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PROTEIN TYROSINE-O-SULFATION IN THE RETINA

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Abstract

Tyrosine-*O*-sulfation, a post-translational modification, is catalyzed by two independent tyrosylprotein sulfotransferases (TPSTs). As an initial step towards understanding the role of TPSTs in retinal function, this study was undertaken to determine the extent to which tyrosine-*O*-sulfation of proteins is utilized in the retina. A previously characterized anti-sulfotyrosine antibody was used to determine the presence and localization of tyrosine-*O*-sulfated proteins (TOSPs) in the retina. Using Western blot, RT-PCR and immunohistochemical analyses, we detected TOSPs in the retinas from diverse species, including frog, fish, mouse and human. Some of the variability in the observed sizes of retinal TOSPs in the mouse, at least, may result from differential patterns of glycosylation; however, there seem to be species-specific sulfated retinal proteins as well. TOSPs were detected in most of the retinal layers as well as in the retinal pigment epithelium from human and mouse. Several retinal TOSPs were detected in the inter-photoreceptor matrix, which is consistent with the secreted nature of some sulfated proteins. Transcripts for both TPST-1 and -2 were expressed in both the human and mouse retinas. These data show that retinal protein tyrosine-*O*-sulfation is highly conserved which suggest a functional significance of these proteins to retinal function and structure.

Keywords

post-translation modification; protein sulfation; RPE

INTRODUCTION

Until recently, tyrosine-*O*-sulfation (TOS) of proteins, a post-translational modification, had received little attention. TOS occurs in the trans-Golgi network (Baeuerle and Huttner, 1987) and involves the covalent transfer of a sulfate group from the universal sulfate donor 3'-phosphoadenosine 5'-phosphosulfate to a tyrosine moiety of the nascent target peptide (Lee and Huttner, 1983). The sulfate transfer is catalyzed by two independent tyrosylprotein sulfotransferases (TPST, 3'-phosphoadenylyl-sulfate:protein-tyrosine *O*-sulfotransferase, EC 2.8.2.20) (Lee and Huttner, 1983; Moore, 2003). The two TPST enzymes differ in their acidic

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pH optima, degree of stimulation by Mn^{2+} and Mg^{2+} (Mishiro et al., 2006) and have differential substrate affinities. Compared with TPST-2, TPST-1 exhibits significantly lower K_m and V_{max} for the majority of the substrates tested (Mishiro et al., 2006). This is corroborated by the finding that in comparison to TPST-1, TPST-2 has a high degree of substrate preference towards the thyroid stimulating hormone receptor (Sasaki et al., 2007). Furthermore, studies on the expression of TPST-1 and TPST-2 demonstrated significant variations in the levels of expression among different tissues, suggesting distinct physiological functions for TPST-1 and TPST-2 (Mishiro et al., 2006; Ouyang, Lane et al., 1998; Ouyang and Moore, 1998; Beisswanger et al., 1998; Nishimura and Naito, 2007).

Many of the tyrosine-*O*-sulfated proteins (TOSPs) are secreted and may become part of the extracellular matrix through either direct autocrine re-association or association with other members of the extracellular matrix. In the absence of TOS, the normally sulfated protein may have a retarded transport (Friederich et al., 1988) or have an altered ability to interact with other proteins (Costagliola et al., 2002; Hirata et al., 2004).

Although both TPSTs are thought to be expressed universally, there is no information available regarding protein TOS in the retina. In this report, TOS of proteins was examined in retinas from various species. We found that sulfation occurs in retinas from diverse species, including frogs, fish, and humans. However, the sizes of TOSPs varied among these species, suggesting that post-translational glycosylation may contribute to some of the observed size differences. In addition, TPST-1 and -2 transcripts were expressed in both the human and mouse retinas. Furthermore, immunohistologic analysis detected TOSPs in most retinal layers and the retinal pigment epithelium (RPE). Interestingly, the sulfated proteins in the RPE seem to be different than those present in the retina.

MATERIALS AND METHODS

Retina and RPE samples

A healthy human retina from a 61-year-old Caucasian donor was obtained from the Illinois Eye Bank, Chicago, IL. A trephine with a 4-mm inside diameter was used to dissect the fovea from the rest of the tissue, which is considered the peripheral retina in this study. Both samples were frozen in liquid nitrogen before long-term storage at -70°C . After the removal of the retina, the human RPE, without differentiating foveal from peripheral, was peeled and frozen as above until use. The dog retina was a generous gift from Dr. Gustavo Aguirre (University of Pennsylvania, Philadelphia, PA). Squirrel, chicken, frog, toad, fish and rat retinas were a generous gift from Dr. Muna Naash (OUHSC, Oklahoma City, OK). Pig and cow retinas were obtained from local slaughter houses. The mouse retina was from an in-house inbred strain of wild-type (wt, (Xu et al., 2000)) mice. The *rd/rd* (C3H) mice were purchased from Charles River (Redfield, Arkansas). After the extraction of the mouse retina, eye cups were obtained and dissected in halves. Using a dissection microscope the RPE was then peeled. Mouse retinas and RPEs were frozen and stored as above.

All experiments were approved by the local Institutional Animal Care and Use Committees, and conformed to the *National Institute of Health Guide for the Care and Use of Laboratory Animals* and the guidelines of the *Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research*.

Cell lines

RGC-5 (a rat ganglion cell line (Krishnamoorthy et al., 2001)), MC-1 (a rat Müller cell line (Sarthy, 1985)) and 661W cells (a mouse cone photoreceptor cell line (Tan et al., 2004)) were

all grown as described before (Kanan et al., 2007). ARPE-19 cells were grown as described by Vogel et al. (Vogel et al., 2007).

Preparation of the soluble fraction of the inter-photoreceptor matrix

The protocol for preparation of the soluble fraction of the inter-photoreceptor matrix (S-IPM) was a slight modification of that described before ((Johnson and Hageman, 1989; Hollyfield et al., 1990)). Briefly, 20 mouse retinas from in-house inbred strain of mice (Xu et al., 2000) were extracted and incubated in PBS containing protease inhibitors (Complete mini, EDTA-free, Roche, Nutley, New Jersey) for 2 min. Then retinas were collected by mild centrifugation (200 g for 2 min in an Eppendorf microfuge, Westbury, NY) and the supernatant was centrifuged again at 20,000 g in the microfuge for 10 min. The resulting supernatant was considered as the S-IPM. The remainder of the sample was extracted as described below and named "IS-IPM⁺" since it contained the insoluble part of the IPM and the rest of the retina.

Western blot analysis

Western blots were processed as described before (Tan et al., 2001) and images were obtained using a Kodak image station (Carestream Molecular Imaging, Rochester, NY). Briefly, retinal protein extracts were prepared by homogenization of frozen tissue as has been described (Tan et al., 2001). Retinal extracts were combined with Laemmli sample buffer (Laemmli, 1970) containing 0.2 M Tris (pH 6.8), 1 mM EDTA, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.005% bromophenol blue, and 5% β -mercaptoethanol. After SDS-PAGE, gels were transferred to PVDF membrane (Immunoblot; Bio-Rad, Hercules, CA) and membranes were probed for sulfated tyrosines with the PSG2 antibody (Hoffhines et al., 2006) at 200 ng/mL. Membranes were processed and imaged as detailed in (Tan et al., 2001). The PSG2 antibody was kindly provided by Dr. Kevin Moore (Oklahoma Medical Research Foundation, Oklahoma City, OK).

The specificity of the PSG2 antibody was demonstrated by Hoffhines et al. (Hoffhines et al., 2006) and confirmed by Western blot analysis (Supplemental Figure 1). Forty micrograms of total mouse retinal extracts were loaded into each of two lanes and subjected to SDS-PAGE. The proteins were transferred to PVDF membrane. The two lanes were separated by cutting the membrane; one lane was probed with PSG2 and the other was probed with PSG2 in the presence of 4 mM sulfotyrosine. As shown in Supplemental Figure 1, four of the protein bands detected with PSG2 (black arrows) were not detected when sulfotyrosine was included. However, a protein band at ~27 KDa (black arrowhead) was not competed out, suggesting that it is not a sulfated protein.

The anti-TPST-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA or from Abnova Corporation, Taipei, Taiwan) and anti-TPST-2 antibodies (Abcam, Inc., Cambridge, MA) were used at 1 μ g/ml. The Santa Cruz anti-TPST-1 antibody (sc-25031) is a goat anti-peptide antibody raised against a peptide mapping near the N-terminus of human TPST-1. The Abnova anti-TPST-1 antibody (H00008460-A01) is a mouse anti-partial recombinant GST-tagged human TPST-1. The Abcam anti-TPST-2 antibody (ab59958) is a rabbit anti-human TPST-2 antibody raised against a peptide mapping to the N-terminus conjugated to KLH.

Peptide:N-Glycosidase F treatment of retinal extracts

Peptide:N-Glycosidase F (PNGaseF) was obtained from ProZyme (San Leandro, CA) and reactions with PNGaseF were carried out as recommended by supplier.

Reverse Transcription-PCR

Total RNA was isolated from mouse and human retinas or the MCF-7 human breast cancer cell line by using the Trizol reagent (Invitrogen, Carlsbad, CA). Complimentary DNA was synthesized using Superscript III (Invitrogen). Genomic DNA contamination was prevented by treatment of the samples with RQ1 RNase free DNase (Promega, Madison, WI) and verified by the absence of amplification in samples that were not reverse transcribed to cDNAs. Amplified samples were separated on 1% agarose gels in the presence of ethidium bromide and alongside the 1 Kb plus DNA ladder (Invitrogen). Amplification of the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a control as described before (Kanan et al., 2008).

Several cell lines, including MCF-7, were tested for the presence of sulfated proteins by using Western blot analysis with the PSG-2 antibody (data not shown). Reverse transcription (RT)-PCR confirmed that the MCF-7 cell line had both TPSTs present, thus it was chosen as a positive control.

RT-PCR primers:

Human Tpst1 Forward 5' CCACAGACTGAGCAAGTGG 3'

Reverse 5' CAATACACTTAAACAGGCACACG 3'

Product size 483 bp

Human Tpst2 Forward 5' CATGGAGGTAGGCAAGGAGA 3'

Reverse 5' CCTTTCAGATTGGCTGGTGT 3'

Product size 465 bp

Mouse Tpst1 Forward 5' AGCTGGCTTTGACCTGAACA 3'

Reverse 5' TTAGGAGGATTGGCGTATGG 3'

Product size 402 bp

Mouse Tpst2 Forward 5' AGTGACACAGTCCTGCACCA 3'

Reverse 5' TGCTTGGGTTAGTCCAGCTT 3'

Product size 424 bp

Immunohistochemistry

Sections of a human retina were a generous gift from Dr. Joe Hollyfield (Cleveland Clinic, Cleveland, OH). The eye was obtained from a 57-year-old Caucasian male donor who had no history of vision problems. Eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate. Sections were processed as described previously (Al-Ubaidi et al., 1997). Mouse retinal sections, from an in-house inbred strain of wild-type (wt, (Xu et al., 2000)) mice, were prepared and processed as described in Al-Ubaidi et al. (Al-Ubaidi et al., 1997). Sections were incubated with 1µg/ml PSG2 antibody.

RESULTS

Retinal TOS is a highly conserved, post-translational modification

To determine whether any retinal proteins are sulfated and whether sulfation of retinal proteins is biologically relevant, the degree of conservation of TOS as a post-translational modification was investigated (Figure 1). Western blot analysis of retinal extracts from various species probed with PSG2, the anti-sulfotyrosine antibody (Hoffhines et al., 2006), revealed the presence of sulfated proteins in the retinas from all species tested. These proteins ranged in size from 30 to 250 kDa (Figure 1). The retinas from human, squirrel and chicken had the most complex patterns of sulfated proteins. However, each species has at least one major and multiple minor sulfated proteins. The human fovea and peripheral retina exhibited similar patterns of TOSPs, suggesting that sulfated proteins are present in both rods and cones (Figure 1C). However, it is difficult to judge from only observed migration on SDS-PAGE whether the proteins (e.g. at 50KDa (white asterisk)) observed in both the fovea and the periphery are the same. Since TOSPs were detected in the retinas of animals that are evolutionally millions of years apart, this may suggest a strong biological significance for protein tyrosine sulfation.

Retinal TOSPs are glycosylated

All mammalian retinas used in the preparation of the Western blot (Figure 1) expressed TOSP (s) between 100 and 150 kDa in size, suggesting that these protein(s) may be common in most mammals. However, other sulfated proteins were detected only in certain species. These bands may represent the same proteins with differences in the degree and pattern of glycosylation that would influence the apparent molecular mass as observed on the Western blot. Consistent with this possibility, several TOSPs appeared as diffuse bands (Figure 1). To determine the contribution of glycosylation to the observed molecular mass of sulfated proteins, mouse retinal extracts were subjected to PNGaseF treatment before separation by SDS-PAGE. As shown in Figure 2A, PNGaseF treatment increased the mobility of both of the high molecular weight sulfated proteins. Interestingly, the peptides after PNGaseF treatment did not collapse to sizes similar to that observed in other species, suggesting that the variability in the sizes of TOSPs in the mouse retina is not resulting from differential glycosylation patterns.

The separation of the human retinal sample shown in Figure 1 into foveal and peripheral fractions was intended to determine whether there are rod- or cone-specific TOSPs. To determine whether some cell-specific TOSPs were present in the retina and since it is difficult to fractionate the different cell types from the retina in quantities sufficient for Western blot analysis, three established retinal cell lines were used. Extracts from RGC-5 (a rat ganglion cell line (Krishnamoorthy et al., 2001)), MC-1 (a rat Müller cell line (Sarthy, 1985)), and 661W (a mouse cone photoreceptor cell line (Tan et al., 2004)), were used. The mouse retina and all three cell lines expressed a major common protein band of approximately 125 kDa that was recognized by the PSG2 antibody (arrow, Figure 2B). However, there were minor cell-type specific protein bands (asterisks in each lane, Figure 2B) that were not observed when total retinal extracts were used. This is probably due to the relatively low contribution of their respective cell types to total retinal proteins. Furthermore, the lower band (immediately above 100 kDa) observed in total retinal extracts does not appear in the cell line lanes (Figure 2B), suggesting that it is produced by cell types other than cones, Müller or ganglion cells. Of specific interest is the appearance of TOSPs in extracts from 661W cells (Figure 2B) that may be a cone-specific product. Alternatively, the expression of these proteins may have been altered as a result of the growth of these cells in culture.

To further investigate the differential distribution of TOSPs in different cell types of the retina, extracts from one-month-old *rd/rd* retinas were analyzed. The *rd/rd* retina, which has a mutation in the β -subunit of phosphodiesterase, exhibits rod photoreceptor degeneration as

early as postnatal day (P) 7 and complete rod photoreceptor loss after P21. Therefore, any TOSP band(s) that is observed in extracts from *wt* retinas but disappears in extracts from degenerated *rd/rd* retinas should be a rod-specific, sulfated protein. There is a TOSP band that is shared by *wt* and *rd/rd* retinas (black arrowhead, Figure 2C). However, two differences were observed when these extracts were probed with PSG2. The first is the presence of a band in *wt* retinal extracts that was absent from *rd* retinal extracts (asterisk, Figure 2C). Since the rod photoreceptors have degenerated by one month of age in the *rd* retina, and since they constitute ~60% of the total number of cells in the retina, it is possible to assume that this TOSP is expressed in rods. Alternatively, this protein may be produced by inner retinal cells and may have been lost during the re-organization of the retina as a consequence of the degeneration. The second difference is the appearance of a protein band in extracts from *rd/rd* retinas (black arrow, Figure 2C), which may be expressed by one of the remaining retinal cell types and only becomes apparent because of the increased contribution of those cell types after the disappearance of the rods.

Contribution of sulfated proteins to retinal extracellular matrix

Some TOSPs are known to be secreted and may or may not associate with the cells that produce them. To determine whether any TOSPs are secreted from retinal cells, 661W conditioned medium (661W CM) was collected, concentrated and used in Western blot analyses. Concentrated unconditioned medium (un-CM) was used as a control. As seen in Figure 3A, probing with PSG2 detected two high molecular weight, secreted protein bands in the conditioned medium. The lower band was present in the total retinal extract as well as in extracts from 661W cells (Figure 2B). However, the higher band (asterisk, Figure 3A) is not observed in either retinal or 661W extracts (Figure 2B), suggesting that this is a secreted protein and a possible member of the extracellular matrix. Absence of that protein in the retinal extract may be a reflection of the small contribution of cones to the total number of cells in the retina. There may also be a third protein band found at ~90 kDa (white asterisk, Figure 3A) that seems diffused. The lower band that was seen in the 661W extracts at ~70 kDa (Figure 2B) was not found likely due to the distortion of the gel by the large amounts of albumin in the medium (gray arrowhead, Figure 3A). Finally, the lower band (black arrow, Figure 3A) that appears in the 661W CM, is also found in the un-CM lanes and is probably a component of the fetal bovine serum.

The inter-photoreceptor matrix (IPM) is a unique milieu that surrounds the outward extension of the photoreceptors. It is essential for the trafficking of nutrients and retinoids between the retina and the RPE, retinal attachment, photoreceptor alignment, and the cellular interactions involved in outer segment (OS) shedding and phagocytosis by the RPE (Hollyfield, 1999). To prepare IPM fractions, freshly isolated retinas from mice were rinsed in PBS (pH 7.2) and the eluted proteins constituted the soluble fraction (S-IPM) while the remainder contained the insoluble fraction (IS-IPM⁺) of the IPM and the remainder of the retina. Western analysis using PSG2 was performed on both fractions and total retinal extracts from *wt* mice. As shown in Figure 3C, two major protein bands between 100 to 150 kDa were recognized by the PSG2 antibody in the S-IPM. The source of these proteins could be either the retina or the RPE. As expected, the TOSPs detected in total retinal extracts seem to be a combination of those observed in both the S-IPM and the IS-IPM⁺. It is clear that the top band is retained in the IS-IPM⁺, suggesting that this protein may be associating with the plasma membrane after being secreted. Other minor TOSPs were also detected in the S-IPM. One TOSP (arrowhead, Figure 3C) seems enriched in the S-IPM fraction while the other two (asterisks, Figure 3C) are not detected in total retinal extracts. The latter two protein bands may have become obvious due to their increased contribution to the total protein content in the S-IPM. However, comparing the Western blot probed with PSG2 (Figure 3C) to the Coomassie Blue stained sister gel (Figure 3B) clearly shows that only a minor fraction of the proteins in the IPM underwent TOS.

To determine whether any of the TOSPs observed in the S-IPM originated from the RPE, Western analysis was performed on extracts from mouse RPE, mouse S-IPM, and mouse retina (Figure 4). By only using the criterion of co-migration, we determined that the S-IPM contained TOSPs that likely originated from the retina itself (Figure 4, black arrows). Likewise, S-IPM also contained a TOSP that seemed to originate from the RPE (Figure 4, white arrowhead). The rest of the proteins that appear in the RPE lane seem to be unique to the RPE (asterisks, Figure 4). Minor amounts of the protein identified by an asterisk in Figure 4 appear in the S-IPM lane. Since extracted retinas are always contaminated by small amounts of RPE, it is likely that the presence of this protein species in the retina and S-IPM lanes is solely due to the contamination of these samples by the RPE.

Human RPE expresses a different subset of sulfated proteins

To determine whether the human RPE differentially expresses a subset of TOSPs, Western analysis was performed on protein extracts from human retina, human RPE, and the ARPE-19 cell line using the PSG2 antibody. The human RPE expressed a different subset of TOSPs than the retina as judged by migration on SDS-PAGE (Figure 5). Compared to human RPE cells, ARPE-19 cells expressed a different subset of TOSPs (notice the two lower molecular weight species present in the extracts of adult human RPE, Figure 5). However, there is a protein band at ~125 KDa (arrow, Figure 5) that seems to be present in both retina and RPE. Since the ARPE-19 cells are not fully differentiated (day 5) this observation suggests that TOSPs may be developmentally regulated, at least in the RPE. Alternatively, these cells may have changed their characteristics as a result of their passage in culture.

Localization of TOSPs in human and mouse retinas

Figure 6 shows the results of immunohistochemical (IHC) labeling of TOSPs in sections of human and mouse retinas with the PSG2 antibody. In the human sections (Figure 6A-C), labeling was observed in the RPE, photoreceptor outer segments (OS), photoreceptor inner segments (IS), photoreceptor outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), ganglion cell layer (GCL) and inner limiting membrane (white arrowhead, Figure 6B&C). There seems to be an area clearly labeled between the OS and RPE that contained TOSPs (green arrow and inset, Figure 6C). These TOSPs could either be in the tips of the OS adjacent to the RPE or in the RPE processes that happened to separate upon section processing. Alternatively, this protein(s) could be in the IPM. Finally, the sclera (Sc) was clearly labeled in the presence of the antibody. In the absence of the PSG2 antibody (Figure 6A), labeling is observed in a blood vessel (white arrow) and to a lesser degree in the Sc.

To compare the cellular localization of TOSPs in the human retina to that of the mouse, IHC analysis was performed on *wt* mouse retinal sections using PSG2 antibody. As shown in Figure 6E, PSG2 antibody recognized retinal TOSPs in the Sc, RPE, OS, IS, ONL, INL, and GCL. Minor signal was observed in OPL and IPL. Although a stronger signal was observed in OPL and IPL of the human retina, the pattern of distribution of sulfated proteins was similar between both human and mouse retinas.

Expression of TPST-1 and TPST-2 in human and mouse retinas

It is clear that human and mouse retinas express several TOSPs. However, since there are two enzymes that can sulfate proteins, experiments were performed to determine whether both of these enzymes are present in retinas of both species. Two sets of primers, one for each of the two enzymes in each species, were designed across introns to eliminate the potential amplification from genomic DNA. Furthermore, all RNA samples were treated with DNase and some PCR amplifications were performed in the absence of reverse transcription (–RT) (Figure 7). In that case only primer dimers were observed in the *Tpst2* lanes (Figure 7). RNA extracted from MCF-7, the human breast cancer cell line, was used as a positive control (Figure

7) as described in the Experimental section. Although the primers for TPST-2 were less efficient in generating a robust product, it is clear that the human and mouse retinas contained transcripts for both enzymes (Figure 7). The identities of the amplified products were confirmed by subcloning and sequencing (data not shown). Since RT-PCR was not quantitative in nature (e.g. real time) it is not possible to conclude that there are more *Tpst1* transcripts than *Tpst2* in the human retina solely based on the intensity of the amplified products. Detection of TPST-1 and/or TPST-2 in the retina by Western or IHC analyses using commercially available antibodies was unsuccessful due to the lack of specificity of the antibodies (data not shown).

DISCUSSION

As the first step towards understanding the role of TOS in retinal function, an antibody that recognizes sulfotyrosine residues in proteins was used to determine the presence and localization of retinal TOSPs. This study demonstrated the evolutionary conservation of retinal TOS across diverse species. Some TOSPs seem to be species-specific as determined by the pattern of migration on SDS-PAGE (Figure 1). Several retinal TOSPs were observed in the IPM, which is consistent with the secreted nature of TOSPs. Furthermore, transcripts for both TPST-1 and -2 were expressed in human and mouse retinas.

Identification of retinal TOSPs will be instrumental to deciphering the role of sulfation in retinal function. Furthermore, it will be interesting to determine the retinal substrates for each of the two TPSTs and determine whether those targets are preferentially sulfated by one of the two TPSTs. It is also of interest to know the fate of retinal TOSPs when one or both of the TPSTs is absent. Friederich et al. (Friederich et al., 1988) showed that when the sulfation of the *Drosophila melanogaster* protein vitellogenin II was inhibited, the transport of the protein was retarded (Friederich 1988). However, inhibition of sulfation of P-selectin glycoprotein ligand-1 (PSGL-1), glycoprotein Ib α , factor V, factor VIII, C4, and type III and V collagens did not affect their surface expression or secretion (for reviews see (Monigatti et al., 2006; Moore, 2003)). Instead, sulfation of these proteins is important for their protein-protein interactions. This conclusion was strongly supported by the co-crystallization of sulfated proteins with their ligands. It has been shown that when thrombin and hirugen were co-crystallized, all three sulfated tyrosines were involved in an extensive intermolecular hydrogen-bonded network with α -thrombin (Skrzypczak-Jankun et al., 1991; Chen et al., 1995). Sulfation of tyrosine 385 on the thyroid stimulating hormone is required for its high-affinity binding and activation of the receptor, but not by thyroid-stimulating autoantibodies (Costagliola et al., 2002). Similarly, tyrosine sulfation of PSGL-1 is required for high affinity binding to P-selectin (Hirata et al., 2004; Rodgers et al. 2001; Pouyani and Seed, 1995; Sako et al., 1995; Wilkins et al., 1995). Sulfation has also been shown to play a role in optimal receptor-ligand interactions such as chemokine/chemokine receptor binding, optimal proteolytic processing of gastrin, and proteolytic activation of extracellular proteins such as factor V and VIII (Moore, 2003). However, the only published evidence showing that lack of sulfation in humans can lead to a phenotype is from cases of mild-to-moderate hemophilia A that result from missense mutations in the factor VIII gene. These mutations result in a Tyr1680Phe substitution in the von Willebrand factor binding site at the junction of the B and A3 domain (Jacquemin et al., 2000; Higuchi et al., 1990; Moore, 2003). It was later shown that optimal binding to von Willebrand factor requires sulfation of Tyr1680 in factor VIII (Leyte et al., 1991).

It is interesting to observe that a sulfated protein becomes obvious in extracts of *rd/rd* retinas immediately after the completion of the degenerative process. One explanation could be that this protein may be produced by the remaining cells in the *rd/rd* retina and only becomes obvious after the rods degenerated. Alternatively, it may be expressed in response to the re-organization that occurs in the *rd/rd* retina after degeneration of the rods. At present, it is difficult to favor either of the two possibilities. Similar situation may be present with the

sulfated proteins expressed in ARPE-19 versus the fully differentiated RPE. Some of the sulfated proteins expressed in ARPE-19 cells are different from those expressed in differentiated RPE. Since ARPE-19 cells are not fully differentiated (day 5) this observation may suggest that TOSPs may be developmentally regulated, at least in the RPE. Alternatively, these cells may have changed their characteristics as a result of their passage in culture.

Another interesting observation is the strong label for TOSPs observed between the OS and RPE in the human retina (green arrow, Figure 6C). These sulfated proteins can be in the tips of the OS adjacent to the RPE or in the RPE processes that happened to separate upon section processing or in the IPM. Regardless of where these proteins are located, this suggests a role for these proteins in RPE-OS interactions and a potential role in the shedding process.

Although only a small subset of mostly large molecular weight TOSPs are observed on Western blots of total retinal extracts, other sulfated protein species are obvious when cell lines representing different retinal cell types were used. This suggests that there are several less-abundant TOSPs present in the retina.

In summary, this study demonstrates for the first time that protein sulfation occurs in the retina and suggests a biological significance based on evolutionary conservation. Future studies will focus on identifying the sulfated proteins by proteomics and determining whether the lack of sulfation could lead to retinal diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Al-Ubaidi MR, Mangini NJ, Quiambao AB, Myers KM, Abler A, Cheng C-J, Tso MOM, Butel JS, Hollyfield JG. Unscheduled DNA replication precedes apoptosis of photoreceptor cells expressing SV40 T antigen. *Exp Eye Res* 1997;64:573–585. [PubMed: 9227276]
2. Baeuerle PA, Huttner WB. Tyrosine sulfation is a trans-Golgi-specific protein modification. *J Cell Biol* 1987;105:2655–2664. [PubMed: 3121635]
3. Beisswanger R, Corbeil D, Vannier C, Thiele C, Dohrmann U, Kellner R, Ashman K, Niehrs C, Huttner WB. Existence of distinct tyrosylprotein sulfotransferase genes: molecular characterization of tyrosylprotein sulfotransferase-2. *Proc Natl Acad Sci USA* 1998;95:11134–11139. [PubMed: 9736702]
4. Chen Z, Li Y, Mulichak AM, Lewis SD, Shafer JA. Crystal structure of human alpha-thrombin complexed with hirugen and p-amidinophenylpyruvate at 1.6 Å resolution. *Arch Biochem Biophys* 1995;322:198–203. [PubMed: 7574675]
5. Costagliola S, Panneels V, Bonomi M, Koch J, Many MC, Smits G, Vassart G. Tyrosine sulfation is required for agonist recognition by glycoprotein hormone receptors. *EMBO J* 2002;21:504–513. [PubMed: 11847099]

6. Friederich E, Baeuerle PA, Garoff H, Hovemann B, Huttner WB. Expression, tyrosine sulfation, and secretion of yolk protein 2 of *Drosophila melanogaster* in mouse fibroblasts. *J Biol Chem* 1988;263:14930–14938. [PubMed: 3139664]
7. Friederich E, Fritz HJ, Huttner WB. Inhibition of tyrosine sulfation in the trans-Golgi retards the transport of a constitutively secreted protein to the cell surface. *J Cell Biol* 1988;107:1655–1667. [PubMed: 3182933]
8. Higuchi M, Wong C, Kochhan L, Olek K, Aronis S, Kasper CK, Kazazian HH Jr, Antonarakis SE. Characterization of mutations in the factor VIII gene by direct sequencing of amplified genomic DNA. *Genomics* 1990;6:65–71. [PubMed: 2105906]
9. Hirata T, Furukawa Y, Yang BG, Hieshima K, Fukuda M, Kannagi R, Yoshie O, Miyasaka M. Human P-selectin glycoprotein ligand-1 (PSGL-1) interacts with the skin-associated chemokine CCL27 via sulfated tyrosines at the PSGL-1 amino terminus. *J Biol Chem* 2004;279:51775–51782. [PubMed: 15466853]
10. Hoffhines AJ, Damoc E, Bridges KG, Leary JA, Moore KL. Detection and purification of tyrosine-sulfated proteins using a novel anti-sulfotyrosine monoclonal antibody. *J Biol Chem* 2006;281:37877–37887. [PubMed: 17046811]
11. Hollyfield JG. Hyaluronan and the functional organization of the interphotoreceptor matrix. *Invest Ophthalmol Vis Sci* 1999;40:2767–2769. [PubMed: 10549633]
12. Hollyfield JG, Rayborn ME, Landers RA, Myers KM. Insoluble interphotoreceptor matrix domains surround rod photoreceptors in the human retina. *Exp Eye Res* 1990;51:107–110. [PubMed: 2373175]
13. Jacquemin M, Lavend'homme R, Benhida A, Vanzieleghem B, d'Oiron R, Lavergne JM, Brackmann HH, Schwaab R, VandenDriessche T, Chuah MK, Hoylaerts M, Gilles JG, Peerlinck K, Vermynen J, Saint-Remy JM. A novel cause of mild/moderate hemophilia A: mutations scattered in the factor VIII C1 domain reduce factor VIII binding to von Willebrand factor. *Blood* 2000;96:958–965. [PubMed: 10910910]
14. Johnson LV, Hageman GS. Characterization of isolated cone matrix sheath substructure. *Investigative Ophthalmology and Visual Sciences* 1989;30(490)
15. Kanan Y, Kasus-Jacobi A, Moiseyev G, Sawyer K, Ma JX, Al Ubaidi MR. Retinoid processing in cone and Muller cell lines. *Exp Eye Res* 2008;86:344–354. [PubMed: 18163989]
16. Kanan Y, Moiseyev G, Agarwal N, Ma JX, Al Ubaidi MR. Light induces programmed cell death by activating multiple independent proteases in a cone photoreceptor cell line. *Invest Ophthalmol Vis Sci* 2007;48:40–51. [PubMed: 17197514]
17. Krishnamoorthy RR, Agarwal P, Prasanna G, Vopat K, Lambert W, Sheedlo HJ, Pang IH, Shade D, Wordinger RJ, Yorio T, Clark AF, Agarwal N. Characterization of a transformed rat retinal ganglion cell line. *Brain Res Mol Brain Res* 2001;86:1–12. [PubMed: 11165366]
18. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
19. Lee RW, Huttner WB. Tyrosine-O-sulfated proteins of PC12 pheochromocytoma cells and their sulfation by a tyrosylprotein sulfotransferase. *J Biol Chem* 1983;258:11326–11334. [PubMed: 6577005]
20. Leyte A, van Schijndel HB, Niehrs C, Huttner WB, Verbeet MP, Mertens K, van Mourik JA. Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J Biol Chem* 1991;266:740–746. [PubMed: 1898735]
21. Mishiro E, Sakakibara Y, Liu MC, Suiko M. Differential enzymatic characteristics and tissue-specific expression of human TPST-1 and TPST-2. *J Biochem(Tokyo)* 2006;140:731–737. [PubMed: 17028309]
22. Monigatti F, Hekking B, Steen H. Protein sulfation analysis--A primer. *Biochim Biophys Acta* 2006;1764:1904–1913. [PubMed: 16952486]
23. Moore KL. The biology and enzymology of protein tyrosine O-sulfation. *J Biol Chem* 2003;278:24243–24246. [PubMed: 12730193]
24. Nishimura M, Naito S. Tissue-specific mRNA expression profiles of human carbohydrate sulfotransferase and tyrosylprotein sulfotransferase. *Biol Pharm Bull* 2007;30:821–825. [PubMed: 17409530]

25. Ouyang Y, Lane WS, Moore KL. Tyrosylprotein sulfotransferase: purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. *Proc Natl Acad Sci USA* 1998;95:2896–2901. [PubMed: 9501187]
26. Ouyang YB, Moore KL. Molecular cloning and expression of human and mouse tyrosylprotein sulfotransferase-2 and a tyrosylprotein sulfotransferase homologue in *Caenorhabditis elegans*. *J Biol Chem* 1998;273:24770–24774. [PubMed: 9733778]
27. Pouyani T, Seed B. PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. *Cell* 1995;83:333–343. [PubMed: 7585950]
28. Rodgers SD, Camphausen RT, Hammer DA. Tyrosine sulfation enhances but is not required for PSGL-1 rolling adhesion on P-selectin. *Biophys J* 2001;81:2001–2009. [PubMed: 11566773]
29. Sako D, Comess KM, Barone KM, Camphausen RT, Cumming DA, Shaw GD. A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. *Cell* 1995;83:323–331. [PubMed: 7585949]
30. Sarthy PV. Establishment of Muller cell cultures from adult rat retina. *Brain Res* 1985;337:138–141. [PubMed: 3891020]
31. Sasaki N, Hosoda Y, Nagata A, Ding M, Cheng JM, Miyamoto T, Okano S, Asano A, Miyoshi I, Agui T. A mutation in *Tpst2* encoding tyrosylprotein sulfotransferase causes dwarfism associated with hypothyroidism. *Mol Endocrinol* 2007;21:1713–1721. [PubMed: 17456791]
32. Skrzypczak-Jankun E, Carperos VE, Ravichandran KG, Tulinsky A, Westbrook M, Maraganore JM. Structure of the hirugen and hirulog 1 complexes of alpha-thrombin. *J Mol Biol* 1991;221:1379–1393. [PubMed: 1942057]
33. Tan E, Ding XQ, Saadi A, Agarwal N, Naash MI, Al Ubaidi MR. Expression of cone-photoreceptor-specific antigens in a cell line derived from retinal tumors in transgenic mice. *Invest Ophthalmol Vis Sci* 2004;45:764–768. [PubMed: 14985288]
34. Tan E, Wang Q, Quiambao AB, Xu X, Qtaishat NM, Peachey NS, Lem J, Fliesler SJ, Pepperberg DR, Naash MI, Al Ubaidi MR. The relationship between opsin overexpression and photoreceptor degeneration. *Invest Ophthalmol Vis Sci* 2001;42:589–600. [PubMed: 11222515]
35. Vogel JS, Bullen EC, Teygong CL, Howard EW. Identification of the *RLBP1* gene promoter. *Invest Ophthalmol Vis Sci* 2007;48:3872–3877. [PubMed: 17652763]
36. Wilkins PP, Moore KL, McEver RP, Cummings RD. Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. *J Biol Chem* 1995;270:22677–22680. [PubMed: 7559387]
37. Xu X, Quiambao AB, Roveri L, Pardue MT, Marx JL, Röhlich P, Peachey NS, Al-Ubaidi MR. Degeneration of cone photoreceptors induced by expression of the *Mas1* proto-oncogene. *Exp Neurol* 2000;163:207–219. [PubMed: 10785460]

Figure 1. Western analysis of sulfated proteins in retinal extracts from different species using the PSG2 antibody. **A&C**, Western blots using PSG2 antibody; **B&D**, sister gels stained with Coomassie Blue. Thirty micrograms of protein extracts were loaded in each lane. After reducing SDS-PAGE and transfer, the blot was probed with PSG2.

Figure 2.

Characterization of sulfated retinal proteins. **A.** Western blot (with PSG2) of sulfated proteins in *wt* mouse retina after treatment with PNGaseF. Thirty micrograms of proteins were loaded in each lane and gel was run under reducing conditions. **B.** Western blot of sulfated proteins in permanent cell lines representing different retinal cell types. Thirty micrograms of protein extracts were loaded in each lane. The asterisks denote proteins that are unique to the cell type and the arrow points to a sulfated protein that seems to be shared by all cell lines. 661W, a cone photoreceptor cell line; MC-1, a rat Müller cell line; RGC-5, a rat ganglion cell line. Mouse retina, first lane, was used as a reference. **C.** Western blot of sulfated proteins in *wt* and *rd/rd* mouse retinas. Thirty micrograms of proteins were loaded in each lane. The black arrowhead points to the common band; the black arrow points to a band that is only present in the *rd/rd* retina; and the asterisk points to the lower band that is only present in *wt* retinas. All blots were probed with PSG2.

Figure 3.

Sulfated proteins in the inter-photoreceptor matrix. **A.** Western blot of sulfated proteins in 661W conditioned medium. Thirty micrograms of mouse retinal protein extracts were loaded in the first lane. The other two lanes each contained concentrated proteins from 50 mL of media. 661W CM, 661W conditioned medium; un-CM, unconditioned regular medium. Gel was run under reducing conditions. The black asterisk denotes a protein band that is not observed in retinal extracts. The gray arrowhead points to the albumin present in the media. The black arrow points to a band that is a component of the fetal bovine serum medium. **B. & C.** Western blots to identify sulfated proteins in the inter-photoreceptor matrix (IPM) of the mouse retina. **B.** shows a Coomassie Blue stained gel and **C** shows a sister gel that was transferred to a membrane and probed with PSG2 antibody. S, soluble fraction; IS, insoluble fraction. IS-IPM⁺, contained the insoluble part of the IPM as well as the rest of the retina.

Figure 4.

Western blot analysis of sulfated proteins in mouse RPE and S-IPM. Fifty micrograms of protein extracts were loaded in each lane, the gel ran under reducing conditions and transferred to a PVDF membrane. The blot was probed with PSG2. Black asterisks denote bands unique to RPE; the white asterisk denotes a band that may be present in S-IPM but originated in the RPE; and the white arrowhead points to a band that is shared between RPE and S-IPM while little black arrows mark protein bands similar in retina and S-IPM.

Figure 5.

Western blot analysis for sulfated proteins in extracts from human retina, human RPE and the ARP-19 cell line. Thirty micrograms of extracts were loaded in each lane, the gel was run under reducing conditions and then transferred to a PVDF membrane. The blot was probed with PSG2. The arrow points to a protein species that may be present in all samples, albeit at much reduced levels in the human retina.

Figure 6.

Cellular localization of sulfated proteins in the human and mouse retinas. Sections of peripheral human retina were incubated in the absence (A) or presence (B&C) of PSG2 antibody. C. is the same image in B. without the DAPI staining of nuclei to better visualize the staining of the different layers of the retina with PSG2. The small white arrow in A is pointing to a blood vessel; the white arrowheads in B&C are pointing to either ganglion cell processes or inner limiting membrane and the green arrow in C is pointing to a junctional layer between OS and RPE that is heavily labeled. Inset in C is an enlargement of the area marked by the green arrow. All images were photographed at the same magnification and exposure time. Although the RPE seems to autofluoresce (A), there is more intense signal observed in the presence of PSG2 antibody (B&C). The asterisks are marking choroidal blood vessels. D&F, Nomarski images of *wt* mouse retinal sections showing the different layers. E. Fluorescent staining of a normal mouse retinal section with PSG2 antibody that recognizes sulfotyrosines on proteins. G. A section similar to that shown in E was incubated with PSG2 and 4 mM sulfotyrosine and imaged at the same settings as E. The specificity of the immunohistochemistry is confirmed by the lack of staining resulting from competition by sulfotyrosine. Similar result (as in G) is obtained

when the primary antibody is eliminated. Sc, sclera; RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 7.

Detection of TPST-1 and TPST-2 transcripts in mouse and human retinas. A representative gel showing the DNA bands amplified in a RT-PCR reaction using cDNAs prepared against total RNAs from human and mouse retinas. Reactions were performed in triplicates on samples before (–RT) or after (+RT) reverse transcription. *HPRT* was used as a control, while PCR reactions without RT served as negative controls. Product sizes (bp), human TPST-1, 483; human TPST-2, 465; mouse TPST-1, 402; mouse TPST-2, 424. In the absence of reverse transcription (–RT). Primer dimers were observed in the *Tpst2* lanes in the absence of reverse transcription.