Chapter 86
Identification of Tyrosine *O* Sulfated Proteins in Cow Retina and the 661W Cell Line

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Abstract Lack of tyrosine *O* Sulfation compromises both rod and cone electroretinographic responses emphasizing the importance of this post-translational modification for vision. To identify tyrosine sulfated proteins in retina, cow retinal lysates were subjected to immunoaffinity purification using an anti-sulfotyrosine antibody. The tyrosine sulfated proteins were eluted from the column using a sulfotyrosine pentapeptide and identified using mass spectrometry. Similarly, tyrosine sulfated proteins secreted by the 661W cell line were identified. Proteins identified were vitronectin, fibronectin, fibulin 2, nidogen, collagen V alpha 2, complement component 3 and C4 and fibrinogen beta. All proteins were subjected to analysis by 'Sulfinator' to determine potential sulfated tyrosines.

Keywords Tyrosine sulfation · 661W · Retina · PSG2 · Posttranslational modification

86.1 Introduction

Tyrosine sulfation is a post-translational modification of proteins that is utilized in all ocular tissues (Kanan et al. 2009, 2012) and plays a very important role in vision (Sherry et al. 2010, 2012). Eliminating tyrosine sulfation reduces scotopic electroretinographic responses to 25% of normal and photopic responses to 15% of normal (Sherry et al. 2010). Besides these functional deficits, ultrastructural examination reveals rod outer segments abnormalities (Sherry et al. 2010). To

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identify the tyrosine sulfated proteins that may be responsible for these effects, we immunoaffinity purified tyrosine sulfated proteins from neural retina using the anti-sulfotyrosine antibody PSG2 (Hoffhines et al. 2006). Column elution using a sulfated pentapeptide was followed by mass spectrometry. Since all sulfated proteins are secreted, some of the tyrosine sulfated proteins produced by the cell line 661W (Tan et al. 2004) were identified by fractionating conditioned media followed by western blotting with PSG2. Proteins that immuno-reacted were identified by in-gel digestion of excised bands followed by mass spectrometry.

86.2 Materials and Methods

86.2.1 Preparation of Bovine Retinal Lysates

Bovine eyes were obtained from Country Home Meat Slaughter House (Edmond, OK). Neural retinas were isolated and lysates were prepared in buffer A (25 mM MOPS, 100 mM NaCl, pH 7.5). Bradford assay was performed and lysate concentrations were adjusted to 4 mg/ml in buffer A prior to loading onto the column.

86.2.2 PSG2 Affinity Purification of Tyrosine O Sulfated Proteins

Ten mg of extracts were filtered using a 0.45 μm syringe filter (Millipore, Billerica, MA) and loaded onto the PSG2-Affi-Gel-10 HPLC column (Hoffhines et al. 2009). Column was washed with buffer A, wash buffer 1 (25 mM MOPS, 200 mM NaCl), wash buffer 2 (25 mM MOPS, 400 mM NaCl) and eluted with elution buffer (25 mM MOPS, 400 mM NaCl, 4 mM sulfated pentapeptide). Eluted samples were concentrated with acetone precipitation. The tyrosine-sulfated pentapeptide LDYSDF was synthesized by Bio-Synthesis Inc. (Lewisville, TX).

86.2.3 Mass Spectrometry

Column fractions were separated by SDS-PAGE to remove the sulfated pentapeptide and gel lane was cut into 1 mm slices and subjected to in-gel trypsin digestion, reduction and alkylation followed by LC MS/MS analysis (ABI MDS Sciex Qstar Elite, (Life Technologies, Grand Island, NY). MS/MS data were collected using ABI Analyst QS 2.0 software and submitted to MASCOT (Matrix Science) server for protein identification against the NCBInr protein database.

86.3 Results

86.3.1 Tyrosine O Sulfated Proteins Identified by Immunoaffinity Purification

About 10% of eluted proteins were run on a gel and immunoblotted with PSG2 (Hoffhines et al. 2006). Identified proteins ranged from 250 kD to 37 kDa in size (Fig. 86.1a). The remaining eluate was acetone precipitated, fractionated by SDS-PAGE and subjected to mass spectrometry. Since tyrosine sulfated proteins transit the secretory pathway (Moore et al. 2003), only membrane or secreted proteins were included in Table 86.1. Identified targets belonged to multiple protein families such as serpins, extracellular matrix proteins and complement proteins. Since the major function of tyrosine sulfation is protein-protein interaction (Zhu et al. 2011; Costagliola et al. 2002; Ramachandran et al. 1999), immunoaffinity may have pulled down non-tyrosine sulfated proteins that co-purified due to their direct or indirect interaction with tyrosine-sulfated proteins. Therefore, each of the identified proteins was subjected to prediction of sulfated tyrosines using the software 'Sulfinator' (Monigatti et al. 2002). Seven of the proteins were predicted to be tyrosine sulfated (Table 86.2). Fibronectin and fibrinogen have previously been shown to be tyrosine sulfated (Liu and Suiko 1987; Hortin et al. 1986) and we have recently shown fibulin 2 and vitronectin to be tyrosine sulfated (Kanan et al. 2014a, 2014b). Complement component 3 and fibrinogen beta have not been previously shown to be tyrosine sulfated. Sulfinator did not predict tyrosine sulfated sites on retinol binding protein 3 (IRBP), pigment epithelium-derived factor (PEDF) precursor, collagen (type I, alpha 1), neuronal membrane glycoprotein M6-b, isoform 2 and fibrinogen alpha. The presence of these proteins suggests that they may be interacting partners to some of the identified tyrosine sulfated proteins.

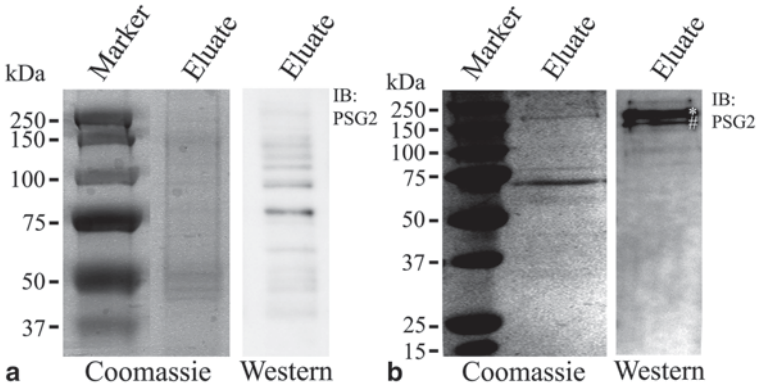

Fig. 86.1 Tyrosine sulfated proteins in cow retinal extracts and 661W cells. **a** Immunoaffinity column purification of tyrosine *O* sulfated proteins from cow retinal lysates. SDS-PAGE and coomassie blue staining of cow retinal lysates eluted from the column and western blotted with PSG2. **b** Tyrosine *O* sulfated proteins from cone derived cell line 661W. SDS-PAGE and coomassie blue staining of 661W conditioned media and western blotting with PSG2

Table 86.1 List of proteins identified in cow retinal lysates after immunoaffinity purification and mass spectrometric analysis. Eleven proteins were identified in cow retinal lysates by MALDI-MS/MS analysis of PSG2 immunoaffinity column eluent

	Protein	Mascot score	%Coverage
1	Retinol binding protein 3 (IRBP)	1500	35
2	Fibulin 2	1281	24
3	Pigment epithelium-derived factor precursor	1002	39
4	Complement component 3	925	22
5	Vitronectin	766	35
6	Fibrinogen beta	692	17
7	Collagen, type I, alpha 1	563	35
8	Fibronectin	472	7
9	Complement C4	407	14
10	Neuronal membrane glycoprotein M6-b, isoform 2	318	16
11	Fibrinogen alpha	92	4

Table 86.2 List of potential sulfated tyrosines residues in cow retina as identified by Sulfinator. Citations are provided for the tyrosine sulfation sites that were experimentally identified

Protein	Site	Sequence	Reference
Vitronectin	75	LPEDE**Y**GFHDY	Novel
Vitronectin	80	YGFHD**Y**SDAQT	Novel
Fibulin 2	197	DPERH**Y**EDPYS	Kanan et al. (2014a)
Fibulin 2	201	HYEDP**Y**SYDQE	Kanan et al. (2014a)
Fibulin 2	203	EDPYS**Y**DQEVA	
Complement component 3	1559	DDFDE**Y**IMVIE	Novel
Vitronectin	75	LPEDE**Y**GFHDY	Kanan et al. (2014b)
Vitronectin	80	YGFHD**Y**SDAQT	Kanan et al. (2014b)
Vitronectin	275	FKGNH**Y**WEYVF	Kanan et al. (2014b)
Vitronectin	278	NHYWE**Y**VFQQQ	Kanan et al. (2014b)
Fibrinogen beta	33	QFPTD**Y**DEGQD	Novel
Fibrinogen beta	259	ETSEM**Y**LIQPE	Novel
Fibrinogen beta	273	KPYRV**Y**CDMK	Novel
Fibronectin	877	QPGVQ**Y**NITIY	Liu and Suiko (1987)
Fibronectin	882	YNITI**Y**AVEEN	Liu and Suiko (1987)
Complement C4	944	REEMV**Y**ELNPL	Hortin et al. (1986)
Complement C4	1414	EAEED**Y**EDYEY	Hortin et al. (1986)
Complement C4	1417	EDYED**Y**EYEDL	Hortin et al. (1986)
Complement C4	1419	YEDYE**Y**EDLLA	Hortin et al. (1986)

86.3.2 Tyrosine Sulfated Proteins in 661W-Conditioned Media

661W-conditioned media was SDS-PAGE fractionated and immunoblotted with PSG2 revealing two major proteins between 150–250 kDa (Fig. 86.1b). These proteins were excised and subjected to MALDI MS/MS analysis. The top band (marked by an asterisk, Table 86.3) was a mixture of three proteins and the bottom

Table 86.3 List of proteins identified in 661W-conditioned media. Four proteins were identified from conditioned media of 661W cells following SDS-PAGE and MALDI-MS/MS analysis

	Protein	Mascot score	% Coverage
1.*	Fibronectin	1019	11
2.*	Fibulin 2	643	14
3.*	Nidogen-2	408	7
4.#	Collagen, type V, alpha 2	524	15

Table 86.4 List of sulfated tyrosines residues in cone derived cell line, 661W. All four proteins have been experimentally proven to contain sulfated tyrosines and citations provided

Protein	Site	Sequence	Reference
*Fibronectin	875	QPGVQ**Y**NITIY	Liu and Suiko (1987)
*Fibronectin	880	YNITIYAVEEN	Liu and Suiko (1987)
*Fibulin 2	192	DSERQ**Y**EDPYS	Kanan et al. (2014a)
*Fibulin 2	196	YEDP**Y**SYDQEV	Kanan et al. (2014a)
*Fibulin 2	198	EDPYS**Y**DQEVA	Kanan Y et al. (2014a)
#Nidogen 2	94	PRETQ**Y**VDDDF	Paulsson et al. (1985)
#Nidogen 2	317	EDSFH**Y**YDENE	Paulsson et al. (1985)
#Nidogen 2	318	DSFHY**Y**DENEE	Paulsson et al. (1985)
#Nidogen 2	327	EEDVE**Y**PPVEP	Paulsson et al. (1985)
#Collagen, type V, alpha 2	34	QENDE**Y**DEEIA	Fessler et al. (1986)
#Collagen, type V, alpha 2	1238	DIMGH**Y**DENMP	Fessler et al. (1986)

band (denoted by #, Table 86.3) was identified as collagen, type V, alpha 2. Sulfinator predicted all the four proteins to be tyrosine sulfated (Table 86.4), which has been experimentally confirmed (Liu and Suiko 1987; Kanan et al. 2014a; Paulsson et al. 1985; Fessler et al. 1986).

86.4 Discussion

We have previously shown the importance of tyrosine sulfation for vision (Sherry et al. 2010; Sherry et al. 2012) and here we identify some of the tyrosine sulfated proteins in ocular tissues. We subjected cow retinal extracts to immunoaffinity purification with PSG2. Eleven proteins were identified and seven of those were predicted to be tyrosine sulfated. The rest of the proteins may have been co-purified with their tyrosine sulfated interacting partners since tyrosine sulfation enhances protein-protein interactions (Zhu et al. 2011; Costagliola et al. 2002; Ramachandran et al. 1999). Of the identified proteins, fibulin 2 and vitronectin were also detected in cow RPE to be tyrosine *O* sulfated (Kanan et al. 2014a, b).

Since the retina is composed of six different classes of cells, identified proteins may belong to any and/or all cell types. Therefore, we used the cone derived cell line 661W to identify tyrosine sulfated proteins that may be cone specific. We identified fibronectin, fibulin 2, nidogen 2 and collagen V proteins from this cell line. These proteins were experimentally shown to be tyrosine sulfated (Liu and Suiko 1987; Kanan et al. 2014a; Paulsson et al. 1985; Fessler et al. 1986).

This is the first report of the identification of tyrosine sulfated protein in the retina and in 661W cells. Further experiments will identify the cell type that produces these proteins in the retina. The function of tyrosine sulfation in these proteins and how it affects vision will only be revealed using '*In-Vivo* knock-in' mutants that will have the tyrosine sulfated residues mutated to phenylalanines.

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