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Immunocytochemical evidence of Tulp1-dependent outer segment protein transport pathways in photoreceptor cells

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ABSTRACT

Tulp1 is a protein of unknown function exclusive to rod and cone photoreceptor cells. Mutations in the gene cause autosomal recessive retinitis pigmentosa in humans and photoreceptor degeneration in mice. In *tulp1*−/− mice, rod and cone opsins are mislocalized, and rhodopsin-bearing extracellular vesicles accumulate around the inner segment, indicating that Tulp1 is involved in protein transport from the inner segment to the outer segment. To investigate this further, we sought to define which outer segment transport pathways are Tulp1-dependent. We used immunohistochemistry to examine the localization of outer segment proteins in *tulp1*−/− photoreceptors, prior to retinal degeneration. We also surveyed the condition of inner segment organelles and rhodopsin transport machinery proteins. Herein, we show that guanylate cyclase 1 and guanylate cyclase activating proteins 1 and 2 are mislocalized in the absence of Tulp1. Furthermore, arrestin does not translocate to the outer segment in response to light stimulation. Additionally, data from the *tulp1*−/− retina adds to the understanding of peripheral membrane protein transport, indicating that rhodopsin kinase and transducin do not co-transport in rhodopsin carrier vesicles and phosphodiesterase does not co-transport in guanylate cyclase carrier vesicles. These data implicate Tulp1 in the transport of selective integral membrane outer segment proteins and their associated proteins, specifically, the opsin and guanylate cyclase carrier pathways. The exact role of Tulp1 in outer segment protein transport remains elusive. However, without Tulp1, two rhodopsin transport machinery proteins exhibit abnormal distribution, Rab8 and Rab11, suggesting a role for Tulp1 in vesicular docking and fusion at the plasma membrane near the connecting cilium.

1. Introduction

Disc membranes in the photoreceptor cells of the vertebrate retina are continually renewed by the addition of membranes at the base of the outer segment (OS) and the removal of older discs from the distal end. OS-bound proteins are sorted and trafficked from the endoplasmic reticulum (ER) and trans-Golgi network (TGN) as cargo in vesicles from the inner segment (IS) toward the OS on a continual basis (Papermaster et al., 1985; Besharse and Wetzel, 1995). Rhodopsin, the photopigment molecule in rod photoreceptors, and other OS-resident proteins are reliant upon this polarized protein trafficking, which involves interactions between proteins of the transport machinery and proteins present on the cargo vesicles (Papermaster et al., 1985; Besharse and Wetzel, 1995; Aroeti et al., 1998). The complexity of this interaction is underscored by the fact that a defect in only one of these components can result in a loss of polarity in protein trafficking and ultimately photoreceptor cell death (Hagstrom et al., 1999; Deretic, 2006).

Retinitis pigmentosa incorporates a group of heterogeneous inherited retinal degenerations (Berson, 1993). One early onset form of retinitis pigmentosa is caused by mutations in *TULP1* (Banerjee et al., 1998; Gu et al., 1998; Hagstrom et al., 1998; Paloma et al., 2000). Tulp1 is a cytoplasmic protein expressed exclusively in photoreceptors, localizing to the IS, connecting cilium (CC), perikarya, and terminals (Hagstrom et al., 1999). Tulp1 associates with cellular membranes and the cytoskeletal elements (Hagstrom et al., 1999, 2001; Ikeda et al., 2000; Xi et al., 2005) and appears to play a role in intracellular protein trafficking (Hagstrom et al., 1999, 2001; Xi et al., 2005, 2007; Grossman et al., 2009). *tulp1*−/− mice develop a rapid photoreceptor degeneration, similar to that in patients with retinitis pigmentosa due to *TULP1* mutations (Hagstrom et al., 1999). In the young *tulp1*−/− retina prior to

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photoreceptor degeneration, there is an accumulation of vesicles in the interphotoreceptor matrix surrounding the IS. These vesicles show rhodopsin immunoreactivity, likely representing misrouted transport carriers for opsin (Hagstrom et al., 1999). Indeed, rod and cone opsins are mislocalized throughout the photoreceptor. Importantly, this mistargeting occurs early in development, indicating a primary defect, and not a secondary effect due to a generalized degenerative process (Hagstrom et al., 1999; Grossman et al., 2009). However, other OS proteins are not mislocalized in the *tulp1*−/− retina, indicating a specialized role for Tulp1 in OS protein transport (Hagstrom et al., 1999).

Proposed OS protein transport pathways have been deduced from a handful of mutant mouse models (Yang et al., 1999; Baehr et al., 2007; Zhang et al., 2007; Karan et al., 2008b). Therefore, it is critical to study additional mouse mutants which present phenotypes showing OS protein transport defects. To define the transport pathways affected in the Tulp1 mutant mouse, we analyzed the localization of several categories of OS-resident proteins in *tulp1*−/− retinas as compared to wild-type (wt) retinas. We also surveyed several IS proteins known to be critical in the transport of OS proteins.

2. Materials and methods

2.1. Mice

The generation of *tulp1*−/− mice has been described previously (Hagstrom et al., 1999) and is maintained on a C57BL/6 background. Mice were euthanized by CO_2 inhalation followed by cervical dislocation. All experiments on animals were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic and were performed in compliance with the National Institutes of Health guidelines.

2.2. Preparation of retinal sections

Mouse eyes were prepared as previously described (Xi et al., 2007). Briefly, after removal of the cornea and lens, the posterior poles were fixed in 4% paraformaldehyde in PBS for 3 h. The eyes cups were then immersed through a graded series of sucrose solutions as follows: 10% for 1 h, 20% for 1 h and 30% overnight. The posterior pole was embedded in OCT freezing medium, flash frozen on powderized dry ice and immediately transferred to −80 °C. The tissue was sectioned at 10-μm thickness using a cryostat (Leica, Wetzlar, Germany) at −30 °C.

2.3. Immunofluorescent staining

For each genotype (wt and *tulp1*−/−), a minimum number of eight sections from six different mice was examined for each antibody. Retinal sections were blocked in 5% bovine serum albumin and 1% normal goat serum with 0.1% Triton X-100 for 1 h before incubation with primary antibodies overnight at 4 °C. A panel of well-characterized antibodies against retinal proteins was used for immunostaining. Table 1 contains a complete list of antibodies, immunogens, sources, host species and dilutions used. After washing 3 times in PBS, sections were incubated in fluorescent secondary antibodies at room temperature for 1 h. Secondary antibodies were: Alexa Fluor® 488 goat anti-rabbit IgG and goat anti-mouse IgG; Alexa Fluor® 594 goat anti-rabbit IgG and goat anti-mouse IgG (Invitrogen, Carlsbad, California). The sections were then rinsed 3 times with PBS followed by a light rinse in dH_2O and coverslipped with Vectashield® mounting media with DAPI (Vector Laboratories, Burlingame, California). Sections were imaged using an Olympus BX-61 fluorescent microscope (Olympus, Tokyo, Japan) equipped with a CCD monochrome camera (Hamamatsu Photonics, Bridgewater, New Jersey).

2.4. Light adaptation

To study the translocation of OS proteins in response to light exposure, eight mice were separated into two groups. At the onset of lights off in the vivarium, both groups were transferred into a chamber under constant darkness. All animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg) and the pupils were dilated with a mixture of 1% cyclopentolate-HCl, 2.5% phenylephrine, and 0.25% tropicamide. At approximately 1 h prior to the time of lights on, one group was transferred to a lighting chamber and exposed to 1000 lux for 60 min, while the control group remained in the light-proof chamber. After 60 min, both groups were sacrificed, and the eyes extracted and processed as described above.

3. Results

3.1. tulp1−/− mice form outer segments

The retinas of postnatal day (P)16 *tulp1*−/− mice contain the normal complement of retinal cell types including photoreceptors. However, at this age, the OS of the *tulp1*−/− photoreceptor is slightly disorganized (Fig. 1E) as compared to wt (Fig. 1A) (Hagstrom et al., 1999). The presence of OSs in *tulp1*−/− photoreceptors is confirmed by the expression and confinement of peripherin/rds to the OS (Fig. 1C and G). In contrast, rhodopsin is severely mislocalized to all cellular compartments in *tulp1*−/− photoreceptors (Fig. 1F) as compared to wt (Fig. 1B). Peripherin/rds and rhodopsin are both integral membrane proteins that are incorporated into nascent OS discs (Lee et al., 2006); however, unlike rhodopsin, peripherin/rds is clearly able to be targeted to the OS in the absence of Tulp1 (Fig. 1G). These results reveal that OS proteins are affected differently in *tulp1*−/− mice, providing evidence that Tulp1 functions in selective OS transport pathways.

3.2. Outer segment proteins that are normally localized in tulp1−/− retinas

Since rhodopsin is mislocalized in *tulp1*−/− photoreceptors, we sought to determine whether additional OS proteins rely on the presence of Tulp1 for their transport to the OS compartment. To this end, we surveyed a panel of OS-resident proteins in the *tulp1*−/− retina as compared to the wt retina. Fig. 2 presents the results of the localization of several well-studied phototransduction and structural OS proteins. All studies were conducted at P16, an age at which photoreceptor development is complete in wt mice, but precedes photoreceptor cell death in *tulp1*−/− mice (Hagstrom et al., 1999; Grossman et al., 2009).

Fig. 2A shows a wt mouse retinal section stained with antibodies against rod OS membrane protein 1 (ROM1). ROM1 is an integral membrane protein localized to the rim of the OS discs similar to peripherin/rds, where the two proteins form heterotetramers (Lee et al., 2006). In the *tulp1*−/− retina, ROM1 staining is restricted to the OS, indicating that Tulp1 is not involved in the transport of this disc structural protein (Fig. 2B). Rhodopsin kinase (GRK1) is a G-protein coupled receptor kinase that participates in the inactivation of rhodopsin through phosphorylation (Smith et al., 1983). It is a peripheral membrane protein that acquires its membrane association from post-translational processing in the ER (Inglese et al., 1992). In the wt and *tulp1*−/− retina, GRK1 is clearly confined to the OS (Fig. 2C and D). Also properly localized in the *tulp1*−/− retina is another peripheral