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Nora Madaras April 6, 2021

Mapping the Interaction Between Oligodendrocyte-Enriched G Protein-Coupled Receptor 37 and Myelin-Associated Glycoprotein

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Mapping the Interaction Between Oligodendrocyte-Enriched G Protein-Coupled Receptor 37 and Myelin-Associated Glycoprotein

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G protein-coupled receptor 37 (GPR37) is an orphan G protein-coupled receptor (GPCR) that is most highly expressed in myelinating oligodendrocytes. GPR37-knockout mice exhibit increased susceptibility to demyelination and a significant down-regulation of myelin-associated glycoprotein (MAG), a component of myelin, in the brain. We have previously demonstrated that GPR37 and MAG co-immunoprecipitate, indicating a physical interaction between the two proteins. In order to determine which regions of these proteins are necessary to facilitate the physical interaction, we performed a series of co-immunoprecipitation experiments with several truncated variants. These studies revealed that the N-terminal domain of MAG is not required for the interaction, nor the N-terminal and C-terminal domains of GPR37, suggesting that the physical interaction is facilitated by the transmembrane domain regions of each protein. Furthermore, we found that each of the seven individual transmembrane domains of GPR37 co-immunoprecipitates with MAG. This work forms an important basis for understanding the structural determinants of the interaction between GPR37 and MAG and manipulating this interaction for use in future experimental models. Ultimately, GPR37 is a promising potential drug target for demyelinating disorders, and studying its interaction with MAG is important for understanding the role of GPR37 in disease pathology and potential treatments.

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Table of Contents

Introduction

Orphan G protein-coupled receptors as potential drug targets

G protein-coupled receptors (GPCRs) are a diverse class of receptors that are expressed widely throughout the body and can be activated by a variety of signals, including light, proteins/peptides, small organic compounds, and carbohydrates.[1] Due to their diversity, tissue specificity, cell-surface localization, and roles as the initiators of many intracellular signaling pathways, GPCRs make excellent drug targets and represent an ongoing area of intensive research in pharmacology.

Receptors categorized as “orphan” GPCRs are those for which an endogenous ligand is not yet known. Therefore, while there is huge potential for drug discovery at these receptors, there remains much unknown about their signaling and function. Research continues to elucidate the structure, signaling, and protein interactions of orphan receptors to further our understanding of their function and identify their potential as drug targets.

G protein-coupled receptor 37 (GPR37)

G protein-coupled receptor 37 (GPR37) is an orphan GPCR that was first described and cloned in 1997.[2,3] It shares highest sequence homology with another orphan receptor, GPR37-like 1 (GPR37L1), and both are highly expressed in the central nervous system.[4] GPR37 in particular has raised interest due to its misfolding and neuronal aggregation in autosomal recessive juvenile Parkinson’s disease (AR-JP) and its identification as a substrate of the E3 ubiquitin ligase parkin,[5] which is mutated in AR-JP.

GPR37 and GPR37L1 were first identified as having high homology with peptide-activated endothelin receptors and bombesin-like receptors. However, screens with ligands such as endothelins and bombesin-like peptides have failed to induce activation of the

receptors,[4] so the search for the endogenous ligands of GPR37 and GPR37L1 has continued. Several ligands have previously been reported for GPR37, including the invertebrate peptide known as “head activator”[6] as well as bioactive lipid neuroprotectin D1.[7] However, these data have yet to be replicated. Our group has previously reported that both GPR37 and GPR37L1 are activated by prosaposin and its active peptide fragment prosaptide,[8] but attempts by other groups to replicate these data have varied in success,[9-12] and therefore prosaposin and prosaptide are not universally recognized as endogenous ligands for these receptors. Thus, there is currently no consensus on the endogenous ligands of GPR37 and GPR37L1, and they remain classified as orphan receptors.

GPR37 in oligodendrocytes

Although highly homologous, GPR37 and GPR37L1 exhibit distinct patterns of expression in the central nervous system. While GPR37L1 is enriched in astrocytes,[13] GPR37 is most highly expressed in oligodendrocytes.[14] It has been shown that expression of GPR37 increases with oligodendrocyte maturation and progression of myelination, and that the receptor is expressed in mature myelinating oligodendrocytes,[14] suggesting an association with the myelination process. Furthermore, compared to wild-type mice, GPR37 knockout mice exhibit increased demyelination and faster remyelination when fed cuprizone, a demyelinating agent used to model multiple sclerosis.[15] Taken together, these findings indicate a role for GPR37 in the myelination process and in myelin stability in the central nervous system, identifying it as a potential drug target for demyelinating disorders.

Myelin-associated glycoprotein (MAG)

Previous work in our lab has identified potential interacting partners for GPR37 and GPR37L1 by analyzing the protein expression in GPR37/GPR37L1 double knockout (DKO) mouse brains. Proteomic data revealed significant downregulation of myelin-associated glycoprotein (MAG) in DKO mouse brain, and Western blot analysis confirmed that this downregulation was specific to GPR37 knockout mice.[15] MAG is a transmembrane glycoprotein expressed in myelinating glial cells that is known to enhance long-term stability of myelin on axons.[16] We have further shown that GPR37 robustly co-immunoprecipitates with MAG when co-expressed in HEK 293T cells, indicating a physical interaction between the two proteins.[15] Given the localization of GPR37 and MAG to the oligodendrocyte plasma membrane, their physical association, and the decreased myelin stability and significant downregulation of MAG expression in brains of GPR37 knockout mice, we hypothesize that GPR37 plays a role in myelin stability via its functional interaction with MAG. However, it remains unknown whether the physical interaction observed between GPR37 and MAG is related to their functional interaction and the regulation of MAG by GPR37 in vivo.

Structural studies

Although we do not yet know the relationship between the physical and functional interactions of GPR37 and MAG, studying the physical interaction can provide insight into their modulation of each other in vivo. It is also useful to understand the physical components of the interaction in order to determine whether and how the physical interaction plays a role in the observed functional interaction, and how this association affects the downstream signaling and overall function of the receptor in oligodendrocytes specifically and the brain more generally. Furthermore, knowledge of the structural determinants of the physical interaction between

GPR37 and MAG is necessary for the manipulation and disruption of the interaction, which can be used for further investigation into the implication of GPR37 in disease (such as demyelinating disorders), its signaling, and its role as a potential drug target.

The purpose of this study was to dissect the structural components of the physical association between GPR37 and MAG. This was accomplished by co-transfecting various truncated forms of GPR37 and MAG into HEK 293T cells and performing co-immunoprecipitation experiments to detect a physical interaction and therefore determine which regions of each protein are essential or non-essential for the interaction. GPR37 and MAG are both embedded in the plasma membrane, with the N-terminal regions in the extracellular space and the C-terminal regions in the cytoplasm (Fig. 1). Thus, it is likely that the physical association between the two is facilitated by co-localized regions (i.e. N-terminal domains of each, C-terminal domains of each, or transmembrane domains of each). Based on this logic, we constructed variants of GPR37 and MAG that lack a specific domain or represent only a small portion of the full protein and used these variants in co-immunoprecipitation experiments.

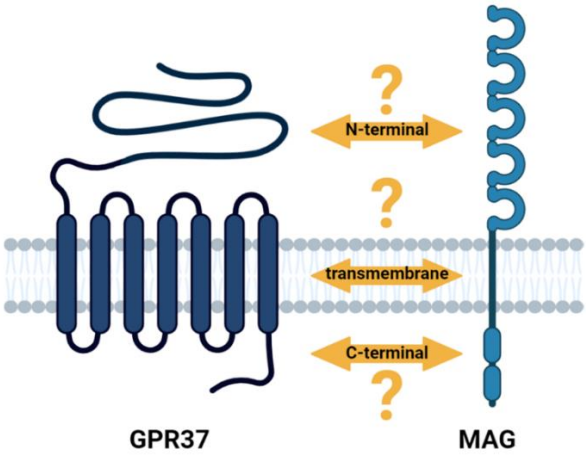

Figure 1. ***Diagram of full-length GPR37 and MAG*****.** GPR37 is characterized by a very large extracellular domain and MAG by an N-terminus consisting of five immunoglobulin (Ig) domains. The goal of these experiments was to determine whether the physical interaction between GPR37 and MAG is facilitated by the N-terminal, C-terminal, or transmembrane domain regions of the two interacting proteins.

Materials and methods

Cell culture and transfection

Human embryonic kidney (HEK 293T) cells were maintained in complete medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C incubator at 5% CO_2. Cells were passaged when 90-100% confluent, approximately every 3 days. To passage, the media was aspirated from a 10 cm plate of confluent cells, and 1 mL trypsin was added. After a 30 second incubation, 8-9 mL complete medium was added and the mixture was triturated with the pipette to create a homogeneous cell suspension, 1 mL of which was added to a new 10 cm plate containing 9 mL complete medium.

HEK 293T cells were transfected when 70-80% confluent using MIRUS TransIT-LT1 according to the manufacturer's protocol. Constructs used: empty vector (EV), MAG, MAGΔNT-Flag, GPR37-GFP, 37Δ210-Flag, 37NT-Flag, 37NTΔ259-Flag, 37TM(1-7)-Flag

Cell lysate preparation

Cells were harvested 24 hours after transfection in low-salt harvest buffer (10 mM HEPES [pH 7.25], 50 mM NaCl, 5 mM EDTA in ultrapure water) + 1% HALT protease/phosphatase inhibitor (Thermo Fisher) and 1% Triton X-100 (Sigma), then rotated slowly at 4 °C overnight to lyse the cells and solubilize the proteins. Solubilization consists of incubating cell lysate with a detergent (Triton X-100) in order to extract proteins from membranes. To prepare input samples, solubilized cell lysates were combined with 2x Laemmli buffer to reduce and denature the proteins.

Co-immunoprecipitation studies

To specifically isolate a protein of interest from cell lysates, we used microbeads in combination with an antibody specific for that protein. This is termed immunoprecipitating or "pulling down" the protein of interest, which in these experiments was either MAG or a Flag-tagged protein. Solubilized cell lysates were incubated with either anti-Flag M2 affinity gel agarose beads (Sigma), or with Pierce Protein A/G agarose microbeads (Thermo Fisher) in combination with anti-MAG antibody (Cell Signaling), for 1 hour (Fig. 2a) and then subjected to centrifugation to pellet the beads (Fig. 2b). Beads were washed 4 times with low-salt harvest buffer + 1% Triton X-100 to minimize non-specific binding, then resuspended in 2x Laemmli sample buffer to elute proteins from beads (Fig. 2c). The immunoprecipitation of the protein of interest targeted by the antibody also pulls down proteins that physically interact with the protein of interest, and these interacting partners can then be detected via Western blot.

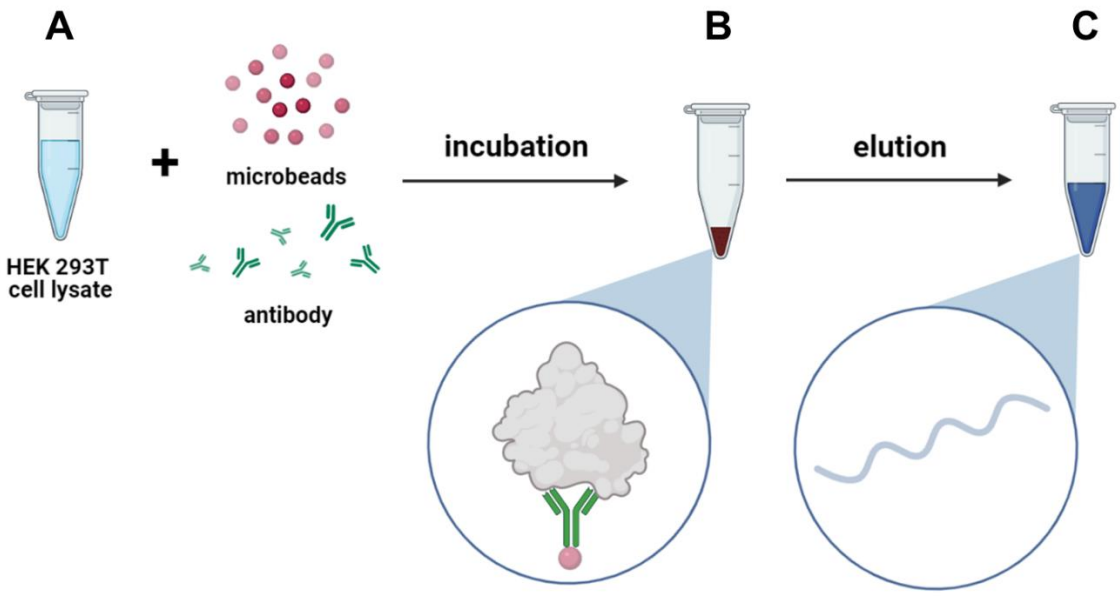

Figure 2. ***Co-immunoprecipitation protocol.*** (A) HEK 293T cell lysates were incubated with agarose microbeads and antibody at 4 °C for 1 hour. (B) Tubes were then centrifuged to pellet beads. Beads were washed with low-salt harvest buffer + 1% Triton X-100, then (C) resuspended in 2x Laemmli sample buffer to elute proteins from beads and prepare them for separation via gel electrophoresis.

Western blot analysis

The Western blot protocol allows specific proteins to be identified in cell lysate or immunoprecipitation (IP) samples. Proteins are separated by molecular weight via gel electrophoresis, transferred to a nitrocellulose membrane, and probed with primary antibody specific for the protein of interest. Blots are then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody that binds the primary antibody, and the membrane is exposed to chemiluminescent substrate (a solution containing a substrate of HRP). By detecting the chemiluminescent reaction between HRP and its substrate, the protein of interest can be visualized as bands on the blot.

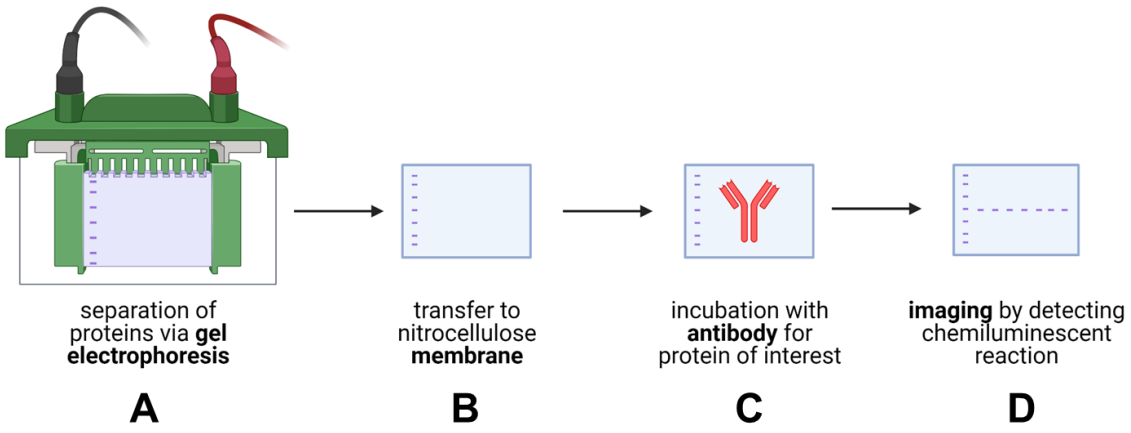

Figure 3. ***Western blot protocol.*** (A) Proteins were separated by molecular weight via gel electrophoresis, then (B) transferred to nitrocellulose membranes. (C) Specific antibodies were used to probe for the protein of interest, which were then (D) visualized as bands on the blot by utilizing a chemiluminescent reaction with the antibody.

Proteins were separated and analyzed via SDS-PAGE. Input and IP samples were run on 4-20% Tris-glycine gels (Bio-Rad) at 130V for 45 minutes, then transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% milk (5% nonfat milk in 50 mM NaCl, 10 mM HEPES [pH 7.25], 0.1% Tween-20 [Sigma] in ultrapure water) for 1 hour, then incubated with primary antibody overnight at 4 °C. The following day, membranes were washed 3 times with PBS + 0.1% Tween-20 and then incubated with secondary antibody in 5% milk for 1 hour at

room temperature, after which they were again rinsed 3 times with PBS. To visualize the proteins via chemiluminescence, membranes were incubated with SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher) for 3-4 minutes and imaged using the Li-Cor Odyssey Fc imaging system. Antibodies used: β-tubulin (Cell Signaling; 1:5,000), GPR37 (Mab Technologies; 1:5,000), MAG (Cell Signaling; 1:5,000), Flag-HRP (Sigma; 1:5,000), anti-rabbit-HRP secondary antibody (Cytiva; 1:5,000)

Results

The N-terminus of MAG is not required for the physical interaction with GPR37.

We have previously demonstrated that full-length MAG co-immunoprecipitates with full-length GPR37[15] (Fig. 4a). To assess whether the N-terminus of MAG is required for this interaction to occur, HEK 293T cells were co-transfected with full-length, GFP-tagged GPR37 and MAGΔNT, a variant of MAG that lacks the extracellular N-terminal domain (Fig. 4b). Western blot analyses (Fig. 4c, 4d) revealed that full-length GPR37 co-immunoprecipitates with MAGΔNT, indicating a physical interaction between these two forms. This suggests that the N-terminal region of MAG is not required for the physical association with GPR37.

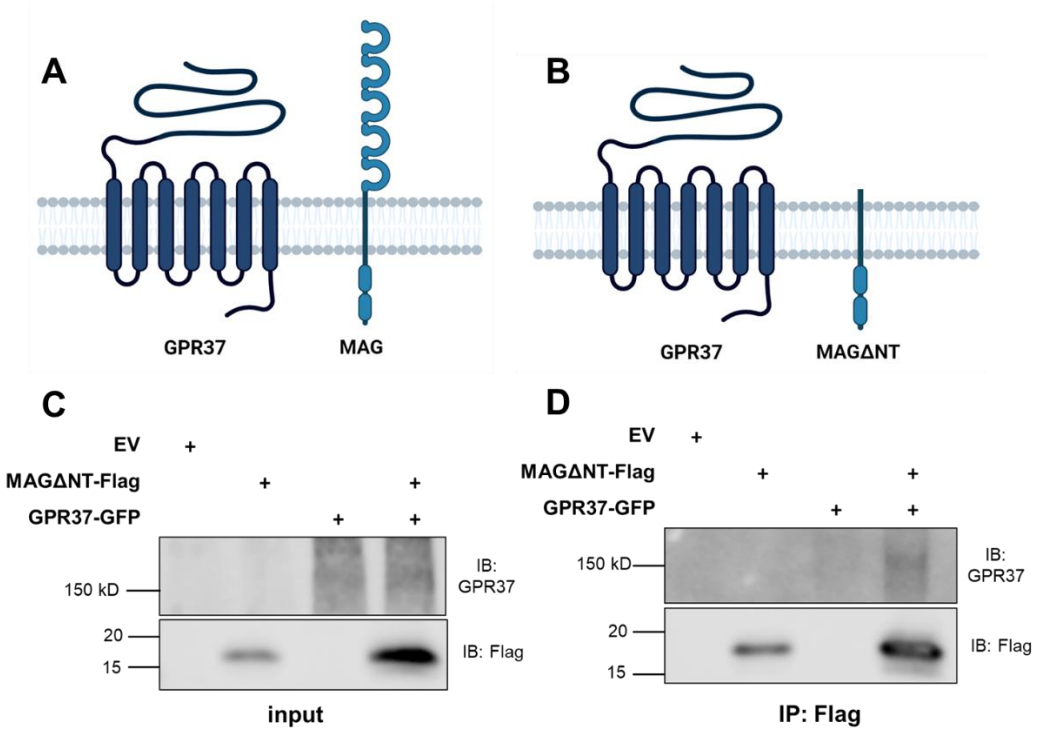

Figure 4. ***The N-terminus of MAG is not required for the physical interaction with GPR37.*** (A) We have shown previously that full-length GPR37 co-immunoprecipitates with full-length MAG. (B) HEK 293T cells were co-transfected with full-length, GFP-tagged GPR37 and Flag-tagged MAGΔNT, a variant of MAG lacking the N-terminal domain. (C) Anti-Flag agarose beads were added to cell lysates to specifically pull down MAGΔNT, and GPR37 was detected in the IP sample (D), indicating a physical interaction between GPR37 and MAGΔNT. (n=3) EV = empty vector, IP = immunoprecipitation, IB = immunoblot

Most of the GPR37 N-terminus is not required for the physical interaction with MAG.

After finding that the N-terminus of MAG was not necessary for co-immunoprecipitation with GPR37, we questioned whether any portion of the GPR37 N-terminus is necessary to facilitate the interaction. We have shown previously that a variant of GPR37 with the first 210 amino acid residues of the N-terminal domain removed (known as 37Δ210) exhibits even higher localization to the cell surface than full-length GPR37.[17] Given that the full N-terminus of GPR37 is 262 residues long, this construct expresses a version of GPR37 with approximately 80% of the N-terminus removed. To determine whether this truncation inhibits the ability of GPR37 to co-immunoprecipitate with MAG, HEK 293T cells were co-transfected with full-length MAG and Flag-tagged 37Δ210 (Fig. 5a). Western blot analyses (Fig. 5b, 5c) revealed that full-length MAG co-immunoprecipitates with 37Δ210, indicating a physical association. This suggests that residues 1-210 of GPR37, representing most of the N-terminal domain, are not required for the physical interaction with MAG.

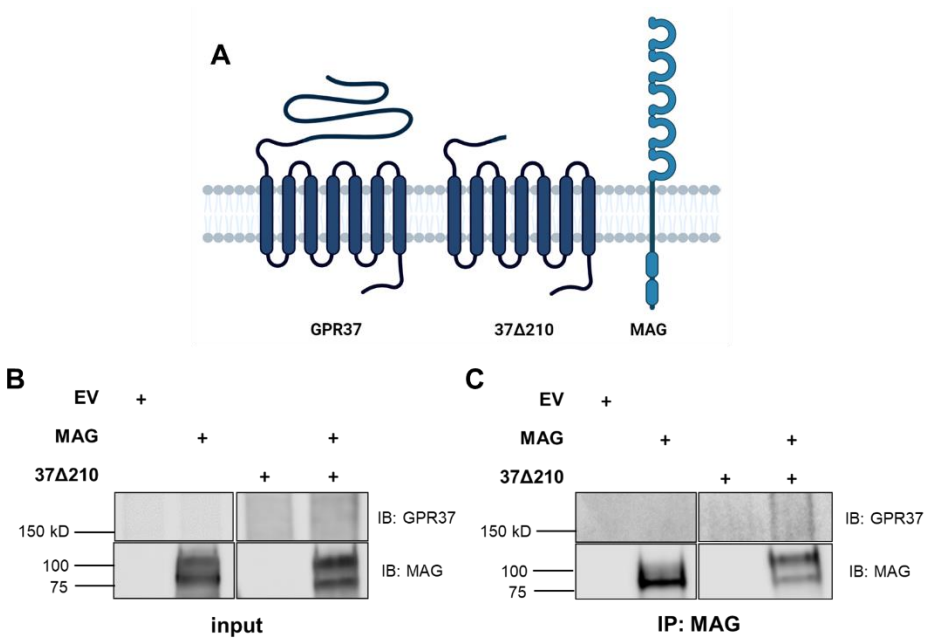

Figure 5. ***Most of the GPR37 N-terminus is not required for the physical interaction with MAG.*** (A) Full-length MAG was co-transfected in HEK 293T cells with Flag-tagged 37Δ210, a form of GPR37 lacking the first 210 amino acid residues of the N-terminal domain. (B) Agarose beads and anti-MAG antibody were added to cell lysates to specifically pull down MAG, and 37Δ210 was detected in the IP sample (C), indicating a physical interaction between MAG and 37Δ210. (n=2)

The N-terminus and first transmembrane domain of GPR37 are sufficient for the physical interaction with MAG.

To assess whether the C-terminal domain of GPR37 is required for the interaction with MAG, HEK 293T cells were co-transfected with full-length MAG and Flag-tagged 37NT, a variant of GPR37 containing only the N-terminus and first transmembrane domain (Fig. 6a). Western blot analyses (Fig. 6b, 6c) revealed that full-length MAG co-immunoprecipitates with 37NT, indicating a physical association. These data suggest that the C-terminus and transmembrane domains 2-7 of GPR37 are not required for the physical association with MAG, and that the N-terminal domain and TM1 are sufficient to facilitate an interaction. This was interesting to find considering that we previously found that the N-terminus of MAG is not required. So, the question became whether it is the N-terminus or TM1 in this construct that is facilitating the physical interaction with MAG.

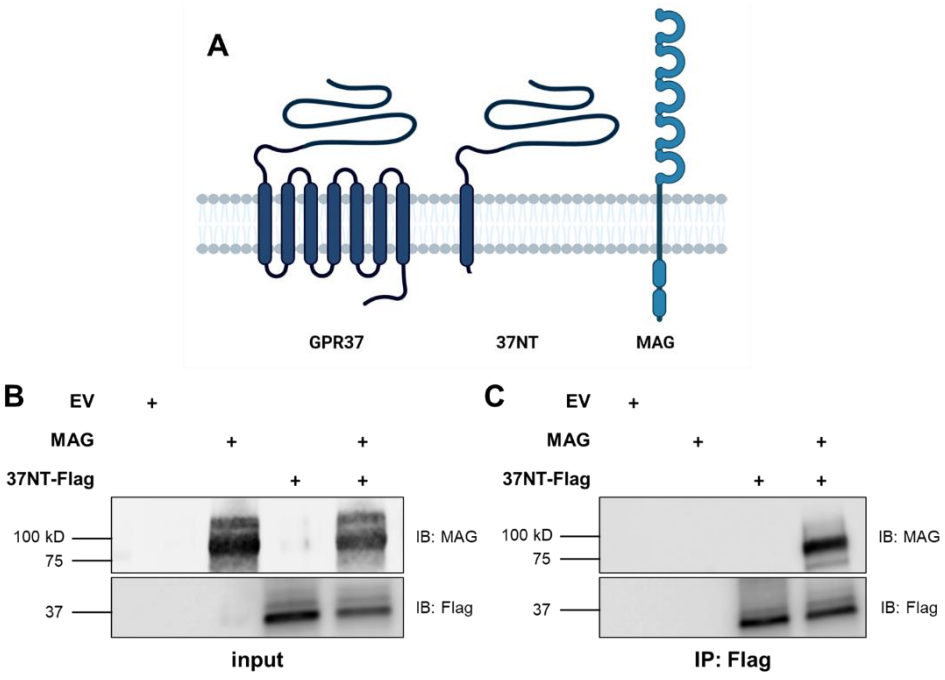

Figure 6. ***The N-terminus and first transmembrane domain of GPR37 are sufficient for the physical interaction with MAG.*** (A) Full-length MAG was co-transfected in HEK 293T cells with Flag-tagged 37NT, a form of GPR37 containing only the N-terminus and the first transmembrane domain. (B) Anti-Flag agarose beads were added to cell lysates to specifically pull down 37NT, and MAG was detected in the IP sample (C), indicating a physical interaction between MAG and 37NT. (n=3)

The first transmembrane domain of GPR37 is sufficient for the physical interaction with MAG.

We found that 37NT, a construct containing the N-terminus and first transmembrane domain of GPR37, is able to co-immunoprecipitate with MAG. To determine whether the GPR37 N-terminus is required for the physical interaction with MAG, or whether GPR37-TM1 is sufficient, HEK 293T cells were co-transfected with full-length MAG and Flag-tagged 37NTΔ259, a small portion of GPR37 that includes the first transmembrane domain and a small portion of the first intracellular loop (Fig. 7a). Western blot analyses (Fig. 7b, 7c) revealed that full-length MAG co-immunoprecipitates with 37NTΔ259, indicating a physical interaction. This suggests that the first transmembrane domain of GPR37 is sufficient to facilitate the physical association with MAG, and that the N-terminal domain of GPR37 is not required.

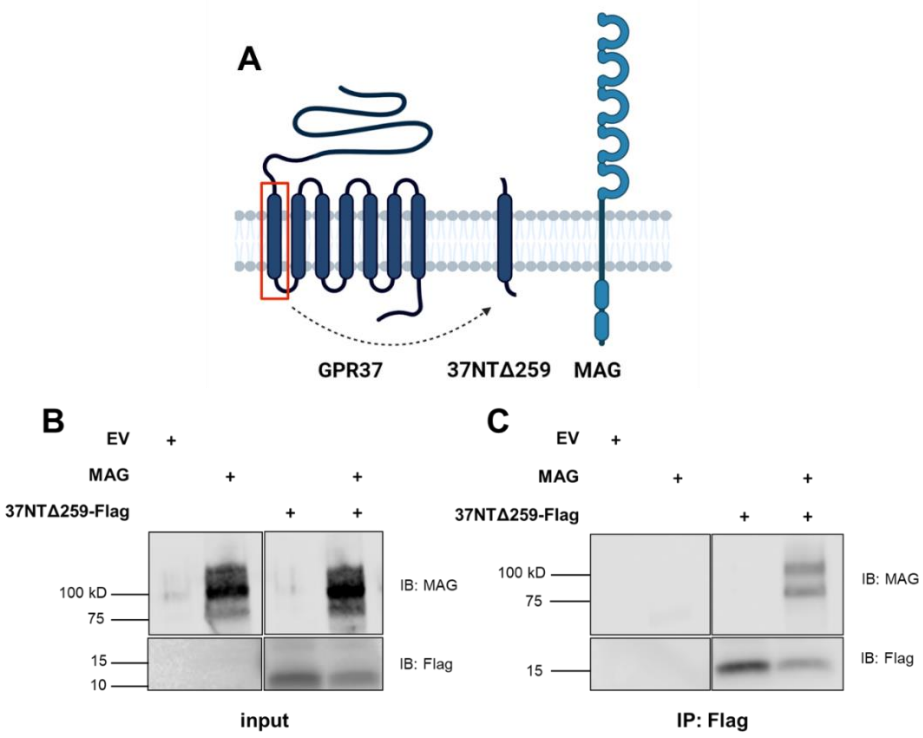

Figure 7. ***The first transmembrane domain of GPR37 is sufficient for the physical interaction with MAG.*** (A) Full-length MAG was co-transfected in HEK 293T cells with Flag-tagged 37NTΔ259, a portion of GPR37 containing only the first transmembrane domain and a small portion of the first intracellular loop. (B) Anti-Flag agarose beads were added to cell lysates to specifically pull down 37NTΔ259, and MAG was detected in the IP sample (C), indicating a physical interaction between 37NTΔ259 and MAG. (n=4)

All seven transmembrane domains of GPR37 co-immunoprecipitate with MAG.

To assess whether the interaction with MAG is specific to TM1 of GPR37, or if it can occur with more than one transmembrane region of the receptor, co-immunoprecipitation studies were performed using Flag-tagged 37TM (GPR37 transmembrane domain) constructs. HEK 293T cells were co-transfected with full-length MAG and each individual 37TM (Fig. 8a). Western blot analyses (Fig. 8b) revealed that MAG co-immunoprecipitates with each 37TM, indicating a physical interaction. These data suggest that the physical association between GPR37 and MAG is not specific to GPR37-TM1 but can occur between MAG and all seven transmembrane domains of the receptor. Furthermore, this supports the idea that the interaction likely occurs in the transmembrane domain region, because any single transmembrane domain of GPR37 was sufficient to observe a physical interaction with MAG.

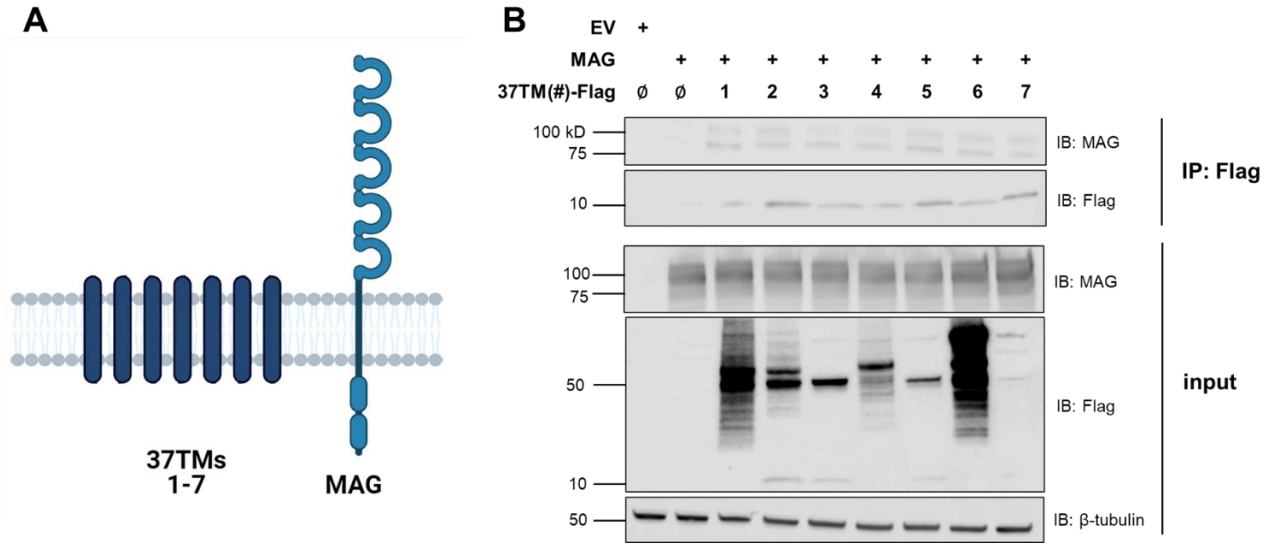

Figure 8. ***All seven transmembrane domains of GPR37 co-immunoprecipitate with MAG.*** (A) Full-length MAG was co-transfected in HEK 293T cells with each individual Flag-tagged transmembrane domain of GPR37. (B) Anti-Flag agarose beads were added to cell lysates to specifically pull down the transmembrane domains, and MAG was detected in the IP sample with each TM, indicating a physical interaction between MAG and each transmembrane domain of GPR37. (n=4)

Discussion

GPR37 is enriched in mature myelinating oligodendrocytes,[14] and GPR37-knockout (37KO) mice show decreased myelin stability in the brain,[15] indicating a role for the receptor in myelination and myelin stability. One of the most significantly downregulated proteins in 37KO mouse brain is myelin-associated glycoprotein (MAG),[15] a component of myelin found in the oligodendrocyte membrane that facilitates long-term adhesion of the myelin sheath to the axon. Furthermore, our group has previously shown that GPR37 and MAG co-immunoprecipitate when co-expressed in HEK 293T cells.[15] Based on these data, we hypothesize that the interaction between GPR37 and MAG acts to enhance the stability of MAG and contributes to the stability of myelin overall. We do not yet know whether the physical association observed between these proteins is related to the in vivo regulation of MAG by GPR37. However, structural studies such as this one are an important step in specifically mapping the physical determinants of the interaction, which allows for investigation of the activity of the receptor by manipulating the physical interaction in experimental models, both in vitro and in vivo.

To elucidate which structural components of GPR37 and MAG are necessary for the physical association to occur, we performed a series of co-immunoprecipitation experiments with various truncated forms of both proteins. Taken together, the data presented here demonstrate that the physical interaction between MAG and GPR37 is most likely facilitated by the transmembrane domain regions of the proteins. We first found that the N-terminal domain of MAG is not necessary for the interaction with GPR37 (Fig. 4), and removing about 80% of the GPR37 N-terminus also did not hinder their association (Fig. 5). A construct of GPR37 containing only the N-terminus and TM1 (37NT) was also found to associate with MAG (Fig. 6). We further confirmed that the first transmembrane domain of GPR37 is sufficient for the physical interaction with MAG by performing successful co-immunoprecipitation of MAG and 37NTΔ259, a segment of the receptor containing only the first transmembrane domain (TM1)

and a small portion of the first intracellular loop (Fig. 7). Based on the known architecture of these proteins, we conclude that the C-terminus of MAG is also unlikely to be involved in the interaction, since the C-termini of the receptor and MAG are both localized to the cytoplasmic side of the plasma membrane. This conclusion could be further supported in the future by co-transfecting GPR37 with a variant of MAG lacking the C-terminal domain and demonstrating co-immunoprecipitation of these forms. Lastly, we showed that a single transmembrane domain of GPR37 was sufficient to confer interaction with MAG. However, the physical association with MAG was not found to be specific to GPR37-transmembrane domain 1 (37TM1), but rather all seven TMs of GPR37 were observed to be able to co-immunoprecipitate with MAG (Fig. 8). While it is peculiar that MAG does not show a specific affinity for one or several TMs in particular in these experiments, these data do confirm that a single transmembrane domain region of GPR37 is sufficient to facilitate a physical association with MAG.

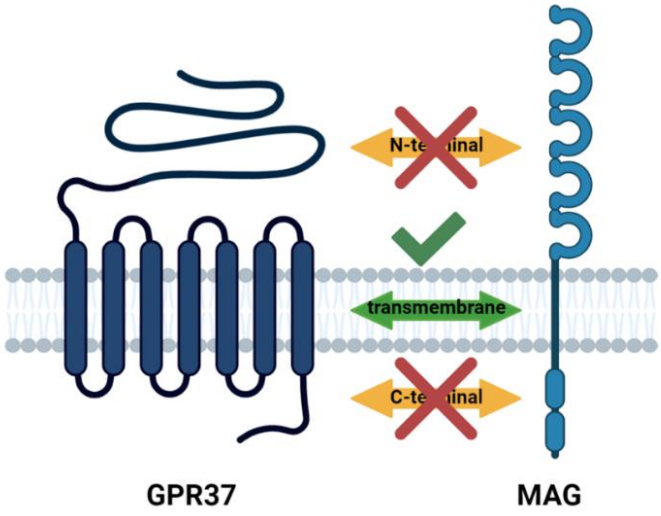

Figure 9. ***The physical interaction between GPR37 and MAG is facilitated by the transmembrane domain regions.*** We found that neither the N-terminus of MAG or the N- or C-terminus of GPR37 is required for the physical interaction. Furthermore, each individual transmembrane domain of GPR37 was shown to co-immunoprecipitate with MAG, indicating a transmembrane interaction.

The physical association of all seven GPR37 transmembrane domains with MAG in our assays raises the question of whether the interaction between GPR37 and MAG is truly specific, or whether MAG has a general capacity to interact with GPCRs. Previous studies performed by our group revealed that MAG is able to co-immunoprecipitate with GPR37 but not the M1 muscarinic acetylcholine receptor, another GPCR highly expressed in oligodendrocytes,[15] indicating that there is at least some degree of specificity to the interaction between GPR37 and MAG. To further verify that this association is specific to GPR37 transmembrane domains and not GPCR TMs in general, similar co-immunoprecipitation experiments can be performed with MAG and transmembrane domains from other, less-related GPCRs. It is possible that the over-expression of the GPR37 transmembrane domains, which consist of mainly hydrophobic residues, induced association with the hydrophobic MAG transmembrane domain in our experiments, when certain 37TMs would not naturally associate with MAG in vivo, and thus the question of which 37TMs have a more specific affinity for MAG requires further study. This can be investigated by creating variants of GPR37 with point mutations in a single transmembrane domain region and performing co-immunoprecipitation with MAG. These results would indicate whether the loss of interaction with a particular GPR37 transmembrane domain more significantly hinders the ability of MAG to physically associate with the receptor, and could more specifically localize the critical region of the receptor that facilitates the physical interaction. Another experimental method that could be employed is the co-transfection of full-length GPR37 and MAG along with each individual GPR37 transmembrane domain construct, followed by co-immunoprecipitation of the full-length proteins. The overexpressed 37TMs would compete with with full-length GPR37 for physical association with MAG, so a more significant decrease in full-length GPR37/MAG co-immunoprecipitation in the presence of a particular 37TM could indicate that this TM may have a higher affinity for MAG.

The experimental design of these studies creates constraints on our ability to draw certain conclusions. First of all, co-immunoprecipitation simply shows that two proteins have some sort of physical association; it is not confirmation that they are directly binding. We do not yet know whether GPR37 and MAG bind directly to each other, or if there are other intermediate binding partners that cause them to be pulled down together without being in direct contact. The co-immunoprecipitation studies presented here and previously[15] demonstrate the ability of MAG and GPR37, and truncated variants, to form a complex, but we cannot conclude necessarily that they bind directly to each other. To assess whether GPR37 and MAG directly bind, purified version of both proteins could be used. If purified GPR37 and MAG pull down together in the absence of other proteins, a binding interaction can be more confidently concluded. Secondly, it has not yet been shown that the physical association observed is involved in the regulation of MAG by GPR37 in vivo. Thus, we cannot assume that the physical interaction is the stabilizing force on MAG expression. In order to determine this, future research should investigate this protein-protein interaction in an in vivo context. If a point mutant of GPR37 can be found that lacks interaction with MAG, this mutant can be knocked-in to mice in order to study the effects of the loss of this interaction on MAG expression, GPR37 signaling, and myelination. If the knock-in of this mutant were to lead to down-regulation of MAG, this would provide evidence that it is indeed the physical interaction between GPR37 and MAG that is important for regulating MAG stability.

There are several potential mechanisms by which GPR37 could stabilize MAG, whether the physical association described here is involved in this process or not. First, downstream effects of GPR37 signaling could lead to changes in MAG expression by altering transcription of the *Mag* gene. Secondly, GPR37 signaling could change protein modifications on MAG (i.e. phosphorylation, glycosylation, ubiquitination) that alter the pattern of MAG degradation. This type of regulation of MAG has been demonstrated with another GPCR, very large G-protein

coupled receptor 1, which inhibits MAG ubiquitination via $G_{\alpha s}/G_{\alpha q}$ signaling, promoting the stability of MAG by reducing its degradation.[18] It is possible that GPR37 stabilizes MAG via a similar mechanism of signaling resulting in protein modifications, but there is not yet evidence to support this. Alternatively, the physical interaction between GPR37 and MAG may in fact contribute to MAG stability, by a direct binding interaction or the formation of a complex with other proteins, resulting in reduced degradation of MAG. These are all hypothetical mechanisms that remain to be tested. Additionally, it is unknown whether the regulation of MAG and GPR37 is uni- or bi-directional. We have observed the in vivo regulation of MAG by GPR37,[15] but it is possible that MAG also regulates GPR37 expression and function, which could be determined by experiments examining GPR37 expression, localization and functionality in a MAG-knockout mouse line.

These studies build upon the finding that GPR37 and MAG have a physical association, which we have previously demonstrated via co-immunoprecipitation.[15] The data presented here indicate that the interaction is likely facilitated by the transmembrane domain regions of the proteins, bringing us closer to understanding the specifics of the structural association. To provide further evidence, a chimera GPR37 containing transmembrane domains of another, non-related GPCR could be used in co-immunoprecipitation studies with MAG. If the association between MAG and GPR37 were disrupted when the transmembrane domains pertained to a different GPCR, we could then conclude that the TMs of GPR37 specifically are not only sufficient, but necessary, for the interaction with MAG. When the details of the interaction are elucidated enough to be able to manipulate and disrupt the interaction between GPR37 and MAG in vitro and in vivo, these models will serve as useful tools to study the implication of GPR37 and MAG in demyelinating disorders, as well as the signaling, pharmacology, and overall function of the receptor.

The capacity of GPR37 to interact with a single-pass transmembrane protein like MAG is not unique among GPCRs. Based on characterizations of other GPCR-protein interactions, there is reason to hypothesize that the interaction of GPR37 with MAG may have some effect on the function and pharmacology of the receptor. Various single transmembrane domain interacting proteins are known to modulate the signaling, pharmacology, folding, and subcellular localization of GPCRs.[19] For example, a class of single-transmembrane proteins known as receptor-activity modifying proteins (RAMPs) are known to physically associate with class B GPCRs to modify receptor function, with effects such as changes in receptor trafficking and ligand selectivity.[20] One class B receptor in particular, the secretin receptor, has been shown to interact physically with RAMP3 via the transmembrane domain region, with TMs 6 and 7 of the receptor associating with the RAMP3 TM.[21] This represents a similar transmembrane domain-facilitated physical interaction to the one proposed here between GPR37 and MAG. This work in mapping the interaction between GPR37 and MAG contributes to the growing field of transmembrane protein interactions with GPCRs, which provides important context for understanding the modulation of GPCR function.

There is much that remains unknown about GPR37, and at this time it is still considered an orphan receptor. However, our work and that of other groups has shown that GPR37 is a promising potential drug target for diseases ranging from Parkinson's disease to multiple sclerosis. For demyelinating disorders in particular, it may prove to be a good target due to its enrichment in myelinating oligodendrocytes specifically, and its interaction with myelin proteins such as MAG.

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Figures 2 and 3 and all GPR37/MAG diagrams created in BioRender.com