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Proteomic Screening Method for Phosphopeptide Motif Binding Proteins Using Peptide Libraries

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Abstract

Phosphopeptide binding domains mediate the directed and localized assembly of protein complexes essential to intracellular kinase signaling. To identify phosphopeptide binding proteins, we developed a proteomic screening method using immobilized partially-degenerate phosphopeptide mixtures combined with SILAC and microcapillary LC/MS/MS. The method was used to identify proteins that specifically bound to phosphorylated peptide library affinity matrices, including pTyr, and the motifs pSer/pThr-Pro, pSer/pThr-X-X-X-pSer/pThr, pSer/pThr-Glu/Asp or pSer/pThr-pSer/pThr in degenerate sequence contexts. Heavy and light SILAC lysates were flowed over columns containing these phosphorylated and non-phosphorylated (control) peptide libraries respectively, and bound proteins were eluted, combined, digested and analyzed by LC/MS/MS using a hybrid quadrupole-TOF mass spectrometer. Heavy:light peptide ion ratios were calculated, and peptides that yielded ratios greater than ~3:1 were considered as being from potential phosphopeptide binding proteins since this ratio represents the lowest ratio from a known positive control. Many of those identified were known phosphopeptide-binding proteins, including the SH2 domain containing p85 subunit of PI3K bound to pTyr, 14-3-3 bound to pSer/pThr-Asp/Glu, polo-box domain containing PLK1 and Pin1 bound to pSer/pThr-Pro and pyruvate kinase M2 binding to pTyr. Approximately half of the hits identified by the peptide library screens were novel. Protein domain enrichment analysis revealed that most pTyr hits contain SH2 domains, as expected and to lesser extent SH3, C1, STAT, Tyr phosphatase, Pkinase, C2 and PH domains, however, pSer/pThr motifs did not reveal enriched domains across hits.

Introduction

Phosphopeptide binding domains mediate the directed and localized assembly of multi protein complexes in response to activation of protein kinases. Src homology 2 (SH2) domains, protein tyrosine binding (PTB) domains, and the C2 domain of PKC- δ , have been shown to recognize phosphotyrosine (pTyr)-containing peptide sequences (1, 2). Among others, 14-3-3 proteins, WW domains, and the Polo-box domain of PLK, have been shown to recognize phosphoserine (pSer)- and phosphothreonine (pThr)-containing peptide sequences (3, 4). The broad structural diversity of these known phosphopeptide binding modules suggests that the ability to recognize phosphorylated residues arose multiple times throughout evolution. Therefore, it is conceivable that additional undiscovered phosphopeptide binding modules with unique folding properties exist. It has recently been

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shown that more than 6,000 proteins can be phosphorylated at nearly 36,000 phosphorylation sites at Ser, Thr and Tyr residues, a number that represents ~50% of known proteome (5). Recently, we identified a phosphohistidine residue in mammalian cells, suggesting additional roles for phosphorylation in eukaryotic cells (6).

Peptide libraries have proven to be a useful tool for studying phosphopeptide binding proteins. Traditional peptide library screening, used to elucidate phosphopeptide binding motifs, has shown that different phosphopeptide binding domains recognize pTyr, pSer, or pThr within specific peptide sequence contexts (7–10). Additionally, immobilized partially degenerate peptide libraries biased towards optimal kinase motifs have successfully been used to screen for proteins out of *in vitro* translated pools of cDNA that specifically bind to pSer or pThr peptides. Such techniques were used to identify the Polo-box domain of PLK as a novel phospho-serine/threonine binding domain that recognizes the sequence motif S-[pT/pS]-(P/X) (11).

While many studies focus on determining phosphorylation sites on proteins from cells and tissues sources (12–14), less work has focused on proteins that interact with specific phosphorylation sites using proteomics (15). Quantification of proteins using mass spectrometry has become routine with the introduction of stable isotope labeling strategies (16–20), label-free techniques (21–23) in addition to combinations of targeted methods with stable isotope labeling (24–26). In this study, we have combined immobilized peptide library technology with stable isotope labeling of amino acids in cell culture (SILAC) and microcapillary liquid chromatography tandem quadrupole time-of-flight (TOF) mass spectrometry (LC/MS/MS) to conduct a screen for phosphopeptide binding proteins from HeLa (human cervical cancer) cell lysates. For immortalized cells, SILAC is a method of choice since labeling efficiency is very high after five cell passages and mixing of the sample occurs early in sample processing prior to a single tryptic digestion thus resulting in low risk of experimental error. While the heavy and light labeled proteins are indistinguishable to the cell and have no effect on morphology or growth rates, they remain distinguishable by mass spectrometry by a six Dalton mass difference, and relative abundances can be determined from the respective MS signal intensities (16, 27). In our screen, light versions of peptides represent control proteins and heavy versions of peptides represent potential hits if above a conservative threshold ratio (~3:1, because this threshold represents the lowest ratio of known positive controls).

Experimental Section

Peptide library and column construction—Phosphorylated and non-phosphorylated versions of the following peptide libraries were constructed: pTyr = biotin-Z-Z-Gly-Gly-Gly-X-X-X-X-X-pTyr-X-X-X-X-X-Gly-Gly, pSer/pThr-Pro = biotin-Z-Z-Gly-Gly-Gly-X-X-X-X-X-(pSer/pThr)-Pro-X-X-X-X-Gly-Gly, pSer/pThr-X-X-X-pSer/pThr = biotin-Z-Z-Gly-Gly-Gly-X-X-X-(pSer/pThr)-X-X-X-(pSer/pThr)-X-X-X-Gly-Gly, pSer/pThr-Asp/Glu = biotin-Z-Z-Gly-Gly-Gly-X-X-X-X-X-(pSer/pThr)-(Asp/Glu)-X-X-X-X-Gly-Gly, where (pSer/pThr) represents a 50/50 mixture of phosphoserine and phosphothreonine, (Asp/Glu) represents a 50/50 mixture of aspartic acid and glutamic acid, Z indicates aminohexanoic acid, and X denotes all amino acids except Cysteine (Tufts University Sequencing Facility). Streptavidin beads (GE Healthcare) were incubated with a five-fold molar excess of each biotinylated library in TBST (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween) for 1 hour at 4°C. Peptide-conjugated beads were packed onto disposable 1 ml chromatography columns (Bio-Rad Laboratories) (250 µL beads/column) and were rapidly washed with five times with TBST

SILAC cell culture—HeLa cells were grown in DMEM media that lacked Arginine and Lysine (Gibco) and nourished with dialyzed fetal bovine serum. The media was supplemented with heavy $^{13}\text{C}_6$ -L-Arginine and $^{13}\text{C}_6$ -L-Lysine (Cambridge Isotope Laboratories) or with $^{12}\text{C}_6$ -L-Arginine and $^{12}\text{C}_6$ -L-Lysine (Sigma-Aldrich). Cells were passaged five times in “heavy” or “light” SILAC media to ensure complete labeling.

Cell lysis—SILAC labeled HeLa cells were lysed in buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 4 $\mu\text{g/mL}$ of the protease inhibitors aprotinin, leupeptin, and pepstatin.

Screens for phosphopeptide binding proteins—5 mg of the SILAC cell lysates were flowed over the peptide library columns. The “heavy” lysate was applied to the phosphorylated peptide library column, and the “light” lysate (control) was applied to the non-phosphorylated peptide library column. Lysate that had flowed through the column was re-applied to the column two additional times for maximal binding. Columns were then rapidly washed five times with TBST, and bound proteins were eluted with 250 μL of 20 mM sodium phenylphosphate pH 7.8 (for the pTyr peptide library screen only) or 250 μL of 30 mg/mL free phosphorylated or non-phosphorylated peptide library for all other screens. The entire binding/elution protocol was conducted at 4°C . Eluates from the phosphorylated and non-phosphorylated columns were combined, the volume was condensed using a Speedvac (Thermo Fisher Scientific), and the entire SILAC sample was run on one lane of an SDS-PAGE gel for fractionation prior to mass spectrometry.

Mass spectrometry data acquisition and identification—The entire SDS-PAGE lane containing the SILAC labeled proteins was cut into ten separate pieces (fractions) and each gel piece was reduced with 55mM dithiothreitol at 56°C , alkylated with 10mM iodoacetamide at RT and digested overnight with modified sequencing grade trypsin (Promega) at pH=8.3 (50mM ammonium bicarbonate) at 37°C with shaking. The peptide mixtures were then extracted from each of the gel pieces with 20mM ammonium bicarbonate followed by 40% acetonitrile/2% formic acid and injected onto a reversed-phase self-packed magic C_{18} (Michrom Bioresources) 75 μm id $\times$ 10 cm length Picofrit column (New Objective). Tandem mass spectrometry (LC/MS/MS) was performed using a QSTAR Excel Q-TOF mass spectrometer (AB/SCIEX) operated in positive ion data dependent (IDA) mode with one MS survey scan followed by three MS/MS scans using a two minute exclusion window. The Ultimate HPLC (Dionex) using split flow was operated at a 275 nL/min flow rate with a gradient of 3% B to 38% B over 55 minutes followed by a 4 minute wash at 95% B and a 12 minute 1% B column re-equilibration. The buffers consisted of 0.1% acetic acid/0.9% acetonitrile/99% water (A buffer) and 0.1% acetic acid/0.9% water/99% acetonitrile (B buffer) using HPLC grade solvents. MS/MS data and peak lists were extracted using ProteinPilot 2.0 software (Applied Biosystems). Proteins were identified using the Paragon search algorithm (28) by searching the MS/MS scans against the reversed and concatenated Swiss-Prot protein database (release 54.5, 289473 sequence entries, ftp.ncbi.nih.gov/blast/db/FASTA/) at a 95% confidence interval (unused ProtScore ≥ 1.3) with trypsin specificity in “Thorough ID” mode with all ‘biological modifications’ considered including differential modifications for +6.00 Da on Arg and Lys residues for SILAC. The peptide false discovery rate (FDR) for the screen was determined to be ~1.4% based on the number of reversed protein database hits above the scoring threshold. A minimum of two peptide spectra were required for identification.

Determination of SILAC ratios—Quantitative heavy:light SILAC ratios from the MS scans were determined using ProteinPilot 2.0 software (28). The SILAC ratios for each protein identified was plotted by protein number versus the average heavy:light ratio for

each peptide identified per protein. SILAC ratios greater than a threshold $\sim 3:1$ were considered significant phosphopeptide binding proteins because that threshold represents the lowest known positive control. Proteins with ratios less than $\sim 3:1$ were considered to be non-specific background. All four screening experiments were performed with biological replicates and similar results were achieved. The most robust replicates (highest number of identified phosphopeptide binding proteins) are presented here.

Domain Analysis

Pfam domain enrichment was calculated for the batch of hits from each screen using the Sanger Institute online database (<http://pfam.sanger.ac.uk/search#tabview=tab1>) using a high stringency gathering threshold. The domains (families were not included) across each phosphopeptide binding protein were plotted using Excel 2007.

Protein Interaction Networks

Protein-protein interaction (PPI) networks were created using String v8.3 (string-db.org/) for the hits from each phosphopeptide binding protein using high confidence (0.700) with all active prediction methods. Proteins connected by lines are predicted to directly interact in biological systems based on the literature as well as experimental genetic and proteomic experiments.

Results

The overall design of the screen is summarized in Fig. 1. One subset of HeLa cells was labeled with $^{12}\text{C}_6$ -Arginine and $^{12}\text{C}_6$ -Lysine, and a second subset with $^{13}\text{C}_6$ -Arginine and $^{13}\text{C}_6$ -Lysine. Light SILAC lysates were flowed over a non-phosphorylated peptide library affinity matrix, and heavy SILAC lysates were flowed over a corresponding phosphorylated peptide library affinity matrix. Bound proteins were eluted with sodium phenyl phosphate (in the case of the pTyr peptide library screen) or non-immobilized pSer/pThr peptide library (in the case of the pSer/pThr peptide library screens). Eluted proteins from the non-phosphorylated and phosphorylated peptide library columns were combined, fractionated by SDS-PAGE, digested with trypsin, and analyzed by microcapillary LC/MS/MS to determine peptides (proteins) that specifically bind to phosphopeptide containing peptide libraries.

Since trypsin cleaves proteins at the C-terminus of arginine and lysine residues, tryptic digestions of lysates from our light and heavy cells yielded peptide pairs that usually contained only single lysine or single arginine residues, resulting in a six Da mass difference between the peptides from heavy and light-labeled cells. However, multiple Arg or Lys residues were accounted in the data analysis, if present. The ratio of the MS signal intensities of the heavy and light version of each peptide in a pair (SILAC ratio) was used to calculate relative abundance. The average ratio for all of the peptide pairs from each identified protein was used to determine the relative binding affinity of that protein for the phosphorylated versus non-phosphorylated peptide library.

We screened four different sets of partially degenerate peptide libraries for phosphopeptide binding proteins. Each peptide library set consisted of a phosphorylated and otherwise identical non-phosphorylated version of the same library. The first peptide library screened was a phosphotyrosine library with complete degeneracy in all positions except for a locked-in pTyr or Tyr residue in the middle position. The second was a pSer/pThr-Pro peptide library biased towards the optimal substrate motifs for the cyclin-dependent kinases and MAP kinases. The third was a pSer/pThr-X-X-X-pSer/pThr peptide library biased towards the optimal substrate motif for GSK3, and the fourth peptide library was designed based on

optimal substrates for casein kinase 2, pSer/pThr-Glu/Asp. The final peptide library was designed with adjacent phosphorylation sites, pSer/pThr-pSer/pThr. All of the peptide libraries were constructed with a flexible linker and an N-terminal biotin tag, allowing for immobilization on streptavidin beads. These libraries were selected because they represented enriched motifs from an early large-scale phosphorylation study (12). An example spectrum from the MS results from the pTyr peptide library screen peptide from p85, the SH2 domain-containing regulatory subunit of phosphatidylinositol-3 kinase (PI3K), shows a >3:1 heavy:light ratio, consistent with its known ability to bind to pTyr containing peptides (7, 8).

SILAC ratios for all identified peptides from a given protein were calculated using commercially available ProteinPilot software to automate screen results for a high-throughput analysis. Results from screens using the four peptide libraries are shown in Fig. 2A–D. As predicted, the majority of the proteins identified by LC/MS/MS exhibited ~1:1 SILAC ratios, consistent with these being non-specific background proteins. As validation of our approach, multiple known phosphopeptide binding proteins exhibited >3:1 SILAC ratios, consistent with preferential binding to the phosphorylated peptide library in accordance with previously published literature. Additionally, several proteins that lack known phosphopeptide binding domains also showed >3:1 SILAC ratios, and are identified as being novel candidate phosphopeptide binding proteins.

The results from the pTyr peptide library screen are shown in Fig. 2A. Twenty-three total hits were observed including sixteen known pTyr peptide binding proteins with >3:1 SILAC ratios. The following proteins exhibiting >3:1 SILAC ratios have an abundance of SH2 domains and serve as positive controls in the screen: STAT1, STAT3, STAT5, SHP-1, SHP-2, p85, GRB2, RasGAP, PLC- γ -1, Crk, Crk-like protein, SHC1, and Vav-2. Pyruvate kinase M2 showed the strongest interaction and is known to interact to pTyr peptides through its 1,6-fructose-bisphosphate (FBP) binding pocket (29). Additionally, PKC- δ shows a 15:1 SILAC ratio and is known to bind pTyr peptides via its C2 domain (1). Talin-1 yields a 3:1 SILAC ratio, consistent with its PTB-like domain mediating pTyr peptide binding (30). Seven candidate novel pTyr binding proteins were also identified with >3:1 SILAC ratios. These proteins could either bind directly to pTyr peptides or associate with other pTyr binding proteins. Fig. 3A shows a Pfam domain enrichment analysis for the pTyr binding screen hits demonstrating the strong preference for SH2 domains with much less contribution from SH3 domains, STAT, pleckstrin homology (PH) and C1/C2 domains. Fig 3B displays the PPI network for the pTyr binding proteins showing that many SH2 domain-containing proteins have been shown to interact *in vivo*. This network is designed to show the interaction of the pTyr hits in cells, however, this does not indicate that we are pulling down a protein complex. Since most pTyr binding proteins contain SH2 domains, they have the ability to bind to the pTyr peptide library column individually. The results from the pSer/pThr-Pro peptide library screen are shown in Fig. 2B and resulted in five hits from the screen. Two known pSer/pThr-Pro binding proteins exhibited >3:1 SILAC ratios. Pin1 is a prolyl isomerase that binds to pSer/pThr-Pro motifs via its WW domain (31). Polo-like kinase-1 is a mitotic kinase known to bind pSer/pThr-Pro motifs via its polo box domain (11). An interesting candidate novel phosphopeptide binding protein identified with this library screen by showing a 15:1 SILAC ratio is the cell cycle and apoptosis regulatory protein 1 (CARP-1) which has been implicated in cell cycle progression and cell proliferation (32).

Results from the pSer/pThr-X-X-X-pSer/pThr (six hits) and pSer/pThr-Asp/Glu (four hits) peptide library screens are summarized in Fig. 2C and Fig. 2D, respectively. Although we did not identify the one known pSer/pThr-X-X-X-pSer/pThr motif binding protein (β -TRCP) in our screen (33), it is possible that the proteins we did identify as exhibiting SILAC ratios of >3:1 are novel phosphopeptide binding proteins such as MRE11A, the

uncharacterized protein C14orf93 and WASP family protein member 2. It is possible that the interaction of pSer/pThr-X-X-X-pSer/pThr with β -TRCP is transient or too weak to survive the IP conditions of our screen since it prefers Asp and Gly surrounding the first phosphorylated residue. Using milder detergents and/or shorter incubation times may help capture its binding, however, it may require increased concentration of peptide library containing Asp and Gly neighboring the first pSer/pThr site. One interesting hit from this screen is AHA1, a co-chaperone protein that stimulates HSP90 ATPase activity (34). 14-3-3 ϵ , a known pSer/pThr binding protein, exhibits a 3:1 SILAC ratio in the pSer/pThr-Asp/Glu peptide library screen. Additionally, PP2A- α , LanC-like protein, and Peroxiredoxin-3 show >3:1 SILAC ratios. However, the finding that PP2A- α and LanC-like protein both bind to all three of the pSer/pThr containing peptide libraries suggests that they bind to pSer/pThr without demonstrating specificity for the peptide motifs utilized in the screen. PP2A could be binding to the column through the catalytic site, which utilizes pSer/pThr as substrates for its phosphatase activity. Table 1 shows the Pfam domain enrichment and PPI networks for hits of the degenerate pSer/pThr library screens. The number of protein domains and known interactions between the hits of these phosphopeptide binding screens were sparse. This may be due to difficulty of protein binding regions to attach to the pSer/pThr residues in the same way that they can attach to the pTyr residues partially due to the smaller structure of Ser and Thr residues. In addition, it could be possible that additional requirements for flanking sequence or elements of 3-D structure may be required for proteins to bind phosphoserine and phosphothreonine residues. Supplemental Tables 1–4 contain the entire list of phosphopeptide binding proteins with SILAC ratios for the four screens described above.

Conclusions

The successful isolation of several proteins with known phosphopeptide binding domains from cell lysates shows the utility of using peptide library affinity matrices in combination with SILAC as a method to identify phosphopeptide binding proteins. Previous peptide library screens for phosphopeptide binding proteins laboriously consisted of screening hundreds of small pools of *in vitro* translated proteins and analyzing potential binders by SDS-PAGE followed by silver staining (10). SILAC enabled the rapid screening for phosphopeptide binding proteins from cell lysates. In addition to being high-throughput, an important advantage of the screen over the previous screening strategy is the more accurate and sensitive mass spectrometry based analysis method. Also, the use of cell lysates rather than *in vitro* translated proteins to search for phosphopeptide binders ensured the inclusion of proteins that rely on post-translational modification or cellular folding machinery for correct binding conformation.

It is important to note that only proteins expressed at the time of cell lysis are included in the screen. Therefore, lysates from other cell lines or cells treated with various stimuli would likely yield different results. Also, it is possible that modification of the peptide libraries by the kinases and phosphatases active within the cell lysate may have occurred. To minimize such modifications and to ensure the integrity of our pTyr peptide library, we added phosphotyrosine phosphatase inhibitors to the cell lysis buffer and performed the binding experiments at 4°C.

Overall, the screen of four different phosphopeptide libraries identified 38 phosphopeptide binding proteins including 16 potential novel binders. This screening approach can be used to study protein-phosphopeptide interactions under different stimuli and drug treatments to gain insight into mechanistic signaling events with high-throughput. Analogous approaches can be also be used to identify proteins that bind to other phospho-motifs as well as other types of modified amino acids. In addition, the combination of various growth factors and

kinase inhibitor drugs with newer generation mass spectrometers should collect more phosphopeptide binding proteins relevant to disease states in follow-up studies using the techniques presented here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Keywords

HeLa	Henrietta Lacks cells
pSer	phosphoserine
pThr	phosphothreonine
pTyr	phosphotyrosine
SILAC	stable isotope labeling of amino acids in cell culture
SH2	Src homology 2
SH3	Src homology 3
Pfam	Protein families database

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Figure 1.

A proteomic screen to identify phosphopeptide binding proteins from cell lysates. Biotinylated partially degenerate peptide libraries were incubated with streptavidin beads and packed onto columns to form the phosphorylated and non-phosphorylated peptide library affinity matrices. Heavy SILAC lysates were flowed over the phosphorylated peptide library column while light SILAC lysates were flowed over the non-phosphorylated peptide library column. Bound proteins were eluted with 20 mM sodium phenylphosphate (in the pTyr peptide library screen) or free peptide library (in the pSer/pThr peptide library screens), digested with trypsin, and analyzed by LC/MS/MS. Proteins were identified and heavy:light ratios were calculated using commercially available automated software. An example spectrum from the pTyr phosphopeptide binding screen showing that p85 specifically binds to the pTyr peptide library.

A.**pTyr binding proteins** **B.****pSer/pThr-Pro binding proteins**

C. pSer/pThr-XXX-pSer/pThr binding proteins

D. pSer/pThr-Asp/Glu binding proteins

Figure 2.

Results from the four screens for phosphopeptide binding proteins yielding greater than ~3:1 heavy:light SILAC ratios. Each protein identified by LC/MS/MS is represented as a black diamond. The names of known (previously published) phosphopeptide binding proteins are shown in blue, the names of candidate novel phosphopeptide binding proteins are shown in red and the phosphopeptide binding proteins across multiple libraries are shown in black. (A). Screen results for the pTyr peptide library. (B). Screen results for the pSer/pThr-Pro peptide library. (C). Screen results for the pSer/pThr-X-X-X-pSer/pThr peptide library. (D). Screen results for the pSer/pThr-Asp/Glu peptide library.

Table 1

The Pfam domain enrichment results for protein hits to the pSer/pThr-Pro, pSer/pThr-X-X-X-pSer/pThr and pSer/pThr-Asp/Glu phosphopeptide library screens.

<u>Protein Binding Screen</u>	<u>Protein Hits</u>	<u>Pfam Domains</u>				
pSer/pThr-Asp/Glu		AhpC-TSA	1-cysPrx_C	Metallophos	14-3-3	
	PRDX3	1	1			
	LANCI					
	PP2AA			1		
	1433E					1
pSer/pThrXXXpSer/pThr		Mre11_DNA_bind	Aha1_N	Metallophos	WH2	
	MRE11	1		1		
	PP2AA			1		
	AHSA1		1			
	LANCI					
pSer/pThr-P	WASF2					1
		WW	Rotamase	Metallophos	Pkinase	SAP
	Pin1	1	1			
	CCAR1					
	PP2AA			1		
	PLK1				1	1
	LANCI					