RNA Editing Underlies Temperature Adaptation in K^+ Channels from Polar Octopuses

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RNA Editing Underlies Temperature Adaptation in K^+ Channels from Polar Octopuses

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To operate in the extreme cold, ion channels from psychrophiles must have evolved structural changes to compensate for their thermal environment. A reasonable assumption would be that the underlying adaptations lie within the encoding genes. Here, we show that delayed rectifier K^+ channel genes from an Antarctic and a tropical octopus encode channels that differ at only four positions and display very similar behavior when expressed in *Xenopus* oocytes. However, the transcribed messenger RNAs are extensively edited, creating functional diversity. One editing site, which recodes an isoleucine to a valine in the channel's pore, greatly accelerates gating kinetics by destabilizing the open state. This site is extensively edited in both Antarctic and Arctic species, but mostly unedited in tropical species. Thus adenosine-to-inosine RNA editing can respond to the physical environment.

Action potentials, during which the electrical potential across a cell membrane rapidly rises and falls, are the nervous system's basic unit of communication. In 1949, Hodgkin and Katz, using the squid giant axon, showed that the action potential's falling phase has steeper temperature dependence than that of the rising phase and that the membrane's return to the resting potential is particularly sensitive to temperature (*1*). Accordingly, they hypothesized that ectotherms would be forced to regulate the waveform of their action potentials to accommodate the thermal environment. As it emerged that the rising phase was due to voltage-dependent Na^+ channels, and the falling phase to voltage-dependent K^+ channels (*2*–*3*), the early observations suggested that potassium channel gating is more temperature sensitive than is sodium channel gating. This assertion has since been demonstrated (*4*). These observations also suggested that the closing kinetics of potassium channels, which determine the rate of return to rest, should be especially temperature sensitive, and this has also been shown (*5*). If K^+ channel kinetics did not adapt to temperature, the cold would make action potentials disproportionately broad and severely limit repetitive firing. Accordingly, potassium channel kinetics should be a prime target for regulation in organisms adapted to the extreme cold. This study shows that they are, but by an unsuspected mechanism.

To identify mechanisms of cold adaptation, we compared potassium channel orthologs from a tropical and an Antarctic octopus. Despite the enormous body of work on squid axons, we chose octopus as a comparative model because individuals generally have small home ranges, and species inhabit widely different thermal environments, from the poles to the equator. The Antarctic octopus was a *Pareledone sp*., which was collected from McMurdo Station (*6*), where the waters, at –1.8°C, are in equilibrium with sea ice and have been so for the past 28 to 38 million years (*7*). For comparison, we used *Octopus vulgaris* which we collected from a Puerto Rican reef at 30°C. Over the year, the temperature at this location fluctuates between ~25° and 35°C. We sequenced the ortholog of the squid delayed rectifier from each species (*8*).

On the basis of conventional natural selection, we hypothesized that the channels' genes would have evolved mutations to help tune them to their respective environments. Suprisingly, the primary sequences encoded by the two genes were virtually identical, differing at only four positions (fig. S1). They also shared 95% identity with the widely studied *Loligo* ortholog, indicating that the basic genetic template for the delayed rectifier is remarkably similar in coleoid cephalopods. To test whether the four differences affected function, each channel was expressed in *Xenopus* oocytes and characterized electrophysiologically at 2°, 15°, and 25°C. Experiments focused on opening and closing kinetics, which are the most temperature-sensitive properties, but also on the voltage dependence of opening and the rate of inactivation. Functionally, the two channels were virtually identical (Fig. 1A and table S1). As with the squid delayed rectifier, they opened rapidly in response to depolarization and exhibited a steep voltage-dependence between –20 and 20 mV. Upon repolarization, they closed quickly, following a simple exponential time course. When held at a constant depolarizing potential, both channels inactivated slowly, over the course of several seconds. There were some subtle differences. Antarctic channels opened slightly faster (Fig. 1A), but this difference was only significant at potentials near the threshold for activation. The steepness factor (Z) of their steady-state voltage dependence was also slightly smaller (table S1). Overall, however, in spite of their drastically different environments the genomically encoded channels displayed essentially the same behavior. At their respective native temperatures, Antarctic channels would open about 14 times slower and close about 60 times slower than would tropical channels (see Q_{10}s in table S1). Either the Antarctic octopus barely compensates for the cold, or post-transcriptional mechanisms are important.

Adenosine deamination, the most common form of RNA editing, is carried out by a family of enzymes known as ADARs (adenosine deaminases that act on RNA). ADARs convert adenosine (A) to inosine (I) (*9*). Because inosine is

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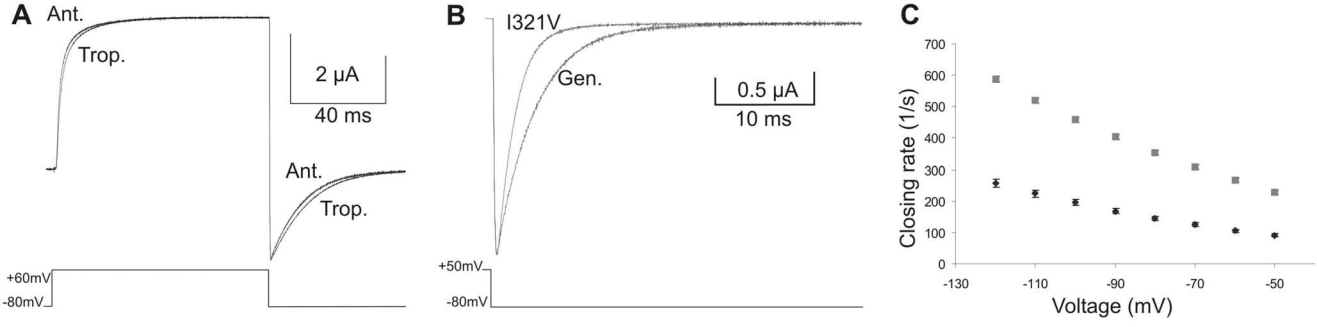


Fig. 1. RNA editing, but not gene-level differences, changes channel function. (**A**) Current traces for Antarctic and tropical K_v1 genomic channels in response to a voltage step from –80 mV to +60 mV. Traces have been scaled in order to show the near identity in opening and closing kinetics. (**B**) Representative current traces focusing on closing kinetics for genomic Antarctic and I321V edited channels. Currents were activated by a stimulus to +50 mV for 20 ms, but only their decay after a return to –80 mV is shown. (**C**) Channel closing kinetics over a range of repolarization voltages after an activating step to +50 mV. Error bars, SEM; n = 16 oocytes for genomic and 9 for I321V. ◆ indicate genomic Antarctic and ■ indicate I321V edited. All data was recorded from voltage-clamped *Xenopus* oocytes injected with cRNA for the appropriate construct, and the temperature was maintained at 15°C.

read as guanosine by the translational machinery, codons in mRNAs can change (*10*). A-to-I editing is common in the nervous system, having been identified in mRNAs encoding ion channels and receptors (*11*–*13*). Extensive editing by this mechanism has been described in squid, in which it modifies diverse mRNAs, including the squid delayed rectifier K^+ channel (*14, 15*). We compared cDNA sequences to check whether the octopus channels were edited as well. Our preliminary cloning results showed site-specific A/G variation in the electropherograms of direct sequences, which is a hallmark of A-to-I RNA editing. To investigate further, we cloned and sequenced 50 individual cDNAs from the stellate ganglia of each species and then looked for A or G variation that was not present in the gene sequence. Each species showed extensive editing. For the Antarctic octopus, there were 18 editing sites, 9 of which caused amino acid changes (fig. S1). For the tropical octopus, there were 15 editing sites, 10 of which caused amino acid changes. Of the 12 nonsilent sites, 5 were species specific, and 4 of the shared sites were edited to greatly different extents (Fig. 2A). Thus, in these channels greater species diversity is generated by RNA editing than by gene mutations.

Do the RNA edits alter channel function? Four editing sites were selected for further study because they were edited exclusively, or to a much greater extent, in one species or the other; N105G and I321V were considered candidates for cold adaptation, whereas N40S and S54G were considered candidates for warm adaptation. [In edited codons, the genomically encoded amino acid was recoded; for example, N105G indicates that asparagine at position 105 was replaced by glycine. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.] N105G is only found in the Antarctic channel, and N40S is only found in the tropical channel. The other two sites showed greater than 50% differences in editing, with I321V highly edited in the Antarctic channel, and S54G highly edited in the tropical channel (Fig. 2A). I321V lies in the fifth transmembrane span (S5), within the pore domain (Fig. 2B) (*16*). The rest of the sites are located in the T1 domain (Fig. 2B), a region that is important for tetramerization (*17*, *18*). At the genomic level, all of these positions except S54G are nearly invariant among Kv1 channels, suggesting that they are functionally important.

To characterize their effects, each of the four edits were singly introduced into the unedited channel background (genomic) and studied at 15° and 25°C. Three of the four editing sites produced clear functional changes to a number of channel properties (table S1). The tropical edits (N40S and S54G) had similar effects: Both slowed channel opening by about 50%, and both increased the rate of inactivation by 30% for N40S and by 60% for S54G (fig. S2). These effects on gating were somewhat surprising, given that both sites are in the cytoplasmic tetramerization domain, which is thought to be physically separate from the membrane-bound voltage sensor and pore (*16*, *19*). However, others have reported that the T1 domain influences function (*20*, *21*). The Antarctic edits also altered function. N105G caused subtle changes in activation and deactivation kinetics. The most pronounced effect of all, however, was due to I321V, which more than doubled the rate of closure (Fig. 1, B and C) and produced a positive shift in the voltage dependence of opening (table S1). This effect was dominant, speeding closing when combined with other editing sites that mimicked natural editing patterns (table S1 and fig. S3) (*6*).

Because I321V was highly edited in the Antarctic species and dramatically accelerated deactivation kinetics, which underlie the action potential's afterhyperpolarization, it was a good candidate for cold adaptation. This position lies in the S5 helix, an interface between the voltage sensor and the ion conduction pathway (*16*, *22*, *23*). At negative voltages, the voltage sensor is thought to force the gate shut via contact between the S4-S5 linker and the S6 helix. At positive potentials, the voltage sensor moves away, allowing the channel to open (*22*, *24*, *25*). The open state is not stable, however, and channels flicker between open and closed conformations (*26*, *27*). Because I321V causes the channel to close faster and also shifts its voltage sensitivity to more positive potentials, we hypothesized that it destabilizes the open state. This possibility was examined directly by recording single-channel events. Representative records for both unedited and I321V Antarctic channels show that I321V channels close more frequently (Fig. 3, A and C). To quantify this phenotype, recordings were idealized by using an algorithm, and the durations of open and closed events were determined (*28*). The average open duration for the unedited Antarctic channels was twice that of I321V channels. The durations of closed events did not change, suggesting that I321V selectively affects the open to closed state transition. This idea was reinforced by a simple five-state model (Fig. 3, E and F): By doubling

A

Site	nt.	Amino acid change	Domain	Editing percentages	
				Antarctic	Tropical
1	119	N40S	T1	0	76
2	124	S42G	T1	0	10
3	160	S54G	T1	10	68
4	175	T59A	T1	0	10
5	314-315	N105G	T1	92	0
6	348	K116R	T1	16	0
7	379	K127E	T1	88	78
8	403	I135V	T1	96	80
9	499	I167V	S1	96	78
10	508	M*170V	S1	98	84
11	961	I321V	S5	92	30
12	1114	I372V	P/S6	90	82

B

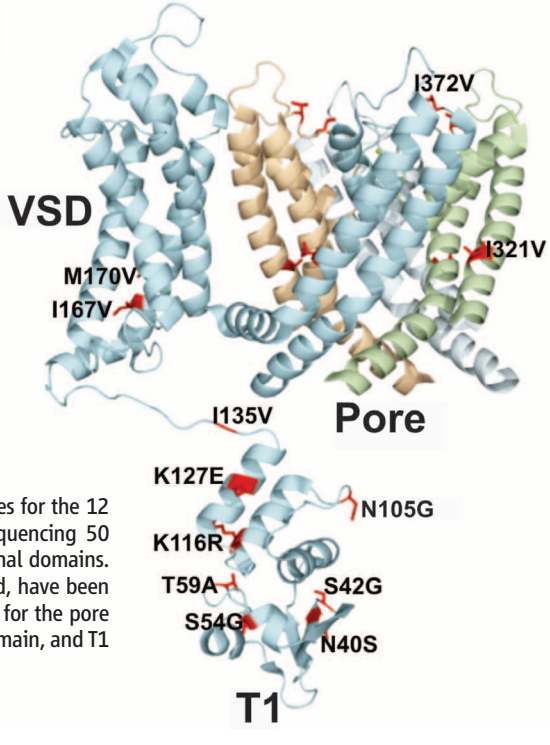


Fig. 2. mRNAs encoding octopus K_v1 channels are extensively edited. (**A**) Editing percentages for the 12 nonsilent sites found in the Antarctic and tropical octopus K_v1 channels calculated by sequencing 50 individual cDNA clones for each channel. (**B**) Octopus editing sites occur in different functional domains. Homologous positions to those altered by RNA editing in octopus K_v1 channels, shown in red, have been mapped on the $K_v1.2$ crystal structure (*23*). One full subunit of the tetramer is shown (blue); for the pore region, all four subunits are shown, each in a different color. VSD indicates voltage-sensing domain, and T1 indicates tetramerization domain.

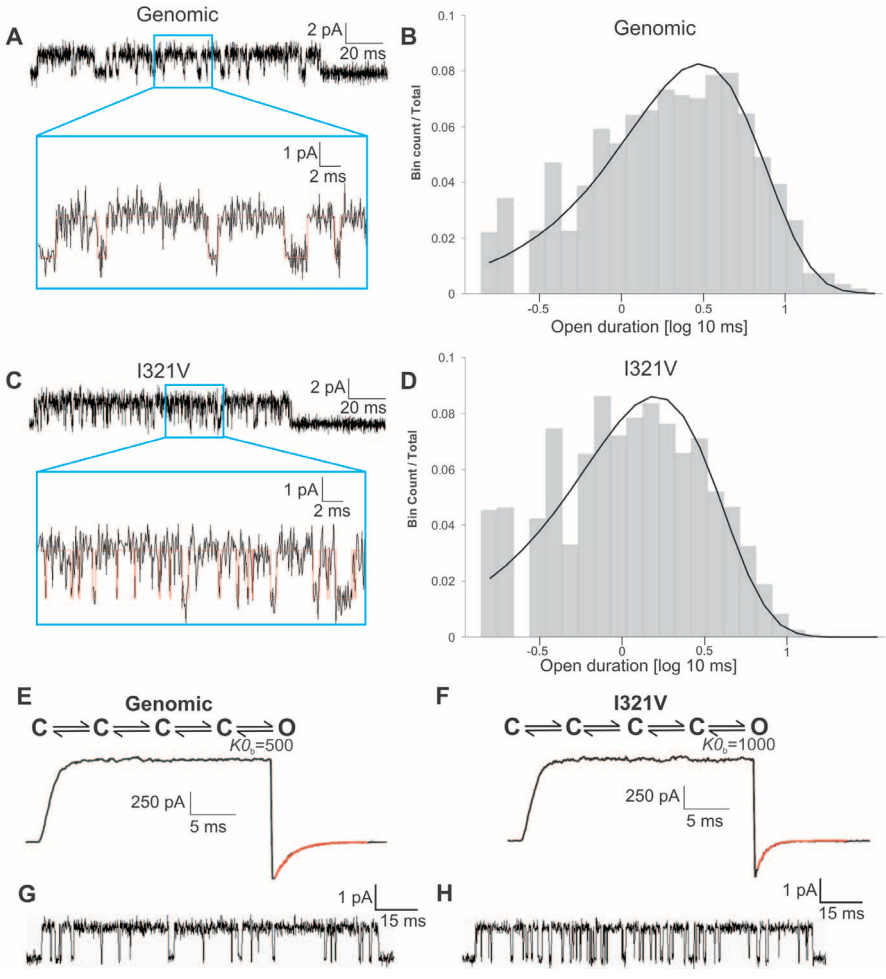
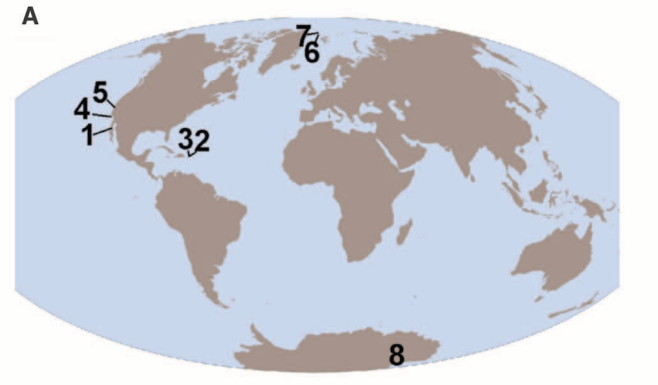


Fig. 3. The editing site I321V speeds channel-closing kinetics by destabilizing the open state. Current traces from cell-attached patches containing single (**A**) Antarctic genomic and (**C**) I321V K_v1 channels. Channels opened in response to a voltage step from −80 mV to +60 mV. I321V channels close more frequently. Overlapping red lines (insets) show idealizations of traces as a series of open and closed events. (**B** and **D**) Duration distributions of open events for genomic Antarctic and I321V channels. The average duration of open events in the genomic channel (3.42 ms) was about twice that of I321V channels (1.72 ms). (**E** and **F**) Simple five-state models for genomic Antarctic and I321V channel gating and simulations generated from the models. Doubling the backward rate constant for the final transition recapitulates both the macroscopic difference in closing kinetics and the single channel behavior (**G** and **H**) between genomic and I321V channels. Rate constants were assumed to depend exponentially on voltage: $k = k_0 e^{(zFV/RT)}$, where F is the Faraday's constant, V is voltage, R is the universal gas constant, and T is the temperature. For the above models: $k_{1f} = 700$, $z = 0.3$; $k_{1b} = 50$, $z = 1.6$; $k_{2f} = 1200$, $z = 1.8$; $k_{2b} = 25$, $z = 1.1$; $k_{3f} = 600$, $z = 0.8$; $k_{3b} = 150$, $z = 1.5$; $k_{4f} = 3000$, $z = 0.02$; k_{4b} variable, $z = 0.2$. A stochastic simulation of 1000 channels pulsed from −80mV to +60mV and back to −80mV showed faster closing with the I321V model. The maximum open probability was similar: 0.59 versus 0.55 for genomic and I321V, respectively. Closing rates (single exponential fit, overlapping red line) were similar to those obtained from room temperature experimental data: 625 and 1250 (s^{-1}) simulated kinetics versus 610 and 1423 (s^{-1}) experimental kinetics for genomic and I321V, respectively. For the single-channel simulations, the average open duration using the I321V model was half that for the genomic model.



Collection sites and water temperatures for octopus species

	Species	Collection site	Water temp. (°C)
1	*Octopus digueti*	Estuary, Baja California	37
2	*Octopus defilippi*	Reef flat, Rio Grande, PR	32
3	*Octopus vulgaris*	Inshore reef, Luquillo, PR	28
4	*Octopus bimaculata*	Near shore rock ledge, Catalina Is., CA	18
5	*Octopus rubescens*	Near shore, Monterey, CA	10
6	*Benthoctopus piscatorum*	Benthic trawl, north Svalbard, Norway	0
7	*Bathypolypus arcticus*	Benthic trawl, north Svalbard, Norway	-1
8	*Pareledone sp.*	McMurdo station, Antarctica	-2

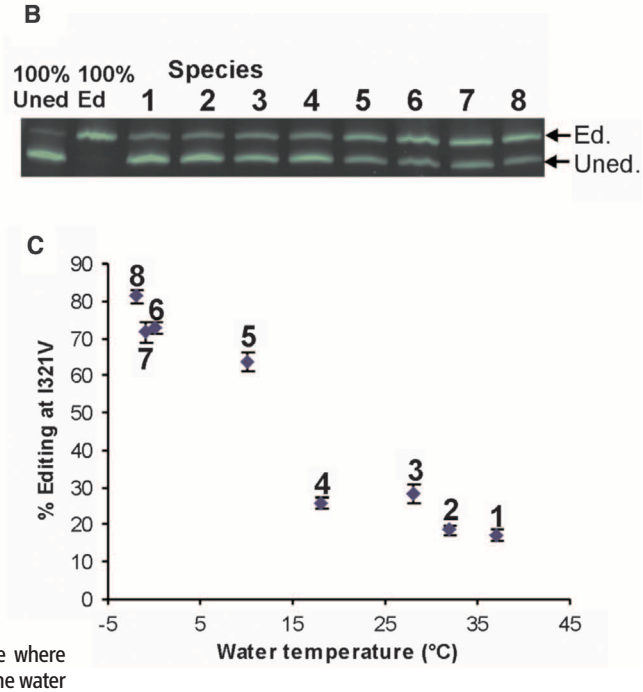


Fig. 4. The extent of editing at I321V correlates with the water temperature where octopus species were captured. (**A**) Collection sites for eight octopus species and the water temperatures and habitats at the time of capture. (**B**) Representative poison primer extension assay showing the amount of I321V editing among the eight species. (**C**) I321V editing percentages for the eight species, based on poison primer extension assays, versus water temperature at capture site. Error bars, SEM; $n = 4$ assays.

the backward rate constant for the final transition, both the macroscopic difference in closing kinetics (Fig. 3, E and F) and the single-channel difference in open duration (Fig. 3, G and H) could be recapitulated. In agreement with experimental data, the model predicts nearly identical activation kinetics and open probability for the two channels (Fig. 3, E and F). Thus, it appears that once open, I321V channels are poised to close rapidly, but the opening kinetics and other properties are preserved. In an axon, we predict that the major effect of I321V channels would be to accelerate the after-hyperpolarization, and thus shorten the refractory period, increasing repetitive firing rates.

If editing of codon I321 is indeed a cold adaptation, then we might expect other cold-adapted octopuses to make the same edit. To test this idea, we collected two Arctic species from benthic trawls northwest of the Svalbard archipelago, where water temperatures at ~0°C were similar to those in Antarctica. We also collected two more tropical species, one from Puerto Rico and one from a desert lagoon in Baja California, and two temperate species from California (Fig. 4A). Editing levels at I321V, as determined by a primer extension assay, correlated very well with the environmental temperature where the species were captured (Fig. 4, B and C). We also quantified all editing sites in the six new species using direct sequencing. Although many of the other editing sites were edited differentially among the eight species, I321V correlated most closely with temperature (table S2).

In Coleoid Cephalopods, and in other higher metazoans, A-to-I RNA editing adds a layer of complexity to the proteome. A clear advantage to this strategy is that it allows options: Different isoforms can be expressed in response to different conditions. Exactly how organisms exercise these options is largely unknown. *Drosophila* and rodents use editing to fine-tune protein function temporally, over the course of development (*29*, *30*), and spatially, in different brain regions (*12*). Here, we present evidence that RNA editing can respond to an external pressure: temperature. Although still maintaining the basic K^+ channel plan, octopuses can make fast-closing versions, and the extent of their expression can be graded. A basic question that remains is whether octopuses use editing for rapid acclimation or long-term adaptation. For each possibility, the biochemical mechanisms that impart temperature sensitivity to the editing process would be different. Others have shown that the RNA structures that drive editing evolve and generate species-specific patterns, suggesting a plausible mechanism for adaptation (*31*). Acclimation could arise from temperature-sensitive RNA structures, or temperature-dependent expression of other factors that control ADAR's access to specific editing sites.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1212795/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S3
References (*32*–*35*)

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Crystal Structure of a Lipid G Protein–Coupled Receptor

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The lyso-phospholipid sphingosine 1-phosphate modulates lymphocyte trafficking, endothelial development and integrity, heart rate, and vascular tone and maturation by activating G protein–coupled sphingosine 1-phosphate receptors. Here, we present the crystal structure of the sphingosine 1-phosphate receptor 1 fused to T4-lysozyme ($S1P_1$-T4L) in complex with an antagonist sphingolipid mimic. Extracellular access to the binding pocket is occluded by the amino terminus and extracellular loops of the receptor. Access is gained by ligands entering laterally between helices I and VII within the transmembrane region of the receptor. This structure, along with mutagenesis, agonist structure-activity relationship data, and modeling, provides a detailed view of the molecular recognition and requirement for hydrophobic volume that activates $S1P_1$, resulting in the modulation of immune and stromal cell responses.

G protein–coupled receptors (GPCRs) convert exogenous signals into a cellular response by initiating a variety of intracellular signaling cascades. The sphingosine 1-phosphate receptor subtype 1 ($S1P_1$) (*1*) belongs to a subclass of the GPCR family originally termed the endothelial differentiation gene (EDG) family of lipid receptors. The family was later renamed to reflect activation by two distinct lipids, S1P and lysophosphatidic acid (*2*). The S1P receptor family comprises five members ($S1P_{1-5}$) with high sequence identity within the ligand-binding region, including the conserved sphingolipid binding pocket. Activation of the $S1P_1$ receptor through exogenous ligands, both physiological and pharmacological, results in inhibition of

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