Protein-phosphotyrosine proteome profiling by superbinder-SH2 domain affinity purification mass spectrometry, sSH2-AP-MS

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Recently, “superbinder” SH2 domain variants with three amino acid substitutions (sSH2) were reported to have 100-fold or greater affinity for protein-phosphotyrosine (pY) than natural SH2 domains. Here we report a protocol in which His-tagged Src sSH2 efficiently captures pY-peptides from protease-digested HeLa cell total protein extracts. Affinity purification of pY-peptides by this method shows little bias for pY-proximal amino acid sequences, comparable to that achieved by using antibodies to pY, but with equal or higher yield. Superbinder-SH2 affinity purification mass spectrometry (sSH2-AP-MS) therefore provides an efficient and economical approach for unbiased pY-directed phospho-proteome profiling without the use of antibodies.

Keywords: Affinity purification / Anti-phosphotyrosine / EGF receptor / HeLa / Phospho-proteomics / Phosphotyrosine / SH2 domain / Superbinder / Technology

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Phosphorylation of proteins is a crucial mechanism for regulating cell signaling processes. Monitoring protein phosphorylation in a given cell or tissue is vital for a better understanding of biological mechanisms. Currently, measurement of phospho-proteins requires the enrichment of phosphopeptides from total peptides. A common technique for phosphotyrosine (pY) peptide enrichment makes use of commercially available anti-pY antibodies conjugated to beads [1]. The utility of recombinant SH2 domains for affinity purification (AP) of pY-containing polypeptides is well established [2]. Recently, SH2 variants containing three amino acid substitutions were engineered to have higher affinities for pY-containing polypeptides than wild-type SH2 [3]. Here we show that one such “superbinder” SH2 domain (sSH2) derived from Src, as shown in Fig. 1B, is able to enrich pY peptides as efficiently as anti-pY antibody. It represents an economical alternative to the antibody-based approach to phospho-proteome analysis of cells and tissues.

The construction of His-tagged Src SH2 domains was described previously [3]. Briefly, DNA encoding human Src SH2 (codons 114 - 252) was cloned into pETM11 vector, and subjected to site-directed mutagenesis (QuikChange II; Stratagene). Wild-type (WT) and triple-mutant SH2 sequences were expressed as hexa-histidine-tagged proteins in E. coli BL21 (DE3). WT and sSH2 variant proteins were purified with Ni-NTA beads (Qiagen) under native conditions, according to manufacturer’s instructions. Routinely, 200 μL Ni-NTA
beads were used for purification from 500 mL E coli cultures, yielding roughly 2 μg protein/μL beads (Supporting Information Fig. S1A).

HeLa cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium. To obtain maximum protein phosphorylation in cells, HeLa cells were treated with phosphatase inhibitor sodium pervanadate (0.5 mM) 10 min before harvesting [4]. For EGF stimulation of cells, EGF (Invitrogen) at a final concentration of 100 ng/mL was added to medium for 10 min prior to harvest.

The workflow of pY enrichment by Src sSH2 is shown in Fig. 1A. A detailed step-by-step experimental protocol including MS data analysis methodology is provided in Supporting Information.

Purification of total peptide from HeLa was described previously [1, 5]. Briefly, cells were lysed in 8M urea buffer (20 mM HEPES pH 8.0, 8 M urea and phosphatase inhibitors (Thermo Sci. Cat#88662)), digested with trypsin, and then peptides purified by using C18.

Src sSH2 was used as a prototype to test whether an immobilized recombinant superbinder SH2 domain variant protein could be used to purify pY peptides from a trypsin-digested total cell extract. First we optimized elution conditions to release bound pY peptide, but not SH2 proteins. Total peptide (2 mg as protein) isolated from pervanadate treated HeLa cells was incubated in batch mode with 50 μg Src sSH2 beads in 0.5 mL Affinity Purification (AP) buffer (50 mM MOPS pH 7.2, 10 mM dibasic sodium phosphate, 50 mM NaCl) for 3 h at 4°C. After washing beads three times with AP buffer, bound pY peptides were tested for elution by using three different 150 microlitre solutions, each introduced in 3 × 50 μL steps: (1) 50 mM phenyl phosphate in AP buffer (PPT) [6]; (2) 1% trifluoroacetic acid (TFA) [1, 5]; and (3) 8 M urea. Peptides were isolated from the three eluents by adsorption to C18 resin, then analyzed by LC-MS/MS (Q-Exactive, ThermoFisher). pY peptide intensity was quantified with MaxQuant software [7], by measuring MS1 extracted ion current (XIC). Phosphorylation site localization probabilities were required to exceed 75% and false discovery rate <1%. A concern with this approach was contamination of the LC-MS platform with sSH2. SDS-PAGE was used to assess release of sSH2 during pY peptide elution. Competitive displacement of pY peptides under native conditions by using PPT had the least sSH2 in eluents (Supporting Information Fig. S1A, lane 3), and also yielded the greatest number of pY peptide identifications (data not shown). Analysis of pY peptides derived from EGF receptor (EGFR) indicated eight EGFR pY sites (Supporting Information Fig. S3) after PPT elution, but only three in TFA eluates (Supporting Information Fig. S1B). Eight molar urea elution showed very poor pY peptide enrichment, and a high amount of sSH2 release, which may have interfered with the subsequent C18 peptide purification step. Therefore, Src sSH2 affinity capture with PPT elution was used for subsequent experiments.

Next we tested whether using more sSH2 would capture more pY peptides from a given amount of starting material. 2 mg peptide from pervanadate treated HeLa cells was incubated with Src sSH2 ranging from 5 to 300 μg (Supporting Information Fig. S1C). 50 μg of Src sSH2 pulled down the highest number of pY peptides. A possible reason is that under test conditions (2 mg starting material) 50 μg Src-mSH2 has relatively optimal capacity to bind pY peptides, and more sSH2 (i.e. 150 μg and 300 μg tested) provides increased surface area for non-specific interaction with non-phosphorylated peptides. 

![Figure 1. Phospho-tyrosine(pY) peptide enrichment with “superbinder” Src SH2 domain (sSH2).](image)
Comparison of purified pY peptides with those remaining in flow through fractions showed that depletion by using 50 µg Src sSH2 missed only 0.4% of total pY peptides (Fig. 1C; Supporting Information Table S1). When increasing amounts of starting material, ranging from 1 to 9 mg were tested, pY peptides captured by 50 µg Src sSH2 also increased (Supporting Information Fig. S1D). This indicates 50 µg Src sSH2 was not saturated for pY peptide binding when 2 mg starting material was used. Since 50 µg sSH2 represents a convenient volume of beads (10 to 25 µL), and with sufficient pY peptide binding capacity for the scale of experiment under investigation, this amount of sSH2 was used in subsequent experiments.

Next, we compared the sSH2 affinity purification mass spectrometry (sSH2-AP-MS) protocol with a widely used anti-pY-based approach [1]. pY peptides captured with anti-pY antibody (Cell Signaling Technology; item #5636) were eluted by using 0.15% TFA [1]. Anti-pY beads (10 µL) pulled down less pY peptide as measured by total MS1 signal, than the sSH2-AP-MS protocol involving 50 µg Src sSH2 (Fig. 1C and Supporting Information Table S1), but anti-pY captured more unique pY peptides (471) than Src sSH2 (414; Fig. 2A). The identity of about 80% of the pY peptides was same in both methods (Fig. 2B and Supporting Information Fig. S1E). The two methods showed very slight preferences for pY-proximal sequences. For example, a total of nine EGFR pY peptides, Src sSH2 captured more pY peptide than anti-pY, however, pY1100 peptide was only detected by anti-pY.

Src and Grb2 SH2 domains have different pY sequence binding preferences, such as pYEEI for Src SH2 and pYXNX for Grb2 [4]. We questioned if Src and Grb2 sSH2 domains, that is, Src and Grb2 SH2 variants containing three analogous amino acid substitutions, retain different pY sequence binding preferences. To test this, we examined whether a combination of the two sSH2 domains was more efficient than either domain used alone, at capturing diverse pY peptides. Figure 3A shows purified SH2 domains. Figure 3B shows that the amount of pY peptide, as measured by total MS signal pulled down by 50 µg of the indicated domains. In rank order, according to maximal pY peptide recovery: Src sSH2 >> Src SH2 (wild type, WT) ≈ Grb2 sSH2 > Grb2 SH2 (WT). The mixture of 25 µg each Grb2 and Src sSH2 domains purified less pY peptide than 50 µg Src sSH2 alone (Figs. 3B and 2A). These results indicate the combination of two mutant SH2 domains did not increase pY peptide amount. This suggests that under conditions of excess sSH2 binding capacity, minor differences in pY proximal sequence preference do not have a pronounced effect on pY peptide recovery. pY peptide sequences in the top pY peptide signals from different affinity reagents (i.e. Src sSH2, Grb2 sSH2, anti-pY) are quite similar (Supporting Information Fig. S2B and Fig. 2B), again indicating the amount of pY peptide present in samples, not the sequence context of pY sites is a decisive factor for pY peptide identified under condition of excess SH2 domain.

Src sSH2 was tested in a functional study of EGFR activation for pY enrichment in samples with different pY levels (Fig. 3C and Supporting Information Table S2). Src sSH2 captured pY peptides from cells in resting condition (i.e. low tyrosine phosphorylation levels), such as no EGFR (lane 1) or with EGFR expression but no EGF stimulation (lane 2); as well as in conditions of activated EGFR (high tyrosine phosphorylation levels), such as in EGF (Lane 3) or EGF/VO 

4 treatment, recovery of EGFR phosphopeptide containing pY1172 was increased dramatically, whereas pY216 of GSK3β, a site typically phosphorylated in the absence of cell stimulation, changed only slightly.

This experimental condition was also tested in different cell lines such as lung cancer and leukemia cell lines. Routinely 50 to 200 pY peptides could be identified in a sample depending on its phosphorylation levels (data not
Figure 3. Phospho-tyrosine peptides purified by wild-type (SH2) and superbinder (sSH2) domains derived from Grb2 and Src, from cells treated with epidermal growth factor (EGF) and/or phosphatase inhibitor pervanadate. (A) Stained protein gel of indicated purified His-tagged SH2 domains. Protein amounts were estimated by comparison with bovine serum albumin standard run on the same gel (not shown). (B) Comparison of MS signals associated with pY peptides captured by indicated SH2 domains used alone (lanes 1–4) or in combination (lane 5). (C) pY peptides purified by Src sSH2 from HEK-Trex-EGFR cells, which express tetracycline (Tet)-inducible EGF receptor (pY peptide in Supporting Information Table S2). Shown in the top row are the number of non-redundant pY peptides purified from HEK-Trex-EGFR without and with Tet-induced EGFR expression; and/or EGF stimulation and pervanadate treatment, as indicated. In rows 2 and 3 from the top, MS signals associated with the indicated EGFR and GSK3 pY peptides are shown. The lower three panels are western blots that verify, respectively, induction of EGFR expression in response to Tet (anti-EGFR); actin as loading control (anti-Actin); and stimulation of cellular protein-pY by EGF and pervanadate treatments (Anti-pY).

Recently, Bian et al. used Src sSH2 protein to capture pY peptides [8]. We compared ~500 pY sites identified by our protocol (Supporting Information Tables S1 and S2) with two databases: 351922 pS/pT/pY sites from Phospho-SitePlus (http://www.phosphosite.org/), and 19 570 pY sites from Bian et al [8]. This indicated ~20 new pY sites (Supporting Information Fig. S2C, Tables S1 and S2). Our results are comparable to other studies of HeLa cell pY proteomes conducted on a similar scale. For example Boersema et al. [9] used an anti-pY approach along with stable isotope dimethyl labeling to identify nearly 1000 pY sites from analyses of 4 mg vanadate-treated HeLa cell samples. In their large-scale comprehensive (i.e. pS/pT/pY) phospho-proteome analysis Olsen et al. [10] identified > 100 pY sites in HeLa cells. Our current method uses a simple one-step affinity purification protocol without antibodies and one LC-MS/MS run, which is suitable for small amounts of start material, a practical situation when dealing with patient samples.

In conclusion, we have established an efficient and economical protocol for phospho-tyrosine peptide enrichment. Our results indicate Src-mSH2 is a good replacement for anti-pY antibody for pY peptide profiling, and the combination of both reagents will provide more coverage for pY profiling or pYomics study.

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References


