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Tubby-like Protein 1 (TULP1) Interacts with F-actin in Photoreceptor Cells

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Abstract

Purpose—TULP1 is a photoreceptor-specific protein of unknown function that, when mutated, can cause retinitis pigmentosa in humans and photoreceptor degeneration in mice. Toward a better understanding of the role of TULP1 in retinal disease, its subcellular localization was sought and the TULP1 protein binding partners identified.

Methods—Immunocytochemistry and subcellular fractionation were used to determine the localization of TULP1 and actin in COS7 cells and photoreceptor cells. Immunoprecipitation from retinal lysates followed by liquid chromatography tandem mass spectrometry and in vitro binding assays was used to identify TULP1-binding partners. Phospholipid binding assays were performed with a commercially available kit.

Results—TULP1 localizes at or near the plasma membrane and associates with the membranous fraction of COS7 cells, probably through binding phosphorylated phospholipids. In addition, TULP1 partitions to the aqueous phase during Triton X-114 extraction. Immunoprecipitation from retinal lysate identified F-actin as a possible TULP1-binding partner. Co-sedimentation assays further support an interaction between TULP1 and actin. In photoreceptor cells, actin and TULP1 colocalize at the inner segment, connecting cilium, and outer limiting membrane.

Conclusions—TULP1 is a cytoplasmic protein that associates with cellular membranes and the cytoskeleton. TULP1 and actin appear to interact and colocalize in photoreceptor cells of the retina. TULP1 may be involved in actin cytoskeletal functions such as protein trafficking that takes place at or near the plasma membrane from the inner segment through the connecting cilium into the outer segment of photoreceptor cells.

TULP1 is a member of the TULP family of four proteins (TUB and TULP1, -2, and -3), characterized structurally by the highly conserved C-terminal half of the protein.[1] TULP proteins are localized primarily to nervous tissues with TUB and TULP3, which are widely distributed throughout the central nervous system, and TULP1 and TULP2, which are restricted largely to the retina and testis, respectively.[1–6] In the retina, TULP1 is found exclusively in photoreceptor cells, localizing primarily in the inner segments and connecting cilium and to a lesser extent in the perinuclear cytoplasm and synaptic termini.[6–9] The functions of these proteins are not known; however, the strong conservation of TULP proteins through evolution suggests that they function in a basic pathway in cells in which

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they are expressed. Other than members of the TULP family, database searches do not reveal significant homology with known proteins or functional motifs.

TULP proteins play a central role in cellular function, as two of the family members have been linked to neurosensory disease phenotypes. Mice homozygous for a mutation in the *Tub* gene exhibit retinal and cochlear degeneration as well as adult-onset obesity associated with insulin resistance.[4,10,11] Mutations in human *TULP1* have been shown to cause retinitis pigmentosa (RP), a heterogeneous group of inherited retinal diseases in which the rod and cone photoreceptor cells degenerate, leading to blindness.[12–15] Unlike the *tubby* phenotype in mice, patients with *TULP1* mutations are not obese and have no hearing impairment; however, they have a more severe visual handicap in comparison to patients with RP caused by defects in other retinal genes.[12,16] Similarly, loss of TULP1 function in mice, introduced by gene knockout, also causes photoreceptor degeneration.[7,9] The degeneration in mice resembles the phenotypic characteristics reported in patients with RP due to *TULP1* mutations, in that it is an early-onset, progressive, panretinal degeneration involving both rod and cone photoreceptor cells.

Given their association with ocular disease, the predominant quest surrounding TULP proteins is to determine their physiological roles. Toward this end, there have been several studies performed to identify the function of TUB, as it is the founding member of the TULP family and its phenotype involves both neurosensory and neuroendocrine deficits. A wide array of postulated cellular functions include vesicular trafficking,[17] mediation of insulin signaling,[18] gene transcription,[19] G-protein signaling,[20] and ribosomal RNA synthesis.[5] However, there is no direct evidence pertaining to the function of TULP1.

As a step toward understanding how TULP1 maintains the health of photoreceptor cells, we determined the subcellular localization of TULP1 and pursued the identification of TULP1 interacting proteins in the retina. In the present study, we showed that TULP1 is a cytoplasmic protein that associates with membranes through binding phospholipids and provide evidence that TULP1 interacts with the cytoskeletal protein F-actin. We also demonstrated that TULP1 and actin colocalize in photoreceptor cells of the retina and in cultured COS7 cells. These data provide the first direct evidence that TULP1 may be involved in actin cytoskeletal functions, such as membrane trafficking or protein movement, and support our previous hypothesis that TULP1 may be involved in photoreceptor protein trafficking.

Methods

Construction of Plasmids

For transfection experiments, wild-type human *TULP1* cDNA was obtained from total human retinal RNA (Superscript II RT; Invitrogen, Carlsbad, CA). The primer pair 5′-GGAAGATCTCATGCCTCTGCGGGATGAA-3′ and 5′-GTAGAATTCGCTCGCAAGCCAGCTTCCC-3′ was used to amplify full-length *TULP1*. The amplified fragment was digested with *Bgl*II and *Eco*RI, gel purified, and cloned into the pEGFP-N1vector (BD-Clontech, Palo Alto, CA). Correct fusion of this construct and the following constructs were confirmed by automated DNA sequencing.

For fractionation experiments, TULP1 cDNA was amplified from pEGFP-N1-TULP1 plasmid DNA by using primers 5′-CCCAAGCTTTCATGCCTCTGCGGGATGAA-3′ and 5′-CCCAAGCTTAGGAGGAGAGGGCCCCGTCT-3′. The cDNA was digested and subcloned into vector pcDNA3.1/myc-HisA (Invitrogen) between the *Hin*dIII and *Xho*I sites.

For production of recombinant human TULP1, the primer pair 5′-GGAATTCCATATGCCTCTGCGGGATGAAAC-3′ and 5′-CCGCTCGAGCTCGCAAGCCAGCTTCCCGT-3′ was used to amplify full-length *TULP1*. The fragment was subcloned into the *Nde*I and *Xho*I sites of the expression vector pET28b (Novagen, Madison, WI).

Production of Recombinant TULP1

Wild-type human recombinant TULP1 was expressed in *Escherichia coli* strain BL21 (DE3) after transformation of the expression plasmid pET28b-TULP1 by using standard methods. Kanamycin-resistant colonies were grown in LB medium and induced with 1 mM isopropyl-1-thio-*β*-galactopyranoside. After induction, the culture was centrifuged and the resultant pellet washed with PBS, resuspended in lysis buffer with protease inhibitors and broken down with an ultrasonic homogenizer. The homogenate was centrifuged, and the supernatant was incubated with Ni-NTA-Agarose beads (Qiagen, Valencia, CA). The beads were washed with lysis buffer plus 50 mM imidazole and eluted with lysis buffer plus 150 mM imidazole. Purified protein was desalted in a NAP-10 column (GE Healthcare, Piscataway, NJ).

Cell Culture and Immunocytochemistry

COS7 cells were maintained in DMEM medium containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and antibiotics. Cells grown to ~40% confluence were transfected with TULP1-GFP plasmid DNA by the calcium phosphate precipitation method.

Approximately 24 to 36 hours after transfection, COS7 cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 for 10 minutes. For actin staining, cells were first incubated in blocking buffer for 30 minutes followed by phalloidin (1:50 dilution in PBS; Molecular Probes, Eugene, OR) for 30 minutes. Nuclei were stained with propidium iodide after treatment with RNase and the cells were then cover slipped to slides for microscopic analysis.

Cells were examined with a confocal microscope (TCP-SP; Leica, Deerfield, IL) with a 60× oil immersion objective lens. A series of confocal sections (0.5-μm interval) were taken through the cells for consecutive 3-dimentional reconstructions.

Subcellular Fractionation and Triton X-114 Phase Separation

Subcellular fractions were obtained by sequential differential centrifugation as follows. COS7 cells were collected 24 to 36 hours after transfection into 500 μL of Hepes, $MgCl_2$, EDTA (HME) buffer, plus protease inhibitors and allowed to swell for 5 minutes on ice. Cell suspensions were then homogenized in a homogenizer (Dounce; Bellco Glass, Vineland, NJ) and centrifuged briefly at 500*g* to pellet whole cells, cell nuclei, and large debris (nuclear pellet). The supernatant was then centrifuged at 50,000*g* for 60 minutes to pellet the membranous and cytoskeletal fraction, with the remaining supernatant containing cytosolic proteins.

Triton X-114 phase separation was performed as described.[21] Briefly, COS7 cells were lysed in Triton X-114 cell lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, and 1% precondensed Triton X-114 containing protease inhibitors), and the solution was centrifuged at 10,000*g* at 4°C. The supernatant was incubated at 37°C for 3 minutes and centrifuged for 1 minute at 10,000*g* at room temperature. The aqueous phase was transferred and reextracted by adding 100 μL of lysis buffer and repeating the incubation and centrifugation steps. The detergent phase was retained and reextracted by adding 600 μL Tris-buffered

saline [TBS] buffer. TULP1 was resolved by SDS-PAGE and identified by immunoblot with polyclonal M-tulp1N antibodies, generated against the N-terminal half of mouse TULP1.[8]

Phospholipid Binding Assay

Nitrocellulose strips containing spots of equal amounts (100 picomoles) of different phospholipids (PIP-strips; Echelon Biosciences, Salt Lake City, UT) were purchased, and binding assays were performed as recommended by the manufacturer. Briefly, the PIP-strips were blocked with 3% BSA and incubated with His-TULP1 fusion protein overnight at 4°C. Bound His-TULP1 protein was detected by M-tulp1N antibodies and visualized by secondary antibodies coupled to peroxidase followed by chemiluminescence detection.

Immunoprecipitation

Two bovine retinas were homogenized in 5 mL of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, and 0.1% NP-40) supplemented with protease inhibitors. The suspension was incubated at 4°C for 2 hours with gentle rotation, centrifuged at 16,000*g* for 20 minutes, and the supernatant removed from the pellet. The supernatant was centrifuged again at 16,000*g* for 20 minutes, and the final supernatant removed. The retinal lysates were used for immunoprecipitation (IP) analysis with the polyclonal antibody M-tulp1N.[8] Approximately 100 μg of M-tulp1N antibody was incubated 1 hour with 60 μL of protein A beads. The beads were then washed and resuspended with 600 μL of 0.2 M sodium borate (pH 9.0) followed by the addition of 4 mg of dimethylpimelimidate. After a 1.5-hour incubation, the antibody coupling reaction was stopped with 0.2 M ethanolamine (pH 8.0). The antibody-coupled beads were then washed with 100 mM glycine (pH 3.0) and then with PBS and was finally resuspended in 60 μL of PBS. Retinal lysate protein (1 mg), determined by the Bradford method, was added to 50 μL of M-tulp1N coupled to the protein A beads and the antigen-binding reaction performed overnight at 4°C. As a negative control, protein A beads alone were allowed to bind to retinal lysate. The binding reactions were stopped by centrifugation at 1000*g* for 1 minute, the supernatants removed, and the beads washed and resuspended in SDS-PAGE sample buffer. Immunoprecipitated antigens were eluted from the antibody-coupled beads by boiling 3 minutes and then were analyzed by SDS-PAGE on a 10% gel. The gel was stained with colloidal Coomassie blue (Gel Code Blue; Pierce, Rockford, IL) and used for protein identification.

Protein Identification by Mass Spectrometry

Identification of proteins by liquid chromatography tandem mass spectrometry (LC MS/MS) was performed as described previously.[22–24] Briefly, gel bands were excised, stain washed away, proteins digested in the gel with trypsin, and peptides extracted for mass spectrometric analysis. LC MS/MS was performed on a commercial system (Cap LC; Micromass, Manchester, UK) with a quadrupole time-of-flight mass spectrometer (QTOF2; Waters Corp., Milford, MA). Proteins were identified from MS/MS data (ProteinLynx Global Server; Waters Corp., and Mascot; Matrix Science search engines, and the Swiss Prot [http://www.expasy.org; provided in the public domain by Swiss Institute of Bioinformatics, Geneva, Switzerland] and National Center for Biotechnology [National Institutes of Health, Bethesda, MD] protein sequence databases).

Western Blot Analysis

Western blot analyses were performed as previously described.[8,22] Briefly, proteins were separated on SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with TULP1 primary antibodies, followed by peroxidase-conjugated secondary antibodies, and detected by chemiluminescence.

Actin Co-sedimentation Assay

Actin co-sedimentation assays were performed with a commercially available kit according to the manufacturer's instructions (Cytoskeleton). In brief, purified recombinant TULP1 was incubated with 40 μg freshly polymerized actin for 30 minutes. After incubation, the solution was centrifuged to pellet actin and TULP1 bound to actin. After solubilization of the pellet fraction, equal volumes of the pellet and supernatant fractions were analyzed by SDS-PAGE and Coomassie blue staining. α-Actinin and BSA were used as positive and negative controls, respectively.

Immunohistochemistry

All experiments on animals were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice eyes were fixed in 4% paraformaldehyde in PBS for 30 minutes followed by immersion in 10% sucrose for 1 hour, 20% sucrose for 1 hour, and 30% sucrose overnight. Frozen retinal sections were cut at 10-μm thickness. Sections were blocked in 5% goat serum before incubation with antibodies. TULP1 was stained using M-tulp1N antibodies at 4°C overnight followed by incubation with goat anti-rabbit Alexa Fluor-568 (Molecular Probes) for 1 hour. Actin was stained with fluorescent phalloidin (Molecular Probes), and nuclei with 4′,6′-diamino-2-phenylindole (DAPI). Sections were examined with a fluorescence microscope (Microphot-2; Nikon, Tokyo, Japan) equipped with a charge-coupled device digital camera (SPOT2; Diagnostic Imaging, Sterling Heights, MI).

Results

Localization of TULP1 in Cultured Cells

To determine the subcellular distribution of TULP1, we performed transfection assays in COS7 cells, with a C-terminal green fluorescent protein (GFP) fusion protein (TULP1-GFP). COS7 cells were transiently transfected with the fusion construct and analyzed between 24 and 36 hours after transfection. Inspection of transfected cells using confocal microscopy revealed that TULP1-GFP staining was confined to two regions of the cell. As shown in the middle row in Figure 1, one population of TULP1-GFP was localized to the plasma membrane and processes of the cells. A second population of TULP1-GFP localized to the nucleolus. These results are consistent with previous reports showing that in cultured cells TULP proteins localize to the nucleus.[5] To determine whether GFP caused an artifactual localization, GFP was localized in control cells transfected with the GFP expression vector alone. GFP fluorescence was observed in both the cytoplasm and nucleus, as expected.[25]

Because TULP1-GFP localized at or near the plasma membrane, further assays were performed to determine whether TULP1 associates with cellular membranes. First, independent cellular fractionation experiments of TULP1-transfected COS7 cells and TULP1-GFP-transfected COS7 cells were performed. Separate experiments were performed to determine whether results were due to the addition of the GFP tag. Subcellular fractionation experiments revealed that the majority of TULP1 was found in the membranous fraction as shown in Figure 2A, and only a small amount was present in the cytosolic fraction, indicating an association with either membranes or the cytoskeleton. Results of both transfection experiments were identical. Second, transfected COS7 cells were extracted with Triton X-114 followed by phase separation into detergent and aqueous phases. Membrane proteins partition in the detergent phase, whereas most soluble proteins are found in the aqueous phase. Figure 2B shows that TULP1 was located in the aqueous phase, indicating that this protein is not an integral membrane protein.

To test whether membrane lipids are involved in the membrane association, an in vitro phospholipid-binding assay was performed (Fig. 2C). TULP1 bound specifically to a number of phosphorylated phosphoinositides—namely, phosphatidylinositol (3)-phosphate [PtdIns(3)P], phosphatidylinositol (4)-phosphate [PtdIns(4)P], phosphatidylinositol (5)-phosphate [PtdIns(5)P], phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2], phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], and phosphatidylinositol (3,4,5)-bisphosphate [PtdIns(3,4,5)P_3]. In addition, TULP1 bound phosphatidylserine and phosphatidic acid. No binding was observed to lysophosphatidic acid, lysophosphocholine, phosphatidylethanolamine, or phosphatidylcholine. The results of this assay are not quantitative but yielded information about the relative affinity of TULP1 for various lipids.

Identification of Actin as a Binding Partner of TULP1

To identify proteins that interact with TULP1 in photoreceptor cells, a TULP1-containing complex was immunoprecipitated from bovine retinal homogenates using M-tulp1N antibodies, which immunoreact with bovine TULP1.[22] Four independent IP experiments were performed, and Figure 3 shows a representative SDS-PAGE result. The bands from lane four were excised and the proteins were identified by LC MS/MS. The major band at ~70 kDa was identified in all four independent IP experiments as TULP1 by MS/MS sequence analysis of six tryptic peptides covering 13.5% of the protein. The band at ~45 kDa was identified in all four independent IP experiments as F-actin by MS/MS sequence analysis of 12 tryptic peptides covering 25.5% of the protein. The identity of eight additional proteins was determined from other bands, including microtubule associated protein 1B, clathrin heavy chain, interphotoreceptor retinoid-binding protein, dynamin-1, rab gerynl gerynl transferase, dynein intermediate chain, pyruvate kinase, and tubulin.[22] Because these IP data suggest that TULP1 interacts with actin and the fractionation studies suggest that TULP1 associates with the cytoskeleton, we sought corroborative evidence of a specific interaction between TULP1 and actin.

Localization of TULP1 with Actin In Vivo

To determine whether actin colocalizes with TULP1 in vivo, we first examined wild-type mouse retina, the tissue in which TULP1 is normally found. In immunohistochemical studies, TULP1 was found to be concentrated in the inner segments and connecting cilium of photoreceptor cells and, to a lesser extent, in the perinuclei and at the outer plexiform or synaptic layer (Fig. 4). This is consistent with our previous reports.[7,8] Figure 4 also shows the presence of actin throughout several retinal layers including the retinal pigment epithelium, the inner segment, the outer limiting membrane, and the outer and inner plexiform layers. The most prominent localization of actin in photoreceptor cells is at the outer limiting membrane. Our data indicate that TULP1 and actin colocalize primarily at the inner segments, the outer limiting membrane, and to a lesser extent the outer plexiform layer, providing additional support for a physiological interaction in photoreceptors.

To extend the localization studies performed in mouse retinal sections, we performed immunohistochemistry on COS7 cells transfected with TULP1-GFP and stained with phalloidin (Fig. 5). The staining pattern detected by confocal microscopy showed TULP1 to be localized in the nucleolus, at or near the plasma membrane, and in the processes of these cells. Labeling of actin revealed staining that coincided with the TULP1-GFP signal at or near the plasma membrane. TULP1 colocalized with circumferential actin in COS7 cells. These results are consistent with those shown in Figure 1.

TULP1 Binding of Actin In Vitro

To further probe an interaction between TULP1 and actin, in vitro co-sedimentation assays were performed with actin and purified recombinant TULP1. Actin-binding proteins co-

sedimented with actin filaments and formed a pellet after high-speed centrifugation. Figure 6 shows that in the absence of actin, TULP1 was located entirely in the soluble fraction whereas, in the presence of actin, TULP1 was located in the pellet fraction. α-Actinin, a known actin-binding protein, was used as a positive control and pelleted with actin; whereas BSA was used as a negative control and remained in the soluble fraction. This result is consistent with the data obtained from the immunoprecipitation and fractionation experiments and provides supporting evidence of an interaction between TULP1 and actin.

Discussion

TULP1 is a photoreceptor-specific protein of unknown function which when mutated causes retinitis pigmentosa in humans[12–15] and photoreceptor degeneration in mice.[7,9] Given its role in retinal disease, we reasoned that determining the subcellular localization and identifying TULP1 binding partners would be a critical step toward understanding the function of TULP1 in maintaining the health of the photoreceptor cell and its participation in the disease process of photoreceptor degeneration.

Our initial experiment was to localize TULP1 in cultured cells. We observed that TULP1 localizes at or near the plasma membrane. We pursued this observation biochemically by showing in fractionation studies that most of the TULP1 associates with the membrane and cytoskeletal fractions, whereas a lesser amount is found in the cytoplasmic fraction. In addition, extraction with the detergent Triton X-114 indicates that TULP1 partitions entirely in the aqueous phase, a property unique to hydrophilic soluble proteins. Taken together, our results suggest that TULP1 is a cytoplasmic protein that associates with membranes and/or the cytoskeleton. TULP proteins are not predicted to be membrane proteins, based on their amino acid sequences. However, a similar observation has been reported for TUB, demonstrating an association with the plasma membrane by binding to phosphatidylinositol(4,5)-bisphosphate.[20] Because TULP1 and TUB are from the same family of proteins, we performed an in vitro lipid-binding assay to test whether membrane lipids are involved in the membrane association observed for TULP1. We found that TULP1 binds phosphorylated phospholipids, similar to TUB, although with less specificity. Phosphoinositides (PIPs) are known to serve as second messengers in signal-transduction cascades and also to play a critical role in vesicular protein transport and organization of the cytoskeleton.[26] Proteins essential for trafficking between compartments bind to PIPs at defined locations within the cells; moreover, different PIP species are concentrated at distinct sites within the cell, suggesting a mechanism by which the direction of traffic may be controlled.[26] Our data indicate that TULP1 associates with membranes and further suggest that it is likely bound at the plasma membrane through an association with negatively charged PIPs.

To identify TULP1 interacting proteins directly, we isolated a TULP1-containing complex from the retina and identified F-actin as a binding partner in each of four independent IP experiments. In vitro co-sedimentation assays supported the binding as specific rather than the result of contamination or background. A TULP1 and actin interaction is intriguing and plausible given that most of the TULP1 appears to be associated with the membranous and cytoskeletal fraction of cells. To pursue this interaction further, we localized TULP1 and actin by immunocytochemistry in mouse retinal sections and COS7 cells.

We previously reported that TULP1 is distributed throughout the photoreceptor inner segments, connecting cilium, outer limiting membrane, perikarya, and synaptic terminals, but is excluded from the outer segments and nuclei.[7,8] Results of the present study are consistent with those reports. Our data also demonstrate that actin is present throughout the photoreceptor inner segments and synaptic termini, with prominent staining at the level of

the outer limiting membrane. Earlier studies localizing actin in photoreceptor cells using immunoelectron microscopy revealed that actin filaments are specifically organized in photoreceptors. Actin is present at the base of the outer segments where nascent discs are formed, along the axoneme of the connecting cilium, under the inner segment plasma membrane, and at the outer limiting membrane corresponding to the zonula adherens junction between photoreceptors and Müller glial cells.[27–31] Our immunocytochemical analysis using fluorescent phalloidin is not of sufficient resolution to identify actin in all these specialized structures and areas of the cell. However, our data indicate that TULP1 and actin colocalize in the inner segments, outer limiting membrane, and synaptic layer of photoreceptors, providing additional evidence for a physical interaction.

In previous studies, we provided evidence that TULP1 functions in the transport of rhodopsin from its site of synthesis in the inner segment through the connecting cilium to its final destination in the outer segment of the photoreceptor cell.[7,8] This finding is based on the photoreceptor distribution of TULP1 and the photoreceptor disease phenotype in *tulp1*$^{-/-}$ mice. As described, TULP1 is localized to specialized compartments of the photoreceptor involved in protein transport. In addition, *tulp1*$^{-/-}$ mice exhibit early-onset, progressive photoreceptor degeneration with involvement of both rods and cones. Before degeneration in *tulp1*$^{-/-}$ retinas, the rod and cone opsins, normally targeted to the outer segment, are mislocalized and an abnormal accumulation of rhodopsin-bearing extracellular vesicles is found surrounding the ellipsoid region of the inner segments indicating a misrouting of rhodopsin transport carriers.[7,8] These results led us to hypothesize that TULP1 may function in trafficking proteins from the inner segment through the connecting cilium to the outer segment. The actin cytoskeleton is widely believed to play an important role in intracellular protein transport.[32,33] Numerous studies implicate actin in several processes associated with protein movement including vesicle assembly and polarized transport. In photoreceptor cells, the actin cytoskeleton is proposed to have various functions. Its presence at the base of the outer segments where nascent discs are formed indicates a possible involvement in disc morphogenesis. Actin also forms filaments that run underneath the inner segment plasma membrane and through the connecting cilium parallel to the axonemal microtubules, probably functioning in protein transport. Its presence in these regions of the cell is believed to provide the structural bases for the actin-based motor protein myosin VIIa to traffic proteins, including rhodopsin, to the outer segment.[27] Many lines of evidence indicate that myosin VIIa plays a prominent role in the translocation of proteins along actin filaments through the connecting cilium.[27,34–36] Furthermore, a recent study performed in frog photoreceptors has shown that a key step in the transport of rhodopsin, the tethering of rhodopsin transport vesicles to the inner segment plasma membrane, involves interactions between the vesicles, PIPs, and Moesin, a PIP-binding ERM protein, to bring the vesicles into contact with the actin cytoskeleton for trafficking.[37] Herein, we provide evidence that TULP1 associates with the plasma membrane, possibly through an association with negatively charged PIPs, binds with actin, and colocalizes with actin at the inner segment and connecting cilium, suggesting that TULP1 may be involved in protein vesicle transport.

In photoreceptors, understanding of the molecular processes that regulate protein trafficking and the proteins involved in these pathways are limited. We know that the photoreceptor is a highly polarized and compartmentalized neuron, both in function and morphology, and large amounts of proteins synthesized in the inner segment must be directionally transported to the outer segment. It is well documented in frog rod photoreceptors that this process includes the sorting of vesicles at the trans-Golgi network, the directional translocation of the vesicles through the inner segment, and the delivery of them to the apical inner segment plasma membrane where they dock and fuse for incorporation through the connecting cilium and into the outer segments.[38,39] Although untested, this process is likely to be similar in

mammalian photoreceptors. There are several possible roles for actin and TULP1 in photoreceptor protein transport that are not mutually exclusive. First, actin could organize and constrain the trafficking machinery, whereas TULP1 could serve as an adapter protein involved in selecting cargo for inclusion in transport vesicles. There is a substantial amount of evidence that PIPs are involved in adaptor recruitment to intracellular membranes. Different types of PIPs can be rapidly generated on membranes providing the cell with a means for recruiting the adaptors onto the donor membrane for vesicle generation. Second, TULP1 may act as part of a protein scaffold at the interface between cellular membranes and the cytoskeleton, linking transport vesicles with actin. Organization of such a complex may require TULP1, or TULP1 may be enlisted to an already established complex that uses actin as the support structure for vesicular movement. Third, TULP1 could be involved in regulating vesicle formation or be a part of the complex involved in vesicle docking and fusion with target membranes.

The subcellular localization and interaction studies presented herein support a physical interaction between TULP1, actin, and biological membranes. Our previous study identified several other potential TULP1 binding partners, such as dynamin-1, dynein intermediate chain, and microtubule-associated protein 1B, which are involved in various aspects of vesicle transport or protein movement, suggesting that TULP1 may be part of a complex involved in protein transport.[22] Further studies are needed to confirm these interactions. However, our results presented herein support a role for TULP1 in protein trafficking in the photoreceptor cells of the retina and provide evidence that defective protein transport pathways may be a pathologic mechanism responsible for retinal degeneration. Future experiments to identify where in a transport pathway TULP1 may function will help to clarify a poorly understood aspect of photoreceptor biology relevant to human retinal disease.

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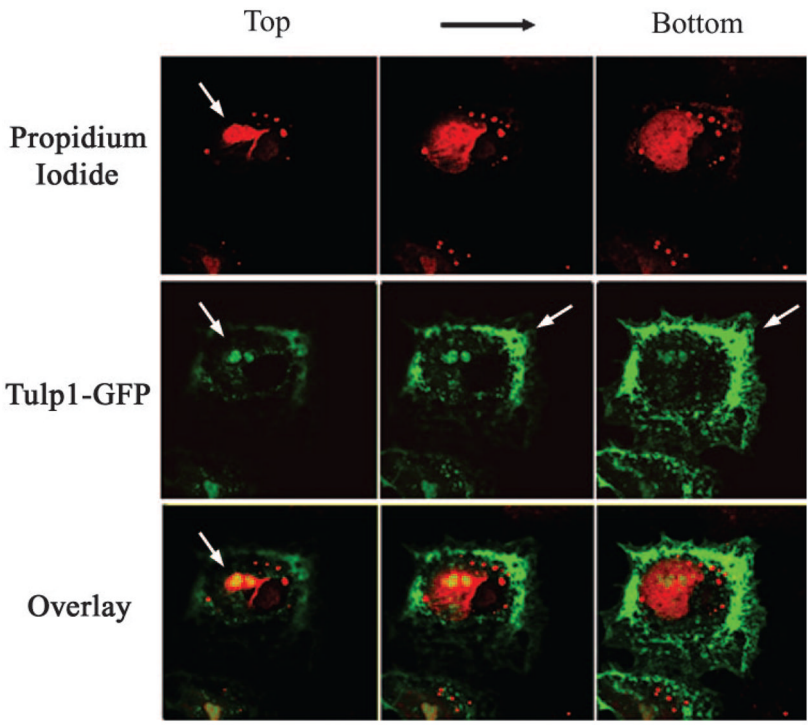

Figure 1.
Subcellular localization of TULP1 in cultured cells. Laser scanning confocal microscopy images of the subcellular localization of TULP1-GFP (*green*) transiently transfected into COS7 cells. Nucleic acids were labeled with propidium iodide (*red*). Cells were sectioned in the *x*-*y* plane from top to bottom in 0.5-μm increments. Three planes are shown at 1.0-μm intervals. TULP1 is localized to the plasma membrane, cell processes, and nucleoli of the cells. *Arrows*: locations of the nucleoli and plasma membrane.

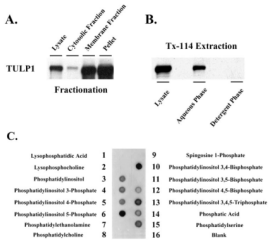

Figure 2.

Biochemical association of TULP1 with membranes. (**A**) Western blot probed with TULP1 antibodies. After a high-speed centrifugation of COS7 cells transfected with TULP1, most of the TULP1 was located in the membranous fraction, indicating an association with either membranes or the cytoskeleton. TULP1 is also detected to a lesser extent in the cytosolic fraction as well as in the cell lysate and the pellet fraction composed of nonlysed cells and debris. (**B**) Western blot probed with TULP1 antibodies. COS7 cells transfected with TULP1 were extracted with Triton X-114 followed by phase separation. Membrane proteins partition into the detergent phase, whereas most soluble proteins are found in the aqueous phase. TULP1 was located entirely in the aqueous phase, indicating that this protein is not an integral membrane protein. (**C**) Protein overlay of purified recombinant TULP1 onto a nitrocellulose membrane spotted with different phospholipids. Bound protein is visualized by immunoblotting with TULP1 antibodies. TULP1 binds primarily to negatively charged phosphatidylinositols.

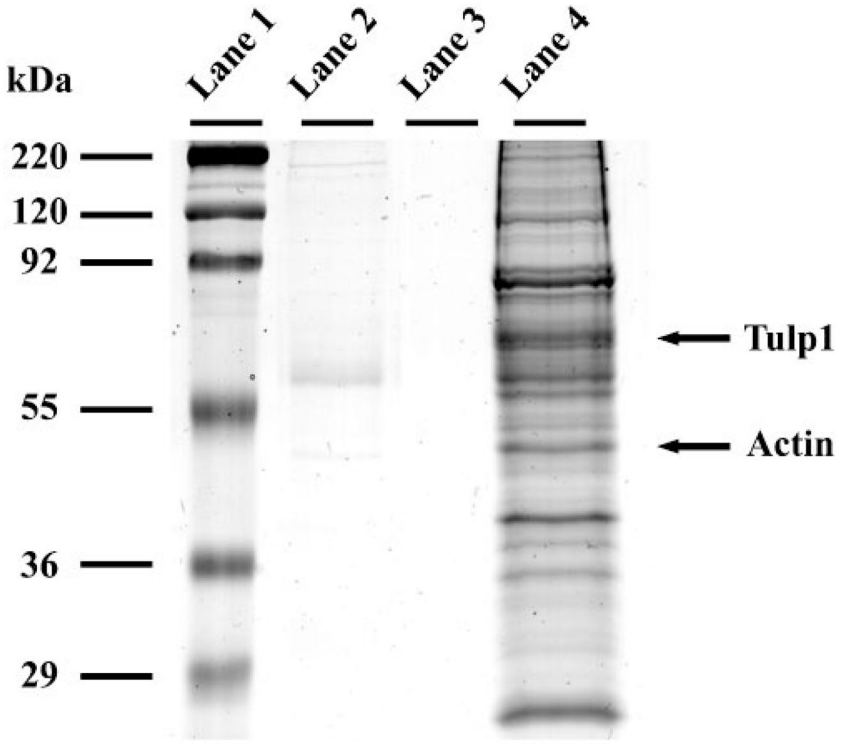

Figure 3.

Immunoprecipitation of a TULP1-containing complex from the retina. A gel code blue stain of an SDS-PAGE gel separating retinal lysates incubated with TULP1 antibodies. *Lane 1*: molecular marker; *lane 2*: immunoprecipitated products after binding with protein A beads without TULP1 antibodies; *lane 3*: blank; *lane 4*: immunoprecipitated products incubated with TULP1 antibodies coupled to protein A beads demonstrating a pattern of bands absent in the control lane. The bands were excised and digested with trypsin and the peptides identified by LC MS/MS. The major band at ~70 kDa was identified as TULP1. The major band at ~45 kDa was identified as actin.

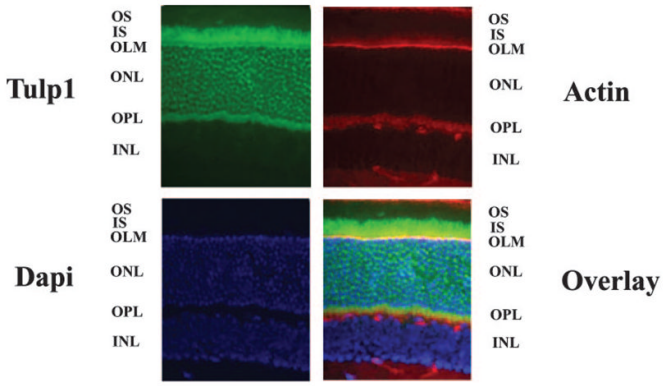

Figure 4.

TULP1 colocalized with actin in retinal sections. Immunofluorescent localization of TULP1 (*green*) and actin (*red*) in 10-week-old wild-type mouse retinal sections. Nuclei were labeled with DAPI (*blue*). TULP1 and actin colocalized to the inner segment, the outer limiting membrane, and the outer plexiform layer of photoreceptor cells. OS, outer segments; IS, inner segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer.

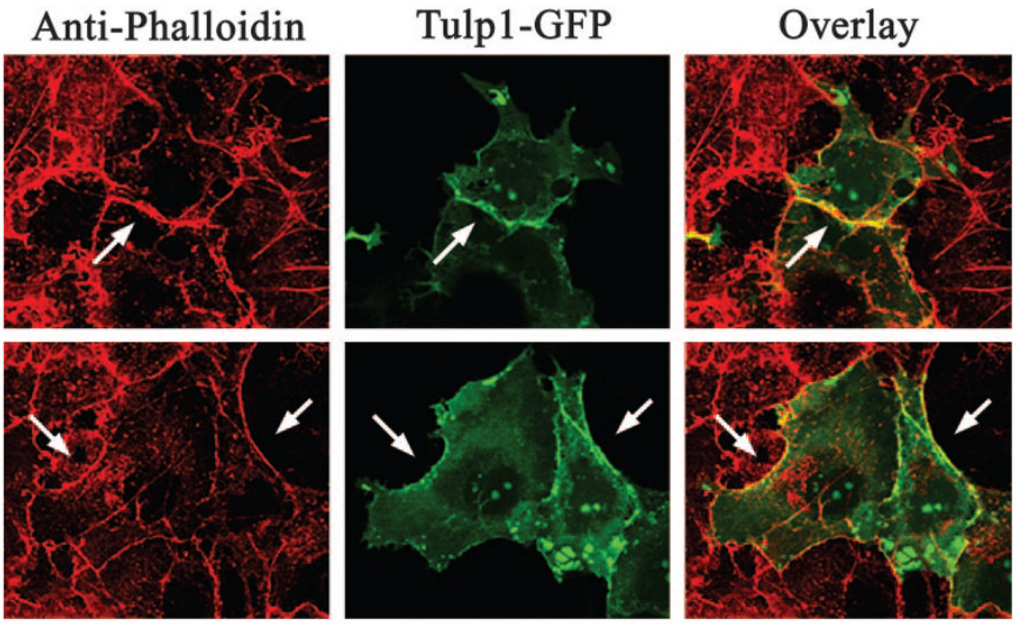

Figure 5.
TULP1 colocalized with actin in cultured cells. Confocal microscopy image of the subcellular co-localization of TULP1-GFP (*green*) transiently transfected into COS7 cells and actin, which is detected with phalloidin (*red*). TULP1 and circumferential actin colocalize to the plasma membrane, as indicated by the *arrows* in the overlay image.

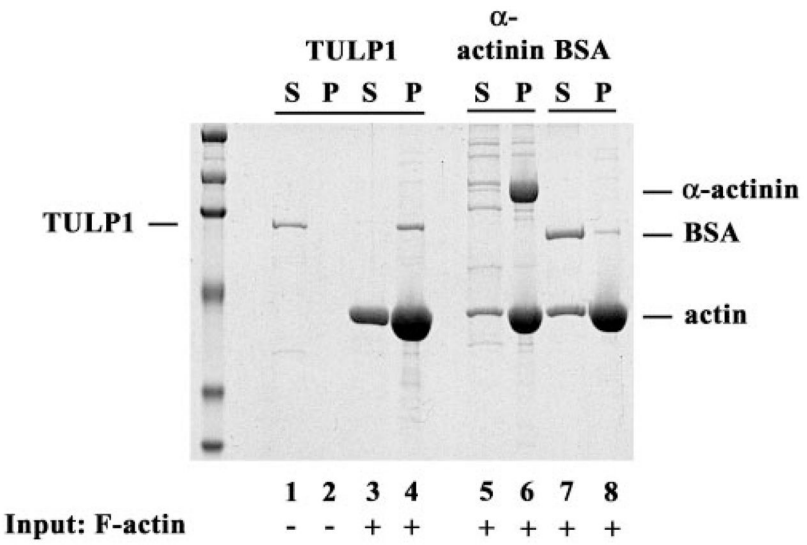

Figure 6.

TULP1 binds actin in vitro. Co-sedimentation assay combining actin and purified recombinant TULP1 revealed that in the absence of actin, TULP1 was located in the soluble fraction after centrifugation (*lane 1*). However in the presence of actin, TULP1 was located in the pellet fraction, indicating a direct interaction between the two proteins (*lane 4*). *α*-Actinin, a known actin binding protein, was used as a positive control and pelleted with actin (*lane 6*), whereas BSA, known not to be an actin-binding protein, was used as a negative control and remained in the soluble fraction (*lane 7*). S, supernatant; P, pellet.