Label-free Comparative Study Of Phosphatase Inhibitor-Induced Phosphoproteomics Using Targeted Analysis

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Overview

- Phosphopeptide enrichment by Fe(III) immobilized metal affinity chromatography (IMAC)
- Small inner diameter (20 μm i.d., 40 cm length) monolithic columns provide more sensitive and reliable label-free relative quantitation results with LC-FTICR analyses
- Pipeline for discovering statistically changing features in label-free LC-MS experiments using improved alignment and clustering algorithms
- Targeted LC-MS/MS experiments to obtain sequence information for "interesting" features
- Data-directed analysis to compare changes in protein phosphorylation in Hela cells treated with and without 10 nM calyculin (a potent inhibitor of PP1, PP2A and other members of this family)
- Peak matching between targeted LC-MS/MS experiments and LC-MS feature list to identify peptides having significant abundance changes

Introduction

Post-translational modifications play key roles in protein function, activity, localization and interactions. Members of the phosphoprotein phosphatase (PPP) structural family of Ser/Thr protein phosphatases, which includes PP1, PP2A and calcineurin, control the phosphorylation state and cellular function of a wide variety of proteins. PP1 and PP2A are essential proteins, and malfunctions of PPP enzymes have been implicated in numerous disease states. In spite of this, our knowledge of substrates and pathways regulated by these enzymes has lagged behind that of protein Ser/Thr kinases. One promising step to reveal this information would be to generate both qualitative and quantitative phosphoproteomics comparisons for cells treated with or without phosphatase inhibitors. This data could then be used to interrogate the mechanisms underlying phosphatase-associated events. Toward this end, we present here a label-free technology for comparative phosphoproteomics study, based on inhibition of the PPP structural family of Ser/Thr protein phosphatases as a model system.

Previous phosphoproteomics studies have provided a large number of protein phosphorylation sites under static conditions. However, to investigate regulated phosphorylation under different cellular conditions, both qualitative and quantitative phosphoproteomics have to be used. At present, quantitative phosphoproteomics has only been performed in a few cases and with different stable isotopic labeling approaches using ^{3}D or ^{13}C[1,2]. However, this kind of study can often increase sample complexity due to incomplete labeling and frequently makes phosphopeptide sequencing even more challenging. In addition, due to the isotopic effects, the light-/heavy-labeled peptide pairs do not always co-elute during LC separation, making a quantitative comparison between control and treated samples less reliable than expected.

Label-free approaches for quantitative proteomics have been hindered due to several concerns including non-linear responses and strongly biased detection of co-eluting species due to ionization suppression effects, especially in the circumstance of complex biological matrixes. Based on our recently developed capabilities for improved quantitation[3] and data alignment algorithms[4], we have developed a label-free relative quantitation technology using data-directed analysis for phosphopeptide identification and applied this technology to the comparison of changes in protein phosphorylation in Hela cells treated with or without 10 nM calyculin, a potent inhibitor of PP1, PP2A and other members of this family. Our technology mainly focuses on the analysis of phosphopeptides that show statistically significant changes and facilitates the very time-consuming process for phosphopeptide sequencing. In addition, both qualitative and quantitative phosphorylation changes, which are equally important to understand the dynamic operation of signaling cascades, can be revealed from this type of analysis.

Methods

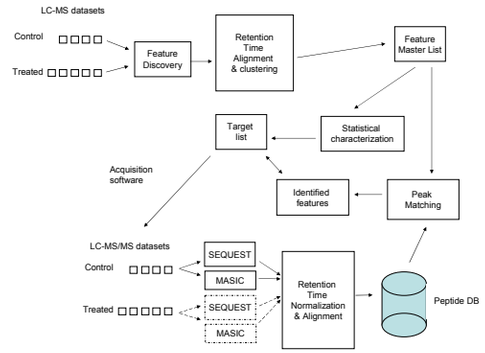

Fig. 1. Targeted pipeline. LC-MS experiments were performed on the control and the IMAC enriched samples. Features in the samples are deconvoluted to monoisotopic mass and time centers. Using MSWARP, we aligned all analyses to a reference sample and performed clustering on mass and time dimensions to group common features and create a master list of MS features clustered together. Statistical filters (e.g., t-test) were applied to consolidated lists to generate a target list, which were then acquired by re-analyzing samples in targeted LC-MS/MS analysis mode. LC-MS/MS experiments were analyzed by SEQUEST and MASIC, normalized for retention time differences and combined into a mass and time tag database. Peak matching between LC-MS feature list and the mass and time tag database was used to provide identification for differentially expressed peptides.

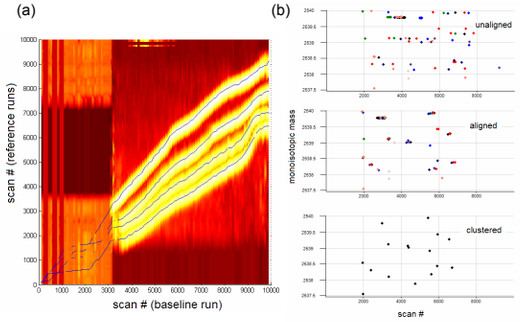

Fig. 2. Alignment and clustering of LC-MS analyses. (a) Heatmap showing similarity scores and alignment functions between four LC-MS analyses of treated samples compared to one baseline treated sample. Obviously, there is a need for elution time alignment between experiments. (b) An example of a zoomed in mass and retention time region, before alignment, after alignment and after clustering.

Results

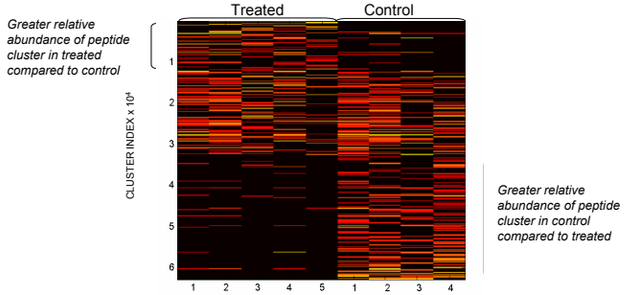

Fig. 3. Heatmap of the expression patterns from treated and control samples; the color is proportional to the intensity of the observation of that cluster in an experiment (e.g., black indicates low intensity peptides; red, greater intensity, etc.).

Here, the features are sorted by decreasing value of probability for over abundance in treated samples. As can be seen, the higher scoring peptides show greater abundance in the treated samples while the lower scoring peptides show higher abundance in the control samples. Higher scoring features were exported to the target list used in subsequent LC-MS/MS experiments.

Table 1. Examples of phosphopeptides that have shown statistically significant changes in phosphatase inhibitor treated samples

Protein Name	Phosphopeptide	Max. Xcorr	Control (C) (observed in #of replicates)	Treated (T)	T/C Ratio	T-test value
Vimentin[a]	DGQVINETS*QHHDDLE	2.72	4/4	5/5	9.55	8.00E-06
Heterogeneous Nuclear[a] Ribonucleoprotein K (hnRNP-K)	RDYDDMS*PR	2.71	4/4	5/5	33.48	3.85E-03
Elongation Factor 2[a]	AGETRFT*DTR	2.69	4/4	5/5	14.52	2.53E-04
CDK1[a]	IEKIGEGT*Y*GVVYKGR	3.56	4/4	5/5	0.335	1.61E-03
Kinesin Light Chain 1l	ASS*LNVLNVGGK	2.68	4/4	5/5	10.20	1.50E-03
Transcription Factor JunB	SRDAT*PPVS*PINMEDQER	3.53	4/4	5/5	2.74	1.60E-02
Rho Guanine Nucleotide Exchange Factor 10	SDLSSSS*GSLS*LSHGSSSLEHR	2.97	4/4	5/5	0.60	1.26E-02
Ran Guanine Nucleotide Exchange Factor(RCC1)	RRS*PPADAIPK	2.74	4/4	5/5	8.46	3.27E-05
Phosphatidylinositol 4-kinase Type II	VAAAAGSGPS*PPGS*PGHDRER	3.12	4/4	5/5	1.68	6.59E-03
NuMa1 Protein	KRVS*LEPHQGPGT*PESK	3.04	4/4	5/5	1.67	7.43E-04
Catenin Delta 1	HYEDGYPGGSDNYGSLS*R	3.68	0/4	5/5	1090.74[b]	
Kinase Suppressor Of RAS-1	SKS*HESQLGNR	2.61	0/4	5/5	201.60[b]	

[a] proteins known to be regulated by PPP family.
[b] Using the minimum abundance for the identified phosphopeptides in control to calculate the T/C ratio.

Conclusions

- Label-free comparative phosphoproteomics study using targeted analyses effectively revealed both qualitative (new) and quantitative (up- or down-regulated) phosphorylation changes induced by phosphatase inhibition. More than 110 phosphopeptides showed statistically significant changes with the treatment and over 20 phosphopeptides were uniquely identified in the treated sample.
- Improved quantitation results were obtained by using a 20 μm i.d. monolithic column with high separation capability (40 cm long) operated at ~20 nL/min.
- Successful quantitative detection of changing features in label-free experiments was accomplished using improved alignment and clustering algorithms.
- The AMT tag approach was extended and improved by employing peak matching between targeted LC-MS/MS experiments and LC-MS feature lists to identify peptides that had changed in abundance.
- Data-directed analysis provides an improved focus on peptides showing statistically significant changes and reduces the time required for manual examination of phosphopeptide spectra. Moreover, data-directed MS3 experiments can be more effectively implemented.
- Many of the phosphopeptides identified in this study contain novel phosphorylation sites and represent proteins not previously known to be regulated by PPP family phosphatases. This provides new information about phosphorylation sites in these proteins and how their phosphorylation status is regulated in a direct or indirect manner by PPP enzymes.

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References

1. Stover, D. R. *et. al.* 2004, *Clin. Proteomics* 1, 069-080
2. Gruhler, A. *et. al.* 2005, *Mol. Cell. Proteomics* 4, 310 – 327
3. Luo, QZ *et. al.* 2006, *J. Proteome Res.* April 1, ASAP Article DOI: 10.1021/pr050424y.
4. Jaitly, N. *et. al.* 2006, accepted by *Anal. Chem.*

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