPA with the Q3.29A mutant showed a lack of ionic interactions in the LPA1 and LPA2 receptors but retention of ionic interactions in the LPA3 receptor (Table 1). The Q3.29A mutation produced a more pronounced decrease in activation in LPA1 and LPA2 than in LPA3 (Fig. 1, D–F, and Table 4). In LPA3 alanine replacement shifted the EC\(_{50}\) from 57 ± 11 to 76 ± 23 nM while reducing E\(_{\text{max}}\) by 34%. In LPA2, replacement of Q3.29 with alanine increased the EC\(_{50}\) 4-fold and decreased the E\(_{\text{max}}\) by 49%. The Q3.29A mutant of LPA1 showed no activation. The Q3.29E mutation, which changes a glutamine residue conserved in LPA-specific EDG receptors to a glutamate that is conserved in S1P-specific EDG receptors, decreased the potency of LPA in all three receptor subtypes with the largest potency shift occurring in the LPA3 receptor (Fig. 1, D–F, and Table 4). This is reflected in the LPA complexes, which show ionic interactions comparable to wild type in the LPA1 and LPA2 receptors, but absent in the LPA3 receptor (Table 1). The Q3.29E mutation decreased the LPA-induced maximal activation of LPA1 by 24% but had negligible effect on the E\(_{\text{max}}\) of LPA2 and LPA3. The impact of the mutation W4.64A in LPA1, LPA2, and LPA3 on Ca\(^{2+}\) mobilization in transiently transfected RH7777 cells is shown in Fig. 2, D–F. This mutation had no impact on the activation of LPA1 and LPA2; however, in LPA3 this mutation decreased the potency (460 ± 66 versus 57 ± 11 nM for wild type) and maximal activation (71% of wild type E\(_{\text{max}}\)) of receptor activation by LPA (Table 4). This result corresponds to the observed impact of mutating W4.64 to alanine in the receptor models (Fig. 2, A–C). Ionic interactions similar to wild type were observed in LPA1 and LPA2 but were completely absent in LPA3 (Table 1).

**Characteristics of LPA\(_1\), LPA\(_2\), and LPA\(_3\) Receptor Mutations at Partially Conserved Sites**—The charged residue at the top of TM5 varies in the three receptors (Fig. 3, A–C). LPA1 contains aspartic acid at position 5.38, whereas LPA2 and LPA3 both have a cationic amino acid, arginine. The R5.38A mutant of LPA2 showed a decrease in the number of close cationic interactions as did the R5.38N mutant of LPA3 (Table 1). Both are expected to show increases in EC\(_{50}\) values, whereas the alanine mutation of the anionic D5.38 in LPA1 is unlikely to be detri-
mental. We evaluated the impact of mutations in TM5 on receptor activation in transiently transfected RH7777 cells. The mutations R5.38A in LPA2 and R5.38N in LPA3 strongly decreased receptor activation, increasing EC$_{50}$ by 11- and 8-fold, respectively; however, the alanine mutation of the corresponding position in LPA1, D5.38A, had no impact on receptor activation by LPA (Fig. 3, D–F, and Table 4). The mutation D5.38R in LPA1, which replaces an aspartate residue in LPA1 with an arginine residue that occurs at this position in LPA2 and LPA3, showed enhanced activation by LPA relative to wild type LPA1, shifting the $E_{\text{max}}$ to 132% of wild type LPA1 and decreasing EC$_{50}$ from 186 to 149 nM (Table 4).

In TM7, LPA1 and LPA2 both contain lysine at position 7.36, which is predicted to be oriented away from the phosphate-binding pocket (Fig. 4, A and B). The modeled alanine mutations at this position show similar interactions between the LPA phosphate group and the cationic residues in the receptor (Table 1), suggesting that EC$_{50}$ values should be similar to wild type values. The K7.36A mutant of LPA1 showed enhanced maximal activation by LPA (Fig. 4D), whereas the K7.36A mutant of LPA2 showed diminished activation by LPA, causing a 49-fold increase in EC$_{50}$ (Fig. 4E and Table 4). This apparent inconsistency between model-derived hypothesis and experimental result may result from either incorrect position of K7.36 in the LPA2 receptor model or an indirect role for K7.36. The K7.36 residue in the LPA2 model forms an ion pair with D1.32. Mutation of K7.36 in LPA2 may therefore have an impact on overall receptor structure that our 1-ns molecular dynamics simulations are not long enough to capture. LPA3 contains two cationic amino acids in TM7, R7.36 and K7.35 (Fig. 4C). Computational modeling of the K7.35A LPA3 mutant showed a loss of all cationic interactions with the LPA phosphate group, whereas the R7.36A mutant was predicted to retain the three cationic interactions with the LPA phosphate group observed in the wild type receptor (Table 1). An EC$_{50}$ increase is expected only for the K7.35A LPA3 mutant. The alanine mutation of R7.36A did not diminish receptor activation by LPA; however, alanine mutation of the adjacent residue, K7.35A, diminished activation of LPA3 by LPA, shifting the EC$_{50}$ from 57 ± 11 to 598 ± 105 nM (Fig. 4F and Table 4).

Impact of TM3 Mutations on Ligand Selectivity Between LPA and S1P—Residue 3.29 is a conserved glutamine in LPA-specific EDG receptors and a glutamate in S1P-specific EDG receptors. We previously reported that the Q3.29E mutant of LPA1 showed diminished activation by LPA but gained responsiveness to S1P; the reciprocal E3.29Q mutant of S1P1 responded to
LPA rather than S1P (24). We evaluated the effects of Q3.29A and Q3.29E mutants on activation by S1P in LPA1–3 as measured by Ca\(^{2+}\)/H\(_{11001}\) mobilization in transiently transfected RH7777 cells; the results of these experiments are summarized in Table 5. S1P did not activate the Q3.29A mutant of LPA1. Wild type LPA1 showed only weak activation at the highest S1P concentration tested (10\(\mu M\)), but the Q3.29E mutant was activated by 10\(\mu M\) S1P to 39% of the maximal LPA-induced Ca\(^{2+}\) response (Fig. 5A). Unexpectedly expression of wild type LPA2 and LPA3 receptors allowed the RH7777 cells to be activable by S1P. Wild type LPA2 was activated by as little as 300 nM S1P, and a 10\(\mu M\) concentration yielded 43% of the maximal LPA-induced Ca\(^{2+}\) response. This response was enhanced in the Q3.29E LPA2 mutant, which was activated by 10\(\mu M\) S1P to 64% of the maximal LPA-induced Ca\(^{2+}\) response. The Q3.29A mutant of LPA2 showed almost no activation by S1P (Fig. 5B). Wild type LPA3 was activated by 10\(\mu M\) S1P to 40% of the LPA-induced maximal LPA-induced Ca\(^{2+}\) response. The mutation Q3.29E enhanced the response to 10\(\mu M\) S1P to 62% of the LPA-induced maximal LPA-induced Ca\(^{2+}\) response, and the mutation Q3.29A diminished receptor activation in response to S1P to 30% of the LPA-induced maximal LPA-induced Ca\(^{2+}\) response (Fig. 5C).

To further investigate the weak agonism we observed of S1P for LPA2 and LPA3, we examined the effect of expression of these receptors on calcium mobilization in response to dihydrosphingosine 1-phosphate (DH-S1P), sphingosine, and dihydrosphingosine as well as in response to S1P (Fig. 6, A and B). Whereas S1P has been reported to release Ca\(^{2+}\) and activate intracellular targets mediating an antiapoptotic response, DH-S1P lacks these effects (34–36). Both LPA2 and LPA3 were completely unresponsive to the non-phosphorylated sphingoid bases sphingosine and dihydrosphingosine, highlighting the importance of the phosphate moiety for receptor recognition. DH-S1P was less potent than S1P in activating LPA3 (Fig. 6A).
and was ineffective in activating LPA₂ (Fig. 6B), indicating that although both receptors prefer S1P to dihydrosphingosine 1-phosphate, LPA₃ may have a greater tolerance for a saturated hydrophobic tail than does LPA₂.

We also examined the Ca²⁺ response to S1P in clonally derived RH7777 cells, which had been stably transfected with wild type LPA₁–₃ (supplemental Fig. 1, A–C). LPA₃ stable transfectants responded to as little as 1 μM S1P with a measurable Ca²⁺ response, whereas the LPA₂ stable transfectants required 3 μM S1P to elicit a response; LPA₁ stable transfectants barely responded to S1P except at the highest (10 μM) concentration tested. Cells stably expressing LPA₁, LPA₂, and LPA₃ were activated by 10 μM S1P to 25, 28, and 44% of the maximal LPA-induced Ca²⁺ response, respectively. The relative responsiveness to S1P conferred by expression of LPA₁, LPA₂, or LPA₃ (data not shown). Radioligand binding studies with LPA and S1P are technically difficult due to the hydrophilic nature of the ligand, which tends to form micelles and partition into the phospholipid bilayer, causing high levels of nonspecific binding and background (39). The relatively high amounts of S1P required to elicit a Ca²⁺ response in our assays suggests that S1P might be a low affinity agonist of LPA₂ and LPA₃. Our inability to detect specific binding is likely due to high nonspecific binding as well as the low affinity of S1P for LPA₂ and LPA₃.

Ligand-induced activation of GPCRs may result in receptor internalization and down-regulation of receptor surface expression. To further investigate whether S1P was directly interacting with LPA₂, we examined ligand-induced receptor internalization by using anti-FLAG flow cytometric analysis to compare cell surface expression of FLAG-tagged LPA₂ in transiently transfected RH7777 cells exposed to vehicle, LPA, S1P, or ATP for 30 min (Fig. 7). Treatment with ATP, which produces robust calcium transients in RH7777 cells through non-LPA receptors, had no effect on surface expression of LPA₂.

During the course of the current study, we attempted radioligand binding assays with the LPA receptors using radiolabeled S1P. Although we were able to detect specific binding of radiolabeled S1P to HEK293T cells transfected with S1P, we could not detect specific binding of radiolabeled S1P to cells transfected with wild type LPA₁, LPA₂, or LPA₃ (data not shown). Radioligand binding studies with LPA and S1P are technically difficult due to the hydrophilic nature of the ligand, which tends to form micelles and partition into the phospholipid bilayer, causing high levels of nonspecific binding and background (39). The relatively high amounts of S1P required to elicit a Ca²⁺ response in our assays suggests that S1P might be a low affinity agonist of LPA₂ and LPA₃. Our inability to detect specific binding is likely due to high nonspecific binding as well as the low affinity of S1P for LPA₂ and LPA₃.
Residues Involved in Ligand Activation of LPA Receptors

Treatment with LPA resulted in a marked decrease in LPA$_2$ surface expression from 34 to 21%. A decrease of similar magnitude of receptor internalization to 23% was measured after treatment with S1P, indicating ligand-induced receptor internalization in support of the agonist properties of S1P on LPA$_2$.

**DISCUSSION**

In the present study, using a combination of computational homology modeling and site-directed mutagenesis experiments, we undertook a comprehensive analysis of residues in LPA$_1$, LPA$_2$, and LPA$_3$ that are computationally predicted to interact with the glycerophosphate moiety of LPA; the complex hydrophobic tail interactions were ignored. The transmembrane domains of LPA$_{1-3}$ show a high (81%) homology with each other, whereas major sequence diversity is present in the amino and carboxyl termini. Although most of the residues that we mutated are conserved in the three LPA receptor subtypes, contrary to the hypothesis that assigns similar roles to these residues in ligand recognition, in most cases we found fundamental differences in their impact on potency and efficacy in the different receptor subtypes. These differences can be rationalized with our computational modeling studies of the wild type and mutant receptors docked to LPA. The modeling results agree completely with the experimental findings and indicate previously unrecognized differences between the LPA receptor subtypes. The present results show that mutations of even strictly conserved residues that interact with the polar head group of the ligand may have very different effects on receptor-ligand interactions within the spatial geometry of the ligand-binding pocket (Table 1).

We found only one residue whose mutation to alanine exerts an identical effect in the three LPA GPCRs of the EDG family. In all three EDG family LPA receptors, R3.28 ion pairs with the phosphate of LPA, and mutation to alanine of this residue abolished activation by submicromolar concentrations of LPA. This residue is also conserved in the S1P-preferring members of the EDG family and has been found to abolish ligand activation when mutated to alanine in S1P$_1$ and S1P$_4$ (21, 24). Thus, R3.28 is a residue that is required for ligand recognition of both LPA and S1P in the EDG family of receptors by making a salt bridge with the phosphate group.

An additional alanine mutation, Q3.29A, was identified that displayed a qualitatively similar but quantitatively distinct role across the three EDG family LPA receptors. Glutamine 3.29 is predicted to interact with the hydroxyl group of LPA. Mutation to alanine of this residue abolished activation of LPA$_1$ by LPA and dramatically decreased activation of LPA$_2$ and, to a lesser extent, LPA$_3$, pointing to the similar role but different impact of this conserved residue in LPA$_{1-3}$. The modeling studies indicate that residues remaining in the ligand-binding pocket of LPA$_3$ after mutation of Q3.29 to alanine are better able to compensate for the loss of the interaction between Q3.29 and LPA than those in LPA$_2$ and LPA$_1$. We have previously observed a similar compensating effect for loss of an ion pair in our studies of the S1P$_1$ receptor: the mutation K5.38A resulted in wild type behavior due to optimization of other ion pairing interactions in the ligand-binding pocket (22).

The present study identified a new interaction between W4.64 of LPA$_3$ and LPA. This interaction seems to be unique to LPA$_3$ among the LPA-specific EDG family receptors; the W4.64A mutation substantially reduced the ability of LPA$_3$ to be activated by LPA but had almost no effect on the activation of LPA$_1$ or LPA$_2$. W4.64 is conserved in all of the EDG receptors and has been shown to form cation-π interactions with the ammonium group of S1P in S1P$_1$ and S1P$_4$ (21). W4.64 is also conserved in the cannabinoid receptors, CB1 and CB2. In CB2, mutation of W4.64 to phenylalanine or tyrosine retained binding to an aromatic, uncharged agonist, whereas mutation to alanine or leucine resulted in loss of agonist binding (41). Taken together, these results indicate that W4.64 is found near the ligand-binding pocket of both EDG and cannabinoid receptors and may interact with either charged or aromatic ligands.

K5.38 is conserved in the EDG family S1P receptors and has been shown to ion pair with the phosphate of S1P in the S1P$_1$ and S1P$_4$ complexes, although it is essential for S1P recognition only in S1P$_1$ (21, 22). Our results indicate that in LPA$_3$ and LPA$_3$ an arginine at position 5.38 ion pairs with the phosphate of LPA, whereas an aspartate residue at this position in LPA$_1$ does not contribute to ligand binding. Replacement of this aspartate with arginine (D5.38R) in LPA$_3$ confers increased receptor activation by LPA, underscoring the importance of this polar inter-
action not only in the S1P4 receptor but also in LPA2 and LPA3 receptors.

We previously demonstrated that in S1P1 a positively charged lysine in TM7, K7.34, forms critical interactions with the phosphate of S1P (20). In LPA1–3, position 7.36 is occupied by a positively charged residue: in LPA1 and LPA2 this residue is a lysine; in LPA3 it is an arginine. The mutation K7.36A in LPA1 enhances activation of LPA1, but diminished activation of LPA2 by LPA (Fig. 4, D and E), and the mutation R7.36A in LPA2 had no effect on receptor activation by LPA. However, alanine mutation of the adjacent residue in LPA3, K7.35, markedly diminished activation by LPA (Fig. 4F). The models show that the cationic residue at position 7.35 (Fig. 4C) is oriented toward the phosphate group of LPA, but the cationic residue at position 7.36 (Fig. 4, A and B) is not. This apparent discrepancy seems to point to another potential role for K7.36 in LPA2 where a different interaction occurs.

The LPA2-mediated responses to S1P were corroborated by S1P-induced LPA2 receptor internalization (Fig. 7). Furthermore, DH-S1P, a ligand with a saturated hydrophobic tail, could activate LPA2, but not LPA3, further supporting the selectivity of the individual receptor subtype in this cross-responsiveness between the three related natural ligands (Fig. 6). The importance of the phosphate head group in this response was underscored by the fact that sphingosine and dihydrosphingosine both failed to activate either receptor.

In the report that originally identified LPA3 as an LPA receptor, S1P at a concentration of 1 μM failed to generate significant increases of a serum response element–driven reporter gene (42). The original reports that identified LPA3 as an LPA receptor indicated that S1P was not a ligand for LPA3. 1 μM S1P did not activate LPA3 expressed in HEK293T cells as measured by GTPγS binding assays (43), 1 μM S1P did not elicit Ca2+ transients in SF9 cells expressing LPA3 (44), and S1P did not compete with [3H]LPA for binding to LPA3-expressing SF9 cells (44). A plausible explanation for the discrepancy between our results, which indicate that S1P is a weak agonist of LPA2 and LPA3, and results from those original studies is that our Ca2+ assays are more sensitive than the radioligand binding assays, GTPγS binding assays, and reporter gene assays used in those studies. Other factors may also influence the cellular responses measured under different conditions, such as the formulation of the ligand with BSA carrier (45) and the cell lines used.

S1P has been shown to function as a low affinity receptor for LPA (46). S1P1-transfected HEK293 cells were activated by relatively high concentrations of LPA (20–50 μM) as measured by receptor phosphorylation, cellular morphogenetic differentiation, and P-cadherin expression. Lower concentrations of LPA (2.5 μM) induced S1P1-mediated mitogen-activated protein kinase activation (46). We do not know the biological significance of the weak agonism of the LPA receptors by S1P that we observed. Certainly it raises caution in interpreting experimental data and ascribing cellular responses to a particular receptor when concentrations in excess of 300 nM S1P are used. In our assays, the concentrations of S1P required to activate LPA2 and LPA3 partially overlap with physiological ranges that have been reported for S1P in human plasma (703 ± 41 nM) or mouse plasma (1310 ± 190 nM) (47).

It has been suggested that human platelets possess a receptor that responds to both LPA and S1P (48–50). Specific binding of [3H]S1P to platelets was inhibited by LPA (48), and the platelet aggregation response to LPA was desensitized by S1P (48–50). Similarly to the relatively high S1P concentrations required to activate LPA2 and LPA3 in our Ca2+ mobilization assays, the platelet aggregation response to LPA was desensitized only by preincubation with relatively high concentrations of S1P (5–40 μM) (48–50); preincubation with 1 μM S1P had no effect (48). S1P is a relatively weak platelet-aggregating agent compared with LPA (49–51), and platelets contain LPA1–3 transcripts (50). The weak agonism of S1P toward LPA2 and LPA3 observed in the present study supports the possibility that the cross-de-
sensitization reported for platelets might be mediated by an EDG family LPA receptor.

The structure of LPA can be described as having a polar head group, a glycerol backbone, and a hydrophobic tail. Recently the residues deeper in the transmembrane helices of S1P₁ that interact with the hydrophobic tail of S1P have been computationally identified and experimentally validated, thus mapping the hydrophobic ligand-binding pocket of S1P₁ (40). That study will serve in the future as a template in modeling the hydrophobic interactions in the other EDG receptors. The present study focused on amino acid residues that interact with the polar head group of LPA. These are the most thoroughly characterized receptor-ligand interactions among the EDG family of LPA receptors and have been shown to be critical for receptor activation (23, 24, 28); modifications to the polar head group have been shown to be critical for receptor activation (23, 24, 28); modifications to the polar head group.

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