

Using Phage Display to Select Antibodies Recognizing Post-translational Modifications Independently of Sequence Context*

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Many cellular activities are controlled by post-translational modifications, the study of which is hampered by the lack of specific reagents due in large part to their ubiquitous and non-immunogenic nature. Although antibodies against specifically modified sequences are relatively easy to obtain, it is extremely difficult to derive reagents recognizing post-translational modifications independently of the sequence context surrounding the modification. In this study, we examined the possibility of selecting such antibodies from large phage antibody libraries using sulfotyrosine as a test case. Sulfotyrosine is a post-translational modification important in many extracellular protein-protein interactions, including human immunodeficiency virus infection. After screening almost 8000 selected clones, we were able to isolate a single specific single chain Fv using two different selection strategies, one of which included elution with tyrosine sulfate. This antibody was able to recognize sulfotyrosine independently of its sequence context in test peptides and a number of different natural proteins. Antibody reactivity was lost by antigen treatment with sulfatase or preincubation with soluble tyrosine sulfate, indicating its specificity. The isolation of this antibody signals the potential of phage antibody libraries in the derivation of reagents specific for post-translational modifications, although the extensive screening required indicates that such antibodies are extremely rare. *Molecular & Cellular Proteomics* 5: 2350–2363, 2006.

Much protein activity is regulated by post-translational modifications (PTMs),¹ over 200 of which have been de-

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¹ The abbreviations used are: PTM, post-translational modification;

scribed (1), with changes in enzymatic activity, protein interaction partners, subcellular localization, and targeted degradation being some of the most important regulated activities. A number of different approaches have been used to study PTMs, most of which rely on either specific isolation of the protein of interest and identification of the PTMs that affect that particular protein (2) or isolation of all proteins containing a specific PTM of interest and subsequent identification of the isolated proteins. There are two basic methods to isolate or identify all proteins containing a specific PTM: chemical derivatization or specific affinity reagents. The former rely on the specific chemical reactivity of the PTM to chemical modification, usually by the addition of an easily recognized tag, such as the biotinylation of nitrosylated cysteines (3) or phosphorylated serine/threonine residues (4), allowing them to be either purified or identified by virtue of specific mass shifts. The latter, specific affinity reagents that recognize PTMs, comprise a number of different molecule types, including IMAC using iron or gallium to bind proteins containing phosphotyrosines (5–8) or phosphorylated threonines or serines (9, 10); lectins, which recognize glycosylated proteins; and antibodies recognizing tyrosine modifications. These have all been used in proteomics studies (11–17). Although antibodies are by far the most common specific affinity reagents, there are very few that recognize PTMs independently of the proteins to which they are attached. Nitrosylated tyrosine and phosphorylated tyrosine are two exceptions for which antibodies have been used in proteomics studies (13–17), and antibodies against phosphoserine/threonine have also been identified (18), indicating that the utility of such reagents exists if they can be derived. However, it has proven generally difficult to isolate antibodies that recognize PTMs within a broad context. In part this is likely to reflect the relatively small size of the recognized epitope, but perhaps more importantly, the ubiquity of such PTMs, especially on secreted and membrane-bound proteins,

TPST, tyrosylprotein sulfotransferase; V, variable; VH, heavy chain variable; VL, light chain variable; scFv, single chain Fv; OVA, ovalbumin; IPTG, isopropyl β -D-thiogalactopyranoside; AP, alkaline phosphatase; DMF, dimethylformamide; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; SMCC, succinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate; AmpGlu, 50 μ g/ml ampicillin, 3% glucose.

renders them non-immunogenic in intact immune systems. Furthermore it is possible that some post-translational modifications, such as phosphotyrosine, are intrinsically more antigenic either because the modified side chain is further from the peptide backbone than, for example, phosphoserine, or because substituted aromatic residues are in general more antigenic than modified residues (such as serine and threonine) that resemble metabolic intermediates.

One widely distributed PTM is tyrosine sulfation (19–22) (Fig. 1A). This has been found to drive extracellular protein-protein interactions with that between PSGL-1 and P-selectin (23–26), in which the sulfated tyrosine residues of PSGL-1 are positioned to form hydrogen bonds with P-selectin (27), serving as the archetype. The tyrosine sulfated chemokine receptors CCR5, CXCR4, and CCR2 also rely on their sulfate groups to increase binding affinity for both chemokines and human immunodeficiency virus (28–32) as do the tyrosine sulfated complementarity-determining regions of some human immunodeficiency virus-neutralizing antibodies, the efficacy of which is reduced with elimination of this PTM (33, 34). The modification is created by the transfer of sulfate from adenosine 3-phosphate 5-phosphosulfate to the hydroxyl group of the polypeptide tyrosine to be modified (35). In man, two enzymes, tyrosylprotein sulfotransferase (TPST)-1 and TPST-2, catalyze this reaction, and transgenic mice lacking each of these enzymes have been made (36, 37). Lack of TPST-1 causes reduced body weight and increased postimplantation fetal death (37), whereas TPST-2 deficiency results in male infertility (36). The lack of a simple, rapid assay for tyrosine sulfate has impeded investigation of this modification, and immunization attempts to derive antibodies recognizing sulfated tyrosines have been unsuccessful² although an antibody whose binding epitope includes tyrosine sulfate has been described (38). This result is not surprising as the presence of tyrosine sulfate on many secreted and membrane-bound proteins should lead the immune system to become particularly tolerant of the modification.

One way to overcome the limitations of intact immune systems in the generation of specific antibodies against non-immunogenic targets is to use phage antibody libraries rather than immunization. In this technique large numbers ($\geq 10^9$) of different antibodies are displayed on the surface of filamentous phage, and specific binders are selected on the basis of their binding abilities (for reviews, see Refs. 39–44). The fact that this technology is completely *in vitro*, using either natural rearranged (45–49) or synthetic V genes (50–53), overcomes the intrinsic biases of the immune system. Although phage antibodies have been selected against large numbers of different polypeptide and chemical targets, including specific peptides (54–57), there have been no descriptions of the use of this technology to select antibodies against post-translational modifications.

To determine whether phage display could be used to select useful antibodies recognizing PTMs, we used a number of different selection strategies to isolate an anti-tyrosine sulfate antibody from a large recombinatorial library of phage-displayed single chain Fv (scFv) fragments (47). We characterized almost 8000 clones after two or three rounds of selection and identified a single scFv able to recognize tyrosine sulfate in multiple sequence contexts that was able to maintain its recognition specificity when converted into a full-length IgG.

EXPERIMENTAL PROCEDURES

Synthesis of the Antigens—Pep1 and Pep2 were synthesized on an Applied Biosystems 431A peptide synthesizer at 0.1 mmol scale using standard dicyclohexylcarbodiimide (Novabiochem) couplings with *N*-hydroxybenzotriazole (Novabiochem). Unless otherwise specified, all reagents for antigen synthesis were purchased from Aldrich. The syntheses began with trityl-protected cysteine preloaded onto 2-chlorotrityl resin (Novabiochem) and proceeded using Fmoc-protected amino acids with standard side chain-protecting groups (Novabiochem). The peptides were simultaneously cleaved and deprotected at room temperature with a 3.5-h incubation in trifluoroacetic acid with 5% thioanisole, 5% water, 2.5% ethanedithiol, and 4.5% phenol. The peptides were then precipitated in cold ether and purified via reverse-phase HPLC.

Synthesis of Pep1S and Pep2S was more complex and was carried out manually. 2-Chlorotrityl resin carrying 0.1 mmol of trityl-protected cysteine was swollen in dimethylformamide (DMF) for 10 min and drained. The preloaded cysteine had a free amino group, but all subsequent cycles required removal of an Fmoc group. To accomplish this, the swollen resin was covered with 20% piperidine in DMF for 10 min, drained, covered again with 20% piperidine in DMF, drained, washed twice with 20 ml of DMF, and then washed six times with 20 ml of dichloromethane. The coupling solutions were prepared by dissolving 0.5 mmol of Fmoc-protected amino acid in 1 ml of DMF to which was added 1 ml of 0.45 M *N*-hydroxybenzotriazole/*O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate solution. Diisopropylethylamine (150 μ l) was added for a final concentration of 7%. The coupling solution was then added to the resin and allowed to sit for 6–12 h. To avoid deletions, every amino acid subsequent to the sulfated tyrosine residue was double coupled. After the synthesis was complete, the peptides were cleaved and deprotected simultaneously in 90% trifluoroacetic acid at 2 °C. The peptides were then precipitated in cold ether and purified via reverse-phase HPLC.

A portion of the antigenic peptides was biotinylated via the carboxyl terminal cysteine. Pep1, Pep2, and Pep1S were dissolved in buffer to 800 μ M, whereas Pep2S was dissolved to 100 μ M. Equimolar amounts of EZ-link polyethylene oxide-iodoacetylbiotin (Pierce) were added to the peptides, and the solutions were held in the dark at room temperature for 90 min. The reactions were then stored at –20 °C. Biotinylation of the peptides was confirmed by mass spectrometry.

The reagent sulfo-SMCC (Pierce) was used to cross-link a portion of the antigenic peptides with the primary amines of BSA and ovalbumin (OVA). BSA and OVA were dissolved in 20 mM Na₂HPO₄, 30 mM NaCl, pH 7.2, to a final concentration of 8 mg/ml. According to the manufacturer's instructions, sulfo-SMCC was dissolved in water and added to BSA in 35-fold excess and to OVA in 20-fold excess. The reactions were allowed to proceed at room temperature for 1 h at which point unreacted sulfo-SMCC was removed on a concentrator with a 10,000 molecular weight cutoff (Ambion). Pep1, Pep1S, and Pep2 were dissolved in PBS and added to the modified BSA and OVA

² W. Huttner, personal communication.

in 3-fold excess, whereas Pep2S was added in the substoichiometric ratio of 0.2. This mixture of peptide and protein was held at room temperature for 12 h and then frozen. Although the heterogeneous glycosylation of BSA and OVA made it difficult to obtain a clean mass spectrum, a series of new, heavier peaks separated by the mass of the antigenic peptide could be seen (data not shown).

Selections—The immunotubes (Nunc) were coated with antigen overnight at 4 °C with 10 µg of antigen in 1 ml of PBS. The immunotubes were then washed with PBS, blocked with 2% fish gelatin in PBS, and left at 37 °C for 1 h. Prior to addition to the immunotubes, 1-ml aliquots of the phage-displayed scFv library (47) (10^{13} phage/ml) were blocked for 30 min with 1 nmol of free Pep1 or Pep2 and 3 mg of BSA or OVA. After blocking, the antigen-coated immunotubes were washed three times with PBS, and the blocked library aliquots were added for a 3-hour incubation at room temperature. The immunotubes were then washed twice with 0.1% Tween 20 in PBS and twice with PBS. The bound phage were eluted with a 30-min incubation in 1 mg/ml trypsin at 37 °C.

Eluted phage from the first and second rounds of selection were amplified for further rounds of selections. 10% of the eluted phage were used to infect 900 µl of DH5αFT at the midlog phase, while the remainder was frozen. The bacteria had been grown in 2× YT (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, adjusted to pH 7)/15 µg/ml tetracycline, 3% glucose. (Except during scFv expression, all bacterial growth took place in the presence of 3% glucose.) To carry out the infection, the phage and bacteria were mixed together and held at 37 °C for 30 min without shaking at which point the bacteria were infected with the KM13 helper phage (59) in the same manner and grown overnight at 30 °C in 2× YT 50 µg/ml ampicillin, 50 µg/ml kanamycin. DH5αFT infected with the third round output were plated on 2× YT AmpGlu plates. Selections using tyrosine sulfation elution were carried out similarly except that instead of trypsin, 10 mM tyrosine sulfate was used for elution.

Identification of Output Phage That Expressed an SV5 Tag—Bacterial clones from the final round were picked using a Q-bot colony picker (Genetix), inoculated into 384-well plates containing 2× YT AmpGlu, and grown overnight at 30 °C without shaking. After growth, the cultures were replicated onto UV light-sterilized nitrocellulose sheets, which were then placed on a fresh 2× YT AmpGlu plate, bacterial spots up, and incubated at 30 °C overnight. The following day, 384 colonies per sheet were visible as discrete spots. The colonies were induced by transferring the sheets to a fresh plate of 2× YT Amp, 100 µM IPTG and incubating at 25 °C for 3–6 h. After induction, the nitrocellulose sheets were placed on damp paper towels next to an open container of chloroform, and both sheets and chloroform were covered with a plastic box for 15 min. The sheets were then subjected to a 16-h incubation in lysis buffer (100 mM Tris, pH 7.8, 150 mM NaCl, 5 mM MgCl₂, 1.5% (w/v) BSA, 1 µg/ml pancreatic DNase I, 40 µg/ml lysozyme) and two washes in TNT buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.05% (v/v) Tween 20). Damp tissues were used to physically remove all traces of the colonies, and the sheets were washed a third time in TNT buffer. After washing, the sheets were submerged for 30 min in blocking buffer (TNT, 3% (w/v) BSA) and then transferred to blocking buffer containing a 1:500 dilution of SV5 antibody (60) for 1 h. The sheets were washed for 10 min each in TNT with 0.1% BSA, TNT with 0.1% BSA and 0.1% Nonidet P40, and then TNT with 0.1% BSA. The secondary antibody, goat α-mouse conjugated to alkaline phosphatase (Dako), was diluted 1:2000 in blocking buffer and incubated with the sheets for 2 h followed by washes as carried out after the primary antibody. The sheets were developed with 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine and nitroblue tetrazolium chloride (Pierce) with positive (SV5-positive) colonies identified as blue.

Identification of scFv Fragments That Bound Background Components of the Selection—SV5-positive colonies were picked into 96-well plates filled with 100 µl/well 2× YT Amp/Glu and incubated at 37 °C until they reached midlog phase. The bacteria were then spun down, and the medium was replaced with 100 µl/well 2× YT Amp/IPTG (0.1 mM). After an overnight induction at 25 °C, a crude isolate of scFv fragments was prepared. The cells were pelleted, 80 µl of medium was collected per well, the remainder was discarded, and the cells were resuspended in 20 µl of periplasmic buffer (20% sucrose in 50 mM Tris, pH 8, with 1 mM EDTA). The cells were then incubated at 4 °C for 15 min and pelleted, and 10 µl of the supernatant was added to the 80 µl of previously collected induction medium. This crude prep contains scFv released into the growth supernatant as well as that retained in the periplasm. This was then diluted 2-fold with 1% fish gelatin in PBS and held at 4 °C until used (less than 4 h).

These scFv preparations were used in ELISAs to determine whether the scFv fragments bound to background components of the immunotube selections. A 1-h incubation at 37 °C with a combination of BSA and OVA (in PBS with a total concentration of 12 µg/ml) was used to coat Immunosorb plates (Nunc). After the incubation, the antigen was discarded, and the wells were washed with 200 µl of PBS. The plates were then blocked for 1 h with 200 µl/well 4.5% fish gelatin in PBS. The blocking solution was discarded, the wells were washed twice with 200 µl of PBS, and 100 µl of the crude scFv solution was added. The scFv fragments were allowed to incubate at room temperature for 90 min before removal and washing with 200 µl/well PBS twice. 100 µl of SV5 diluted 1:2000 in PBS with 1% fish gelatin was added, and after 1 h at room temperature, the antibody solution was removed, and the wells were washed twice with 200 µl/well PBS. The tertiary antibody, goat α-mouse conjugated to horseradish peroxidase (Dako), was diluted 1:4000 in PBS with 1% fish gelatin, and 100 µl was added to each well. After 1 h at room temperature the antibody solution was removed, and the wells were washed with three 200-µl aliquots of PBS, 0.1% Tween 20 and then three 200-µl aliquots of PBS. The plates were developed with 3,3',5,5'-tetramethylbenzidine (Pierce). ELISAs for background binding that used the alkaline phosphatase reporter were run in a similar fashion. The only differences were in the tertiary antibody and development of the signal. For the tertiary antibody, a 1:2000 dilution of goat α-mouse conjugated to alkaline phosphatase (Dako) in PBS with 1% fish gelatin was used. After incubation and washes as for the horseradish peroxidase reporter, the signal was developed using *p*-nitrophenyl phosphate (Pierce).

Identification of scFv Fragments That Bound Tyrosine Sulfate—The initial ELISAs to detect binding to tyrosine sulfate were run essentially as the ELISAs to detect background binding. The only difference was the adsorption of a sulfated antigen rather than a background component of the selection. Upon identification of a single scFv that specifically recognized sulfated tyrosine residues, the protein was purified as below.

Expression and Partial Purification of the Active scFv—XL-1 Blue competent cells (Stratagene) transformed with scFv (in pDAN5 (47)) were grown overnight in 3 ml of 2× YT AmpGlu. This starter culture was used to inoculate a 500-ml culture of 2× YT AmpGlu. After growth to midlog cells were pelleted, resuspended in 2× YT Amp with 250 µM isopropyl α-D-thiogalactoside (Invitrogen), and incubated overnight at 25 °C. The cells were then pelleted, and the medium was filtered through a 0.22-µm filter (Nalgene) and dialyzed against phosphate-buffered saline with a 10,000 molecular weight cutoff dialysis cassette (Pierce). The scFv was stored at 4 °C. The presence of scFv was confirmed by Western blotting SDS-PAGE.

Sulfatase Treatment of Tyrosine Sulfated Proteins—The following tyrosine sulfated proteins were purchased from Sigma: IgM (mouse), fibrinogen (rat), thyroglobulin (pig), fibrinogen fraction 1s (bovine),

fibrinogen fraction IV (bovine), hirudin (*Hirudo medicinalis*), and vitronectin (human). Human IgM was purchased from Chemicon International. The proteins were divided into 2 × 0.5-mg aliquots and dissolved in 0.067 M sodium acetate at pH 5.5. 1.5 mg of the abalone sulfatase (Sigma) was added to one of the tubes, and both samples were placed in a heating block at 37 °C overnight. The enzyme reaction was stopped by placing the tubes on ice for 30 min followed by dilution in PBS. Only 20 units of hirudin and 5 µg of vitronectin were used, and the sulfatase amount was reduced accordingly. Proteins were analyzed by SDS-PAGE to assess the level of degradation.

scFv, scFv-Alkaline Phosphatase (AP), and Ig ELISAs—A 96-well Maxisorp microplate (Nunc) was coated with antigen at 4 °C overnight. For peptides, the plates were first coated with streptavidin (10 µg/ml) and then loaded with biotinylated Pep1, Pep1S, Pep2, Pep2S, or 100 µl of BSA (10 µg/ml)-tagged Pep1, Pep1S, Pep2, and Pep2S. For other antigens, proteins were coated at 10 µg/ml in 100 µl in PBS or sulfatase buffer if proteins were treated with sulfatase. After an overnight incubation at 4 °C the plate was washed three times with PBS and blocked with 100 µl of 4.5% fish gelatin (Sigma) in PBS or wonder block (0.3% BSA, 0.3% milk, 0.3% fish gelatin). After 1 h at room temperature the plate was washed with PBS three times, and 100 µl of the partially purified scFv solution was added to each well for 1.5 h. The plate was washed as above, and 100 µl of the secondary antibody, mouse anti-SV5, was added to each well and left for 1 h. The secondary antibody was diluted in PBS 1:2000 with 1% fish gelatin. The plate was then washed, and 100 µl of the tertiary antibody, goat anti-mouse conjugated to alkaline phosphatase (Sigma), was added to each well. The tertiary antibody was diluted 1:2000 in PBS containing 1% fish gelatin. After 1 h the plate was washed three times with PBS containing 0.05% Tween 20 (Sigma) and then washed three additional times with PBS. Finally 100 µl of alkaline phosphatase substrate buffer (Bio-Rad or Pierce) was added to each well. After a significant color change occurred the plate was quantified by reading at 450 nm.

In the case of the AP fusions, 1 µg of purified scFv-AP was added per well in 100 µl of 0.1× wonder block rather than the scFv, and no additional antibodies were added. The IgG ELISAs were done similarly except that 1 µg/well IgG was used, and the signal was detected using anti-human AP in a 1:2000 dilution (Santa Cruz Biotechnology).

Construction and Purification of scFv-AP Protein—The scFv gene was excised from the phage display vector and cloned into pEP-AP vector using BssHII and NheI restriction enzymes. The pEP-AP vector is a derivative of pET22b in which the gene encoding alkaline phosphatase was subcloned from the pSKAP/S vector (61) into pET22b in such a way that scFv fragments could be directly cloned in frame from the pDAN5 phage display library vector using BssHII and NheI.

scFv-AP protein was purified after growth in 250 ml of autoinduction medium (62) at 18 °C for 40 h. The bacterial pellet was resuspended in 10 ml of PBS and homogenized using EmuFlex C5 (Avestin Inc.). After centrifuging at 20,000 × g for 20 min to remove cell debris, the His₆-tagged scFv-AP protein was purified by immobilized metal affinity chromatography using the Biologic LP system (Bio-Rad) following the manufacturer's instructions.

Construction, Expression, and Purification of IgG—The aTyrS IgG was generated from the selected scFv clone as described previously (81). Briefly the VH gene was amplified from its scFv expression phagemid clone with the primer pairs 25STVH5' (GTA CCA ACG CGT GTC CAG TCT CAG GTG CAG CTG GTG GAG TCT) and 25STVH3' (GTC TCC TGA GCT AGC TGA GGA GAC GGT GAC CAG GGT) by PCR, and the purified DNA fragment was digested with MluI and NheI and ligated into human IgG1 expression vector N5KG1Val-Lark (a kind gift from Dr. Mitch Reff, IDEC Pharmaceuticals, San Diego, CA), and clones containing the correct VH gene were identified by DNA

sequencing. The Vk gene of the clone was PCR-amplified from the same phagemid vector with the primer pairs 25STVK5' (TAC TCG CAG CAA GCG GTG CAC GAT GTG CAA TTG TGT TGA CAC AGT CTC C) and 25STVK3' (ATT ATA CGA AGT TAT GGT CGA CCC CGT ACG TTT GAT ATC CAC TTT GGT C) and cloned into the pCR-2.1 vector (Invitrogen). Clones containing the correct Vk gene were identified by DNA sequencing. The Vk gene was excised from pCR-2.1 vector with DralII and BsiWI and ligated into DralII- and BsiWI-digested N5KG1Val-Lark DNA containing the appropriate VH gene. Clones containing the correct VH and Vk genes were identified by DNA sequencing, and vector DNA was used to transfect Chinese hamster ovary DG44 cells by electroporation. Stable cell lines were established by selection in G418 and expanded into 1-liter spinner flasks. Supernatant containing IgG was collected and purified on a protein G column (GE Healthcare). The affinity-purified IgG was assessed by native and reduced SDS-PAGE, and protein concentration of the final stock was determined by A_{280 nm}.

Western Analyses with scFv-AP and IgG—Antigens were separated by polyacrylamide gel electrophoresis using a 4–12% gradient Novex acrylamide gel (Invitrogen) and electrotransferred onto nitrocellulose using a semiwet electroblotter. The antigens were loaded in the following amounts: *Escherichia coli* cell extract, 30 µg; sulfatase, 28 µg; the three fibrinogens, 10 µg; C4, 0.75 µg; and vitronectin, 10 µg. Prior to analysis, the blot was blocked using wonder block solution for at least 30 min. 200 µg of IgG or 50 µg of scFv-AP was diluted in 10 ml of 1× wonder block and incubated with the transferred blot for 1 h. The blot was then washed for 10 min with PBS with Tween 20 (twice) followed by 10 min with PBS (twice). The bound IgG was detected using alkaline phosphatase-labeled anti-human (Santa Cruz Biotechnology) antibodies after similar washings. Alkaline phosphatase activity was detected using 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine and nitroblue tetrazolium chloride (Pierce).

Tyrosine Sulfate Competition ELISA Assay—Bovine fibrinogen IV was biotinylated using the EZ-link sulfo-*N*-hydroxysuccinimide LC-LC biotinylation kit (Pierce). 5 µg of biotinylated antigen was incubated with 2 mg of scFv-AP for 1 h in the presence or absence of competing compounds (tyrosine sulfate, tyrosine phosphate, and tyrosine) at 5 mM. After incubation with antibody, the biotinylated antigen with bound scFv-AP was transferred to successive wells using the King-Fisher magnetic particle processor (ThermoFisher). 10 µl of streptavidin-coated magnetic beads (Dyna) was used for each sample and incubated for 10 min. 3× PBS with Tween 20 and 3× PBS washes were subsequently carried out, and the AP signal after washing was detected using the phosphatase substrate kit (Pierce). The ELISA with the IgG was carried out similarly except that 5 µg of antigen and 1 µg of IgG were used; after the first wash, the complex was incubated with 1:2000 dilution of anti-human AP for 1 h and washed again prior to measurement. The absorbance at 405 nm reported represents the final value obtained after background subtraction.

RESULTS

Synthesis of the Peptide Antigen—Two peptide sequences were synthesized with and without a sulfate group on the central tyrosine (Fig. 1B). The sequence for Pep1 was based on the sulfation site of PSGL-1, whereas Pep2 was a synthetic non-natural sequence designed to differ from Pep1. Synthesis of the non-sulfated peptides was readily accomplished on an Applied Biosystems 431A peptide synthesizer using standard chemistry. The sulfated peptides were more difficult and required double couplings. Despite the use of double couplings, both sulfated peptides were produced in low yield. After purification via reverse-phase HPLC, the pep-

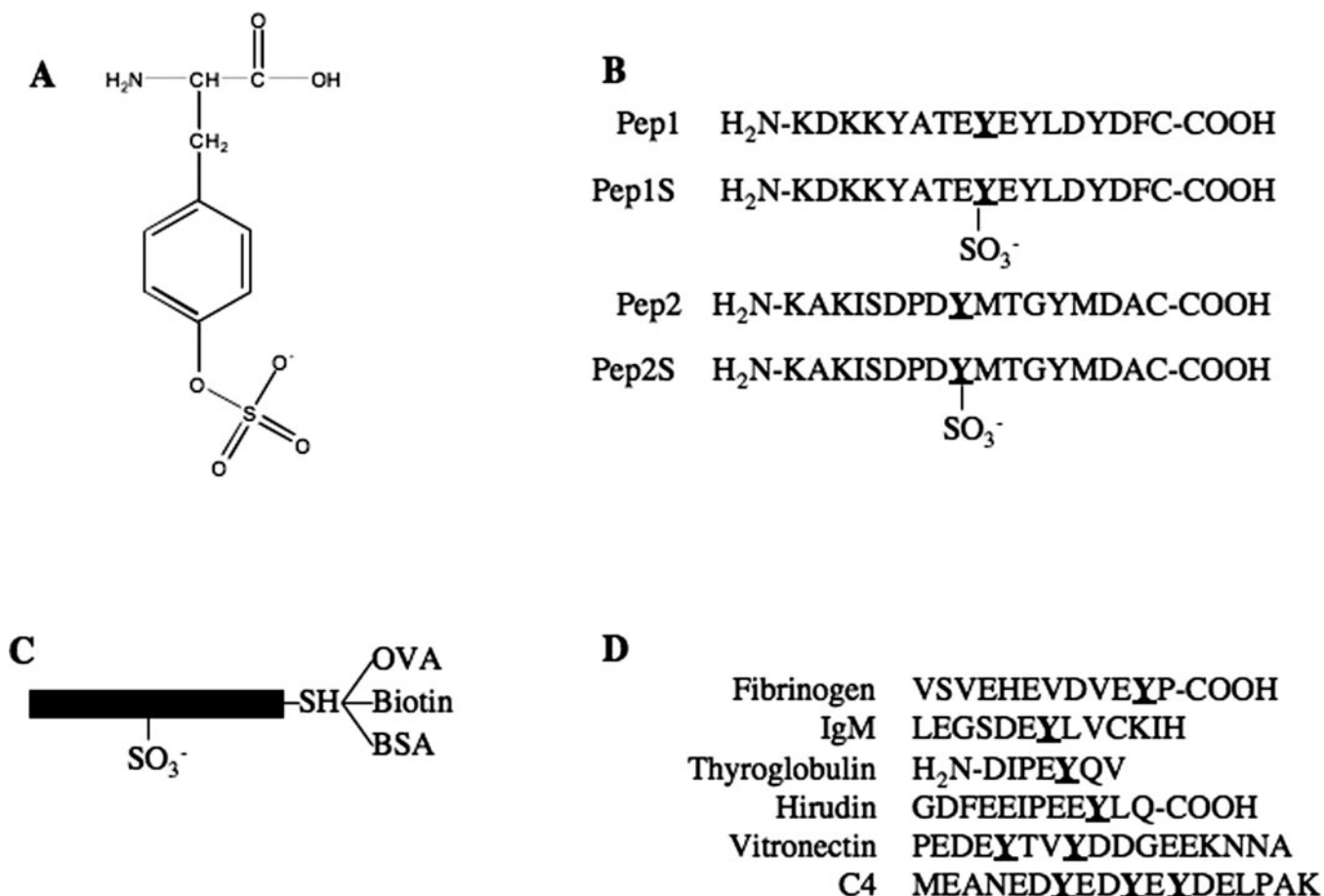


FIG. 1. *A*, the structure of the tyrosine sulfate post-translational modification. *B*, the sequences of the two peptides used for selection. The tyrosine that is sulfated is indicated in *bold* and *underlined* with the SO₃ attached. *C*, each of the peptides was coupled to ovalbumin, BSA, or biotin via the carboxyl-terminal SH group. *D*, the amino acid sequences flanking known tyrosine sulfate modification sites, in *bold* and *underlined*, for the different proteins used in this study.

ptide antigens were coupled to BSA, OVA, or biotin as shown in Fig. 1C. The peptides were named first for their carrier (BSA or OVA) followed by the peptide (BSA-1) and then for the presence or absence of the tyrosine sulfate modification (BSA-1S).

scFv Selections—As it has been observed previously that different selection procedures with the same antigen and the same library yield different scFv fragments (63, 64), 14 different selection strategies were adopted (see Table I) using the antigens described above. By varying carrier (BSA, ovalbumin, or biotin), peptide (peptide 1 or 2), and elution method (trypsin or tyrosine sulfate) in the different selections, including between different rounds and including non-sulfated peptide linked to the same carrier as blocking agent during the selection, it was hoped that antibodies recognizing tyrosine sulfate, the common component of each selection, would be isolated.

Immunotubes (Nunc) were first coated with one of the antigens described above (direct adsorption for the BSA and ovalbumin conjugates; capture on streptavidin-coated immunotubes for the biotin conjugates). After blocking with fish

gelatin (65), 10¹³ phage displaying scFv fragments were added to the immunotubes in a solution containing 4.5% fish gelatin and a non-sulfated form of the antigen as blocking agents. After a 3-h incubation and washing, the retained phage were eluted with either trypsin, which digests the scFv from the phage (59), or 10 mM tyrosine sulfate. Trypsin would be expected to release all phage that had bound to the target via the scFv (as opposed to nonspecifically), whereas it was hoped that tyrosine sulfate would elute tyrosine sulfate-specific phage. In addition to the immunotube selections, selections were also carried out in solution using streptavidin magnetic beads to harvest phage that had bound to biotinylated peptides captured on the magnetic beads.

Identification of an scFv That Binds in a Tyrosine Sulfate-dependent Manner—An initial examination of 288 clones randomly chosen from the stringent and non-stringent selections (strategies 1 and 2) revealed that none were specific for tyrosine sulfate (data not shown), and many appeared to consist of truncated clones. We have observed previously the domination of selections by truncated clones when the target antigen is present at very low concentrations or the selection

TABLE I

Different selection strategies used, indicating the antigen used for each round and the method of elution

Biotin, biotinylated peptide; 1S, sulfated peptide 1; 2S, sulfated peptide 2. The elution method applied to all selection rounds in the strategy described.

	Round 1	Round 2	Round 3	Elution
Strategy 1: stringent				
Selection 1	BSA-1S	OVA-2S	Biotin-1S	Trypsin
Selection 2	OVA-1S	Biotin-2S	BSA-1S	Trypsin
Selection 3	Biotin-1S	BSA-2S	OVA-1S	Trypsin
Strategy 2: non-stringent				
Selection 4	BSA-1S	OVA-1S		Trypsin
Selection 5	OVA-1S	Biotin-1S		Trypsin
Selection 6	Biotin-1S	BSA-1S		Trypsin
Strategy 3: specific elution				
Selection 7	BSA-1S	OVA-1S		Tyr sulfate
Selection 8	BSA-2S	OVA-2S		Tyr sulfate
Selection 9	OVA-1S	BSA-1S		Tyr sulfate
Selection 10	OVA-2S	BSA-2S		Tyr sulfate
Strategy 4: solution interaction				
Selection 11	Biotin-1S	Biotin-1S		Trypsin
Selection 12	Biotin-1S	Biotin-2S		Trypsin
Selection 13	Biotin-2S	Biotin-1S		Trypsin
Selection 14	Biotin-2S	Biotin-2S		Trypsin

TABLE II

Summary of clone screening

The number of clones identified at each stage of the selection procedure for each strategy is indicated.

	No. picked	SV5-positive	Background-negative	Sulfate-dependent binding	
				Phage ELISA	scFv ELISA
Strategy 1: stringent; Strategy 2: non-stringent	3810	1295	855	9	1
Strategy 3: specific elution	573	243	174	24	1
Strategy 4: solution interaction	3528	2117	1863	0	

is not straightforward. In the selection strategy described, the only common target was the tyrosine sulfate, a relatively small epitope. As a result truncated clones, containing exposed hydrophobic surfaces and binding relatively nonspecifically, were probably able to dominate. We thus decided to screen a larger number of clones (Table II). Almost 8000 clones split between the different selection strategies were picked (clones from the first two strategies were pooled). However, rather than immediately screen all scFv fragments against the (precious) sulfated peptide antigens, we subjected these clones to two preliminary tests: first whether they were in frame with the SV5 peptide tag and second whether these scFv fragments were able to bind to a background component of the selection (peptide, carrier, or blocking agent). The scFv library had been constructed with an SV5 tag at the carboxyl terminus of the scFv (47). Screening for SV5 reactivity identified those clones in which SV5 was correctly translated at levels above the detection limit, indicating a minimum expression level that would exclude poorly expressed clones. Although we hoped this would enrich for full-length clones, well ex-

pressed in-frame deletions (e.g. of a VH or VL domain) should also be enriched by this method.

SV5-positive clones were detected using an immunarray approach (66). After overnight growth in microtiter plates, the colonies were replicated onto UV light-sterilized nitrocellulose using a Q-bot picking robot. This nitrocellulose was then placed, bacterial side up, onto a fresh agar plate. After overnight growth, scFv expression was induced by moving the nitrocellulose sheet to a fresh agar plate containing 0.25 mM IPTG. The colonies were then lysed, and the nitrocellulose was probed with SV5 (60) and goat anti-mouse conjugated to alkaline phosphatase. 34–60% of clones were positive for SV5 expression, depending upon the selection strategy used.

Clones positive for SV5 binding were then tested for the ability to bind to a mixture of BSA, ovalbumin, and fish gelatin, the carrier and blocking proteins, respectively. ELISAs using this protein mixture as an antigen found that 12–34% of the SV5-positive clones could bind to a background component of the selection, and these were also excluded from subsequent analysis.

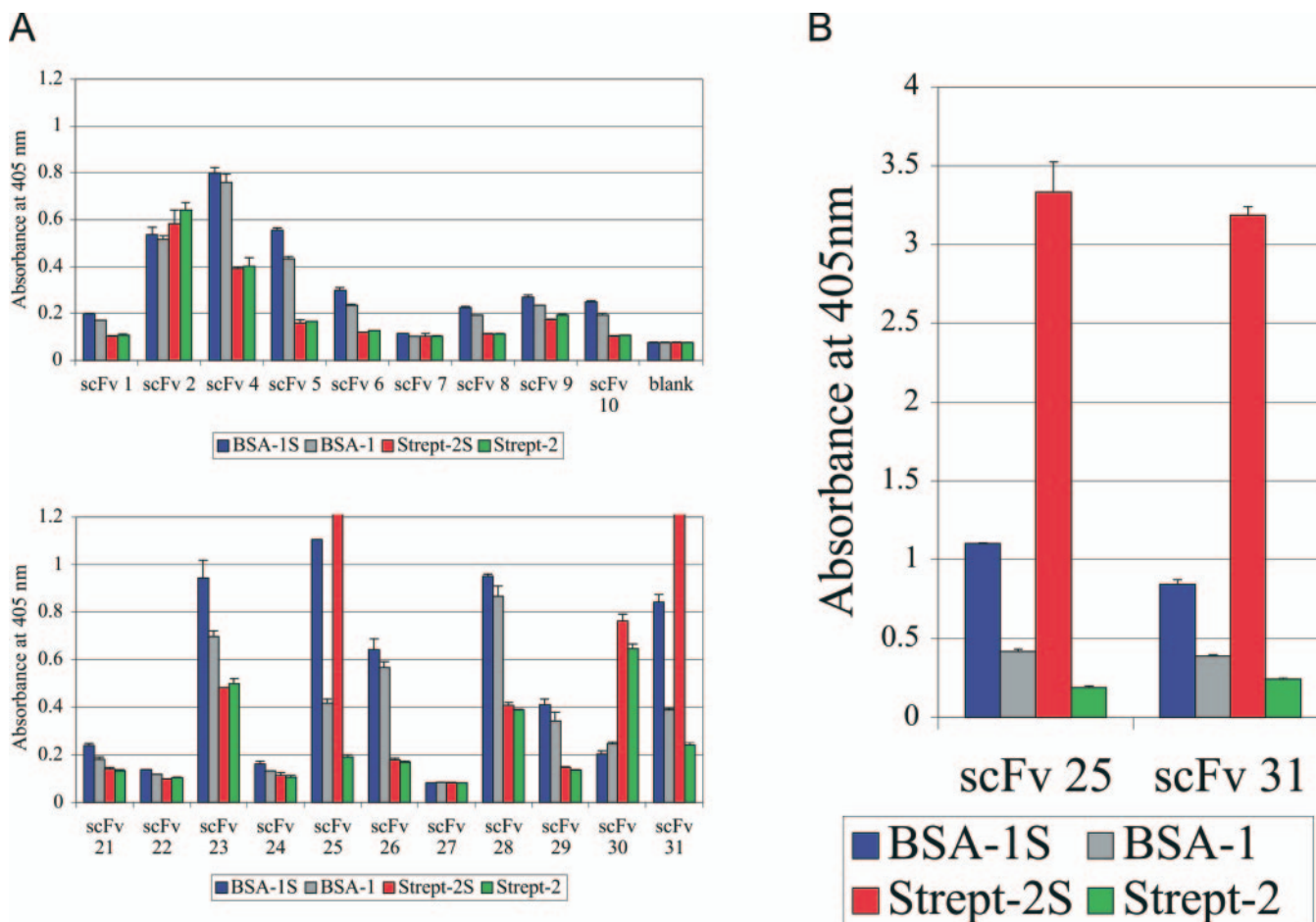


FIG. 2. A, soluble scFv ELISA results for 22 of the putatively positive clones identified by phage ELISA. B, confirmatory ELISA with the two clones scFv 25 and scFv 32 identified as binding in a sulfate-dependent manner. BSA-1S and BSA-1 represent peptide 1 coupled to BSA in the sulfated and non-sulfated forms, respectively.

The remaining 2892 scFv fragments were tested for their ability to bind to tyrosine sulfate specifically and independently of sequence context as phage antibodies. A series of ELISAs were carried out using BSA-1, BSA-1S, BSA-2, and BSA-2S as antigens, and 31 clones appeared to bind to one or another of the peptides in a sulfate-dependent manner (data not shown). These 31 clones were further tested for sulfate-specific binding as soluble scFv fragments, rather than phage, and only two were identified as being able to bind to both Pep1S and Pep2S in a sulfate-dependent fashion (Fig. 2). One of these (scFv 25) was derived from the Strategy 1/2 pool, whereas the second (scFv 31) was derived from the tyrosine sulfate elution. In addition, two clones (scFv fragments 2 and 9) bound all four targets equally well, and some appeared to recognize all four targets with a preference for one or other of the peptides (scFv fragments 4, 23, 28, and 30), whereas others were relatively specific for one of the peptides rather than the modification (scFv fragments 5, 6, 8, 10, 26, and 29). The linker between the peptide antigens and the carrier proteins was the only common structure between all four proteins, and it is possible that this was the epitope

recognized by those clones recognizing all four targets. In retrospect, this result could have been predicted as the libraries were not blocked with soluble linker, and linkers were not switched between selections in the way that carrier proteins were.

The two scFv fragments recognizing peptides in a sulfate-dependent fashion were retested on sulfated and non-sulfated peptides (Fig. 2B), and their reactivity was confirmed. Both clones were sequenced and found to be identical.

Characterization of Binding to Natural Targets—To further characterize the binding specificity of this antibody, we also evaluated the ability of the scFv to recognize naturally tyrosine sulfated proteins (20) by carrying out ELISAs with three proteins known to be tyrosine sulfated: fibrinogen (67), thyroglobulin (68), and rat IgM (69). These proteins were purchased from commercial sources and not otherwise tested for the presence of the sulfotyrosine modification. The scFv recognized all three proteins, the only common feature of which was the tyrosine sulfate modification. This can be removed with abalone sulfatase (70, 71), and this treatment was shown to significantly reduce the scFv ELISA signal (Fig. 3a). How-

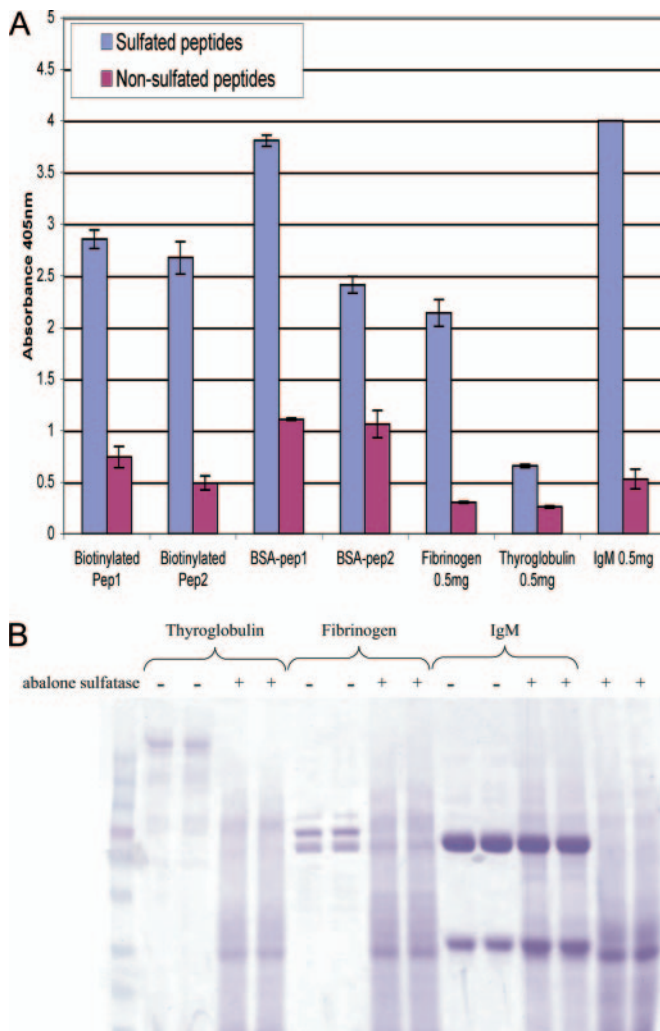


FIG. 3. A, ELISA reactivity of the soluble scFv against the sulfated and non-sulfated peptides and three proteins known to be sulfated. B, polyacrylamide gel electrophoresis of the proteins used in the ELISA in A, showing the effects of sulfatase treatment.

ever, examination of the proteins by SDS-PAGE before and after sulfatase treatment showed the presence of a significant smear from the sulfatase and a reduction in the amounts of thyroglobulin and fibrinogen (especially thyroglobulin), whereas the levels of rat IgM remained relatively constant. Rat IgM had the highest signal with the antibody and the greatest signal reduction after treatment with sulfatase (Fig. 3b), indicating that signal reduction was probably due to loss of sulfation, whereas in the case of thyroglobulin and fibrinogen, some of the signal loss was probably also due to proteolysis.

As the purified scFv was poorly expressed, two recombinant forms of greater stability were created: full-length IgG and an scFv-AP fusion protein (61, 72–75). These are both dimeric and were found to be stable for months at 4 °C. In general, the IgG was easier to use and tended to give much stronger signals, whereas the scFv-AP fusion had the advantage that there was no need for a secondary antibody. ELISA

with the IgG showed that it recognized hirudin from leeches, three different forms of fibrinogen (fraction 1s (bovine), fibrinogen fraction IV (bovine), and fibrinogen (rat)), and complement C4 (Fig 4A) and that the signal was lost upon sulfatase treatment. Interestingly we noted a correlation between the ELISA signals obtained for the different proteins and the number of sulfated tyrosines: C4 and vitronectin gave the highest ELISA signals and possessed three and two sulfated tyrosines, respectively (Fig. 1D). The scFv-AP conjugate was used to analyze binding to human vitronectin (which could not be tested with the IgG as it gave a strong nonspecific signal with the secondary anti-human antibody) and confirm binding to human IgM (Fig. 4B). However, as can be seen from Fig. 4C, with the exception of IgM and C4, there was again significant proteolysis upon sulfatase treatment.

To further demonstrate the specificity of the antibody for the tyrosine sulfate modification, a magnetic bead-based ELISA (KingFisher, ThermoFisher Inc.) was developed with the goal to see whether antibody binding could be inhibited by soluble tyrosine sulfate. Biotinylated bovine fibrinogen IV, the anti-sulfotyrosine antibody, and PBS, 5 mM tyrosine sulfate, 5 mM tyrosine phosphate, or 5 mM tyrosine were incubated together for 1 h. After this period, the amount of antibody that was bound to the biotinylated fibrinogen was assessed by adding streptavidin magnetic beads followed by washing. As can be seen in Fig. 5A, incubation with 5 mM tyrosine sulfate reduced the signal to background levels, whereas tyrosine phosphate and tyrosine had no effect whatsoever, indicating the specificity of the binding. Similar results were obtained with the scFv-AP fusion (Fig. 5B), and a control experiment with lysozyme and an anti-lysozyme antibody showed that signal inhibition was not due to nonspecific inhibitory effects caused by tyrosine sulfate (data not shown). This inhibition was further studied by titrating the amount of tyrosine sulfate required to inhibit binding. As can be seen in Fig. 5C, at 10 mM tyrosine sulfate almost full inhibition was observed, whereas half-maximal inhibition was seen at ~1.25 mM.

We also determined whether the antibody was able to recognize the tyrosine sulfate modification in Western blots. Analyses with the various fibrinogens and C4 were carried out with the IgG followed by anti-human AP, whereas the vitronectin was assayed using the scFv-AP conjugate as it gave a very high background with the secondary antibody. Each of the proteins was clearly recognized by the anti-tyrosine sulfate antibody with a signal intensity not correlated to the intensity of the Coomassie Blue band, suggesting that the visible bands are differentially sulfated. These proteins were treated with the abalone sulfatase treatment, modulated to reduce proteolysis for each protein (Fig. 6A). It can be seen that after sulfatase treatment the Western signal for each protein was abolished or significantly reduced (Fig. 6B) without affecting the integrity of the protein itself. As *E. coli* does not express any sulfated proteins, lacking the enzymatic machinery to do so (20), we tested the extent of nonspecific

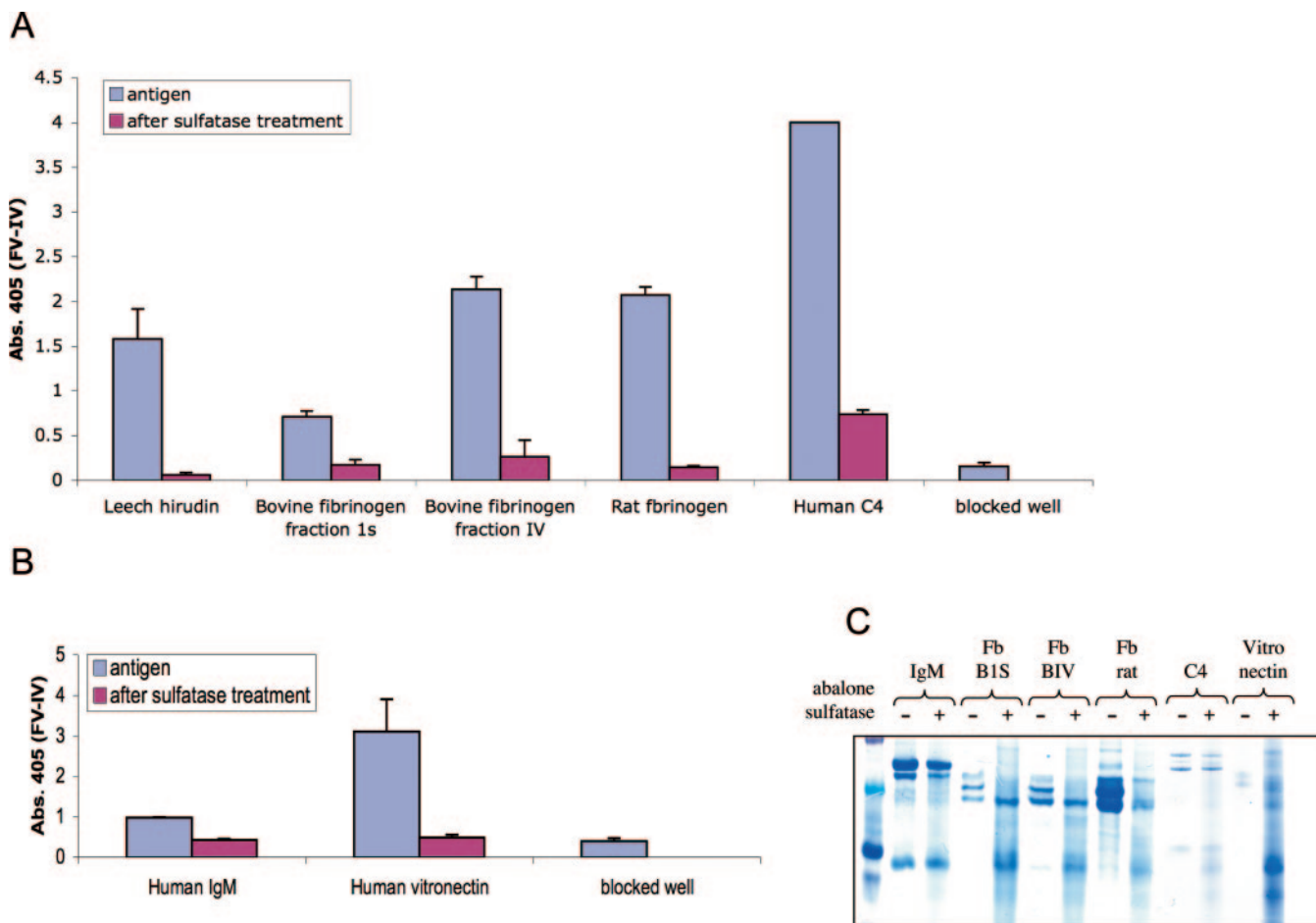


FIG. 4. A, ELISAs carried out with full-length IgG against a number of sulfated proteins. B, ELISAs carried out with the scFv-AP fusion protein. C, PAGE of the analyzed proteins before and after sulfatase treatment. Fb, fibrinogen; B1S, bovine fraction 1s; BIV, bovine fraction IV.

binding of the antibody by probing an overloaded *E. coli* extract. As can be seen in lane 1, no signal was obtained whatsoever, indicating that the antibody was unable to react with the diverse array of *E. coli* proteins expressed under normal growth conditions.

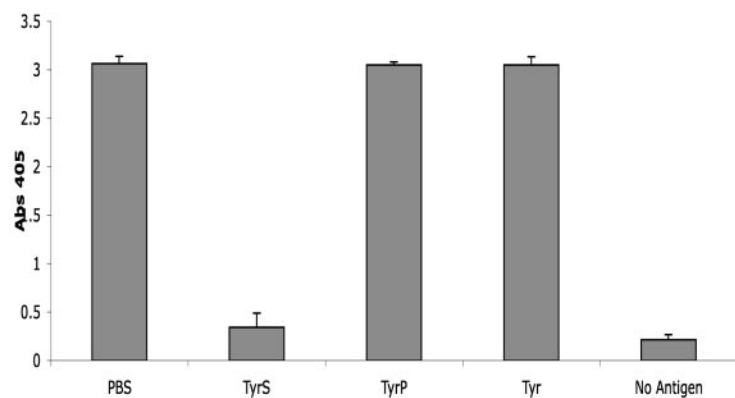
DISCUSSION

Antibodies that recognize PTMs, independently of the protein to which they are bound, have proved to be as useful as they are rare. By using a generic PTM-specific antibody for immunoprecipitation, all proteins containing that PTM can be identified and monitored under different experimental conditions (13–17). Unfortunately it has proved extremely difficult to isolate such antibodies by immunization, reflecting the innate tolerance immune systems have for such ubiquitous protein modifications.

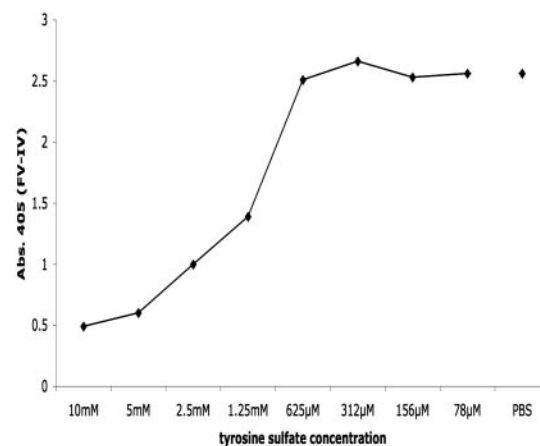
Phage display overcomes this innate tolerance by providing *in vitro* selection conditions in which the normal restrictions of the intact immune system no longer apply. In the case of libraries made from synthetic V genes (50–53, 76), endogenous germ line V genes form the basis for the diversity, but the

crucial third heavy chain hypervariable loop is created from random oligonucleotides that have never undergone editing by the immune system. As a result there is no reason why they should not be able to recognize self-antigens. In libraries made from natural VH and VL genes (45–49), although the rearranged V genes have been subject to immune tolerance, this has only occurred within the context of the original VH/VL pairing, which provides specificity, and not on the individual rearranged V genes themselves. As antibodies in such libraries are made up of random VH/VL combinations, almost all of which are novel, many new specificities are formed that allow the selection of antibodies against vast arrays of different targets, including self-antigens. Although phage antibody libraries have been widely used to select antibodies against such targets (45–53), it is surprising that no attempts have been made (or at least published) to select antibodies against post-translational modifications. The results described here suggest a possible explanation: rather than screening 96–384 clones, which almost always yields a multiplicity of different clones for most targets, we had to screen almost 8000 different clones before finding two identical clones with the desired

A: IgG



C: titration



B: scFv-AP

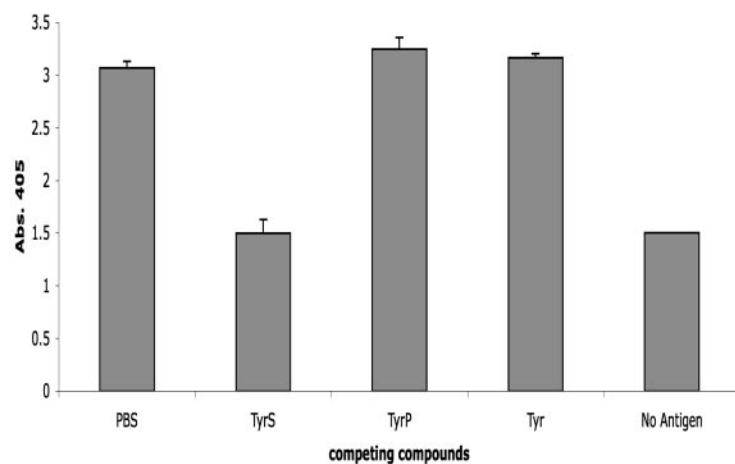


FIG. 5. A, inhibition of IgG binding to bovine fibrinogen IV upon incubation with tyrosine sulfate (*TyrS*) but not tyrosine or tyrosine phosphate (*TyrP*). B, same as A except the scFv-AP fusion was used. C, titration of the inhibition of IgG binding to fibrinogen IV by increasing concentrations of tyrosine sulfate. Abs, absorbance.

characteristics. The fact that this was the only clone and it was isolated using two very different selection strategies suggests that it may have been the only one with this specificity in this very large library (47). This indicates that such antibodies are far more rare than ones recognizing other targets, and extensive screening is required to find them.

An examination of antibody binding site structures has revealed three main topographies, cavity, grooved, and flat, binding haptens, peptides, and proteins, respectively (77–82), with rare antibodies showing alternative topographies such as long finger-like HCDR3 projections (83, 84). With the exception of polypeptide modifications, such as ubiquitination (85), most PTMs can be considered to be small haptens. However, unlike free haptens, they are attached to polypeptides and so are unlikely to be able to penetrate sufficiently deeply to be recognized by antibody binding site cavities. Similarly they probably cannot be recognized by antibodies with flat or grooved topologies without components of the supporting

polypeptide chain also being recognized. These exacting structural requirements probably explain why such antibodies are so rare.

A number of different lines of evidence are presented in this study to demonstrate the specific characteristics of this antibody, which, either as scFv, scFv-AP fusion, or full-length IgG is able to recognize a number of different proteins known to be tyrosine sulfated. These proteins have no sequence identity, and the only factor in common is the sulfated tyrosine residue (Fig. 1D). Although binding could be abrogated by treatment with abalone sulfatase, it is clear that the protein used (Sigma) was contaminated with some protease activity, and as a result, loss of binding was a combination of both activities. However, by careful titration, this effect could be reduced (Fig. 6), and loss of antibody reactivity could be observed with a maintenance of protein integrity. The antibody was also able to recognize two synthetic tyrosine sulfated peptides far more effectively than the unsulfated forms,

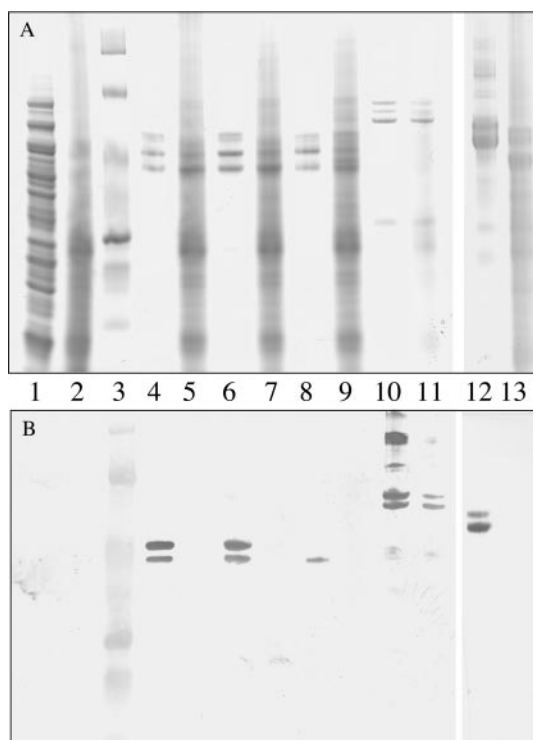


FIG. 6. A, polyacrylamide gel electrophoresis of different proteins treated (lanes 5, 7, 9, 11, and 13) and not treated (lanes 1, 4, 6, 8, 10, and 12) with abalone sulfatase. B, the same proteins as in A analyzed by Western blotting using the IgG (lanes 1–11) or scFv-AP fusion (lanes 12 and 13). Lane 1, *E. coli* extract; lane 2, abalone sulfatase; lane 3, markers; lanes 4 and 5, fibrinogen 1s; lanes 6 and 7, fibrinogen IV; lanes 8 and 9, rat fibrinogen; lanes 10 and 11, human C4; lanes 12 and 13, vitronectin.

and signal intensity appeared to be dependent upon the sulfation state of the recognized protein. Finally and most convincingly, we were able to inhibit binding of the antibody with free tyrosine sulfate but not tyrosine phosphate or tyrosine alone. Titration of this inhibition showed that half-maximal inhibition was obtained at 1.25 mM, indicating that this antibody is also able to bind free tyrosine sulfate albeit at a relatively low affinity.

Although we believe that the application of this method to other PTMs is likely to be successful, we expect that the frequency of such antibodies in libraries similar to the one used will be equally low, requiring similar extensive screening for identification. In the experiments reported here, successful elution was carried out using either trypsin or tyrosine sulfate. Trypsin is a generic method that removes the scFv by proteolysis (59). Although such elution is dependent upon the binding activity of the displayed scFv, it is not necessarily specific for the post-translational modification. By using high concentrations of tyrosine sulfate as the elutant, we expected that the eluted scFv fragments would be specific for the modification. It is striking in this regard that the frequency of the specific scFv in the tyrosine sulfate eluted population (0.17%) was considerably higher than that using trypsin (0.026%), reflect-

ing the fact that elution with tyrosine sulfate resulted in fewer clones. This suggests that the use of high concentrations of the soluble form of the PTM is likely to be the most effective elutant with fewer clones eluted and available for screening but a higher proportion of positives. However, it should be pointed out that, due to the protuberant nature of the amino acid side chain, sulfotyrosine, like phosphotyrosine, may be a particularly antigenic post-translational modification within the context of both intact immune systems and phage antibody libraries. If this is the case, selection of antibodies with similar properties recognizing alternative PTMs may be more challenging.

This library (47) was based on natural rearranged human VH and VL genes. Rather than using a general library, it may be possible to create specific libraries targeted to recognize post-translational modifications independently of context as has been recently proposed for other target types (77) using this or other generic PTM antibodies as scaffolds. Within this context, the determination of the structure of this antibody in complex with a tyrosine sulfate-containing protein may provide useful information, including the identification of amino acids involved in binding, the mutation of which may allow the selection of antibodies with higher affinities either for the generic modification or specifically modified sites as has been carried out recently with a hapten binding antibody (86).

Although a computational analysis of the human genome suggests that up to one-third of the proteins that enter the secretory pathway may be tyrosine sulfated (87), less than 70 have been shown to carry tyrosine sulfate experimentally (20). This reflects the difficulties in identifying sulfotyrosine residues, which has been traditionally carried out by thin-layer chromatographic isolation of tyrosine [^{35}S]sulfate from hydrolysates of radiolabeled proteins (24) or MS (58) with the presence of sulfate groups often inferred rather than proven. The isolation of the antibody described here should considerably simplify further study of this post-translational modification, allowing a greater understanding of its distribution and role in different physiological processes.

Addendum—We have recently shown that the scFv-AP version of the antibody is also able to recognize heparin cofactor II.³

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