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DETECTION AND PURIFICATION OF TYROSINE-SULFATED PROTEINS USING A NOVEL ANTI-SULFOTYROSINE MONOCLONAL ANTIBODY*

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Abstract

Protein-tyrosine *O*-sulfation is a post-translational modification mediated by one of two Golgi tyrosylprotein sulfotransferases (TPST-1 & TPST-2) 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 independent of sequence context. We show that it can detect tyrosinesulfated 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 indicates that certain sperm/epididymal proteins are undersulfated in *Tpst2*−/− mice. This indicates that TPST-1 and TPST-2 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.

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[1]The abbreviations used are: TPST, tyrosylprotein sulfotransferase; MOPS, 3-(Nmorpholino)propanesulfonic acid; HRP, horseradish peroxidase; ABTS, 2, 2′-azino-di(3-ethylbenzthiazoline-6-sulfonate), HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride: sY, sulfotyrosine; pY, phosphotyrosine; HBSS, Hank's balanced salt solution; TBS, Tris-buffered saline; MBS, MOPS-buffered saline.

Protein tyrosine-*O* sulfation is a post-translational modification that occurs in most eukaryotes (1-3). In the mouse and man tyrosine sulfation is mediated by one of only two tyrosylprotein sulfotransferases (TPST-1 and TPST-2, EC 2.8.2.20) (4-6). These enzymes catalyze the transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the universal sulfate donor, to tyrosine residues in polypeptides (7). TPSTs are type II transmembrane proteins that reside in the transGolgi network and have luminally-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 tyrosinesulfated 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: 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) that is expressed on leukocytes is required for cell-cell interactions mediated by P- and L-selectin in the vasculature (10-12). In the co-crystal of the lectin-EGF domain of P-selectin and a recombinant glycosulfopeptide mimetic of the N-terminal domain of PSGL-1, the sulfate groups at Tyr^{48} and Tyr^{51} 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 (GPCR) function after Farzan *et al* showed that CCR5, a major HIV co-receptor, is tyrosine-sulfated (14). Sulfation of one 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 HIV co-receptor function. Similar studies indicate that tyrosine sulfation of the N-terminal domains of other chemokine receptors (CXCR4, CCR2B, CX3CR1, CCR8, CXCR3), as well as other GPCRs (C5a, C3a, SIP1, and the FSH, LH, and TSH receptors), is required for optimal binding of their cognate ligands (2,15-17).

Likewise tyrosine sulfation is required for optimal proteolytic processing of progastrin (18), proteolytic activation of coagulation factors V and VIII by thrombin (19-21), proteolysis of the complement C4α chain by C1s (22), binding of glycoprotein Ibα to thrombin (23), binding of glycoprotein Ibα (24,25) and factor VIII (26) to von Willebrand factor (26), binding of cholecystokinin to the CCK-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 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 tyrosinesulfated 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 mAbs using the strategy employed by Glenney *et al* to generate anti-phosphotyrosine monoclonal antibodies (29). 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 independent 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 P-selectin glycoprotein ligand-1 (PSGL-1) and human platelet P-selectin were purified as previously described (30,31). Purified bovine factor X_1 and X_2 were provided by Charles Esmon (Oklahoma Medical Research Foundation) and human heparin cofactor II (HCII) expressed in *E. coli* (32) was a gift from Douglas Tollefsen (Washington Univ., St. Louis, MO). Purified human plasma C4 was purchased from Advanced Research Technologies (San Diego, CA) and human plasma HCII and mouse fibrinogen were from Haematalogic 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 HPLC-purified (>95% purity) by Biosynthesis Inc. (Lewisville, TX). The anti-phosphotyrosine mAb PY20 was purchased from MP Biomedicals.

Identification of an Antibody Binding to Sulfotyrosine

A single chain Fv (scFv) phagemid library, an expanded version of the 1.38×10^{10} library (33), was used to select antibodies that bind to a protein containing sulfated tyrosines. The immobilized target protein was the purified dimeric 19.ek.Fc recombinant protein (13) that contains three sulfated tyrosines within the N-terminal 19 amino acids of human PSGL-1 (QATE(sY)E(sY)LD(sY)DFLPETEPP) and is fused to human immunoglobulin G1 Fc via an enterokinase cleavage site. Several scFv clones were isolated following multiple rounds of panning procedure (33). The purified psg2 scFv clone was identified as an scFv whose binding to 19.ek.Fc was inhibited in the presence of increasing concentrations of the murine PSGL-1 Fc fusion protein, mPSGL-1 Fc, which contains sulfated tyrosines within a different sequence context (34). The psg2 scFv was converted to a full length, intact IgG4-λ antibody (designated PSG2) and expressed in mammalian CHO cells as described (35).

PSG2 Purification

PSG2-CHO cells were expanded from a single vial of frozen cells in αMEM (Mediatech) containing 10% heat-inactivated dialyzed fetal bovine serum (FBS, Sigma), 2 mM L-glutamine, 100 nM methotrexate, 1 mg/ml G418, 100 units/ml penicillin, and 100 μg/ml streptomycin into 850 cm^2 roller bottles. At ≈ 90% confluence serum-containing media was removed, the monolayer washed with warm PBS and the media replaced with αMEM, 2 mM Lglutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Conditioned media was harvested and replaced with fresh media twice a week until senescence. Conditioned media was clarified by centrifugation, benzamidine added to 5 mM final concentration, sterilized by passage through a 0.2 μm filter, and then frozen at −20°C. To purify PSG2, conditioned media was batch adsorbed to Protein G Sepharose overnight at room temperature, the resin was packed into a column, washed extensively with 0.1 M NaCl, 20 mM MOPS, pH 7.5 (MBS), and bound mAb eluted with ImmunoPure® Gentle Ag/Ab Elution Buffer (Pierce). Eluted mAb was exhaustively dialyzed against MBS, clarified by centrifugation (48,000 × g, 30 min), and stored at −80°C. The purified IgG is >95% pure as assessed by SDS-PAGE. The purified material co-elutes with IgG standard on a TosoBioSep G3000SWXL size-exclusion column (7.8 × 300 mm) using 150 mM NaCl, 20 mM sodium phosphate, pH 6.7 as mobile phase.

Epitope mapping

Peptide spot synthesis was performed as previously described (36). The arrays were defined on the membranes by coupling a β-alanine spacer and peptides were synthesized using standard DIC/HOBt coupling chemistry as described previously (37,38). Activated amino acids were spotted using an Abimed ASP 222 robot and the washing and Fmoc deprotection steps were done manually. Following the final synthesis cycle, the membranes were treated with acetic anhydride and side chains deprotected resulting in an array of peptides that are N-terminally acetylated and attached via the C-terminus. Membranes were washed in methanol, blocked with 1% casein in Tris-buffered saline (TBS), and then incubated with 1 μg/ml of PSG2 in TBS for 1 h with gentle shaking. The membranes were washed 4 times for 2 min in TBS and then probed with an HRP-conjugated anti-Fc antibody in TBS/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 (BioRad). For Coomassie staining, 1 μg of purified proteins were loaded, while for Western blotting, 10 −20 ng of proteins were loaded. For Western blots, PSG2 or control IgG4-λ mAb was used as primary antibody at 30 ng/ml, followed by 200 ng/ml of anti-human IgG-HRP as secondary antibody. Bound secondary antibody was detected using ECL Plus (Amersham) followed by either autoradiography or imaging on a Storm 860 scanner (Molecular Dynamics).

ELISAs

Microtiter plates (Immulon 1B, Dynex) were coated with bovine factor X_1 or factor X_2 (5 μg/ml, 100 μl/well) in sodium carbonate, pH 9.6 overnight at 4°C. Plates were washed 3 times with 100 mM NaCl, 20 mM TrisHCl pH 6.9 (TBS) containing 0.05% Tween-20 and then blocked with TBS/1% BSA for 2 h and then washed twice with TBS/0.05% Tween-20. Tyrosine sulfate, tyrosine phosphate or various peptides in 0.1 M NaCl, 20 mM MOPS, pH 7.5 (MBS) 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 5 times with TBS/0.05% Tween-20 and 1:5000 dilution of anti-human IgG-HRP in TBS-T was added (100 μl/well) and incubated for 1 h. Plates were washed 5 times with TBS/0.05% Tween-20 and then ABTS peroxidase substrate (KPL) was added (100 μl/well). Plates were read at 405 nm using a Versamax microplate reader (Molecular Devices).

Pervanadate Treatment of NRK Cells

Rat NRK cells (ATCC: CRL-6509) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine 10% fetal bovine serum at 37°C 5% CO_2. A fresh stock solution of pervanadate ions (30 mM) was prepared by combining 100 μl of a 100 mM Na_3VO_4, 10 μl of 30% H_2O_2, and 223 μl of PBS, followed by incubation in the dark for 10 min at room temperature. NRK cells were then incubated for 30 min at 37°C 5% CO_2 in complete media containing 100 μM pervanadate or media alone. After 30 min the media was removed and the cells detached and collected by centrifugation (90 × g, 5 min). Cells were washed once with PBS, collected by centrifugation, and cell pellets lysed with 1% Triton X-100 in 100 mM NaCl, 20 mM HEPES, pH 7.2, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 160 μg/ml benzamidine, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 mM Na_3VO_4. 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 N_2 and stored at −80°C until use.

Glycan Array Screening

The PSG2 mAb was used to screen a glycan microarray (Glycan Array v3.5) 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 previously described (39). In total, 206 unique glycans are represented on the array. Briefly, biotinylated glycosides (40) were bound to streptavidin-coated microtiter plates in replicates of 4. Purified bovine factor X_1 and factor X_2 were biotinylated using sulfo-NHS-LCbiotin (Pierce) and included on the array (30 pmol/well) as negative and positive controls, respectively. Pre-coated plates were washed three times with 100 μl of wash buffer (20 mM TrisHCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween-20). PSG2 mAb (10 μg/ml) in wash buffer containing 1% BSA was added to each well and incubated for 1 h. The plates were washed and 25 μl of anti-human IgG-FITC (2 μg/ml) in binding buffer was added and incubated for 1 h. The wells were washed three times, 25 μl wash buffer was added to each well, and bound secondary antibody detected using a Victor-2TM 1420 Multi-label Counter (PerkinElmer Life Sciences) at excitation 485/emission 535.

Generation of Tpst double knockout mice

Tpst1 null ($Tpst1^{tm1Klm}$, MGI:2183366) and *Tpst2* null ($Tpst2^{tmlklm}$, MGI:3512111) mice were generated and characterized as previously described (41,42). The *Tpst1* and *Tpst2* genes reside on chromosome 5 and the physical distance between the two is ≈18.4 Mb. To generate the *Tpst* DKOs, *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 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 previously described (41) and all animal procedures were approved by the Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation. A detailed description of these mice will be published elsewhere.

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 (HBSS) was coated onto 35-mm tissue culture plates at 4°C overnight and blocked with 1% human serum albumin (HSA) in HBSS for 2 h at room temperature. Human neutrophils were isolated as previously described (44), resuspended at 4×10^6 cells/ml in HBSS/1% HAS, and perfused over the P-selection surface at a wall shear stress of 1 dyn/cm^2. Neutrophil adhesion was allowed to equilibrate for 4 min and then non-adherent cells were flushed from the system with HBSS/1% HSA. After an additional 4 min of perfusion, control human IgG_4-λ mAb (50 μg/ml) or PSG2 mAb (50 μg/ml) was added to the perfusate. Neutrophil adhesion was visualized using phase-contrast video microscopy and the number of rolling neutrophils was determined each 20 s.

PSG2 Affinity Chromatography and Peptide Sequencing

Epididymides were collected from ten sexually mature wild type mice and homogenized in cold 0.1M NaCl, 20 mM MOPS, pH 7.5 (MBS) containing a cocktail of protease inhibitors (Complete Mini, Roche) using a dounce homogenizer. A post-nuclear supernatant was obtained (800 × g, 10 min) which was then subjected to centrifugation (100,000 × g, 60 min) to prepare a soluble protein fraction. The soluble fraction (8.5 ml, ≈28 mg total protein) was applied to a PSG2-Affigel-10 column (4 mg mAb/ml resin, 0.9 × 5 cm) at 50 μl/min. The column was washed extensively with MBS, then 100 mM NH_4OAc, and the column was eluted with 4 mM of the sulfated peptide LD(sY)DF in 100 mM NH_4OAc at 30 μl/min. Flow-through and elution fractions were monitored by SDS-PAGE followed by PSG2 Western blotting or silver staining.

Epididymal/sperm proteins eluted from the PSG2 column were concentrated, electrophoresed on 4-15% Tris-HCl SDS-polyacrylamide gels under non-reducing conditions, and separated proteins visualized with colloidal Coomassie staining. Gels were washed with H_2O and then bands were excised and washed again with H_2O followed by 60% acetonitrile in H_2O then dryed in a SpeedVac. The gel pieces were reduced (10 mM DTT in 50 mM NH_4HCO_3, 1 h at 56°C) and alkylated (55 mM iodoacetamide in 50 mM NH_4HCO_3, 45 min at 22°C in the dark). After washing with 50 mM NH_4HCO_3 the gel pieces were dehydrated with 60% acetonitrile in H_2O then dryed. Gel pieces were re-swollen with trypsin in 50 mM NH_4HCO_3 and incubated overnight at 37°C. Peptides were eluted from the gel slices by repeated (3×, 60 min) extraction with 60% acetonitrile/0.1% TFA in H_2O. Eluates were pooled, dried down, and re-dissolved in 2% acetonitrile in 0.1% TFA prior to LC-MS/MS analyses.

Mass Spectrometry Analysis

Nano-LCMS/MS experiments were performed on a Thermo Electron LTQ-FT hybrid linear ion trap/7T FTICR mass spectrometer, equipped with a Thermo Electron Nanospray ion source, Surveyor MS pump, and micro-autosampler. The tryptic peptide mixture was separated on a 12 cm × 50 μm ID PicoFrit column packed with C18 reversed-phase resin (Magic C18AQ, 100 Å pore size, 3 μm particle size, Michrom BioResources Inc.). The column was equilibrated before sample injection for 10 min at 2% solvent B (0.1% (v/v) formic acid in acetonitrile and 98% solvent A (0.1% (v/v) formic acid in H_2O) at a flow rate of 140 nl/min. The column was developed with a linear gradient from 2 to 50% solvent B in 30 min at a flow rate of 320 nl/min. The LTQ-FT mass spectrometer was operated in the data-dependent acquisition mode using the TOP10 method: a full-scan MS acquired in the FTICR mass spectrometer followed by 10 MS/MS experiments performed with the LTQ on the ten most abundant ions detected in the full-scan MS. All tandem mass spectra (MS/MS) from each LC-MS run were searched against the NCBI protein database using the Mascot search engine. Searches were performed with tryptic specificity allowing four missed cleavages and a tolerance on the mass measurement of 10 ppm in MS mode and 0.5 Da for MS/MS ions. Possible structure modifications allowed were carbamidomethylation of Cys, sulfation of Tyr, oxidation of Met, and deamidation of Asn and Gln.

RESULTS

Sulfotyrosine is Required for PSG2 Binding to Synthetic Pentapeptides

To determine if 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, (TE(sY)EF and LD(sY)DF, sY = sulfotyrosine) synthesized *in situ* on PEG-modified cellulose membranes and PSG2 binding was assessed by Western blotting. We observed that PSG2 bound both peptides (Fig. 1A, first and last columns). For both pentapeptides, binding was lost when the sulfotyrosine was changed to any other amino acid, including tyrosine. However, binding was not affected by single amino acid substitutions at any other position with the exception that PSG2 binding was diminished if a Lys was substituted at the P1' position relative to the sulfotyrosine (Fig. 1A).

A similar analysis was performed on an array of 67 pentapeptide sequences selected using a random sequence generator that were synthesized with either sulfotyrosine or tyrosine in the third position. PSG2 bound to 54 of 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 one or more Glu or Asp residues and all 23 peptides bound PSG2 strongly. Thus, the presence of Glu or Asp ± 2 to the sulfotyrosine is a strong positive predictor of PSG2 recognition. Five of 13 of the peptides that did not bind PSG2 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.

PSG2 Binds to Tyrosine-Sulfated Proteins

We next examined the ability of PSG2 to bind various sulfotyrosine-containing proteins using Western blotting. Bovine coagulation factor X is a two-chain serine protease zymogen that exists in two chromatographically separable isoforms in bovine plasma, factor X_1 and X_2. Factor X_2 contains a sulfotyrosine at position 18 of the activation peptide, whereas factor X_1 does not (45). Western blotting of factor X_1 and X_2 revealed that PSG2 binds to factor X_2, but not factor X_1 (Fig. 2A). A parallel analysis under reducing conditions shows that PSG2 binds to the factor X_2 heavy chain, but not to the heavy chain of factor X_1, or to the light chains of factors X_1 or X_2 (Fig. 2A). PSG2 did not bind to the heavy or light chain of factor X_a that 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 (α, β, and γ). Human C4 has been shown to have 3 sulfotyrosine residues near the C-terminus of the α subunit (46-48). As expected, PSG2 bound to the α subunit of C4, but not to the β or γ subunit (Fig. 2B). A similar analysis was also 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 lack TPST activity (Supplemental 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 *Tpst* double knockout livers were analyzed by Western blotting with PSG2. We observed that PSG2 binds to multiple protein species from wild type liver, but none from *Tpst* double knockout liver (Fig. 3A). Thus, PSG2 does not recognize a variety of proteins that lack sulfotyrosine.

PSG2 Does Not Bind Tyrosine-Phosphorylated Proteins

Given the high degree of structural similarity between sulfotyrosine and phosphotyrosine, we sought to determine if PSG2 could discriminate between these two amino acids in the context of peptides and proteins in two analytical formats.

We first assessed the ability of sulfotyrosine, phosphotyrosine, or sulfotyrosine- and phosphotyrosine-containing peptides to inhibit PSG2 binding to bovine factor X_2 in an ELISA format (Table 1). In these assays PSG2 bound avidly to immobilized bovine factor X_2, but not factor X_1. We observed that sulfotyrosine was 13-fold more efficient as an inhibitor of PSG2 binding to factor X_2 than was phosphotyrosine. However, the IC_{50} for inhibition of PSG2 binding was >1000-fold lower for the sulfopeptide (LD(sY)DF) than the corresponding phosphopeptide (LD(pY)DF). In addition, the sulfopeptide (LD(sY)DF) was ≈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 while 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 blots.

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 (49). To further assess the PSG2 specificity, we screened a glycan microarray to determine if PSG2 could recognize various sulfated glycans. The array contains 206 distinct glycosides, including 16 sulfated glycosides and their unsulfated counterparts. In addition, bovine factor X_2 and X_1 were included as positive and negative controls, respectively. We 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 if 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 on P-selectin-coated surfaces *in vitro* and to activated endothelium *in vivo* (50,51). 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 dyn/cm^2 to initiate neutophil rolling for 4 min, then cell-free buffer was allowed to flow for another 4 min to remove any non-adherent cells. Then either human IgG_4-λ mAb or PSG2 mAb was added to the perfusate and the number of rolling cells per field was counted every 20 s. The results show that infusion of PSG2 into the flow system results in rapid and complete detachment of rolling neutrophils, but that control IgG_4-λ 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 if 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 Affigel-10 column and the column was washed extensively and eluted with 4 mM LD(sY)DF in 100 mM NH_4OAc. Flow-through and elution fractions were monitored by SDS-PAGE followed by PSG2 Western blotting or silver staining. We observed that flow-through fractions were effectively depleted of PSG2 reactive proteins (data not shown). Analysis of the eluted fractions revealed a very close correlation between the bands detected by silver staining and PSG2 Western blotting, indicating that these proteins were markedly enriched (Fig. 5).

The major bands indicated in Figure 5 were excised from the gels and subjected to in-gel tryptic digestion. The digests were analyzed by nano-LC-MS/MS using a Thermo Electron LTQFT hybrid linear ion trap/7T FTICR mass spectrometer. This analysis showed that the three high molecular weight bands at > 205 kDa (Bands A-C) corresponded to mouse fibrinogen (Table 2). An example highlighting the identification of mouse fibrinogen by nano-LC-MS/MS analysis is shown in Figure 6. The tryptic peptide mixture from the gel band B was injected into the nano-LC C18 column, separated using a linear gradient from 2 to 50% solvent B in 30 min (Fig. 6A), and analyzed with the LTQ-FT mass spectrometer using data-dependent acquisition mode. The tryptic peptides were first analyzed in the ICR cell (Fig. 6B) with high resolution and high mass accuracy, and then sequenced in the linear ion trap. All MS and MS/MS spectra were used to search 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 (insert). The identification of a tryptic peptide from the fibrinogen β chain by tandem mass spectrometry is shown (Fig. 6C). At an elution time of 20.97, a precursor ion was observed in the FTICR survey scan (Fig. 6C, insert) 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 (CID) to produce the MS^2 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 MS^2 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 $\alpha_2\beta_2\gamma_2$ 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-chains (Fig. 7). LC-MS/MS analysis of the broad 50-70 kDa band (Band D) identified this protein as mouse lumican (Table 2).

Profiling Tyrosine-Sulfated Proteins in Mouse Epididymis

We recently reported that *Tpst2*−/− males are infertile, whereas *Tpst1*−/− males have normal fertility (42). The phenotype we observe in *Tpst2*−/− males is that of normal spermatogenesis but abnormal sperm function, suggesting that the infertility is due to defective tyrosine sulfation of one or more proteins involved in sperm function. To explore this question, PSG2 Western blot analyses have been performed on detergent extracts of the whole epididymis from wild type, *Tpst1*−/−, and *Tpst2*−/− mice (Fig. 8). These extracts contain proteins from both the epididymis itself as well as sperm, because the epididymis is normally filled with sperm. This analysis revealed that there are multiple PSG2 reactive sperm/epididymal proteins and that there are distinct differences in the profile of these proteins in wild type vs. *Tpst* knockouts. A comparison of the PSG2 profiles shows the absence of 2-3 PSG2 reactive bands in the ≈40-50 kDa range in *Tpst1*−/− mice compared to wild type and *Tpst2*−/− mice and the absence of a major band at 31 kDa in *Tpst2*−/− compared to wild type and *Tpst1*−/− mice. These results lead reasonably to the preliminary conclusion that certain sperm/epididymal proteins are differentially sulfated by TPST-1 and TPST-2.

DISCUSSION

Protein-tyrosine *O*-sulfation was first described in bovine fibrinopeptide B in 1954 (52). 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 explore the role of tyrosine sulfation in their function. In this report we have described the isolation of a novel monoclonal antibody against tyrosine-sulfated peptides and proteins, called PSG2, and an in-depth assessment of its specificity and utility as a discovery tool.

Our studies reveal 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, Supplemental Table 1). These data also show that a Lys residue at the P1' position relative to the sulfotyrosine in various peptides markedly impairs or eliminates PSG2 binding. Furthermore, our hapten inhibition studies (Table 1) show that the sulfopeptide LD(sY)DF is ≈570 times more potent as an inhibitor of PSG2 binding than 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, Supplemental Table 1). Among the 67 pentapeptides analyzed in our study, 23 contained one 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 presence of Glu or Asp residues within ± 5 residues is the dominant sequence feature of known tyrosine sulfation sites.

We also provide convincing evidence that PSG2 can effectively discriminate between tyrosine phosphate and tyrosine sulfate. First, tyrosine sulfate inhibited PSG2 binding to bovine factor X_2 with an IC_{50} 13 times lower than that of tyrosine phosphate. Furthermore, the sulfated peptide, LD(sY)DF is >1000 times more potent as an inhibitor of PSG2 binding than the phosphorylated peptide LD(pY)DF.

PSG2 binds to sulfotyrosine residues in a variety of native tyrosine-sulfated proteins and can detect tyrosine-sulfated proteins in complex biological samples in Western blot assays. The specificity of PSG2 for sulfotyrosine is supported by the fact that PSG2 recognizes the subunits in bovine factor X_2 and human C4 that have been documented to contain sulfotyrosine residues (Fig. 2). The specificity of PSG2 is further highlighted by our studies documenting that PSG2 does not bind to a variety of proteins in crude extracts of *Tpst* double knockout liver or to a variety of tyrosine-phosphorylated proteins in Western blots (Fig. 3). Furthermore, we show that PSG2 does not bind to a variety of sulfated glycans. Taken together these data show that PSG2 binds with exquisite specificity to tyrosine-sulfated proteins and peptides independent of sequence context.

In this report, we also show that PSG2 is a useful reagent for examining the role of sulfotyrosine in protein function. The interaction of human P-selectin with PSGL-1 on human neutrophils is known to be dependent on the sulfation of tyrosine residues within the N-terminus of PSGL-1 (10-12,53). Our studies showed that PSG2 caused very rapid and complete detachment neutrophils rolling on P-selectin-coated plates (Fig. 4).

In a proof-of-concept study we used PSG2 affinity chromatography to purify tyrosine-sulfated proteins from a soluble fraction of whole mouse epididymis. A mixture of polypeptides that included three high molecular weight proteins of >205 kDa and a diffuse polypeptide of ≈50-70 kDa were purified. These were identified using LC-MS/MS as fibrinogen and lumican, respectively. The isolation of these proteins is remarkable. Human lumican, a class II small leucine-rich proteoglycan has been shown to be tyrosine-sulfated (54) and we have recently found that mouse lumican is sulfated as well (Yonghao Yu and Julie A Leary, submitted for publication). Using a novel subtractive mass spectrometry strategy, we determined that mouse lumican was stoichiometrically sulfated at Tyr^{20}, Tyr^{21}, Tyr^{23}, and Tyr^{30}. Second, fibrinogen has been shown to be tyrosine-sulfated in multiple mammalian species, but not in the mouse (52,55). Sulfation in many mammalian fibrinogens occurs on fibrinopeptide B, the N-terminal peptide cleaved from fibrinogen β chain by the action of thrombin during blood coagulation. Analysis of the sequences of the three mouse fibrinogen chains using the Sulfinator software tool, predicts tyrosine sulfation of Tyr^{24} of the β-chain and Tyr^{299}, Tyr^{305}, and Tyr^{318} of the γ chain. However, Western blot analysis shows that PSG2 binds to the β chain, but not the α or γ chains of mouse fibrinogen (Fig. 7). This result suggests that 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 profile of these proteins in wild type and *Tpst* knockouts. These differences suggest that certain sperm/epididymal proteins may be differentially sulfated by TPST-1 and TPST-2. These observations support the conclusion that the phenotypic differences between *Tpst1* and *Tpst2* knockout mice are due to differences in the macromolecular substrate specificities of TPST-1 and TPST-2.

Kehoe *et al* recently reported the production of an anti-sulfotyrosine antibody using phage display (56). 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 towards two of the three subunits of C4, as well as some other bands of higher molecular weight that were not seen on an accompanying Coomassie stained gel. In contrast, PSG2 recognizes only the C4 α subunit (Fig. 2) consistent with previous work demonstrating that only the α subunit of human C4 is tyrosine-sulfated (46-48). Likewise, the analyses of bovine fibrinogen by Kehoe *et al* showed reactivity towards 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 get clean blots of 20-30 ng of purified protein using 30 ng/ml of primary antibody (Fig. 2, Supplemental Fig. 1). This suggests that PSG2 has a substantially higher affinity and specificity than their reagent.

In summary, we described an anti-sulfotyrosine antibody that binds with high affinity and exquisite specificity to sulfotyrosine residues in peptides and proteins independent 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 in our understanding of the role of tyrosine sulfation in biologic systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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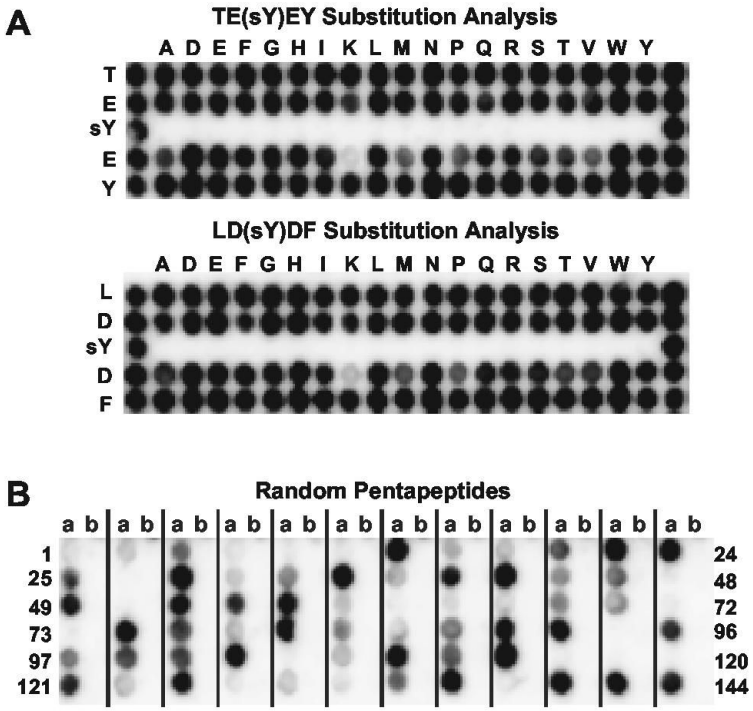

FIGURE 1.

PSG2 Epitope Mapping. Peptides were synthesized on PEG-modified cellulose membranes and PSG2 binding was assessed by Western blotting as described in Experimental Procedures. A. Single amino acid substitution analysis was performed on two pentapeptides modeled on the N-terminus of human PSGL-1. The first and last columns are the respective sulfated peptides. In the other columns, each position was substituted with the amino acid indicated in single letter code. B. Sixty-seven pentapeptide sequences selected using a random sequence generator were synthesized with either sulfotyrosine (columns labeled a) or tyrosine (columns labeled b) in the third position. The sequences of the peptides on the array are shown in Supplemental Table 1.

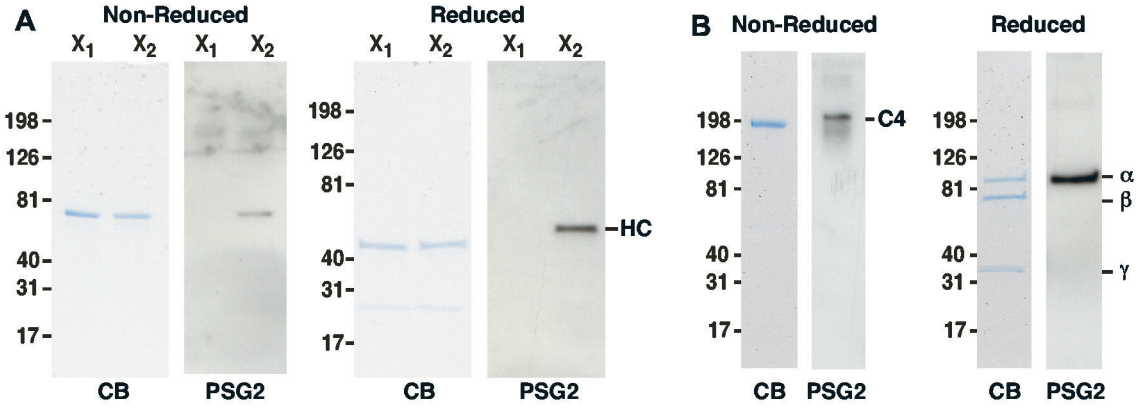

FIGURE 2.

PSG2 Western Blotting of Purified Tyrosine-Sulfated Proteins. Bovine factor X_1 and X_2 (panel A) and human C4 (panel B) were run on 4-15% SDS-polyacrylamide gels and stained with Coomassie blue (CB) or transferred to PVDF and subjected to Western blotting with PSG2 as described in Experimental Procedures. In lanes stained with Coomassie blue (CB) 1 μg of the proteins were loaded. In lanes subjected to Western blotting 10 ng of factors X_1 and X_2 (0.18 pmol) or C4 (0.05 pmol) were loaded. HC = heavy chain.

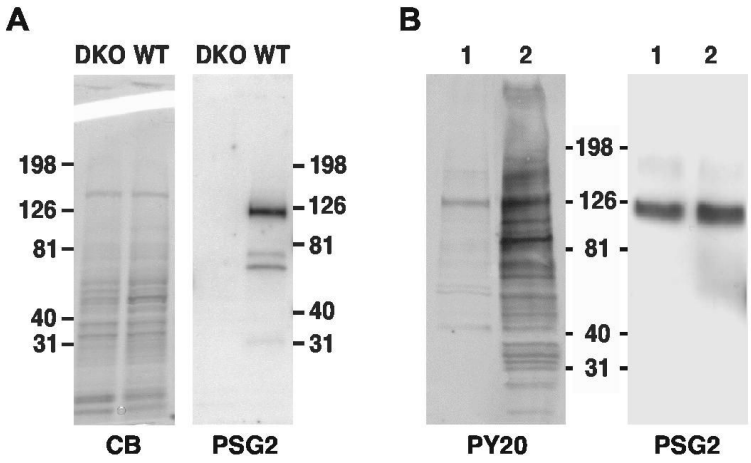

FIGURE 3.

PSG2 Western Blotting of Cell and Tissue Extracts. A. Detergent extracts of wild type and *Tpst* double knockout liver (10 μg total protein) were run on 4-15% SDS gels and stained with Coomassie blue (CB) or transferred to PVDF and subjected to Western blotting with PSG2 as described in Experimental Procedures. B. Rat NRK cells were treated with buffer (lanes 1) or 100 μM pervanadate (lanes 2). The cells were solubilized in detergent, run on 4-15% SDS gels, transferred to PVDF, and subjected to Western blotting with the anti-phosphotyrosine mAb PY20 or PSG2 as described in Experimental Procedures.

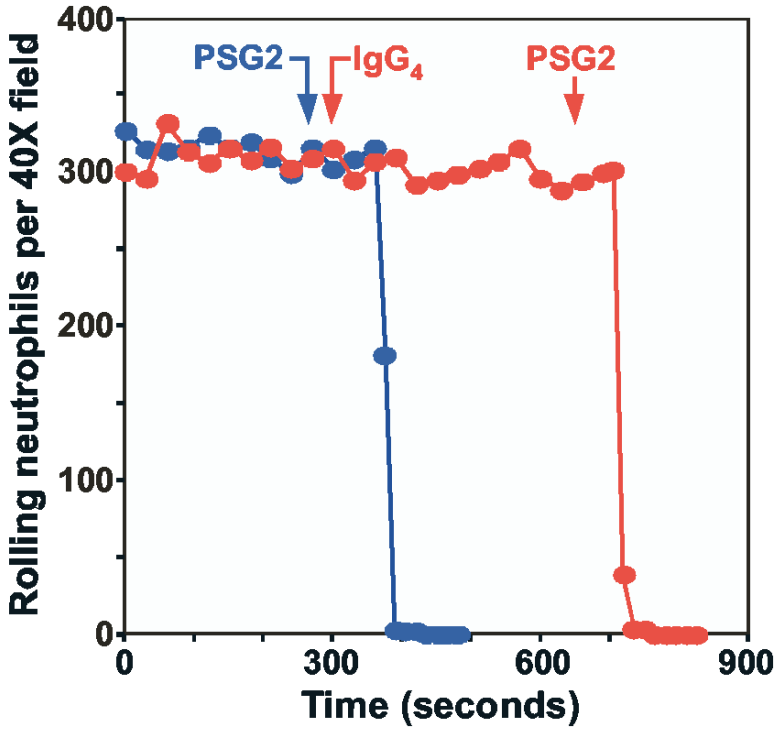

FIGURE 4.

PSG2 Blocks Neutrophil Adhesion to P-selectin Under Flow. P-selectin was coated onto plates and neutrophils were perfused over the surface at 1 dyn/cm^2. 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) control human IgG_4-λ (50 μg/ml) and then PSG2 (50 μg/ml) was sequentially added to the perfusate at the indicated time points. In the other experiment (blue line) PSG2 was added to the perfusate first.

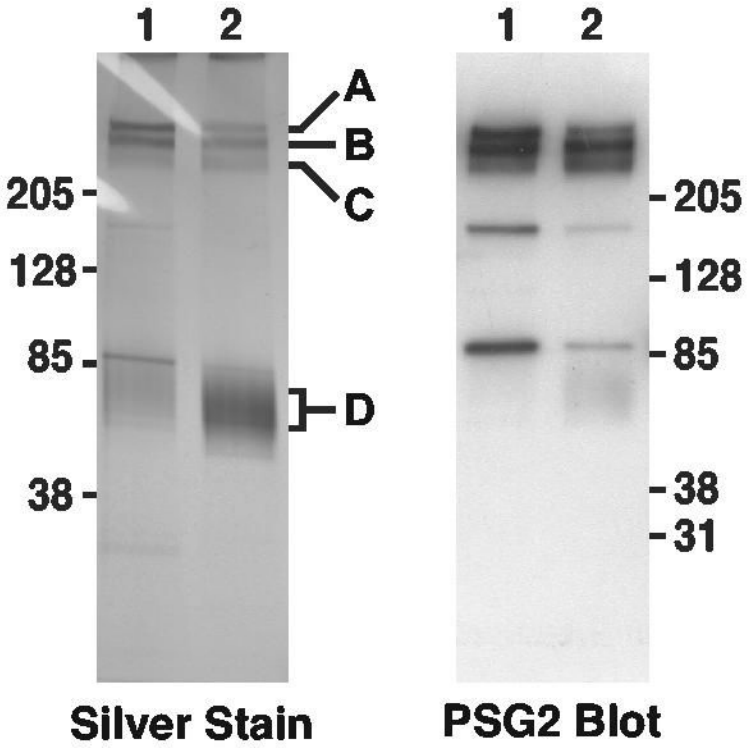

FIGURE 5.

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 which were then either silver stained (left panel) or transferred to PVDF membrane and probed with PSG2 (right panel). The bands subjected to in-gel tryptic digestion and LC-MS/MS sequencing are labeled (A-D).

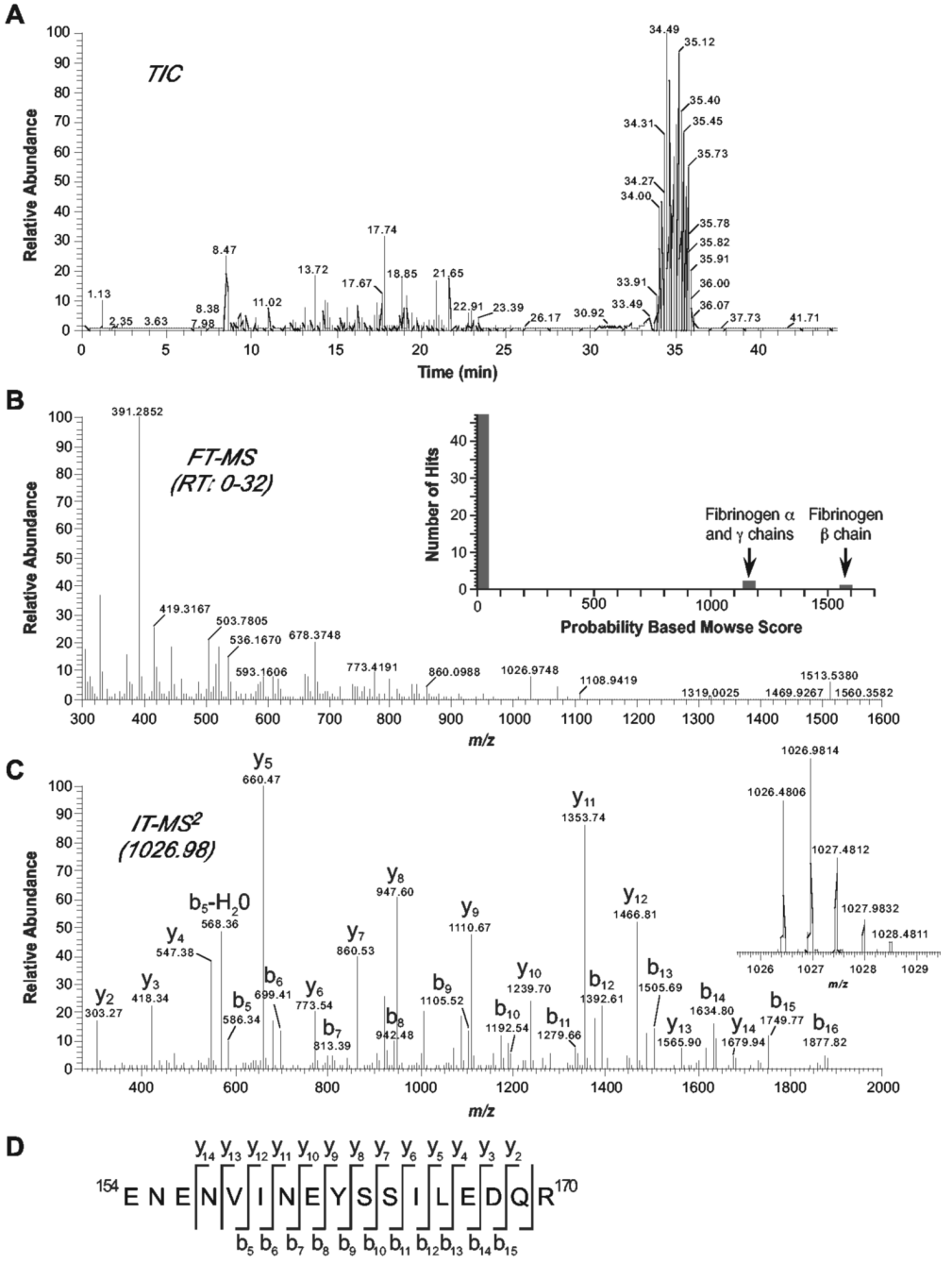

FIGURE 6.

LC-MS/MS Sequencing. A. Total ion chromatogram (TIC) of the tryptic digest from the protein gel band B shown in Figure 5. B. FTICR mass spectrum of peptides eluted in the first 32 minutes of the LC gradient. The insert shows the MASCOT histogram of the score distribution for the proteins identified in the gel band B. C. MS^2 scan of the m/z 1026.98 ion. The insert shows the precursor mass scan at 20.97 min using the FTICR (single scan from 300 to 1400 m/z with 100,000 resolution at 10^6 target ions). For illustration purposes, only the 1026 to 1029 m/z mass region of the $[M+2H]^{2+}$ ion is shown. D. Peptide sequence (154-170) from the fibrinogen β chain identified by MASCOT and manually validated from the MS^2 spectrum of the m/z 1026.98 ion.

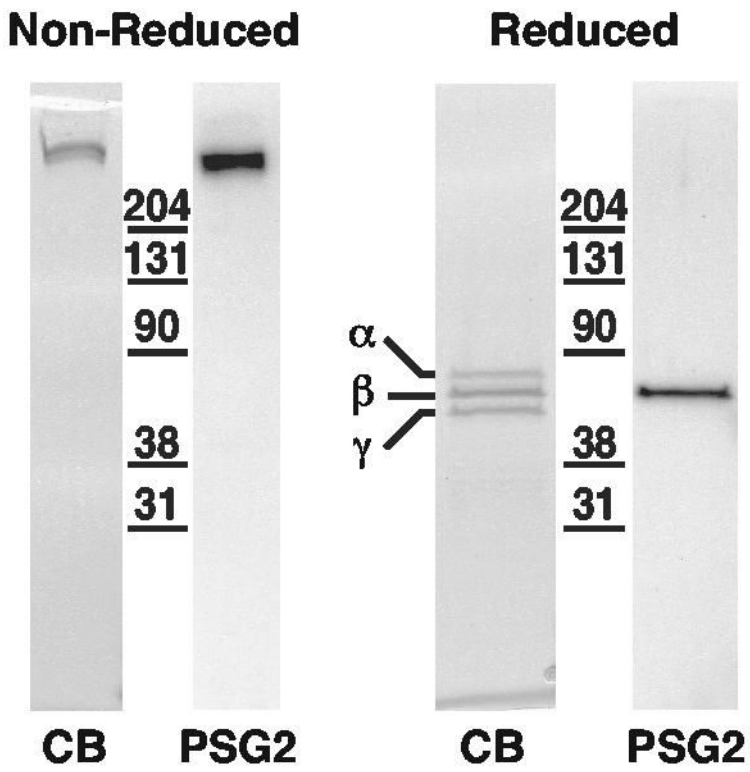

FIGURE 7.

PSG2 Western Blots of Mouse Fibrinogen. Purified mouse fibrinogen was run on 4-15% SDS gels, transferred to PVDF, and subjected to Western blotting with PSG2 as described in Experimental Procedures. In lanes stained with Coomassie blue (CB) 2 μg of protein were loaded. In lanes subjected to Western blotting with PSG2, 10 ng was loaded.

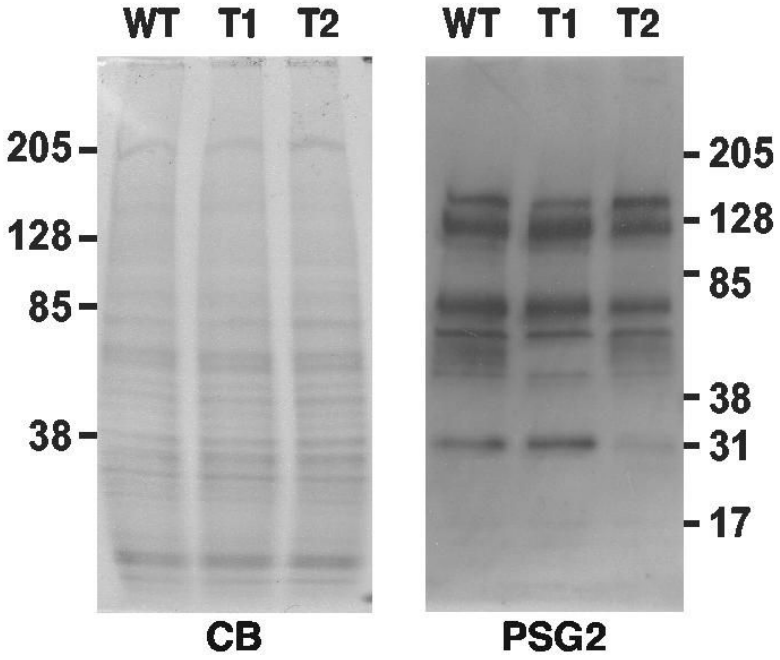

FIGURE 8.

PSG2 Western Blotting of Epididymal/Sperm Proteins. Detergent extracts of whole epididymis from wild type, *Tpst1*−/−, and *Tpst2*−/− mice (15 μg total protein) were run on 4-15% SDS-polyacrylamide gels and stained with Coomassie blue (CB) or transferred to PVDF and subjected to Western blotting with PSG2 as described in Experimental Procedures.

TABLE 1

IC_{50} for Inhibition of PSG2 Binding to Bovine Factor X_2 by Amino Acids and Peptides. Binding of PSG2 to bovine factor X_2 immobilized on microtiter plates was determined in the presence of increasing concentration of the indicated amino acid or pentapeptide as described in Experimental Procedures. Results represent the mean ± SD of 4 independent experiments.

Hapten	IC_{50} (mM)
pY	7.5 ± 1.2
sY	0.57 ± 0.31
LDYDF	> 2.0
LD(pY)DF	1.2 ± 0.98
LD(sY)DF	0.001 ± 0.0003

TABLE 2

Summary of LC-MS/MS Sequencing of Proteins Purified From Mouse Epididymis. Proteins purified by PSG2 affinity chromatography from a soluble fraction of wild type mouse epididymis were run on 4-15% SDS-polyacrylamide gels and the bands labeled A to D in Figure 5 were subjected to in-gel tryptic digestion and LC-MS/MS sequencing as described in Experimental Procedures.

Protein	NCBI gi number	MW (kDa)	Sequence coverage (%)	Number of unique peptides identified	RMS error (ppm)	Location on gel
Fibrinogen α chain	33563252	61.8	38	30	4	B (A, C)*
Fibrinogen β chain	33859809	55.4	61	38	5	B (A, C)*
Fibrinogen γ chain	19527078	50.0	54	28	4	B (A, C)*
Lumican	6678740	38.6	34	17	5	D

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