Detection and Purification of Tyrosine-sulfated Proteins Using a Novel Anti-sulfotyrosine Monoclonal Antibody*

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Protein tyrosine O-sulfation is a post-translational modification mediated by one of two Golgi tyrosylprotein sulfotransferases (TPST1 and TPST2) that catalyze the transfer of sulfate to tyrosine residues in secreted and transmembrane proteins. Tyrosine sulfation plays a role in protein–protein interactions in several well defined systems. Although dozens of tyrosine-sulfated proteins are known, many more are likely to exist and await description. Advancing our understanding of the importance of tyrosine sulfation in biological systems requires the development of new tools for the detection and study of tyrosine-sulfated proteins. We have developed a novel anti-sulfotyrosine monoclonal antibody (called PSG2) that binds with high affinity and exquisite specificity to sulfotyrosine residues in peptides and proteins independently of sequence context. We show that it can detect tyrosine-sulfated proteins in complex biological samples and can be used as a probe to assess the role of tyrosine sulfation in protein function. We also demonstrate the utility of PSG2 in the purification of tyrosine-sulfated proteins from crude tissue samples. Finally, Western blot analysis using PSG2 showed that certain sperm/epididymal proteins are undersulfated in Tpst2−/− mice. This indicates that TPST1 and TPST2 have distinct macromolecular substrate specificities and provides clues as to the molecular mechanism of the infertility of Tpst2−/− males. PSG2 should be widely applicable for identification of tyrosine-sulfated proteins in other systems and organisms.

II transmembrane proteins that reside in the trans-Golgi network and have luminaly oriented catalytic domains (4–6, 8). Thus, tyrosine sulfation occurs only on soluble and transmembrane proteins that transit the Golgi en route to either secretion or incorporation into the plasma membrane. Accordingly, all of the native tyrosine-sulfated proteins described to date fall into one of these two categories (2).

Consensus features for tyrosine sulfation have been proposed based on the amino acid sequences flanking known sulfation sites coupled with in vitro studies on the sulfation of various synthetic peptides (PROSITE accession number PS00003). In addition, a software tool for prediction of tyrosine sulfation sites in proteins called Sulfinator has been developed (9). However, the positive predictive value of these features and the Sulfinator tool is not known. Some known tyrosine sulfation sites do not fulfill proposed consensus features, and some are not predicted by Sulfinator. Thus, unlike some other post-translational modifications, there is no way to reliably predict sites of sulfation.

Tyrosine sulfation plays a role in protein–protein interactions in several well defined systems. For example, tyrosine sulfation of P-selectin glycoprotein ligand-1 (PSGL-1; CD162) expressed on leukocytes is required for cell–cell interactions mediated by P- and L-selectins in the vasculature (10–12). In the co-crystal of the lectin/epidermal growth factor domain of P-selectin and a recombinant glycosulfopeptide mimetic of the N-terminal domain of PSGL-1, the sulfate groups at Tyr48 and Tyr51 are involved in direct protein–protein contacts with P-selectin (13).

A great deal of recent interest has focused on the role of tyrosine sulfation in G-protein-coupled receptor function after Farzan et al. (14) showed that CCR5, a major human immunodeficiency virus coreceptor, is tyrosine-sulfated. Sulfation of 1 or more tyrosine residues in the N-terminal extracellular domain of CCR5 is required for optimal binding of CCL3, CCL4, and CCL5 and for optimal human immunodeficiency virus coreceptor function. Similar studies indicated that tyrosine sulfation of the N-terminal domains of other chemokine

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and Table 1.

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**The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; HCl, heparin cofactor II; mAb, monoclonal antibody; pY, phosphotyrosine; sY, sulfotyrosine; scFv, single chain Fv; MOPS, 3-(N-morpholino)propanesulfonic acid; MBS, MOPS-buffered saline; TBS, Tris-buffered saline; NRK, normal rat kidney; LC-MS/MS, liquid chromatography-tandem mass spectrometry; FTICR, Fourier transform ion cyclotron resonance; PVDF, polyvinylidene difluoride.

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receptors (CXCR4, CCR2B, CX3CR1, CCR8, CXCR3) as well as other G-protein-coupled receptors (C5a; C3a; SIP1; and the follicle-stimulating, luteinizing, and thyroid-stimulating hormone receptors), is required for optimal binding of their cognate ligands (2, 15–17).

Likewise, tyrosine sulfation is required for optimal proteolytic processing of proaggrin (18), proteolytic activation of coagulation factors V and VIII by thrombin (19–21), proteolysis of the complement C4 α-chain by C1s (22), binding of glycoprotein Ib to thrombin (23), binding of glycoprotein Ibα (24, 25) and factor VIII (26) to von Willebrand factor (26), binding of cholecystokinin to the cholecystokinin A receptor (27), and optimal binding of hirudin to thrombin (28). However, for many of the known tyrosine-sulfated proteins, there is no information on the role of the sulfotyrosine residue(s) in protein function. Many more tyrosine-sulfated proteins are likely to exist and await description. However, the pace of discovery has been very slow.

One of the major barriers to developing a full understanding of the importance of tyrosine sulfation in biological systems has been a lack of a facile means to identify additional tyrosine-sulfated proteins and probes to explore the role of tyrosine sulfation in protein function. An antibody reagent able to detect sulfotyrosine residues would be highly desirable. We attempted to generate anti-sulfotyrosine monoclonal antibodies using the strategy employed by Glenney et al. (29) to generate anti-phosphotyrosine monoclonal antibodies. However, we failed to generate detectable antibody responses to sulfotyrosine in mice.

Here, we describe the identification and characterization of a novel anti-sulfotyrosine antibody generated using phage display technology. We show that this antibody binds with high affinity and exquisite specificity to sulfotyrosine residues in peptides and proteins independently of the sequence context. Furthermore, we show that it can detect tyrosine-sulfated proteins in complex biological samples, and we demonstrate its utility in affinity purification of tyrosine-sulfated proteins from crude tissue samples.

Experimental Procedures

Materials—Human neutrophil PSGL-1 and human platelet P-selectin were purified as described previously (30, 31). Purified bovine factors X₁ and X₂ were provided by Charles Esmon (Oklahoma Medical Research Foundation), and human heparin cofactor II (HCII) expressed in Escherichia coli (32) was a gift from Douglas Tollefsen (Washington University, St. Louis, MO). Purified human plasma C₄ was purchased from Advanced Research Technology (San Diego, CA), and human plasma HCII and mouse fibrinogen were from Hematologic Technologies, Inc. (Essex Junction, VT). Phosphotyrosine (pY) and sulfotyrosine (sY) were purchased from Sigma and Bachem, respectively. The pentapeptides LDYDF, LD(sY)DF, and LD(pY)DF were synthesized and high pressure liquid chromatography-purified (>95% purity) by Biosynthesis Inc. (Lewisville, TX). Anti-phosphotyrosine monoclonal antibody (mAb) PY20 was purchased from MP Biomedicals.

Identification of Antibody Binding to Sulfotyrosine—A single chain Fv (scFv) phagemid library, an expanded version of the 1.38 × 10¹⁰ library (33), was used to select antibodies that bind to a protein containing sulfated tyrosines. The immobilized target protein was the purified dimeric 19ek.Fc recombinant protein (13), which contains three sulfated tyrosines within the N-terminal 19 amino acids of human PSGL-1 (QATE(sY)E-(sY)L/D(sY)DFLPETEP) and is fused to human IgG₁, Fc via an enterokinase cleavage site. Several scFv clones were isolated following multiple rounds of a panning procedure (33). The purified PSG2 scFv clone was identified as an scFv fragment whose binding to 19.ek.Fc was inhibited in the presence of increasing concentrations of the murine PSGL-1/Fc fusion protein, which contains sulfated tyrosines within a different sequence context (34). The PSG2 scFv fragment was converted to a full-length intact IgG₄ antibody (designated PSG2) and expressed in mammalian hamster ovary cells as described (35).
membranes were washed four times for 2 min with TBS and probed with horseradish peroxidase-conjugated anti-Fc antibody in TBS and 1% casein. After washing with TBS, bound protein was visualized using SuperSignal West reagent (Pierce) and a digital camera.

**SDS-PAGE and Western Blotting—**All samples were electrophoresed on 4–15% Tris-HCl/SDS-polyacrylamide gels (Bio-Rad). For Coomassie Blue staining, 1 μg of purified proteins was loaded, whereas for Western blotting, 10–20 ng of proteins was loaded. For Western blotting, the PSG2 or control IgG4 mAb was used as primary antibody at 30 ng/ml, followed by 200 ng/ml horseradish peroxidase-conjugated anti-human IgG as secondary antibody. Bound secondary antibody was detected using ECL Plus (Amersham Biosciences), followed by either autoradiography or imaging on a Storm 860 scanner (GE Healthcare).

**Enzyme-linked Immunosorbent Assays—**Microtiter plates (Immulon 1B, Dynex) were coated with bovine factor X1 or X2 (5 μg/ml, 100 μl/well) in sodium carbonate, pH 9.6, overnight at 4 °C. Plates were washed three times with TBS containing 0.05% Tween 20, blocked with TBS and 1% bovine serum albumin for 1 h, and washed twice with TBS containing 0.05% Tween 20. Tyrosine sulfate, tyrosine phosphate, or various peptidoglycans were added (50 μl/well) to triplicate wells. PSG2 (60 ng/ml, 50 μl/well) in MBS was then added to all wells. After 1 h, plates were washed five times with TBS containing 0.05% Tween 20, and a 1:5000 dilution of horseradish peroxidase-conjugated anti-human IgG in the same buffer was added (100 μl/well) and incubated for 1 h. Plates were washed five times with TBS containing 0.05% Tween 20, and then 2.2’-azidodiethylaminoethyl (3-ethylbenzthiazolone 6-sulfonate) peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.) was added (100 μl/well). Plates were read at 405 nm using a VersaMax microplate reader (Molecular Devices Corp.).

**Pervanadate Treatment of Normal Rat Kidney (NRK) Cells—**NRK cells (ATCC CRL-6509) were grown to confluence in Dulbecco’s modified Eagle’s medium with 2 mM l-glutamine and 10% fetal bovine serum at 37 °C and 5% CO2. A fresh stock solution of pervanadate ions (30 mM) was prepared by combining 100 μl of 100 mM Na2VO4, 10 μl of 30% H2O2, and 223 μl of phosphate-buffered saline, followed by incubation in the dark for 10 min at room temperature. NRK cells were then incubated for 30 min at 37 °C and 5% CO2 in complete medium alone or containing 100 μM pervanadate. After 30 min, the medium was removed, and the cells were detached and collected by centrifugation (90 × g, 5 min). Cells were washed once with phosphate-buffered saline and collected by centrifugation, and cell pellets were lysed with 1% Triton X-100 in 100 mM NaCl, 20 mM HEPES, pH 7.2, 10% glycerol, 1 mM phenylmethylsulfonil fluoride, 10 μg/ml aprotinin, 160 μg/ml benzamidine, 10 μg/ml antipain, 10 μg/ml leupeptin, and 1 mM Na2VO4. Lysates were incubated in the dark for 20 min at 4 °C and then clarified by centrifugation (14,000 × g, 10 min, 4 °C). Protein content was determined using the BCA protein assay (Pierce), and lysates were snap-frozen in liquid N2 and stored at −80 °C until used.

**Glycan Array Screening—**The PSG2 mAb was used to screen a glycan microarray (Glycan Array version 3.5) at the Protein-Glycan Interaction Core of the Consortium for Functional Glycomics (Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center) as described previously (39). In total, 206 unique glycans are represented on the array. Briefly, biotinylated glycoconjugates (40) were bound to streptavidin-coated microtiter plates in replicates of four. Purified bovine factors X1 and X2 were biotinylated using sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce) and included on the array (30 pmol/well) as negative and positive controls, respectively. Precoated plates were washed three times with 100 μl of wash buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, and 0.05% Tween 20). The PSG2 mAb (10 μg/ml) in wash buffer containing 1% bovine serum albumin was added to each well and incubated for 1 h. The plates were washed, and 25 μl of fluorescein isothiocyanate-conjugated anti-human IgG (2 μg/ml) in wash buffer was added and incubated for 1 h. The wells were washed three times; 25 μl of wash buffer was added to each well; and bound secondary antibody was detected using a VICTOR2™ 1420 multilabel counter (PerkinElmer Life Sciences) at excitation and emission wavelengths of 485 and 535 nm, respectively.

**Generation of Tpst Double Knock-out Mice—**Tpst1-null (Tpst1tm1Klm, MGI:2183366) and Tpst2-null (Tpst2tm1Klm, MGI:3512111) mice were generated and characterized as described previously (41, 42). The Tpst1 and Tpst2 genes reside on chromosome 5, and the physical distance between the two is ≈18.4 megabases. To generate the Tpst double knock-out mice, Tpst1−/− males were mated with Tpst2−/− females to generate obligate in trans compound heterozygotes. Male and female offspring from this cross were mated, and their offspring were screened by PCR for the presence of the wild-type and mutant alleles at both loci as described (41, 42). Male and female Tpst1−/−, Tpst2−/+ offspring were then interbred to generate double homozygotes. Animals were mated, housed, and fed as described previously (41).

**Adhesion of Neutrophils to P-selection under Flow Conditions—**Adhesion assays were performed as described previously (43). Briefly, 2 ml of 1 μg/ml human platelet P-selectin in Hank’s balanced salt solution was coated on 35-μm tissue culture plates overnight at 4 °C and blocked with 1% human serum albumin in Hank’s balanced salt solution for 2 h at room temperature. Human neutrophils were isolated as described previously (44), resuspended at 4 × 106 cells/ml in Hank’s balanced salt solution and 1% human serum albumin, and perfused over the P-selection surface at a wall shear stress of 1 dyne/cm². Neutrophil adhesion was allowed to equilibrate for 4 min, and then non-adherent cells were flushed from the system with Hank’s balanced salt solution and 1% human serum albumin. After an additional 4 min of perfusion, the control human IgG4 (50 μg/ml) or PSG2 (50 μg/ml) mAb was added to the perfusate. Neutrophil adhesion was visualized by phase-contrast video microscopy, and the number of rolling neutrophils was determined every 20 s.

**PSG2 Affinity Chromatography and Peptide Sequencing—**Epididymides were collected from 10 sexually mature wild-type mice and homogenized in cold MBS containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science) using a Dounce homogenizer. A post-nuclear supernatant was
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obtained (800 x 10 min), which was then subjected to centrifugation (100,000 x 10 min) to prepare a soluble protein fraction. The soluble fraction (8.5 ml, ~28 mg of total protein) was applied to a PSG2-Affi-Gel-10 column (4 mg of mAb/ml of resin, 0.9 x 5 cm) at 50 µl/min. The column was washed extensively with MBS and then with 100 mM NH₄HOAc, and the column was eluted with the sulfated peptide LD(sY)DF (4 mM) in 100 mM NH₄HOAc at 30 µl/min. Flow-through and elution fractions were monitored by SDS-PAGE, followed by PSG2 Western blotting or silver staining.

Sperm/epididymal proteins eluted from the PSG2 column were concentrated and electrophoresed on 4–15% Tris-HCl/SDS-polyacrylamide gels under nonreducing conditions, and separated proteins were visualized by colloidal Coomassie Blue staining. Gels were washed with H₂O, and bands were excised, washed again with H₂O and then with 60% acetonitrile in H₂O, and dried in a SpeedVac. The gel pieces were reduced in 10 mM dithiothreitol in 50 mM NH₄HCO₃ for 1 h at 56 °C and alkylated in 55 mM iodoacetamide in 50 mM NH₄HCO₃ for 45 min at 22 °C in the dark. After washing with 50 mM NH₄HCO₃, the gel pieces were dehydrated with 60% acetonitrile in H₂O and dried. Gel pieces were re-swollen with trypsin in 50 mM NH₄HCO₃ (0.1% (v/v) formic acid in H₂O) at a flow rate of 140 nl/min. The column was developed with a linear gradient of 2–50% solvent A last lanes over 30 min at a flow rate of 320 nl/min. The column was washed extensively with MBS and then with 100 mM NH₄OAc, and the gel pieces were visualized by colloidal Coomassie Blue

**RESULTS**

**Sulfotyrosine Is Required for PSG2 Binding to Synthetic Pentapeptides**—To determine whether PSG2 recognition of peptides requires sulfotyrosine, single amino acid substitution analysis was performed on two pentapeptides modeled on the N terminus of human PSGL-1. The first and last lanes are the respective sulfated peptides. In the other lanes, each position was substituted with the amino acid indicated. 8, 67 pentapeptide sequences selected using a random sequence generator were synthesized with either sulfotyrosine (lanes a) or tyrosine (lanes b) in the third position. The sequences of the peptides on the array are shown in supplemental Table 1.

A similar analysis was performed on an array of 67 pentapeptide sequences selected using a random sequence generator and synthesized with either sulfotyrosine or tyrosine in the third position. PSG2 bound to 54 of the 67 sulfotyrosine-containing pentapeptides analyzed, but not to the corresponding peptides containing unmodified tyrosine (Fig. 1B). These data show that sulfotyrosine is required for binding of PSG2 to synthetic peptides and that PSG2 recognition of sulfated peptides is independent of sequence context.

Of the random pentapeptides that bound PSG2, 29 were judged to be strong binders and 25 weak binders (supplemental Table 1). Twenty-three of 67 of the pentapeptides contained 1 or more Glu or Asp residues, and all 23 peptides bound PSG2 strongly. Thus, the presence of Glu or Asp within ±2 residues of the sulfotyrosine is a strong positive predictor of PSG2 recognition. Five of 13 of the peptides that did not bind PSG2...
Anti-sulfotyrosine Antibody

PSG2 Does Not Bind to Tyrosine-sulfated Proteins—We next examined the ability of PSG2 to bind various sulfotyrosine-containing proteins by Western blotting. Bovine coagulation factor X is a two-chain serine protease zymogen that exists in two chromatographically separable isoforms in bovine plasma, factors X\(_1\) and X\(_2\). Factor X\(_1\) contains a sulfotyrosine at position 18 of the activation peptide, whereas factor X\(_2\) does not (45). Western blotting of factors X\(_1\) and X\(_2\) revealed that PSG2 bound to the heavy chain of factor X\(_2\), but not to the heavy chain of factor X\(_1\) (Fig. 2A). A parallel analysis under reducing conditions showed that PSG2 bound to the heavy chain of factor X\(_2\), but not to the light chain of factor X\(_1\) or to the light chains of factors X\(_1\) and X\(_2\) (Fig. 2A). PSG2 did not bind to the heavy or light chain of factor Xa, which lacks the activation peptide (data not shown).

Western blot analysis was also performed on purified human complement C4. C4 is a plasma protein containing three disulfide-linked subunits (\(\alpha\), \(\beta\), and \(\gamma\)). Human C4 has been shown to have 3 sulfotyrosine residues near the C terminus of the \(\alpha\)-subunit (22, 46, 47). As expected, PSG2 bound to the \(\alpha\)-subunit of C4, but not to the \(\beta\)- or \(\gamma\)-subunit (Fig. 2B). A similar analysis was performed on purified human PSGL-1 and HCII, a single chain plasma glycoprotein serine protease inhibitor. We observed that PSG2 recognized human PSGL-1 and plasma-derived HCII, but not recombinant HCII expressed in E. coli, which lacks TPST activity (supplementary Fig. 1). Thus, PSG2 recognizes a variety of tyrosine-sulfated proteins in Western blot assays.

To further document the exquisite specificity of PSG2 for tyrosine-sulfated proteins, detergent extracts of wild-type and \(\text{Tpst}\) double knock-out livers were analyzed by Western blotting with PSG2. We observed that PSG2 bound to multiple protein species from wild-type liver, but none from \(\text{Tpst}\) double knock-out liver (Fig. 3A). Thus, PSG2 does not recognize a variety of proteins that lack sulfotyrosine.

PSG2 Does Not Bind to Tyrosine-phosphorylated Proteins—Given the high degree of structural similarity between sulfotyrosine and phosphorysine, we sought to determine whether PSG2 could discriminate between these 2 amino acids in the context of peptides and proteins in two analytical formats. We first assessed the ability of sulfotyrosine, phosphorysine, or sulfotyrosine- and phosphorysine-containing peptides to inhibit PSG2 binding to bovine factor X\(_2\) in an enzyme-linked immunosorbent assay format (Table 1). In these assays, PSG2 bound avidly to immobilized bovine factor X\(_2\), but not to factor X\(_1\). We observed that sulfotyrosine was 13-fold more efficient as an inhibitor of PSG2 binding to factor X\(_2\) than was phosphorysine. However, the IC\(_{50}\) for inhibition of PSG2 binding was >1000-fold lower for the sulfopeptide LD(sY)DF than for the corresponding phosphopeptide LD(pY)DF. In addition, the sulfopeptide was \(\approx 570\)-fold more potent than sulfotyrosine in inhibiting PSG2 binding to factor X\(_2\).

In a second approach, extracts of NRK cells were prepared and analyzed by Western blotting using either PSG2 or the benchmark anti-phosphotyrosine mAb PY20 (29). We observed that, although treatment with pervanadate caused an accumulation of tyrosine-phosphorylated proteins recognized by PY20, none of these proteins was recognized by PSG2 (Fig. 3B). Thus, PSG2 does not recognize a variety of tyrosine-phosphorylated proteins in Western blot assays.

PSG2 Does Not Bind to Sulfated Glycans—The majority of sulfate that is covalently incorporated into proteins in mammalian cells is linked to glycosaminoglycan and N- or O-glycans (48). To further assess the PSG2 specificity, we screened a glycan microarray to determine whether PSG2 could recognize various sulfated glycans. The array contains 206 distinct glycosides, including 16 sulfated glycosides and their unsulfated counterparts. In addition, bovine factors X\(_2\) and X\(_1\) were included as positive and negative controls, respectively. We contained Lys at the P1’ position relative to the sulfotyrosine. Thus, the presence of Lys at the P1’ position appears to be a negative predictor of PSG2 recognition. It is of note that PSG2 bound weakly to the tripeptide A(sY)A and the dipeptide (sY)A, but failed to detect sulfotyrosine alone and the A(sY) dipeptide.

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observed that PSG2 bound to none of the sulfated glycans on the array (supplemental Fig. 2). These data indicate that PSG2 does not recognize sulfated carbohydrates.

**PSG2 Blocks Adhesion of Neutrophils to P-selection under Flow Conditions** —To determine whether PSG2 would be a useful reagent to assess the role of sulfotyrosine in proteins, we tested its ability to block PSGL-1 function. PSGL-1 is a homodimeric cell-surface mucin that mediates rolling adhesion of neutrophils to P-selectin-coated surfaces in vitro and to activated endothelium in vivo (49, 50). It is well documented that PSGL-1 must be tyrosine-sulfated to function as a P-selectin ligand (10–12). Human neutrophils were perfused over plates coated with human P-selectin at 1 dyne/cm² to initiate neutrophil rolling for 4 min, and then cell-free buffer was allowed to flow for another 4 min to remove any non-adherent cells. Either the human IgG₄ mAb or PSG2 mAb was added to the perfusate, and the number of rolling cells/field was counted every 20 s. The results show that infusion of PSG2 into the flow system resulted in rapid and complete detachment of rolling neutrophils, but that the control IgG₄ mAb had no effect (Fig. 4). This demonstrates that PSG2 is able to abolish binding of PSGL-1 to P-selectin.

**Affinity Purification of Tyrosine-sulfated Proteins from Mouse Epididymis** —We next sought to determine whether PSG2 would be useful for affinity purification of tyrosine-sulfated proteins. A soluble protein fraction from wild-type epididymis was prepared and applied to a PSG2-Affi-Gel-10 column, and the column was washed extensively and eluted with 4 mM LD(sY)DF in 100 mM NH₄OAc. Flow-through and elution fractions were monitored by SDS-PAGE, and the column was eluted with 4 mM LD(sY)DF as described under “Experimental Procedures.” Results represent the means ± S.D. of four independent experiments.

<table>
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<tr>
<th>Hapten</th>
<th>IC₅₀ (mM)</th>
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<tr>
<td>pY</td>
<td>7.5 ± 1.2</td>
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<tr>
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**TABLE 1**

IC₅₀ for inhibition of PSG2 binding to bovine factor X₂ by amino acids and peptides

Binding of PSG2 to bovine factor X₂ immobilized on microtiter plates was determined in the presence of increasing concentrations of the indicated amino acid or pentapeptide as described under “Experimental Procedures.” Results represent the means ± S.D. of four independent experiments.

**FIGURE 4.** PSG2 blocks neutrophil adhesion to P-selectin under flow conditions. P-selectin was coated on plates, and neutrophils were perfused over the surface at 1 dyne/cm². Adhesion was allowed to equilibrate, and then non-adherent cells were flushed from the system. Rolling neutrophils were counted every 20 s. In one experiment (red line), the control human IgG₄ mAb (50 µg/ml) and then PSG2 (50 µg/ml) were sequentially added to the perfusate at the indicated time points. In another experiment (blue line), PSG2 was added to the perfusate first.

**FIGURE 5.** Affinity purification of proteins from mouse epididymis. Proteins from the soluble fraction of wild-type mouse epididymis were applied to a PSG2 affinity column, and the column was eluted with 4 mM LD(sY)DF peptide. Early (lanes 1) and late (lanes 2) fractions from the peptide elution were run on 4–15% SDS-polyacrylamide gels, followed by silver staining (left panel) or transfer to PVDF membrane and probing with PSG2 (right panel). Bands A–D were subjected to in-gel tryptic digestion and LC-MS/MS sequencing.
TABLE 2
Summary of LC-MS/MS sequencing of proteins purified from mouse epididymus

<table>
<thead>
<tr>
<th>Protein</th>
<th>NCBI gi no.</th>
<th>Molecular mass (kDa)</th>
<th>Sequence coverage %</th>
<th>No. of unique peptides identified</th>
<th>r.m.s. error ppm</th>
<th>Location on gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen α-chain</td>
<td>33563252</td>
<td>61.8</td>
<td>38</td>
<td>30</td>
<td>4</td>
<td>B (A, C)</td>
</tr>
<tr>
<td>Fibrinogen β-chain</td>
<td>33859809</td>
<td>55.4</td>
<td>61</td>
<td>38</td>
<td>5</td>
<td>B (A, C)</td>
</tr>
<tr>
<td>Fibrinogen γ-chain</td>
<td>19527078</td>
<td>50.0</td>
<td>54</td>
<td>28</td>
<td>4</td>
<td>B (A, C)</td>
</tr>
<tr>
<td>Lumican</td>
<td>6678740</td>
<td>38.6</td>
<td>34</td>
<td>17</td>
<td>5</td>
<td>D</td>
</tr>
</tbody>
</table>

*The fibrinogen α-, β-, and γ-chains were also identified in gel bands A and C.

accuracy and then sequenced in the linear ion trap. All mass and tandem mass spectra were searched against the NCBI Protein Database using the Mascot search engine. The Mascot histogram showing the score distribution for the proteins identified in gel band B are shown in Fig. 6B (inset). The identification of a tryptic peptide from the fibrinogen β-chain by MS/MS is shown (Fig. 6C). At an elution time of 20.97 min, a precursor ion was observed in the FTICR survey scan (Fig. 6C, inset) with a 2+ charge state and a monoisotopic m/z of 1026.4806. This ion was automatically isolated in the linear ion trap and fragmented by collision-induced dissociation to produce the tandem mass spectrum. The tryptic peptide (Glu^{154}–Arg^{170}) from the fibrinogen β-chain was unambiguously identified based on the very accurate monoisotopic mass of the [M + 2H]^2+ ion (Δm = 0.58 ppm) and the predominant series of b and y fragment ions present in the tandem mass spectrum. A large number of unique peptides were identified for all three different subunits of mouse fibrinogen using the same methodology (Table 2). Fibrinogen is multichain protein with an α₂β₂γ₂-subunit structure. To assess which subunit might be tyrosine-sulfated, PSG2 Western blot analysis was performed on purified mouse fibrinogen. This showed that PSG2 recognized the β-chain, but not the α- or γ-chain (Fig. 7). LC-MS/MS analysis of the broad band at 50–70 kDa (band D) identified this protein as mouse lumican (Table 2).

Proteins purified by PSG2 affinity chromatography from a soluble fraction of wild-type mouse epididymis were run on 4–15% SDS-polyacrylamide gels, and bands A–D (Fig. 5) were subjected to in-gel tryptic digestion and LC-MS/MS sequencing as described under “Experimental Procedures.”

**DISCUSSION**

Protein tyrosine O-sulfation was first described in bovine fibrinopeptide B in 1954 (51). Since that time, tyrosine sulfation has become better understood as more tyrosine-sulfated proteins have been described and as investigations on the importance of sulfotyrosine in the function of dozens of proteins have been conducted. However, despite this progress, we still lack a broad understanding of the importance of tyrosine sulfation in protein function and biology. Many basic questions about this post-translational modification and the enzymes that catalyze its formation remain unanswered. First, the number of tyrosine-sulfated proteins in the human or mouse proteome is unknown. Second, little is known about how the two TPST isoenzymes differ with respect to their macromolecular substrate specificities. Third, for most of the proteins known to be tyrosine-sulfated, the role for sulfotyrosine in the function of that protein remains unknown. Progress in the field has been hampered by the lack of reagents to identify new tyrosine-sulfated proteins and to explore the role of tyrosine sulfation in their function. In this study, we have described the isolation of a novel monoclonal antibody (called PSG2) against tyrosine-sulfated peptides and proteins and an in-depth assessment of its specificity and utility as a discovery tool.

Our experiments have revealed some interesting details of the fine specificity of PSG2. Our epitope mapping data show that PSG2 bound to various sulfated pentapeptides, the A(sY)A tripeptide, and the (sY)A dipeptide immobilized on membranes. However, PSG2 did not detectably bind to A(sY) or sulfotyrosine alone (Fig. 1B and supplemental Table 1). These data also show that Lys at the P1’ position relative to the sulfotyrosine in various peptides markedly impaired or eliminated PSG2 binding. Furthermore, our hapten inhibition experiments (Table 1) show that the sulfopeptide L(D(sY)DF was ~570-fold more potent as an inhibitor of PSG2 binding than was free sulfotyrosine. Taken together, these data show that the PSG2 epitope comprises some structural elements of the P1’ amino acid, in addition to the sulfotyrosine side chain.

Our analysis of PSG2 binding to sulfated pentapeptides showed that the presence of an acidic residue is a positive predictor of “strong” PSG2 recognition (Fig. 1B and supplemental Table 1). Among the 67 pentapeptides analyzed in our study, 23 contained 1 or more Glu or Asp residues, and all 23 bound PSG2 avidly. It is very interesting to note the similarity of the sequence context required for avid PSG2 binding to peptides and that for TPST substrate recognition. Remember that the
Anti-sulfotyrosine Antibody

A

TIC

Relative Abundance

Time (min)

B

FT-MS

(RT: 0-32)

Relative Abundance

Probability Based Mowse Score

Number of Hits

m/z

391.2852

Fibrinogen α and γ chains

Fibrinogen β chain

C

IT-MS²

(1026.98)

Relative Abundance

m/z

D

154 ENENVINEYSSLIEDQ R170

b₅-H₂O

b₄ 547.38

b₃ 698.91

b₂ 773.54

b₁ 963.48

b₀ 1105.52

b₁₀ 1239.70

y₂ 303.27

y₃ 418.34

y₄ 547.38

y₅ 947.50

y₆ 1110.67

y₇ 1353.74

y₈ 1466.81

y₉ 1505.89

y₁₀ 1634.80

y₁₁ 1749.77

y₁₂ 1877.82

y₁₃ 1679.94

y₁₄ 1505.09

y₁₅ 1505.89

y₁₆ 1505.09

y₁₇ 1679.94

y₁₈ 1877.82

y₁₉ 1749.77

y₂₀ 1505.09

y₂₁ 1505.89
presence of Glu or Asp residues within ±5 residues is the dominant sequence feature of known tyrosine sulfation sites.

We have also provided convincing evidence that PSG2 can effectively discriminate between tyrosine phosphate and tyrosine sulfate. First, tyrosine sulfate inhibited PSG2 binding to antin sequence feature of known tyrosine sulfation sites.

To further investigate the specificity of PSG2, we performed LC-MS/MS sequencing on the tryptic digest of fibrinogen bands. The results showed that PSG2 specifically recognizes phosphorylated Tyr305 and Tyr318 of PSGL-1 on human neutrophils. Our experiments also demonstrate that PSG2 binds to a variety of sulfated and phosphorylated proteins in complex biological samples in Western blot assays.

In conclusion, PSG2 offers a novel tool for examining the role of sulfotyrosine in protein function. It can be used to study the interaction of human P-selectin with PSGL-1 on human neutrophils and to identify sulfotyrosine-containing proteins in biological samples. This work provides new insights into the role of sulfotyrosine in protein function and opens up new avenues for the development of therapeutic strategies targeting sulfotyrosine.

Y. Yu and J. A. Leary, submitted for publication.
Anti-sulfotyrosine Antibody

fibrinopeptide B of mouse fibrinogen is tyrosine-sulfated like other mammalian fibrinogens. These data convincingly demonstrate that a high degree of enrichment of tyrosine-sulfated proteins can be achieved in a single step using PSG2 affinity chromatography.

We also used PSG2 Western blotting to compare putative tyrosine-sulfated proteins in wild-type, Tpst1−/−, and Tpst2−/− mice. This analysis revealed that there are multiple PSG2-reactive sperm/epididymal proteins and that there are distinct differences in the profiles of these proteins in wild-type and Tpst knock-out mice. These differences suggest that certain sperm/epididymal proteins may be differentially sulfated by TPST1 and TPST2. These observations support the conclusion that the phenotypic differences between Tpst1 and Tpst2 knock-out mice are due to differences in the macromolecular substrate specificities of TPST1 and TPST2.

Kehoe et al. (54) reported recently the production of an anti-sulfotyrosine antibody using phage display. Although their reagent was not as well characterized as PSG2, a couple of direct comparisons can be made. They reported Western blot analyses of human C4 using an intact anti-sulfotyrosine IgG. This showed reactivity toward two of the three subunits of C4, as well as toward some other bands of higher molecular mass that were not seen on an accompanying Coomassie Blue-stained gel. In contrast, PSG2 recognized only the C4 α-subunit (Fig. 2), consistent with previous work demonstrating that only the α-subunit of human C4 is tyrosine-sulfated (22, 46, 47). Likewise, the analyses of bovine fibrinogen by Kehoe et al. showed reactivity toward two of the three fibrinogen subunits. However, only the β-subunit of bovine fibrinogen has been shown to be tyrosine-sulfated. In addition, in their Western blot analyses, the amount of purified proteins analyzed (1–10 μg) and the concentration of primary antibody used (20 μg/ml) were 2–3 orders of magnitude higher than was required in our analyses. We obtained clean blots of 20–30 ng of purified protein using 30 ng/ml primary antibody (Fig. 2 and supplemental Fig. 1). This suggests that PSG2 has a substantially higher affinity and specificity than their reagent.

In summary, we have described an anti-sulfotyrosine antibody that binds with high affinity and exquisite specificity to sulfotyrosine residues in peptides and proteins independently of the sequence context. The antibody can detect tyrosine-sulfated proteins in complex biological samples and can be used to purify and identify tyrosine-sulfated proteins. This novel reagent is a useful probe to explore the role of tyrosine sulfation in protein function, should be widely applicable for identification of tyrosine-sulfated proteins in other systems and organisms, and will facilitate a rapid expansion of our understanding of the role of tyrosine sulfation in biological systems.

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REFERENCES