Targeted Phosphoproteomics Analysis of Immunoaffinity Enriched Tyrosine Phosphorylation in Mouse Tissue

Application Note

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Abstract

Tyrosine kinases play a prominent role in transmitting and regulating various cell signaling pathways. Deregulation of this key PTM drives inappropriate proliferation and cell survival. MS-based global phosphoproteomics related studies have significantly evolved in recent years, but complexity of the signaling pathways and sub-stoichiometry levels of tyrosine phosphorylation sites, when compared to phosphoserine and phosphothreonine residues, limit extensive mapping of the phosphotyrosine (pTyr) repertoire[1]. Targeted phosphoprotein identification approaches offer a comprehensive solution to study selected sites involved in specific biochemical processes. More recently, coupling of peptide-level anti-pTyr immunoaffinity purification (IP) with LC/MS/MS has proven to be a good approach for profiling tyrosine phosphorylation[2,3]. This Application Note demonstrates a targeted pathway-level enrichment workflow from various mouse tissues, and shows the strength of the Agilent data analysis software including Spectrum Mill and GeneSpring for a pathway-centric based analysis of the phosphoproteome repertoire.

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Introduction

Phosphotyrosine (pTyr) based signal transduction is an innovative molecular system that evolved in cellular life approximately 600 million years ago. It has become an essential part of metazoan biology, playing a critical role in various signaling and cell-to-cell communication pathways[4].

Tyrosine kinase activity deregulation plays a critical role in human cancers by altering oncogenic signaling pathways. Unbiased cellular signaling maps can provide a deeper insight into disease biology and such maps can be generated by using tyrosine-specific antibody immunoaffinity-based enrichment followed by LC/MS analysis.

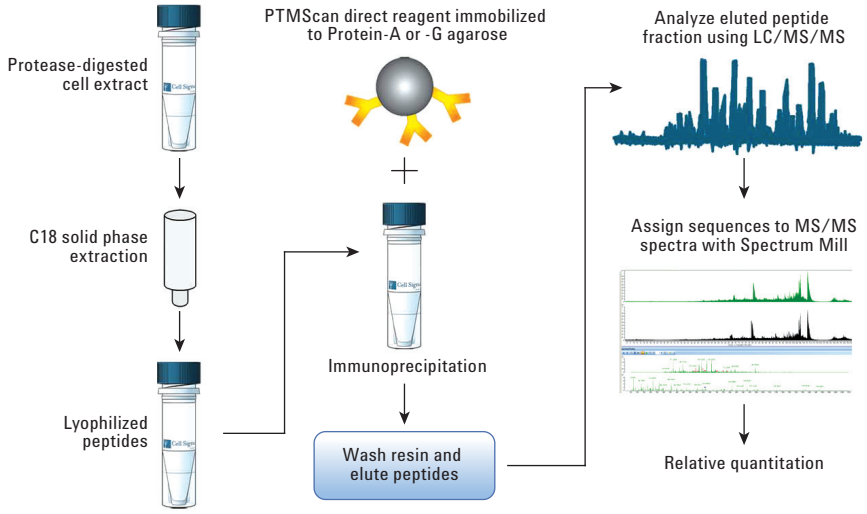

Figure 1A. Workflow of immunoaffinity enrichment for phosphotyrosine peptide mixtures.

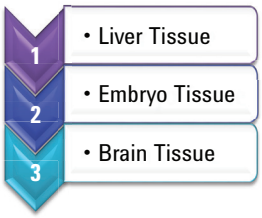

Figure 1B. Mouse tissues processed for immunoaffinity enrichment of phosphotyrosine peptides.

Experimental

Sample preparation

Tissue samples were lysed under denaturing conditions (9 M urea, 20 mM HEPES pH 8.0). The resulting soluble protein (5 mg) was digested with trypsin, and the resulting peptide mixtures were desalted over a C18 StageTip column, and dried under vacuum. Peptides were resuspended in immunoaffinity purification (IAP) buffer (50 mM MOPS pH 7.2, 10 mM KH_2PO_4, 50 mM NaCl), and pTyr target peptides were immunoprecipitated using p-TYr-1000 antibody coupled to protein A agarose beads (Cell Signaling Technology, #8803) for 2 hours. Peptides were released by 0.15 % TFA and the resulting peptides were desalted over a C18 StageTip column and subsequently dried under vacuum.

Employing the immunoaffinity enrichment strategy with the p-TYr-1000 motif antibody summarized in Figure 1A, target phosphotyrosine peptides were enriched from mouse tissues including liver, brain, and embryo. The enriched peptide mixtures from mouse liver tissue were subjected to long (140 minutes) linear gradient separations of acetonitrile in 0.1 % formic acid delivered at 300 nL/min over a C18 reverse phase LC system using a microfluidic device.

LC/MS analysis

In this study, phosphoproteomics data were acquired on the Agilent 6550 iFunnel Q-TOF MS using data-dependent acquisition. The 6550 iFunnel Q-TOF spectrometers incorporate iFunnel technology to achieve high sensitivity for the detection of peptides.

The microfluidic HPLC-Chip allows routine and robust nano-scale separations to be interfaced to the mass spectrometer. The HPLC-Chip system was comprised of an Agilent 1200 Series LC system consisting of a binary nano pump, thermostatted wellplate sampler, and binary capillary pump. Enriched phosphopeptide mixtures from each individual tissue were analyzed in duplicate. For the protein discovery workflow, a 140-minute gradient method was used with an Agilent Polaris chip (G4240-6230), comprised of a 360-nL enrichment column and a 150 mm, 75 µm analytical column. Both were packed with Agilent Polaris C18-A, 180Å pore size, and a surface area of 200 m^2/g.

Data analysis

The raw data were extracted and searched using Spectrum Mill (v 4.0) against the Uniprot *Mus musculus* protein database. The enzyme parameter was limited to full tryptic peptides with a maximum miscleavage of 1. Ox-Met, and phosphorylation on Ser, Thr, or Tyr were defined as variable modifications for phosphoproteome data. A maximum of two modifications per peptide was used and validated at the spectral level using 1.2 % FDR as the criteria. Phosphorylation sites were localized to a particular amino acid within a peptide using the variable modification localization score in the Agilent Spectrum Mill software[5].

Results from Spectrum Mill were imported for evaluation in Mass Profiler Professional 12.6.1(GX-MPP-PA), a data analysis and visualization software. Pathway analysis was conducted using MPP'S optional Pathway's Architect software module.

Results and Discussion

LC/MS/MS data were acquired in both centroid and profile modes. Figure 2 shows the total ion chromatogram. Acquired spectra were then searched with Spectrum Mill and Mascot (Matrix Science, UK) against the *Mus musculus* Swissprot FASTA protein database and resulted in a cumulative identifications of 3,138 unique phosphotyrosine peptides across various mouse tissues, including liver, brain, and embryo. A stringent validation criterion of 1 % FDR at the spectral level was applied. Higher sensitivity levels of peptide detection with dual-stage ion funnel technology resulted in the identification of hundreds of target phosphotyrosine peptides in the combined data set. A screenshot of Spectrum Mill with a representative dataset is shown in Figure 3, with enriched brain-derived neurotrophic pathway (BDNF) along with pTYr enriched proteins from curated databases indicated in yellow (Figure 4).

HPLC-Chip conditions	
HPLC-Chip	Agilent Polaris-HR-Chip-3C18
Mobile phase A	0.1 % formic acid in water
Mobile phase B	0.1 % formic acid in 90 % acetonitrile in water
Loading	2 µL/min with 3 % B
Analytical flow rate	300 nL/min
Q-TOF analytical gradient (140 minutes)	3 % B initial to 30 % B at 97 minutes, 45 % B at 100 minutes, 60 % B at 120 minutes, 100% B at 125 minutes, 3 % B to 140 minutes
Injection volume	3 µL volume with a concentration of 10 fmol per injection
Agilent 6550 Q-TOF conditions	
Instrument mode	Extended dynamic range (2 GHz) with low mass range (1700 *m/z*)
Drying gas	11 L/min, 250 °C
Acquisition rate	8 (MS) and 20 (maximum rate in MS/MS) spectra/sec
Acquisition range	300–1,700 (MS) and 50–1,700 (MS/MS)
Collision energy	slope of 3.2 and intercept 1 (+2) or slope of 3 and intercept 3.1 (+3) or slope of 3.6 and intercept -4.8 (+3 and higher)
Isolation	Medium (~4 *m\z*)
Data dependent acquisition	20 precursors per cycle using precursor abundance based acquisition rate with accumulation time limit enabled; active exclusion after one spectrum for 0.5 minutes

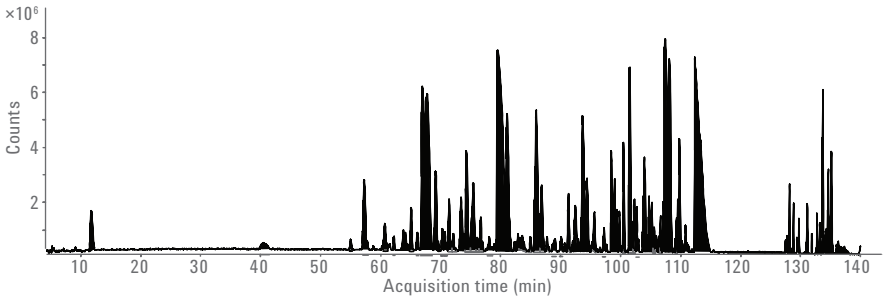

Figure 2. TIC chromatogram of pY enriched brain peptide mixture.

z	Spectrum Intensity	Score VML	# STY	# sty Sites	# Loc sty Sites	# Ambig sty Sites	Variable Sites	Sequence	Accession #	Protein Name
2	4.16e+007	1.60	3	1	1	0	Y12y	(K) IGEGTyGVVYK (A)	P24923	Cell division control protein 2 homolog 1 (Fragment)
2	2.05e+006	99	1	1	1	0	Y699y	(K) AADGyVKPQIK (Q)	P42232	Signal transducer and activator of transcription 5B
2	6.57e+006	3.47	3	1	1	0	Y798y	(K) VVQEYIDAFSDyANFK (-)	P18433	Receptor-type tyrosine-protein phosphatase alpha
2	4.07e+007	7.17	3	1	1	0	Y321y	(R) IYQyIQSR (F)	Q13627	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
2	1.34e+006	99	1	1	1	0	Y694y	(K) AVDGyVKPQIK (Q)	Q95115	Signal transducer and activator of transcription 5A
3	4.14e+006	1.69	4	1	1	0	Y187y	(R) VADPDHDHTGFLTEyVATR (W)	P46196	Mitogen-activated protein kinase 1
3	8.53e+005	4.76	2	1	1	0	Y635y	(K) NAIKVPIVINPNAyDNLAIYK (S)	Q9H792	Pseudopodium-enriched atypical kinase 1
2	3.80e+006	7.41	2	1	1	0	Y483y	(R) GPLDGSPyAQVQR (P)	Q63HR2	Tensin-like C1 domain-containing phosphatase
2	3.54e+006	3.78	4	1	1	0	Y307y	(R) VYTyIQSR (F)	Q5ZIU3	Dual specificity tyrosine-phosphorylation-regulated kinase 2
3	2.32e+006	0.87	4	1	0	1	Y250y	(R) IADPEHDHTGFLTEyVATR (W)	P40417	Mitogen-activated protein kinase ERK-A
3	7.70e+005	1.53	7	1	1	0	Y253y	(R) NTyNQTALDIVNQFTTSQASR (E)	Q8WXE0	Caskin-2
3	1.54e+006	-0.10	2	1	0	1	Y100y	(R) VFDKDGNGyISAAELR (H)	P62146	Calmodulin-alpha (Fragment)
3	1.51e+006	8.47	4	1	1	0	Y183y	(R) HTDDEMTGyVATR (W)	Q90336	Mitogen-activated protein kinase 14A
2	7.56e+005	0.00	2	1	0	1	Y389y	(R) IIEDNEyTAR (E)	Q95M30	Tyrosine-protein kinase HCK
2	1.13e+006	2.29	4	1	1	0	Y1106y	(R) NEEENIySVPHDSTQGK (I)	P83509	Rho GTPase-activating protein 35
4	8.31e+004	0.00	6	1	0	1	Y168y	(R) TTGIVLDSGDGVTHNVPIyEGYALPHAIMR (L)	P10995	Actin, alpha skeletal muscle 2
3	1.26e+006	1.38	5	1	1	0	Y792y	(R) LDTASSNGyQRPGSVVAAK (A)	A6NI28	Rho GTPase-activating protein 42
2	6.27e+005	-0.10	5	1	0	1	Y570y	(R) yMEDSTYYK (A)	Q00944	Focal adhesion kinase 1
2	6.60e+005	3.07	10	1	1	0	Y551y	(R) SSGLGQyGSPSGQTSSSTR (Q)	Q8VHD8	Hornerin

Figure 3. Spectrum Mill screenshot displaying variable phosphorylation sites, phosphopeptide sequences. and protein names.

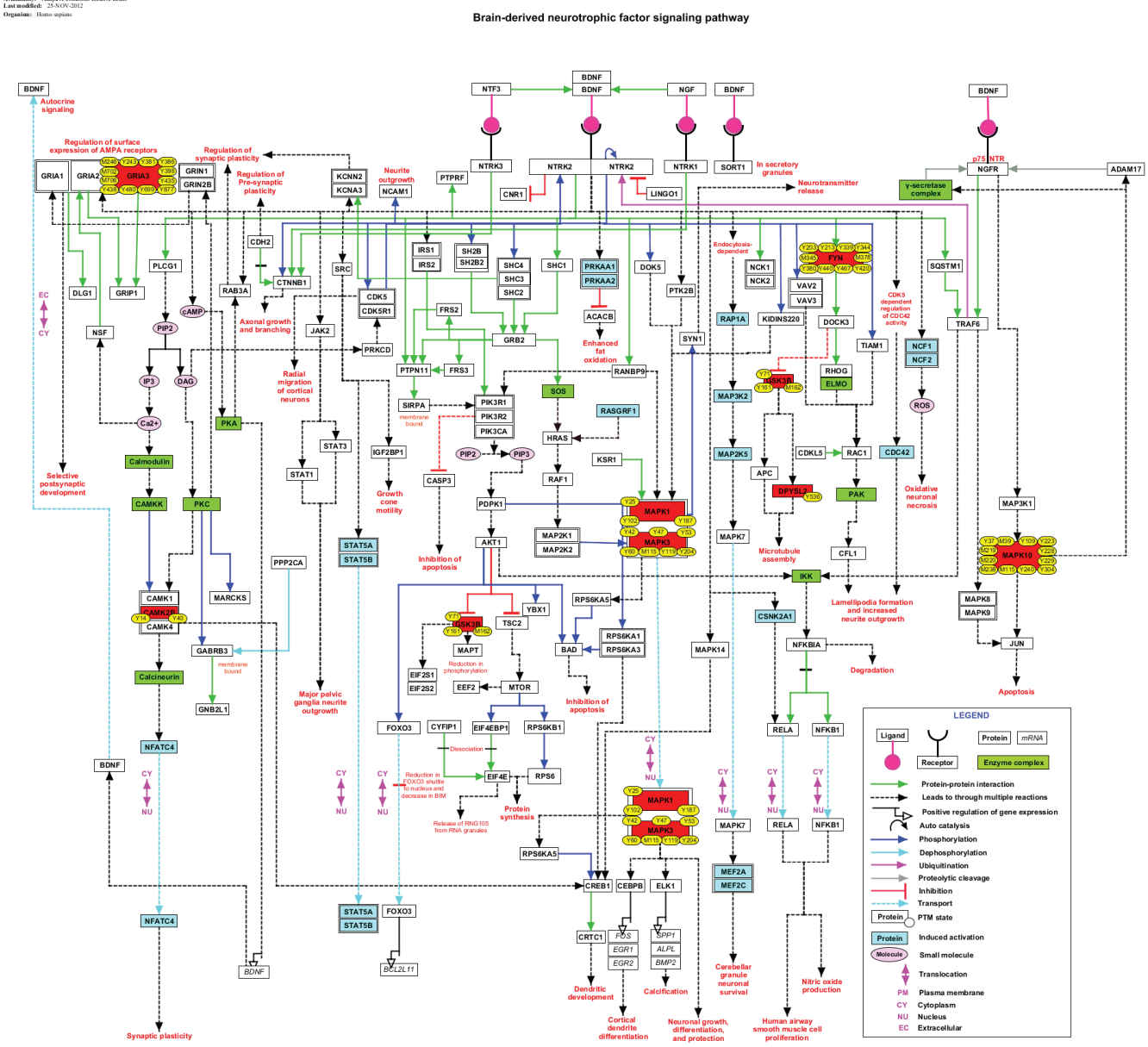

Figure 4. Brain-derived neurotrophic pathway, specifically highlighting the enriched pY sites (yellow bubbles).

Classes of phosphorylated proteins

Scaffold software was used to extract the GO terms for *Mus musculus* proteins from their closest GO-annotated orthologs in the Swiss-Prot database. Phosphoproteins were present in all parts of the mouse tissues (Figure 5A), and are distributed among a wide variety of metabolic and regulatory enzymes.

Identified peptides included moderately abundant cellular protein tyrosine kinases for example, tyrosine-protein kinase BTK, mitogen-activated protein kinase 3, and A-kinase anchor protein SPHKAP, along with low-abundant transcriptional regulators including transcriptional activator GLI3, histone-lysine N-methyltransferase, ETS-related transcription factor EI, and catenin alpha (Figure 5B). Our results demonstrate the ability of the immunoaffinity, LC/MS enrichment strategy, to identify the phosphotyrosine repertoire.

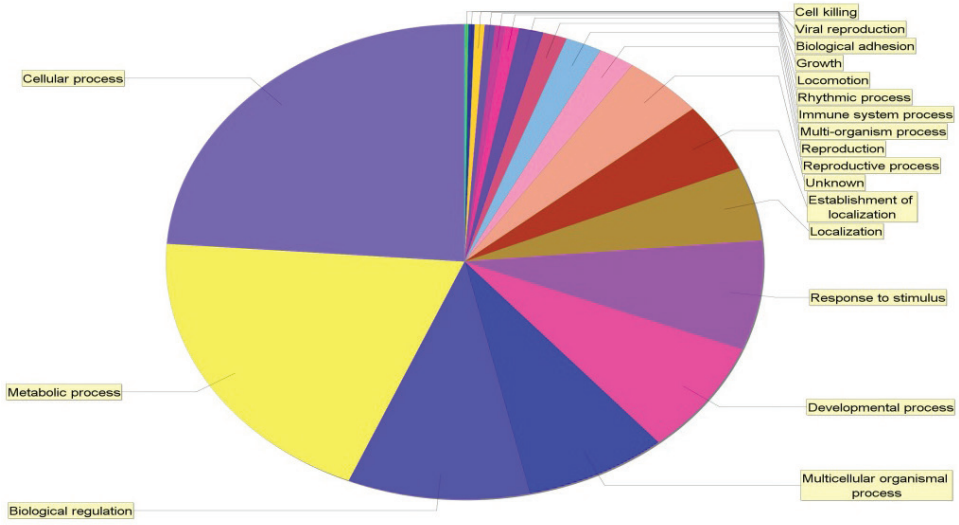

Figure 5A. Molecular Function assigned by Scaffold based GO annotations.

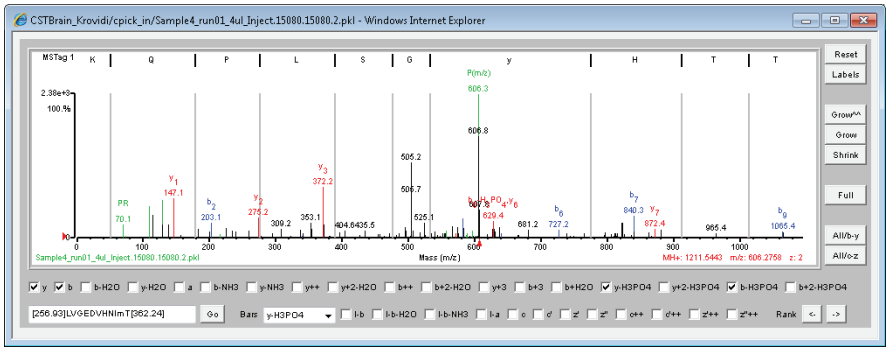

Figure 5B. Spectrum Mill output: MS/MS spectrum of phosphotyrosine peptide to Myelin basic protein isoform 1.

Conclusions

1. Immunoaffinity enrichments were performed from various mouse tissues including liver, brain and embryo.
2. Nano LC/MS experiments were performed to identify the enriched phosphotyrosine repertoire.
3. Higher peak capacities were obtained with the Polaris chip based chromatographic separations resulted in a very large number of peptide and protein identifications.
4. Discovery proteomics-based approach employing p-Try-1000 antibody identified several thousands of phosphotyrosine specific peptides.
5. Pathway analysis helps organize the phosphotyrosine profiling results from the list of peptides/proteins to biologically relevant signaling pathways and has been used to illustrate the signaling role of enriched phosphoproteins from various mouse tissues.

References

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