Topological analysis of peripherin/*rds* and abnormal glycosylation of the pathogenic Pro^{216} → Leu mutation

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Peripherin/*rds* is an integral membrane glycoprotein found in the rim regions of vertebrate photoreceptor cell discs. The protein is believed to be involved in both formation and maintenance of the characteristic flattened morphology of the outer segment discs and its essential nature is demonstrated by the wide range of retinal degenerative disorders in which the protein has an involvement. Little structural data has been determined for peripherin/*rds*, but a topological model of the protein has been proposed. In this paper, we present the first direct evidence for the topology of the protein through the use of scanning glycosylation mutagenesis. Both the topological data and the observation that only the Asn^{229} site is efficiently glycosylated in this *in vitro* transcription/translation system support the common hypotheses. Additionally, expression of the Pro^{216} → Leu mutant demonstrates an abnormal glycosylation pattern, which may explain the mechanism by which this mutation precipitates a retinal degenerative phenotype.

Key words: peripherin-2, photoreceptor outer segment, retinal degeneration, retinitis pigmentosa, scanning glycosylation mutagenesis.

INTRODUCTION

Peripherin/*rds* is a 39 kDa integral membrane glycoprotein localized exclusively in the rim regions of photoreceptor cell discs [1,2]. The protein has been shown to interact non-covalently with a related disc rim protein ROM-1 (rod outer membrane 1) [3] to form a mixture of homo- and hetero-tetrameric core complexes [4,5]. These 'dimers of dimers' associate further through Cys^{150}-mediated intermolecular disulphide bridges to form higher-order oligomers, which are believed to be involved in photoreceptor disc rim maintenance [6,7].

Peripherin/*rds* has long been implicated in the maintenance of the characteristic shape of the photoreceptor outer segment discs owing to the phenotypes associated with mutations in the protein. Recent results demonstrate that the protein is capable of inducing vesicular flattening in an *in vitro* system [8]. The human peripherin/*rds* gene is therefore of great clinical significance as illustrated by its involvement in a wide range of degenerative retinal disorders, including retinitis pigmentosa [9]. A detailed knowledge of the structure of the protein would therefore be beneficial in the search for therapies for this form of retinal degeneration. A most important first step in this structural appreciation would be a topological delineation of the protein in the lipid bilayer.

Examination of the hydrophobicity of the bovine peripherin/*rds* sequence, using Kyte–Doolittle plots [10], predicts that there are four hydrophobic regions of sufficient length to span the bilayer ([11]; Figure 1). Antibodies directed against the C-terminal region of peripherin/*rds* interact on the cytosolic face of the membranes, thereby orienting this region of the protein [1]. On the basis of these two pieces of evidence, Connell and Molday [11] proposed the topological model shown in Figure 1.

This paper describes the topological investigation of peripherin/*rds* using the technique of scanning glycosylation mutagenesis. This procedure involves the use of native and engineered acceptor sequons for *N*-glycosylation (Asn-Xaa-Ser/Thr) within putative intracellular and extracellular domains of polytopic membrane proteins to determine the location of these sites.

The major requirement for *N*-glycosylation is that the acceptor site must be exposed to the luminal face of the endoplasmic reticulum and therefore have an extracellular location in the mature protein. This restriction is due to the strict compartmentalization of *N*-glycosylating enzymes [12]. In addition, the sequon must reside a minimum of 12–14 residues away from a transmembrane domain. This corresponds to a minimum distance of 40–45 Å (1 Å ≡ 0.1 nm) away from the membrane surface, and this restraint is believed to be imposed by the position of the oligosaccharyltransferase active site 30–40 Å above the luminal membrane surface. This precise distance constraint is known as the '12 + 14 rule' [13], as the acceptor site must be a minimum of 14 residues from the end of the transmembrane segment preceding the loop, and 12 residues from the start of the following transmembrane segment [14]. Efficient *N*-glycosylation therefore requires a minimum loop size of 26 residues (R. A. F. Reithmeier, personal communication).

In addition to providing the first direct experimental evidence for the topology of peripherin/*rds*, using the technique of scanning glycosylation mutagenesis, this study demonstrates the *N*-glycosylation status of both wild-type and mutant peripherin/*rds* in this *in vitro* system. Furthermore, elucidation of the abnormal *N*-glycosylation of the Pro^{216} → Leu mutant demonstrates how this mutation may precipitate retinal degeneration.

EXPERIMENTAL

Plasmid construction

The construct denoted pRDSF contains the murine peripherin/*rds* cDNA [15] inserted into the pBluescript-KS (Stratagene) expression vector, under the control of the T7 promoter. In this construct, the FLAG® antigenic tag (Sigma) was incorporated by PCR mutagenesis, downstream of the peripherin/*rds* cDNA before the stop codon.

Abbreviation used: ROM-1, rod outer membrane 1.

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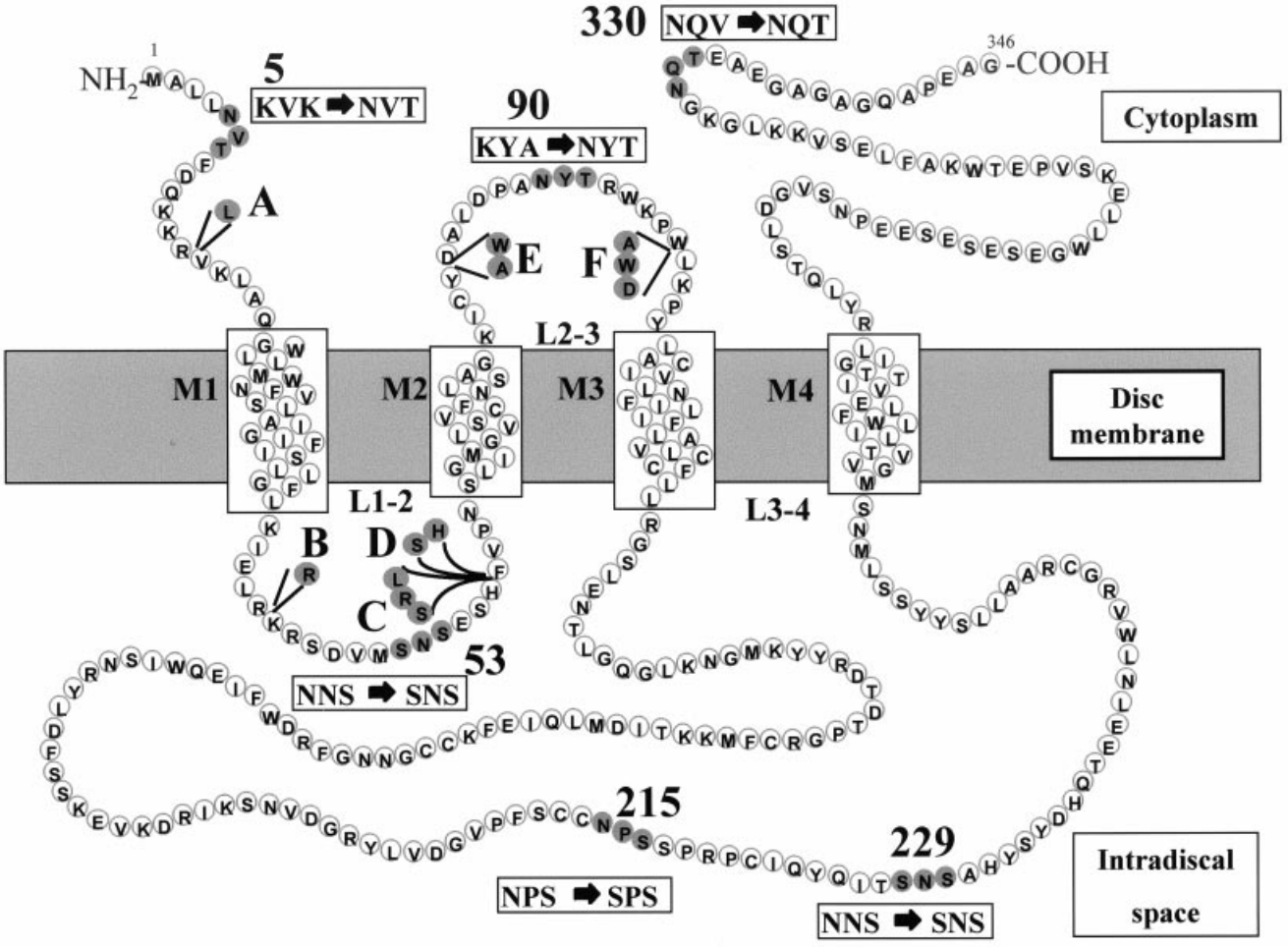

Figure 1 Mutagenesis strategy

The proposed topological model of human peripherin/*rds* [10], detailing the mutagenesis strategy employed in this study. The mutation of endogenous sites at Asn^{53}, Asn^{215} and Asn^{229} and engineered sites at Asn^{5}, Asn^{90} and Asn^{330} is highlighted in addition to the insertions A–F.

In vitro transcription/translation

In vitro transcription and translation were performed using a TNT® coupled reticulocyte lysate system (Promega). Reactions were performed for 90 min at 30 °C in a final volume of 25 μl, using T7 RNA polymerase (Promega), 1 μg of template DNA and 2 μl of [^{35}S]methionine (10 mCi/ml, DuPont NEN), according to the manufacturer's instructions. Where appropriate, reactions were supplemented by the addition of 2.5 μl (5 equivalents) of canine pancreatic microsomal membranes (Promega; 1 equivalent is the amount of membranes required to cleave the signal sequence of preprolactin by 50 %). *N*-Glycosylation was competitively inhibited by the inclusion of 32 μM Ac-Asn-Tyr-Thr-NH_2 [12].

Site-directed mutagenesis

All scanning *N*-glycosylation mutants were produced using the QuikChange™ mutagenesis kit (Stratagene), according to the manufacturer's instructions. Insertional mutagenesis was performed with modifications to the standard protocol as described below [16]. Template DNA, 10 × reaction buffer, primers (Sigma-GenoSys) and milli-Q water (Millipore) were combined according to the standard protocol, and heated to 95 °C for 2 min. The thermal cycler was then turned off and the samples allowed to cool slowly to room temperature (20 °C), before the addition of dNTPs, *Pfu* Turbo (Stratagene) and mineral oil (Sigma) on ice. The reactions were then slowly heated to 68 °C in a water bath and, following incubation at 68 °C for 5 min, were subjected to 18 cycles of 95 °C for 1 min (denaturation), 55 °C for 1.5 min (annealing) and 68 °C for 8 min (extension).

Mutations were confirmed by double-stranded automated DNA sequencing (A.B.I.).

Strategy

An overview of the strategy and details of the mutant constructs produced in this study are detailed in Figure 1 and Table 1.

Removal of endogenous *N*-glycosylation sites

Initially the wild-type pRDSF construct was used as a template for three QuikChange™ mutagenesis reactions which each involved an amino acid substitution within an endogenous sequon, resulting in the loss of that sequon from the mutated protein. The removal of the Asn^{53}, Asn^{215} and Asn^{229} acceptor sites therefore

Table 1 Table of mutants

The mutants of peripherin/*rds* constructed in the present study. For each mutant, the native and engineered *N*-glycosylation sites within that mutant are listed, along with any other mutations within the sequence. In addition, the templates used in the mutagenesis reaction are detailed. Information on the oligonucleotides used in this study may be obtained from the corresponding author. A summary of the results obtained with these mutants is also included, using the following symbols; −, *N*-glycosylation not detected; +, *N*-glycosylation detected; ++, double *N*-glycosylation detected.

Mutant	Native sequons	Engineered sequons	Other mutations	Template	*N*-glycosylation (see Results)
pRDS: −53	Asn^{215}, Asn^{229}	–	–	pRDSF	+
pRDS: −215	Asn^{53}, Asn^{229}	–	–	pRDSF	+
pRDS: −229	Asn^{53}, Asn^{215}	–	–	pRDSF	–
pRDS: +53	Asn^{53}	–	–	pRDS: −215	–
pRDS: +215	Asn^{215}	–	–	pRDS: −53	–
pRDS: +229	Asn^{229}	–	–	pRDS: −53	+
pRDS:P216L	Asn^{53}, Asn^{215}, Asn^{229}	–	$Pro^{216} \rightarrow Leu$	pRDSF	++
pRDS: +215*	Asn^{215}	–	$Pro^{216} \rightarrow Leu$	pRDS: +215	+
pRDS:NULL	–	–	–	pRDS: +229	–
pRDS: +5	–	Asn^{5}	–	pRDS:NULL	–
pRDS: +90	–	Asn^{90}	–	pRDS:NULL	–
pRDS: +330	–	Asn^{330}	–	pRDS:NULL	–
pRDS:InsA	–	Asn^{5}	Insert A	pRDS: +5	–
pRDS:InsB	Asn^{53}	–	Insert B	pRDS: +53	–
pRDS:InsBC	Asn^{53}	–	Insert B, Insert C	pRDS:InsB	–
pRDS:InsBCD	Asn^{53}	–	Insert B, Insert C, Insert D	pRDS:InsBC	+
pRDS:InsE	–	Asn^{90}	Insert E	pRDS: +90	–
pRDS:InsEF	–	Asn^{90}	Insert E, Insert F	pRDS:InsE	–

resulted in three constructs, termed pRDS: −53, pRDS: −215 and pRDS: −229, which each possessed two remaining sequons. One of the remaining native acceptor sites was then removed from each construct by the same procedure. The three resulting constructs, each of which possessed only a single acceptor site, were termed pRDS: +53, pRDS: +215, and pRDS: +229.

Finally, the pRDS: +229 construct underwent further mutagenesis to remove the remaining site and thus produce a null mutant, termed pRDS:NULL.

Introduction of engineered *N*-glycosylation sites

Following the removal of all endogenous *N*-glycosylation sites the resultant null mutant (pRDS:NULL) was used as a template for the introduction of engineered acceptor sites. Three constructs were produced, containing single acceptor sites at Asn^{5}, Asn^{90}, and Asn^{330}. The resulting constructs were termed pRDS: +5, pRDS: +90, and pRDS: +330 respectively.

Extension of extramembranous domains

Insertional mutagenesis was performed on the pRDS: +N5, pRDS: +N53, and pRDS: +N90 constructs, such that the enclosed acceptor sites fulfilled the requirements for *N*-glycosylation. Initially, insertions A, B, and E were performed, yielding products termed pRDS:InsA, pRDS:InsB and pRDS:InsE respectively. The pRDS:InsB construct was then used as a template for the introduction of insert C and pRDS:InsE was used as the template to introduce insert F. The resulting constructs were termed pRDS:InsBC and pRDS:InsEF. Finally, insertion D was performed using pRDS:InsBC as a template and the resulting construct was termed pRDS:InsBCD.

Substitution of leucine for Pro^{216}

Mutagenesis reactions were performed using the pRDSF and pRDS: +215 constructs as templates for the amino acid substitution $Pro^{216} \rightarrow Leu$. The resulting constructs were termed pRDS:P216L and pRDS: +215*.

RESULTS

The production of peripherin/*rds* containing the FLAG® epitope as a 38 kDa species, and the ability of this immunodetectable product to undergo *N*-glycosylation has been demonstrated previously [8]. In addition, the *N*-glycosylation of this product is also visible in Figure 2(c) by an increase in the molecular mass of the expressed protein.

Expression of mutants with endogenous *N*-glycosylation sites removed

Following the successful production of constructs containing two (pRDS: −53, pRDS: −215, and pRDS: −229), one (pRDS: +53, pRDS: +215, and pRDS: +229) and no *N*-glycosylation sequons (pRDS:NULL), all these proteins were expressed *in vitro*. Expression of pRDS: −53, pRDS: −215 and pRDS: +229 yielded products of approx. 38 kDa in the absence of membranes, which displayed an increase in apparent molecular mass of approx. 3 kDa upon addition of microsomal membranes (Figures 2a and 2b). Inclusion of the glycosylation inhibitor Ac-Asn-Tyr-Thr-NH_2 resulted in an almost complete loss of the glycosylated product. Expression of pRDS: −229, pRDS: +53, pRDS: +215 and pRDS:NULL also yielded a product with an apparent molecular mass of 38 kDa, although no band shift was observed in the presence of membranes or Ac-Asn-Tyr-Thr-NH_2 (Figures 2a and 2b).

Expression of mutants containing engineered sequons

In vitro expression of the mutant constructs pRDS: +5, pRDS: +90, and pRDS: +330, which all contain engineered sites, gave similar results. In all cases, a product of 38 kDa was observed in the absence of membranes, in the presence of membranes, and in the presence of membranes with Ac-Asn-Tyr-Thr-NH_2 (Figure 2d).

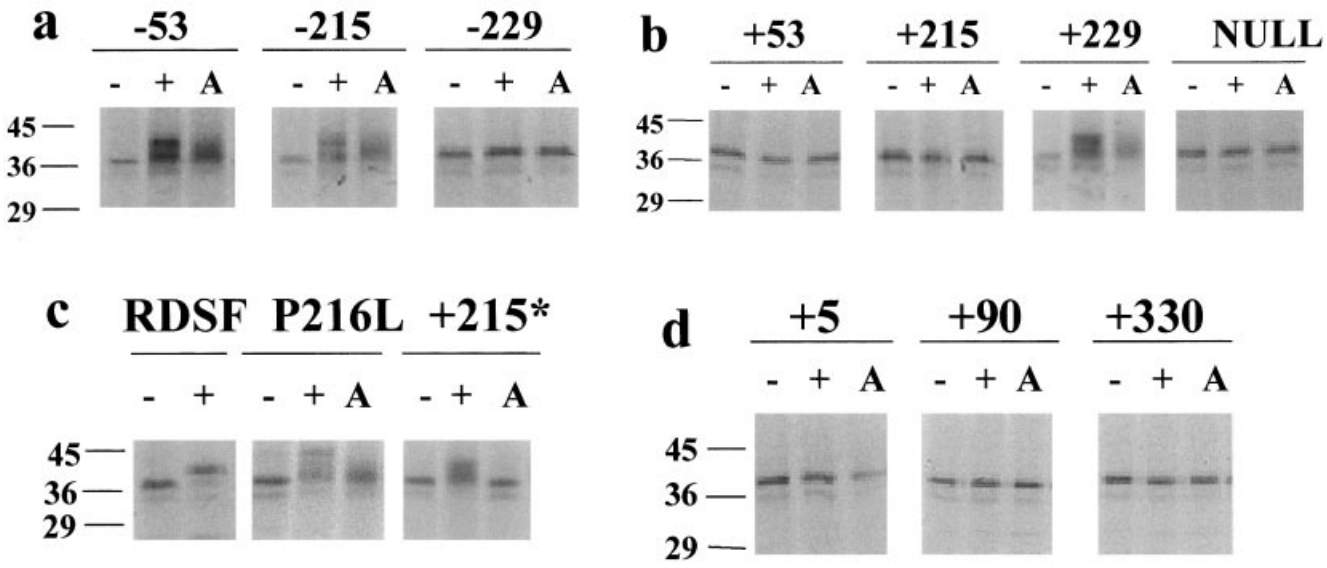

Figure 2 ***In vitro*** **expression of substitution mutants**

Autoradiographs of 10% SDS/PAGE gels showing products from the *in vitro* expression of *N*-glycosylation substitution mutants. Mutants displayed are (**a**) pRDS: −53, pRDS: −215, pRDS: −229; (**b**) pRDS: +53, pRDS: +215, pRDS: +229, pRDS:NULL; (**c**) pRDS, pRDS:P216L, pRDS: +215*; and (**d**) pRDS: +5, pRDS: +90, pRDS: +330. Samples are shown from expression reactions of these mutants in the absence of membranes (−), in the presence of membranes (+) and in the presence of membranes and Ac-Asn-Tyr-Thr-NH_2 (A). Numbers to the left of gels indicate molecular mass (in kDa).

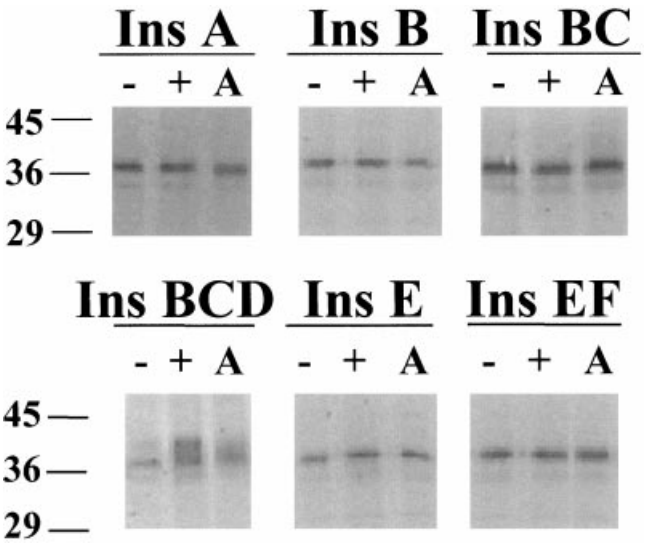

Figure 3 ***In vitro*** **expression of insertion mutants**

Autoradiographs of 10% SDS/PAGE gels showing products from the *in vitro* expression of *N*-glycosylation substitution mutants. Mutants displayed are pRDS:InsA, pRDS:InsB, pRDS:InsBC, pRDS:InsBCD, pRDS:InsE and pRDS:InsEF. Samples are shown from expression reactions of these mutants in the absence of membranes (−), in the presence of membranes (+) and in the presence of membranes and Ac-Asn-Tyr-Thr-NH_2 (A). Numbers to the left of gels indicate molecular mass (in kDa).

Expression of mutants containing insertions

The expression products of pRDS:InsA, pRDS:InsB, pRDS:InsBC, pRDS:InsE and pRDS:InsEF all existed as species of approx. 38 kDa in the absence of membranes (Figure 3). Additionally, no change in molecular mass was obtained upon expression of these constructs in the presence of membranes, or membranes with Ac-Asn-Tyr-Thr-NH_2.

However, expression of the pRDS:InsBCD construct yielded a product of 38 kDa in the absence of membranes (Figure 3), which displayed an increase in apparent molecular mass upon expression in the presence of membranes. This increase, of approx. 3 kDa, was almost completely abolished upon inclusion of Ac-Asn-Tyr-Thr-NH_2.

Expression of mutants containing modifications of Pro^{216}

In vitro expression of the peripherin/*rds* P216L mutant (pRDS:P216L) also yielded a product with an apparent molecular mass of approx. 38 kDa in the absence of membranes (Figure 2c). Expression in the presence of membranes yielded a product of approx. 43 kDa, and this increase of approx. 5 kDa was not observed upon expression in the presence of membranes and Ac-Asn-Tyr-Thr-NH_2.

In contrast, the expression of pRDS: +215*, which contains only the Asn^{215} sequon in addition to the $Pro^{216} \rightarrow$ Leu mutation, yielded a product that behaved slightly differently. In the absence of membranes, expression yielded a product of approx. 38 kDa (Figure 2c). However, in the presence of membranes, a product of approx. 41 kDa was observed. Upon inclusion of Ac-Asn-Tyr-Thr-NH_2 this band shift, of approx. 3 kDa, was almost entirely lost.

DISCUSSION

Rationale

On the basis of hydropathy analysis of peripherin/*rds* [11], a strategy for scanning glycosylation mutagenesis was employed to allow the topology of recombinant peripherin/*rds* in the membrane to be determined.

As murine peripherin/*rds* contains three endogenous sequons for *N*-glycosylation at residues Asn^{53}, Asn^{215} and Asn^{229}, these were deemed suitable to provide information on the two proposed extracellular loops of the protein (L1-2 and L3-4). It was only necessary, therefore, to introduce engineered sites into the N- and C-termini, and the proposed intracellular L2-3 loop, to allow complete analysis of all regions of the protein (Figure 1). In order to determine the ability of a specific sequon to accept an oligosaccharide attachment, however, each mutant must possess only a single sequon. The sequential removal of endogenous sites was, therefore, necessary to produce mutants containing only a single potential glycosylation site. The subsequent production of a null mutant would allow the insertion of single engineered sites in other regions of the protein.

Engineered sites were designed in accordance with the requirements for *N*-glycosylation, thereby utilizing favourable local amino acids and, where possible, sufficient spacing from the membrane to comply with the 12 + 14 rule. In addition, sites were chosen which could be produced by minimal amino acid substitutions. However, it was not always possible to introduce an engineered site that fulfilled all the requirements for *N*-glycosylation. In order to allow efficient *N*-glycosylation of all sequons, a series of six sequential amino acid insertions (A–F) was designed such that the Asn^{5}, Asn^{53} and Asn^{90} sites were repositioned a sufficient distance away from the membrane to comply with the 12 + 14 rule. The insertions, of no greater than 9 bp, were designed to introduce amino acids, characteristic of that region, that should have no significant structural effect on the resulting protein.

Native *N*-glycosylation status of peripherin/*rds*

The sequential removal of endogenous *N*-glycosylation sequons allowed characterization of the glycosylation status of recombinant peripherin/*rds* expressed *in vitro*, which should be indicative of the *in vivo* situation. Expression of pRDS: −53, the mutant form of peripherin/*rds* lacking the Asn^{53} sequon, yielded a product of 38 kDa, characteristic of peripherin/*rds* expression (Figure 2a). Upon inclusion of membranes, this product exhibited a band shift of approx. 3 kDa, comparable with that of wild-type peripherin/*rds*, which was abolished in the presence of the *N*-glycosylation inhibitor Ac-Asn-Tyr-Thr-NH_2. It therefore appears that the Asn^{53} site does not contribute to the glycosylation shift observed with wild-type peripherin/*rds* and is not glycosylated in the native protein.

Similarly, the expression product of pRDS: −215, which lacks the Asn^{215} sequon, also existed as a 38 kDa product that exhibited a similar band shift caused by *N*-glycosylation (Figure 2a). It therefore appears that the N^{215} site is also not *N*-glycosylated in the wild-type protein.

These results are confirmed by the expression of mutant peripherin/*rds* solely containing the Asn^{53} or Asn^{215} site (pRDS: +53, pRDS: +215), which both yielded products that appeared incapable of *N*-glycosylation (Figure 2b). The inability of these two sites to accept an oligosaccharide complies with the strict requirements for *N*-glycosylation, as the Asn^{53} site resides in a loop of only 21 amino acids, which is sub-optimal for *N*-glycosylation [14]. Additionally, the presence of Pro^{216} within the pRDS: P216L protein at the Xaa position within this sequon will block glycosylation at Asn^{215}.

Expression of peripherin/*rds* lacking the Asn^{229} site (pRDS: −229) yielded a 38 kDa species that failed to display a band shift in the presence of membranes (Figure 2a). The removal of the Asn^{229} site therefore appears to completely abolish glycosylation of the protein, implying that the Asn^{229} site is entirely responsible for the glycosylation shift observed with wild-type protein. This was confirmed by the finding that, upon expression of pRDS: +229, which contains the Asn^{229} sequon alone, a product of 38 kDa that undergoes a glycosylation shift comparable with wild-type peripherin/*rds* was observed, confirming the extracellular location of this site (Figure 2b). The *N*-glycosylation of recombinant peripherin/*rds*, solely at the Asn^{229} site in this system coupled with the highly conserved nature of this site in all species, and the strict requirements for glycosylation at specific sequons, implies an identical glycosylation status for wild-type peripherin/*rds* in the native system. Indeed, in agreement with this study, published results of the transgenic analysis of peripherin/*rds* *N*-glycosylation [17] demonstrate that the Asn^{229} site alone is *N*-glycosylated in transgenic mice.

Topological examination of peripherin/*rds*

The complete topological analysis of peripherin/*rds* has been possible using the information about the position of the L1-2 and L3-4 loops obtained from the endogenous sequons, in addition to the information supplied by the engineered sequons in the N- and C-termini, and the putative intracellular loop.

The observed ability of the Asn^{229} sequon to undergo *N*-glycosylation in this system firmly orients the large L3-4 loop in an extracellular environment, in agreement with the proposed topological model. However, the other endogenous *N*-glycosylation site within this loop, Asn^{215}, does not display any such modification, presumably due to the presence of Pro^{216} at the Xaa position within this sequon. This hypothesis was confirmed by the observation that a mutant solely containing the Asn^{215} sequon, which is unable to undergo *N*-glycosylation (pRDS: +215, Figure 2b), does display a glycosylation band shift upon conversion of this proline residue into a more favourable leucine residue (pRDS + 215*, Figure 2c). This again demonstrates that the L3-4 loop must reside extracellularly.

In order to demonstrate that the L1-2 loop resides in an extracellular environment, it was necessary to demonstrate *N*-glycosylation at the Asn^{53} sequon. Unfortunately, the Asn^{53} sequon does not naturally undergo *N*-glycosylation (Figure 2b), presumably because it resides in a loop of sub-optimal size. In order to enable this site to undergo glycosylation, the insertion of additional amino acids into the L1-2 loop was necessary. In addition, these insertions must flank the Asn^{53} site on either side, to ensure adequate spacing from the membrane on both sides of the sequon. The mutagenic incorporation of insertions B or C did not affect the inability of the Asn^{53} sequon to undergo *N*-glycosylation (Figure 3). However, upon incorporation of insert D, the mutant product was observed to be *N*-glycosylated (Figure 3). This is a significant result as it illustrates three important pieces of information. First, this is the first reported evidence of the L1-2 loop possessing an extracellular location. Secondly, these results also allow accurate definition of the polypeptide chain with respect to the bilayer. Owing to the precise constraints of the 12 + 14 rule, the requirement for insertion D before glycosylation of the protein successfully localizes the membrane-aqueous border to be within two amino acids of the proposed model. Finally, this result also illustrates that the Asn^{53} site is not utilized in the wild-type protein due to the sub-optimal size of the L1-2 loop.

The introduction of engineered *N*-glycosylation sites into peripherin/*rds* was performed in order to gather topological information about the N- and C-termini, and L2-3 loop of the

protein. This was not possible for the engineered Asn^{5} and Asn^{90} sites, without subsequent insertional mutagenesis. Both the pRDS: +5 and pRDS: +90 constructs yielded a 38 kDa product that was unable to undergo *N*-glycosylation (Figure 2d). This is in agreement with the requirements for *N*-glycosylation as Asn^{5} contravenes the 12 + 14 rule and Asn^{90} is in a loop of sub-optimal size. The C-terminally engineered Asn^{330} sequon does yield topological information about peripherin/*rds*. As this sequon fulfils all the other requirements for *N*-glycosylation, the inability of the pRDS: +330 expression product to glycosylate must be attributed to an intracellular location for this region of the protein (Figure 2d). This result is therefore in agreement with the immunocytochemical localization of the C-terminus to an intracellular environment by Molday et al. [1].

In order to gain topological information about the locations of the N-terminus and the L2-3 loop, it was necessary to introduce amino acids in order to allow sufficient spacing of the Asn^{5} and Asn^{90} sequons from the membrane. The insertion of one amino acid into the N-terminus (insertion A) was sufficient to enable the Asn^{5} site to comply with the 12 + 14 rule. However, despite fulfilling all other requirements, this sequon was still unable to undergo *N*-glycosylation, indicative of an intracellular localization (Figure 3).

Similarly, the insertion of residues into the L2-3 loop (insertions E and F) sufficiently spaced the Asn^{90} glycosylation site from the membrane such that this sequon fulfils all the requirements for *N*-glycosylation. The observed inability of this mutant to undergo *N*-glycosylation therefore also provides experimental evidence of the intracellular location of this loop (Figure 3).

It must also be noted that *in vitro* expression of the pRDS: NULL construct, which contained no endogenous *N*-glycosylation sequons, yielded a 38 kDa product, which was unable to undergo *N*-glycosylation, as expected (Figure 2b).

Taken together, these results confirm the proposed topological model of peripherin/*rds*, localizing the N- and C-termini and the L2-3 loop intracellularly, and the L1-2 and L3-4 loops on the extracellular face of the membrane. The accuracy of this model is also demonstrated in the present study.

Significance of Pro^{216} in peripherin/*rds* structure

One of the ever increasing number of pathogenic mutations within peripherin/*rds* is $Pro^{216} \rightarrow$ Leu [18]. The relevance of this mutation, which has been studied in transgenic mice [19], is that it resides in the Xaa position of the Asn^{215} sequon, and has been predicted to prevent *N*-glycosylation at this site. As already described, the mutation of Pro^{216} to a more favourable amino acid (leucine) in a mutant construct possessing only a single *N*-glycosylation sequon at Asn^{215} allowed glycosylation of the protein (Figure 2c), demonstrating that Pro^{216} is responsible for inhibition of glycosylation at Asn^{215}. This implies that, in peripherin/*rds* solely containing the $Pro^{216} \rightarrow$ Leu mutation, *N*-glycosylation would occur at both the Asn^{215} and Asn^{229} sites.

Throughout the present and related studies [17], the *N*-glycosylation of wild-type peripherin/*rds* has been consistently observed as an increase in molecular mass of approx. 3 kDa. In contrast, upon inclusion of membranes with the $Pro^{216} \rightarrow$ Leu mutant construct (pRDS:P216L), the product displayed an increase in molecular mass of approx. 5 kDa, which is abolished by inclusion of Ac-Asn-Tyr-Thr-NH_2 (Figure 2c). This provides evidence for the presence of attached oligosaccharides at both Asn^{229} and Asn^{215}.

This mutation has been shown to induce photoreceptor degeneration and outer segment shortening in transgenic mice [19] and also to abolish the ability of wild-type peripherin/*rds* to induce vesicular flattening in an *in vitro* system [8]. The pathogenic nature of this mutation may therefore be explained by the double glycosylation of the mutant protein. The attachment of two bulky carbohydrate chains to the protein may result in steric hindrance or altered conformation in the protein. This would therefore explain the dysfunctionality of the mutant protein and the resulting pathogenic phenotype. Future studies investigating the ability of this mutant to interact both with itself and with ROM-1 would prove advantageous in increasing our understanding of the biochemistry and pathogenicity of peripherin/*rds*.

SUMMARY

The work described in this communication provides strong evidence in support of the proposed topological model of peripherin/*rds* [11]. Delineation of the structure of peripherin/*rds* should allow further investigation into determining the structure–activity relationship of the protein and also uncover further the mechanism behind the degenerative effects of pathogenic mutations such as $Pro^{216} \rightarrow$ Leu.

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