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Rhodopsin: the Functional Significance of Asn-Linked Glycosylation and Other Post-translational Modifications

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Abstract

Rhodopsin, the G-protein coupled receptor in retinal rod photoreceptors, is a highly conserved protein that undergoes several types of post-translational modifications. These modifications are essential to maintain the protein's structure as well as its proper function in the visual transduction cycle. Rhodopsin is *N*-glycosylated at Asn-2 and Asn-15 in its extracellular N-terminal domain. Mutations within the glycosylation consensus sequences of rhodopsin cause autosomal dominant retinitis pigmentosa, a disease that leads to blindness. Several groups have studied the role of rhodopsin's *N*-linked glycan chains in protein structure and function using a variety of approaches. These include the generation of a transgenic mouse model, study of a naturally occurring mutant animal model, *in vivo* pharmacological inhibition of glycosylation, and *in vitro* analyses using transfected COS-1 cells. These studies have provided insights into the possible role of rhodopsin glycosylation, but have yielded conflicting results.

Keywords

rhodopsin; glycosylation; retina; post-translational modifications; retinal diseases

INTRODUCTION

G-protein coupled receptors (GPCRs) are seven-pass membrane-spanning proteins that facilitate a variety of fundamental cellular processes and are essential for the maintenance of cellular homeostasis (1,2). They constitute the largest family of cell-surface receptors and are highly homologous structurally, although they recognize a large variety of ligands.

GPCRs are generally classified into three distinct families (A, B and C) based on sequence similarity, although a fourth specialized class (*i.e.*, the Frizzled class), also has been characterized (1–7). Alternatively, GPCRs can be grouped into two classes based upon the location of the ligand-binding region (7). One class contains a ligand-binding pocket that is

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formed from the transmembrane portion of the protein, while a second class has a ligand-binding domain that is formed mainly from the amino terminus of the protein (7).

Rhodopsin (RHO), the most extensively studied GPCR (Figure 1), belongs to the largest of the GPCR families, family A (8), and its ligand-binding domain is buried within the transmembrane portion of the molecule (7). RHO is the rod photoreceptor “visual pigment”, consisting of a protein portion (“opsin”, a single, 348 amino acid polypeptide chain) and a chromophore (11-*cis* retinaldehyde, derived from vitamin A). It is the first component of the visual transduction pathway and is activated by absorption of light in the rod photoreceptor cells of the retina (9–11). The light-activated RHO interacts with and activates its cognate G (GTP-binding) protein, transducin, which subsequently initiates a canonical second-messenger signaling pathway. RHO is highly conserved in vertebrate species, and RHO-like proteins can also be found in lower vertebrates. For example, a RHO-like protein, Rh1, is found in the visual system of the fly, *Drosophila melanogaster* (12–16). Other proteins structurally homologous to RHO include bacteriorhodopsin, proteorhodopsin, and xanthorhodopsin, found in species of archaea, marine bacteria and eubacterium, respectively (17–19). RHO, as well as the analogous visual pigment found in cone photoreceptor cells and the aforementioned RHO-like proteins, are unique among the GPCRs in that they are light-sensitive, undergoing a conformational change to activate their respective G-proteins upon light stimulation (17). Though they are structurally similar and respond to light, this is the extent of the similarities between the vertebrate opsins and other RHO-like proteins. Functionally, the bacterial RHO-like proteins act as proton pumps (17), while RHO, cone visual pigments, and Rh1 are involved in the translation of a light stimulus into a neural signal.

The rod photoreceptor is a polarized cell that consists of a synaptic region, a cell body, an inner segment (IS) and an outer segment (OS) (Figure 2). The “molecular machinery” involved in biosynthesis, energy metabolism, and membrane trafficking, such as the endoplasmic reticulum (ER), Golgi apparatus, and mitochondria, reside within the IS, which connects to the OS via the so-called connecting cilium. The OS is comprised of membranous discs surrounded by a plasma membrane. Over 90% of the total protein in the OS is RHO, which resides both within the membrane of the discs and in the surrounding plasma membrane (1,6,20–23)

RHO is synthesized in the rough ER, transported through the Golgi apparatus and targeted to the plasma membrane, remaining membrane bound throughout its journey through the IS to the OS (11,20,23). In common with other GPCRs, RHO has been shown to form both dimers and higher-order oligomers in the membrane (1). This formation of higher-order complexes is presumably of structural importance to the rod cell. Fotiadis et al. (1) speculated that formation of these complexes may be essential to proper RHO-transducin binding and signaling.

EXPRESSION

The regulation of RHO expression is essential for maintaining a healthy retinal environment. Overexpression of wild-type RHO protein by as little as 23% can lead to degeneration of the retina (24). This degenerative phenotype is of unknown origin, but it is thought that as a result of increasing opsin synthesis, the photoreceptor cell is functioning at its maximum translational capacity (24). Such a situation would be unsustainable and might ultimately lead to the cell death observed.

In addition, the effects of losing either one or both copies of the RHO gene (*rho*) have been studied using a mouse model lacking RHO protein expression due to targeted gene disruption (25). The loss of ~50% of the protein leads to a developmental delay in the size of the OS. However, by 30 days of age the retinas of these animals are indistinguishable from those of animals that have both copies of endogenous opsin. Upon the loss of both alleles of *rho*, there

is no detectable protein expressed, no formation of OS, and by P90 there is almost complete loss of rod photoreceptor cells (25)

THE DOMAINS OF RHODOPSIN

RHO has four specialized domains: the cytoplasmic, intradiscal, ligand-binding, and transmembrane domains (8,26). Each domain is responsible for assisting in the maintenance of either the proper structure of RHO, its trafficking through the cell, or its role in phototransduction.

RHO's cytoplasmic C-terminal domain is essential in regulating both the trafficking of RHO and in forming proper interactions with other proteins in the phototransduction cascade (27–31). When activated by a photon of light, RHO undergoes a conformational change which, upon binding to transducin, activates that G-protein by promoting GTP/GDP exchange (32, 33,34). Activation of transducin is followed by activation of a cGMP phosphodiesterase, resulting in hydrolysis of cGMP to 5'-GMP. The subsequent closure of cyclic nucleotide-gated (CNG) ion channels in the OS plasma membrane hyperpolarizes the cell and begins the neural transmission process (35). Although studies on a transducin knockout mouse model system demonstrated that transducin is required for rod function, the survival of the rod cells is not affected, indicating that RHO does not need to be a functional molecule to support the survival of the cell (36).

During the process of shutting off photo-activated RHO, the protein arrestin binds to the phosphorylated C-terminal tail as well as cytoplasmic loops I and II of RHO (37,38). Binding of arrestin to cytoplasmic loop III is thought to be directly correlated to the phosphorylation state of RHO's C-terminus (38). This binding of arrestin to RHO blocks RHO's interaction with transducin (37,39,40). In a mouse model in which arrestin is knocked out, there was an abundance of photo-activated RHO, but initial analysis of retinal morphology showed that the overall structure of the retina was not affected by the presence of this activated, phosphorylated RHO (41). However, upon careful examination of the rod photoreceptor cells, it was found that there was both disorganization and a decrease in the length of the OS (41). Interestingly, animals that were reared in darkness showed less prominent retinal abnormalities, suggesting that a RHO molecule that is unable to be turned off after light stimulation can be quite detrimental to the health of the retina (in contrast to an un-activated molecule). In *Drosophila* as well as in the RHO mutant transgenic mouse model K296E, it was found that formation of a persistent RHO/arrestin complex was implicated in photoreceptor cell death, and when this complex was prevented from forming, degeneration was also prevented (42–45). These results support the hypothesis generated from the arrestin knock-out mouse model; namely that a constitutively activated form of RHO is harmful to the retina (41). The C-terminus of RHO also has been shown to be essential for RHO's proper transport through the secretory system. Upon a loss of its five highly conserved C-terminal amino acid residues (QVS(A)PA), RHO is improperly transported through the secretory system, specifically the *trans*-Golgi network (20,28).

The intradiscal/extracellular domain of RHO contains the N-terminus as well as several extracellular loops between the transmembrane domains. A number of mutations within this domain have been shown to lead to autosomal dominant retinitis pigmentosa (ADRP), a retinal degenerative disease that is characterized by night blindness and an initial loss of peripheral vision. As the disease progresses there is an eventual total loss of vision (46,47). It is thought that mutations at residues within the intradiscal domains lead to protein misfolding and subsequent accumulation of the protein in the secretory system, leading ultimately to the disease phenotype (48,49).

Deletion mutations within the intradiscal loops show differing phenotypes (50). Several mutants produce phenotypes that are defective in regenerating the RHO chromophore. Also, many of the mutants led to expression of RHO with incorrect glycosylation patterns. These phenotypes can arise from deletions in any of the loops, highlighting the contribution of each in generating and maintaining the proper protein structure. In contrast, the cytoplasmic loops are thought to function more in regulating RHO's biochemical functions (50).

The ligand-binding domain, where the opsin apoprotein binds 11-*cis* retinaldehyde to form RHO, is within the seventh transmembrane portion of the molecule (51–54). Upon light stimulation, the 11-*cis* retinaldehyde isomerizes leading to structural changes in RHO and subsequently to transducin activation (55–57).

The transmembrane domains of RHO contain several residues that have been shown to be essential for the maintenance of protein stability and function. Mutations within the first and second transmembrane domains can lead to ADRP (G51A/V and G89D) or congenital night blindness (CNB) (G90D), another disorder that results in loss of rod vision (58–62). It was found that mutations within the first transmembrane segment (G51A/V) allowed for the proper folding of RHO, but still led to the disease through a destabilizing effect on the molecule. The mutations within the second transmembrane domain (G89D and G90D) led to defects in the interaction of RHO with transducin (58–60,63).

RHODOPSIN MUTATIONS AND THE MANIFESTATION OF ADRP

Several RHO mutations lead to retinal degenerations such as ADRP, autosomal dominant CNB and autosomal recessive retinitis pigmentosa (ARRP) (20,48,53,64–70). A documented case of ARRP resulted from a mutation in the RHO gene that led to a protein that was missing the sixth and seventh transmembrane domains, including the 11-*cis* retinal binding site (70). RHO mutants associated with CNB appear to arise as a result of the expression of a constitutively active form of opsin that can catalytically activate the G-protein transducin in the absence of chromophore and in the absence of light (63,69).

Generally speaking, RHO mutants that lead to ADRP have been grouped into three classes that exhibit strikingly different protein characteristics (48,71,72). Class I mutations reside near the C-terminus of the protein or within the first transmembrane segment. *In vitro* they resemble the wild-type protein in both expression levels and signaling capability (48,65,66). However, the mutations seem to interfere with the targeting of the protein to the OS. Class II mutants differ from the Class I mutants in that they are unable to stably associate with 11-*cis* retinal and they are expressed at low levels (65,66,73). These mutations have been shown to reside primarily in the transmembrane, intradiscal and cytoplasmic domains and are responsible for 85% of the observed disease causing mutations (20). It is believed that Class II mutants lead to defective folding and/or stability of the protein, as there is often an observed accumulation of the mutant protein in the secretory system (48,65). The third group, Class III, consists of mutant proteins that form only a small amount of pigment (protein + chromophore) and are usually retained in the ER or form aggresomes (72).

POST-TRANSLATIONAL MODIFICATIONS

RHO undergoes several post-translational modifications (PTMs) that have been shown, both *in vitro* and *in vivo*, to be essential for proper structure and function. Below is a description of RHO's PTMs in their general order of occurrence during protein synthesis and transport; the early post-translational modifications in the ER are in no particular order.

1. Binding of 11-cis Retinaldehyde

In the ER, the opsin apoprotein can be covalently linked at Lys-296 (K296) to the chromophore, 11-*cis* retinaldehyde, via the formation of a Schiff base to form the visual pigment, RHO (53, 54,74–79) (Figure 1, number 1). The change in the spatial conformation of the 11-*cis* retinaldehyde and RHO upon light stimulation allows RHO to properly interact with and activate its G-protein. A mutation at K296 (K296E) has been shown to lead to RP (42,80). Histologic analysis of a mouse model carrying this mutation revealed progressive photoreceptor degeneration, although RHO was localized properly to the OS and the mutant was able to bind to arrestin (80). Further studies showed that retinal degeneration resulting from this mutation does not occur through continued activation of transducin and the phototransduction cascade, but rather through the formation and accumulation of the mutant RHO/arrestin complexes (42).

2. Disulfide Bond Formation

Of structural importance to RHO is the formation of an intramolecular disulfide bond between Cys-110 and Cys-187 to connect helices IV and V (6,81) (Figure 1, number 2). This modification occurs early in the process of RHO's folding process and transport in the ER and helps maintain the structure of RHO in its favored conformation. Misfolded RHO mutants, such as P23H, have been found to have an abnormal Cys110-Cys187 bond, however, this incorrect bond was not solely responsible for the misfolding (81).

3. Palmitoylation

While the protein is in the ER, the C-terminal tail of RHO is palmitoylated at Cys-322 and 323 (Figure 1, number 3) (82,83). This modification has been shown to be essential in the tethering of the C-terminal tail to the membrane as well as in the formation of an interacting domain with transducin (1,6,11,53,84,85). A study using palmitoylation-deficient RHO found that there was a loss of visual sensitivity as well as an increase in the phosphorylation of RHO upon light stimulation. However, the overall morphology of the retina was little affected and researchers determined that palmitoylation was not necessary for the proper expression, stability, or transport of RHO (85).

4. Acetylation

Acetylation occurs on the N-terminal methionine residue of RHO (Figure 1, number 4) (6, 86,87). This modification is required to neutralize the positively charged lysine residues and facilitates the activation of transducin (86).

5. Phosphorylation

Upon the termination of RHO signaling, the protein undergoes phosphorylation via rhodopsin kinase (RK) (Figure 1, number 5) (6,88). Phosphorylation of RHO occurs at the C-terminus on six to seven Ser/Thr residues *in vitro*, though the three favored sites *in vivo* are three serine residues (S336, S338 and S343) (6,38,89,90). Protein kinase C may also play a role in phosphorylating RHO, though its phosphorylating role in the photoreceptors is unclear (89, 91). Without RHO phosphorylation by RK, arrestin binding is impaired, thereby preventing RHO from returning to its basal conformational state (11,35,37,88). Activated RHO will continue to activate transducin if phosphorylation does not occur (9,11,35,89,92,93). Studies with a RK knockout mouse found that without RHO phosphorylation, there was slowed deactivation of RHO upon light exposure (35). In addition to defective RHO signaling, the retinas of RK knockout mice raised in normal, cyclic light have shorter OSs and reduced levels of RHO and other photoreceptor proteins. This OS shortening was less noticeable in animals that were transferred from these lighting conditions to complete darkness suggesting that prolonged activation of RHO leads to retinal degeneration.

6. Ubiquitinylation (Ubiquitination)

RHO can be ubiquitinated while in the secretory system or after it has been transported to the plasma membrane (71,94) (Figure 1, number 6). The exact site(s) of ubiquitinylation on RHO are unknown, but are thought to be on one or several cytoplasmic lysine residues. Obin et al. (94) suggest that ubiquitinylation could modulate the transducing properties of RHO. This role for ubiquitinylation is plausible because seven out of the nine cytoplasmic (and possibly ubiquitinated) lysines are within regions that participate in protein binding and activation, receptor folding and stabilization, or receptor phosphorylation and arrestin binding (94). Other possible roles of ubiquitinylation include, but are not limited to, controlling the levels, activities, trafficking and phosphorylation of RHO (94).

7. Asn (N)-linked Glycosylation

N-linked glycosylation is a highly conserved post-translational modification that occurs on GPCRs (6,95–97). Vertebrate RHO undergoes *N*-linked glycosylation at its N-terminus on Asn-2 and Asn-15 (Figure 1, number 7) (98,99). A protein sequence alignment of RHO from several different species (generated using ClustalW <http://www.ebi.ac.uk/Tools/clustalw/>) highlights the high degree of conservation of these glycosylated residues (Figure 3). Invertebrate RHO *N*-linked glycosylation has not been extensively studied, with the exception of the fruit fly, *Drosophila melanogaster* (12–16). Another fly species, *Calliphora*, has putative *N*-glycosylation sites, though these have not been well characterized (100). Studies on squid (101) and octopus (102) RHO have also found putative *N*-linked glycosylation sites at residues other than those found in vertebrates. Extensive studies of mammalian RHO have not found other sites of glycosylation (*N*- or *O*-linked) on the molecule (98,102–105); however, *O*-linked glycosylation has been found on octopus RHO (102).

RHO is core glycosylated in the rough endoplasmic reticulum (rER), a process that involves a transfer of a Glc₃Man₉GlcNAc₂ chain (where Glc is glucose, Man is mannose, and GlcNAc is N-acetyl-glucosamine) from a dolichylpyrophosphate donor to two residues, Asn-2 and Asn-15, that are within the *N*-glycosylation consensus sequence (AsnXSer/Thr, where X can be any amino acid, except Pro) (13,15). The glycan moiety undergoes further processing and trimming during its transport through the Golgi apparatus. Upon exit from the secretory system, Asn-2 and Asn-15 typically will have relatively short oligosaccharide chains consisting primarily of three Man and three GlcNAc residues, although 1–2 additional Man residues also may be present on the arm of the glycan not “capped” by a terminal GlcNAc (98,104). Frog (*Rana pipiens*) RHO also has been found to possess more complex *N*-glycan chains, in addition to those found on bovine RHO, including the additional sugars galactose (Gal) and sialic acid (NeuAc) (106). Interestingly, the sialylated glycan chains were only found on the Asn-2 site.

Several potential roles for RHO's *N*-linked glycosylation have been suggested. It is important to note that these roles are not necessarily mutually exclusive. First, while in the secretory pathway and during post-Golgi sorting, glycosylation may be a determinant in the fate of the protein (107). Glycosylation is thought to be essential for the binding of chaperones to assist in protein folding as well as in translocation through the secretory system (107,108). The interaction with the chaperone is influenced by the composition of the glycosylated moieties on a protein (107–110). Without the proper chaperone binding, the protein will be unable to fold properly and subsequently will be targeted for degradation. Thus, glycan chain composition is a significant determinant of whether or not the protein will be targeted for ER-associated degradation (ERAD) (107). The relative activities of glycosidases in the secretory pathway play a role in regulating the timing of ERAD by protecting newly synthesized proteins from early degradation (107).

Studies using *Drosophila melanogaster* found that although glycosylation is not required for the association of RHO with its chaperone, it is required for the exit of fly opsin from the intracellular secretory pathway (13). As opposed to vertebrate opsins, which are *N*-glycosylated at two residues, the fly opsin is *N*-glycosylated at only one, Asn-20 (16). Fly opsin also differs from vertebrate RHO in that it is not glycosylated in its mature form (16). Therefore, in order for fly opsin to exit the secretory system, the sugar moieties must be removed. This indicates that the glycosylation of fly opsin is necessary for the maturation and transport of the protein, but not for rhabdomeric membrane assembly or for its role in the phototransduction cascade.

A second role of RHO glycosylation is in protein trafficking. It has been shown in two different systems that glycosylation may be essential for the proper targeting of RHO to the rod OS (43,111). A third role relates to the proper morphogenesis and assembly of discs in the rod OS (48). With regard to this latter role, it was shown that blocking core *N*-glycosylation of RHO in the *Xenopus laevis* retina *in vitro* did not interfere with intracellular RHO trafficking to the site of OS membrane assembly; however, once there, the nonglycosylated RHO could not be assembled into nascent disc membranes with normal morphology (112). Instead, vesicular membranous blebs formed within the space between the IS and OS of rod photoreceptor cells (termed the “inter-segmental space”). However, once the core glycan is added to RHO, interfering with subsequent post-translational processing of the *N*-linked glycan chains (*e.g.*, with the glycosidase inhibitors swainsonine or castanospermine) does not perturb disc morphogenesis (113). This suggests that, although glycan chains are required for normal disc assembly in vertebrate rod photoreceptors, the exact nature or chemical composition of the glycan chains is not rigidly specified. It has been proposed that the sugar moieties on RHO may play an active role in disc membrane assembly, involving at least three possible mechanisms (114): one is a direct carbohydrate-carbohydrate interaction between the oligosaccharides of RHO on the two opposing membrane surfaces that ultimately will come together to form an OS disc; a second is glycan-directed allosteric change in the conformation of the N-terminal domains of RHO molecules on those opposing membrane surfaces, thereby promoting van der Waals interactions between them; and third is the interaction of RHO’s oligosaccharide chains with other molecular species, such as endogenous lectins.

A fourth potential role for glycosylation could be in stabilizing the position of RHO in the disc membranes (115,116). Fifth, glycosylation may be needed for proper ROS shedding and/or phagocytosis of the shed ROS by the adjacent retinal pigment epithelium (RPE) (111,117–120).

Several groups have studied RHO glycosylation using pharmacological and biochemical methods *in vitro* and *in vivo*. Plantner et al. (121) incubated bovine retinas with radiolabeled monosaccharides *in vitro* in the presence of tunicamycin (TM), which is a general inhibitor of protein *N*-glycosylation. In that study it was found that lack of glycosylation did not inhibit newly synthesized opsin incorporation into ROS membranes. However, in a subsequent *in vitro* study mentioned above (112) using *Xenopus laevis* retinas, it was found that nonglycosylated, newly synthesized opsin was trafficked efficiently through the inner segment to the site of ROS disc membrane assembly at the base of the ROS, but did not get incorporated into the ROS *per se*. Rather, it was incorporated into disc membrane precursors that were abortively assembled and blebbed off into the inter-segmental space, a fact that was only realized by employing EM and EM autoradiography (112). The discrepancy between these two studies is best explained by the comigration (due to similar physical properties, *e.g.*, buoyant density) on sucrose gradients of these abortively assembled membranes and *bona fide* ROS membranes. However, although a parallel study using frog (*Rana pipiens*) retinas found that blocking RHO glycosylation with TM resulted in the lack of assembly of opsin into new ROS membranes (122), this appeared to be due to a defect in intracellular trafficking, with

accumulation of the nonglycosylated opsin in the ER. Hence, different (and somewhat conflicting) results are obtained, depending on the particular biological system employed.

Other investigators have used site-directed mutagenesis and a heterologous expression system to examine the role of RHO glycosylation. Kaushal et al. individually mutated Asn-2 or Asn-15, or both sites (Asn-2, -15) to render them glycosylation-incompetent and transfected these constructs into COS cells (105). This study showed that the mutation at Asn-2 did not block intracellular transport of the mutant protein to the plasma membrane of the cell, nor did it prevent the ability of the mutant protein to bind the retinaldehyde chromophore stably, suggesting that it would be functionally competent. In contrast, the Asn-15 mutant and the double mutant were unable to bind chromophore efficiently, and they were not able to translocate to the cell surface; instead, they remained in the perinuclear region, presumably in the ER. These latter findings are consistent with the findings of Fliesler and Basinger (122) using the *in vitro* frog retina system, but conflict with the results of the studies by Plantner et al. (121) and Fliesler et al. (112) mentioned above.

Mutations within the glycosylation consensus sequence for either Asn-2 (T4K/R) or Asn-15 (N15S, T17M) are associated with instances of retinitis pigmentosa (123–127). Also, mutations within either of the consensus sequences have been shown to interfere with the glycosylation of RHO at their respective glycosylation sites. A naturally occurring point mutation in the first (Asn-2) glycosylation consensus sequence of RHO (a Thr to Arg change at amino acid position 4, T4R), which prevents glycosylation at Asn-2, occurs in an English mastiff dog strain (124). Using this animal model, it was found that expression of the mutant (T4R) opsin protein was deleterious, in that the dogs displayed increased sensitivity to light-induced retinal damage (124). Also, the glycosylation-deficient mutant protein was shown to regenerate RHO poorly with 11-*cis* retinaldehyde and its stability was compromised. Consistent with the Kaushal et al. study (105), lack of glycosylation at Asn-2 did not affect the intracellular transport of this protein in photoreceptors. In fact, the T4R protein is targeted properly to the OS. The results from the T4R model contradict the TM/*Rana pipiens* (122) study, although presumably both sites of glycosylation in the latter model were affected.

The T17M transgenic mouse model expresses T17M human RHO (126,128). This mutation is within the glycosylation consensus sequence for Asn-15 and leads not only to a loss of glycosylation at this site, but also results in concomitant electrophysiological dysfunction and severe retinal degeneration (126).

CURRENT RESEARCH ON RHODOPSIN GLYCOSYLATION

To gather further insight into the importance of RHO glycosylation in the formation and maintenance of ROS in the native (*in vivo*) retinal environment, we have generated several lines of a novel RHO glycosylation mutant mouse model, designated as “No-Glycosylation” (NOG) mice (129). Site-directed mutagenesis was used to generate a construct that has both asparagines (Asn-2, -15) mutated to glutamines, obliterating both sites of *N*-glycosylation. In this animal model we found that, even in the presence of endogenous (wild-type) RHO, there were ROS histological and ultrastructural abnormalities, including a marked reduction both in OS length and ONL thickness (Figure 4B) as well as vesiculation and disorganization of OS membranes (Figure 4D, asterisks), that were not present in the age-matched non-transgenic cohort (*cf.* Figures 4A and 4C) (129). Retinal function was also compromised, as assessed by electroretinography (ERG), which correlates well with the observed histological and ultrastructural defects (129). Both the reduction in function and the structural anomalies of the retina in the NOG animals worsened with age (129). It remains to be seen what phenotype is obtained when the NOG transgene is expressed on a *rho*^{-/-}

background. However, based upon the studies summarized above (105,112,122), one would predict no rod OSs would form, resulting in severe retinal degeneration.

In summary, each of the post-translational modifications that RHO undergoes is either essential for the proper morphogenesis and maintenance of rod OS structure or for the fine-tuning of protein function. Due to its high degree of conservation, it is apparent that *N*-linked glycosylation plays an essential role in RHO's function. The NOG animal model supports the idea that glycosylation is essential for proper disc and outer segment structure. Studies from vertebrate model systems, in contrast to those using *Drosophila*, indicate that RHO glycosylation is essential for the protein's stability and light-dependent function.

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Figure 1. Rhodopsin and its sites of post-translational modifications

RHO is a 7-pass G-protein coupled receptor (GPCR) that resides both within the disks of the rod outer segments and within the plasma membrane surrounding those disks. The protein undergoes several post-translational modifications that are indicated here in their order of occurrence during the synthesis and intracellular transport of RHO: (1) Schiff base formation upon binding of the chromophoric ligand, 11-*cis* retinaldehyde, to Lys-296 [white square]; (2) disulfide bond formation between two cysteine residues [grey hexagons]; (3) palmitoylation at two cytoplasmic cysteines [grey spotted circles]; (4) acetylation at its N-terminal methionine [black circle]; (5) phosphorylation at three C-terminal residues [grey diamonds]; (6) ubiquitinylation of an unknown number of cytoplasmic lysines [white spotted circles]; and (7) N-linked glycosylation at its extracellular N-terminus [grey and black diagonal circles]. Cytoplasmic loops I, II, and III are also depicted.

Figure 2. Schematic of the rod photoreceptor cell

It is a polarized neuronal cell type that contains a synaptic region, cell body, inner segment, connecting cilium and outer segment. RHO is synthesized in the rER in the inner segment and is translocated through the Golgi to the base of the outer segments where the formation of new disks and plasma membrane occurs. The RHO protein is found both within the disk and the plasma membrane of the cell.

Figure 3. Conservation of the asparagines (N) involved in N-linked glycosylation

Sequence alignment of the first 50 amino acid residues of RHO from several vertebrate species is shown. The alignment was generated using ClustalW

(<http://www.ebi.ac.uk/Tools/clustalw/>). The glycosylated residues at N-2 and N-15 are in grey and are designated by an arrow at the top of the alignment.

(*), indicates that the residue is identical; (:), indicates that the residue is conserved; (.) indicates that the residue is semi-conserved.

Figure 4.

Histological and ultrastructural appearance of retinas from non-transgenic mice (A,C) and NOG transgenic mice on a *rho*^{+/-} genetic background (B,D). Magnification: A and B, 40x; C and D, 10,000x. Asterisks (panel D) indicate OS structural abnormalities. Abbreviations: RPE, retinal pigment epithelium; OS, outer segment layer; IS, inner segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer.