Multiplexing Kinase Substrate Motif Antibodies for Expanded Coverage of the Serine/Threonine Kinome

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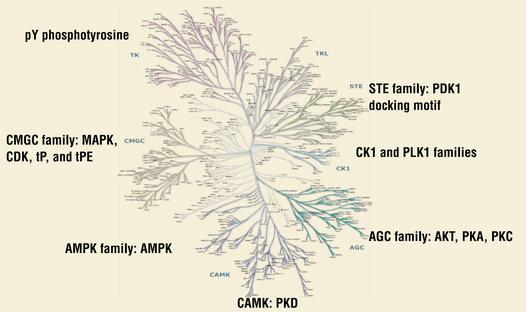
Introduction

The post-translational modification of proteins by phosphorylation has been shown to regulate many aspects of cellular function from growth and differentiation to basic metabolism, autophagy, and apoptosis. In an effort to identify these sites of phosphorylation, substrate motif antibodies with high affinity to known substrates of kinases have been used to probe kinase-substrate interactions. We describe the results of a phosphopeptide immunoaffinity based enrichment strategy focused on substrates to basophilic kinases in the AGC family (AKT, PKA, PKC, & PKD) and cyclin dependent kinase, CDK; including substrates to the proline-dependent motifs of PXsP, SIP, tPE, and tP. We have performed phosphopeptide profiling studies using these motif antibodies in combination to systematically survey the phosphoproteome in a variety of mouse tissues.

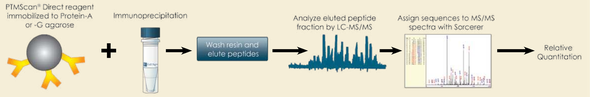
METHODS: Phospho-Motif Kinome Coverage

Phosphopeptides were isolated from 5 mg of trypsin digested mouse embryo tissue using the following motif antibody groups; Mix 1 all: AKT, AMPK, ATM/ATR, Cdk, CK, MAPK, PDK1, PKA, PKC, PKD, PLK, tP, tPE, tXR, and pY (phosphotyrosine); Mix 2 Basophillic group; Mix 3 Proline-directed group; Mix 4 Atypical group. Phosphopeptides were eluted from the affinity matrix with TFA, desalted, and analyzed by LC-MS/MS using an LTQ-Orbitrap Velos with an LC gradient from 0% to 32% acetonitrile over 72 minutes. MS/MS spectra were searched against an NCBI mouse database with 5% FDR and a TPP probability of 0.95. An in-house label-free quantification algorithm was used to quantify all identified phosphopeptides by their MS1 signal among the tissue samples.



METHODS: PTMScan® Work Flow

Motif Antibodies:
Mix 1 - 17 Motif Antibodies **Mix 2** - Basophillic Motifs **Mix 3** - Proline-directed Motifs **Mix 4** - Atypical Motifs



PTMScan workflow from immunoaffinity purification of phosphopeptides to LC-MS/MS analysis.

METHODS: Motif Antibody Groups

Equimolar total amounts of motif antibodies pooled in the 4 groups above were conjugated to Protein-A agarose (Roche) and incubated with identical 5 mg aliquots of trypsin-digested mouse embryo-derived peptides for 90 minutes. Peptides were washed in 2X IAP buffer and 2X water prior to elution with 0.15% TFA. The peptides were then desalted and dried prior to resuspension for LC-MS/MS on an Orbitrap Elite with a 90 minute 0-32% MeCN gradient.

Phospho-Motif Antibodies Groups

Motif Antibody	Motif	Antibody Number	Mix 1 All	Mix 2 Basophilic	Mix 3 Proline Directed	Mix 4 Atypical
Akt Substrate	RXX(s/t)	9614	X	X		
Akt Substrate	RXRXX(s/t)	23CST2	X	X		
PKA Substrate	(K/R)(K/R)X(s/t)	9624	X	X		
PKC Substrate	(K/R)XaX(K/R)	2261	X	X		
PKD Substrate	LXRXX(s/t)	4381	X	X		
CDK Substrate	(K/R)sPX(K/R)	2324	X	X		
MAPK Substrate	PXsP	2325	X		X	
tP Motif	tP, tPP	BL4180	X		X	
tPE Motif	tPE, tP	C32G12	X		X	
PLK Binding motif	StP	A7907	X		X	
tXR Motif	tXR, tPR	2351	X		X	
PDK1 Docking Motif	(F/Y)(s/t)(F/Y)	9634	X		X	
tP Motif	tP, tPP	BL4180	X		X	
ATM/ATR Substrate	(s/t)QG	6966	X			X
ATM/ATR Substrate	sQ	9607	X			X
CK Substrate	t(D/E)X(D/E)	BL4176	X			X
Phosphotyrosine	y	9411	X			X

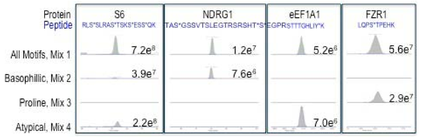
RESULTS: Qualitative

Each motif mix was run as an LC-MS/MS analytical replicate (with the exception of the Proline-directed mix). Sequest results for each replicate injection are presented with the combined non-redundant total for each mix. In total 5,631 unique phosphopeptides were identified.

Motif Antibody Groups	Analytic Replicates	Redundant	Non-Redundant	Collapsing Redundancy: Combined Non-Redundant	Collapsing Redundancy: Non-Redundant Proteins
All Motif	1	2395	2247	2735	1066
	2	2339	2179		
Basophillic	1	1553	1421	1829	741
	2	1515	1398		
Proline Directed	1	1654	1528	1528	624
Atypical	1	1579	1445	2078	695
	2	1593	1204		

RESULTS: Extracted Ion Chromatograms

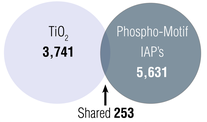
Representative peptides were chosen to illustrate the specificity of the motif antibodies to immunoprecipitate phosphopeptides containing the kinase substrate motif. Peptide from S6 m/z = 724.6277, z = 3; peptide from NDRG1 m/z = 869.7114, z = 3; peptide from eEF1A1 m/z = 600.7867, z = 2; peptide from FZR1 m/z = 558.7579, z = 2. All peak area measurements from Excalibur with relative intensity scale fixed for each peptide. Site of phosphorylation denoted with asterisk (*).



RESULTS: PTMScan Multi-Motif Antibody Capture Complementary with TiO_2 Enrichment

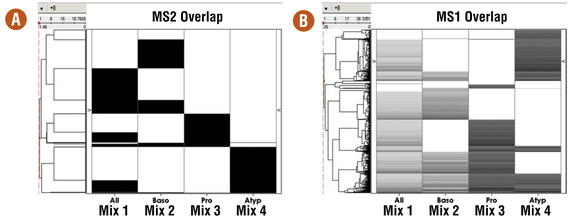
Nonredundant phosphopeptides identified from identical mouse embryo tissue by multi-motif IAP and TiO_2 enrichment have a 6% overlap. This result demonstrates that the two methods are highly complementary with respect to phosphopeptide enrichment. TiO_2 enrichment was performed by Eric Soderblom (see oral presentation Thursday, Exhibit Hall A, at 2:50 p.m.- PTMs: Advances in Isolation, Derivatization, and Separation and reference 1).

Overlap of TiO_2 and Motif Groups (Non-Redundant Peptide Sequences)



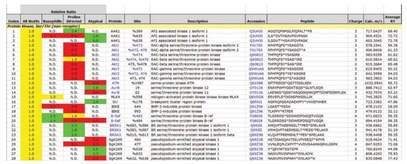
RESULTS: Analysis by MS1 Compared to MS2

Heat map of phosphopeptides identified by MS2 or by MS1 detection for the four motif antibody groups. Phosphopeptides identified by MS2 are shown in black (**Panel A**) and illustrate that there is little overlap between between motif groups and individual peptide identification. In contrast, many more peptides are shared by the various motif groups (**Panel B**) upon inspection of the corresponding, clustered MS1 features. Peptide intensities are presented in grey scale.



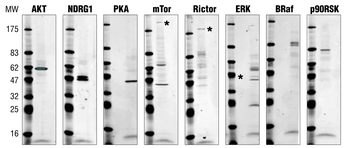
RESULTS: PhosphoScan® Tabulated Results

PTMScan® tabulated results integrate LC-MS/MS data with bioinformatics and label-free quantitation showing identified phosphopeptides with site of modification, their parent proteins with functional descriptions, relative phosphopeptide quantitation and corresponding raw and normalized fold-change ratios. Included in a full report are details on each identified phosphopeptide including charge, m/z, MS2 chromatogram scan number, ΔCN, Xcorr, RSP, and PP (peptide probability) metrics.



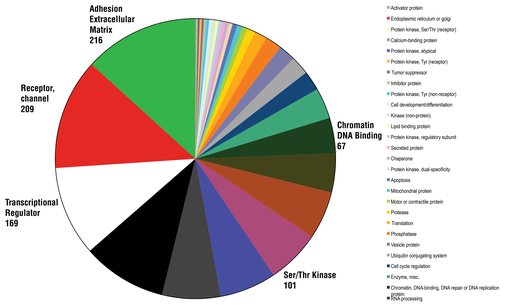
RESULTS: Western Blotting Validation

A selected number of targets identified from the LC-MS/MS studies were validated using site-specific antibodies to phospho-AKT (S473), NDRG1 (T346), PKA (T197), mTor (S2448), Rictor (T1135), ERK 1/2 (T202/Y204), B-Raf (S445), and p90RSK (S380). 20 µg of mouse embryo lysate was run per lane on 4-20% gradient gels (Invitrogen), transferred to nitrocellulose, and probed with site-specific antibodies. Images were developed with the LI-COR® near infrared imaging system. *Indicates targeted protein band.



RESULTS: Proteins Identified by Multi-Motif PhosphoScan®

The parent proteins from which the identified phosphopeptides in the all motif mix 1 were classified according to their GO (gene ontology) terms. In total there were 1,066 proteins identified from all motif mix 1.



Summary

- The multiplexing of kinase substrate motif antibodies is a novel approach to identifying sites of serine, threonine, and tyrosine phosphorylation cell-wide.
- This study shows that a large number of unique phosphopeptides can be identified from tissue; moreover, this technology is highly complementary with other phosphopeptide enrichment techniques such as TiO_2.

References

1. Soderblom, E.J., et al. 2011, Quantitative Label-Free Phosphoproteomics Strategy for Multifaceted Experimental Designs, *Anal. Chem.* 83, 3758-3764.

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