was 100% bootstrap support for the separation of the mouse species in both tissues.
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10. All RNA pools were hybridized 4 to 6 times to the same set of filters in order to estimate interexperimental errors and to minimize their effects through the combined analysis of several experiments. Signals that were at least 5 times above background and not influenced to more than 25% by neighboring spots were further analyzed. A gene was regarded as differently expressed if it fulfilled two criteria: (i) The difference in signal between two species was at least two-fold; and (ii) the signal between the two species was significantly different as determined by a paired *t* test. Sixteen differently expressed genes were analyzed by Northern blots, and 1 out of 12 that were detected by the Northern analyses yielded results contradictory to the arrays, whereas the remaining 11 showed expression patterns that were both qualitatively and quantitatively similar in all three species to that detected by the arrays. Details of experimental procedures are available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5566/340/DC1and on http://email.eva.mpg.de/~khaitovi/supplement1.html.
11. The distance between two expression profiles of two species in a given tissue was determined by summing up the absolute ratios of the included genes given by the formula: $\sum_{i=1}^{n} \left| \log_2 \sum \frac{x_i^j}{x_i^k} \right|$, where $n$ is the number of included genes, and is the normalized intensity of gene $i$ as measured in species $j$. In order to avoid the contribution of genomic differences, only those differently expressed genes were considered that did not show the same expression difference in two or more tissues. The resulting distance matrix was used to build neighbor joining trees (*19*) as implemented in the PHYLIP package (*20*). The data are available at http://email.eva.mpg.de/~khaitovi/supplement1.html
12. We retrieved nonmitochondrial nucleotide sequences from *M. spretus* (10 sequences) and *M. caroli* (11 sequences) from GenBank and compared them with the corresponding *M. musculus* sequence. The average number of substitutions at silent sites was estimated to be 0.025 (± 0.006) for *M. spretus* and 0.045 (± 0.008) for *M. caroli*.
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24. We thank C. Allen and T. Insel of the Yerkes Primate Center, Atlanta, for chimpanzee tissues; T. Arendt and U. Überham of the Paul Flechsig Institute, Leipzig, for human and mouse tissue dissections; E. Kuhn at the Biomedical Primate Research Centre, Rijswijk, for autopsies; H. Zischler and K. Mätz-Rensing from the German Primate Center, Göttingen, for macaque samples; C. Wittekind and U. Gütz from the University of Leipzig for liver resections; E. Edel from the University of Leipzig for human blood samples; F. Bonhomme and A. Orth of the Université Montpellier II for the mouse tissues; J. Retief at Affymetrix for help and support; K. Chowdhury from the Max-Planck-Institute of Biophysical Chemistry, Göttingen, and K. Krohn and Petra Süptitz from the Interdisziplinäres Zentrum für Klinische Forschung (IZKF), Leipzig, for the help with microarray experiments; T. Kitano for analyzing the mouse divergence; D. Kuhl for initial advice in the project; and many people at Max Planck Institute for Evolutionary Anthropology for stimulating discussions. We also thank the Bundesministerium für Bildung und Forschung, the Saxonium Ministry for Science, and the Max Planck Gesellschaft for financial support.

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# A MADS-Box Gene Necessary for Fruit Ripening at the Tomato *Ripening-Inhibitor* (*Rin*) Locus

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**Tomato plants harboring the *ripening-inhibitor* (*rin*) mutation yield fruits that fail to ripen. Additionally, *rin* plants display enlarged sepals and loss of inflorescence determinacy. Positional cloning of the *rin* locus revealed two tandem MADS-box genes (*LeMADS-RIN* and *LeMADS-MC*), whose expression patterns suggested roles in fruit ripening and sepal development, respectively. The *rin* mutation alters expression of both genes. Gene repression and mutant complementation demonstrate that *LeMADS-RIN* regulates ripening, whereas *LeMADS-MC* affects sepal development and inflorescence determinacy. *LeMADS-RIN* demonstrates an agriculturally important function of plant MADS-box genes and provides molecular insight into nonhormonal (developmental) regulation of ripening.**

The maturation and ripening of fleshy fruits is a developmental process unique to plants and affects the quality and nutritional content of a significant portion of the human diet. Although specific fruit-ripening characteristics vary among species, ripening can be generally described as the coordinated manifestation of changes in color, texture, flavor, aroma, and nutritional characteristics that render fruit attractive to organisms receiving sustenance in exchange for assisting in seed dispersal (*1*, *2*).

Fruit species are classically defined as one of two ripening types, climacteric and nonclimacteric, where the former display a burst in respiration at the onset of ripening, in contrast to the latter. Climacteric fruit typically increase biosynthesis of the gaseous hormone ethylene, which is required for the ripening of fruit such as tomatos, bananas, apples, pears, and most stone fruit. Nonclimacteric fruit, including strawberries, grapes, and citrus fruits, do not require climacteric respiration or increased ethylene for maturation. Molecular ripening research has focused primarily on ethylene, but little is known of control before ethylene induction, nor of common regulatory mechanisms shared by climacteric and nonclimacteric species (*3*).

The tomato is a model for analysis of ripening due originally to its significance as a food source and diverse germplasm, and more recently, the availability of molecular tools (*4*) and efficient transformation (*5*). A number of tomato-ripening mutants have been useful for research and breeding (*3*). Especially interesting is the recessive *ripening-inhibitor* (*rin*) mutation that inhibits all measured ripening phenomena, including the respiratory climacteric and associated ethylene evolution, pro-vitamin A carotenoid accumulation, softening, and production of flavor compounds (*6*). The *rin* mutant exhibits ethylene sensitivity, including the seedling triple response (*7*), floral abscission, and petal and leaf senescence. Nevertheless, *rin* fruit do not ripen in response to exogenous ethylene, yet they display induction of at least some ethylene-responsive genes, indicating retention of fruit ethylene sensitivity (*8*). We interpret these results to mean that the *RIN* gene encodes a genetic regulatory component necessary to trigger climacteric respiration and ripening-related ethylene biosynthesis in addition to requisite factors whose regulation is

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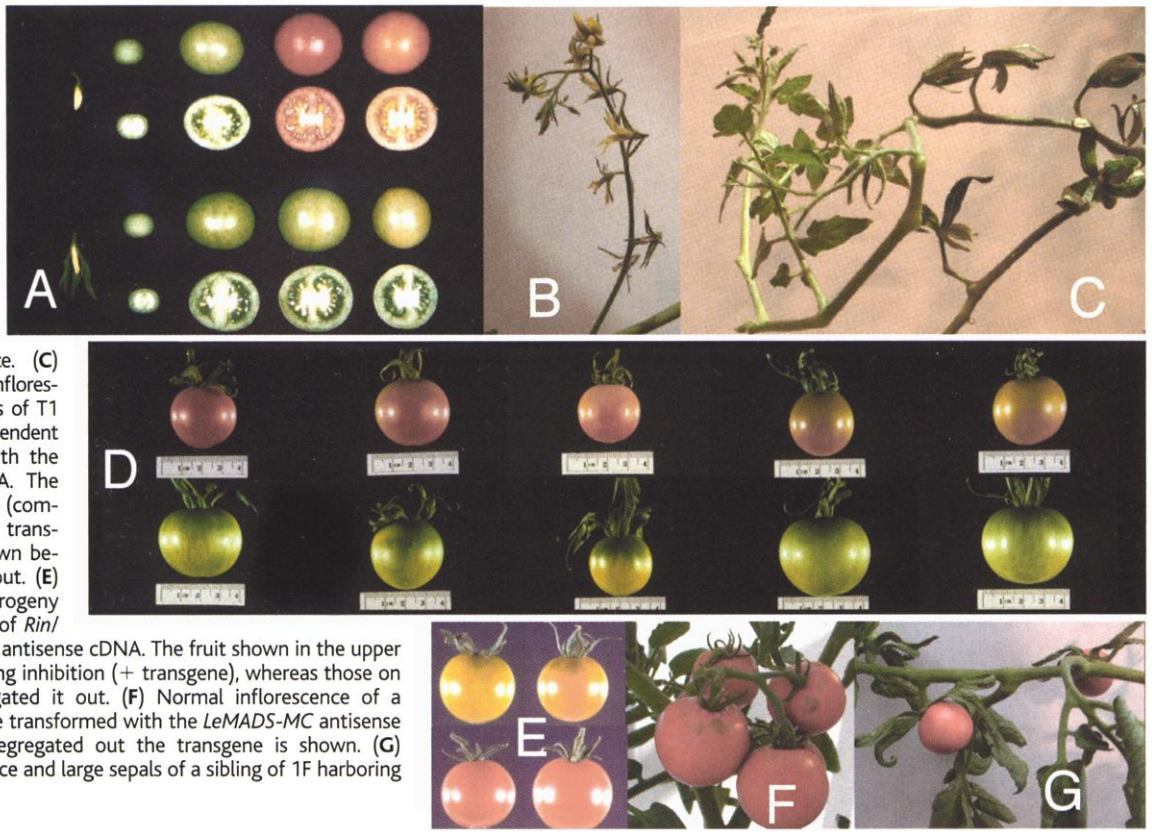


**Fig. 1.** Normal, mutant, and transgenic lines. (**A**) Developmental series of normal (cultivar Ailsa Craig *Rin/Rin*, top) and mutant (nearly isogenic for *rin/rin*, bottom) fruit. The fruit shown on the right were mature green when exposed to 10 parts per million (ppm) ethylene for 12 hours and photographed 48 hours later. (**B**) *Rin/Rin* determinate inflorescence. (**C**) *rin/rin* indeterminate inflorescence. (**D**) Fruit and sepals of T1 progeny from five independent transformants (*rin/rin*) with the *LeMADS-RIN* sense cDNA. The progeny shown at the top (complemented) contain the transgene, whereas those shown below have segregated it out. (**E**) Fruit and sepals of T1 progeny from two transformants of *Rin/Rin* with the *LeMADS-RIN* antisense cDNA. The fruit shown in the upper panel demonstrate ripening inhibition (+ transgene), whereas those on the bottom have segregated it out. (**F**) Normal inflorescence of a representative *Rin/Rin* line transformed with the *LeMADS-MC* antisense cDNA. A T1 that has segregated out the transgene is shown. (**G**) Indeterminate inflorescence and large sepals of a sibling of 1F harboring the transgene.

outside the sphere of ethylene influence. As such, *RIN* acts upstream of both ethylene and nonethylene-mediated ripening control. We previously reported mapping of the *rin* locus to tomato chromosome 5 (*9*). Here, we report cloning and characterization of two MADS-box genes at the *rin* locus. MADS-box genes encode transcription factors that, in plants, primarily regulate floral development (*10*, *11*), yet have never before been demonstrated to regulate ripening.

The only reported mutation at the *rin* locus arose spontaneously in a breeding line developed by H. Munger (at Cornell University) (*12*). In addition to ripening inhibition, the mutant exhibits large sepals and a loss of inflorescence determinacy (Fig. 1, A to C). Genetic complementation of only the ripening phenotype of the original *rin/rin* mutant, with a recessive large sepal mutant (*macrocalyx*, *mc*), suggested that the lesion at *rin* affected two adjacent loci controlling ripening and sepal development, respectively (*12*).

Previous mapping indicated that *rin* resides on a tomato 365-kb yeast artificial chromosome (YAC) clone, [Yrin9 (*9*)], and the analysis summarized in Fig. 2 confirmed this hypothesis (*13*). Yrin9 was used as a hybridization probe to identify putative *rin* cDNAs (*13*). Gene-expression analysis revealed two Yrin9-derived cDNA clones with altered transcripts in mutant fruit (Fig. 3A). C34 showed constitutive expression during maturation of normal fruits, whereas C43 was induced coincident with ripening. Both sequences hybridized to mRNA that was identically expressed in maturing *rin* fruit. This result, in combination with increased transcript size for both probes in *rin*, suggested that the *rin* lesion resulted in the fusion of gene sequences represented by C34 and C43. Reverse transcription–polymerase chain reaction (RT-PCR) of *Rin/Rin* and *rin/rin* fruit RNA (*14*) confirmed this hypothesis (Fig. 3B). Sequencing of both cDNAs, in addition to the *rin* RT-PCR product (*15*), verified that a deletion occurred in the mutant, yielding a chimeric RNA (Fig. 3C). PCR of *Rin/Rin* genomic DNA (*16*) indicates that 2.6 kb separates the C43 and C34 coding regions (Fig. 3C).

C34 and C43 sequencing revealed that both are members of the MADS-box family of transcription factors (*15*). MADS-box genes encode proteins characterized by the conserved MADS-box DNA binding domain and have been isolated from numerous eukaryotic organisms (*17*). Plant MADS-box genes are primarily associated with the regulation of floral development (*10*, *11*, *18*). The best described plant MADS-box family is from *Arabidopsis*, with at least 47 genes identified, although many remain to be defined functionally (*19*).

We named the genes corresponding to C43 and C34, *LeMADS-RIN* and *LeMADS-MC*, respectively, based on analyses described herein.

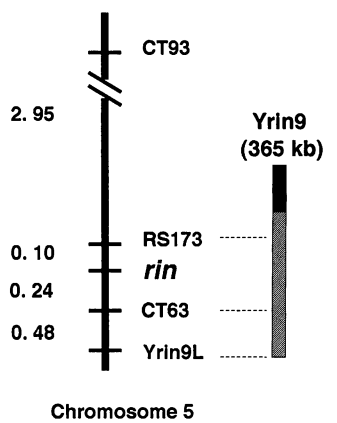


**Fig. 2.** Genetic map of the *rin* region of chromosome 5. Mapping of the YAC end (Yrin9L) and a subclone of Yrin9 (RS173) provided genetic evidence that Yrin9 contains *rin*.

To define the activities of each gene, sense and antisense constructs of full-length cDNAs under the direction of the CaMV35 promoter were created (*13*). Transgene integration into the *Rin/Rin* and *rin/rin* genomes was performed by *Agrobacterium*-mediated transferred DNA (T-DNA) transformation (*5*), and 10 to 50 independent transformants were recovered for each construct.

The expression of *LeMADS-RIN* in the *rin/rin* genotype resulted in complementation of the ripening deficiency but did not

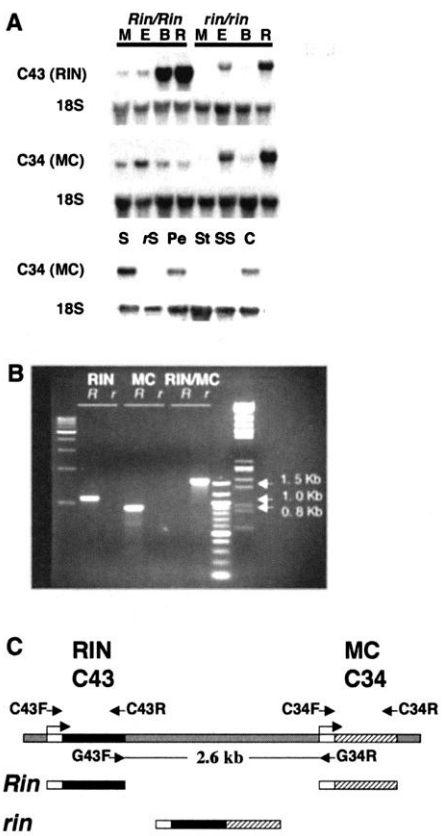


**Fig. 3.** Characterization of *LeMADS-RIN* and *LeMADS-MC*. (**A**) RNA gel-blot hybridizations with 3′–untranslated region probes of *LeMADS-RIN* (C43) and *LeMADS-MC* (C34). Total RNA was isolated from normal (*Rin/Rin*) and mutant (*rin/rin*, identical age post-pollination as normal) fruit pericarp tissue. Mature green (M), M after 12 hours of exposure to 10 ppm ethylene (E), "breaker" showing initial external signs of lycopene (red pigment) accumulation (B), and ripe [B + 7 days (R)]. *LeMADS-MC* was additionally probed to RNA from normal sepals (S), petals (Pe), stamen (St), styles/stigma (SS), and carpels (C) of anthesis flowers. *r*S denotes *rin/rin* sepals. A tomato ribosomal (18*S*) probe confirmed equal RNA loading. (**B**) Chimeric MADS-box transcript in the *rin* mutant. RT-PCR of total RNA from R-stage *Rin/Rin* and *rin/rin* fruit pericarp using primers to *LeMADS-RIN* (RIN), *LeMADS-MC* (MC), or both (RIN/MC) (*14*). (**C**) Schematic of MADS-box genes at the *rin* locus in addition to RNAs defined in (B). The arrows above the diagram indicate primers used in (B) (*14*), whereas those below it were used to estimate intergenic distance (*16*). C43F and C34R were used to amplify *rin/rin* RNA. The gray boxes represent nontranscribed regions. The black and crosshatched boxes correspond to the *LeRIN-MADS* and *LeMADS-MC* transcribed regions, respectively. The white boxes indicate MADS domains. The right-angle arrows denote transcription start sites.

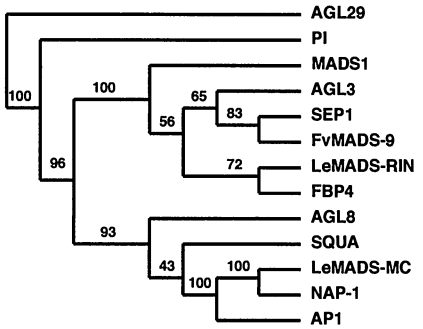


**Fig. 4.** Relation of RIN and MC to other plant MADS-box proteins. Maximum parsimony map of LeMADS-RIN, LeMADS-MC, *Arabidopsis* (AP1, SEP1, AGL3, AGL8, PI, AGL29), *Antirrhinum* (SQUA), petunia (FBP4), pepper (MADS1), tobacco (NAP1-2), and strawberry (FvMADS-9) MADS-box sequences (*13*).

affect the sepal or indeterminacy phenotypes (Fig. 1D). This result demonstrates that *LeMADS-RIN* encodes the *RIPENING-INHIBITOR* gene and, furthermore, the function of this gene is specific to ripening control. Although homeotic conversion of tomato sepals to carpeloid structures by ectopic expression of the tomato *AGAMOUS1* gene (TAG1) demonstrated the obvious link between tomato carpel development and eventual ripening (*20*), elucidation of the *rin* mutant establishes that a MADS-box gene directly regulates ripening. Consistent with the complementation results, antisense repression of *LeMADS-RIN* in normal (*Rin/Rin*) tomatoes resulted in a phenotypic mimic of the ripening aspect of *rin* with no effect on sepals or determinacy (Fig. 1E).

Antisense of *LeMADS-MC* resulted in indeterminate inflorescences with large sepals and normally ripening fruit (Fig. 1, F and G). Together, these results confirm that *LeMADS-MC* affects tomato inflorescence determinacy and sepal development, whereas *LeMADS-RIN* is a previously undescribed regulator of ripening.

Phylogenetic analysis of *LeMADS-MC* revealed substantial similarity to the *Arabidopsis* APETALA1 (AP1) protein in addition to putative AP1 orthologs from additional species (Fig. 4). *AP1* is a class A MADS-box gene whose expression is restricted to the outer whorls (sepals and petals) of flowers, and gene knockouts of *AP1* lead to the homeotic conversion of sepals to leaf or bractlike organs, loss of petals in most flowers, and inflorescence indeterminacy (*21*). *LeMADS-MC* is expressed in sepals, petals, and carpels (Fig. 3A) more similar to the *SQUAMOSA* (*SQUA*) AP1 ortholog from *Antirrhinum* (*22*). *LeMADS-MC* expression is absent in *rin* mutant sepals, presumably because the chimeric gene is directed by the *LeMADS-RIN* promoter. Our strongest repression of *LeMADS-MC* causes homeotic conversion from sepals to leaflike structures (Fig. 1G), although no effect on petals was observed. This phenotype contrasts the loss of petals in *ap1*, yet is similar to the phenotype of the *squamosa* mutant of *Antirrhinum*. In summary, phylogeny, gene expression, and both the *rin* mutant and antisense of *LeMADS-MC* suggest that *LeMADS-MC*, *SQUA*, and *AP1* are orthologs.

*LeMADS-RIN* phylogenetic analysis revealed the greatest similarity to *FPB4* of petunia and a pathogen-infected pepper fruit EST, MADS1 (Fig. 4). The most similar *Arabidopsis* genes were SEP1 and AGL3. SEP1 is required for manifestation of B and C class organ-identity functions (petals, stamen, and carpel development) and distinct from *LeMADS-RIN*, where only the ripening aspect of carpel development is affected (*23*). The exact function of AGL3 remains uncertain, although expression has been observed in all aerial tissues (*24*). Functional characterization of AGL3 and continued isolation and analysis of tomato MADS-box genes should elucidate these relations. Gene expression analysis indicates that *LeMADS-RIN* is expressed primarily in fruits, and its induction coincides with ripening. Unlike many previously characterized tomato fruit-ripening genes, *LeMADS-RIN* expression is not significantly influenced by ethylene (Fig. 3A).

The *rin* mutation reveals a function for plant MADS-box genes as regulators of fruit ripening. LeMADS-RIN is required to initiate climacteric respiration and associated ethylene biosynthesis in addition to ripening factors that cannot be complemented by supplemental ethylene. Consequently, LeMADS-RIN is upstream of ethylene in the regulatory cascade and may represent a global developmental regulator of ripening potentially shared among climacteric and nonclimacteric species. In support of this hypothesis, we have isolated a cDNA (FvMADS-9) from strawberries (a nonclimacteric fruit), using *LeMADS-RIN* as a probe (*25*). FvMADS-9 displays fruit-specific expression (*25*) and clusters close to LeMADS-RIN in phylogenetic analysis (Fig. 4).

LeMADS-RIN is closely related to sequences derived from petunias and peppers (Fig. 4) but for which no functional characterization has been reported. The near identity of these sequences from additional members of the Solanaceae suggests potential involvement in ripening. Interestingly, unlike tomatos and peppers, petunia fruit are not fleshy and undergo a maturation process ending in senescence, dehydration, and dehiscence similar to that of *Arabidopsis* siliques. Functional analysis of FBP4 could lead to additional insights regarding maturation of different fruit types.

Ripe fruits serve as a significant portion of the human diet, directly affecting human

nutrition and health. *LeMADS-RIN* represents a molecular bridge between the extensively studied phenomena of floral development and fruit ripening/ethylene response with regard to the cascade of ethylene-regulated events associated with climacteric ripening being dependent on a member of the floral development–associated MADS-box family. This discovery opens a new research frontier in fruit ripening. For example, because MADS-box genes are known to act as multimers (*26*), one could logically predict that additional MADS-box genes might affect ripening.

From a practical perspective, the *rin* mutation is widely used in tomato hybrid cultivars to yield fruit with a long shelf life and acceptable quality. Tomatoes heterozygous for the *rin* allele remain firm and ripen over a protracted period (presumably due to reduced levels of functional RIN protein) permitting industrial-scale handling and expanded delivery and storage opportunities. *LeMADS-RIN* is a rare example of a gene whose effects are documented a priori, suggesting excellent potential for practical genetic modification of fruit ripening and quality characteristics.

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15. GenBank accession numbers are C34 (LeMADS-MC), AF448521; C43 (LeMADS-RIN), AF448522; and C43/34 (*rin* mutant), AF448523.
16. Primers were designed to amplify the region separating the C34 and C43 transcribed regions, as shown in Fig. 3C. The primers used were designated as G34R 5′TCTTAATTCAACTTTTCCTCTTCCCATCTTC3′ and G43F 5′TTGTCAAGAAGAGTATGGCAATATTGATAACAC3′.
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# Alteration of Lymphocyte Trafficking by Sphingosine-1-Phosphate Receptor Agonists


**Suzanne Mandala,[1] Richard Hajdu,[1] James Bergstrom,[1] Elizabeth Quackenbush,[2] Jenny Xie,[2] James Milligan,[1] Rosemary Thornton,[1] Gan-Ju Shei,[1] Deborah Card,[1] CarolAnn Keohane,[1] Mark Rosenbach,[1] Jeffrey Hale,[3] Christopher L. Lynch,[3] Kathleen Rupprecht,[3] William Parsons,[3] Hugh Rosen[1]***



**Blood lymphocyte numbers, essential for the development of efficient immune responses, are maintained by recirculation through secondary lymphoid organs. We show that lymphocyte trafficking is altered by the lysophospholipid sphingosine-1-phosphate (S1P) and by a phosphoryl metabolite of the immunosuppressive agent FTY720. Both species were high-affinity agonists of at least four of the five S1P receptors. These agonists produce lymphopenia in blood and thoracic duct lymph by sequestration of lymphocytes in lymph nodes, but not spleen. S1P receptor agonists induced emptying of lymphoid sinuses by retention of lymphocytes on the abluminal side of sinus-lining endothelium and inhibition of egress into lymph. Inhibition of lymphocyte recirculation by activation of S1P receptors may result in therapeutically useful immunosuppression.**


T and B lymphocytes migrate from their sites of lymphopoiesis (thymus and bone marrow), traverse the blood stream, and enter the appropriate secondary lymphoid organ (SLO) where they may either enter a specialized compartment to await antigen, or egress from lymph nodes (*1*). Egressing lymphocytes cross an endothelial barrier from the ablumi-

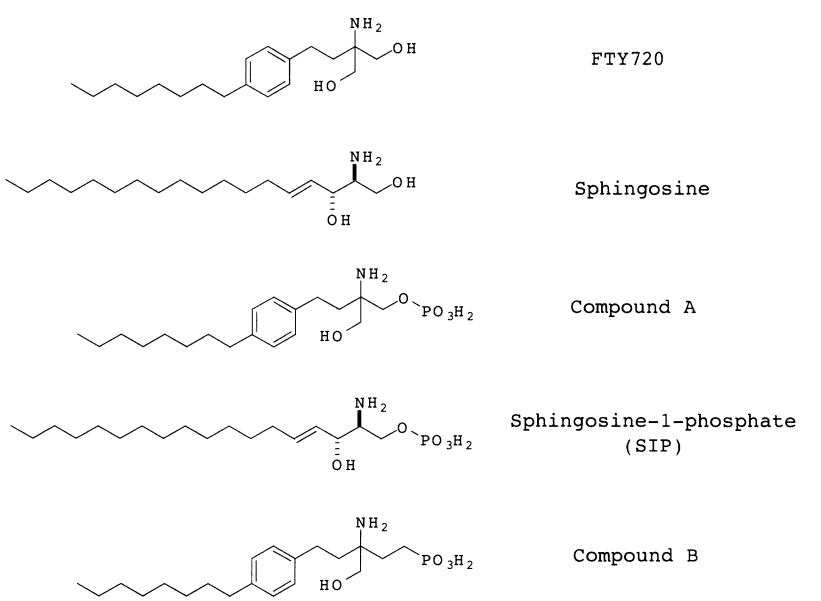


**Fig. 1.** The structures of FTY720, S1P and related synthetic compounds.