Sphingosine 1-Phosphate Receptors Are Essential Mediators of Eyelid Closure during Embryonic Development*

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The fetal development of the mammalian eyelid involves the expansion of the epithelium over the developing cornea, fusion into a continuous sheet covering the eye, and a splitting event several weeks later that results in the formation of the upper and lower eyelids. Recent studies have revealed a significant number of molecular signaling components that are essential mediators of eyelid development. Receptor-mediated sphingosine 1-phosphate (S1P) signaling is known to influence diverse biological processes, but its involvement in eyelid development has not been reported. Here, we show that two S1P receptors, S1P2 and S1P3, are collectively essential mediators of eyelid closure during murine development. Homozygous deletion of the gene encoding either receptor has no apparent effect on eyelid development, but double-null embryos are born with an “eyes open at birth” defect due to a delay in epithelial sheet extension. Both receptors are expressed in the advancing epithelial sheet during the critical period of extension. Fibroblasts derived from double-null embryos have a deficient response to epidermal growth factor, suggesting that S1P2 and S1P3 modulate this essential signaling pathway during eyelid closure.

During mammalian embryogenesis, eyelid development begins with the appearance of a protruding ridge surrounding the developing eye. This is followed by the formation of a loose aggregate of epithelial cells that extend from a leading edge to cover the exposed eye to ultimately fuse the upper and lower lids until the eye is closed (1). The eye remains fused until a separation event occurs some weeks later. In humans, this separation event occurs in utero by gestational week 20 (2), long before birth. However, mice are born with their eyelids still fused because the separation event does not occur until approximately postpartum day 12 (1). This process was thought to serve as a protective function until complete maturation of the retina and was described in detail as early as 1921 (3). However, the mechanistic details have only recently begun to emerge.

Characterization of the molecular pathways underlying the process of eyelid closure and fusion has been facilitated almost entirely by the use of genetic knock-out mice. A number of genetic deletions have been reported to cause defects in eyelid development and result in the “eyes open at birth” (EOB) phenotype. This has revealed the identity of several components of known signaling pathways that are critical mediators of the keratinocyte migration and epidermal extension that are required for eyelid closure (4).

Several reports have identified the EGF family of ligands and their cognate receptors. EOB defects are seen in mice with mutation of the EGF receptor (EGFR) (5, 6) or of EGFR ligands such as HB-EGF (7) and TGFα (8, 9).

Similarly, deficiencies in other growth factor receptor signaling pathways have also been associated with EOB. These include TGFβ (10) and FGF (11, 12). Interestingly, the involvement of G protein-coupled receptor signaling in eyelid closure was recently revealed. Loss of the orphan receptor GPR48/LGR4 results in an EOB phenotype, likely produced by disruption of EGFR signaling (13, 14).

Several downstream pathways are known to be essential for eyelid development. These include the MAPK pathway as exemplified by numerous studies involving genetic deletion of the protein kinase MEKK1 (15–18). Additionally, defects in the transcription factor c-Jun and c-Jun kinases also result in defects in eyelid closure (15, 19). Moreover, loss of Rho-associated kinase 1 (ROCK-1), an essential regulator of the actin cytoskeleton, also causes the EOB phenotype (20). All of these processes are likely to involve EGF signaling pathways in some way, but the mechanisms are not completely resolved.

Sphingosine 1-phosphate (S1P) is a potent lipid signaling molecule that acts as a high-affinity ligand for a family of five G protein-coupled receptors (S1P₁–S1P₅) (21, 22). These recep-

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2 The abbreviations used are: EOB, eyes open at birth; EGFR, EGF receptor; S1P, sphingosine 1-phosphate; MEF, mouse embryonic fibroblast; E, embryonic day; SphK, sphingosine kinase.
tors have differential but overlapping expression patterns and are involved in many developmental, physiological, and pathological processes. Studies involving genetic knock-out mice have been particularly illuminating (23) and have identified roles for S1P receptors in diverse processes such as lymphocyte trafficking (24), blood vessel maturation (25), regulation of neuronal excitability (26), neonatal viability (27), neural protection (28), systemic inflammation (29), and maintenance of vestibulocochlear organs (30). It is thought that the overlapping expression pattern may provide some functional redundancy for critical roles of S1P signaling. Here, we show that two of these receptors, S1P2 and S1P3, act as redundant but cumulatively essential mediators of epithelial sheet extension during eyelid development, likely by transducing EGF signaling.

EXPERIMENTAL PROCEDURES

Materials—Human EGF was obtained from Cell Signaling Technology (catalog no. 8916L.C). S1P was obtained from Enzo Life Sciences (catalog no. BML-SL140-0001), resuspended in methanol, and stored as a 1 mM stock solution. S1P was stabilized with 10% fatty acid-free bovine serum albumin (catalog no. A7030, Sigma-Aldrich) before dilution to working concentration. Sphingosine kinase inhibitor 2 was obtained from Cayman Chemical (catalog no. 10009222). The antibodies used were as follows: rabbit anti-ERK1/2 (catalog no. 9102), rabbit anti-phospho-ERK (catalog no. 4267), rabbit anti-EGFR-ERK Tyr-992 (catalog no. 2235), rabbit anti-phospho-EGFR Tyr-1045 (catalog no. 2237), and rabbit anti-phospho-EGFR-EGFR Tyr-1068 (catalog no. 3777) (Cell Signaling Technology); mouse anti-β-actin (catalog no. A2228, Sigma-Aldrich); and HRP-labeled goat anti-mouse IgG (catalog no. 62-6520) and HRP-labeled goat anti-rabbit IgG (catalog no. 62-6120) (Invitrogen).

Animal Husbandry—Mice were housed in ventilated cages in the vivarium at The Scripps Research Institute. Deletions of the genes encoding receptors S1P2 (S1pr2) and S1P3 (S1pr3) were described previously (27, 31). S1pr2−/− and S1pr3−/− null mice were back-crossed to congenicity (N12) into a BALB/cByJ strain. S1pr2−/−;S1pr3−/− double-null mice were generated by crossing S1pr2−/−;S1pr3−/− females with S1pr2−/−;S1pr3−/− males. MEFs were maintained as a monolayer culture on tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells from the third to fourth passages were used for analyses.

Cell Viability Assay—MEFs were seeded into 96-well plates at 20,000 cells/well, incubated overnight, serum-starved for 4 h, and treated overnight (16 h) with EGF or S1P. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (33).

Cell Proliferation Assay—MEFs were grown on poly-l-lysine-coated coverslips at low density, serum-starved for 4 h, treated with or without EGF overnight in the presence of BrdU (catalog no. 00-0103, Invitrogen), fixed with 70% ethanol, labeled with an anti-BrdU antibody (Millipore), and stained with propidium iodide (Invitrogen). Positive nuclei were counted relative to the total number of propidium iodide-labeled nuclei.

Western Blot Analysis—Cells were grown in 6-well tissue culture dishes and treated as indicated for 5 min. After washing with ice-cold 1× phosphate-buffered saline, lysates were collected by addition of ice-cold lysis buffer (1× radioimmune precipitation assay buffer and Complete protease inhibitor mixture (Roche Diagnostics)) for 15 min at 4 °C on a rotator and then dislodged with a cell scraper. 10 μg of total lysate protein was separated on a 4–12% SDS-polyacrylamide gel, transferred, and blocked overnight. The blot was then incubated with anti-β-actin (1:10,000), anti-ERK1/2 (1:1000), anti-phospho-ERK (1:1000), anti-EGFR (1:1000), anti-phospho-EGFR Tyr-992 (1:1000), anti-phospho-EGFR Tyr-1045 (1:1000), or anti-phospho-EGFR Tyr-1068 (1:1000) antibody; washed; incubated with secondary antibody (1:10,000); and subsequently visualized using the West Femto kit (Thermo Scientific). Quantitations were performed with ImageJ software and represent averages of two independent experiments.

RESULTS

S1pr2−/−;S1pr3−/− Mice Have an Eyelid Closure Defect—Previous studies in our laboratory involving genetic deletion of S1pr2 and S1pr3 in mice have utilized mixed background strains (27, 30, 31). During the course of these studies, we observed the sporadic occurrence of a degenerative eye phenotype in S1pr2−/−;S1pr3−/− mice (data not shown). To characterize this defect in the absence of variable extragenic modifiers, these knock-out mice were bred to congenicity onto a BALB/cByJ background and then bred to generate S1pr2−/−;S1pr3−/− double-null offspring.

Histology—Pregnant dams were deeply anesthetized by iso-flurane inhalation and killed by cervical dislocation. Embryos were harvested, decapitated, fixed overnight in 4% paraformaldehyde, embedded in paraffin using standard techniques, sectioned at 10 μm, and stained with hematoxylin and eosin.

In Situ Hybridization—In situ hybridization was performed essentially as described (32). Briefly, tissues were fresh-frozen, sectioned at 18 μm, fixed with 4% paraformaldehyde, acetylated, and hybridized to bromodeoxyuridine-labeled antisense probes corresponding to the full-length open reading frames of S1pr2 and S1pr3.

Preparation of Mouse Embryonic Fibroblasts (MEFs) —MEFs were prepared as described previously (31) from embryonic day (E) 12 embryos generated by crossing S1pr2+/−;S1pr3+/− females with S1pr2−/−;S1pr3−/− males. MEFs were maintained as a monolayer culture on tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells from the third to fourth passages were used for analyses.
Upon closer examination of the neonates, we observed that \textit{S1pr2}^{-/-};\textit{S1pr3}^{-/-} pups were uniformly characterized by the EOB phenotype (Fig. 1, G–I), which resulted in eye inflammation and subsequent eyelid fusion. This was due to a failure in eyelid closure that normally occurs in mice at E16 (Fig. 2, A–D) (4). Consistent with the postnatal phenotype, there was com-

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|c|}
\hline
Genotype & Frequency per mouse & Frequency per eye & No. of null alleles & Frequency per mouse & Frequency per eye \\
\hline
\textit{S1pr2}^{-/-};\textit{S1pr3}^{++} & 0/4 (0%) & 0/8 (0%) & 0–2 & 0/112 (0%) & 0/224 (0%) \\
\textit{S1pr2}^{++};\textit{S1pr3}^{-/-} & 0/30 (0%) & 0/60 (0%) & & & \\
\textit{S1pr2}^{-/-};\textit{S1pr3}^{++} & 0/14 (0%) & 0/28 (0%) & & & \\
\textit{S1pr2}^{++};\textit{S1pr3}^{++} & 0/23 (0%) & 0/46 (0%) & & & \\
\textit{S1pr2}^{++};\textit{S1pr3}^{-/-} & 0/27 (0%) & 0/54 (0%) & & & \\
\textit{S1pr2}^{-/-};\textit{S1pr3}^{-/-} & 0/14 (0%) & 0/28 (0%) & & & \\
\hline
\textit{S1pr2}^{-/-};\textit{S1pr3}^{++} & 3/23 (13.04%) & 3/46 (6.52%) & 3 & 7/38 (18.42%) & 7/76 (9.21%) \\
\textit{S1pr2}^{++};\textit{S1pr3}^{++} & 4/15 (26.67%) & 4/30 (13.33%) & & & \\
\hline
\textit{S1pr2}^{-/-};\textit{S1pr3}^{-/-} & 16/16 (100%) & 31/32 (96.88%) & 4 & 16/16 (100%) & 31/32 (96.88%) \\
\hline
\end{tabular}
\caption{Frequency of occurrence of eye defects in adult mice}
\end{table}

Upon closer examination of the neonates, we observed that \textit{S1pr2}^{-/-};\textit{S1pr3}^{-/-} pups were uniformly characterized by the EOB phenotype (Fig. 1, G–I), which resulted in eye inflammation and subsequent eyelid fusion. This was due to a failure in eyelid closure that normally occurs in mice at E16 (Fig. 2, A–D) (4). Consistent with the postnatal phenotype, there was com-
S1pr2 and S1pr3 Genes Are Expressed in the Eyelid Epithelial Sheet—Spatial distribution of S1pr2 and S1pr3 mRNAs was evaluated in E15.5 embryos by in situ hybridization (Fig. 3). Both transcripts were enriched in the nascent epithelial sheets at the eyelid rim. The absence of labeling in knock-out embryos confirmed probe specificity.

S1pr2 and S1pr3 Mediate EGF Signaling in MEFs—Because EGFR activity has been shown to be essential for eyelid closure in mice (7, 34), we investigated whether loss of S1pr2 and S1pr3 affects EGF signaling in embryonic cells. MEFs isolated from double-heterozygous embryos (S1pr2+/−;S1pr3+/−) showed a dose-dependent increase in viability in response to exogenously administered S1P or EGF (Fig. 4A). This response was absent in cells obtained from double-null embryos (S1pr2−/−;S1pr3−/−). Similar results were obtained with a BrdU incorporation proliferation assay (Fig. 4B).

To confirm that the S1P receptors are mediators of EGF signaling, we examined the consequence of loss of S1pr2 and S1pr3 on the activation of ERK by EGF (Fig. 4C). In the presence of S1pr2 and S1pr3, MEFs exhibit 3.8- and 7.4-fold increases in ERK phosphorylation when treated with S1P (1 μM) and EGF (100 ng/ml), respectively. This response was attenuated in S1pr2/S1pr3-null MEFs, which exhibited only 1.6- and 4.6-fold increases. This corresponds to 59% (S1P) and 38% (EGF) decreases in activity due to loss of S1P receptors. The S1P-mediated response remaining in the S1pr2/S1pr3-null MEFs is likely due to the presence of S1pr1, which is reported to be functionally expressed in primary MEFs (35).

These results confirm previous studies showing that S1pr2 and S1pr3 are the primary mediators of S1P signaling in MEFs (27) and demonstrate that loss of S1P receptors results in the complete failure of eyelid closure in S1pr2−/−;S1pr3−/− embryos at E16.5 and intermediate phenotypes in S1pr2+/−;S1pr3−/− and S1pr2−/−;S1pr3+/− embryos (data not shown).

In wild-type mice, eyelid closure is mediated by the formation of an actin-rich leading edge structure of the epithelial root, resulting in the extension of epithelial sheets from the rims of the eyelids beginning at E15.5 (4, 7, 20). The eyelid closure defect seen in S1pr2−/−;S1pr3−/− mice was secondary to a 1-day delay in leading edge formation on the eyelid rim, leading to a failure in epithelial sheet extension (Fig. 2, E–H).
attenuation EGF activity. This attenuation suggests that EGFR activation results in the transactivation of S1P receptors, likely via the activation of sphingosine kinase (SphK), as reported previously (36–40). To test this hypothesis, we evaluated the contribution of SphK by stimulating wild-type MEFs with EGF after pretreating the cells with 10 μM SphK inhibitor 2, a specific inhibitor of SphK activity (41). This resulted in a 36% reduction in EGF-induced ERK1/2 phosphorylation relative to vehicle-pretreated cells (Fig. 5, A and B). As expected, ERK1/2 phosphorylation induced by exogenous S1P was not attenuated by inhibition of SphK, indicating that downstream signaling was not affected by the inhibitor (Fig. 5, A and B).

**DISCUSSION**

Eyelid development is a complex process involving multiple molecular mediators. Although the involvement of S1P signaling has not been previously reported, many of the essential regulators of eyelid closure have known relationships with S1P receptors. ROCK-1, which is critical for cytoskeletal remodeling during epithelial sheet extension (20), is a downstream effector of S1P2 and S1P3 (43, 44). Furthermore, activation of the MAPK pathway is a well-characterized response to S1P receptor activation (45–47). Interestingly, EGF signaling has been shown to activate SphK, resulting in the production of S1P (36–40). These studies, in combination with our current findings, suggest a mechanism by which S1P2 and S1P3 may regulate eyelid closure (Fig. 5D). In this model, EGFR activation causes the production of S1P and the stimulation of S1P2 and S1P3, resulting in the activation of downstream signaling that induces epithelial sheet extension.

Because it has been previously reported that activation of G protein-coupled receptors can induce the ADAM protease-dependent cleavage of HB-EGF to generate ligand for EGFR (42), we investigated whether this occurs in our system. Treatment of wild-type MEFs with S1P did not cause any detectable increase in EGFR phosphorylation (Fig. 5C).

**FIGURE 4.** S1pr2−/−;S1pr3−/− MEFs have deficient responses to S1P and EGF. A, fibroblasts from heterozygous embryos responded to S1P and EGF treatment with dose-dependent increases in viability. These responses were absent in cells obtained from S1pr2−/−;S1pr3−/− littersmates. There was no statistically significant change in viability of knock-out cells under any treatment condition. B, fibroblasts obtained from S1pr2−/−;S1pr3−/− embryos were deficient in proliferative response to EGF. Cells from heterozygous mice responded to EGF (100 ng/ml) with a small but significant increase in BrdU-labeled nuclei, whereas homozygous null cells did not. Error bars represent S.E. C, EGF signaling was attenuated in the absence of S1P2 and S1P3. In heterozygous MEFs, there were 3.8- and 7.4-fold increases in activation of ERK1/2 when treated with S1P and EGF, respectively. These responses were reduced by 59% (S1P) and 38% (EGF) in S1pr2/S1pr3-null MEFs. pERK, phospho-ERK.

**FIGURE 5.** S1P2 and S1P3 are activated downstream of EGFR activation. A, wild-type MEFs were pretreated with vehicle or SphK inhibitor 2 (SKI-II) for 15 min; treated with vehicle, EGF (100 ng/ml), or S1P (1 μM) for 5 min; and collected for Western analysis. pERK, phospho-ERK. B, quantitation of Western blots revealed that SphK inhibition reduced EGF-mediated ERK1/2 phosphorylation by 36% but had no effect on S1P-mediated ERK1/2 phosphorylation (average of two experiments). C, wild-type MEFs were treated with vehicle, EGF (100 ng/ml), or S1P (1 μM) for 5 min and collected for Western analysis. Although EGF treatment caused a marked increase in EGFR phosphorylation at each of three relevant tyrosine residues, S1P treatment resulted in no detectable EGFR phosphorylation (representative of two experiments). D, proposed model for the involvement of S1P signaling in eyelid closure.
S1P Signaling Mediates Eyelid Closure

It is worth noting that the observed EOB phenotype is due to a 1-day delay in leading edge formation rather than a complete loss of function. This is likely due to a requirement for S1P/EGF signaling to initiate the early events in the protruding edge at ~E15, whereas additional signaling systems (perhaps TGFβ and/or FGF) may provide compensatory signaling at ~E16. By this point, presumably a developmental window with additional cellular machinery required for epithelial sheet extension has closed, preventing eyelid fusion. Alternatively, S1P/EGF signaling may be essential both for initiation of the protruding edge and for propagation of epithelial sheet extension.

Further corroboration for the essential role of S1P signaling in eyelid closure was provided in a recent report that revealed that Sphn2−/− mice display a similar EOB phenotype (63). Because Sphn2 is known to function as an S1P transporter (64), loss of this gene reduces the availability of the ligand for S1P2 and S1P3, resulting in a reduction of receptor activation and providing a phenocopy of the results reported here.

In summary, this study has revealed a novel role for S1P receptor signaling during development and provided the first demonstration of a truly redundant biological function for two S1P receptor subtypes. The mechanism underlying this process is likely via the activation of S1P2 and S1P3 downstream of EGF/FGF signaling, which in turn activates overlapping G protein-mediated intracellular pathways.

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