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Type IV collagen is an activating ligand for the adhesion G protein-coupled receptor GPR126

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Abstract

GPR126 is an orphan heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) that is essential for the development of diverse organs. We found that type IV collagen, a major constituent of the basement membrane, binds to Gpr126 and activates its signaling function. Type IV collagen stimulates the production of cyclic adenosine monophosphate in rodent Schwann cells, which require Gpr126 activity to differentiate, and in human embryonic kidney 293 (HEK 293) cells expressing exogenous Gpr126. Type IV collagen specifically bound to the extracellular N-terminal region of Gpr126 containing the CUB and pentraxin domains. Gpr126 derivatives lacking the entire N-terminal region were constitutively active, suggesting that this region inhibits signaling, and that ligand binding relieves this inhibition to stimulate receptor activity. A new zebrafish mutation that truncates Gpr126 after the CUB and pentraxin domains disrupted development of peripheral nerves and the inner ear. Thus, our findings identify type IV collagen as an activating ligand for GPR126, define its mechanism of activation, and highlight a previously unrecognized signaling function of type IV collagen in basement membranes.

Introduction

Receptors on the cell surface control a myriad of processes in development, physiology, and behavior. G protein-coupled receptors (GPCRs) have a characteristic heptahelical transmembrane (7TM) region, and they comprise the largest group of receptors in vertebrates (1). GPCRs receive diverse inputs, such as photons, small molecules, neuropeptides, and proteins (2). Agonists induce a conformational change in GPCRs that activates the associated heterotrimeric G proteins (3). Different GPCRs are coupled to distinct G proteins that activate specific downstream second-messenger systems by binding

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to effector proteins, such as adenylyl cyclases and phospholipase C (4). Reflecting the diversity of their ligands and downstream effectors, GPCRs have an enormous number of critical functions in receiving signals between neighboring and distant cells, and in sensing environmental cues through olfaction, taste, and vision. In addition, approximately 30% of all Food and Drug Administration-approved drugs specifically target GPCRs (5). Many GPCRs remain “orphan” receptors with no known activating ligands, and there is intense interest in defining the functions of these receptors and their signaling pathways.

The adhesion GPCRs (aGPCRs) are a recently defined subfamily that combines characteristics of adhesion molecules and 7TM receptors (6). The aGPCRs share with other GPCRs the heptahelical domain, but are distinguished by long, heavily glycosylated N-terminal regions that contain different adhesion domains, such as cadherin, EGF, and thrombospondin motifs, among others. A hallmark of the aGPCR family is the presence of a conserved GPCR proteolytic site (GPS) within the GPCR autoproteolysis-inducing (GAIN) domain (7, 8). The GAIN domain is always located in the extracellular region, just N-terminal to the first of the seven transmembrane helices. During receptor processing, autoproteolysis in the GAIN domain cleaves aGPCRs into N-terminal and C-terminal regions that remain non-covalently associated at the cell surface (7, 9-11). Three distinct models have been proposed to explain the role of the GAIN domain in aGPCR activation. In the tethered inverse agonist model, the cleaved N-terminal region interacts with the 7TM region to antagonize receptor signaling; the receptor is activated when a ligand binds the N-terminal region to relieve this antagonism. The main evidence for this model derives from studies showing that deletion constructs lacking the N-terminal region are constitutively active receptors, as has been reported for the aGPCRs GPR56 (12, 13), CD97 (14), BAI2 (15), and BAI1 (16). Analysis of the aGPCR LAT-1 in the nematode led to the tethered agonist model, in which ligand binding allows the GAIN domain to interact with the 7TM region in a way that activates receptor signaling (17). A third model, based on work on the aGPCR CD97, proposes that the N-terminal region binds to activating ligands and that GAIN domain autoproteolysis leads to removal of the N-terminal region and elimination of signaling (18). Finally, there is also emerging evidence that the N-terminal regions of some aGPCRs, cleaved at the GAIN domain or in other regions, may act as soluble factors with other activities, independent of G-protein activation (13-15).

The majority of aGPCRs are orphan receptors. Many studies have identified proteins that bind to different aGPCRs, but most of these are not known to activate receptor signaling, and many may have a purely adhesive function (10). For example, the aGPCR latrophilin-1 has three known classes of high-affinity ligands [Teneurin-2 (19), FLRT proteins (20), and the Neurexin proteins (21)], but receptor activation has not been observed upon ligand treatment. The best-defined aGPCR-ligand pair is type III collagen and GPR56. Type III collagen can bind to GPR56 and activate $G\alpha_{12/13}$ to control neuronal progenitor migration (22). Moreover, type III collagen knockout mice exhibit an abnormal cortical development phenotype similar to that of GPR56 mutants (23), and mutations in the N-terminal region of GPR56 that cause disease in humans can prevent binding to type III collagen (24). Identification of activating ligands for other aGPCRs will elucidate their functions and enable systematic studies to resolve the various models of aGPCR activation.

Analyses in zebrafish and mice have defined Gpr126 as an orphan aGPCR that functions in diverse essential processes, including the myelination of peripheral nerves, trabeculation of the developing heart, and formation of the semi-circular canals of the inner ear. A zebrafish screen for disruptions in myelin formation discovered mutations in *gpr126* (25, 26). Analysis of the zebrafish mutant phenotype revealed that *gpr126* is essential for Schwann cells to initiate myelin formation, and subsequent characterization of knockout mice demonstrated that this function is conserved in mammals (26, 27). Treatment of mutant zebrafish with forskolin to increase cAMP rescues the mutant phenotype (26, 28), providing evidence that Gpr126 may signal through a cAMP second-messenger cascade. Additional studies provide evidence that Gpr126 signals through a pathway involving G α _s, adenylyl cyclases, and Protein Kinase A (28, 29). Zebrafish *gpr126* mutants also have defects in morphogenesis of the semi-circular canals in the inner ear, and this phenotype could also be rescued by increasing cAMP (30). In addition to defects in Schwann cell development and myelination, Gpr126 mutant mice have abnormalities in the developing heart, and they die during embryogenesis or shortly after birth (27, 31). A recent study using morpholinos in zebrafish reported that targeting the translation start site of Gpr126 disrupted heart development, ear development, and myelination, whereas a morpholino that blocked splicing of a downstream exon caused only myelination and ear defects that are characteristic of previously characterized *gpr126* mutants (32). The heart defects were rescued by a construct encoding most of the N-terminal region of Gpr126, but not the 7TM region (32). This N-terminal construct did not rescue the inner ear or myelination phenotypes. The authors suggested that heart development requires a function of the Gpr126 N-terminal region that is independent of G-protein signaling, but the domains involved and the mechanism of action were not defined.

Here we identified type IV collagen as an activating ligand for Gpr126 and defined the activation mechanism for this essential aGPCR, highlighting a previously unrecognized signaling function of type IV collagen in basement membranes.

Results

Gpr126 binds specifically to type IV collagen

To identify ligands for Gpr126, we expressed a secreted, Fc-tagged protein containing the N-terminal fragment of zebrafish Gpr126 (Gpr126-NT-Fc, Fig. 1A) in human embryonic kidney 293 (HEK293) cells. Previous studies show that Gpr126 and type IV collagen in the extracellular matrix are involved in myelination (26, 34, 35), and a recent paper identified type III collagen as an activating ligand for Gpr56 (22). To search for specific interactions between Gpr126 and different collagen proteins, we performed a series of pulldown assays with the Gpr126 N-terminal Fc fusion protein and purified collagen proteins, types I, II, III, IV, and V. Biotinylated collagen proteins were incubated with streptavidin-agarose and conditioned media from transfected HEK293 cells expressing Gpr126-NT-Fc. Bound proteins were analyzed by immunoblot with an antibody specific to Fc to determine if any collagen proteins interacted with the Gpr126 N-terminal region. Gpr126-NT-Fc robustly interacted with type IV collagen, but not the other four collagen types (Fig. 1B). A control protein representing only the Fc tag of the fusion protein did not bind to any of the collagen

proteins or to BSA (fig. S1). In reciprocal experiments, biotinylated collagen proteins and media containing Gpr126 NT-Fc were incubated with Protein A-agarose. Again, Gpr126-NT-Fc interacted robustly with type IV collagen, but not with the other four collagen types or BSA (Fig. 1D). These results demonstrate that the N-terminal region of Gpr126 specifically binds to type IV collagen.

Type IV collagen activates Gpr126 signaling

Previous genetic and biochemical studies show that Gpr126 signals through $G\alpha_s$ to stimulate cAMP in vivo and in heterologous cell systems (26-29). In addition, forskolin promotes myelination in cultured Schwann cells (36), and Schwann cells express *gpr126* and secrete type IV collagen prior to the onset of myelination (26, 37). In combination, our results and these previous studies suggested that type IV collagen may be an autocrine signal that increases cAMP concentration in Schwann cells. To investigate this possibility, we treated Schwann cells purified from rat sciatic nerve with type IV collagen or PBS for 1 hour. Treatment with type IV collagen significantly increased cAMP concentrations in Schwann cells (Fig. 2A), supporting the possibility that type IV collagen is an endogenous autocrine inducer of cAMP signaling in Schwann cells.

To further analyze the interaction between type IV collagen and Gpr126, we used a heterologous overexpression system. HEK293 cells were transfected with a full-length Gpr126 expression vector or a control plasmid and treated with PBS or type IV collagen. Treatment with type IV collagen significantly increased cAMP concentrations in cells transfected with Gpr126, but had no effect on control cells lacking the receptor (Fig. 2B). Transfection with Gpr126 caused a smaller but significant increase in cAMP concentration in the absence of collagen treatment (Fig. 2B). Ligand-independent signaling has been commonly observed in similar experiments with other GPCRs, because overexpression increases the population of receptors in the active conformation even in the absence of ligand (38).

To test the temporal response of Gpr126-expressing cells to collagen IV, we performed a time course study. In response to 3 $\mu\text{g/mL}$ of type IV collagen, cAMP concentration increased in Gpr126-expressing HEK293 cells as early as 10 min, reached peak concentrations after 60 min, and declined after 120 min (Fig. 2C). The reduction at later time points could be caused by internalization of the receptor after ligand binding, similar to the aGPCR CD97 and its ligand CD55 (18). There is also evidence that Gpr126 couples to $G\alpha_i$ (29), and it is possible that extended activation of the receptor leads to inhibition of cAMP by coupling to different effectors.

In a dose-response analysis, we treated Gpr126-expressing HEK293 cells with a range of type IV collagen concentrations and measured cAMP concentrations after 1 hour. Gpr126-expressing cells increased cAMP production in response to concentrations of type IV collagen as low as 0.06 $\mu\text{g/mL}$ and produced near-maximal amounts of cAMP at concentrations above 0.6 $\mu\text{g/mL}$ (Fig. 2 D). Analysis of the data determined that the concentration that induced a half-maximal response (EC_{50}) was 0.2 $\mu\text{g/mL}$, corresponding to approximately 0.7 nM.

To explore the possibility that other collagen proteins activate Gpr126 signaling, we treated HEK293 cells transfected with the Gpr126 expression plasmid or a control vector with PBS or 3 µg/mL collagen type I, II, III, IV, or V for 1 hour. As shown above, type IV collagen robustly increased cAMP concentration in Gpr126-expressing cells, but the other four collagen proteins had no effect (Fig. 2E). In summary, these results indicate that type IV collagen specifically binds the N-terminal region of Gpr126, and that this interaction activates the receptor, leading to an increase in the second messenger cAMP.

The CUB and Pentraxin domains of Gpr126 bind to type IV collagen

The N-terminal region of Gpr126 contains several conserved elements, including the CUB, PTX, hormone binding, and GAIN domains (39, 40). We focused on the CUB and PTX domains because these domains in other proteins can bind to various collagen-like proteins, including type IV collagen (41-44). We analyzed the binding activity of two complementary Fc fusion proteins (Fig. 3A). CUBPTX-Fc contained a signal peptide, the CUB domain, and the PTX domain followed by an Fc tag (amino acids 41-336 of Gpr126). The complementary protein, CUB PTX-Fc, contained a signal peptide, the rest of the Gpr126 N-terminal region, and an Fc tag (amino acids 337-827 of Gpr126). The fusion proteins were harvested from conditioned media of transfected HEK293 cells and analyzed in pulldown experiments with biotinylated collagen type IV and streptavidin-agarose. Both Gpr126NT-Fc and CUBPTX-Fc strongly bound collagen type IV, in contrast to CUB PTX-Fc, which displayed no detectable interaction (Fig 3B).

To quantitatively compare the affinities of Gpr126NT-Fc and CUBPTX-Fc for type IV collagen, we used surface plasmon resonance binding assays. Collagen type IV was coupled to the surface of the sensor chip, and the affinity-purified Gpr126-NT-Fc protein was introduced at various concentrations, with the surface being regenerated between trials. Gpr126NT-Fc bound to collagen IV with high affinity in duplicate trials (Fig. 3C). The mean calculated K_D value was 1.3 nM, similar to the EC_{50} for activation (Fig. 2D), with association and dissociation constants of $3.06 \pm 0.05 \times 10^5$ (1/Ms) and $4.04 \pm 0.8 \times 10^{-4}$ (1/s), respectively. In similar experiments, CUBPTX-Fc also bound to collagen IV with high affinity in duplicate trials (Fig. 3D). The mean calculated K_D value was 1.2 nM, with association and dissociation constants of $2.78 \pm 0.4 \times 10^6$ (1/Ms) and $3.33 \pm 1 \times 10^{-3}$ (1/s), respectively. These results indicate that the region of Gpr126 containing the CUB and PTX domains binds type IV collagen with high affinity and specificity.

The N-terminal region of Gpr126 controls its signaling activity

Functional studies of aGPCRs have led to different models about the role of the N-terminal region in receptor activation (12-17, 45). One model is that the cleaved N-terminal region antagonizes receptor signaling activity, so that truncation of the N-terminus can create a constitutively active receptor (12-16). To test whether this scenario might apply to Gpr126, we created FLAG-tagged Gpr126 constructs representing the full-length and N-terminally cleaved receptor (Fig. 4A). Both constructs were expressed at similar amounts, as indicated by immunostaining of transfected cells with an antibody against FLAG (fig. S2). In lysates of transfected cells (Fig. 4B, right lanes), analysis of Gpr126-FLAG detected multiple bands, including a 35 kDa protein corresponding to the C-terminal region after GPS

cleavage (bottom arrow), and higher molecular weight species representing modified and uncleaved forms of Gpr126. Multiple proteins were also detected in samples from cells transfected with Gpr126 NT-FLAG (Fig. 4B), including the 35 kDa protein and an abundant species with an apparent molecular weight of approximately 60 kDa (top arrow). The 60 kDa protein could be a ubiquitinated form, which is observed in the active species of other GPCRs, including the adhesion GPCR GPR56 (12, 46, 47). We also determined the extent of surface expression for each construct using a standard surface biotinylation assay, in which surface proteins were biotinylated and then pulled down with streptavidin beads (Fig. 4B, left lanes). The 35kDa protein produced from cells transfected with Gpr126-FLAG and Gpr126 NT-FLAG was expressed at the surface at similar amounts, consistent with similar experiments on GPR56 showing that the cleaved form of the receptor predominates at the cell surface (12). In control analyses of the same lysates, we detected no surface-biotinylated actin protein (Fig. 4B lower panel).

We next examined the activity of the Gpr126 NT-FLAG receptor and its ability to respond to type IV collagen. As in the experiments described above, treatment with type IV collagen increased cAMP concentrations in HEK293 cells expressing the full-length, FLAG-tagged receptor (Fig. 4C), whereas cells transfected with a control vector showed no response to type IV collagen. Cells expressing Gpr126 NT-FLAG showed a markedly high concentration of cAMP (Fig. 4C), which did not increase further after addition of type IV collagen (Fig. 4C). These experiments provide evidence that the N-terminal region of Gpr126 inhibits signaling in absence of ligand.

The *gpr126*^{st86} mutation truncates Gpr126 after the CUB and PTX domains and disrupts myelination and inner ear development, but not the heart

In a genetic screen for mutations that disrupt myelinated axons, we recovered a new allele of *gpr126*, *gpr126*^{st86}, that introduces a premature stop codon at position 406 and truncates the protein between the pentraxin and hormone binding domains (Fig. 5, A and B). The amount of *gpr126* mRNA was similar in wild-type and *gpr126*^{st86/st86} homozygotes, and therefore it is possible that the mutant allele produced a truncated protein containing the CUB and PTX domains (fig. S3). Like other strong mutant alleles of *gpr126*, homozygous *gpr126*^{st86/st86} mutants lack expression of *mbp* encoding myelin basic protein, in Schwann cells and have swollen ears (Fig. 5, C and D). Heart development appeared to be normal in *gpr126*^{st86/st86} mutants (Fig. 5D). Like *gpr126*^{st49/st49} mutants (26, 28), homozygous *gpr126*^{st86/st86} mutants survived to adulthood, further confirming that the mutation did not cause lethal heart abnormalities and allowing us to examine the possible maternal functions of *gpr126*. To examine the phenotype caused by loss of maternal (M) and zygotic (Z) function, we crossed a heterozygous *gpr126*^{st86/+} male to a *gpr126*^{st86/st86} homozygous mutant female. The resulting *Mgpr126*^{st86/+} mutants had a wild-type phenotype, and *MZgpr126*^{st86/st86} mutants resembled *Zgpr126*^{st86/st86} mutants, having swollen ears and normal hearts (Fig. 5D). If the previous proposal is correct that a region of Gpr126 has a function in heart development in zebrafish (32), our analysis suggests that this function is fulfilled by a protein fragment containing the first 405 amino acids of Gpr126—the region encoded 5' to the premature stop codon in the *gpr126*^{st86} mutation.

Discussion

Our analysis indicates that type IV collagen is a specific agonist of Gpr126. Type IV collagen bound to the N-terminal region of Gpr126 with high affinity and high specificity, unlike the other collagen proteins tested. The region of Gpr126 containing the adjacent CUB and pentraxin domains mediated the interaction with type IV collagen. Treatment of Schwann cells and Gpr126-expressing HEK293 cells with type IV collagen stimulated the production of cAMP, providing evidence that type IV collagen activates Gpr126 signaling. Finally, constructs lacking the N-terminal region of Gpr126 were constitutively active, providing evidence that the N-terminal region antagonizes receptor signaling activity and that type IV collagen binding relieves this repression to trigger downstream signaling.

In combination with previous work, our studies provide strong evidence that type IV collagen is an endogenous activating ligand for Gpr126. Type IV collagen is a component of the extracellular matrix in nerves, heart, and the inner ear at stages when Gpr126 is required for the development of these organs (48-50). In particular, previous work supports a role for type IV collagen in the myelination of peripheral nerves, which is the best-defined function of Gpr126 in fish and mammals (26, 27). After they associate with axons, but prior to myelination, Schwann cells secrete type IV collagen, laminin, and other components to form a continuous basal lamina (35, 51). Classic studies using Schwann cell and neuron co-cultures demonstrate that Schwann cell myelination is dependent on the secretion of type IV collagen and proper formation of the basal lamina (52, 53). For example, Schwann cells cultured with neurons in medium lacking ascorbate proliferate, but do not form myelin (53). Addition of ascorbate, a cofactor necessary for the post-translational modification and secretion of collagen, promotes type IV collagen secretion, basal lamina formation, and myelination (53). Addition of a reconstituted matrix, composed mainly of collagen type IV and laminin, to cultures lacking ascorbate also induces Schwann cells to ensheath axons, produce myelin proteins, and generate myelin (54). Addition of type I collagen does not promote myelination in these cultures, demonstrating the specificity of the requirement for type IV collagen—an observation that parallels our analysis of the specificity of the interaction between type IV collagen and Gpr126.

Our analysis, together with previous studies showing that Schwann cells synthesize the basal lamina that is essential for their myelination, suggests that type IV collagen serves an autocrine signal that promotes myelination. Just prior to myelination, promyelinating Schwann cells associate with the axon on one surface, and the basal lamina on the other. This polarization is essential for myelination (55). Our analysis suggests that type IV collagen signaling occurs to initiate myelination after the ECM is established. In addition to the ECM, axonal signals, including Nrg1-Type III, are needed to promote myelination (56). In light of previous work showing that changes in cAMP affect Schwann cell's response to Nrg1 (57), an attractive possibility is that activation of Gpr126 by type IV collagen changes the response of the Schwann cell so that axonal neuregulin signals trigger myelination instead of proliferation, as occurs at earlier stages.

The GAIN domain is the main structural feature that unites the aGPCRs and distinguishes them from other GPCRs. Nonetheless, the role of the GAIN domain and the autoproteolytic

cleavage that it mediates is so far unclear. Our results demonstrate that removal of the N-terminal region of Gpr126 leads to robust, ligand-independent signaling. Thus, our work on Gpr126 parallels studies of GPR56 (12, 13), BAI2 (15), BAI1 (16), and CD97 (14), which are also constitutively activated upon deletion of the region from the GAIN cleavage to the N-terminus. Collectively, these studies support the tethered inverse agonist model for aGPCR signaling, in which the N-terminal region suppresses signaling in a way that is relieved upon ligand binding. Binding of the activating ligand may involve removal of the cleaved N-terminal region or alteration of its interaction with the 7TM domain. A mode of activation that involves cleaving the peptide backbone may lead to irreversible activation of the receptor that persists even after ligand is no longer present, similar to the activation of the GPCR protease-activated receptor 1 (PAR-1) by the protease thrombin (58). Our data, however, showed that Gpr126 signaling is transient, with cAMP concentration peaking 1-2 hours after addition of type IV collagen and then declining. As in the case of GPR56, it is possible that Gpr126 signaling is inactivated by interaction with β -arrestin 2 and ubiquitination. Prolonged stimulation of GPCRs with ligand can also cause receptor internalization, many times facilitated by the arrestin proteins, leading to signal termination (18, 59, 60). It is also possible that, like the β 2-adrenergic receptor, Gpr126 signaling initiates a feedback loop in which PKA phosphorylates the receptor, weakening its association with G_s and strengthening its association with G_i , which inhibits adenylyl cyclase and reduces cAMP (61). Like the β 2-AR, Gpr126 has predicted PKA phosphorylation sites in the third intercellular loop and the C-terminal tail, and there is also evidence that Gpr126 couples to G_s and G_i (29). We propose that type IV collagen binding to Gpr126 initially activates only G_s , and that G_i only comes into play to attenuate signaling after increased or extended action of G_s . This would explain the transient rise of cAMP that begins shortly after addition of type IV collagen (Fig. 2C) and the reduction of cAMP in *Gpr126* mutant nerves (29). The transient nature of Gpr126 signaling is also supported by our previous study showing that Gpr126 is only required at the onset of myelination, so that myelin maturation and maintenance can proceed independently of Gpr126 signaling (28).

There is emerging evidence that the cleaved N-terminal regions of some aGPCRs may have functions independent of the 7TM. For example, the cleaved N-terminal fragment of BAI1 (Vasculostatin) can inhibit angiogenesis by binding integrins to disrupt migration and proliferation of endothelial cells (62, 63). Similarly, a recent study proposed that an N-terminal fragment of Gpr126 has a function that is independent of the 7TM domain and is required for heart development in zebrafish (32). Although this function is proposed to be distinct from the G_{α_s} /cAMP pathway that acts in myelination and inner ear development, the mechanism by which an N-terminal fragment might act in heart development has not been defined. The precise domains of Gpr126 involved in the putative heart function have also not been defined, but analysis of truncation constructs showed that a protein containing the first 783 amino acids of Gpr126 could rescue heart defects in some *gpr126* morphant embryos, but not myelination defects (32). In the same study, a construct containing amino acids 1-572 did not rescue the heart defects, implicating the GAIN and hormone binding domains in this putative heart function (32). In contrast, our analysis of a new mutation in *gpr126* shows that animals with a premature stop codon upstream of the hormone binding and GAIN domains resemble previously characterized *gpr126* mutant alleles, with

myelination and ear defects, but normal heart development. Thus, our analysis suggests that if the N-terminal region of Gpr126 has a function in heart development, it is mediated by the region containing the CUB and pentraxin domains. We have shown that this region of Gpr126 binds to type IV collagen with high specificity and affinity, suggesting that any 7TM-independent function of this N-terminal fragment may involve collagen binding. To further address the previous proposal that this region has a 7TM-independent function in heart development, it will be important to confirm the morpholino studies with corresponding mutations in *gpr126*.

Together with previous studies, our analysis of the interaction between type IV collagen and Gpr126 emphasizes the close relationship between aGPCRs and components of the ECM. Based on sequence similarity of the 7TM domain, Gpr126 and human GPR56 belong to the same subclass of aGPCR (6), collagen proteins activate both receptors (22), and deletion of the N-terminal region leads to ligand-independent signaling in both cases (12, 13). There are, however, important differences between Gpr126 and GPR56 and their collagen ligands. Type IV collagen forms a meshwork, whereas type III forms fibrils (64), Gpr126 and GPR56 couple to different G proteins and activate different intracellular second messengers (65), and type IV collagen acts as an autocrine signal for Gpr126-expressing Schwann cells, whereas type III collagen is a paracrine signal for migrating neurons expressing GPR56 (22). By defining type IV collagen as an activating ligand for Gpr126, our results have increased the understanding of aGPCRs and highlighted an important signaling function of the ECM that may apply to other members of this versatile family of GPCRs and their functions in diverse processes throughout the organism.

Materials and Methods

Constructs

The Gpr126NT-Fc was cloned into pFUSE-hIgG2-Fc2 vector (Invivogen) using homologous recombination method with the GenScript CloneEZ kit per manufacturer's protocol. Target DNA was amplified by PCR (primers: forward 5'-GTCACGAATTCGATATCGGCCATGGTTCAGAGCTGCCAGAGTAGCACC-3' and reverse, 5'-TGGGCAAGGTGGGCACTCCACAGATCTGAGGTGATTACACAAGCAGAC-3') and inserted into BglIII linearized vector using CloneEZ kit. The same method was used for CUBPTX-Fc (primers: forward, 5'-CTTGCACTTGTACGAATTCGTGCAATGTGGTGCTCACCGAC-3' and reverse, 5'-TGGGCAAGGTGGGCACTCCACATCCCAGTCAACGACATTACC-3') and CUB PTX-Fc (primers: forward, 5'-CTTGCACTTGTACGAATTCGCATTCCTACTGGACTATCCCA-3' and reverse, 5'-TGGGCAAGGTGGGCACTCCACGAGGTGATTACACAAGCAGAC-3'). C-terminal FLAG tag Gpr126 was cloned into p3XFLAG-CMV-14 vector (Sigma Aldrich) using standard cloning techniques and restriction sites HindIII and ClaI (primers: forward, 5'-AAATTTAAGCTTATGATTTTCGTTTCATCAGTGGTCCG-3' and reverse, 5'-AAATTTATCGATAATTGCAGGGTACTATCTGCATTACT-3'). To create the Gpr126 NT construct, the signal peptide was first cloned into p3XFLAG-CMV-14 vector

using HindIII and ClaI restriction sites (primers: forward, 5'-AAATTTAAGCTTATGATTTTCGTTTCATCAGTGGTCGG-3' and reverse, 5'-AAATTTATCGATGACAACGCGGTCATAGATGCAGCC-3'). The Gpr126 NT fragment was amplified (primers: forward, 5'-ATCTGCCTTTCCACCTCAGTGGCAACACACTTTGGCATCCTAATGGAT-3' and reverse, 5'-TGGTACCGATATCAGATCTATCGATGATTGCAGGGTACTATCTGCATT-3') and inserted into the signal peptide containing p3XFLAG-CMV-14 vector at the XhoI site using the Clone EZ kit. A recent paper (30) noted that the reference sequence for the zebrafish *gpr126* gene codes for Cys⁸⁰⁴, but that the wild-type zebrafish strains they analyzed encoded Trp at that position. Our analysis also showed that wild-type fish strains code for Trp at this position, and our constructs all contained Trp at this position. The sequences of all constructs were verified.

Purification of Fusion proteins

HEK293 cells were transfected with empty pFUSE vector or expression vectors for our different Gpr126-Fc fusion proteins using Lipofectamine 2000 following the manufacturer's standard protocol. Cells were incubated at 37 °C for 3 days. The conditioned media was collected and concentrated using Amicon Ultra 15 centrifugation filters. Concentrated conditioned media was then incubated overnight at 4°C with Protein A agarose to purify fusion proteins. Proteins were then eluted from beads with 1 M NaCl or 0.1M glycine buffers and dialyzed into HEPES buffered saline [10 mM HEPES and 150 mM NaCl, pH 7.5]. Protein purity was determined by SDS-PAGE followed by silver stain, and the concentration determined by Bradford assay.

Pull down assays

Purified collagen proteins were obtained from Sigma Aldrich and were biotinylated using the Pierce EZ-link biotinylation kit. Equal quantities by weight of biotinylated collagen and Gpr126 NT-Fc were mixed and incubated overnight with streptavidin or Protein A agarose. Beads were washed in HEPES buffered saline [10 mM HEPES and 150 mM NaCl, pH 7.5] then eluted by addition of Laemmli sample buffer. Samples were resolved by SDS-PAGE followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking buffer (2% nonfat dry milk, [50 mM NaCl, 20 mM HEPES, and 0.1% Tween 20] for 30 min and then incubated with primary antibody for 1 hour at room temperature. In cases where the primary antibody was not directly coupled to HRP, membranes were washed in blocking buffer and incubated with HRP-conjugated secondary antibody (GE healthcare) for 30 min. After washing to remove HRP-coupled antibodies, blots were visualized via ECL reagent (Pierce) followed by exposure to film.

cAMP measurements

HEK293 cells were transfected by standard protocol using Lipofectamine 2000 with empty vector or expression plasmid containing various Gpr126 constructs. Cells were treated with collagen for 1 hour unless otherwise indicated. The cells were lysed in 0.1 M HCl and, the intracellular cAMP concentration measured with an ELISA-based kit (EMD Millipore). To

determine the EC₅₀, the dose-response data were analyzed with a four-parameter Hill equation using GraphPad Prism software.

Surface Plasmon Resonance (SPR)-based Biosensor Analysis

The interaction of Gpr126 variants with type IV collagen was analyzed by SPR using a BIACORE 3000 biosensor system (GE Healthcare). Protein dilutions were performed in HBS-N (10mM HEPES at pH 7.4, 150 mM NaCl) running buffer. Type IV collagen was immobilized onto CM5 biosensor chip by amine coupling chemistry using *N*-hydroxysuccinimide (NHS) and *N*'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). To investigate binding of Gpr126NT-Fc or CUBPTX-Fc, proteins were diluted in HBS-N buffer and injected over the Type IV collagen surface. The experiments were performed at 25 °C using a flow rate of 30 µl/min. For each experiment, at least 5 different concentrations of Gpr126NT-Fc or CUBPTX-Fc were injected over each experimental and control flow cell. Dissociation was allowed to occur at the same flow rate for 300 sec, followed by running buffer alone for 2 min. The surface was regenerated with 0.5 N NaCl for 30 sec, followed by 1.0M NaCl using a flow rate of 100 µl/min, followed by running buffer alone for 2 min to enable stabilization of the baseline. Biosensor experiments were repeated a minimum of two times. All data were corrected for nonspecific binding by subtracting the signal measured in a control cell lacking immobilized ligand. Both data processing and kinetic fitting were performed using Scrubber software, version 2 (BioLogic Software, Pty., Australia) or BIAevaluation software 4.1 (Biacore).

Purification and culture of neonatal rat Schwann cells

Rat Schwann cells were acutely isolated from neonatal (P2) rat sciatic nerves by immunopanning, using the method of Cheng and Mudge (66). Briefly, isolated sciatic nerves were digested with collagenase/dispase and then incubated on sequential negative panning plates containing antibodies against CD45 (to deplete macrophages) and Thy 1.1 (to deplete fibroblasts). Schwann cells were then purified to about 99% purity by adherence to an O4 (anti-sulfatide) plate, and removed by trypsin. For cAMP assays, acute Schwann cells were plated on poly-D-lysine coated coverslips in defined, serum-free media (DMEM/SATO containing insulin, sodium pyruvate, L-glutamine, Thyroxine, B27, and N-acetyl-cysteine) in the absence of forskolin or growth factors. Cells were grown overnight prior to testing.

Fish strains and Genotyping

Zebrafish embryos were raised at 28.5°C and staged as described (67). The *gpr126*^{st86} mutation was identified in an ENU-based screen for mutations that disrupted *mbp* mRNA expression, following previously described methods for mutagenesis and screening (25). The *gpr126*^{st86} mutation failed to complement the previously described *gpr126*^{st49} allele (26), and sequencing the *gpr126* gene from mutants and wildtype siblings identified the lesion. To genotype individual animals in the phenotypic analyses, the *gpr126*^{st86} mutation was scored by a PCR test of genomic DNA samples. We used primers to amplify genomic DNA spanning the lesion (5'-TGTAAGACTTTGGGTTGATTTGG-3' and 5'-CATTGAGATATTATCGCCGTAGC-3'), and digested the products with the restriction

enzyme DpnII. The *gpr126^{st86}* mutation disrupts one of the two DpnII sites in the wild-type sequence.

Surface Biotinylation Assay

HEK293 cells transfected with Gpr126-FLAG or Gpr126 NT-FLAG were washed with PBS and suspended in PBS containing 2mM Sulfo-NHS-Biotin reagent (Pierce). The biotinylation reaction was incubated for 2 hours on ice. Cells were gently washed in 100 mM glycine solution to quench the reaction and then lysed in buffer with 1% Triton X-100, 300 mM NaCl, and 50 mM Tris. The lysate was cleared by high-speed centrifugation. After a portion was set aside to analyze total cell lysates, the remainder of the lysate was incubated with streptavidin agarose overnight at 4 degrees to pull down biotinylated proteins. Beads were washed, suspended in sample buffer [5 M Urea, 1% SDS, 10% glycerol, 5% β -mercaptoethanol, 50 mM Tris], and then resolved by SDS-PAGE followed by transfer to nitrocellulose membranes. Gpr126-FLAG surface expression was assessed by Western blot analysis using a FLAG antibody. As a control cytoplasmic protein, we analyzed the samples in immunoblots with an actin antibody (Thermo Scientific, MS-1295).

Analysis of FLAG constructs

For immunoblots, transfected HEK293 cells transfected with Gpr126-FLAG or Gpr126 NT-FLAG were lysed directly in a Urea based sample buffer [5 M Urea, 1% SDS, 10% glycerol, 5% β -mercaptoethanol, 50 mM Tris] and then sonicated on ice. For microscopy, HEK293 cells were transfected with empty vector, Gpr126-FLAG, or Gpr126 NT-FLAG, plated onto coverslips, and allowed to attach overnight. Cells were then fixed with 4% paraformaldehyde, washed, and incubated with an antibody against FLAG (1:2000) overnight at 4 °C. Cells were washed extensively and then incubated with Alexa Fluor goat anti-mouse 488 secondary (1:500) for 1 hour at room temperature. Slides were viewed using the 40 $\times$ objective of a Zeiss LSM 5 Pascal confocal microscope and images were acquired with a constant setting for comparison across conditions using Zeiss LSM software. Mean gray value was used to compare expression of different FLAG-tagged constructs in transfected cells, measured using Image J software; FLAG-positive cells were traced and the mean gray value and area determined by the software. To determine transfection efficiency, cells were visualized with a Zeiss Axio Imager M2 microscope; DAPI stained nuclei were counted to determine the total number of cells, and transfected cells counted by visualizing FLAG antibody staining.

qRT-PCR Analysis

Total RNA was extracted from individual embryos using the RNeasy Micro Kit (Qiagen). Embryos were sorted according to the ear phenotypes prior to lysis and confirmed using a PCR-restriction digest assay as described above. cDNA was made using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen). qPCR was performed on the Bio-Rad CFX384 Real-Time PCR Detection System with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). *gpr126* transcripts were detected using the forward primer 5'-TGTCGTTGACTGGGATCATT-3' and reverse primer 5'-CGGTTCCCTGGTGAAAGAGTT-3'. Values were normalized to the expression of *ef1a*.

Statistical Analysis

Single comparisons for statistical significance were done using an unpaired *t*-test with GraphPad Prism software. Statistical significance was deemed $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Type IV collagen binds specifically to the N-terminal region of Gpr126

(**A**) Schematic diagram showing the conserved domains of Gpr126 including signal peptide (SP), CUB, pentraxin (PTX), hormone binding domain (HBD), GPCR autoproteolysis-inducing domain (GAIN) and seven transmembrane (7TM) region in the wild-type protein and the corresponding N-terminal fusion protein Gpr126NT-Fc. The amino acids mediating the autocatalytic cleavage of the GAIN domain are noted (HLT) and the position of the cleavage indicated by the line in the GAIN domain. (**B and C**) Conditioned media containing Gpr126NT-Fc fusion protein was mixed with biotinylated collagen or BSA and incubated with streptavidin agarose. Interactions (**B**) were detected by immunoblot using an antibody against Fc (Anti-Fc-HRP) and total input (**C**) was detected as a control. (**D to E**) In the reciprocal pull-down experiments, biotinylated collagen or BSA protein was mixed with Gpr126NT-Fc fusion protein in conditioned media and incubated with Protein A agarose. As in (**B**) and (**C**), interactions and total input were detected by immunoblot using an antibody against biotin. Blots are representative of 3 experiments.

Figure 2. Type IV collagen stimulates cAMP signaling in cells expressing Gpr126

(A) cAMP concentration in rat Schwann cells treated with PBS or of type IV collagen (3 $\mu\text{g/mL}$) for 1 hour. Data are mean fold change $\pm$ S.E.M. from 3 experiments. $**P = 0.0013$. (B) cAMP concentration in HEK293 cells transfected with empty vector or Gpr126 and treated with PBS or type IV collagen (3 $\mu\text{g/mL}$) for 1 hour. Data are mean fold change $\pm$ S.E.M. from 5 experiments. $***P = 0.0004$, $***P = 0.0001$. (C) cAMP concentration in Gpr126-transfected HEK293 cells treated with type IV collagen (3 $\mu\text{g/mL}$) for up to 480 min. Data are mean fold change $\pm$ S.E.M. from two experiments. (D) Fold cAMP concentration in Gpr126-transfected HEK293 cells treated for 1 hour with increasing doses of type IV collagen. Data are mean fold change $\pm$ S.E.M. from two experiments. EC_{50} value, 0.2 $\mu\text{g/mL}$ (E) cAMP concentration in HEK293 cells transfected with Gpr126 treated for 1 hour with PBS or collagen I, II, III, IV, or V (3 $\mu\text{g/mL}$) for 1 hour. Data are mean fold change $\pm$ S.E.M. from 3 experiments. $**P = 0.0035$.

Figure 3. A region of Gpr126 containing CUB and Pentraxin domains mediates the high affinity interaction with type IV collagen

(A) Schematic diagram depicting truncated versions of the Gpr126NT-Fc fusion protein either containing (CUBPTX-Fc) or lacking (Δ CUB Δ PTX-Fc) the CUB and PTX domains.

(B) Pull down experiments using conditioned media containing Gpr126NT-Fc,

CUB Δ PTX-Fc, or CUBPTX-Fc incubated with biotinylated type IV collagen and streptavidin agarose. Blot is representative of 4 experiments. (C to D) Biacore sensograms detecting the interaction of type IV collagen with Gpr126NT-Fc and CUBPTX-Fc. SPR monitored the binding of various concentrations of fusion proteins to immobilized collagen IV on a biosensor chip. The association and dissociation constants (K_a , K_d) for each trial are shown in the figure panels. Data are representative of two experiments; the values for association and dissociation constants are reported as mean $\pm$ SD in the text.

Figure 4. The N-terminal region of Gpr126 inhibits receptor signaling activity

(A) Schematic diagram showing the C-terminally FLAG tagged constructs representing full-length (Gpr126-FLAG) and N-terminally truncated Gpr126 (Gpr126 NT-FLAG). (B) Upper panel shows FLAG immunoblot analysis of surface expression and total lysate of HEK293 cells transfected with tagged full-length (WT) or N-terminally truncated (NT) Gpr126. Lower panel shows control blot probed for actin, a cytoplasmic protein. Blot is representative of 2 experiments. (C) cAMP concentration in HEK293 cells transfected with empty vector, Gpr126-FLAG, or Gpr126 NT-FLAG and treated with PBS or type IV collagen (3 μ g/mL) for 1 hour. Data are mean fold change $\pm$ S.E.M. from 5 experiments.

** $P = 0.0037$; *** $P = 0.0001$; N.S., $P = 0.36$.

Figure 5. The *gpr126*^{st86} mutation truncates Gpr126 after the PTX domain and disrupts myelination and ear development

(A) Schematic representation of Gpr126 showing conserved domains and the mutations in *gpr126*^{st86} and previously characterized *gpr126*^{st49} (26). (B) Sequence chromatogram of heterozygous sibling and *gpr126*^{st86} mutant. STOP in the amino acid code marks a premature stop codon in the mutant. (C) Expression of *mbp* in wild-type (*gpr126*^{st86/+}) and mutant (*gpr126*^{st86/st86} and *gpr126*^{st86/st49}) larvae. Arrowheads mark *mbp* (or lack of it) in the lateral line nerve at 5 days post-fertilization. (D) Ear morphology in maternal (M) *gpr126*^{st86/+} mutants (which have wild-type morphology), maternal-zygotic (MZ) *gpr126*^{st86/st86} mutants, and zygotic (Z) *gpr126*^{st86/st86} mutants. Arrowheads mark the normal pericardium; arrows mark ears. Scale bars, 100 μ m.