Role of the Second Intradiscal Loop of Peripherin/*rds* in Homo and Hetero Associations†

Xi-Qin Ding, Heidi M. Stricker, and Muna I. Naash*

The Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

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ABSTRACT: Peripherin/*rds* (P/*rds*) is a disk rim protein that assembles into homo and hetero complexes with its nonglycosylated homologue, Rom-1, to maintain the integrity of the photoreceptor outer segment. Mutations in the *rds* gene have been identified in a variety of human retinal degenerative diseases. More than 70% of these mutations are located in the second intradiscal (D2) loop, highlighting the functional importance of this region. This study examines the involvement of different regions of the D2 loop in protein associations using a GST pull-down assay and a heterologous coexpression system. The pull-down assay suggests an association of the N-terminal portion (Phe^{120}−Phe^{187}) of the D2 loop with Rom-1 as well as with other P/*rds* molecules. Through peptide competition experiments, the region between Cys^{165} and Asn^{182} of the D2 loop has been identified as the domain for these associations. In a COS-1 cell heterologous expression system, coexpression of the D2 loop along with the intact P/*rds* and Rom-1 hindered the association of the two full-length proteins. In contrast to the homo association of P/*rds* molecules, it seems that the hetero association of P/*rds* with Rom-1 has a more stringent structural requirement. This work defines the crucial domain of the D2 loop, which mediates homo and hetero associations, specifically the regions that lay between Cys^{165} and Asn^{182}. Elucidation of the molecular mechanisms behind the protein−protein associations of P/*rds* and its partners may reveal the pathogenic defects arising from the most common mutations in this gene.

Peripherin/*rds* (P/*rds*)[1] is a membrane-bound glycoprotein localized along the rim region of disks found in rod and cone outer segments (OSs) (*1*, *2*). The importance of this protein was established by a naturally occurring null mutation in the *rds* gene, identified as *retinal degeneration slow* (*rds*). Mice homozygous for this mutation fail to develop OS structures (*3*, *4*), while heterozygous *rds* mice form highly disorganized OSs (*5*, *6*). The role of P/*rds* in photoreceptor integrity has been demonstrated through the link between mutations in the *rds* gene and human retinal degenerative diseases such as autosomal dominant retinitis pigmentosa (ADRP) and several forms of macular dystrophy (MD) (*7*−*10*). Although P/*rds* possesses a clear role in maintaining the structure of rods and cones, insights into its molecular mechanism through protein interactions are quite limited. Currently, P/*rds* is conjectured to play a significant role in disk morphogenesis and stability (*1*, *11*), disk shedding (*1*, *12*), and membrane fusion (*13*, *14*).

Cloning and identification of P/*rds* orthologs suggests that these proteins share several distinctive features, including four hydrophobic transmembrane domains, a large intradiscal (D2) loop containing seven highly conserved cysteines, and a highly charged cytosolic C-terminal segment (*4*, *15*, *16*). Over 70% of the disease-causing mutations in P/*rds* are located in the D2 loop, providing evidence of the importance that this region plays in proper protein function. Early hydrodynamic studies have shown that P/*rds* assembles noncovalently with itself to form homotetramers and with its nonglycosylated homologue, Rom-1, to form hetero-tetramers (*11*, *17*).

P/*rds* and Rom-1 share several characteristics including sequence homology, predicted membrane topology, and OS localization (*17*−*19*). The noncovalently associated hetero-tetramers of P/*rds* and Rom-1 can join via disulfide bonds to generate higher order molecular complexes of undefined sizes (*20*). Indeed, tetramerization and oligomerization are vital mechanisms in the maintenance of the disk rim structure, when considering that any impairment of this process has been observed to result in photoreceptor degradation and visual loss (*21*−*24*). Although the nature of homo and hetero complexes of P/*rds* and Rom-1 has been well-documented, the sites of their interactions have yet to be determined.

This study maps the region of P/*rds* that is necessary for homo and hetero protein associations. The functional importance of the N-terminal portion of the D2 loop in P/*rds* associations was determined by a GST pull-down assay. Through peptide competition experiments, a region between Cys^{165} and Asn^{182} in the P/*rds* D2 loop was identified as a domain for proper homo and hetero protein association. Coexpression of the D2 loop along with P/*rds* and Rom-1

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* To whom correspondence should be addressed. Telephone: (405) 271-2388. Fax: (405) 271-3548. E-mail: muna-naash@ouhsc.edu.

[1] Abbreviations: ADRP, autosomal dominant retinitis pigmentosa; DTT, dithiothreitol; IP, immunoprecipitation; IPTG, isopropyl-β-D-thiogalactopyranoside; MD, macular dystrophy; OS, outer segment; PMSF, phenylmethylsulfonyl fluoride; P/*rds*, peripherin/*rds*.

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Table 1: Primers Used in the Construction of the GST-P/*rds* Fusion Proteins[a]

primer name	regions amplified	sequence
P/*rds*-D2 (F)	$Phe^{120}-Asn^{256}$	5′-ggatcctttctgttgcggggctcc-3′
P/*rds*-D2 (R)		5′-ctcgagattcagcagagcggccct-3′
P/*rds*-D2-N (F)	$Phe^{120}-Phe^{187}$	5′-ggatcctttctgttgcggggctcc-3′
P/*rds*-D2-N (R)		5′-ctcgaggaagtccaggtagcgatt-3′
P/*rds*-D2-C (F)	$Phe^{187}-Asn^{256}$	5′-ggatcctcctccaaggaggtcaaa-3′
P/*rds*-D2-C (R)		5′-ctcgagattcagcagagcggccct-3′
P/*rds*-CT (F)	$Trp^{273}-Gly^{346}$	5′-ggatcctggctctttgaggtgagc-3′
P/*rds*-CT (R)		5′-ctcgagccatcagccagcctctgg-3′

[a] Restriction enzyme sites (*Bam*H I and *Xho* I) are underlined. Forward (F) and reverse (R) primers are indicated.

interfered with the interactions between the two proteins. Identification of this domain on P/*rds* contributes to our understanding of the molecular basis of homo and hetero complex formation of P/*rds* and gives insight into the underlying mechanisms behind the pathogenic defects associated with mutations in this gene.

EXPERIMENTAL PROCEDURES

Plasmids. cDNA sequences encoding the full-length P/*rds* and Rom-1 were obtained by polymerase chain reaction (PCR) amplification of the mouse retinal expression library (λ ZAP II) using the appropriate primers. The PCR fragment was then inserted into the *Bam*H I and *Xba* I sites of the expression vector pcDNA3.1 (Invitrogen Inc., Rockville, MD). In addition, a minigene encoding the P/*rds* D2 loop ($Phe^{120}-Asn^{256}$, P/*rds*-D2) was constructed by inserting a D2 loop PCR product into the multiple-cloning sites of the pcDNA3.1 expression vector. The D2 loop cDNA was amplified by PCR using mouse *rds* cDNA as a template. The amplification product was designed to contain a restriction site (*Bam*H I) at the 5′ end for subcloning, a consensus sequence for the initiation of translation (GCCGCCACC), and codons for methionine and glycine upstream of the D2 loop sequence. At the 3′ end of the amplification product was a stop codon and another restriction site for subcloning (*Xho* I). Authenticity of all constructs was confirmed by sequence analysis.

The GST fusion protein constructs included the P/*rds* full-length D2 loop, ($Phe^{120}-Asn^{256}$, GST-P/*rds*-D2), the N-terminal portion of the D2 loop ($Phe^{120}-Phe^{187}$, GST-P/*rds*-D2-N), the C-terminal portion of the D2 loop ($Phe^{187}-Asn^{256}$, GST-P/*rds*-D2-C), and the C-terminal tail of P/*rds* ($Trp^{273}-Gly^{346}$, GST-P/*rds*-CT) (Figure 2A). These constructs were generated from the full-length *rds* cDNA clone by PCR amplification using the appropriate primers (Table 1). The PCR fragments were inserted into the *Bam*H I and *Xho* I sites of the pGEX-4T-2 vector (Amersham Biosciences, Piscataway, NJ).

Expression and Purification of the GST Fusion Proteins. Expression and purification of the fusion proteins were carried out as previously described by Frangioni et al. (*25*) Briefly, *Escherichia coli* BL21 (DE3) cells possessing the pGEX-4T-2 vector harboring the GST-P/*rds* fusion protein constructs were grown in Luria broth (LB) medium containing ampicillin (50 μg/mL). Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM when the culture reached an optical density of 0.6 at 600 nm. The culture was harvested 2 h after induction. Bacteria were recovered through centrifugation at 6000*g* for 15 min, and the pellets were washed twice with PBS. Cells were then resuspended in ice-cold STE buffer [10 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA, 100 μg/mL of lysozyme, and 1.0 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by sonication at moderate intensity for 30 s. The lysates were solubilized in 1% Triton X-100 for 10 min at 4 °C, and the cellular debris was removed by centrifugation at 16000*g* for 30 min. The fusion protein was affinity-purified using glutathione-Sepharose resin (Amersham Biosciences, Piscataway, NJ) per the protocol of the manufacturer. Purified proteins were separated by 10% SDS−PAGE and stained with Coomassie Brilliant Blue. Specific expression of the fusion proteins were examined via Western blot analysis using an anti-GST antibody (Amersham Biosciences, Piscataway, NJ) as well as antibodies against different regions of P/*rds*. Protein concentration was quantified with the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Cell Culture and Transfection. COS-1 cells were plated on 100-mm tissue culture dishes at 1×10^6 cells/dish in modified Eagle's medium (Invitrogen Inc., Rockville, MD) supplemented with 10% fetal bovine serum for 20 h before transfection using the Lipofectamine 2000 transfection kit (Invitrogen Inc., Rockville, MD). Coexpression of P/*rds*, Rom-1, and the P/*rds* D2 loop in COS-1 cells was accomplished by cotransfection of the respective cDNAs into the cells. A constant amount (8 μg) of cDNA for P/*rds* and Rom-1 and varying amounts (2−8 μg) of cDNA for the D2 loop constructs were used for transfection. COS-1 cells transfected with the empty plasmid were used as a negative control. Cells were harvested 48 h post-transfection, and protein extracts were prepared for Western blot and immunoprecipitation (IP) analyses.

Preparation of Retinal Protein Extracts. Retinas from wild-type (C57BL/6XFVB) and rom-$1^{-/-}$ mice were used in this study as positive and negative controls, respectively. Procedures were approved by the Institutional Animal Care and Use Committee. Animals were maintained on a 12-h light/dark cycle. After the sacrifice, dissected retinas were homogenized on ice in solubilization buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS, 2.5% glycerol, and 1.0 mM PMSF). After solubilization at 4 °C for 1 h, the homogenates were centrifuged at 100000*g* for 30 min, and supernatants were collected for the determination of protein concentration and subsequently used in the GST pull-down experiments.

GST Pull-Down Binding Assay and Peptide Competition. GST pull-down assays were performed as described by Wu et al. (*26*). Purified fusion proteins were used for the pull-down assay within 24 h after preparation. Mouse retinal extracts (50 μg) were incubated with ~5 μg of fusion protein bound to the glutathione resin in 200 μL of binding buffer (20 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1 mM PMSF) at 4 °C for 2 h. The resin was washed 3 times with 0.5 mL of the binding buffer and eluted with the Laemmli sample buffer. Proteins retained on the resin were analyzed by Western blot analysis using anti-GST, anti-Rom-1, and anti-P/*rds* antibodies.

Three peptides corresponding to distinct regions of the N-terminal portion of the D2 loop, named peptide 1 (P1, Leu^{122}−Lys^{139}, LRGSLESTLAYGLKNGMK), peptide 2 (P2, Tyr^{140}−Met^{158}, YRDTDTPGRCFMKKTIDM), and peptide 3 (P3, Cys^{165}−Asn^{182}, CCGNNGFRDWFEIQWISN) were synthesized (Biosources, Camarillo, CA) and used in the competition binding assay. Mouse retinal extracts were incubated with the GST-P/*rds*-D2 fusion protein in the absence or presence of the peptides at 4 °C for 2 h. A peptide corresponding to the region between Ser^{524} and Asp^{537} in the carboxyl terminus of the rod cyclic nucleotide-gated (CNG) channel α subunit, named peptide 4 (P4, SPDRENSEDASKTD), was included in the competition assay as an unrelated sequence peptide control.

Western Blot and IP Analyses. The polyclonal antibodies against residues Glu^{333}−Gly^{346} from the carboxyl terminus and residues Lys^{190}−Arg^{203} in the D2 loop of P/*rds* were used to detect P/*rds* and the GST fusion proteins. A polyclonal antibody against residues His^{335}−Glu^{351} from the carboxyl terminus of Rom-1 was used to detect Rom-1. A polyclonal antibody against GST was used to detect GST and the GST fusion proteins. Western blotting was performed as previously described (*16*). Protein extracts were solubilized with Laemmli sample buffer (50 mM Tris-HCl, 2% SDS, 100 mM dithiothreitol (DTT), and 10% glycerol), separated on 10 or 15% SDS−PAGE, and transferred onto a PVDF membrane in transfer buffer (25 mM Tris-base, 190 mM glycine, 20% methanol, and 0.03% SDS). The blots were preblocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at 4 °C overnight and incubated with the primary antibodies (anti-P/*rds* or anti-Rom-1 at a 1:1000 dilution and anti-GST at a 1:2000 dilution) for 2 h at room temperature. Blots were then washed 3 times with TBST and incubated with the secondary antibody at a dilution ratio of 1:25 000 for 1 h at room temperature. Super Signal chemiluminescent substrate (Pierce, Rockford, IL) was used for signal detection.

For IP (*27*), 200 μg of retinal protein or cellular protein extract prepared from COS-1 cells cotransfected with P/*rds*, Rom-1, and the D2 loop were incubated with either anti-P/*rds* or anti-Rom-1 antibodies (1:100 dilution) along with protein A-sepharose beads (Sigma−Aldrich, St. Louis, MO) at 4 °C for 4 h in solubilization buffer. After adsorption, the beads were washed with the solubilization buffer 3 times (500 μL for each wash) and bound proteins were eluted with 2× Laemmli sample buffer, followed by gel electrophoresis and immunoblotting with the respective antibodies.

RESULTS

Interactions between P/rds and Rom-1. Protein extracts prepared from mouse retinas and from COS-1 cells cotransfected with cDNAs encoding P/*rds* and Rom-1 were subjected to reciprocal co-IP using anti-P/*rds* and anti-Rom-1 antibodies, respectively. As shown in Figure 1, P/*rds* and Rom-1 from retinal extract (lanes 1 and 2) and from transfected COS-1 cells (lanes 3 and 4) were coprecipitated by both antibodies, which confirms the direct association between these two proteins.

Expression of the GST Fusion Proteins. To identify the domain(s) of P/*rds* that is involved in homo and hetero associations, a series of GST fusion proteins encoding distinct

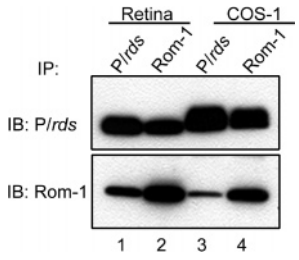

FIGURE 1: Interactions between P/*rds* and Rom-1. Reciprocal co-IP was performed using protein extracts from mouse retinas and from COS-1 cells cotransfected with cDNAs encoding the two proteins. The anti-P/*rds* C-terminus and anti-Rom-1 C-terminus antibodies were used in the IP, and the products were detected by Western blotting with the respective antibodies.

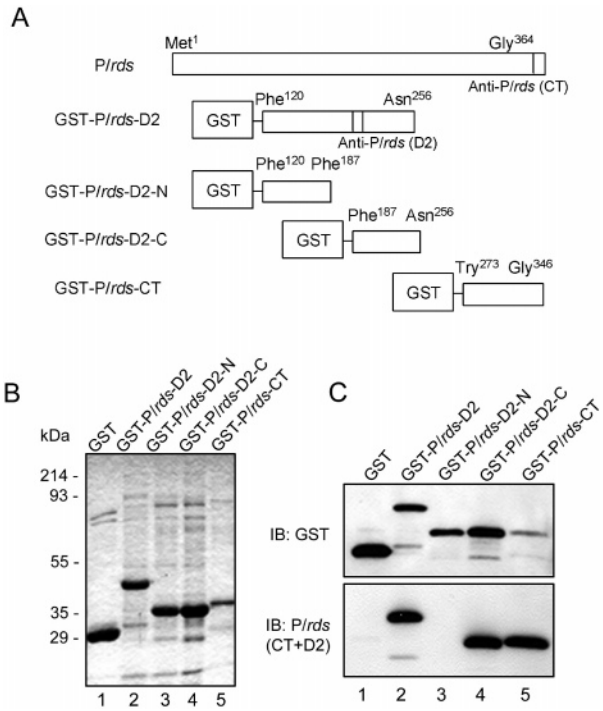

FIGURE 2: Construction and expression of GST-P/*rds* fusion proteins. (A) Diagram showing the structure of GST-P/*rds*-D2, GST-P/*rds*-D2-N, GST-P/*rds*-D2-C, and GST-P/*rds*-CT. (B) Coomassie blue gel staining showing expression of the fusion proteins in *E. coli* BL21 cells. (C) Western blot analysis of the fusion proteins using anti-GST and anti-P/*rds* D2 loop and C-terminus antibodies.

regions of P/*rds* were generated (Figure 2A). Figure 2 shows the expression of the GST fusion proteins by Coomassie Blue staining (Figure 2B) and Western blot analysis using an antibody against GST and different antibodies against the D2 loop and the C-terminal region of P/*rds* (Figure 2C). The GST control migrated to a position corresponding to 29 kDa, while the GST-P/*rds*-D2 fusion protein migrated to 45 kDa, which is comparable to its predicted size (16 + 29 kDa). The GST-P/*rds*-D2-N, GST-P/*rds*-D2-C, and GST-P/*rds*-CT fragments all migrated to 37 kDa, also comparable to their predicted sizes (8 + 29 kDa). A small amount of free GST was detected in the fusion protein preparations, which may reflect partial degradation during the purification procedure or an early termination. The D2 loop antibody does not detect GST-P/*rds*-D2-N because of the site of antigenicity (lane 3 of Figure 2C). The D2 loop antibody recognizes Lys^{190}−Arg^{203} in P/*rds*, while the GST-P/*rds*-D2-N construct covers Phe^{120}−Phe^{187} (Figure 2A).

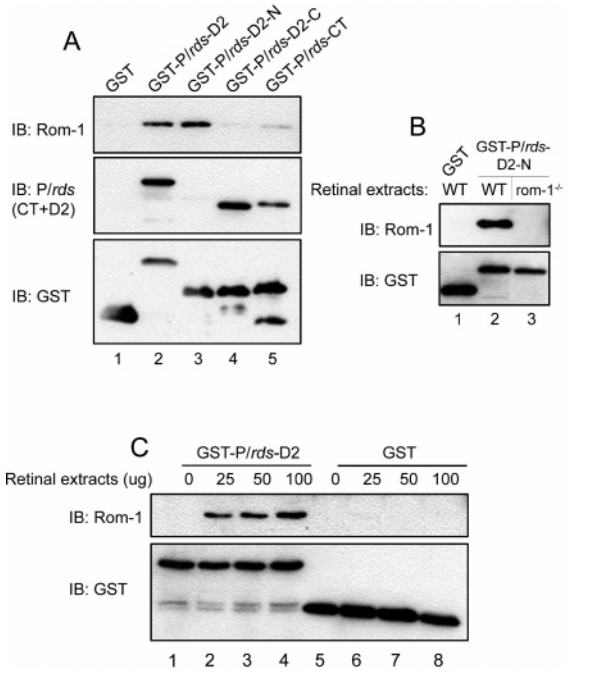

FIGURE 3: P/*rds* D2 loop interaction with Rom-1. (A) Interaction of Rom-1 to GST-P/*rds*-D2 and GST-P/*rds*-D2-N detected in the pull-down assay. The GST fusion proteins attached to the glutathione resin were incubated with mouse retinal extracts (50 μg of protein) at 4 °C for 2 h. Interactions of Rom-1 and presence of the fusion proteins were verified by Western blotting with the respective antibodies. (B) Absence of Rom-1 in the pull-down assay using GST-P/*rds*-D2-N incubated with retinal extracts prepared from rom-$1^{-/-}$ mice. (C). Rom-1 association to GST-P/*rds*-D2 in a concentration-dependent pattern.

Role of the P/rds D2 Loop in Hetero Associations. Purified fusion proteins were subjected to a pull-down assay with mouse retinal extract, which allowed for identification of important regions of P/*rds* involved in hetero association with Rom-1. As seen in Figure 3, Rom-1 was pulled down by GST-P/*rds*-D2 and GST-P/*rds*-D2-N (lanes 2 and 3 of Figure 3A) but not by GST-P/*rds*-D2-C (lane 4 of Figure 3A). A faint signal of Rom-1 interacting with GST-P/*rds*-CT was also detected (lane 5 of Figure 3A), which may suggest a weak binding affinity of the C terminus of P/*rds* to Rom-1 or the presence of an intermediate protein that serves as a link. However, there was no Rom-1 interaction with the GST controls incubated with wild-type mouse retinal extracts (lane 1 of Figure 3A) or in the D2 loop fusion proteins incubated with retinal extract prepared from rom-$1^{-/-}$ mice (lane 3 of Figure 3B). Furthermore, Rom-1 was pulled down by the GST-P/*rds*-D2 loop in a concentration-dependent manner as shown in the pull-down assay with increasing amounts of retinal extracts (lanes 1−4 of Figure 3C). Thus, these data have provided experimental evidence suggesting that the N-terminal portion of the P/*rds* D2 loop is necessary for proper hetero complex association with Rom-1.

Role of the P/rds D2 Loop in Homo Associations. Analogous experiments were performed to define the regions in P/*rds* involved in homo complex formation. Taking advantage of different recognition epitopes of two different P/*rds* antibodies, the interaction of P/*rds* with GST-P/*rds*-D2 was probed with the P/*rds* C-terminus antibody, while the interaction of P/*rds* with GST-P/*rds*-CT was probed with the P/*rds* D2 loop antibody. As shown in Figure 4, P/*rds* was detected in the pull-down assay using GST-P/*rds*-D2 and GST-P/*rds*-D2-N (lanes 2 and 3 of Figure 4A), while no P/*rds* was detected with GST-P/*rds*-CT using the anti-P/*rds* D2 antibody (lane 2 in the top panel of Figure 4B). Lane 5 in Figure 4B shows the detection of P/*rds* by both P/*rds* antibodies in retinal extracts that were used in the pull-down assay. As shown in Figure 4C, the amount of P/*rds* that is pulled down by GST-P/*rds*-D2-N increases as the amount of retinal extracts used in the binding assay increases. To verify whether this interaction occurs through the association of the fusion protein with P/*rds*, Rom-1, or components of the complexes, a pull-down assay was performed using rom-$1^{-/-}$ retinas (Figure 4D). Both GST-P/*rds*-D2 and GST-P/*rds*-D2-N show direct association with P/*rds* from the Rom-1-deficient retinal extracts (lanes 2 and 3 in the top panel of Figure 4D). However, there was no P/*rds* detected with GST-P/*rds*-D2-C (lane 4 in the top panel of Figure 4D). These data demonstrate the direct involvement of the N-terminal portion of the D2 loop in P/*rds* homo association and further indicate the importance of this region to form proper complex formation.

Identification of the Domain in the P/rds D2 Loop Required for Protein Associations. To further map the regions in the D2 loop necessary for protein association, a competitive pull-down assay was performed in the presence of peptides corresponding to different regions of the N-terminal portion of the D2 loop (Figure 5A). Retinal extracts were incubated with GST-P/*rds*-D2 or GST-P/*rds*-D2-N in the absence or presence of the peptides at varying concentrations (from 0.01 to 0.3 mM). As a negative control, a peptide (P4) of unrelated sequence was included in the competition pull-down assay. At the concentration of 0.3 mM, interaction of Rom-1 to the fusion protein was abolished by P3, a peptide corresponding to the region between Cys^{165} and Asn^{182} (lane 5 in the top panel of Figure 5B). Significant inhibition of this interaction was also detected by P2, a peptide corresponding to the region between Tyr^{140} and Met^{158} (lane 4 in the top panel of Figure 5B). However, the pull-down of Rom-1 with GST-P/*rds*-D2 was not affected by P1 when compared to the control peptide (P4) (lanes 3 and 6 in the top panel of Figure 5B). Thus, the region between Cys^{165} and Asn^{182} on the P/*rds* D2 loop appears to be critical for Rom-1 interaction. Similar experiments were conducted to map the region for P/*rds* interaction with other P/*rds* molecules. In these assays, P/*rds* interactions with GST-P/*rds*-D2 were abolished by P3 at a concentration of 0.3 mM (lane 5 in the middle panel of Figure 5B), while P1 and P2 at this concentration showed no significant interference with the interaction (lanes 3 and 4 in the middle panel of Figure 5B). These data demonstrate the importance of the region between Cys^{165} and Asn^{182} in P/*rds* for homo and hetero associations. The region between Tyr^{140} and Met^{158} was also shown to be significant for the hetero association with Rom-1, suggesting that these types of hetero complexes may require a larger domain to form a stable complex.

Involvement of the P/rds D2 Loop in Protein Associations in COS-1 Cells. A COS-1 cell heterologous expression system was used to determine the direct association of the D2 loop with P/*rds* and Rom-1. The minigene encoding the P/*rds* D2 loop (Figure 6A) was constructed by PCR amplification using the wild-type P/*rds* cDNA as a template. The PCR product was subcloned into a pcDNA3.1 expression vector and transfected into COS-1 cells. Figure 6B shows

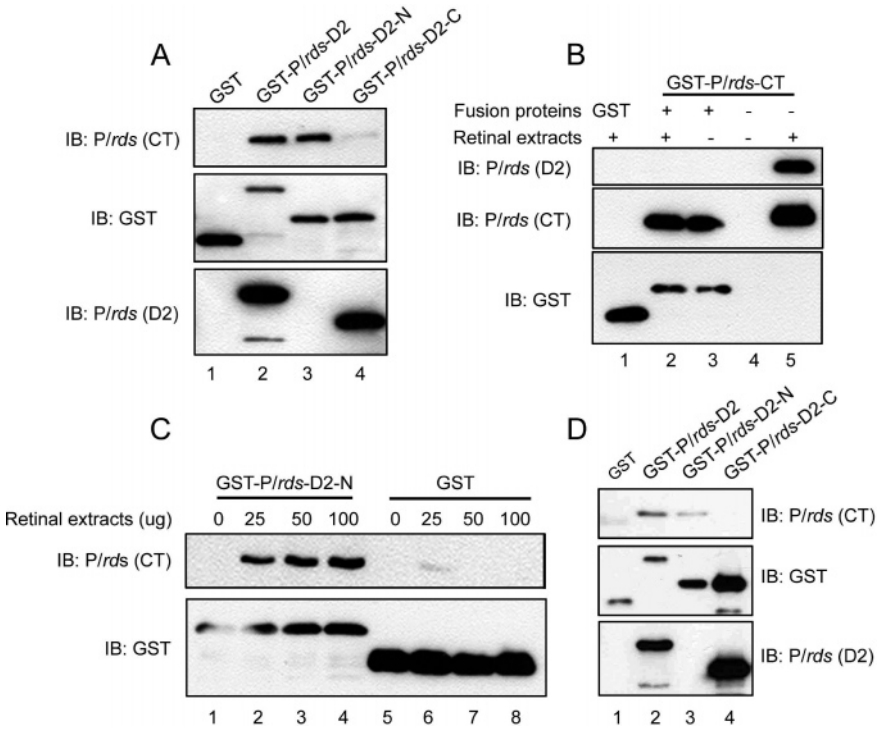

FIGURE 4: P/*rds* D2 loop interaction with other P/*rds* molecules. (A) GST pull-down assay showing binding of P/*rds* to GST-P/*rds*-D2 and GST-P/*rds*-D2-N. The D2 loop fusion proteins were incubated with mouse retinal extract, and binding of P/*rds* was detected with the anti-P/*rds* C-terminus antibody. Presence of the fusion proteins in these assays was verified with anti-GST and anti-P/*rds* D2 loop antibodies. (B) C terminus of P/*rds* is not involved in associations with other P/*rds* molecules. GST-P/*rds*-CT was incubated with mouse retinal extract and P/*rds* binding was detected with an anti-P/*rds* D2 loop specific antibody. Lane 5 shows the P/*rds* signal detected in 5 μg of retinal extract, which is only 10% of the total amount of retinal extract used in the pull-down assay. (C) Concentration-dependent binding of P/*rds* to GST-P/*rds*-D2-N. (D) GST pull-down assay showing binding of P/*rds* to GST-P/*rds*-D2 and GST-P/*rds*-D2-N using rom-1$^{-/-}$ retinal extracts.

Western blot detection (15% SDS−PAGE) of the D2 loop alone or with P/*rds* and/or Rom-1 in COS-1 cells. Protein extracts prepared from cells expressing the D2 loop showed a band at 16 kDa, as predicted. The samples prepared from cells coexpressing the D2 loop and the relevant recombinant proteins all migrated to their respective sizes (Figure 6B). In this heterologous expression system, coexpression of the D2 loop did not interfere with the expression levels of the intact proteins, although the expression levels of all three constructs varied between different transfections (data not shown). Interestingly, the D2 loop forms homodimers as detected under nonreducing gel conditions (lane 2 of Figure 6C).

Co-IP was performed to determine if the coexpression of the D2 loop interferes with the association between P/*rds* and Rom-1 (Figure 6D). COS-1 cells were transfected with varying amounts of the D2 loop cDNA along with a constant amount of the cDNAs encoding the intact P/*rds* and Rom-1. The results in Figure 6D show that coexpression of the D2 loop interferes with the association between P/*rds* and Rom-1 (lanes 1 and 3 in the second panel of Figure 6D). Furthermore, the D2 loop coprecipitated with the intact P/*rds* (lanes 1 and 3 in the third panel of Figure 6D), suggesting a direct association between the two. IP of the D2 loop was more abundant in cells transfected with 8 μg of the D2 loop cDNA (lane 3 compared to lane 1 in the third panel of Figure 6D). This mammalian expression system shows a direct involvement of the D2 loop in homo associations of P/*rds*. In contrast, there was no detection of the D2 loop when the anti-Rom-1 antibody was used for the IP (data not shown), which may suggest a more stringent structural requirement for Rom-1 binding.

DISCUSSION

The nature of the association between P/*rds* and Rom-1 to form homo and hetero complexes has been previously documented with both *in vitro* and *in vivo* systems (*17*, *20*, *22*, *28*). The impairment of complex formations of P/*rds* has been linked to several disease-causing mutations in the *rds* gene (*21*−*23*); however, insight into the precise molecular basis underlying these associations is primitive. This study demonstrates the important role of the N-terminal portion of the P/*rds* D2 loop and identifies the region between Cys^{165} and Asn^{182} as the domain for P/*rds* and Rom-1 association.

In the retina, P/*rds* and Rom-1 are believed to exist as hetero and homo complexes; however, there seems to be monomeric forms of both proteins as well as lower order complexes which may undergo dynamic movement between the different states. These free forms of P/*rds* and Rom-1 in retinal extracts should preferentially bind the fusion proteins because the binding interface is normally occupied in the complexes; therefore, the signal detected with retinal extracts should indicate direct binding of the fusion protein to P/*rds* or Rom-1 rather than the components of the complexes. The likely direct association of the D2 loop fusion protein to P/*rds* was further demonstrated by the use of rom-1$^{-/-}$ retinal extracts in the pull-down assay. Surprisingly, D2 loop binding to Rom-1 was not detected in the pull-down assay using COS-1 cell extracts expressing P/*rds* and Rom-1. Possibilities for this phenomenon may include subtle differences between

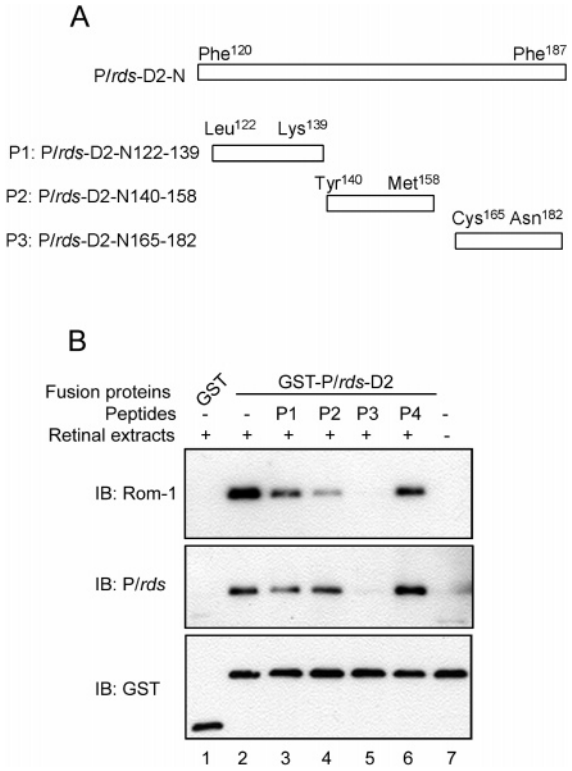

FIGURE 5: Identification of the domain in the P/*rds* D2 loop required for the protein associations. (A) Peptides used in the competitive binding assay. (B) Peptide competition assay identifying the domain for Rom-1 and P/*rds* association. GST-P/*rds*-D2 was incubated with retinal extracts (50 μg of protein) in the presence and absence of the competition peptides (0.3 mM) for 2 h at 4 °C. A peptide (P4) with nonrelated sequence was included as a negative control.

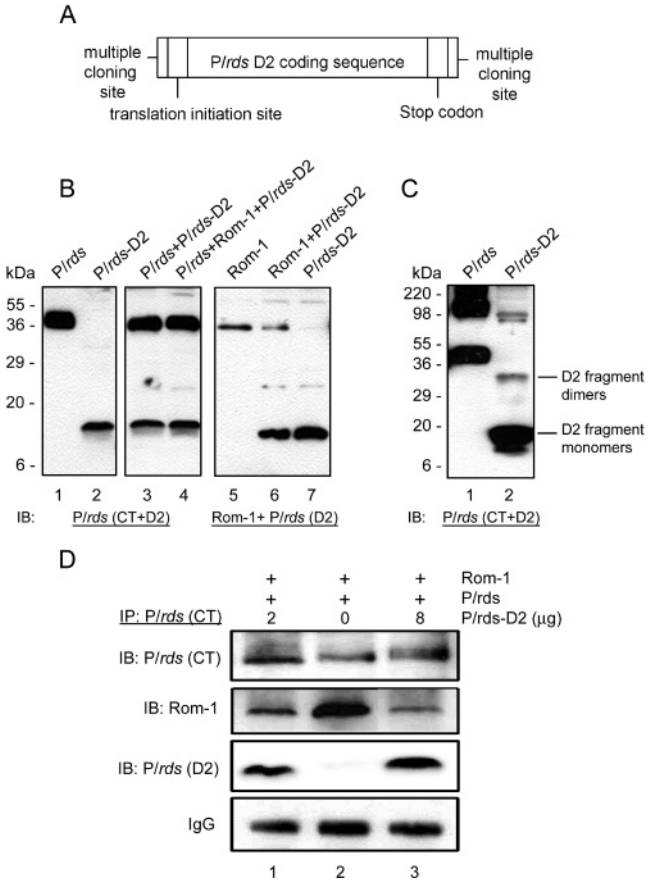

FIGURE 6: Involvement of the P/*rds* D2 loop in protein associations in COS-1 cells. (A) Diagram of the minigene encoding the D2 loop sequence with a translation initiation site and stop codon. (B) Coexpression of the D2 loop with intact P/*rds* and Rom-1 in COS-1 cells detected by Western blot analysis with the respective antibodies. (C) Nonreducing gel analysis showing formation of disulfide-linked homodimers of the D2 loop expressed in COS-1 cells. (D) Effects of coexpression of the D2 loop on the protein interactions in a co-IP assay. Protein extracts prepared from cells expressing the D2 loop, and the intact proteins were subjected to IP analysis with anti-P/*rds* C-terminus antibody. The immunoprecipitants were analyzed with the respective antibodies.

the endogenous and recombinant proteins or the existence of another protein yet to be characterized, present only in the retina, which mediates the association between P/*rds* and Rom-1. Although the participation of a third protein in the complex formation is possible, it seems less likely because researchers over the past decade have yet to discover an intermediate protein in the P/*rds*−Rom-1 complex during their respective course of studies. Peptide competition experiments confirmed an association between the fusion proteins with P/*rds* and Rom-1 and have identified the region between Cys^{165} and Asn^{182} as the domain for these associations. Again, the signal detected by the pull-down assay should reflect the competitive binding at the sites of the fusion protein with P/*rds* or Rom-1 on an individual basis because the binding site is most likely masked in the complex.

Sequence alignment of the N-terminal portion of the D2 loop indicates that the region identified in this study (Cys^{165}−Asn^{182}) is highly conserved in all known P/*rds* sequences (Figure 7). This region is also the site of many disease-causing mutations (*7*−*9*, *29*) (enlarged and bolded residues of Figure 7). Because P/*rds* homo and hetero complex formation is vital for maintaining the integrity of the photoreceptor disk rim, mutations in this region most likely cause structural abnormalities that disrupt protein interactions necessary for proper photoreceptor function resulting in the onset of disease.

Previous studies have shown P/*rds* and Rom-1 to form similar patterns of homo and hetero complexes in photoreceptors compared to those in transfected COS-1 cells (*11*, *17*). We hypothesized that coexpression of the D2 loop in COS-1 cells along with the intact P/*rds* and Rom-1 would interfere with this association. The underlying rationale was to explore the ability of an individual structural domain to compete for a functionally important process. Precedent for this experimental design came from studies in which co-expression of the intracellular loops of β-adrenergic, muscarinic M_3, and CCK receptors with their respective intact proteins were shown to interfere with ligand-stimulated signaling and trafficking (*30*, *31*). On the basis of the aforementioned experimental design, coexpression of the D2 loop along with the two intact proteins in COS-1 cells was established and optimized. In this system, coexpression of the D2 loop diminished the association between P/*rds* and Rom-1. The association of the D2 loop with the intact P/*rds* was confirmed by co-IP.

In comparison with P/*rds* homo association, the data suggest that the hetero association between P/*rds* and Rom-1 must meet a more stringent structural requirement. In the peptide competition experiments, a larger domain between Tyr^{140} and Asn^{182} of the P/*rds* D2 loop was shown to participate in Rom-1 association. Furthermore, in COS-1

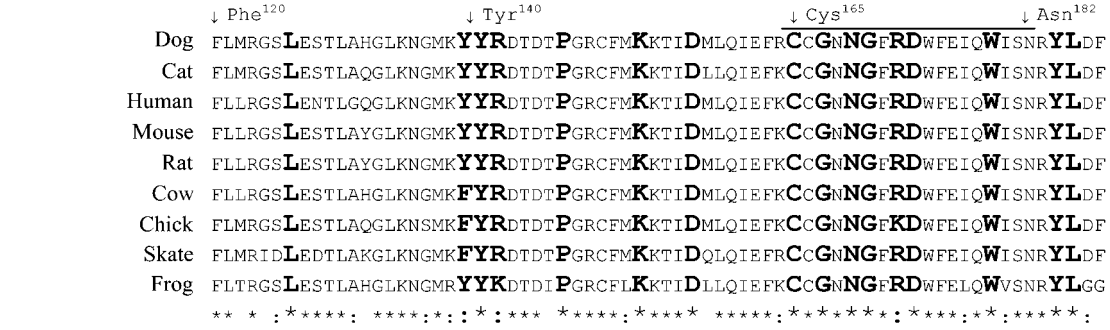

FIGURE 7: Sequence alignment of the N-terminal portion (Phe[120]−Phe[187]) of the P/*rds* D2 loop. Sequences of the N-terminal portion of the D2 loop from all known P/*rds* homologues are aligned. The region between Cys[165] and Asn[182] is identified as the domain for P/*rds* and Rom-1 binding and is marked on the top with a solid line. The residues in which mutations cause human retinal degenerations are bold and enlarged. An asterisk refers to identical residues, and a colon refers to residues with high similarity in all P/*rds* homologues.

cells, while the D2 loop co-immunoprecipitated with P/*rds* by the anti-P/*rds* antibody, it did not co-immunoprecipitate with Rom-1 by the anti-Rom-1 antibody. This suggests that the D2 loop alone in a non-native cellular environment is unable to interact with Rom-1. This finding is consistent with another study conducted by Kedzierski et al. (*28*), where a chimeric protein of Rom-1 containing the P/*rds* D2 loop displayed a more stable interaction with P/*rds* when compared to authentic Rom-1.

The importance of the P/*rds* D2 loop was first recognized by the identification of a large number of disease-causing mutations in this region and later with *in vitro* and *in vivo* studies showing the involvement of this loop in complex formations (*28*, *32*, *33*). Mutations in the conserved cysteines of the D2 loop perturbed dimer formation, association with Rom-1, and subunit assembly (*34*), indicating the important role of these cysteines in maintaining the proper structure of the protein and preserving complex formation. Three of these cysteines, Cys[150], Cys[165], and Cys[166], are located within the N-terminal portion of the D2 loop. The importance of the D2 loop in complex assembly was examined recently by Goldberg et al. (*33*), in which a highly charged hendecapeptide was inserted into specific regions of P/*rds*. No detrimental effects were observed in protein folding and tetrameric subunit assembly by the insertion mutations in nine sites residing outside the D2 loop, while gross protein misfolding and/or a perturbation of subunit assembly were detected in the five insertion mutations within the D2 loop (*33*). It is important to note that three of these five insertion sites were in the N-terminal portion of the D2 loop with two adjacent insertions covering residues Cys[165]−Asn[182].

There was no detection of interactions of Rom-1 or P/*rds* with the C-terminal portion of the D2 loop. Nevertheless, this portion of the D2 loop seems to be important for photoreceptor viability. In fact, several mutations found in patients are located in this region, suggesting its integrity is likely needed for proper protein conformation and complete exposure of the interacting domain in the N-terminal portion of the D2 loop.

The observed biochemical characteristics of the C-terminal tail of P/*rds* in this study are consistent with previous reports (*14*, *35*). This region of P/*rds* has been found to promote membrane fusion activity as well as membrane targeting (*13*, *14*, *35*). Past experiments have shown that the deletion of the C-terminal tail of P/*rds* abolishes the fusogenic activity but does not have any negative effects on protein dimerization or complex assembly with Rom-1 (*14*). In addition to the fusogenic activity and membrane targeting, the C terminus of P/*rds* may be involved in anchoring the protein or connecting the disk rim to the plasma membrane through associations with auxiliary proteins, cytoskeleton proteins, or plasma-membrane proteins. An example of such an interaction has been shown to occur between P/*rds* and the β subunit of the rod CNG channel (*36*).

This study is the first to define the essential domain of the P/*rds* D2 loop required for homo and hetero associations with Rom-1. This domain may represent a crucial structural element required for protein interactions and homo and hetero complex assembly, especially considering that the majority of the disease-causing mutations in P/*rds* are found in this region. Knowledge of the importance of this specific region of P/*rds* in complex formation contributes to our understanding of the mechanisms underlying retinal disease and aids in the development of therapeutic strategies.

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