Chapter 2. Biology of LPA and S1P and Their Receptors

2.1. Overview

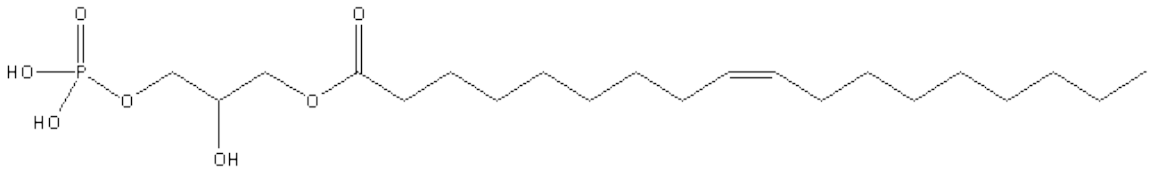
In the body, ligands such as lysophosphatidic acid (LPA) activate G protein-coupled receptors (GPCRs) that, in turn, activate G proteins responsible for cellular messaging. This thesis develops first-principles structural models for the interactions between ligands and a specific class of G protein receptors, the lysophosphatidic acid receptors. Section 2.2 presents lysophosphatidic acid, sphingosine-1-phosphate and their role in the body. Section 2.3 broadly describes GPCRs, while Section 2.4 presents the specific lysophosphatidic acid receptors studied in this thesis.

2.2. Phospholipids

2.2.1. Lysophosphatidic Acid

Lysophosphatidic acid (LPA) refers to a class of phospholipids, the prototypical being 1-acyl-2-*sn*-glycerol-3-phosphate (Structure 2.1), that shows a wide and sometimes contradictory range of cellular effects. Lysophospholipids consist of an alkyl chain on a three-carbon glycerol backbone bound to a phosphate head. The chain length of LPA species can vary from 10 carbons to over 20 carbon atoms and be saturated or unsaturated. This bioactive lipid is a growth factor primarily synthesized by activated platelets, but it is also produced by many other cell types, including ovarian cancer cells

and adipocytes.[1-3] The macro-scale effects of LPA include cell proliferation, chemotaxis, differentiation, and alteration of cell shape.[4-11]



Structure 2.1 Lysophosphatidic Acid *cis*-18:1

LPA initiates a variety of intracellular signaling cascades that lead to the above macro-scale events. DNA synthesis, mitogen-activated protein kinases (MAPK), serum responseive element (SRE), Rho GTPases, phosphoinositide 3-kinase, and phospholipase C (PLC) can all be stimulated by lysophosphatidic acid. Adenylyl cyclase can be either inhibited or stimulated by extracellular LPA. Increases in calcium levels are also sometimes seen upon exposure of cells to lysophosphatidic acid.

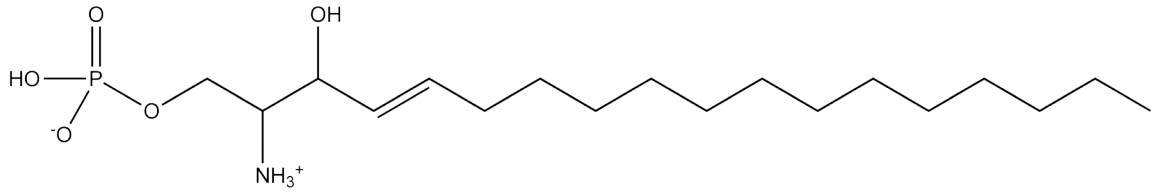
The concentrations of LPA in serum are in the 1-5 μM range,[7,12] although most serum LPA is not free but bound to serum proteins, in particular albumin.[13] LPA has also been isolated in the ascites of ovarian tumors and is seen at higher levels in the blood serum of women with ovarian cancer. LPA concentrations in the fluid surrounding ovarian malignancies are frequently an order of magnitude higher than those seen circulating in the bloodstream of healthy individuals. Lysophosphatidic acid has been explored as a potential biomarker for ovarian cancer, with mixed results.[14-21]

LPA and other lysophospholipids are primarily synthesized *in vivo* through two different methods. The predominant path involves the functionalization of membrane

phospholipids. In this path, membrane microvesicles create an environment where sphingomyelinase and phospholipases (D and/or C) work together to convert the phospholipids into phosphatidic acid (PA). Then secretory type PLA2 converts the PA into LPA. The second mechanism for LPA synthesis, which is an intracellular pathway, involves the reaction of dihydroxyacetone phosphate with a fatty acid-CoA.[22] Early studies suggested that the majority of LPA was synthesized extracellularly, but more recent work intimates that the majority of LPA is produced inside cells.[23]

2.2.2. Sphingosine-1-Phosphate

Sphingosine-1-phosphate (S1P), like LPA, is a lipid with a polar head group and a lipid tail. Unlike LPA, S1P refers to a specific molecule as opposed to a family of lipids (Structure 2.2). The stereochemistry of endogenous S1P is *2S, 3R*. Other stereoisomers have been synthesized and their efficacy examined *in vitro*, but the endogenous isomer has the lowest EC_{50}.[24]



Structure 2.2 Sphingosine-1-phosphate

S1P is synthesized and stored in platelets and released upon their activation.[25] As with LPA, there are two pathways for the synthesis of S1P. Both pathways go through a ceramide intermediate. The *de novo* pathway produces dihydroceramide, which then undergoes desaturation to create ceramide. The second pathway converts sphingomyelin, through sphingomyelinase, into ceramide. After the synthesis of ceramide through either

pathway, ceramidase acts upon the intermediate to manufacture sphingosine. Then any of a number of sphingosine kinases phosphorylate the sphingosine to create sphingosine-1-phosphate.[26] Transportation of S1P throughout the body occurs as a complex of S1P and serum proteins, in this instance, high-density lipoprotein. The serum concentrations of S1P are lower than that of LPA, generally in the range of 0.5-0.8 μM.[25]

2.3. G Protein-Coupled Receptors

Rhodopsin-like GPCRs mediate the effects of LPA in intracellular signaling. Ubiquitous in the body, GPCRs are responsible for a significant amount of intracellular signaling. There are GPCRs that can be activated by light, hormones, lipids, and peptides. The effects of these proteins and the processes by which they are activated are so diverse that GPCRs are one of the most targeted super-families of proteins by the pharmaceutical industry.[5,7,12]

Bovine rhodopsin is the most well studied GPCR, as it is the only member of the superfamily to have a high-resolution X-ray crystal structure.[27] From crystal data, scientists determined that GPCRs are transmembrane proteins with seven helices, three loops extracellular with the N-terminus, and three intracellular loops that, along with the carboxyl terminus, create a binding site for the G protein heterotrimer (Figure 2.1). G proteins are intracellular proteins bound to guanosine phosphates. Common models of G protein signaling hypothesize an inactive GPCR bound to a heterotrimeric G protein, consisting of an α (G_α), a β (G_β), and a γ (G_γ) subunit. The α subunit is bound to guanosine diphosphate (GDP). Upon activation, the GPCR-G protein complex undergoes conformational changes that decrease the affinity of the G protein for GDP. GDP is

released, and the α subunit binds to GTP. The GTP binding to G_α alters the affinity between the G_α and $G_{\beta\gamma}$ subunits. The subunits then interact with other proteins and effector molecules to initiate the synthesis of secondary messengers.

The recent popularity in GPCRs as drug targets has spurred significant research in determining their structure. Unfortunately as with other membrane-bound proteins, GPCRs are generally difficult to crystallize in their natively folded state.[27,28] Many research groups are currently developing computational models in an effort to expedite structure determination. GPCRs are a strong candidate for computational structure prediction because there are clues to the tertiary structure beyond what is available for most other, globular, proteins. Since it is known that all GPCRs are hetpahelical, with each helix approximately 25-35 residues long, the secondary structure guides the prediction process.

There are five known human GPCRs that are activated by LPA, $LPA_{1\text{-}5}$, although another GPCR, $S1P_1$, weakly binds LPA. The following section discusses the receptors $LPA_{1\text{-}3}$ and $S1P_1$ at length. Briefly, receptors $LPA_{1\text{-}3}$ and $S1P_1$ are all in the endogenous differentiation gene (*edg*) family and therefore are related to each other phylogenetically (Table 2.1). Another GPCR, GPR45/PSP24, is a human orthologue of a *Xenopus* LPA receptor but has not been activated by LPA in most experiments.[29,30] LPA is also known to activate an intracellular protein, peroxisome proliferator activated receptor gamma (PPARγ). Less is known about LPA_4 and LPA_5 or about the relationship between LPA and PPARγ.

Since lysophospholipids and sphingolipids affect fundamental cellular functions, the receptors in the *edg* family are seen as potential drug targets. These fundamental

cellular functions influence larger biological processes, including wound healing, angiogenesis, neurogenesis, immunity, and tumorogenesis. There are multiple pathways involved in lysophosphatidic acid signaling through the LPA-activated GPCRs. The excitatory and inhibitory effects of LPA, as well as the lipid's patho-physiological roles, on certain cellular pathways are discussed below.

In order for a receptor to be a viable drug target, the designed ligand must be specific for the targeted receptor. Experimentally, it is difficult to create receptor subtype-specific agonists or antagonists within the LPA family since more than one LPA receptor is often expressed in a given tissue type. The expression patterns for $LPA_{1\text{-}3}$ and $S1P_1$ are described below in Section 2. Studying the receptors computationally will provide a picture of the similarities and differences between the various receptors that bind LPA, and, in particular, allow us to determine how the binding sites are different. Understanding those differences enables us to predict the structures of molecules that could be receptor-subtype specific. This structure prediction motivates the present thesis. Previous homology models have not been able to explain receptor-subtype activity in the LPA family of GPCRs. All of the LPA receptors are activated by Structure 2.1 above, but the GPCRs in the family show selectivity for different chain lengths and have vastly different propensities for inhibition.[31-35] Mutation studies, performed in conjunction with homology modeling, have tentatively isolated residues involved in binding the phosphate head, but the role of the lipid tail in binding and activation has yet to be elucidated.

In order to isolate the binding sites of the LPA receptors, we present a method of structure prediction that does not rely on homology to bovine rhodopsin. We exploit the highly regular nature of GPCRs, *i.e.*, the heptahelical structure in the transmembrane

region, to model them. Chapter 3 details the methods used for predicting and constructing the protein models using LPA_2 as an example system.

2.4. Lysophosphatidic Acid and Sphingolipid Receptors

Lysophosphatidic acid mediates both excitatory and inhibitory intercellular signaling pathways. The nature of the LPA signals depends both on the LPA receptor involved and on the G-proteins to which the receptor couples. At least one LPA receptor couples to each of the major classes of G-proteins: $G_{i/o}$, G_q, $G_{12/13}$, and G_s. All three of the LPA receptors in the *edg* family, as well as $S1P_1$, can couple to $G_{i/o}$. The LPA receptors all couple to G_q, but LPA_3 can also couple to G_s. LPA_1 and LPA_2, on the other hand, couple to $G_{12/13}$.[25]

2.4.1. LPA_1/EDG2/*vzg*-1

The GPCR currently identified as LPA_1 was the first lysophosphatidic acid receptor discovered and the second GPCR found on the endothelial differentiation gene (thus initially called EDG2).[36-38] Concurrently the receptor was found in neural cortical cells during mammalian development and received the name ventricular zone gene-1 (*vzg*-1). Eventually lysophospholipid receptors were renamed according to their high affinity ligand, thus the present name, LPA_1.

LPA_1 is a 364 amino acid, ~41 kDa receptor expressed in almost all mammalian tissue types. The highest concentrations of LPA_1 mRNA are found in neurological tissue. As alluded to above, LPA_1 is expressed in the ventricular zone of the brain in developing fetuses. After birth, LPA_1 is seen in white-matter cells, and its expression coincides with

myelination. The myelinating cells of the peripheral nervous system, Schwann cells, also express LPA_1. The mRNA for LPA_1 is also found in heart, placenta, digestive, reproductive, kidney, thymus, and skeletal tissue.[25]

Murine knockout studies have shown non-redundant functions for LPA_1 in fetal neuroblasts and fibroblasts. A 50% lethality rate was seen in mice lacking the LPA_1 gene. The surviving mice had craniofacial deformities, increased death of Schwann cells, incompletely or malformed skulls, and poor suckling behavior attributed to olfactory defects.[25,39] In addition to neurological defects rooted in embryonic development, LPA_1 may play a role in other neurological afflictions such as chronic pain conditions.[40,41] The study by Inoue *et al.* found no signs of neuropathic pain in LPA_1 deficient mice.

LPA_1 also plays a role in embryonic vascular development, as multiple $lpa_1^{(-/-)}$ embryos developed hemorrhages. However, the survival of some LPA_1 deficient embryos indicates that there are other factors that can compensate for the deficiency and that LPA, acting through LPA_1, is not solely responsible for vascular development.[23]

LPA_1-regulated lipid activity is seen in both healthy and malignant cells. In particular, the LPA_1 receptor may regulate LPA-induced cell migration in pancreatic and colonic cancer cells. Although all three of the *edg* family LPA receptors are expressed in healthy colon and islet cells, LPA_1 is overexpressed in malignant cells from both organs. Yet more evidence implicating LPA_1 in cancer cell motility was seen when Ki16425, an LPA_1/LPA_3 specific antagonist, curtailed cell migration.

2.4.2. LPA_2/EDG4

LPA_2 was discovered through homology searches of orphan GPCR sequences,[42] and it is expressed in human reproductive organs, pancreas, prostate, thymus, spleen, and peripheral blood leukocytes. The human LPA_2 receptor consists of 351 amino acids, while the mouse orthologue contains 348 amino acids. Both receptors have a molecular mass of ~39 kDa.[43]

Knockout mouse studies show that targeted deletion of LPA_2 triggers no obvious phenotypes but that affected mouse embryos do show defective or absent wild-type LPA signaling. The pathology of LPA_2 is mostly seen in malignancies such as colon carcinoma cell lines, DLD1, HT29, and WiDR. Studies into the roles of LPA receptors in colorectal cancer demonstrated marked increases in LPA_2 expression, both at the mRNA and protein levels, in malignant cells when compared to normal tissue from the same patient. LPA_1 expression, while approximately equal to the expression of LPA_2 in normal cells, was significantly decreased in cancerous cell lines indicating that an imbalance in the ratio of LPA_2 expression to LPA_1 expression may be the root of some pathologies. LPA_2 expression is lower than that of LPA_1 in DLD1, but is the only LPA receptor mRNA seen in the other two cell lines. LPA induces cell proliferation and initiates secretion of angiogenic factors in HT29 and WiDR.[44]

Current research on ovarian and ductal breast carcinoma suggests the role of LPA_2 is different than its function in colon cancer, which reiterates the point that the roles of LPA and its GPCRs vary according to concentration, tissue expression, and receptor type.[23,45] Cellular migration, but not invasion, is at least partially regulated by the LPA receptors (LPA_2 and LPA_3) that are expressed in malignant ovarian epithelial cells. In

ductal carcinoma, there is significant expression of LPA_2 relative to healthy gland tissue (within the same patient). Although all examined cancer patients showed higher expression of LPA_2, post-menopausal patients showed a more dramatic increase in LPA_2 protein and mRNA concentrations.[45]

2.4.3. LPA_3/EDG7

LPA_3 consists of 353 amino acids in humans, and the mouse orthologue also contains 353 amino acids. Although LPA_3 is expressed widely, the expression is more limited than that of LPA_1 or LPA_2.[43] It is found in the heart, pancreas, prostate, testis, lung, ovary, and brain of humans. Expression in the mouse is slightly different.[25]

Less is known about the physiology or pathology of LPA_3. It is found in relatively high concentrations in healthy prostate cells, indicating a physiological role. The most striking effects of LPA_3, though, are found in the female reproductive system. The presence of LPA_3 in ovarian cancer cells increases cell migration in multiple cell lines.[23] In recent mouse knockout studies, LPA_3 genetic nulls showed no gross abnormalities, but the female LPA_3 deficient mice showed significant fertility problems. The dams displayed low implantation rates, crowding of the embryos, and implantation proximal to the cervix.[46]

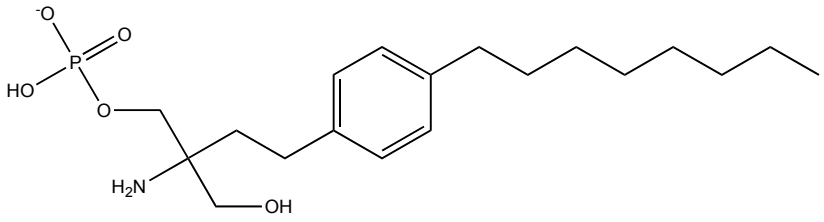
2.4.4. $S1P_1$/EDG_1

$S1P_1$, like LPA_1 and LPA_2, is expressed throughout the body and its activation effects cell shape, growth, and motility. Unlike the LPA receptors, $S1P_1$ is only known to couple to

one G protein, $G_{i/o}$. The downstream signaling events triggered by activation of $S1P_1$ include stimulation of mitogen-activated protein kinases, phospholipase C, Rac, and phosphoinositide 3-kinase. The effects of $S1P_1$ activation upon adenylyl cyclase are strictly inhibitory.[25]

$S1P_1$ was isolated as an orphan GPCR but subsequently recognized as a high-affinity receptor for S1P, with a K_d value of approximately 8 nM. Knockout mouse studies have shown that without $S1P_1$ embryos are not viable. Embryonic maturation, including development of the cardiovascular system, proceeds normally through day 11. After this point, vascular growth is stunted, and all embryos terminate between embryonic days 12 and 14. The current hypothesis asserts that defects in the surrounding smooth-muscle cells impair motility and prohibit vascular maturation.[25]

One drug that targets $S1P_1$, and the related $S1P_3$, has been explored in clinical trials. FTY720-P (Structure 2.3) is known to inhibit lymphocyte egress without diminishing other aspects of the immune response.[47] This physiological response is highly desired in transplant patients, as organ rejection is minimized while the body can still fight infection. Cardiac side effects from FTY720-P, though, minimize the likelihood of it reaching the market.



Structure 2.3 FTY720-P (racemic)

2.5. Conclusion

Because of their roles in fundamental physiological processes, lipids as signaling molecules have become a significant area of study. The majority of lysophospholipid signaling occurs through GPCRs, but details of the method(s) of action are unspecified because of the difficulties in crystallizing membrane receptors. This thesis provides insight into how computational chemistry allows deeper exploration of the roles of lipids in cellular signaling.

2.6. Figures and Tables

LPA_2	49%				
LPA_3	46%	45%			
LPA_4	10%	12%	18%		
$S1P_1$	31%	30%	29%	14%	
BR	17%	15%	14%	17%	16%
	LPA_1	**LPA_2**	**LPA_3**	**LPA_4**	**$S1P_1$**

Table 2.1. The comparative identities of GPCRs that bind to LPA, as well as a comparison to bovine rhodopsin (BR). The identities were calculated by the alignment software ClustalW.[48]

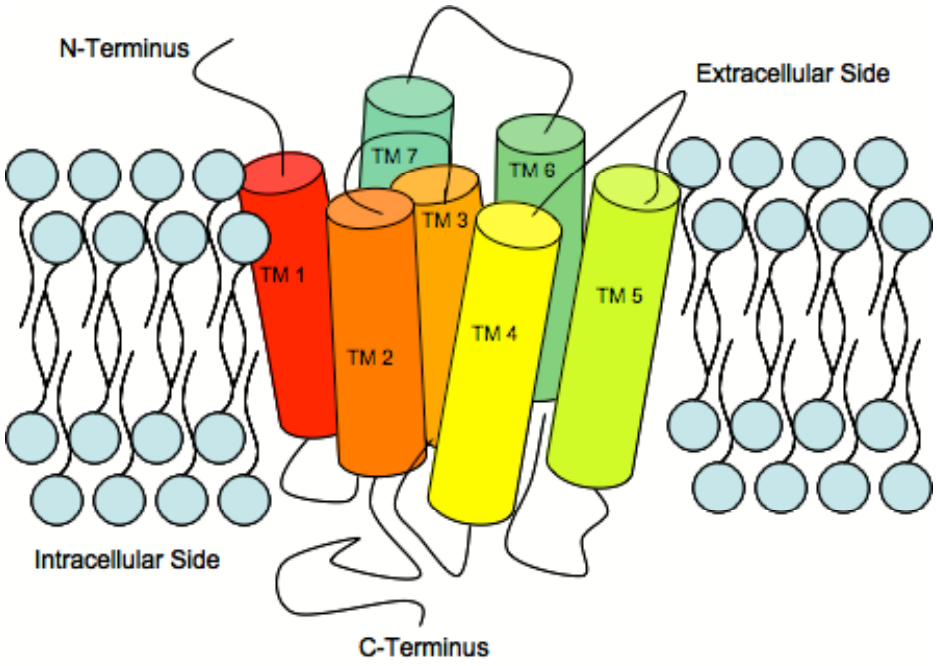


Figure 2.1. A cartoon schematic of a generic GPCR. The schematic shows the topology common to GPCRs, as well as the orientation of a receptor in a cellular membrane.

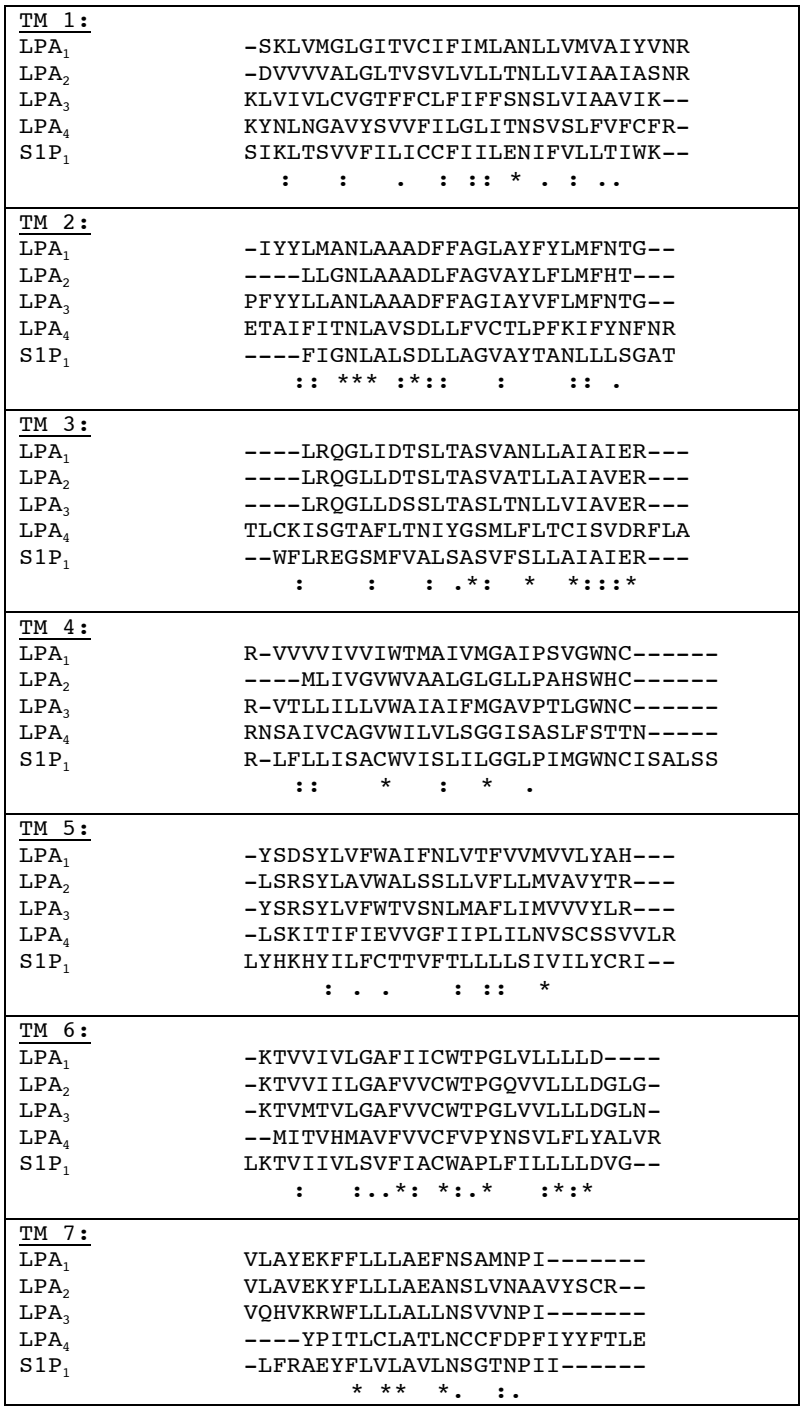


Figure 2.2. The alignment of the predicted transmembrane regions for the LPA receptors and $S1P_1$. LPA_4 shows very little homology to the other receptors in the *edg* family. The asterisks (*) indicate identical residues, and the colons (:) show conservative replacements.

2.7. References

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