Molecular Characterization of Peripherin-2 and Rom-1 Mutants Responsible for Digenic Retinitis Pigmentosa*

Received for publication, December 26, 2000, and in revised form, March 28, 2001
Published, JBC Papers in Press, April 10, 2001, DOI 10.1074/jbc.M011710200

Christopher J. R. Loewen‡, Orson L. Moritz, and Robert S. Molday§§

From the Department of Biochemistry and Molecular Biology and §Department of Ophthalmology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Peripherin-2 and Rom-1 are homologous tetranspanning membrane proteins that assemble into noncovalent tetramers and higher order disulfide-linked oligomers implicated in photoreceptor disc morphogenesis. Individuals who coinherit a L185P peripherin-2 mutation and a null or G113E rom-1 mutation are afflicted with retinitis pigmentosa, whereas individuals who inherit only one defective gene are normal. We examined the expression, subunit assembly, and disulfide-mediated oligomerization of L185P and L185A peripherin-2 and L188P Rom-1 by velocity sedimentation, co-immunoprecipitation, and cross-linking. These mutants formed noncovalent dimers under disulfide-reducing conditions but failed to assemble into core tetramers. Under nonreducing conditions, L185P dimers formed disulfide-linked tetramers but not higher order oligomers. L185P coassembled with wild-type peripherin-2 and Rom-1 to form tetramers and higher order disulfide-linked oligomers characteristic of the wild-type proteins. The G113E Rom-1 mutant expressed 20-fold lower than wild-type Rom-1, indicating that it behaves mechanistically as a null allele. We conclude that Leu$^{185}$ of peripherin-2 (Leu$^{188}$ of Rom-1) is critical for tetramer but not dimer formation and that the core tetramer has 2-fold symmetry. Peripherin-2-containing tetramers are required for higher order disulfide-linked oligomer formation. The level of these oligomers is critical for stable photoreceptor disc formation and the digenic retinitis pigmentosa disease phenotype.

Peripherin-2 and Rom-1 share many structural properties (3, 2, 11). They are 35% identical in sequence and contain four putative membrane-spanning segments, a large intradiscal loop that connects the third and fourth transmembrane segments, and a relatively long C-terminal segment on the cytoplasmic side of the disc membrane (Fig. 1) implicated in membrane fusion (12). The large intradiscal loop contains seven conserved cysteine residues. One cysteine, Cys$^{150}$, forms intermolecular disulfide bonds, whereas the other six cysteine residues contribute to protein folding presumably through intramolecular disulfide bonds (13).

Peripherin-2 and Rom-1 subunits interact noncovalently to form a mixture of homo- and heterotetrameric core complexes (14). Subunit assembly is mediated through interactions involving the large intradiscal loop (13, 15, 16). Peripherin-2-containing core complexes can link together through Cys$^{150}$-mediated disulfide bonds to form higher order oligomers, which are thought to play a key role in the formation and stabilization of the hairpin rim region of outer segment discs (14). In support of this concept, Wrigley et al. (17) have shown that in vivo expression of wild-type (WT)$^1$ peripherin-2 under nonreducing conditions results in the flattening of microsomal vesicles, whereas expression of WT peripherin-2 under disulfide-reducing conditions or expression of several peripherin-2 mutants including the C150S mutant under nonreducing conditions produces characteristic spherical microsomal vesicles.

Mutations in the Prph2 gene have been linked to a variety of human retinal degenerative diseases including autosomal dominant retinitis pigmentosa (RP), macular dystrophy, and related pattern dystrophies (18–22). Most disease-causing missense mutations are located within the large intradiscal loop of peripherin-2. Heterologous expression studies have shown that some of these mutations cause misfolding of peripherin-2 and defective subunit assembly (13, 15). In contrast, there is no clear example of a mutation in Rom-1 that, by itself, causes retinal degeneration (23).

Dyria and colleagues (23, 24), however, identified several families with a digenic form of autosomal dominant RP linked to mutations in both peripherin-2 and rom-1. Individuals who inherited both a L185P mutation in a peripherin-2 allele and a null or G113E mutation in a rom-1 allele are severely affected with RP, whereas family members who inherit only one of these mutations are essentially normal. Biochemical studies have shown that the L185P peripherin-2 mutant assembles with Rom-1 to form core heterotetrameric complexes, but unlike WT peripherin-2, it is unable to self-assemble into core homotetramers (15).

In order to understand the molecular basis of digenic auto-

$^*$ This work was supported by grants from the National Eye Institute (EY 2422) and Foundation Fighting Blindness, Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Holder of a predoctoral fellowship from the Foundation Fighting Blindness, Canada.

¶ Recipient of a predoctoral fellowship from the Foundation Fighting Blindness, Canada.

†† Holder of a Canada Research Chair in Vision and Macular Degeneration. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, 2146 Health Sciences Mall, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada. Tel.: 604-822-6173; Fax: 604-822-5227; E-mail: molday@interchange.ubc.ca.

1 The abbreviations used are: WT, wild-type; RP, retinitis pigmentosa; PBS, phosphate-buffered saline; DTT, dithiothreitol; NEM, N-ethylmaleimide.

---

‡ Recipient of a predoctoral fellowship from the Foundation Fighting Blindness, Canada.

§§ Recipient of a predoctoral fellowship from the Foundation Fighting Blindness, Canada.
somal dominant RP, we have carried out a detailed study of several peripherin-2 and Rom-1 mutants. Here, we show that the L185P peripherin-2 mutant self-assembles into dimers that can further associate through intermolecular disulfide bonds to form tetramers but not higher order oligomers characteristic of WT peripherin-2. The L185P mutant, however, can interact with WT Rom-1 and WT peripherin-2 to form core tetramers and higher order disulfide-linked oligomers. Together, these studies provide new insight into the quaternary organization of the peripherin-2 and Rom-1 complexes and mechanisms that contribute to autosomal dominant digenic RP.

MATERIALS AND METHODS

Plasmids and Heterologous Cell Expression—pcPer (WT peripherin-2), pcPer-L185P (L185P peripherin-2), and pcROM (WT rom-1) have been described (5, 13, 25). pcPer-L185A (L185A), pcPer-L185P (L185P), pcROM-G113E (G113E), and pcROM-G113A (G113A) were constructed by polymerase chain reaction-based mutagenesis (26). pcPerID4, containing a 1D4 epitope tag, TETSQVAPA, at the C terminus was created by ligating an oligonucleotide corresponding to the 1D4 epitope onto the 3’-end of the peripherin-2 cDNA. A Q341P change that abolishes the binding of monoclonal antibody 2B6 was also introduced into this construct by site-directed mutagenesis. pcL185P-ID4 (pcPerID4 with the L185P mutation) was created by cloning the BamHI-SacII restriction fragment from pcPer-L185P into BamHI-SacII-digested pcPerID4.

COS-1 cells (6 × 10^6 cells/100-mm dish) were transfected with a total of 30 μg of plasmid DNA and harvested 72 h post-transfection as described (25).

Hydrodynamic Characterization of L185P Peripherin-2—Hydrodynamic analysis of the L185P mutant under reducing conditions was carried out as described previously for wild-type peripherin-2 (27). Briefly, pcPer-L185P transfected cells were solubilized in PBS containing 1% Triton X-100, 2 mM DTT, and phenylmethanesulfonyl fluoride, and centrifuged at 90,000 χ g for 30 min at 4 °C. The supernatant was used to determine the hydrodynamic properties of the detergent-solubilized complex. The Stokes radius was determined by gel exclusion chromatography on a calibrated Sephacryl S-300 column. Velocity sedimentation analysis was performed by centrifugation at 4 °C in a Beckman SW 50.1 rotor at 49,000 rpm for 13 h on sucrose gradients in H_2O and 28 h for gradients in D_2O. Fractions were collected and analyzed on Western blots. Calibration protein standards (Stokes radius, sedimentation coefficient (Svedbergs), partial specific volume) were cytochrome c (1.7 nm, 1.9 S, 0.728 ml/g), malate dehydrogenase (3.5 nm, 4.32 S, 0.734 ml/g), aldolase (4.6 nm, 7.70 S, 0.742 ml/g), and apoferritin (6.7 nm, 17.7 S, 0.73 ml/g) (28–32).

The molecular mass of the detergent-protein complex and the molecular mass of the protein portion were calculated using the method of Clarke (31). Briefly, the values used for the sedimentation coefficient (sT,m), the density (ρw), and the viscosity (ηw) were experimentally determined values at the run temperature (subscript T) and in sucrose gradients in H_2O or D_2O (subscript m). The sedimentation coefficient (sT,m) was determined from the following relationship,

\[
S_{T,m} = ((r - r_0)ω^2 / μ) \eta_w^2 \rho_w \rho_m
\]  
(Eq. 1)

where \( r \) is the distance of the protein at time \( t \) from the center of rotation, \( r_0 \) is the initial radial position, \( ω \) is the angular rotation, and \( \eta_w^2 \) is the average radial position defined by \( r_{avg} = (r_0 + r)/2 \).

The density \( ρ_{T,m} \) at \( r_{avg} \) was determined by interpolation of the measured densities of the 5% and 20% sucrose/H_2O and sucrose/D_2O solutions. The linearity of the gradients was confirmed by measuring the refractive index of the gradient fractions.

The average viscosity \( η_{T,m} \) experienced by the marker proteins was determined from Equation 2 using the published values for \( \eta_{T,m} \) and \( \eta_{T,m} \) for the standards. The densities \( ρ_{T,m} \) and viscosities \( η_{T,m} \) of water under standard conditions of 20 °C are 0.998 g cm⁻³ and 0.01002 g cm⁻¹ s⁻¹, respectively.

The average viscosity experienced by the peripherin-2-detergent complex was interpolated from plots of \( η_{T,m} \) for the markers versus fraction.

The specific partial volume of the complex \( (\rho_{T,m}) \) was calculated from the sedimentation coefficient in H_2O and D_2O (denoted by the corresponding subscripts H and D) according to Equation 3.

\[
\rho_{T,m} = \left( \frac{ρ_{T,m} \cdot η_{T,m}}{1 - η_{T,m}} \right) \left( \frac{ρ_{T,m} \cdot η_{T,m}}{1 - η_{T,m}} \right)
\]  
(Eq. 2)

The sedentation coefficient \( (s_{T,m}) \) for the complex under standard conditions (20 °C) was determined using Equation 4.

\[
s_{T,m} = \left( \frac{s_{T,m} \cdot η_{T,m}}{1 - η_{T,m}} \right) \left( \frac{s_{T,m} \cdot η_{T,m}}{1 - η_{T,m}} \right)
\]  
(Eq. 3)

The molecular mass of the detergent-protein complex was calculated using the sedimentation coefficient \( (s_{T,m}) \), the partial specific volume \( (ν) \), and the Stokes radius \( (a) \) using Equation 5 (30), where \( N \) is Avogadro’s number.

\[
M_c = (6πN\rho_{T,m}Na_{T,m})(1 - η_{T,m})
\]  
(Eq. 4)

Finally, the molecular mass of the protein portion of the L185P peripherin-2 complex \( (M_p) \) was calculated using Equation 6. An average value for the partial specific volume for protein (0.74 ml/g) and for the detergent Triton X-100 (0.94 ml/g) was used in the calculation (27).

\[
M_p = M_c / (1 + (ν - 0.74)(0.94 - ν))
\]  
(Eq. 5)

Solubilization, Immunoprecipitation, and Velocity Sedimentation Analysis—Transfected COS-1 cells were scraped from plates, washed with PBS, and incubated with PBS in the presence or absence of 20 mM DTT for 90 min at 25 °C. The cells were solubilized with an equal volume of PBS containing 2% Triton X-100, 80 mM NEM, and phenylmethanesulfonyl fluoride (pH 7.4) for 10 min on ice. In some experiments, NEM was substituted with 1 mM DTT in the solubilization buffer. The cell extract was centrifuged at 90,000 × g for 30 min at 4 °C, and supernatant (solubilized fraction) was retained on ice for analysis. Peripherin-2 was purified from the solubilized fraction on a Per26B-Sepharose immunoaffinity matrix as previously described (14).

Purified peripherin-2 was applied to (20% w/w) sucrose gradients prepared in PBS and containing 0.1% Triton X-100 (15). Routinely, 1 mM DTT was included in the gradients for DTT-treated samples analyzed under reducing conditions. After centrifugation for 16 h at 50,000 rpm in a Beckman TLS-55 rotor at 4 °C, the bottom of the centrifuge tube was punctured, and four-drop fractions were collected for analysis by Western blotting. This procedure was used to determine apparent sedimentation coefficients for comparative purposes.

SDS-PAGE, Western blotting, and Cross-linking—Samples were denatured with an equal volume of SDS mixture (4% SDS, 0.02 M Tris-HCl, pH 6.8, 20% glycerol, 0.01% bromphenol blue) in the absence (nonreducing) or presence (reducing) of 5% β-mercaptoethanol and applied to 8 or 10% SDS-polyacrylamide gels as described (14). After electrophoresis, the proteins were transferred to Immobilon-P using a

![Diagram](http://www.jbc.org/Downloaded from http://www.jbc.org/)
Bio-Rad semidy transfer apparatus. Blots were probed with monoclonal antibodies to peripherin-2 (Per2B6) (1), Rom-1 (Rom1C6), or rhodopsin (Rho1D4) (33) and sheep anti-mouse immunoglobulin-peroxidase for detection by ECL. The Rom1C6 monoclonal antibody was generated to a glutathione S-transferase fusion protein containing the intradiscal loop region of Rom-1 (5). Cross-linking was performed on Triton X-100-solubilized samples with 0.001% glutaraldehyde for 15 min at 37 °C.

RESULTS

**Molecular Mass of L185P Peripherin-2 under Disulfide-reducing Conditions**—Previous studies have shown that under reducing conditions (with DTT), the sedimentation coefficient of the L185P peripherin-2 mutant is significantly smaller than that of the tetrameric WT peripherin-2 core complex (15). To determine how the sedimentation behavior of this mutant relates to its molecular mass, we carried out a hydrodynamic study of the COS-1 cell-expressed L185P protein under reducing conditions. As shown in Fig. 2A, the L185P mutant eluted from a calibrated gel exclusion column as a single species with a Stokes radius of 4.83 ± 0.29 nm (n = 5), a value that is significantly lower than the value of 6.2 nm previously measured for the WT peripherin-2 tetramer (27). The sedimentation coefficient and partial specific volume of the L185P mutant were determined by velocity sedimentation in sucrose gradients containing H2O and D2O (Fig. 2B). A single detergent-protein species was observed in each case resulting in a s20,w value of 2.78 ± 0.06 S (n = 8) and a partial specific volume of 0.82 ml/g. There was no difference between the sedimentation profiles of unpurified and immunoaffinity-purified complex (data not shown). Furthermore, since the partial specific volume of L185P detergent-protein complex was similar to the native complex from rod outer segments (0.83 ml/g for native complex (27)), the L185P mutant bound similar amounts of detergent as the native complex, indicating that it was not grossly misfolded. Using the values determined for Stokes radius, sedimentation coefficient, and partial specific volume, a molecular mass of 91 kDa was calculated for the L185P detergent-protein complex according to the method of Clarke (31). Using an average value for the partial specific volume for protein (0.74 ml/g) and the value for the detergent Triton X-100 (0.94 ml/g) and assuming additivity of partial specific volumes for protein and detergent, the molecular mass was estimated to be 54 kDa for the detergent-free L185P protein complex. This value is intermediate between a L185P monomer (39 kDa) and dimer (78 kDa) but considerably less than the experimental value of 135 kDa previously measured for the native peripherin-2 tetramer (calculated Ms = 152,000) from rod outer segments (27).

To determine if the L185P mutant exists as a monomer or a dimer, we carried out a series of immunoprecipitation studies with the L185P mutant and a related L185P-1D4 mutant. The L185P mutant is recognized by monoclonal antibody Per2B6; the L185P-1D4 mutant contains a 9-amino acid C-terminal epitope tag recognized by monoclonal antibody Rho1D4 and a Q341P mutation (Pro at position 341 is found in mouse peripherin-2) that abolishes Per2B6 immunoreactivity.

We first determined if the individually expressed L185P and L185P-1D4 proteins were able to interact to form a stable complex. As shown in Fig. 3A, when detergent-solubilized L185P and L185P-1D4 were mixed together and immunoprecipitated with Per2B6-Sepharose under disulfide-reducing conditions, L185P-1D4 mutant was eluted in the unbound fraction, whereas the L185P mutant was only present in the bound fraction. This result indicates that individually expressed L185P and L185P-1D4 proteins do not associate to form a complex. This experiment also demonstrates that the Per2B6-Sepharose matrix selectively binds the L185P mutant but not the L185P-1D4 mutant.

To determine if L185P and L185P-1D4 mutants can coassemble into a multisubunit complex, COS-1 cells were cotransfected with the L185P and L185P-1D4 plasmids. The cells were treated with DTT to reduce intermolecular disulfide bonds and subsequently solubilized in Triton X-100 in the presence of NEM to block free sulfhydryl groups (14). The detergent-solubilized extract was applied to a Per2B6-Sepharose column, and the unbound and bound (peptide-eluted) fractions were analyzed for L185P and L185P-1D4 by Western blotting. Fig. 3B shows that all of the L185P protein bound to the Per2B6-

FIG. 2. Hydrodynamic analysis of COS-1 cell-expressed L185P peripherin-2 mutant under reducing conditions. COS-1 cells expressing the L185P peripherin-2 mutant were solubilized in Triton X-100 in the presence of DTT. A, gel exclusion chromatography of L185P peripherin-2. *Inset,* calibration curve generated from standards: cytochrome c (cyt), malate dehydrogenase (mal), aldolase (ald), and apoferritin (apo) and the void (V0) and included (V1) volumes. B, velocity sedimentation of L185P peripherin-2 through 5–20% sucrose gradients made up with H2O or D2O. Peripherin-2 was detected on Western blots labeled with the Per2B6 monoclonal antibody and quantified by densitometry.
glutaraldehyde and labeled with the Per2B6 antibody. Molecular stand-
reduced, immunoaffinity-purified L185P peripherin-2 cross-linked with
peripherin-2 (arrowhead) coprecipitated (bound, peptide-eluted) fraction from
Western blots labeled with Per2B6 antibody and Rho1D4.

C lane c, and bound, peptide-eluted fraction (lane b) were analyzed on
Western blots labeled with Per2B6 and Rho1D4 antibodies. B, COS-1
cells coexpressing L185P and L185P-1D4 peripherin-2 were solubilized in
Triton X-100 under reducing conditions and immunoprecipitated with
Per2B6-Sepharose. The COS-1 cell extract (lane a), unbound fraction
(lane b), and bound, peptide-eluted fraction (lane c) were analyzed on
Western blots labeled with Per2B6 and Rho1D4 antibodies. C, the
coprecipitated (bound, peptide-eluted) fraction from B was subjected to
velocity sedimentation, and fractions were analyzed on Western blots
labeled with Rho1D4 antibody. The sedimentation position of L185P
peripherin-2 (arrowhead) is shown for comparison. D, Western blot of
reduced, immunoaffinity-purified L185P peripherin-2 cross-linked with
glutaraldehyde and labeled with the Per2B6 antibody. Molecular stand-
ards are given in kDa.

The isolated L185P-L185P-1D4 complex was further ana-
lyzed by velocity sedimentation (Fig. 3C). The major complex
had an apparent sedimentation coefficient that was similar to
the individually expressed L185P (Table I). Additional complex
was apparent near the bottom of the tube. This most likely
represents L185P-L185P-1D4 aggregates that form during the
immunoaffinity purification procedure.

Finally, we determined if glutaraldehyde could cross-link the
purified L185P mutant into a dimer. As shown in Fig. 3D, a
significant fraction of detergent-solubilized L185P peripherin-2
migrated as a dimer (~67 kDa) after treatment with glutaral-
dehyde. This provides additional evidence that L185P is a
multisubunit complex, minimally a dimer.

Taken together, the hydrodynamic analysis, coimmunopre-
cipitation, and cross-linking studies provide strong evidence that
the L185P peripherin-2 mutant exists as a dimer under
disulfide-reducing conditions.

Velocity Sedimentation of L185A Peripherin-2 and L188P
Peripherin-2 Mutants—To further evaluate the role of leucine at
position 185 on the multisubunit assembly of peripherin-2, we ana-
alyzed the sedimentation behavior of a L185A mutant under
disulfide-reducing conditions. The L185A mutant, like L185P,
sedimented as a single species having an apparent sedimenta-
tion coefficient characteristic of a dimer (Fig. 4A; Table I). This
mutant also coassembled with Rom-1 into a tetrameric core
complex (data not shown).

Rom-1 contains a leucine residue at an equivalent position
(Leu188). The L188P Rom-1 mutant sedimented as a single
species with an apparent sedimentation coefficient of 3.09 S, a
value that is considerably less than that of WT Rom-1 tetramer
and consistent with a dimer (Fig. 4B; Table I).

Interaction of L185P Peripherin-2 and L188P Rom-1—To
determine if L185P peripherin-2 can assemble with L188P
Rom-1, COS-1 cells coexpressing the L185P peripherin-2 and
L188P Rom-1 were treated with DTT, and the detergent-solu-
bilized extract was immunoprecipitated with Per2B6 Sepha-
rose. Fig. 5C shows that a significant fraction of L188P Rom-1
coprecipitated with L185P peripherin-2. This isolated complex
sedimented with an apparent sedimentation coefficient of 3.24
S (Table I), a value intermediate between a L185P homodimer
($s_{20,w} = 3.64$ S) and a L188P homodimer ($s_{20,w} = 3.09$ S) and
consistent with heterodimer formation.

Velocity Sedimentation of L185P Peripherin-2 under Nonre-
ducing Conditions—The WT peripherin-2 core tetramer forms
higher order, disulfide-linked oligomers under nonreducing
conditions (14). To determine if the L185P peripherin-2 dimer
also forms disulfide-linked oligomers, COS-1 cells expressing
the L185P mutant were treated with NEM and solubilized in
Triton X-100 in the absence of DTT. The soluble extract was
subjected to velocity sedimentation, and fractions were ana-
lyzed on nonreducing SDS-polyacrylamide gels (Fig. 5, A and
B). The velocity sedimentation profile showed two distinct
species. One species (a) had an apparent sedimentation coefficient
of 3.62 S, a value similar to the value for reduced L185P (Table
I); this species was composed solely of monomers when ana-
lyzed on nonreducing SDS gels. A second species (b) had an
apparent sedimentation coefficient of 5.22 S, a value that is
similar to that observed for WT tetrameric peripherin-2 core
species (Table I); this species contained disulfide-linked
dimers. These results indicate that two L185P peripherin-2
core dimers can link together through intermolecular disulfide
bonds to form a tetrameric complex. However, this complex
does not form the higher order oligomers observed for WT
peripherin-2 (Fig. 5, C and D).

Interaction of WT Rom-1 with WT and L185P Peripherin-2
under Nonreducing Conditions—Previous studies have shown
that both WT and L185P peripherin-2 assemble with WT
Rom-1 to form heterotetrameric complexes under reducing
conditions (15). To determine if these complexes can form higher
order disulfide-linked oligomers, we examined the sedimenta-
tion behavior of immunoaffinity-purified WT peripheri-
2-Rom-1 and the L185P peripherin-2-Rom-1 complex under
nonreducing conditions. As shown in Fig. 6A, the WT peripheri-
2-Rom-1 complex sedimented as two distinct species. Ap-
proximately 25% of the total complex sedimented as a het-
etetramer (b in Fig. 6A) devoid of intermolecular disulfide
bonds, and 75% sedimented as an intermediate oligomer (c),
presumably an octamer, containing a high proportion of inter-
molecular disulfide bonds.

The sedimentation profile of L185P peripherin-2-Rom-1 com-
xplex is shown in Fig. 6B. Three distinct species were observed:
a heterodimer (a) accounting for ~17% of the complex and
consisting of monomers; a heterotetramer (b) accounting for
23% of the total complex and containing both monomer and
disulfide-dimers; and an intermediate oligomer (octamer) (c)
accounting for 60% of total complex and containing disulfide-
linked dimers. These results show that most of the L185P
peripherin-2 mutant assembles with Rom-1 into higher order
“octameric” disulfide-linked complexes similar to that observed
for WT peripherin-2-Rom-1 complex.

Interaction of L185P with WT Peripherin-2—Individuals
with autosomal dominant RP express both WT and mutant
peripherin-2 alleles. To determine if L185P peripherin-2 mu-
Peripherin-2 and Rom-1 Mutants

Proteins expressed in COS-1 cells were solubilized in Triton X-100, applied to 5–20% sucrose gradients, and sedimented for 16 h at 50,000 rpm and 4 °C in a Beckman TLS-55 rotor under reducing conditions. Fractions were analyzed on Western blots.

| Species | $S_{20,w}$ | Core complex |
|---------|------------|--------------|
| WT peripherin-2$^a$ | 5.36 ± 0.14 (n = 3)$^b$ | Homotetramer |
| WT Rom-1 | 4.56 ± 0.28 (n = 6) | Homotetramer |
| WT peripherin-2–1D4 | 5.14 (n = 1) | Homodimer |
| L185P peripherin-2 | 3.64 ± 0.19 (n = 3) | Homodimer |
| L185A peripherin-2 | 3.69 ± 0.31 (n = 3) | Homodimer |
| L185P-1D4 peripherin-2 | 3.67 ± 0.15 (n = 3) | Heterodimer |
| L185P peripherin-2–L188P Rom-1 | 3.24 (n = 2) | Heterodimer |
| L188P Rom-1 | 3.09 ± 0.23 (n = 5) | Homodimer |

$^a$ Value previously reported (13).
$^b$ $n$ is the number of independent experiments.
$^c$ Value previously reported (15) was recalculated using corrected parameters as described in Ref. 13.

With the L185P peripherin-2 mutant, velocity sedimentation analysis of this complex showed a mixed population of heterodimer (a) and heterotetramer (b) (Fig. 7B). Under nonreducing conditions, the velocity sedimentation profile of L185P peripherin-2–peripherin-2–1D4 complex (Fig. 7C) revealed the presence of higher order disulfide-linked oligomers (c) as well as heterotetramers (b) and heterodimers (a). In control experiments, the individually expressed WT peripherin-2–1D4 exhibited the same sedimentation behavior as WT peripherin-2 (Table I), indicating that the addition of the 1D4 epitope had no effect on subunit assembly.

**Expression of the G113E Rom-1 Mutant in COS-1 Cells**—A subset of individuals with autosomal dominant digenic RP have a G113E rom-1 missense mutation in addition to the L185P peripherin-2 mutation (23). We compared the protein expression level of G113E Rom-1 with that of WT Rom-1 in COS-1 cells (Fig. 8). In four independent experiments, the level of G113E Rom-1 expression was 5 ± 3% that of WT Rom-1. This suggests that low G113E Rom-1 expression contributes to this digenic disease.

**DISCUSSION**

Previous studies have shown that the L185P peripherin-2 mutant expressed in COS-1 cells has a sedimentation coefficient that is considerably smaller than that of the WT peripherin-2 core tetramer (15). We have determined the size of this mutant by hydrodynamic analysis, coimmunoprecipitation, and cross-linking. Although hydrodynamic analysis gave a molecular mass that was intermediate between a monomer and dimer, both coprecipitation and cross-linking provided strong evidence that the detergent-solubilized L185P mutant exists as a dimer under disulfide-reducing conditions. The low value for the molecular mass of L185P dimer determined by hydrodynamic measurements is consistent with the underestimation of the molecular mass of the native tetrmeric peripherin-2–Rom-1 complex reported using this technique (27). This underestimation in molecular mass can arise in part from the assumption that the protein binds the same amount of detergent in H$_2$O and D$_2$O (31). The estimated partial specific volume of the protein can also contribute to an inaccurate value for the molecular mass of the protein. Partial specific volumes in the range of 0.71–0.75 ml/g are generally used for proteins. The true value, however, is dependent not only on the amino acid composition of the protein but also on carbohydrate in the case of a glycoprotein as well as the possible presence of small amounts of bound lipid in the case of membrane proteins (31). WT and L185P peripherin-2 are known to be glycosylated in COS-1 cells (data not shown). The sugar composition of the oligosaccharide chain, however, is not known and therefore could not be factored into the calculations. The contribution of
the carbohydrate to the partial specific volume, however, is likely to be small, since endoglycosidase digestion studies show that peripherin-2 is not heavily glycosylated (2, 25). The peripherin-2 mutant may contain a small amount of tightly bound lipid in the detergent-solubilized state, which can also have a small effect on the partial specific volume. The value of 0.74 ml/g used in this study may represent an underestimation of the actual partial specific volume of the peripherin-2 mutant, which in turn will affect the value for the molecular mass of the peripherin-2 mutant.

The role of the conserved leucine residue at position 185 in subunit assembly was determined by analyzing several mutants. The L185A peripherin-2 mutant behaved like the L185P mutant. It formed a core dimer when individually expressed but assembled into a heterotetramer when coexpressed with Rom-1. This indicates that defective homotetramer formation of the L185P mutant is due to the absence of a leucine at position 185 and not the introduction of a proline residue. The ability of these mutants to interact with Rom-1 to form a native-like tetramer further indicates that the mutants are not grossly misfolded. Rom-1 has a leucine residue at an equivalent position (Leu188) within a conserved segment of the large intradiscal loop (Fig. 1). Replacement of this leucine with proline also prevented Rom-1 from assembling into homotetramers. Together, these studies indicate that Leu185 in peripherin-2 and Leu188 in Rom-1 play essential roles in the association of dimers into core tetramers.

The fact that these mutants exist as dimers and not mono-
FIG. 7. Immunoprecipitation and velocity sedimentation of L185P peripherin-2-WT peripherin-2-ID4 complexes under reducing and nonreducing conditions. A, COS-1 cells coexpressing L185P peripherin-2 and WT peripherin-2-ID4 were reduced with DTT, solubilized in Triton X-100, and immunoprecipitated with Per2B6-Sepharose. The COS-1 cell extract (lane a), unbound fraction (lane b), and bound, peptide-eluted fraction (lane c) were analyzed on Western blots labeled with Per2B6 antibody and Rho1D4. B, the co-precipitated complex from (A) was subjected to velocity sedimentation, and fractions were analyzed on Western blots labeled with Rho1D4 antibody. C, samples were treated as in A except under nonreducing conditions (without DTT), and the purified L185P-WT peripherin-2-ID4 complexes were subjected to velocity sedimentation. Western blots of fractions from a nonreducing SDS gel were labeled with the Rho1D4 antibody. Sedimentation positions of dimer (a), tetramer (b), and oligomer (c) are shown.

FIG. 8. Western blot of G113E Rom-1 and WT Rom-1 individually expressed in COS-1 cells. Detergent-solubilized cell extracts run on SDS gels were analyzed on Western blots labeled with Rom1C6 antibody (lane a, WT Rom-1; lane b, G113E Rom-1).

Peripherin-2 and Rom-1 Mutants

mers provides additional insight into the quaternary structure of the peripherin-2 and Rom-1 core complexes. The interactions responsible for dimer formation are distinct from those responsible for tetramer formation, since dimerization is not dependent on leucine at position 185 in peripherin-2 (or leucine 188 in Rom-1). On this basis, we infer that the WT peripherin-2 and Rom-1 core tetramer exhibits pseudo-2-fold symmetry. This leads to a model in which the core tetramer can be considered as a “dimer of dimers” (Fig. 9). The domain responsible for dimer formation is not known at the present time. However, the ability of L185P peripherin-2 to associate with L185P Rom-1 to form a heterodimer (Fig. 4C) suggests that the dimerization domain of these subunits, like the tetramerization domain, must be structurally similar.

The contribution of leucine and other bulky hydrophobic residues to the formation and maintenance of protein-protein interactions is well documented for intracellular proteins (transcription factors of the bZIP class (GCN4 (34)), the regulatory subunit of PKAIIb (35)), extracellular matrix proteins (tenascins, laminins, and thrombospondin (36)), and integral membrane proteins (glycophorin A (37) and metabotropic glutamate receptor (38)). Recently, the crystal structure of the dimeric extracellular ligand binding region of the metabotropic glutamate receptor, a seven-transmembrane G-protein-coupled receptor, has been solved (38). In this case, four conserved leucines and one isoleucine contribute to the dimer interface. In the intradiscal loop region of peripherin-2 and Rom-1, there are 9 conserved leucine and several conserved isoleucine residues in addition to Leu$^{185}$. It is possible that at least some of these residues also contribute to subunit interactions of peripherin-2 and Rom-1. Interestingly, many of these hydrophobic residues are also conserved in other members of the tetrspamien family of proteins including CD9, CD37, CD53, CD63, CD81/82, CD151, A15, and SJ23 (see Ref. 39 for review; alignment performed using the BLAST conserved domain data base search). This leads to the possibility that these proteins also exist as oligomeric complexes whose subunit interactions are mediated by these hydrophobic residues.

Peripherin-2-containing core tetramers further link together through Cys$^{150}$-mediated intermolecular disulfide bonds to form higher order oligomers (14). In this study, we have shown that two L185P dimers link together to form a disulfide-linked tetramer (Fig. 9), but this complex does not further assemble into the higher order oligomers observed for WT peripherin-2. This indicates that core tetramer formation is not required for intermolecular disulfide bond formation, but it is required for the generation of higher order oligomers. This finding together with previous studies showing that C150S peripherin-2 mutants form core noncovalent tetramers, but not higher order oligomers (14), indicates that higher order oligomerization involves both noncovalent and covalent (disulfide) interactions. The noncovalent interactions involved in higher order oligomerization are likely to be similar to those involved in tetramerization (Fig. 9). Formation of Cys$^{150}$-mediated intermolecular disulfide bonds between adjacent tetramers may initiate a conformational change within the intradiscal loop that facilitates noncovalent interactions responsible for oligomerization.

Disulfide-linked oligomers appear to be important in outer segment disc formation. In vitro expression of WT peripherin-2 under nonreducing conditions has been reported to result in flattened microsomal vesicles, whereas expression of WT peripherin-2 under reducing conditions or expression of C150S peripherin-2 under nonreducing conditions results in round vesicles (17). It is not known if the disulfide-linked L185P tetramers cause the flattening of these membranes or if higher order oligomers are required for this process.

Individuals with autosomal dominant digenic RP express a WT peripherin-2 allele along with the mutant allele. To understand in more detail the molecular basis for this disease, we examined the interaction of L185P peripherin-2 mutant with WT peripherin-2 and WT Rom-1. Co-expression of L185P and

*Peripherin-2 and Rom-1 Mutants*
**Fig. 9. Model for the oligomeric structure of peripherin-2 and the L185P mutant.** WT peripherin-2 monomers (transmembrane domain shown as a cylinder) interact to form a core noncovalent tetramer with a 2-fold axis of symmetry. Leucine at position 185 (Leu185) of the intradiscal loop is required for tetramer formation (tetramerization). Dimer formation (dimerization) may involve interactions between transmembrane, intradiscal, or cytoplasmic domains (omitted for simplicity). The core tetramers link up through Cys185-mediated intermolecular disulfide bonds to form higher order oligomers (only the octamer is shown). The L185P peripherin-2 mutant consists of a core dimer that can link through Cys185-mediated intermolecular disulfide bonds to form a tetramer but not a higher order oligomer. Numbers on cylinders illustrate the 2-fold symmetry of the core tetramer and the concept that it consists of a dimer of dimers (dimerization has been drawn as head-to-head). Rom-1 also forms homotetramers and heterotetramers with peripherin-2 but limits oligomerization to octamers (14). WT peripherin-2 resulted in a mixture of dimeric and tetrameric core complexes that further interacted through intermolecular disulfide bonds to form oligomers. L185P peripherin-2 also assembled with Rom-1 to form predominantly core tetramers as previously shown (15). Like the WT proteins, the L185P peripherin-2–Rom-1 heterotetramers further associated via intermolecular disulfide bonds into disulfide-linked intermediate oligomers, most likely octamers. On the basis of these results, we conclude that the L185P mutant can interact with WT proteins to form higher order oligomers that most likely contribute to the formation and stabilization of outer segment discs.

Individuals affected with autosomal dominant digenic RP linked to the L185P mutation in peripherin-2 coinherit a mutation in rom-1. In initial genetic screens, affected families were found to harbor a 1-base pair insertion at codon 80 or codon 114 of the rom-1 gene, resulting in an early termination of the polypeptide chain and in effect a null allele (24). More recently, Dryja et al. (23) identified a family with digenic RP that has a G113E mutation in rom-1 along with the L185P mutation in peripherin-2. Since the G113E missense mutation occurs in the third putative transmembrane segment of Rom-1 (Fig. 1), we reasoned that introduction of a negatively charged glutamate residue into a hydrophobic membrane-spanning segment might affect the stable expression of Rom-1. Our results show that the level of G113E Rom-1 expression in COS-1 cells is 20-fold lower than WT Rom-1. Due to the low level of G113E Rom-1 expression, we were unable to analyze the properties of this mutant or its interaction with peripherin-2. On the basis of the very low expression level of this mutant, it is reasonable to conclude that the G113E mutant in Rom-1 behaves mechanistically as a null mutation.

Autosomal dominant digenic RP is a complex disease requiring the coinheritance of a L185P peripherin-2 allele and an effective rom-1 null allele (15, 23, 24). Individuals who inherit only one of these mutant alleles are essentially normal. Our data suggest that the level of peripherin-2-containing oligomers is critical in the manifestation of this disease. Since the L185P peripherin-2 mutant is incapable of forming homotetramers and corresponding higher order disulfide-linked oligomers, individuals who inherit this mutation along with a null allele in Rom-1 will have a level of peripherin-2-containing oligomers below the critical threshold level required for the formation of stable outer segments. The presence of disorganized, unstable outer segments will lead to photoreceptor degeneration as observed for heterozygous rd8 mice (7). In the case of individuals who inherit only the L185P peripherin-2 mutation, a significant amount of the L185P will assemble with WT Rom-1 as well as WT peripherin-2 to form “functional” oligomers. This would have the effect of raising the level of oligomers above the threshold needed to form stable outer segments. These individuals would experience little if any photoreceptor degeneration and have essentially normal vision. Individuals who inherit only a rom-1 null allele would be expected to have normal levels of peripherin-2, and half the level of Rom-1. Reduced levels of Rom-1, however, have no appreciable effect on outer segment structure or photoreceptor viability as shown in recent studies of heterozygous rom-1 knockout mice (10). Hence, individuals who inherit only a null allele or a G113E missense mutation in rom-1 would have a sufficient level of peripherin-2-containing oligomers to support photoreceptor outer segment structure and photoreceptor viability.

The L185P peripherin-2 mutant is different from peripherin-2 mutants that cause monogenic autosomal dominant RP. For example, the C214S and C165S peripherin-2 mutants are highly misfolded and do not form core tetramers or higher order oligomers in the presence or absence of Rom-1 (13, 14). Furthermore, expression of the C165Y mutant fails to result in the formation of flattened vesicles (17). Hence, individuals with these mutations would have a significantly decreased level of functional oligomers in the presence or absence of Rom-1, resulting in unstable, disorganized outer segment discs and photoreceptor degeneration. A dominant negative effect of these misfolded mutants, however, may also contribute to photoreceptor degeneration and the autosomal dominant RP phenotype.

**Acknowledgments—** We thank Dr. Andrew Goldberg for constructing the peripherin-2–1D4 cDNA.

**REFERENCES**

1. Molday, R. S., Hicks, D., and Molday, L. (1987) Invest. Ophthalmol. Vis. Sci. 28, 50–61
2. Connell, G. J., and Molday, R. S. (1990) Biochemistry 29, 4691–4698
3. Bascom, R. A., Manara, S., Collins, L., Molday, R. S., Kalnins, V. I., and McInnes, R. R. (1992) Neuron 8, 1171–1184
4. Arikawa, K., Molday, L. L., Molday, R. S., and Williams, D. S. (1992) J. Cell Biol. 116, 659–667
5. Mortiz, O. L., and Molday, R. S. (1996) Invest. Ophthalmol. Vis. Sci. 37, 352–362
6. Sanyal, S., and Jansen, H. G. (1981) Neuroscience 21, 1–26
7. Hawkins, R. K., Jansen, H. G., and Sanyal, S. (1985) Exp. Eye Res. 41, 701–720
8. Goldberg, A. F., Brennan, M. B., Danielson, P. E., Knize, C. A., and Sutcliffe, J. G. (1989) Nature 338, 70–73
9. Connell, G., Bascom, R., Molday, L., Reid, D., McInnes, R. R., and Molday, R. S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 723–726
10. Clarke, G., Goldberg, A. F., Vidgen, D., Collins, L., Pledger, L. I., Schwarz, L., Molday, R. L., Rossant, J., Stuel, A., Molday, R. S., Birch, D. G., and McInnes, R. R. (2000) Nat. Genet. 25, 67–73
11. Travis, G. H., Sutcliffe, J. G., and Bek, D. (1991) Neuron 6, 61–70
12. Boeze-Battaglia, K., Lamba, O. P., Napoli, A. A. Jr., Sinha, S., and Guo, Y. (1998) Biochemistry 37, 9477–9487
13. Goldberg, A. F., Loewen, C. J., and Molday, R. S. (1998) Biochemistry 37, 680–685
14. Loewen, C. J., and Molday, R. S. (2000) J. Biol. Chem. 275, 5370–5378
15. Goldberg, A. F., and Molday, R. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15726–15730
16. Kedzierski, W., Weng, J., and Travis, G. H. (1999) J. Biol. Chem. 274, 29181–29187
17. Wrigley, J. D., Ahmed, T., Nevett, C. L., and Findlay, J. B. (2000) J. Biol.

---

**Peripherin-2 and Rom-1 Mutants**

---

**FIG. 9. Model for the oligomeric structure of peripherin-2 and the L185P mutant.** WT peripherin-2 monomers (transmembrane domain shown as a cylinder) interact to form a core noncovalent tetramer with a 2-fold axis of symmetry. Leucine at position 185 (Leu185) of the intradiscal loop is required for tetramer formation (tetramerization). Dimer formation (dimerization) may involve interactions between transmembrane, intradiscal, or cytoplasmic domains (omitted for simplicity). The core tetramers link up through Cys185-mediated intermolecular disulfide bonds to form higher order oligomers (only the octamer is shown). The L185P peripherin-2 mutant consists of a core dimer that can link through Cys185-mediated intermolecular disulfide bonds to form a tetramer but not a higher order oligomer. Numbers on cylinders illustrate the 2-fold symmetry of the core tetramer and the concept that it consists of a dimer of dimers (dimerization has been drawn as head-to-head). Rom-1 also forms homotetramers and heterotetramers with peripherin-2 but limits oligomerization to octamers (14).
Peripherin-2 and Rom-1 Mutants

Chem. 275, 13191–13194
18. Farrar, G. J., Kenna, P., Jordan, S. A., Kumar-Singh, R., Humphries, M. M., Sharp, E. M., Sheils, D. M., and Humphries, P. (1991) Nature 354, 478–480
19. Kajiwara, K., Hahn, L. B., Mukai, S., Travis, G. H., Berson, E. L., and Dryja, T. P. (1991) Nature 354, 480–483
20. Saga, M., Mashima, Y., Akeo, K., Oguchi, Y., Kudoh, J., and Shimizu, N. (1993) Hum. Genet. 92, 519–521
21. Wells, J., Wroblewski, J., Keen, J., Inglehearn, C., Jubb, C., Eckstein, A., Jay, M., Arden, G., Bhattacharya, S., Fitzke, F. and Bird, A. (1993) Nat. Genet. 3, 213–218
22. Weleber, R. G., Carr, R. E., Murphy, W. H., Sheffield, V. C., and Stone, E. M. (1993) Arch. Ophthalmol. 111, 1531–1542
23. Dryja, T. P., Hahn, L. B., Kajiwara, K., and Berson, E. I. (1997) Invest. Ophthalmol. Vis. Sci. 38, 1972–1982
24. Kajiwara, K., Berson, E. L., and Dryja, T. P. (1994) Science 264, 1604–1608
25. Goldberg, A. F., Moritz, O. L., and Molday, R. S. (1995) Biochemistry 34, 14213–14219
26. Nelson, R. M., and Long, G. L. (1989) Anal. Biochem. 180, 147–151
27. Goldberg, A. F., and Molday, R. S. (1996) Biochemistry 35, 6144–6149
28. Worthington Biochemical Corp. (1996) Worthington Enzyme Manual, pp. 36, 126, Freehold, NJ
29. Fasman, G. D., and Chemical Rubber Co. (1975) Handbook of Biochemistry and Molecular Biology, 3rd Ed., p. L-10, CRC Press, Inc., Cleveland
30. Siegel, L. M., and Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362
31. Clarke, S., and Smigel, M. D. (1989) Methods Enzymol. 172, 696–709
32. Caccio, M., Kumar, N. M., Safarik, K., and Gilula, N. B. (1993) J. Biol. Chem. 270, 18643–18648
33. MacKenzie, D., Arendt, A., Hargrave, P., McDowell, J. H., and Molday, R. S. (1984) Biochemistry 23, 6544–6549
34. O'Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) Science 254, 539–544
35. Li, Y., and Rubin, C. S. (1995) J. Biol. Chem. 270, 1935–1944
36. Kammerer, R. A. (1997) Matrix Biol. 15, 555–565, 567–568
37. MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) Science 276, 131–133
38. Kumishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakamichi, S., Jingami, H., and Morikawa, K. (2000) Nature 407, 971–977
39. Wright, M. D., and Tomlinson, M. G. (1994) Immunol. Today 15, 588–594
Molecular Characterization of Peripherin-2 and Rom-1 Mutants Responsible for Digenic Retinitis Pigmentosa
Christopher J. R. Loewen, Orson L. Moritz and Robert S. Molday

J. Biol. Chem. 2001, 276:22388-22396.
doi: 10.1074/jbc.M011710200 originally published online April 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011710200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 37 references, 14 of which can be accessed free at http://www.jbc.org/content/276/25/22388.full.html#ref-list-1