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ROLE OF TYROSINE-SULFATED PROTEINS IN RETINAL STRUCTURE AND FUNCTION

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Abstract

The extracellular matrix (ECM) plays a significant role in cellular and retinal health. The study of retinal tyrosine-sulfated proteins is an important first step toward understanding the role of ECM in retinal health and diseases. These secreted proteins are members of the retinal ECM. Tyrosine sulfation was shown to be necessary for the development of proper retinal structure and function. The importance of tyrosine sulfation is further demonstrated by the evolutionary presence of tyrosylprotein sulfotransferases, enzymes that catalyze proteins' tyrosine sulfation, and the compensatory abilities of these enzymes. Research has identified four tyrosine-sulfated retinal proteins: fibulin 2, vitronectin, complement factor H (CFH), and opticin. Vitronectin and CFH regulate the activation of the complement system and are involved in the etiology of some cases of age-related macular degeneration. Analysis of the role of tyrosine sulfation in fibulin function showed that sulfation influences the protein's ability to regulate growth and migration. Although opticin was recently shown to exhibit anti-angiogenic properties, it is not yet determined what role sulfation plays in that function.

Future studies focusing on identifying all of the tyrosine-sulfated retinal proteins would be instrumental in determining the impact of sulfation on retinal protein function in retinal homeostasis and diseases.

Keywords

tyrosine sulfation; retina; extracellular matrix; retinal diseases

INTRODUCTION

Tyrosine is a non-essential amino acid that is a precursor for melanin, neurotransmitters, and hormones. As a member of a peptide, a tyrosine may undergo nitration, phosphorylation, or sulfation as post-translational modifications (PTMs) that ultimately influence the function of

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a protein. These tyrosine-specific PTMs (Figure 1) are made possible by the presence of a side chain hydroxyl group. Nitrotyrosine, a non-reversible modification, is formed from the interaction of reactive nitrogen species with peptide bound tyrosines. Nitrotyrosine is undetected in normal subjects, but detected, among others, in keratoconus (Buddi et al. 2002) and diabetes (Pacher et al. 2005). While nitration is a non-enzymatic reaction, both phosphorylation and sulfation occur through the actions of kinases and tyrosylprotein sulfotransferases (TPSTs), respectively. While tyrosine phosphorylation is involved in signal transduction, tyrosine sulfation is important for protein-protein interactions (Moore, 2003). Although tyrosine phosphorylation can be identified by state of the art mass spectroscopy, tyrosine sulfation is labile to mass spectrometry conditions (Wolfender et al. 1999; Önnerfjord et al 2004). Furthermore, unlike phosphorylation, tyrosine sulfation is a permanent modification. There is no known sulfatase that can remove the sulfate from a tyrosine.

Although tyrosine sulfation is widely used by plants and animals (Moore, 2003), its biological significance is not fully understood, partly due to the difficulty in studying this process. What makes this PTM interesting is the fact that tyrosine-sulfated proteins are secreted to either 1) become independent members of the extracellular matrix, 2) to directly re-associate with the cells, or 3) to indirectly re-associate with the cells through interaction with membrane-bound proteins (Figure 2).

Tyrosine sulfation is catalyzed by two independent tyrosylprotein sulfotransferases (TPSTs) that are bound to the trans-Golgi network membrane, with their active sites residing in the lumen (Niehrs and Huttner, 1990; Niehrs et al. 1992). TPSTs transfer the sulfuryl group from the sulfate carrier, 3'-phosphoadenosine 5'-phosphosulfate, onto tyrosine residues in proteins (Figure 2; Suiko et al. 1992). Mice lacking either of the two TPSTs (*Tpst1*$^{-/-}$ or *Tpst2*$^{-/-}$) have been generated and characterized (Borghei et al. 2006; Ouyang et al. 2002; Westmuckett et al. 2008). *Tpst1*$^{-/-}$ mice, on average, weigh approximately 5% less than controls, and the knockout females produce smaller litters because of embryonic deaths (Ouyang et al. 2002). *Tpst2*$^{-/-}$ mice, on the other hand, exhibit a moderate growth delay but achieve normal body weight by the age of 10 weeks. However, the males are infertile (Borghei et al. 2006). Although they exhibit minor developmental phenotypes, the life spans of both *Tpst1*$^{-/-}$ and *Tpst2*$^{-/-}$ mice are normal. However, *Tpst1*$^{-/-}$/*Tpst2*$^{-/-}$ double knockout (*Tpst/DKO*) mice die soon after birth, and few survive beyond weaning (Westmuckett et al. 2008). These mice succumb to cardio-pulmonary insufficiency, demonstrating the importance of tyrosine sulfation for animal viability (Westmuckett et al. 2008).

Function of tyrosine sulfation

Tyrosine-sulfated proteins belong to multiple families, including coagulation factors, chemokine receptors, extracellular matrix proteins, hormone receptors, G-Protein coupled receptors, and serpins (Costagliola et al. 2002; Farrell et al. 1991; Hortin, 1990; Jukkola et al. 1986). In these proteins, the major function of tyrosine sulfation is protein-protein interaction (Costagliola et al. 2002; Nishimura et al. 2010; Rodgers et al. 2001; Stone et. al 2009; Zhu et al. 2011). In proteins, tyrosine sulfation introduces a negative charge, which

can then interact with positively charged amino acids, forming salt bridges (Woods et al. 2007). Some examples of the role of tyrosine sulfation in protein-protein interaction are given below.

Binding of leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) protein to P-Selectin—It has been previously shown that PSGL-1 binding to P-selectin on endothelial cells is responsible for optimal leukocyte rolling in blood vessels, and recruitment of leukocytes to inflamed tissue (Rodgers et al. 2001). Elimination of tyrosine sulfation in PSGL-1, by harvesting leukocytes from TPST knockout animals, resulted in impaired leukocyte rolling compared with rolling of leukocytes harvested from wild-type animals (Westmuckett et al. 2011). Rolling of leukocytes on endothelial cells, the result of rapid and balanced formation and breaking of selectin–ligand bonds, is an important step for the successful recruitment of those leukocytes into the tissue (Boehncke and Schön, 2003). In addition, antibody blocking experiments using anti-PSGL-1 antibody completely eliminated rolling of leukocytes in both TPST and wild-type animals (Westmuckett et al. 2011). Taken together, the results show that optimum binding of PSGL-1 to P-selectin is responsible for leukocyte rolling. Tyrosine sulfation enhances this binding.

Binding of PSGL-1 to enterovirus 71 (EV71)—Tyrosine sulfation of PSGL-1 in leukocytes is responsible for binding Enterovirus 71 (EV71) virus and mediating viral entry into leukocytes (Nishimura et al. 2010). This virus is responsible for a wide range of neurological diseases in children (Nishimura et al. 2010). Elimination of one or more sulfated tyrosines on PSGL-1 by site-directed mutagenesis or inhibition with chlorate resulted in elimination of optimum binding between PSGL-1 to EV71, and prevented viral entry and replication (Nishimura et al. 2010). These results demonstrate that tyrosine sulfation of the amino terminus of PSGL-1 is critical for EV71 infection.

Chemokine receptor binding to chemokines—Further evidence of tyrosine sulfation enhancing protein-protein interaction was discovered in studies of chemokine receptors. Chemokine receptors are G protein-coupled receptors which play an important role in regulating immune and inflammatory responses by mediating leukocyte migration (Nibbs and Graham, 2013).

All of the chemokine receptors have at least 3 tyrosines surrounded by acidic amino acids, a consensus associated with sulfated tyrosines (Kraemer et al. 2013; Zhu et al. 2013). Seven of these receptors, CCR5, CCR2, CCR8, CXCR3, CXCR4, DARC, and CX_3CR1, have been shown to contain 3 sulfated tyrosines at the N-terminus (Bannert et al. 2001; Colvin et al. 2006; Rapp et al. 2013; Simpson 2009; Tan et al. 2013; Veldkamp et al. 2006; Zhu et al. 2011). Using sulfopeptides showing differential sulfation of tyrosine residues, it has been demonstrated that sulfation enhances binding of chemokines to their receptors compared with their unsulfated counterparts: differential sulfation of tyrosine residues modulates the selectivity and binding affinity of the receptor to different chemokines (Simpson et al. 2009).

Binding of CCR5 to HIV-1 gp120/CD4 complexes—Tyrosine sulfation of chemokine receptor CCR5 is also responsible for the binding of HIV-1 gp120/CD4 complexes and virus

entry (Brower et al. 2009; Farzan et al. 1999). The generation of a tyrosine-sulfated peptide derived from the N-terminal region of CCR5 prevented the association of HIV-1 gp120 with CCR5 and thereby prevented HIV-1 entry, a result that was not seen with its unsulfated counterpart (Dorfman et al. 2006).

Hormone-receptor binding—Tyrosine sulfation also plays a role in protein-protein interaction in the three glycoprotein hormone receptors, follicle-stimulating hormone receptor (FSHr), luteinizing hormone receptor (LH), and thyrotrophin receptor (TSHr). Tyrosine sulfation has been detected in the C-terminus of these receptors. Using site-directed mutagenesis, it was shown that tyrosine sulfation of the receptor is needed for efficient hormone binding (Bonomi et al. 2006).

Blood Coagulation—Numerous proteins involved in the blood coagulation process, such as Factors V, VIII, IX, and fibrinogen, are tyrosine-sulfated (Arruda et al. 2001; Farrell et al. 1991; Hirose et al. 1988; Hortin, 1990; Leyte et al. 1991). Studies of Factor VIII have shown that sulfated tyrosine at position 1680 is required for interaction with von Willebrand factor (vWF) (Leyte et al. 1991). Peptides derived from Factor VIII, containing the sulfated tyrosine 1680 and its non-sulfated analogue using a chlorate inhibitor, showed drastically reduced binding to vWF (Leyte et al. 1991). Tyrosine sulfation sites on two other proteins, fibrinogen and hirugen, is also necessary for optimum binding to thrombin. This was determined by studying the binding of fibrin and hirugen peptides that either have sulfated tyrosines, or are unsulfated to thrombin (Meh et al. 2001; Skrzypczak-Jankun et al. 1991).

Tyrosine sulfation of extracellular proteins

Tyrosine sulfation in elastic tissue—Elastic fibers are extracellular matrix (ECM) structures that are composed of elastin, microfibrills, and their associated proteins, such as microfibril-associated glycoproteins (MAGPs), fibulins, and EMILIN-1. Microfibril-associated glycoprotein-1 (MAGP-1) and fibulin 2 have been shown to contain tyrosine-sulfated residues (Trask et al. 2001; Kanan et al. 2014a), while fibrillin 1 and 2 have been predicted to contain numerous tyrosine sulfate residues, according to the Sulfinator (Monigatti et al. 2002) (http://www.pdg.cnb.uam.es/cursos/BioInfo2004/pages/visualizacion/programas_manuales/spdbv_userguide/us.expasy.org/tools/sulfinator/index.html). The Sulfinator is a software tool that is used for the prediction of sulfated tyrosines based upon location within a sequence in the middle of a 25-amino acid window, as well as in domains when several tyrosines are present (Monigatti et al. 2002). Although it is proposed that it's predictions are with an overall accuracy of 98% (Monigatti et al. 2002), we have found that many of the predicted tyrosines are not sulfated and vice versa.

While MAGP-1 has been shown to bind tropoelastin and fibrillin (Clarke and Weiss, 2004; Werneck et al. 2004), fibulin 2 has been shown to bind several ECM proteins such as tropoelastin, fibronectin, nidogen 2, laminin 1, aggrecan, versican, and perlecan (Gu et al. 2000; Olin et al. 2001; Sasaki et al. 1999). While it has not been shown that tyrosine sulfation is responsible for these interactions, given the function of tyrosine sulfation in mediating protein-protein interaction, it is not impossible to conceive that tyrosine sulfation may play a role in overall elastic fiber integrity.

Tyrosine sulfation of collagens—Collagens are the most abundant proteins in the ECM. Procollagen V and Procollagen III have been shown to be tyrosine-sulfated (Fessler et al. 1986; Jukkola et al. 1986). Sulfinator analysis predicts that three human minor fibrillar collagens, Col5a1, Col11a1, and Col11a2, are tyrosine-sulfated (Fang et al. 2012). Since collagens bind to other proteins in the ECM, such as fibronectin (Chandrasekhar et al. 1983; Dzamba et al. 1993), integrins such as α1β1 and α2β1 (Nykvist et al. 2000), morphogenetic protein-2 (BMP-2; Morin et al. 2006), and hepatocyte growth factor (HGF; Schuppan et al. 1998), tyrosine sulfation on collagens may be involved in optimum binding to these ECM proteins.

Other tyrosine-sulfated proteins in the ECM—Class II leucine-rich repeat (LRR) proteins contain numerous sulfated tyrosines in the N-terminal region. These ECM proteins include fibromodulin (Antonsson et al. 1991), lumican (Onnerfjord et al. 2004), and osteoadherin (Onnerfjord et al. 2004). The function of tyrosine sulfation in these proteins is unknown. However, in other proteins, such as PSGL-1 and chemokine receptors CCR5, CCR2, CCR8, CXCR3, CXCR4, DARC, and CX3CR1, the presence of tyrosine sulfation at the N-terminus suggests a role in protein-protein interaction (Kehoe and Bertozzi, 2000). Therefore, its role in class II LRR proteins may be to interact with other proteins.

Function of tyrosine-sulfated proteins in ocular tissues

The analysis of bovine ocular tissues identified the presence of tyrosine-sulfated proteins in the cornea, aqueous humor, lens, iris, vitreous humor, neurosensory retina, retinal pigment epithelium (RPE), and sclera (Kanan et al. 2012). Using the anti-sulfotyrosine antibody PSG2, the vitreous humor was revealed as the richest source of tyrosine-sulfated proteins, while the lens had the fewest tyrosine-sulfated proteins (Kanan et al. 2012). Analysis of mouse neurosensory retinas in models such as *Tpst1*$^{-/-}$, *Tpst2*$^{-/-}$, and *Tpst/DKO*, showed that many tyrosine-sulfated proteins are still retained in each of the single knockout animals. Tyrosine-sulfated proteins are only completely eliminated in the double knockout animals, suggesting some compensation between the two TPST enzymes (Sherry et al. 2012; Sherry et al. 2010). The *Tpst1*$^{-/-}$ mice lived a normal lifespan with a mild developmental visual phenotype, wherein the scotopic “a” and “b” waves were slightly below wild type levels at early ages, but reached normal levels at P90 (Sherry et al. 2012). However, the *Tpst2*$^{-/-}$ mice exhibited similar developmental visual deficits that did not correct with age (Sherry et al. 2012). The rod outer segment structure and synaptic terminals in both *Tpst1*$^{-/-}$ and *Tpst2*$^{-/-}$ single knockout animals were normal (Sherry et al. 2012). Immunohistologic examinations of retinal sections from both single knockouts probed with PSG2 suggested that TPST-1 and -2 differentially sulfate most of their substrates but other substrates may be sulfated by both enzymes (Sherry et al. 2012).

The mild phenotypes observed in the single knockout mice is in contrast to the drastic visual functional and ultrastructural abnormalities observed in *Tpst/DKO* animals (Sherry et al. 2010). Postnatal (P) 21 *Tpst/DKO* mice exhibited 25% of wild-type electroretinographic (ERG) scotopic responses, and only 15% of wild-type photopic responses. Additionally, the rod outer segments were ultrastructurally abnormal, with large inter-discal and intradiscal spacing and projection of the disk membrane into the extracellular space (Sherry et al.

2010). Moreover, the synaptic terminals of the photoreceptors appeared to be structurally disorganized, though they still formed the triad structure seen in rod terminals and flat contacts seen in cone terminals (Sherry et al. 2010). Unfortunately, the *Tpst/DKO* mice do not survive long past weaning (Westmuckett et al. 2008), making it impossible to study the role of tyrosine-sulfated proteins in retinal maintenance in this model.

To study the role of tyrosine-sulfated proteins in retinal function and structure past weaning, it is essential to develop a conditional knockout model wherein TPST1 and 2 are both eliminated in the context of rods, cones, RPE, and other retinal cells using cell-type specific Cre-expressing mice. This will permit the study of the role of tyrosine-sulfated proteins in the function of each retinal cell type, the determination of the expressing cell type (following the identification of all tyrosine-sulfated retinal proteins), and the long term effects of the elimination of tyrosine sulfation on retinal structure and function.

Tyrosine-sulfated proteins and retinal diseases

The first step in determining the role of tyrosine-sulfated proteins in retinal diseases is the identification of all of those proteins. As initial steps towards that goal, extracts of bovine RPE and neurosensory retina were subjected to immunoaffinity column purification using the PSG2 antibody (Kanan et al. 2014a, 2014b). Three proteins, fibulin 2, vitronectin, and opticin, were identified (Kanan et al. 2014a, 2014b). In addition, data mining and tyrosine sulfation prediction analysis of the RPE secretome identified complement factor H (CFH) as another tyrosinesulfated protein (Kanan et al. 2014b). Further *in vivo* and *in vitro* analysis confirmed that all four proteins are tyrosine-sulfated (Kanan et al. 2014a, 2014b).

While the *in vivo* function of fibulin 2 has yet to be identified, this protein belongs to the fibulin family (De et al. 2009), several members of which have been implicated in ocular disease. Fibulin 3 is implicated in Malattia Leventinese or dominant Doyne honeycomb retinal degeneration (Marmorstein et al. 2002; Stone et al. 1999). Fibulin 5 and 6 have been associated with macular dystrophy (Schultz et al. 2003; Stone et al. 2004). Fibulin 1 has been implicated in recessive vitreoretinal dystrophy (Weigell-weber et al. 2003). Genome-wide homozygosity mapping with microsatellite markers mapped the candidate gene to 22q13, where fibulin 1 is located (Weigell-weber et al. 2003). Therefore, fibulin 2 may also be involved in ocular disease.

Analysis of the role of tyrosine sulfation in the function of fibulin 2 showed that fibulin 2 is upregulated in the RPE following retinal detachment (Kanan et al. 2014a), suggesting a role for fibulin 2 in the re-attachment process. Elimination of fibulin 2 sulfation did not influence fibulin 2 secretion, but reduced its ability to regulate cellular growth and migration (Kanan et al. 2014a). This is not surprising, knowing that tyrosine sulfation is important for protein-protein interaction.

Vitronectin and CFH are regulatory proteins of the complement system. While CFH inactivates the complement system by acting as a cofactor in Factor I-induced decay of C3-covertase (Pechtl et al. 2011), vitronectin inhibits the complement system by binding the final product of the activated complement system, membrane attack complex (MAC) C5b-9 (Podack et al. 1984). Mutations in CFH are implicated in some cases of age-related macular

degeneration (AMD) resulting from uncontrolled complement activation (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005). One of the clinical signs of AMD is the presence of drusen in the Bruch's membrane (Pauleikhoff et al. 1992). Vitronectin and C5b-9 are primary components of drusen (Hageman et al. 1999). It has been shown that complement activation of RPE cells results in the upregulation of both C5b-9 and vitronectin (Lueck et al. 2011; Wasmuth et al. 2009). Therefore, vitronectin may serve a protective purpose in drusen, namely, to inactivate the complement cascade by inhibiting the MAC complex. Future experiments should focus on identifying the role of sulfation in the functions of CFH and vitronectin, and whether modulation of tyrosine sulfation influences the uncontrolled activation of the complement system.

Opticin is another tyrosine-sulfated protein (Kanan et al. 2014b). This protein has recently been shown to prevent neovascularization in the oxygen-induced retinopathy model (OIR model) in mouse eyes (Le Goff et al. 2012a). Further studies have shown that the mechanism involves binding of opticin to collagen, thereby preventing the binding of endothelial cells' integrins to collagens (Le Goff et al. 2012b). Future studies should determine the role of sulfation in opticin's ability to regulate neovascularization.

Current and future research focus

Since it impossible to study the role of tyrosine sulfation in adult *Tpst/DKO* mice, due to their limited survival, the best way to study the role of tyrosine sulfation is using a conditional knockout mouse model (Utomo et al. 1999). Conditional *Cre* expression has been demonstrated in rods (e.g. Le et al. 2006b), cones (e.g. Le et al. 2006a), Müller cells (e.g. Ueki et al. 2009), and RPE cells (e.g. Le at al. 2008; Fu et al. 2014). If the two TPSTs are knocked down in specific cells, these animal models can be used to study the role of tyrosine sulfation in these cells. Furthermore, since these mice should not have limited viability, age-related studies could also be performed.

Knockin experiments can also produce animal models in which tyrosine sulfation is eliminated in each known retinal tyrosine-sulfated protein. Although costly, these are informative models. The models can be generated by substituting the sulfated tyrosine with a phenylalanine that lacks the hydroxyl group, hence eliminating sulfation.

CONCLUSION

The study of tyrosine sulfation is in its infancy, and requires considerable effort to identify the involved proteins, their role in human diseases, and, ultimately, the role of tyrosine sulfation in the overall function of proteins and in diseases. Four tyrosine-sulfated retinal proteins have been identified: fibulin 2, CFH, vitronectin, and opticin. The role of sulfation in modulating fibulin function has been elucidated. Future studies should focus on the role of sulfation in CFH and vitronectin function, and should determine the role of tyrosine sulfation in complement activation and AMD.

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Highlights

- Tyrosine sulfation, a post-translational modification, occurs on ECM proteins
- All ocular tissues express tyrosine sulfated proteins but identities are unknown
- Although a common post-translational modification, tyrosine sulfation is understudied

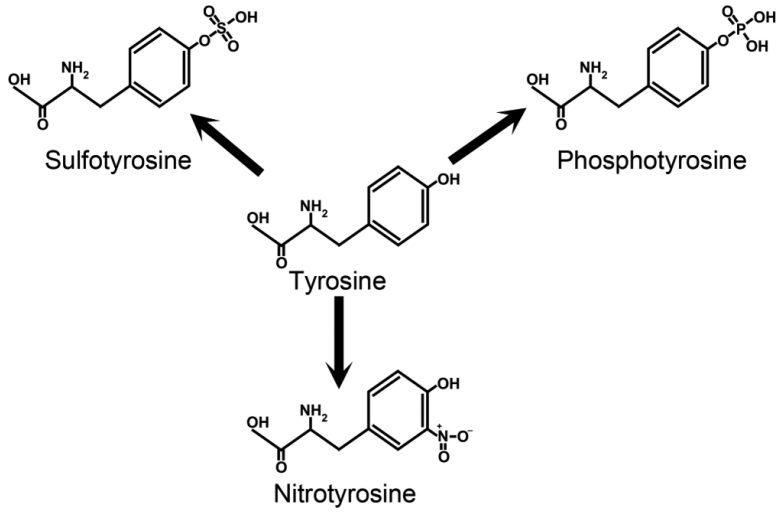

Figure 1. Known biologically significant tyrosine-specific post-translational modification to proteins

Tyrosine undergoes enzymatic sulfation and phosphorylation and non-enzymatic nitration that is mediated by reactive nitrogen species.

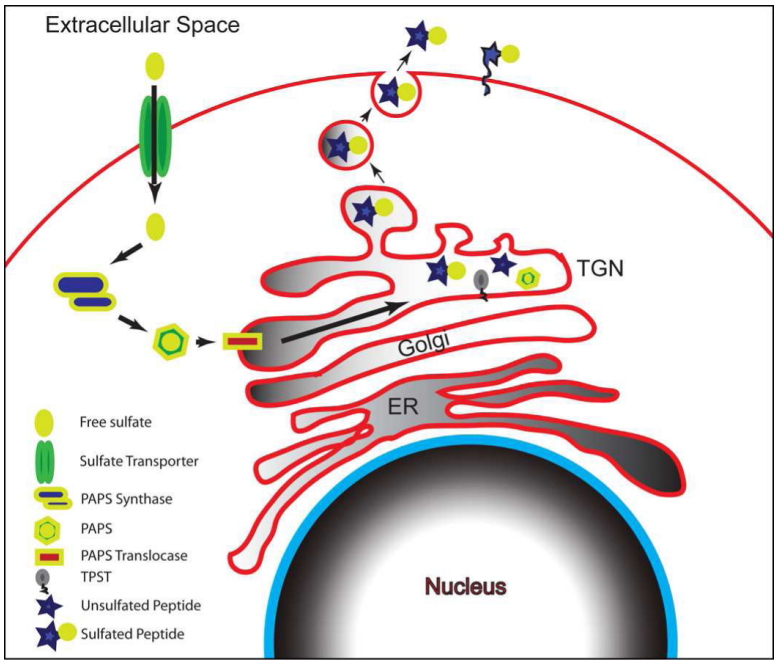

Figure 2. Pathway of tyrosine sulfation

Free sulfate enter the cell via sulfate transporters and are rapidly converted to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) by 3'-phosphoadenosine 5'-phosphosulfate synthase. 3'-phosphoadenosine 5'-phosphosulfate is then translocated into the trans-Golgi through 3'-phosphoadenosine 5'-phosphosulfate translocase. This allows the tyrosylprotein sulfotransferase to then add the free sulfate from the high energy source, 3'-phosphoadenosine 5'-phosphosulfate, to a newly synthesized protein. The tyrosine sulfated protein is secreted to become a free member of the extracellular matrix or re-associate with the producing cell directly or through interactions with membrane proteins.