A Role for the Tubby-Like Protein 1 in Rhodopsin Transport

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PURPOSE. To test the hypothesis that a lack of Tubby-like protein 1 (TULP1) function causes aberrant transport of nascent rhodopsin and to examine the functional relationship between the homologous proteins TULP1 and Tubby by studying mice carrying combined mutations.

METHODS. Subcellular localization of TULP1 and rhodopsin in photoreceptors was determined by immunofluorescence and by postembedding immunoelectron microscopy. Mice carrying different tulp1/tubby allele combinations were examined by histology, electroretinograms (ERGs), and immunofluorescence microscopy.

RESULTS. TULP1 is distributed throughout the photoreceptor cytoplasm but is excluded from the outer segments and the nuclei. In the tulp1+/− mice, ectopic accumulation of rhodopsin occurs at an early age. Both the vesicular profiles in the interphotoreceptor space and the inner segment plasma membranes are immunoreactive for rhodopsin. Mice doubly homozygous for null mutations in the tulp1 and tubby genes initially develop photoreceptors and express a battery of photoreceptor markers at age 14 days. Thereafter their photoreceptors undergo a fulminant degeneration that reaches completion by postnatal day 17. The disease phenotype in the double homozygote is much more severe than either single homozygote. Double heterozygotes are phenotypically normal.

CONCLUSIONS. A lack of TULP1 function results in misrouting of nascent rhodopsin. TULP1 may be a component of the cellular machinery that targets nascent rhodopsin to the outer segments. Comparison of disease phenotypes in the single and double mutants suggests that TULP1 and Tubby are not functionally interchangeable in photoreceptors nor do they form an obligate functional complex. (Invest Ophthalmol Vis Sci. 2001; 42:1955–1962)

TULP1 is a member of the Tubby family of proteins, defined by the highly conserved carboxyterminal half of their primary sequences.1,2,3 This family of proteins includes the prototype Tubby and TULP1, 2, and 3 (Tubby-like protein 1, 2, and 3). Their functions are not understood, but two have been associated with their defects, we explored the functional relationship between TULP1 and Tubby in photoreceptors by examining the retinal phenotypes of mice with combined mutations in these two genes.

METHODS

Animals and Phenotype Analyses

The tulp1 knockout mouse was described previously.4 The tubby mutant mice (C57BL/6J-tub/tub) were purchased from the Jackson Laboratory (Bar Harbor, ME). Single heterozygotes of either mutant were generated by crossing the homozygotes with C57BL/6 wild-type (wt) mice. Mice doubly heterozygous for tubp1 and tubby mutations were generated by crossing homozygous tulp1 and tubby mice. Mice doubly homozygous for tulp1 and tubby were generated by sibling cross between the double heterozygotes. Genotypes of mice were determined by PCR amplification of genomic DNA. A minimum of three (double homozygote) or more animals (other genotypes) was

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examined by light microscopy of retinal sections and by immunostaining for rhodopsin and cone opsin to determine if there was mislocalization. The double heterozygotes were also examined by electoretinography, performed as previously described. The double homozygotes were not examined by electoretinography because of their exceptionally early cell loss. All experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Generation of TULP1 Antibody**

A cDNA fragment corresponding to the N-terminal 201 amino acid residues of mouse TULP1 (GenBank accession no. AF085681) was amplified by PCR from a mouse retinal cDNA pool and inserted in frame into the BamHI/HindIII sites of the expression vector pET28b (Novagen, Madison, WI). The recombinant TULP1 protein was expressed as a His-tagged fusion protein in the Escherichia coli host BL21(DE3)pLysS. The recombinant protein was purified through an Ni2+charged nitriloacetic acid agarose column and was used to immunize rabbits. The specific antibody, designated M-tulp1N, was purified from the antiserum through an affinity column in which the recombinant TULP1 protein was cross-linked to the AminoLink coupling gel (Pierce, Rockford, IL).

**Immunoblotting, Immunofluorescence, and Light Microscopy**

For immunoblotting, tissues were homogenized in Laemmli buffer, and the proteins were separated on SDS-polyacrylamide gels and electroblotted to PVDF membranes. Membranes were incubated with the TULP1 or rhodopsin (rho 1D4) primary antibodies, followed by peroxidase-conjugated secondary antibodies, and detected by chemiluminescence. For immunofluorescence microscopy, mouse eyes were fixed in 2% formaldehyde for 1 hour. The eye cups were cryoprotected in 30% sucrose, quick-frozen in liquid nitrogen, and sectioned at 10-μm thickness. Sections were incubated with primary antibodies, followed by Cy3-conjugated secondary antibodies, and mounted in an aqueous mounting medium. To examine retinal sections by light microscopy, tissues were prepared as above and stained with methylene blue.

**Postembedding Immuno Electron Microscopy**

Mouse eyes were fixed in 2% formaldehyde, 0.1% glutaraldehyde in phosphate-buffered saline (PBS) for 1 hour. The anterior segments and lens were dissected out, and the eye cups were embedded in LR White resin. Thin sections were cut and collected on Formvar-coated nickel grids. Alternatively, eye cups were soaked in 30% sucrose in PBS overnight and frozen in liquid nitrogen. Thin (70 nm) sections were cut on a cryo-ultramicrotome and collected on Formvar-coated nickel grids. Grids carrying the sections were incubated with 0.15 M glycine/PBS and then blocked in 1% fish gelatin in TTBS. A mixture of rho 1D4 and rho 4D2 monoclonal antibodies was used to detect rhodopsin, and M-tulp1N was used to detect TULP1. Incubation with primary antibodies diluted in the blocking solution proceeded overnight. The sections were then washed in TTBS. To detect rhodopsin, the sections were incubated with goat anti-mouse antibody conjugated to 12-nm gold particles (LR White sections) or with unlabeled rabbit anti-mouse secondary antibody followed by incubation with protein A conjugated to 10-nm gold particles (frozen sections). TULP1 was detected by incubating sections with goat anti-rabbit secondary antibody conjugated to 0.8-nm gold particles, followed by silver enhancement using the Aurion silver enhancement reagents (Electron Microscopy Sciences, Fort Washington, PA). Sections were poststained with 5% uranyl acetate, washed through drops of methylcellulose, and air-dried. Sections were viewed and photographed on a JEOL 100CX electron microscope (Peabody, MA).

**RESULTS**

**Ultrastructural Localization of TULP1**

To obtain a TULP1 antibody suitable for ultrastructural studies, we generated a polyclonal antibody, M-tulp1N, directed against the N-terminal 201 amino acid residues of murine TULP1. Figure 1 illustrates the monospecificity of the antibody. On immunoblots, it recognizes a band of approximately 70 kDa that was present in wt mouse retinas but not in wt brain or tulp1−/− retinas (Fig. 1A). The apparent molecular weight is similar to that previously estimated for murine TULP1. On

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932896/ on 06/24/2017)
retinal sections, staining is found only in the wt retina and is restricted to the photoreceptor cell layer. The staining is strongest in the inner segments but is largely absent from the outer segments (Fig. 1B). The weaker punctate signals in the outer nuclear layer do not overlap with the nuclei but appear to be located in the perikarya. These results are similar to our previous finding using a TULP1 peptide antibody and an antiserum raised against the human TULP1 sequence and are also consistent with the results reported by others. Thus, the M-tulp1N antibody is specific for TULP1.

To establish the subcellular localization of TULP1 at the ultrastructural level, immunoelectron microscopy analyses were performed on wt mouse retinal sections. Figure 2 is a sequential montage of immunoelectron micrographs spanning the photoreceptor cell layer. TULP1 is distributed uniformly throughout the photoreceptor inner segments, perikarya, and synaptic terminals but is excluded from the outer segments and the nuclei. Inner retinal layers were not significantly labeled (Fig. 2 and data not shown). In photoreceptors, TULP1 does not appear concentrated in a particular intracellular compartment. These observations are consistent with previous cellular fractionation studies that indicated that TULP1 is present in the cytosol.

**Rhodopsin Mislocalization in Younger tulp1 −/− Mutant Mice**

In our previous study, rhodopsin was found mislocalized in the tulp1−/− photoreceptors at 17 to 21 postnatal days of age. Ectopic localization of rhodopsin can be a secondary event, seen in the late stage of degeneration in some disease models when all outer segments have disappeared. To investigate if the tulp1 mutant photoreceptors are able to transport rhodopsin to the outer segments initially and subsequently lose this ability as the disease progresses, mice at postnatal day 12 were examined. Rhodopsin was also found mislocalized at this early
age (Fig. 3A). In comparison, the rd mouse at postnatal day 12 was found to partition rhodopsin to the outer segments (Fig. 3A), despite having a faster disease course than the tulp1 mutant. Mice at even younger ages were not examined because rhodopsin is known to be present in normal photoreceptor cell bodies before the elaboration of the outer segments. Another outer segment membrane protein, peripherin/RDS, localizes predominantly in the outer segments in the 12-day-old tulp1 mutant, similar to our previous finding in the mutant mice at 17 days of age. Thus, rhodopsin mislocalization in the tulp1 mutant appears to be selective. If rhodopsin mislocalization were a nonspecific secondary event, one might expect both proteins to be affected. Taken together, these data suggest that rhodopsin mislocalization in the tulp1--/-- mouse is an early, and likely primary, event.

On immunoblots, rhodopsin from tulp1--/-- photoreceptors comigrates with rhodopsin from wt photoreceptors (Fig. 3B). This indicates that rhodopsin in tulp1--/-- photoreceptors is similarly glycosylated as rhodopsin in the wt photoreceptors and is therefore likely to have been processed through the Golgi compartment. Mutant rhodopsin that can not proceed to the Golgi apparatus exhibits lower apparent molecular weights.

**Localization of Rhodopsin in the Extracellular Vesicles and Plasma Membranes**

A defect in TULP1 function may cause misrouting of nascent rhodopsin to the lateral inner segment plasma membranes, with subsequent budding and accumulation of extracellular vesicles. This hypothesis predicts that both the vesicular profiles and plasma membranes are laden with rhodopsin. To test this hypothesis, we performed immunogold labeling for rhodopsin on retinal sections. Retinas embedded in LR White resin were initially studied. Rhodopsin labeling is seen only in the outer segments in the wt retina (Fig. 4A). In the tulp1 mutant retina (Fig. 4B), rhodopsin labeling is also found in the extracellular vesicles and inner segment plasma membranes. The labeling density for rhodopsin in the vesicular membranes appears quite high, approaching that in the outer segment membranes. Thus, a substantial fraction of rhodopsin fails to reach the outer segments. Occasional vesicles are seen connected with the inner segment plasma membranes.

Although these data suggest that rhodopsin accumulates aberrantly in the inner segment plasma membranes, the labeling is not of sufficient density to allow unambiguous conclusions. To improve sensitivity, we performed immunolabeling on ultrathin frozen sections and found strong labeling of the plasma membranes surrounding the connecting cilia in wt photoreceptors (Fig. 5A). This membrane domain is known to be immunopositive for rhodopsin in normal photoreceptors detectable only with sensitive procedures. Plasma membranes proximal to the cilia are not significantly labeled (Figs. 5A, 5B). Thus, in our hands the frozen section approach affords higher sensitivity while maintaining specificity. With this approach, the plasma membranes surrounding

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the inner segments in the *tulp1* mutant are found densely labeled (Figs. 5C, 5D), as are the extracellular vesicles (Fig. 5E). Plasma membranes surrounding the perikarya and synaptic terminals in the mutant are also immunopositive for rhodopsin (not shown), as might be expected. There is no apparent accumulation of rhodopsin in intracellular membranous compartments. Mitochondrial membranes, appearing as negative images, are visible and are not labeled (Figs.
tors, and at 14 days of age the photoreceptor nuclear layer and tulp1 outcome was found in mice doubly homozygous for the and TULP1 proteins are not interchangeable. A very different to 6 months of age (not shown). Thus, the functions of Tubby photoreceptors (not shown), and their ERGs were normal up there was no evidence of rhodopsin mislocalization in their double heterozygotes appear normal, and the single homozygotes are in the early stage of degeneration. In the double homozygote, all retinal layers are present at 14 postnatal days. By 17 postnatal days, the photoreceptor layer is reduced to a single row of nuclei.

These data demonstrate that the plasma membranes are the primary sites of rhodopsin accumulation in the tulp1 mutant and suggest that the extracellular vesicles are derived from the plasma membranes.

Retinal Phenotype in the tulp1/tubby Double Mutants

Given the similarities in their primary structures and in the disease manifestations associated with their defects, it is possible that TULP1 and Tubby perform analogous functions in photoreceptors. To explore the functional relationship between these two proteins, we crossed the tubby and tulp1 mutant mice to each other to generate mice that were doubly heterozygous and doubly homozygous for the mutant alleles and analyzed their disease phenotypes by histology and immunocytochemistry for rhodopsin and by ERG. We reasoned that two possible scenarios could be tested by the genetic crosses. First, the two proteins may perform essentially identical functions in the photoreceptors so that they are interchangeable. In this case the severity of disease would be a function of the gene dosage reduction involving these two genes. One would predict that the double heterozygotes should have a disease similar in severity to either one of the single homozygotes, depending on the relative expression levels of these two genes. Alternatively, the two proteins may exist in photoreceptors as an obligate functional complex. In the latter case, loss of either protein would render both proteins nonfunctional. Therefore, either single homozygote should exhibit the same severity of disease as the double homozygotes. We found that the double heterozygotes are phenotypically normal by histology (Fig. 6). There was no evidence of rhodopsin mislocalization in their photoreceptors (not shown), and their ERGs were normal up to 6 months of age (not shown). Thus, the functions of Tubby and TULP1 proteins are not interchangeable. A very different outcome was found in mice doubly homozygous for the tubby and tulp1 mutations. These mice initially develop photoreceptors, and at 14 days of age the photoreceptor nuclear layer retains approximately normal thickness. However, the inner segments are extremely short, and there is no evidence of outer segment formation. Thereafter, the photoreceptors undergo a rapid degeneration so that by 17 days of age, there is only a single layer of photoreceptor cell nuclei remaining (Fig. 6). This rate of decline is much faster than either single homozygote alone and is even faster than that of the rd mouse, which has the fastest rate of degeneration known. Therefore, these two proteins do not exist in an obligate functional complex.

As an initial screen for putative target proteins that might be under transcriptional control by the Tubby proteins, we examined a panel of photoreceptor-specific markers by immunofluorescence to see if the expression of any markers was abolished in the double homozygotes. These markers included rhodopsin, cone opsins, arrestin, rod cGMP phosphodiesterase α-subunit, transducin, cGMP-gated cation channel, and peripherin/RDS. The photoreceptor cell layer of the double homozygotes stained positive for all of these markers at postnatal day 14 (not shown). Because of the grossly abnormal photoreceptor cell morphology, more quantitative assays were deemed not feasible and therefore were not performed.

DISCUSSION

An important finding of this work is that TULP1 is largely excluded from the outer segment and the nuclei of photoreceptors. This finding has two implications for the TULP1 function. First, it does not favor a role for TULP1 in disc morphogenesis or maintenance. Second, Tubby and TULP1 proteins have been suggested to be transcription activators, with nuclear localization of Tubby cited as supporting evidence. Our data show that TULP1 is not a nuclear protein and therefore do not favor a role for TULP1 as a transcription activator. We cannot, however, rule out the possibility that TULP1 may translocate dynamically into the nuclei in response to extracellular cues. We also cannot rule out the formal possibility that epitope masking underlies the absence of staining in the nuclei.

As another test for the putative role of TULP1 and Tubby as transcriptional activators, we examined the expression of a panel of photoreceptor-specific marker proteins in the mutant photoreceptors. We conducted these experiments in the double homozygotes to avoid ambiguities that might arise from the potential functional redundancy of these two proteins. Because the tulp1 and tubby mutants were able to support initial postnatal photoreceptor development and because TULP1 is highly photoreceptor specific, we reasoned that photoreceptor-specific proteins present in mature photoreceptors would be appropriate candidates. Among a panel of seven photoreceptor-specific proteins, no loss of expression was found for any of them in the double homozygotes. Thus, at least for these proteins, transcriptional activation does not appear to be dependent on the Tubby/TULP1 functions.

Rhodopsin is mislocalized in the photoreceptor cell bodies in the tulp1−/− mutant. A loss of polarity in rhodopsin distribution is not sufficient evidence for a primary defect in the pathway that targets nascent rhodopsin to the outer segments. It has been found in the rd5 mice, which do not support any outer segment formation, and in the late stage of degeneration in the rd and RCS models when the outer segments have disappeared. Thus, mislocalization of rhodopsin can be a secondary response to the blocked outer segment formation. Our previous study examined the tulp1−/− retinas at a relatively early age (17–21 postnatal days) when the photoreceptor outer segments are still preserved. This would suggest that rhodopsin mislocalization in this mutant occurs at
the early stage of disease and represents a primary defect. To strengthen this argument, we expanded our studies in the current work to mutant retinas at postnatal day 12 and found that rhodopsin is also mislocalized. Because the rd mouse has a much faster course of disease than the tulp1/−/− mouse and yet at the same age is able to maintain a better partitioning of rhodopsin in the outer segments, we conclude that rhodopsin mislocalization in the tulp1 mutant retinas is indeed an early and primary defect. Rhodopsin distribution in other rodent models of retinal degeneration also supports the notion that rhodopsin mislocalization in the tulp1 mutant is a direct consequence of missing TULP1 function. For example, predominant rhodopsin localization in the outer segments has been found in the rhodopsin P23H transgenic mice and rats25–27 and in the T17M transgenic mice.28 A line of P23H rhodopsin transgenic mice (L line) has a much faster rate of degeneration than the tulp1 mutant. Yet when examined at postnatal day 15, rhodopsin was found predominantly in the outer segments.25,26

We have demonstrated that the ectopic site of rhodopsin accumulation in photoreceptors is the plasma membrane. The extracellular vesicles are also highly enriched for rhodopsin. The origin as well as the significance of these extracellular vesicles remains a subject for discussion. We do not believe the vesicles are simply “debris” from rapid cell death. In most animal models of retinal degeneration spanning the spectrum of severity, accumulation of extracellular vesicles is not part of the pathology. Another observation inconsistent with the debris theory is that the vesicles accumulate transiently, peaking around 17 to 21 postnatal days, and become scarce after 28 postnatal days even as rapid photoreceptor cell death is occurring. The high rhodopsin content of the vesicles rules out the possibility of mitochondrial remnants, as some have suggested. The available data indicate that the vesicles have originated from the plasma membranes. From a review of the literature, we found five other genetic models exhibiting transient vesicular accumulation during photoreceptor degeneration. These are the rds,22,28–31 tubby,32 pcd,33 one line of rhodopsin P347S transgenic mice (the A line),34 and the recently described kinesin-II mutant.35 In the rds mice, rhodopsin-bearing vesicles accumulate just distal to the rudimentary connecting cilia. The rds photoreceptors never elaborate outer segments because these mice lack the normal RDS protein required for outer segment disc membrane assembly.35 These observations indicate that the vesicular elements in the rds retinas are the product of aborted outer segment formation. In the tulp1 mutant, outer segments form, albeit shortened, and contain well-stacked disc membranes. The vesicles do not accumulate at the junction between the inner and outer segments where disc assembly takes place but are located more proximally. Therefore in the tulp1 retinas, the vesicles appear not to have originated from a failure of disc assembly or maintenance. Mechanistically, the origin of extracellular vesicles is probably similar in the tulp1, tubby, pcd, the rhodopsin P347S, and the kinesin-II mutants. The function of the pcd and tubby genes remains unknown. In the case of the rhodopsin mutant, it is well established that the C terminus of rhodopsin contains a signal sequence that targets rhodopsin to the outer segments and that the proline residue at the 347 position is essential.27,33–36,40 It should be noted that in the P347S transgenic mice, the extent to which the C-terminal mutation disrupts rhodopsin transport depends on the ratio of mutant to wt rhodopsins.35 In a lower expressing line (C1 line), the mice exhibit little rhodopsin mislocalization and few extracellular vesicles. When C1 mice were bred to homozygosity or when mice of a higher expressing line (A1 line) were examined, vesicular accumulation was found. Rhodopsin mislocalization in the higher expressing lines was not examined in detail in the earlier work,33 but recent immunoelectronmicroscopy studies confirmed rhodopsin accumulation in the plasma membranes in the A1 line of transgenic mice (our unpublished observation). Thus, the similarity in phenotype between the rhodopsin P347S mutant and the tulp1/−/− mutant lends further credence to the proposal that TULP1 functions in the transport of nascent rhodopsin to the outer segments. It appears that vesicle formation is an outcome as well as an indicator of rhodopsin accumulation in the plasma membrane to high levels. In support of this notion, vesicle formation in these mutants occurs early but transiently, corresponding to the period of peak rhodopsin synthesis during postnatal development.14 The shortened outer segments in these mutants can be readily explained by the reduced amount of nascent rhodopsin reaching the correct destination and being available for disc assembly.

In normal photoreceptors, rhodopsin is sorted into post-Golgi transport vesicles and transported vectorially to specialized membrane microdomains surrounding the base of the connecting cilium. To complete the task of transport, rhodopsin must be sorted into the correct post-Golgi vesicles at the trans-Golgi network, translocate vectorially and be prevented from redistribution once it reaches the target membrane domains. Future studies of TULP1 should examine at which of these steps TULP1 is a required participant.

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References


