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Preparation and analysis of N-terminal chemokine receptor sulfopeptides using tyrosylprotein sulfotransferase enzymes

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Abstract

In most chemokine receptors, one or multiple tyrosine residues have been identified within the receptor N-terminal domain that are, at least partially, modified by post-translational tyrosine sulfation. For example, tyrosine sulfation has been demonstrated for Tyr-3, -10, -14, and -15 of CCR5, for Tyr-3, -14, and -15 of CCR8 and for Tyr-7, -12, and -21 of CXCR4. While there is evidence for several chemokine receptors that tyrosine sulfation is required for optimal interaction with the chemokine ligands, the precise role of tyrosine sulfation for chemokine receptor function remains unclear. Furthermore, the function of the chemokine receptor N-terminal domain in chemokine binding and receptor activation is also not well understood. Sulfotyrosine peptides corresponding to the chemokine receptor N-termini are valuable tools to address these important questions both in structural and functional studies. However, due to the liability of the sulfotyrosine modification, these peptides are difficult to obtain using standard peptide chemistry methods. In this chapter, we provide methods to prepare sulfotyrosine peptides by enzymatic *in vitro* sulfation of peptides using purified recombinant tyrosylprotein sulfotransferase (TPST) enzymes. In addition, we also discuss alternative approaches for the generation of sulfotyrosine peptides and methods from sulfopeptide analysis.

1. INTRODUCTION

With up to one percent of eukaryotic proteins potentially containing sulfotyrosine residues, sulfation of tyrosines is a common posttranslational modification whose biological impact has only just started to be elucidated (Ludeman & Stone, 2014; Moore, 2003; Seibert, Veldkamp, Peterson, Chait et al., 2008). In humans and most mammals there are two isoforms of the enzyme responsible for tyrosine sulfation, these enzymes are tyrosylprotein sulfotransferase 1 and 2 (TPST-1 and TPST-2) (Moore, 2003; Seibert & Sakmar, 2008). TPST-1 and TPST-2 are located in the *trans*-Golgi network and this limits tyrosine sulfation to secreted or membrane proteins (Moore, 2003; Seibert & Sakmar, 2008). Both enzymes utilize the cosubstrate PAPS, or 3'-phosphoadenosine-5'-phosphosulfate, as the sulfate donor to catalyze the sulfation of a tyrosine's phenolic hydroxyl in a substrate protein or peptide as seen in figure 1 (Moore, 2003; Seibert & Sakmar, 2008).

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Early studies of sulfotyrosine containing proteins involved in blood coagulation made it clear that sulfotyrosine posttranslational modifications influence protein-protein interactions (Moore, 2003; Seibert & Sakmar, 2008). Coagulation Factor VIII, or anti-hemophilic factor, contains a sulfotyrosine at position 1680 that is required for a strong interaction of the inactive form of Factor VIII with von Willebrand Factor. Mutation of tyrosine 1680 in Factor VIII to phenylalanine leads to the loss of sulfotyrosine at this position and results in a form of hemophilia A (Leyte, van Schijndel, Niehrs, Huttner et al., 1991). Hirudin, the well-known anticoagulant from the medicinal leech *Hirudo medicinalis*, is an inhibitor of thrombin that binds 10-fold more tightly to thrombin when it contains a sulfotyrosine at amino acid position 63 (S. R. Stone & Hofsteenge, 1986).

Just as coagulation and the coagulation cascade involve numerous protein-protein interactions that include sulfotyrosine residues, leukocyte chemo attraction, including leukocyte rolling, tight adhesion and transendothelial migration, does as well. For example, P-selectin glycoprotein ligand-1 (PSGL-1)'s binding to selectins is essential for leukocyte rolling and tethering, the initial step in the leukocyte extravasation cascade (Carlow, Gossens, Naus, Veerman et al., 2009). This PSGL-1 selectin interaction is, in part, mediated by sulfotyrosine residues located in the N-terminus of mature PSGL-1 (Rodgers, Camphausen, & Hammer, 2001; Wilkins, Moore, McEver, & Cummings, 1995). Resting T-cells expressing PSGL-1 show enhanced chemotaxis towards the homeostatic chemokines CCL19 and CCL21 and increased recruitment to secondary lymphoid organs (Veerman, Williams, Uchimura, Singer et al., 2007). However, these enhancements are not a result of the canonical PSGL-1 selectin interactions, but result from the N-terminus of PSGL-1 binding directly to CCL19 or CCL21 (Veerman et al., 2007; Veldkamp, Kiermaier, Gabel-Eissens, Gillitzer et al., 2015). It is not surprising that the acidic, sulfotyrosine containing N-terminus of PSGL-1 binds CCL19 or CCL21. The N-termini of chemokine receptors are acidic and many contain or are predicted to contain sulfotyrosine residues that enhance affinity for chemokine ligands (Farzan, Babcock, Vasilieva, Wright et al., 2002; Farzan, Vasilieva, Schnitzler, Chung et al., 2000; Ludeman et al., 2014; Seibert & Sakmar, 2008).

Farzan and colleagues were the first to show that specific tyrosine residues in the N-termini of the chemokine receptors CCR5 and CXCR4 were sulfated and that tyrosine sulfation increased the affinity for these receptors' chemokine ligands (Farzan, Babcock, et al., 2002; Farzan, Chung, Li, Vasilieva et al., 2002; Farzan, Mirzabekov, Kolchinsky, Wyatt et al., 1999). Furthermore, they have also shown that tyrosine sulfation of CCR5, which is a major coreceptor for HIV-1, is required for viral entry into host cells (Farzan et al., 1999). Sulfation of CXCR4 in contrast, which also acts as a major HIV-1 coreceptor, is not required for coreceptor function (Farzan, Babcock, et al., 2002).

Chemokines are hypothesized to activate their receptors through a two site, two step binding and activation model in which the chemokine receptor N-terminus binds to the chemokine domain (site one) followed by the chemokine N-terminus binding to a second site on the receptor leading to receptor activation (Crump, Gong, Loetscher, Rajarathnam et al., 1997; Kufareva, Salanga, & Handel, 2015). Farzan and colleagues illustrated the importance of sulfotyrosines for this site one interaction using an inactive CCR5 mutant lacking N-terminal residues 2–17 (Bannert, Craig, Farzan, Sogah et al., 2001; Farzan, Chung, et al., 2002).

CCL3 or CCL3 could only activate a CCR5 2-17 induced intracellular calcium flux when the receptor was rescued by the presence of synthetic CCR5 N-terminal peptides containing sulfated tyrosines but not unsulfated counterparts (Bannert et al., 2001; Farzan, Chung, et al., 2002). Subsequently many researchers have used protein NMR and sulfotyrosine containing peptides corresponding to a chemokine receptor N-terminus to mimic and study the site one interaction between a chemokine receptor and its chemokine ligand (Duma, Haussinger, Rogowski, Lusso et al., 2007; Millard, Ludeman, Canals, Bridgford et al., 2014; Simpson, Zhu, Widlanski, & Stone, 2009; Veldkamp, Seibert, Peterson, De la Cruz et al., 2008; Veldkamp, Seibert, Peterson, Sakmar et al., 2006).

While several investigators have used chemically synthesized sulfotyrosine containing peptides to mimic a chemokine receptor's N-terminus (Bannert et al., 2001; Cormier, Persuh, Thompson, Lin et al., 2000; Duma et al., 2007; Farzan, Chung, et al., 2002; Ludeman et al., 2014; Millard et al., 2014; Simpson et al., 2009; J. H. Tan, Ludeman, Wedderburn, Canals et al., 2013; Q. Tan, Zhu, Li, Chen et al., 2013), our approach differs in that we used recombinant TPSTs to enzymatically sulfate peptides. Moore and colleagues cloned human TPST-1 and TPST-2 and showed recombinant TPSTs from mammalian expression systems had sulfotransferase activity in the presence of the cosubstrate PAPS (Moore, 2003; Y. B. Ouyang & Moore, 1998; Y. Ouyang, Lane, & Moore, 1998). We and others have used TPST-1 and TPST-2 to characterize the sulfation of N-terminal peptides from CCR5 (Seibert, Cadene, Sanfiz, Chait et al., 2002); (Jen, Moore, & Leary, 2009). We have also used TPST enzymes to characterize the enzymatic sulfation of CXCR4 (Seibert, Veldkamp, et al., 2008), a chemokine receptor that plays significant roles in cancer metastasis (Ben-Baruch, 2008; Muller, Homey, Soto, Ge et al., 2001). Using sulfotyrosine containing CXCR4 N-terminal peptides the structural basis for the site one interaction between the CXCR4 N-terminus and CXCL12 was also probed (Seibert, Veldkamp, et al., 2008; Veldkamp et al., 2008; Veldkamp et al., 2006).

Here we describe approaches for utilizing recombinant TPST-1 and TPST-2 to enzymatically sulfate N-terminal chemokine receptor peptides and the characterization of these sulfopeptides. For a more expansive introduction to TPST enzymes and proteins containing sulfotyrosines posttranslational modifications, see the following references (Ludeman et al., 2014; Moore, 2003, 2009; Seibert & Sakmar, 2008; M. J. Stone, Chuang, Hou, Shoham et al., 2009).

2. METHODS

2.1 Expression and purification of TPST-1 and -2 from mammalian cells

TPSTs are type II transmembrane proteins with a single α -helical transmembrane segment that anchors the catalytic domain in the Golgi lumen (Moore, 2003, 2009). To improve heterologous expression and purification soluble recombinant variants of human TPST-1 and TPST-2 have been engineered that are suitable for *in vitro* sulfation of peptide substrates (Y. B. Ouyang et al., 1998). These TPST variants lack the cytoplasmic N-terminus and the transmembrane domain, which are not required for enzymatic activity. Furthermore, an N-terminal transferring signal peptide followed by a protein C epitope were N-terminally fused

to the catalytic domain of both TPSTs (residues 25 to 370 and 25 to 377 of full length TPST-1 and TPST-2, respectively) to aid in expression and purification of the enzymes.

In this section, we provide methods for the expression and purification of recombinant engineered TPST-1 and TPST-2 using transiently transfected HEK293-T cells. With these methods, which were adapted from published procedures (Y. B. Ouyang et al., 1998; Seibert et al., 2002), 100 µg quantities of purified TPST-1 and TPST-2 can be obtained that are sufficient for analytical and semipreparative-scale peptide sulfation reactions (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008; Veldkamp et al., 2008; Veldkamp et al., 2006).

2.1.1 Required materials

HEK293-T cells (ATCC)

pMSH1TH and pMSH2TH expression vectors (Dr. Kevin L. Moore, University of Oklahoma Health Sciences Center)

Cell culture media and supplements (Gibco)

Disposable cell culture materials (Corning or Falcon)

Plasmid purification kit (Qiagen)

LipofectAMINE Plus (Invitrogen)

Complete™ protease inhibitor mixture (Roche)

Anti-protein C resin (Roche)

Disposable plastic column with stopcocks (BioRad)

Centricon YM10 concentrators (Millipore)

SDS-PAGE gels (10%), electrophoresis buffers, staining solution, and electrophoresis system (BioRad)

Analytical-grade reagents (Sigma-Aldrich or Fisher Scientific)

Standard cell culture equipment (cell culture incubator, sterile cell culture hood, centrifuge etc.)

2.1.2 Cell culture and transfection

1. Prepare transfection-grade pMSH1TH and/or pMSH2TH DNA using a plasmid purification kit according to the manufacturer's protocol.
2. Culture HEK293-T cells in DMEM supplemented with 10% FBS using 100 mm cell culture plates and maintain cells at 37 °C with 5% CO₂.
3. Expand HEK293-T cells into as many 100 mm cell culture plates as required, assuming a yield of approximately 4 µg purified TPST-1 or 8 µg purified TPST-2 per plate of transfected HEK293-T cells.
4. Transfect HEK293-T cells with pMSH1TH or pMSH2TH plasmid (4 µg plasmid per plate) using LipofectAMINE Plus™ (20 µl Plus reagent and 30 µl LipofectAMINE reagent per plate) according to the manufacturer's protocol.

5. After 48 h, wash plates with 5 ml PBS each and harvest transfected HEK293-T cells in 2 ml per plate of ice-cold PBS supplemented with Complete[™] protease inhibitor mixture (without EDTA).
6. Collect cells by centrifugation (15 min at 10,000 μ g) and proceed with anti-protein C immunoaffinity purification (section 2.1.3).

2.1.3 Anti-protein C immunoaffinity purification of TPSTs

1. Resuspend transfected HEK293-T cells in 0.5 ml per plate of ice-cold solubilization buffer (20 mM TAPS, pH 9.0, 100 mM NaCl, 1% Triton X-100, Complete[™] protease inhibitor mixture without EDTA), and incubate for 1 to 2 h at 5 °C (on a Nutator).
2. Centrifuge (20 min at 10,000 μ g) to separate solubilized proteins from cell debris. Collect supernatant and continue with step 3 or store at –80 °C.
3. Wash anti-protein C resin (use 30 – 50 μ l of resin per plate of transfected HEK293-T cells) with equilibration buffer (50 mM MOPS, pH 7.5, 100 mM NaCl, 5 mM CaCl₂, 1 % (v/v) Triton X-100).
4. Add 50 mM MOPS, pH 7.5, 5 mM CaCl₂ and 10% glycerol to supernatant, and incubate with washed anti-protein C resin over night at 4°C (on a Nutator).
5. Pour anti-protein C resin slurry into a disposable column.
6. Wash anti-protein C resin with 10 column volumes of ice-cold wash buffer 1 (20 mM MOPS, pH 7.5, 2 M NaCl, 2 mM CaCl₂, 0.1% Triton X-100) followed by 10 column volumes of ice-cold wash buffer 2 (20 mM MOPS, pH 7.5, 150 mM NaCl, 2 mM CaCl₂, 0.1% Triton X-100).
7. Elute bound TPST protein with 10 column volumes of ice-cold elution buffer (20 mM MOPS, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 10% glycerol) and collect eluent fractions.
8. Analyze TPST eluent fractions by SDS-PAGE on 10% gels under reducing conditions and staining with Coomassie brilliant blue R-250 according to standard protein chemistry protocols.
9. Pool TPST containing fractions, concentrate with Centricon YM10 concentrator to yield a final TPST concentration between 0.3 and 0.8 μ g/ μ l, and store aliquots at –80 °C.
10. Determine concentration of purified TPST preparations by densitometry of Coomassie brilliant blue R-250 stained SDS-PAGE gels with BSA as an internal standard.
11. Test the enzymatic activity of purified TPST preparations by tyrosine sulfation assays (see section 2.4) using the PSGL-1 1–15 peptide (QATEY EYLDYDFLPE-NH₂) as a standard substrate.

2.2 Expression and refolding of functional TPST-1 from *E. coli*

For certain structural and functional studies, in particular NMR spectroscopy studies, rather large quantities of sulfotyrosine peptides in the ten-milligram range are required. Due to the low tyrosine sulfation activities of soluble TPST enzymes for peptide substrates, enzymatic peptide sulfation at this scale requires quantities of TPST enzymes that are difficult and expensive to obtain from transfected HEK293 cells. In this section, we describe methods to produce hundred milligram-quantities of a soluble recombinant variant of human TPST-1 by using an *E. coli* expression system. Expression of this TPST-1 variant in *E. coli* results in incorrectly folded protein that accumulates in inclusion bodies. Active enzyme is obtained by solubilization of inclusion bodies followed by functional refolding of TPST-1.

Due to the overlapping substrate specificities of TPST enzymes (Seibert, Veldkamp, et al., 2008), it should be possible to efficiently sulfate most peptide substrates with TPST-1 at sufficiently high enzyme concentrations. However, if TPST-2 is required for sulfation of a specific peptide, a protocol for *E. coli* expression and functional refolding of TPST-2 can be found elsewhere (Teramoto, Fujikawa, Kawaguchi, Kurogi et al., 2013).

2.2.1 Required materials

pMSH1TH expression vectors (Dr. Kevin L. Moore, University of Oklahoma Health Sciences Center) and pET28a(+) expression vector (Novagen) or pET28a-TPST1 expression vector

Transformation competent *E. coli* BL21 (DE3) cells (Novagen)

Luria Broth (LB) (Gibco)

Complete[™] protease inhibitor cocktail (Roche)

Guanidine hydrochloride (GndHCl) (Sigma-Aldrich)

n-dodecyl- β -D-maltoside (DM) (Anatrace)

Reduced glutathione (GSH) and oxidized glutathione (GSSG) (Sigma-Aldrich)

15 ml polycarbonate tubes with conical bottom (Falcon, Greiner or Sarstedt)

Parafilm

YM10 ultrafiltration membranes and ultrafiltration device (Amicon Millipore)

Bradford protein assay (BioRad)

SDS-PAGE gels (10%), electrophoresis buffers, staining solution, and electrophoresis system (BioRad)

Analytical-grade reagents (Sigma-Aldrich or Fisher Scientific)

French press pressure cell

FPLC system with Superdex 200 26/60 size exclusion column (GE Healthcare)

Peristaltic pump with 0.01 inch ID silicone tubing (Rainin)

Standard laboratory equipment (bacterial incubator, centrifuge, ultracentrifuge, UV-VIS Spectrometer, Nutator, magnetic stirrer etc.)

2.2.2 Transformation and expression of TPST-1 as inclusion bodies in *E. coli*

1. Construction of the pET28-TPST1 plasmid for expression of recombinant engineered human TPST-1 in *E. coli*: The cDNA encoding the catalytic domain and stem region of human TPST-1 (amino acids 25 to 370) was amplified by polymerase chain reaction (PCR) using the eukaryotic expression vector pMSH1TH as a template and introducing 5' *NheI* and 3' *XhoI* restriction sites. After *NheI* and *XhoI* cleavage, the PCR fragment was spliced into a pET28a(+) vector, that was modified to introduce a hexa-histidine (H₆) tag followed by a Precision protease cleavage site (LEVLFQ/GP) at the N-terminus of the TPST-1 construct. Due to the subcloning strategy, a total of 26 residues (MGSSHHHHHSSGLEVLFG/GPHMASM) were fused to Gly-25 of TPST-1. The pET28-TPST1 plasmid can be obtained from the authors (after obtaining permission from Dr. Kevin L. Moore) or constructed according to the described strategy.
2. Transform competent *E. coli* BL21 (DE3) cells with the pET28a-TPST1 expression plasmid and grow transformed *E. coli* at 37 °C in LB medium supplemented with 50 mg/ml of kanamycin.
3. Inoculate 1.6 L cultures with 1.6 mL each of a 100 ml over-night starter culture and incubate at 37 °C on a shaker platform.
4. At a cell density corresponding to an OD_{600nm} of approximately 0.4 add IPTG at a final concentration of 0.4 mM to induce TPST-1 expression, and incubate cultures for 3 h at 37 °C.
5. Harvest *E. coli* cells by centrifugation (25 min, 3600 rpm, 4 °C) and resuspend in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0; 10 mM MgCl₂; 1 mM DTT; 1 mM PMSF; 1 tablet per 25ml of Complete™ protease inhibitor cocktail without EDTA) using 3 ml of lysis buffer for each gram of *E. coli* cell pellet. Freeze aliquots of resuspended *E. coli* cells with liquid nitrogen and store at -80°C.

2.2.3 Solubilization of TPST-1 from inclusion bodies

1. Thaw *E. coli* cells containing TPST-1 inclusion bodies (from section 2.2.2) on ice and lyse cells by 3 passages through a French press pressure cell at 15,000 psi.
2. Collect inclusion bodies by centrifugation for 1 h at 10,000 x g and 4 °C and remove the yellow layer of membrane fragments from on top of the white inclusion body pellet.
3. Wash inclusion bodies with ice-cold WB1 (50 mM Tris-HCl pH 8.0; 100 mM NaCl; 10 mM EDTA; 1% (w/v) Triton X-100; 1 mM PMSF; 1 tablet per 25 ml of Complete™ protease inhibitor cocktail without EDTA).
4. Wash inclusion bodies with ice-cold WB2 (20 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA; 1 mM PMSF).

5. Resuspend inclusion bodies in solubilization buffer (SB) (100 mM Tris-HCl, pH 8.0; 6 M guanidine hydrochloride (GndHCl); 5 mM EDTA; 10 mM DTT) using 10 ml SB for 1 g of inclusion bodies and incubate at 5 °C with constant agitation using a Nutator. After 12 h add fresh DTT (10 mM) and continue incubation for 2 h at room temperature.
6. Remove insoluble material by centrifugation (30 min, 125,000 x g, 4 °C) and concentrate supernatant containing solubilized unfolded TPST-1 by ultrafiltration using a 10 kDa cutoff membrane (YM10, Amicon Millipore).
7. Dilute concentrated TPST-1 solution 10-fold with buffer A (100 mM Na-acetate, pH 4.5; 6 M GndHCl; 10 mM DTT), concentrate by ultrafiltration using a 10 kDa cutoff membrane (YM10, Amicon Millipore) to about 30 mg/ml, and store aliquots of this TPST-1 stock solution at –80 °C.
8. Determine protein concentrations by the Bradford method with bovine serum albumin (BSA) as a standard.

2.2.4 Purification of TPST-1 by size exclusion chromatography under denaturing conditions (optional)

—Optionally, unfolded TPST-1 can be purified by size exclusion chromatography under denaturing conditions to separate monomeric from aggregated TPST-1.

1. Equilibrate a Superdex 200 26/60 size exclusion column with buffer B (100 mM Na-acetate, pH 4.5; 6 M GndHCl; 5 mM DTT) at a flow rate of 2 ml/min and with UV detection at 280 nm.
2. Load 500 µl of concentrated TPST-1 stock solution in buffer A to the column and collect eluent in 5 ml fractions.
3. Pool fractions corresponding to monomeric TPST-1, concentrate by ultrafiltration using a 10 kDa cutoff membrane (YM10, Amicon Millipore) to a final concentration of about 30 mg/ml, and store aliquots at –80 °C.
4. Determine protein concentrations by the Bradford method with bovine serum albumin (BSA) as a standard.

2.2.5 TPST-1 Refolding—TPST-1 refolding is achieved by diluting a concentrated (30 mg/ml) stock solution of unfolded TPST-1 in buffer A into a large (200-fold) volume of the refolding buffer. The refolding conditions were first optimized in small-scale (1 ml) refolding trials using a fractional factorial refolding screen (Chen & Gouaux, 1997). Conditions were then adjusted and further optimized for large-scale refolding reactions by successively increasing the reaction volume from 100 ml to 2000 ml.

1. For a 1000 ml scale refolding reaction transfer approximately 5 ml of concentrated TPST-1 stock solution in buffer A (containing approximately 150 mg of unfolded TPST-1) into a 15 ml polycarbonate tube with conical bottom that is kept on ice.
2. Prepare 1000 ml of filtered (0.22 µm) 50 mM Tris-HCl, pH 8.5, 500 mM Guanidinium-HCl, 10 mM NaCl, 0.4 mM KCl, 1 mM EDTA buffer, transfer to a 1500 ml beaker,

add a magnetic stir bar, and refrigerate to 5 °C. Finalize the refolding buffer by flushing with argon and adding 0.14 mM of DDM, 5 mM of GSH, and 2.5 mM of GSSG. Cover the beaker with Parafilm and place on a magnetic stirrer.

3. Assemble the refolding setup in a cold room or a large refrigerator kept at 5 °C. To slowly add the TPST-1 stock solution to the refolding buffer, use a peristaltic pump with 0.01 inch ID silicon tubing. Place one end of the silicon tubing into the TPST-1 stock solution so that it reaches to the bottom of the conical tube. Place the other end of the silicon tubing so that it just touches the surface of the refolding buffer (use P10 tip) (keep covered with Parafilm).
4. To start the refolding reaction, first switch on the magnetic stirrer and adjust the stir speed so that a small vortex forms, that just reaches the bottom of the beaker. Next, start the peristaltic pump and set it to a flow rate between 10 and 20 µl/min. After TPST-1 addition is complete, stop the magnetic stirrer and incubate the refolding mixture for 20 h at 4 °C.
5. Centrifuge (30 min, 125,000 x g, 4 °C) the refolding mixture to remove precipitated protein.
6. Concentrate the supernatant containing refolded TPST-1 by ultrafiltration using a membrane with 10-kDa cut-off (YM10, Amicon Millipore).
7. Dialyze the concentrated refolding mixture against storage buffer (20 mM MOPS, pH 7.5; 150 mM NaCl; 10 mM EDTA; 0.1% Triton X-100; 10% glycerol). Store aliquots at -80 °C.
8. Determine protein concentrations by the Bradford method using BSA as a standard.
9. Analyze TPST-1 preparations by SDS-PAGE on 10% gels and by TPST-1 sulfation assay using PSGL-1 peptide (QATEY EYLDYDFLPE-NH₂) as a standard (section 2.4). (on average, 80% yield of TPST-1 protein with 30 to 50 % specific activity compare to TPST-1 from eukaryotic expression)

2.3 Analysis and purification of PAPS

In our experience, PAPS from commercial sources was generally about 80% pure. However, significantly lower purities have been observed in some cases. A major impurity in any PAPS preparation is 3'-phosphoadenosine-5'-phosphate (PAP), which is formed by spontaneous hydrolysis of PAPS. As a by-product of the TPST-catalyzed sulfotransfer reaction PAP acts as a product inhibitor of tyrosine sulfation (Danan, Yu, Hoffhines, Moore et al., 2008; Danan, Yu, Ludden, Jia et al., 2010), which is one reason why sulfation rates of *in vitro* sulfation reactions decrease over time. Thus, for efficient *in vitro* sulfation of peptide substrates, in particular, if multiple sulfation sites are present, high-purity (80%) PAPS preparations with low (<10%) PAP content are to be used. For these reasons, we found it necessary to routinely analyze all PAPS preparations by ion-pair HPLC, and to re-purify low-purity PAPS preparations by Mono Q anion-exchange chromatography. Furthermore, to minimize the formation of PAP, PAPS preparations should be kept at neutral pH and at very low temperatures (-80 °C).

2.3.1 Required materials

PAPS (3'-phosphoadenosine-5'-phosphosulfate) and PAP (3'-phosphoadenosine-5'-phosphate) can be obtained from various commercial sources (Calbiochem/EMD Biosciences, Sigma/Aldrich, Fluka, R & D Systems)

HPLC-grade solvents (Pierce or Fisher Scientific)

Tetrabutylammonium phosphate (TBAP) (Alltech)

Dowex 50WX8/H⁺ resin (400 – 200 mesh) p.a. (Fluka)

Analytical-grade reagents (Sigma-Aldrich or Fisher Scientific)

HPLC-system with UV-VIS detector

LiChrosphere 100 RP-18 end capped (5 µm, 250 × 4.6 mm) (EMD Millipore) or similar analytical RP-HPLC column

FPLC system with MonoQ HR5/5 column (GE Healthcare)

Standard laboratory equipment (SpeedVacTM, lyophilizer, UV-VIS Spectrometer, etc.)

2.3.2 Preparation of PAPS stock solutions

1. Dissolve lyophilized PAPS in HPLC-grade water at 3 to 4 mM.
2. Measure pH, and neutralize by adding approximately 10 µl of 1 M Tris-HCl, pH 7.5 to 1 ml of PAPS solution if pH is below 7.0.
3. Precisely determine PAPS concentration by UV spectroscopy using a molar extinction coefficient of 15400 M⁻¹cm⁻¹ (at 259 nm and pH 7.0).
4. Analyze PAPS stock solution by ion-pair RP-HPLC (section 2.3.3).
5. Store aliquots at –80 °C.

2.3.3 Analysis of PAPS by ion-pair RP-HPLC—This method was modified after a published procedure by Pennings and van Kempen (Pennings, 1979).

1. Prepare eluent A (10 mM NH₄H₂PO₄, pH 5.5, 5 mM TBAP, in water) and eluent B (100% acetonitrile), filter and degas.
2. Equilibrate an analytical LiChrosphere 100 RP-18 HPLC column with 20% B at a flow rate of 1.5 ml/min. For each HPLC analysis, inject a 20 µl sample onto the column and apply an eluent gradient from 20 to 50% B in 20 min at a flow rate of 1.5 ml/min with UV detection at 260 nm.
3. To test and calibrate the HPLC system, first perform a blank analysis by injecting a water sample. Next analyze a PAP reference, which should elute at ca. 12 min followed by a PAPS reference, which should elute at ca. 16 min.
4. Analyze the PAPS sample and calculate PAPS purity from peak integration data of the HPLC chromatogram.

2.3.4 Purification of PAPS by Mono Q anion exchange chromatography

(optional)—Optionally, if PAPS purity is less than 80% or PAP content exceeds 10%, PAPS can be purified by Mono Q anion exchange chromatography (Burkart, Izumi, Chapman, Lin et al., 2000).

1. Prepare eluent A (water) and eluent B (1 M NH_4HCO_3 , in water), filter and degas.
2. Equilibrate a Mono Q HR5/5 column with 100% A at a flow rate of 1 ml/min.
3. Inject 250 μl sample of PAPS stock solution onto the column and apply an eluent profile of 0% B for 5 min followed by 0 to 100% B in 55 min, and 100% B for 5 min at a flow rate of 1 ml/min with UV detection at 245 nm.
4. Collect 0.5 ml eluent fractions and pool fractions corresponding to PAPS (PAP should elute at ca. 26 min and PAPS at ca. 40 min).
5. Using a vacuum filtration device with a sintered glass filter, wash Dowex 50WX8/ H^+ resin with water followed by methanol and ethanol, and air-dry resin.
6. Neutralize pooled PAPS fractions (from step 4) by stepwise adding washed Dowex 50WX8/ H^+ resin and carefully monitoring the pH to avoid acidification.
7. Filter the PAPS solution, freeze with liquid nitrogen and lyophilize.
8. To convert PAPS to its tetralithium salt, prepare Dowex 50WX8/ Li^+ resin: Treat Dowex 50WX8/ H^+ resin stepwise with 1 M LiCl until the pH is neutral, wash with water followed by ethanol, and air-dry resin.
9. Dissolve PAPS (from step 7) with HPLC-grade water (at 3 to 4 mM) and treat with Dowex 50WX8/ Li^+ resin. Filter the PAPS solution, and neutralize with approximately 10 μl of 1 M Tris-HCl, pH 7.5 for 1 ml of PAPS solution.
10. Analyze PAPS solution by ion-pair RP-HPLC (section 2.3.3), determine PAPS concentration by UV spectroscopy (section 2.3.2), and store aliquots at -80°C .

2.4 *In vitro* sulfation of N-terminal chemokine receptor peptides using TPST enzymes

We have not found fluoride, manganese, or 5'AMP to be essential for TPST-1 or TPST-2 activity. Dithiothreitol (DTT) is also optional as precaution for preventing possible methionine oxidation if peptides contain methionine(s).

2.4.1 Required materials

- 400 mM PIPES pH 6.8
- 10% Triton X-100
- 5 M NaCl
- 1 M DTT (optional)
- 3–4 mM PAPS (>80%, see section 2.3)
- 0.5 mg/mL TPST-1 or TPST2
- Concentrated stock of N-terminal peptide dissolved in water, pH 6.8

2.4.2 Conditions for enzymatic sulfation of peptides

1. Combine the above reagents with a volume of HPLC grade water to produce final concentrations of 40 μ M PIPES, 100 mM NaCl, 0.10% Triton X-100, 10 mM DTT (optional), 400 μ M PAPS, 50–100 μ M peptide and 0.05 mg/mLTPST-1 or TPST-2. Control reactions can leave out the PAPS cosubstrate or TPST enzyme.
2. Incubate at 16 °C.
3. The reaction can be monitored using RP-HPLC and mass spectrometry by removing 50 μ L of the reaction every 12 to 24 hours. See RP-HPLC and mass spectrometry sections for protocols. New peak(s) with increasing intensity in the RP-HPLC chromatograms along with a decreased peak intensity for the unsulfated peptide peak correlate with tyrosine sulfation. See Figure 2.

2.5 Reversed phase-HPLC of sulfopeptides

While successful separation of sulfotyrosine peptides has been described with standard RP-HPLC solvent systems containing 0.1% trifluoroacetic acid, we found that separation at pH 6.5 using 20 mM ammonium acetate as a buffer gave best results. Under these conditions, the potential loss of the sulfotyrosine modification caused by acid catalyzed hydrolysis is minimized (Seibert & Sakmar, 2008; M. J. Stone & Payne, 2015). Furthermore, at pH 6.5 resolution of closely related sulfotyrosine peptides was greatly improved compared to resolution in the standard acidic solvent system.

2.5.1 Required Materials

2:1 Chloroform methanol solution
Bioselect™ C18 SPE columns (218SPE3000, Grace Vydac)
Analytical RP-HPLC Column (218TP54, Grace Vydac)
Semipreparative RP-HPLC Column (218TP510, Grace Vydac)
SpeedVac™ with heater disabled or lyophilizer
RP-HPLC with UV-Vis detector
HPLC buffer A: 20 mM Ammonium Acetate, pH 6.5 in water
HPLC buffer B: 20 mM Ammonium Acetate, pH 6.5, 70% Acetonitrile
0.22 μ m spin filter

2.5.2 Analytical RP-HPLC—RP-HPLC can be used to follow sulfation reaction (monitoring absorbance at 220 nm) for the appearance of new sulfopeptide peaks and the loss of the unsulfated peak (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008). Peaks can be collected to purify individual sulfopeptides.

2.5.2.1 Sample clean up

1. Extract the sulfation reaction or portion thereof with 3.5 to 4 times the reaction volume of 2:1 chloroform/methanol solution. Save the aqueous layer.

2. Equilibrate the SPE column(s) (1 mL HPLC buffer A, followed by 1 mL HPLC buffer B, followed by 3 mL HPLC buffer A)
3. Load the aqueous layer onto the SPE column. Do not exceed 5 mg of total peptide; use additional SPE columns.
4. Wash the SPE column with 1 mL buffer A.
5. Elute the SPE column with 1.5 mL of a 50:50 mixture of HPLC buffers A and B.
6. Strip the SPE column with 100% acetonitrile.
7. SpeedVacTM or lyophilize the elution.

2.5.2.2 RP-HPLC

1. Dissolve the cleaned up, dried sulfation reaction in a volume of water equivalent to the reaction volume from which the sample came. For example, if a 50 μ L aliquot of a sulfation reaction was prepared for HPLC, dissolve the cleaned up, dried sample in 50 μ L of water.
2. Spin filter the dissolved sulfation reaction
3. Analyze 50 μ L of the dissolved, filtered sulfation reaction using the analytical RP-HPLC column with a linear gradient of HPLC buffer B from 5–60% over 40 minutes. If using an HPLC with a detector limited to one wavelength, monitor at 220 nm.
4. Collect eluting fractions corresponding to peptide peaks.
5. Pool peaks and SpeedVacTM or lyophilize.

2.5.3 Semipreparative RP-HPLC—Purification of large amounts of sulfopeptides requires scaling up the RP-HPLC procedure.

1. Semipreparative RP-HPLC follows the same general procedure as the analytical RP-HPLC with some exceptions. A semipreparative column is used with a flow rate of 3 mL/min and with a larger, 1, 5 or 10 mL injection loop.
2. The gradient may also need to be optimized to obtain baseline separation of peaks when purifying large quantities of sulfopeptides (0.5 to 1 mg of total peptide). Start by injecting 50 μ L and incrementally increasing the percent HPLC buffer B at the start of the gradient while decreasing the percent HPLC buffer B at the end of the gradient until peptide peaks are very well resolved. Then incrementally increase the injected volume or total peptide injected. Stop increasing when it appears baseline separation will be lost or column capacity may be exceeded. For example, using a linear gradient from 19–30% HPLC buffer B over 40 minutes up to one milligram of total peptide from a sulfation reaction using the CXCR4 N-terminus as substrate could be separated into the individual resulting sulfopeptides (Seibert, Veldkamp, et al., 2008). See Figure 3.

2.6 Mass spectrometry of sulfotyrosine peptides

Analysis of sulfotyrosine peptides and localization of sulfotyrosine positions in the presence of multiple potential sulfation sites can be a challenging task, in particular, if multiple sulfotyrosines are present in a single peptide chain, which is the case with most N-terminal chemokine receptor peptides. While mass spectrometry analysis of protein phosphorylation on a proteomics scale is well established, this is not the case for protein tyrosine sulfation. Due to the inherent lability of the sulfotyrosine sulfoester bond, partial or complete loss of the sulfotyrosine modification is generally observed as a neutral loss of SO_3 ($M_r = -80$ Da) under standard mass spectrometry conditions. In particular, irrespective of the desorption/ionization method employed, positive ion mode mass spectrometry methods generally lead to complete loss of the sulfotyrosine modification. In negative ion mode mass spectrometry, in contrast, using optimized experimental conditions, a high degree of sulfotyrosine retention has been observed (see (Seibert & Sakmar, 2008) for review). For example, analyzing a N-terminal CXCR4 peptide with three sulfotyrosines in negative ion mode electrospray ionization (ESI) mass spectrometry, we observed complete retention of all three sulfotyrosine modifications. Analysis of the same peptide using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry in negative ion linear mode, on the other hand, resulted in a major peak corresponding to triple sulfated peptide with additional peaks corresponding to the neutral loss of one or two SO_3 (Seibert, Veldkamp, et al., 2008).

The biggest obstacle for sulfotyrosine analysis to date is the lack of a robust and reliable mass spectrometry fragmentation method for sequencing sulfotyrosine peptides and localizing the sulfation sites by tandem mass spectrometry (MS/MS). In particular, collision induced dissociation (CID), which in the past has been the workhorse method for fragment ion generation in MS/MS and therefore is widely available in mass spectrometry labs and core facilities, generally results in extensive loss of sulfate in the observed fragment ions (see (Seibert & Sakmar, 2008) for review). In recent years, novel fragmentation methods such as electron capture dissociation (ECD), electron transfer dissociation (ETD), electron detachment fragmentation (EDD), metastable atom-activated dissociation (MAD), and ultraviolet photodissociation (UVPD) have been developed, which favor peptide backbone fragmentation over fragmentation of labile side chain modifications. Using these fragmentation methods, it was demonstrated that sulfated fragment ions sufficient for sulfotyrosine localization can be produced, in particular, if these methods were adopted for negative ion mode (see (Robinson, Moore, & Brodbelt, 2014; Seibert & Sakmar, 2008) and refs. therein). While these results are promising, most of these methods have so far been used in pilot studies only, and therefore the available data is limited to a small number of model peptides with one or two sulfotyrosines. It remains to be seen, which of these methods are capable of analyzing more challenging peptides and proteins containing multiple sulfotyrosines in highly acidic sequences and at a proteomics scale.

These novel fragmentation methods require highly specialized equipment that is currently not readily available at many institutions. Therefore, we provide alternative methods for sulfotyrosine localization that are based on the generation of peptide fragments by proteolytic cleavage of sulfotyrosine peptides. While this approach can be laborious and is

not suited for sulfotyrosine analysis at a proteomics-scale, it might still be the best option, if a small number of closely related sulfotyrosine peptides of known sequence is to be analyzed, as is the case with identifying the products of an *in vitro* sulfation reaction (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008).

Another robust method for sulfotyrosine localization, that does not rely on sulfotyrosine retention in fragment ions and should be considered as an alternative to the method described by us, was developed by Leary and coworkers (Yu, Hoffhines, Moore, & Leary, 2007). This method is based on the acetylation of unsulfated tyrosine hydroxyl groups using sulfosuccinimidyl acetate, leaving sulfated tyrosine residues unmodified. MS/MS analysis of the derivatized peptides in positive ion mode using CID as the fragmentation method results in complete loss of tyrosine sulfation but leaves tyrosine acetylation intact. Thus, sulfotyrosine residues are identified as unmodified tyrosines whereas unsulfated tyrosines are observed as acetyltyrosines in the fragment ions. Using this method, Leary and coworkers were able to localize sulfotyrosine residues in numerous peptides, including several N-terminal chemokine receptor peptides with multiple sulfation sites (Jen et al., 2009; Yu et al., 2007).

2.6.1 Required materials

Sequencing-grade proteases (Roche)

α -cyano-4-hydroxycinnamic acid (4HCCA) (Sigma)

HPLC-grade solvents (Pierce or Fisher Scientific)

RP-C18 ZipTips (Millipore)

Analytical-grade reagents (Sigma-Aldrich or Fisher Scientific)

Mass spectrometry instrumentation for MALDI-TOF MS and/or ESI MS

2.6.2 Proteolytic cleavage of sulfotyrosine peptides—To localize sulfotyrosine residues in peptides with multiple potential sulfation sites, peptides can be cleaved with specific proteases to generate fragments containing subsets of the sulfation sites (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008). If sulfation sites in fragments are ambiguous, the cleavage process can be repeated for the fragments using proteases with different specificities. In our experience, endoproteases Asp-N or Glu-C are most useful for fragmenting sulfotyrosine peptides, because sulfotyrosine peptides generally contain multiple acidic residues in proximity of the sulfation sites. However, for peptides with complex tyrosine sulfation patterns, specific cleavage with additional proteases such as chymotrypsin, or generating a peptide ladder by using time dependent carboxypeptidase Y digestion might be required.

1. Determine the number of sulfotyrosine residues in the uncleaved (RP-HLPC purified) peptide by mass spectrometry. If the sulfation sites are ambiguous, proceed with step 2.
2. Identify proteolytic cleavage sites in the peptide and design a strategy for fragment generation.

3. Digest peptide (0.1 to 1 μg) with sequencing-grade proteases according to the manufacturer's instructions and take (5 μl) samples after different incubation times.
4. Optionally, to separate cleavage fragments subject samples to analytical RP-HPLC (see section 2.5.2) and collect peak fractions.
5. Dry samples using a SpeedVacTM, dissolve in a small volume (3 μL) of saturated 4HCCA matrix solution and analyze by MALDI-TOF MS (see section 2.6.3). Alternatively, desalt samples using RP C18 ZipTips and analyze by ESI MS.
6. If sulfation sites in fragments are ambiguous, repeat steps 2 to 5 for (RP-HPLC separated) fragments or uncleaved peptide using different protease.

2.6.3 MALDI-TOF mass spectrometry—In our experience, an ultra-thin layer sample preparation method (Cadene & Chait, 2000) using ammonium acetate as an additive gave best results for MALDI-TOF MS of sulfotyrosine peptides (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008). Under optimized measurement conditions, with low laser power in linear negative ion mode, complete sulfate retention was routinely observed in the most abundant ion. However, some loss of sulfate was generally observed, giving rise to additional signals that could be mistaken for contaminating peptide species. Hence, it is crucial to use RP-HPLC purified sulfotyrosine peptides for MALDI-TOF MS analysis. For a more detailed discussion of the characteristic loss-of-sulfate peak patterns observed in MALDI-TOF mass spectra of sulfotyrosine peptides we refer to the following references (Seibert et al., 2002; Seibert & Sakmar, 2008; Seibert, Veldkamp, et al., 2008).

1. Purify the MALDI matrix α -cyano-4-hydroxycinnamic acid (4HCCA) by HCl precipitation.
2. Prepare the ultra-thin layer solution by diluting a saturated solution of 4HCCA in TWA (0,1% TFA in $\text{H}_2\text{O}/\text{ACN}$ 2:1) 1:3 with isopropanol.
3. Prepare a saturated 4HCCA matrix solution in a 2:1 (v/v) mixture of water and acetonitrile with 10 mM ammonium acetate as an additive.
4. Wash sample plate with methanol, followed by water, followed by methanol and let it dry thoroughly.
5. To create an ultra-thin layer of the 4HCCA matrix, evenly distribute 20 μl of the ultra-thin layer solution over the sample plate with the flat side of a 200 μl pipette tip. Wait until approximately 2/3 of the liquid has dried and use a Kimwipe paper to distribute the remaining liquid. Carefully remove white material from the sample plate with a Kimwipe paper wrapped around a finger, leaving a yellowish ultra-thin layer of 4HCCA on the surface.
6. Dilute peptide sample 1:10 in matrix solution and spot a small aliquot (0.5 to 1 μl) of peptide-matrix solution onto the ultra-thin layer-coated sample plate. Immediately when crystals form, remove excess liquid using a 10 μl pipette tip attached to a vacuum line.
7. Perform MALDI-TOF mass measurements in negative linear, delayed extraction mode. The precise measurement parameters will depend on the specific

instrumentation used and require some optimization. As a guideline, the instrument settings for a Voyager DE-STR instrument (Applied Biosystems) can be found elsewhere (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008)

2.6.4 ESI mass spectrometry—Alternatively, sulfotyrosine peptides can be analyzed using ESI MS in negative ion mode. In our experience, using optimized measurement conditions, virtually no loss of sulfate was observed, and therefore, interpretation of negative ion mode ESI mass spectra of sulfotyrosine peptides was straight forward (Seibert, Veldkamp, et al., 2008). If sodium adduct formation is observed, the use of ammonium acetate as an additive should be considered to improve signal intensities.

1. Desalt sulfotyrosine peptide samples by binding to RP-C18 ZipTips according to the manufacturer's protocol. After washing with HPLC-grade water, elute peptides with 70% acetonitrile.
2. Perform ESI mass measurements in negative ion mode. The precise measurement parameters will depend on the specific instrumentation used and require some optimization. As a guideline, the instrument settings for a QSTAR XL hybrid electrospray quadrupole-quadrupole time-of-flight mass spectrometer (Applied Biosystems) equipped with a nano-electrospray ionization source can be found elsewhere (Seibert, Veldkamp, et al., 2008).

2.7 Characterization of sulfopeptides by protein NMR

If uniformly isotopically labeled (^{15}N or $^{15}\text{N}/^{13}\text{C}$) labeled peptides are used in TPST driven sulfation reactions and purified by RP-HPLC, protein NMR, in addition to mass spectrometry, can be used to confirm the location of tyrosine sulfation.

2.7.1 Required material

High Field Nuclear Magnetic Resonance Spectrometer (500 MHz)

RP-HPLC purified [^{15}N or $^{15}\text{N}/^{13}\text{C}$] peptide

RP-HPLC purified [^{15}N or $^{15}\text{N}/^{13}\text{C}$] sulfopeptide

Chemical shift assignments for [$^{13}\text{C}/^{15}\text{N}$] peptide determined using standard assignment strategies (Markley, Ulrich, Westler, & Volkman, 2003)

^{15}N - ^1H heteronuclear single quantum coherence pulse program such as hsqcf3gp19 from the Bruker sequence library.

2.7.2 ^{15}N - ^1H HSQC spectroscopy

1. Prepare separate NMR samples consisting of purified [^{15}N or $^{15}\text{N}/^{13}\text{C}$] unsulfated peptide and [^{15}N or $^{15}\text{N}/^{13}\text{C}$] sulfopeptides in an appropriate NMR buffer. For example, 25 mM deuterated MES, 10 % D_2O , 0.2% NaN_3 , pH 6.8.
2. Collect ^{15}N - ^1H HSQC spectra and overlay each sulfopeptide spectra with that of the unsulfated peptide.

3. Compare the spectra paying particular attention to tyrosine residues and residues proximal to tyrosines. Similarities in chemical shift correlate with the absence of sulfation while differences in chemical shift are indicative of tyrosine sulfation. See Figure 4.

3. CAVEATS AND LIMITATIONS

While making chemokine receptor sulfopeptides enzymatically is one approach, Payne and colleagues have turned what were pitiful methods for chemically synthesizing sulfopeptides into a usable approach (M. J. Stone et al., 2015). A purported strength of the chemical synthesis approach allows for control of where sulfotyrosines are placed in a peptide or the pattern of sulfotyrosines in a peptide containing more than one. Hence a possible limitation, or strength depending on perspective, to using TPSTs to sulfate chemokine receptor peptides is that one is limited to the tyrosine sulfation pattern resulting from enzymatic sulfation versus the control afforded through chemical synthesis. For example, one could not use TPSTs to produce a CXCR4 N-terminal peptide, which contains tyrosines at positions 7, 12 and 21, sulfated at only Y7 as analysis of CXCR4 sulfation kinetics indicates Y21 is sulfated first followed by either 7 or 12 with the remaining tyrosine sulfated thereafter (Seibert, Veldkamp, et al., 2008). Hence, the impact of only sY7 on the interaction of a CXCR4 N-terminal peptide with CXCL12 could not be addressed using this system. However, this limitation can be viewed as a strength because receptor peptides with more physiologically relevant sulfation patterns will be investigated. It is unlikely that it is just a coincidence that TPSTs sulfates an N-terminal CXCR4 peptide at Y21 first and that, in the context of intact CXCR4, sY21 has the most impact on CXCL12 binding (Farzan, Babcock, et al., 2002).

While sulfation of the CXCR4 N-terminus has little impact on its role as an HIV-1 coreceptor, sulfotyrosines in the N-terminus of CCR5 greatly enhance HIV-1 infection or affinity for chemokine ligands (Farzan, Babcock, et al., 2002; Farzan, Chung, et al., 2002; Farzan et al., 1999). A peptide corresponding to residues 2–18 of the CCR5 N-terminus, which contains tyrosines at positions 3, 10, 14 and 15, is sulfated by TPST-1 or TPST-2 first at tyrosines 14 and 15 followed by sulfation at position 10 and lastly at position 3 (Jen et al., 2009; Seibert et al., 2002). But, a CCR5 1–18 peptide is sulfated at Y3 first followed by Y14/Y15 and finally Y10 (Jen et al., 2009). While CCR5 Y3 is sulfated first, sY3 appears least important amongst other sY's in full length CCR5 for HIV-1 infection but sY3 does have a small impact on chemokine binding (Bannert et al., 2001; Farzan et al., 1999). While a CCR5 sY10/sY14 N-terminal peptide is sufficient to rescue chemokine signaling through CCR5 2–17 (Farzan, Chung, et al., 2002) or reconstitute HIV-1 infection in cells expressing CCR5 2–17 (X. Liu, Malins, Roche, Sterjovski et al., 2014), neither study investigated sY3 in the context of an N-terminal CCR5 peptide. As one would expect, knowledge regarding the order of tyrosine sulfation by TPSTs, which in some instances appears to correlate with functional importance, along with the ability to control the location and pattern of sulfotyrosines chemical synthesis will provide the broadest picture of functional importance for individual sulfotyrosine residues.

While lack of control over the location of sulfotyrosines in the sulfopeptides produced enzymatically may be a limitation or perhaps a strength, there is a much more important

caveat to mention. A major caveat to using recombinant TPST enzymes to generate sulfotyrosine containing chemokine receptor peptides or other peptides is the requirement for highly pure PAPS cosubstrate, generally greater than 80 percent pure. This is because PAPS can spontaneously degrade to sulfate and PAP (3'-phosphoadenosine-5'-phosphate) at a rate that increases with increasing temperature. In our experience PAPS is relatively stable over a period of a couple months at -80°C , but will degrade to PAP at 37°C in a matter of a day. Additionally, highly pure PAPS has not always been commercially available and when it is shipping time or conditions can have an impact on PAPS purity. If PAPS purity is less than 80% or PAP content exceeds 10%, PAPS can be purified by Mono Q anion exchange chromatography as described above (Burkart et al., 2000).

4. PERSPECTIVES

Native sources of TPSTs have been used to enzymatically sulfate peptides but producing enzymatically sulfated peptides on a large scale really became realistic upon the cloning and purification of recombinant TPSTs from mammalian cell lines by Moore and colleagues (Y. B. Ouyang et al., 1998; Y. Ouyang et al., 1998). Here we provide a protocol that closely follows Moore and colleague's method for preparing recombinant human TPSTs from mammalian cells and our methods for sulfation of N-terminal chemokine receptor peptide (Y. B. Ouyang et al., 1998; Y. Ouyang et al., 1998). We also present a method for expression and functional refolding of recombinant, enzymatically active TPST-1 from *E. coli*, which we have also used to sulfate N-terminal chemokine receptor peptides and PSGL-1 peptides. The structure of human TPST-2 was recently solved using recombinant TPST-2 from *E. coli* and has provided on the structural information on the TPST's mechanism (Teramoto et al., 2013). The availability of bacterial produced enzymes makes large-scale preparation of sulfopeptides using TPSTs even more practical. An added benefit of using TPSTs to produce sulfopeptides is that the reactions can be monitored overtime and, if multiple tyrosines are present, the order of tyrosine sulfation can be determined. This can be done simply by quantifying absorbance of RP-HPLC peaks (Seibert et al., 2002; Seibert, Veldkamp, et al., 2008) or through monitoring the reactions using more complex mass spectrometry approaches pioneered by Leary and colleagues (Danan et al., 2008; Danan et al., 2010; Jen et al., 2009). Knowledge of tyrosine sulfation order provides information of which sulfopeptides are most physiologically relevant for study and such kinetic data can also provide information on the enzymatic mechanism of TPSTs (Danan et al., 2008; Danan et al., 2010; Jen et al., 2009).

We have used TPSTs to make large quantities of sulfopeptides for structural studies. For example, TPSTs were used to sulfate milligram quantities of CXCR4 N-terminal peptides of natural isotopic abundance or labeled uniformly with N-15 or N-15 and C-13. Because CXCR4 N-terminal peptides promote CXCL12 dimer formation, we used a covalently locked CXCL12 dimer to solve the structure of the CXCL12 dimer with various N-terminal CXCR4 peptides that were either unsulfated, sulfated at position 21 or sulfated at tyrosines 7, 12 and 21 (Veldkamp et al., 2008; Veldkamp et al., 2006; Ziarek, Getschman, Butler, Taleski et al., 2013). Utilizing this structure, small molecule inhibitors that target the CXCR4 sulfotyrosine 21 binding site on CXCL12 have been developed (Smith, Liu, Getschman, Peterson et al., 2014; Veldkamp, Ziarek, Peterson, Chen et al., 2010). The

availability of isotopically labeled sulfopeptides, which is possible when producing sulfopeptides enzymatically but not synthetically, aided in solving a high quality NMR structure of a locked CXCL12 dimer with CXCR4 sulfopeptides. However, Stone and colleagues have shown through their structure of CCL11 bound to a synthetic CCR3 sulfopeptide that isotopic labeling of sulfopeptides is not required for structure determination (Millard et al., 2014). Stone and colleagues provide a comparison of their CCL11/CCR3 sulfopeptide structure to that of our locked CXCL12 dimer with CXCR4 sulfopeptides (Millard et al., 2014), while Handel and colleagues provide analysis of the locked CXCL12 dimer with CXCR4 sulfopeptide structure in the context of their recently determined structure of intact CXCR4 and the viral chemokine vMIP-II (Kufareva et al., 2015; Qin, Kufareva, Holden, Wang et al., 2015).

The classical view of sulfotyrosine is that it is a posttranslational modification that increases affinity between binding partners. We have shown this to be the case for CXCL12 and enzymatically produced CXCR4 N-terminal peptides with affinity increasing with increasing sulfation (Seibert, Veldkamp, et al., 2008). Others have shown similar increases in affinity between chemokines and synthetic sulfotyrosine containing receptor peptides from CCR5 (Duma et al., 2007), CCR3 (Simpson et al., 2009; Zhu, Millard, Ludeman, Simpson et al., 2011), and CCR2 (J. H. Tan et al., 2013). However, a more significant role for sulfotyrosine posttranslational modifications in some chemokine receptors likely involves the role of sulfotyrosines in ligand bias or biased agonism. Many chemokine receptors have two or more chemokine ligands and are excellent examples of ligand bias or biased agonism with CCR7 and its ligands CCL19 and CCL21 being a prototypical example (Zidar, Violin, Whalen, & Lefkowitz, 2009). Different sulfation patterns affect the selectivity of synthetic N-terminal peptides from CCR3 for CCL11/eotaxin-1, CCL24/eotaxin-2, and CCL26/eotaxin-3 and are hypothesized to contribute to ligand biased signaling in intact CCR3 (Zhu et al., 2011). Unlike CCR3 or CCR7, which have multiple and in most cases monomeric ligands (Love, Sandberg, Ziarek, Gerarden et al., 2012; Millard et al., 2014; Veldkamp et al., 2015), CXCR4 has only one natural ligand CXCL12. Peptides and sulfopeptides corresponding to the N-terminus of CXCR4 promote CXCL12 dimer formation and a covalently locked CXCL12 dimer with a nearly identical structure to the wild-type CXCL12 dimer signals through CXCR4 as a partial agonist (Drury, Ziarek, Gravel, Veldkamp et al., 2011; Veldkamp et al., 2008; Veldkamp et al., 2006; Ziarek et al., 2013). Similarly to CCR3, it could be hypothesized that sulfotyrosines in CXCR4 are contributing to bias signaling, but instead of conferring selectivity for one ligand over another the sulfotyrosines are affecting selectivity for an oligomeric state and thereby influencing bias signaling.

Some CC chemokines form dimers, however, these dimers are not thought to activate, even partially, or bind with significant affinity to their chemokine receptors as exemplified by a covalently locked CCL2 dimer (J. H. Tan et al., 2013) or CCL4 (Jin, Shen, Baggett, Kong et al., 2007). CC chemokine monomers are believed to be full receptor agonists; for example, an obligate CCL2 monomer is a CCR2 agonist (J. H. Tan et al., 2013). Yet CCR2 N-terminal sulfopeptides bind more tightly to the covalently locked CCL2 dimer and the obligate monomer than unsulfated peptides (J. H. Tan et al., 2013). In the context of wild-type CCL2 these same sulfopeptide promote a shift in the CCL2 monomer-dimer equilibrium towards monomer (Ludeman et al., 2014; J. H. Tan et al., 2013). Stone and colleagues conclude from

these results that for CC chemokines that dimerize receptor sulfotyrosines promote formation of the receptor agonist or the CC chemokine monomer (Ludeman et al., 2014; M. J. Stone et al., 2015; J. H. Tan et al., 2013). Recall Farzan and colleagues used CCR5 N-terminal sulfopeptides to rescue CCL3 activation of a CCR5 2-17 receptor (Bannert et al., 2001; Farzan, Chung, et al., 2002). Both CCL3 and CCL4 form tight dimers (Czaplewski, McKeating, Craven, Higgins et al., 1999; Lodi, Garrett, Kuszewski, Tsang et al., 1994). Interestingly, the results from Stone and colleagues may suggest another interpretation of Farzan and colleagues' results. The CCR5 N-terminal sulfopeptides may not necessarily be rescuing an inactive CCR5 2-17 receptor so much as these sulfopeptides are promoting formation a receptor agonist, a monomeric chemokine.

While recombinant TPSTs allow for the study of the enzymes themselves and for preparation of sulfopeptides useful as reagents, other approaches to making sulfopeptides or proteins exist. For example, chemical synthesis approaches pioneered by Stone and Payne (M. J. Stone et al., 2015), Liu and Shultz's incorporation of sulfotyrosine in recombinantly expressed proteins through amber codon suppression (C. C. Liu & Schultz, 2006), or simply recombinant expression in mammalian cell lines containing endogenous TPSTs (references in (Seibert & Sakmar, 2008)) have all been reported. Still others have coexpressed both a protein of interest and a TPST in mammalian cells. For example, Farzan and colleagues used this coexpression approach to generate a fusion of CD4-Ig with a small C-terminal CCR5 sulfopeptide mimetic and showed this fusion to be more potent at preventing HIV-1 infection than the best broadly neutralizing HIV-1 antibodies (Gardner, Kattenhorn, Kondur, von Schaewen et al., 2015). They also used a coexpression approach in rhesus macaque vaccine studies by using adeno-associated viruses coding for production of the CD4-Ig with a small C-terminal CCR5 sulfopeptide fusion and TPST-2 (Gardner et al., 2015). We believe the potential exists for using recombinant TPSTs to sulfate purified membrane or secreted proteins through *in vitro* sulfation reactions similar to those used for sulfation of peptides. For example, Handel and colleagues used a disulfide trap to preform a CXCR4/vMIP-II complex for their structure of CXCR4 with the viral chemokine in which the majority of the CXCR4 N-terminus is absent (Qin et al., 2015; Wu, Chien, Mol, Fenalti et al., 2010). This involved coexpression of both CXCR4 and vMIP-II with each containing an additional cysteine for formation of the disulfide trapped complexes in insect cells (Qin et al., 2015; Wu et al., 2010). While coexpression or coinfection of a third gene of interest, a TPST, for sulfation of the CXCR4 N-terminus might generate high enough affinity for the chemokine that the receptor N-terminus adopts an observable conformation, asking insect cells to heterologously express three different genes at the same time may be a daunting task (Sokolenko, George, Wagner, Tuladhar et al., 2012). An alternative approach, which might prove to be equally daunting, would be to utilize recombinant TPSTs to sulfate tyrosines in purified receptors like CXCR4 or CCR5.

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Figure 1.
Reaction catalyzed by TPST enzymes.

Figure 2.

RP-HPLC analysis of CCR5 2–18 sulfation products. A) Characterization of the in vitro sulfation reaction. Peptide CCR5 2–18 (0.1 mg/ml, ~50 μ M) was incubated with a mixture of TPST-1 and TPST-2 (20 μ g/ml each) and in the presence of the sulfation cosubstrate PAPS (400 μ M). After 30 h or 100 h at 16°C 60 μ l aliquots were analyzed by RP-HPLC. In negative-control experiments (100 h incubation time), either the TPST mixture (no TPST) or the PAPS (no PAPS) was omitted. Peaks were labeled a–f in increasing order of hydrophobicity. B) Comparison of TPST-1 and TPST-2. CCR5 2–18 (0.1 mg/ml, ~50 μ M) was incubated for 100 h with TPST-1 (40 μ g/ml) or TPST-2 (40 μ g/ml) in the presence of PAPS (400 μ M). Peak a corresponds to CCR5 2–18, peak b to CCR5 2–18 sY14, peak c to CCR5

2–18 sY15, peak c' to CCR5 2–18 sY10/sY14, peak d to CCR5 2–18 sY14/sY15, peak e' to CCR5 2–18 sY3/sY10/sY15, peak e to CCR5 2–18 sY10/sY14/sY15, and peak f to CCR5 2–18 sY3/sY10/sY14/sY15. (Reproduced from (Seibert et al., 2002). Copyright 2002 National Academy of Sciences, USA).

Figure 3.
Representative RP-HPLC chromatogram for purification of CCR4 sulfopeptides on a semi-preparative scale. See section 2.5.3.

Figure 4.

Protein NMR analysis of CXCR4 sulfopeptides. A) Analytical scale RP-HPLC chromatograms of TPST-1 catalyzed sulfation reactions of 50 μ M CXCR4 1-38 (C24A) without and with 400 μ M PAPS. Peak *a* corresponds to CXCR4 1-38 (C24A), peak *b* to CXCR4 1-38 (C24A) sY21, peak *c* to CXCR4 (C24A) sY12/sY21, peak *d* to CXCR4 (C24A) sY7/sY21 and peak *e* to CXCR4 (C24A) sY7/sY12/sY21. B) ^{15}N - ^1H HSQC spectra of peak *a* and overlays of ^{15}N - ^1H HSQC spectra of peak *b*, *c*, *d* or *e* (red) onto peak *a* (gray).