Identification of Edg1 Receptor Residues That Recognize Sphingosine 1-Phosphate

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Originating from its DNA sequence, a computational model of the Edg1 receptor has been developed that predicts critical interactions with its ligand, sphingosine 1-phosphate. The basic amino acids Arg120 and Arg292 ion pair with the phosphate, whereas the acidic Glu121 residue ion pairs with the ammonium moiety of sphingosine 1-phosphate. The requirement of these interactions for specific ligand recognition has been confirmed through examination of site-directed mutants by radioligand binding, ligand-induced [35S]GTPγS binding, and receptor internalization assays. These ion-pairing interactions explain the ligand specificity of the Edg1 receptor and provide insight into ligand specificity differences within the Edg receptor family. This computational map of the ligand binding pocket provides information necessary for understanding the molecular pharmacology of this receptor, thus underlining the potential of the computational method in predicting ligand-receptor interactions.

EXPERIMENTAL PROCEDURES

Materials—SPP and LPA were from Avanti Polar Lipids (Alabaster, AL). Anti-flag M2 antibody and horseradish peroxidase- or fluorescein isothiocyanate-labeled anti-mouse were purchased from Sigma.

Edg1 Homology Model Development—The Edg1 model was developed using homology modeling in the MOE program (Chemical Computing Group, Montreal, Canada). The corrected Edg1 sequence from GenBank® (accession number AFF43420) was aligned against the transmembrane helices (THs) of a rhodopsin model (Protein Data Bank entry 1boj (17)). The amino-terminal 30 and carboxyl-terminal 50 residues were deleted, because no template structure was available for these regions. Manual realignments were made to remove gaps in the THs. The structure of the seventh TH of the dopamine D2 receptor was obtained from Dr. H. Weinstein (18) and mutated to produce the corresponding TH for Edg1. This modified structure was substituted into the template structure for homology modeling. The preliminary model was...
refined by converting cis-amide bonds in the loops to trans-amide bonds and by manually rotating side chains at polarity-conserved positions (19) to optimize hydrogen bonding between the THs. Geometry optimization was performed with the AMBER94 force field (20) to a 0.1 root mean square gradient. Interhelical hydrogen bonds formed that were retained upon minimization connect helices 1 and 2 (Asn63 to Asp91), 2 and 3 (Asn66 to Ser134), 2 and 4 (Asn66 to Trp168), 2 and 7 (Asp91 to Ser304), 3 and 7 (Ser131 to Ser304), and 4 and 5 (Trp182 to His205). These geometries were evaluated using the criteria from our previous modeling studies using the incorrect Edg1 sequence (GenBank accession number M31210; Refs. 22 and 23), namely the presence of favorable interactions involving the phosphate group. Geometry optimization of the best complex obtained with the correct Edg1 sequence (accession number AFF43420) was performed to a 0.01 root mean square gradient after manual optimization of ion-pairing interactions.

Site-directed Mutagenesis—The amino-terminal FLAG epitope-tagged Edg1 receptor construct (GenBank accession number AF233865) was obtained from Dr. T. Hla. This receptor construct has been shown to behave the same as the wild-type Edg1 receptor (24). Eight mutations of the Edg1 receptor were generated using the ExSite™ (Stratagene) mutagenesis kit. Positive clones were verified by complete sequencing of the receptor insert.

Cell Culture and Transfection—SPP non-responsive RH7777 cells (12) (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO). Cells (10^6) were transfected with 1.5 µg of plasmid DNA and 12 µl of Cytofectene reagent (Bio-Rad) for 16 h. The following day, the transfection medium was replaced with 3 ml of fresh medium. The cells were used the next day for ligand binding and receptor activation assays.

Radioligand Binding Assays—The human embryonic kidney (HEK) 293 cell line (ATCC) does not endogenously express the Edg1 receptor and has been used for the heterologous expression of SPP receptors (6, 25). The Edg1 mutants were tested for SPP binding after transient transfection into HEK293 cells as described previously (6).

Receptor Activation Assays—Functional assays were performed in RH7777 cells by measuring SPP-activated [35S]GTPγS binding. Transfected cells were homogenized in 20 mM HEPES, 50 mM NaCl, 2 mM EDTA (pH 7.5). Nuclei and cell debris were removed by centrifugation at 2,000 x g for 5 min at 4°C. The supernatant was centrifuged at 40,000 x g for 30 min at 4°C. Membranes were washed and resuspended in 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM MgCl2 and stored at −80°C. 5 µg of membrane protein from Edg1 receptor expressing RH7777 cells was incubated in 1.0 ml of binding buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM MgCl2, 10 µM GDP, 2 mM dithiotreitol, 0.1 mM [35S]GTPγS (1191 Ci/mmol; Amersham Pharmacia Biotech)). After incubating for 30 min at 30°C, bound radioactivity was separated by rapid vacuum filtration through a Whatman GF/B glass filter, and determined by liquid scintillation counting of triplicate samples.

Receptor Expression, Localization, and Internalization—For Western blot analysis, lysates from 24-h transfected cells were collected after an 8-h serum starvation, separated on 12.5% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Bio-Rad). The primary antibody was the FLAG epitope tag-specific M2 monoclonal antibody (Sigma, 1:500 dilution), and the secondary antibody was a horseradish peroxidase-labeled goat anti-mouse antibody (Sigma, 1:4000 dilution). The bound antibody was visualized using the SuperSignal chemiluminescent substrate kit (Pierce).

RH7777 cells transfected with empty vector or plasmids containing Edg1 construct inserts were cultured on coverslips in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO). Cells (10^6) were transfected with 1.5 µg of plasmid DNA and 12 µl of Cytofectene reagent (Bio-Rad) for 16 h. The following day, the transfection medium was replaced with 3 ml of fresh medium. The cells were used the next day for ligand binding and receptor activation assays.

RESULTS

Three-dimensional Structure of Edg1 in Complex with SPP—A preliminary homology model of Edg1 was derived from a model of bovine rhodopsin (16) that is consistent with the 9-Å resolution structural data on the rhodopsin molecule (the best structural information available at the time model development began; Ref. 26). The preliminary model was refined by manually optimizing the interhelical network of hydrogen bonds at polarity-conserved positions followed by energy minimization (19). Hydrogen bonds formed that were retained upon minimization connect helices 1 and 2 (Asn63 to Asp91), 2 and 3 (Asn66 to Ser134), 2 and 4 (Asn66 to Trp168), 2 and 7 (Asp91 to Ser304), 3 and 7 (Ser131 to Ser304), and 4 and 5 (Trp182 to His205). The refined model was used in automated docking studies with SPP to generate a model of their complex. The model complex most consistent with the available data on the ligand selectivity of the Edg1 receptor shows three different ion-pairing interactions with the SPP head-group (Fig. 2C). Two of these involve cationic amino acids, Arg230 and Arg252, predicted to be within 2 Å of the anionic phosphate group of SPP. The third involves an anionic amino acid, Glu121, positioned within 2.5 Å of the positively charged ammonium group of the sphingosine backbone. These ion-pairing interactions can provide an explanation for why the Edg1 receptor shows a strong preference for SPP over LPA, a glycerophospholipid that lacks an ammonium substituent. LPA is unable to ion-pair with Glu121, leaving the carboxylate of Glu121 within 5 Å of the anionic phosphate of LPA with no counterion to mitigate the repulsive interaction between the two.

The theoretical model of the complex between Edg1 and SPP has been compared with the recently published 2.8-Å resolu-
conserved residue in that helix, which is given the number 50.

part of the label refers to its position in that helix relative to the most conserved residue in that helix, which appears. The second part of each label refers to its position in that helix relative to the most conserved residue in that helix, which is given the number 50.

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Notably, both structures involve the same residues, 1.50, 2.50, and 7.46, in a hydrogen bonding network. The rhodopsin structure (1F88 in the Protein Data Bank) has the backbone carbonyl of residue 7.46 occupying a central role, hydrogen bonding with both an asparagine (1.50) and a glutamate (2.50) side chain. This arrangement would require a protonated glutamate as shown in Fig. 3A. The Edg1 model has glutamate 2.50 occupying the central role, hydrogen bonding with the asparagine (1.50) and serine (7.46) side chains. The arrangement in the Edg1 model uses glutamate in its anionic form (Fig. 3B), which would not be possible in the rhodopsin sequence, because the side chain of alanine 7.46 cannot donate a hydrogen bond. A second trio of residues in both structures mediates hydrogen bonds among helices 2, 3, and 4. In both structures asparagine 2.45 occupies a central role, hydrogen bonding with side chains of residues serine 3.42 and tryptophan 4.50. The rhodopsin structure includes an additional hydrogen bond between asparagine 2.45 and the side chain of threonine 4.49 that does not appear in the Edg1 model.

Although the Edg1 model shares several features with the crystal structure of rhodopsin, there are also several notable differences between the structures. These may reflect deficiencies in the model, structural differences that arise because of the sequence differences between the two proteins, or differences between the inactive and active forms of the GPCR structures examined. The most notable difference is in the SPP binding pocket predicted by the Edg1 model. The position of the SPP phosphate in the Edg1 model is occupied by the extracellular beta sheet (b4) that forms the top of the retinal binding site in the rhodopsin structure. The residues in rhodopsin (glutamine 3.26, glutamine 3.29, and phenylalanine 7.34) corresponding to the residues predicted by the Edg1 model to ion-pair with the phosphate of SPP (arginine 3.26, glutamine 3.29, and arginine 7.34) are thus oriented more toward neighboring helices or even outside the helical bundle to make room for SPP.

**Experimental Validation of the Edg1 Complex with SPP**

Validation of the theoretical model of the Edg1 complex with SPP involved the generation of eight site mutations and evaluation of the ability of SPP to bind and activate these mutant receptors. Five of the site mutations, R120A, R292A, R292V, E121A, and E121Q, were designed specifically to evaluate the importance of the residues shown by the model to ion-pair with SPP. Two of these mutations, R292V and E121Q, introduced residues that are found in the corresponding positions in the LPA receptors of the Edg family. The other three site mutations, K111A, N101K, and N101I, were designed as controls to verify that changes in charge and other properties near to, but outside, the predicted binding pocket do not have as dramatic an impact on the ability of SPP to bind to, and activate, the mutant receptors. In the model, Lys111 and Asn101 are 20.2 and 7.1 Å, respectively, from the docked phosphate and are not predicted to interact with SPP.

The level of expression and proper targeting of each mutant to the plasma membrane was verified by Western blots and confocal immunofluorescence microscopy using monoclonal antibodies recognizing an amino-terminal FLAG epitope (Fig. 4, A and B). The mutants showed expression levels and localization patterns similar to the wild type Edg1. Fig. 5 shows the results of the radioligand binding assays for the wild type Edg1 receptor and the site mutants. Each mutation of an ion-pairing residue showed specific binding dramatically less than the wild type Edg1 receptor and similar to that of the vector-transfected controls. The binding results to mutants R120A, R292A, R292V, E121A, and E121Q showed no concentration dependence in the nanomolar range (data not shown), preventing Scatchard analysis to calculate $K_d$ values.

Site mutations in a GPCR can have an impact on the activation state of the receptor as well as its ability to interact with its natural ligand. To verify that the mutations have similar
Effects on ligand binding and subsequent receptor activation have not caused constitutive activation, receptor activation was assessed using a ligand-induced [35S]GTP$^\gamma$S binding assay. Fig. 6A demonstrates that four of the mutations to the proposed ion-pairing residues, R120A, R292A, R292V, and E121A, all produce a receptor that SPP is unable to activate at concentrations up to 100 nM. Calculated EC$_{50}$ concentrations for each mutant are shown in Fig. 6B. These results confirm and extend those from the radioligand binding assays that demonstrated that the receptor was unable to specifically bind SPP at a concentration of 1 nM. The fifth mutation involving an ion-pairing residue, E121Q, is a mutation that changes a residue conserved in the SPP binding Edg receptor subfamily (Edg1, -3, -5, -6, and -8) to a residue conserved in the LPA binding subfamily (Edg2, -4, and -7). This mutation, only at the maximal 1 μM ligand concentration, showed a slight but detectable 17% activation relative to the response of the wild type receptor, indicating that the glutamine residue is able to interact somewhat with the protonated 2-amino group of SPP, perhaps by hydrogen bonding. It should be noted that this result is not in conflict with the radioligand binding assay, which was performed at an 8-fold lower concentration than the 8.1 nM $K_d$ for SPP on the Edg1 receptor (6) and gives a higher background in the higher concentration range due to the hydrophobic nature of the ligand. The control mutations K111A, N101K, and N101I showed activation results that were expected based on the radioligand binding results and were similar to that of the wild type Edg1.

Activation of a GPCR is terminated by ligand-induced internalization of the receptor, which can be followed by the translocation of the epitope-tagged receptor from the plasma membrane to the cytoplasm. In complete agreement with the results of the [35S]GTP$^\gamma$S binding assay, mutants of the predicted ion-pairing residues did not show internalization (Fig. 7), indicating that they were not activated during the prolonged 15-min incubation with the 100 nM physiological concentration of the ligand. This is in contrast to the control mutations, which produced receptors that were activated and internalized by SPP treatment under the same conditions.

**DISCUSSION**

Starting from the DNA-derived amino acid sequence, a theoretical model of the Edg1 receptor and its complex with SPP has been developed. The Edg1 receptor model demonstrates an interhelical hydrogen bonding network that is very similar to that found in the recently reported 2.8-Å resolution crystal structure of bovine rhodopsin (27). Docking studies with this model have predicted that SPP best fits into a pocket formed within the helical bundle, a region that has been experimentally identified as the ligand binding pocket for several GPCRs.
The model predicts three ion-pairing interactions critical to the recognition of SPP by Edg1. These include two ion pairs between cationic amino acids, Arg120 and Arg292, and the anionic phosphate of SPP, as well as a single ion pair between an anionic amino acid, Glu121, and the protonated amino group of SPP. The importance of these ion-pairing interactions identified by the model has been confirmed by site-directed mutagenesis and subsequent radioligand binding assays for both specific binding of SPP by the receptor and receptor activation.

There are 42 basic amino acids (19 of them are arginine) and 20 acidic amino acids (10 of them are glutamate) in the cDNA-derived amino acid sequence of Edg1. Thus identification of the critical residues involved in ion pairing by either random or alanine/cysteine-scanning mutagenesis would have been prohibitive.

Several studies have delineated the specificity of ligand recognition by the Edg1 receptor. Non-phosphorylated sphingosine derivatives (sphingosine, sphinganine, ceramide) have been demonstrated not to compete with the binding of SPP and/or not to activate the receptor. Phospholipids lacking a basic amine (sphingomyelin, LPA, lysophosphatidyl inositol) also fail either to compete with SPP binding or to activate the receptor at physiologically relevant concentrations. Additionally, SPP-phosphonate, having one less atom between the cationic and anionic moieties, was unable to compete with SPP (29). Of the many compounds evaluated for Edg1 interaction, only dihydro-SPP (29), sphingosylphosphorylcholine (30), and a phosphonate (SPP-homophosphonate; Ref. 31) that maintains the appropriate chain length between the cationic and anionic moieties have shown the ability to displace SPP from Edg1. Dihydro-SPP and SPP-homophosphonate were almost as effective as SPP itself at displacing radiolabeled SPP, whereas sphingosylphosphorylcholine was 1–2 orders of magnitude less effective.
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 weaker in binding affinity. These studies collectively demonstrate that the anionic phosphate and cationic amino groups, as well as the distance between them, are critical components of the interaction between SPP and Edg1.

The predicted interactions also provide insight into the ligand selectivity differences within the Edg receptor family. Relevant portions of the amino acid sequences of Edg1 through Edg8 have been aligned and are shown in Fig. 8. The SPP-specific receptors, Edg1, -3, -5, -6, and -8, all share an anionic residue that corresponds to the Glu in the Edg1 receptor predicted by the model to interact with the ammonium of SPP. The LPA-specific receptors, Edg2, -4, and -7, instead have a neutral glutamine residue at that position that could interact with the neutral hydroxyl group in LPA.

Our work demonstrates the applicability of computational modeling to correctly predict the critical ionic interactions defining the ligand binding pocket of a GPCR, thus supporting the utility of a model-driven approach to gene function. The model also lays the foundation for the rational design of therapeutic agents targeted at Edg1.

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