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Differential quantification of isobaric phosphopeptides using data-independent acquisition mass spectrometry

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Abstract

Phosphorylation is a post-translational modification (PTM) fundamental for processes such as signal transduction and enzyme activity. We propose to apply data-independent acquisition (DIA) using mass spectrometry (MS) to determine unexplored phosphorylation events on isobarically modified peptides. Such peptides are commonly not quantitatively discriminated in phosphoproteomics due to their identical mass.

Phosphorylation is a reversible modification that regulates many biological events in a cell, including cell cycle, growth, apoptosis and signal transduction pathways¹. Phosphorylation is currently the most studied and characterized post-translational modification (PTM) in biological systems, and likely one of the most abundant in nature. Its misregulation can be detrimental, giving rise to abnormal phenotype and many diseases such as cancer^{2, 3}. Epidermal growth factor (EGF) binds to the EGF receptor on external cell membrane and stimulates cell signalling, modulating the activity of enzymes involved in specific pathways including transcriptional regulators, small GTPases and kinases by stimulating phosphorylation events^{4–6}. Since EGF stimulation influences a complex cell signalling pathways, there are numerous phosphorylation and de-phosphorylation events in response to EGF⁴. Antibody based approaches for characterization of phosphorylations are labor intensive and time consuming to study a large number of protein phosphorylation events.

Mass spectrometry (MS), especially when coupled with nano-liquid chromatography (nLC), has become the method of choice to characterize PTMs in large scale and in an unbiased manner⁷. In MS numerous acquisition methods have been developed to address different needs in protein and peptide characterization. For instance, selected reaction monitoring (SRM) gives the highest sensitivity and selectivity due to its ability to select predetermined precursor and product ions from complex mixtures. On the other hand, methods like data dependent acquisition (DDA) are preferred for discovery proteomics, since the instrument

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total list of identified phosphopeptides are multiple: (i) only part of isobaric peptides have sufficiently intense unique fragment ions for differential quantification; (ii) it is required that both species are identified with comparable scores during database searching, which might not happen as one of the two species could be of low abundance and thus considered as not a confident identification; (iii) there is presently no automated data analysis software, implying that all calculations of relative ratios was tediously manually performed. In addition, 28 isobaric phosphopeptides (14 pairs) we considered had different retention times showing distinct chromatographic profile (Figure 2A). These do not require MS/MS based quantification, as their abundance can be easily discriminated using traditional MS based XIC.

One example of co-eluting and discriminated isobaric phosphopeptides is shown in Figure 2B. The peptide AASPPQSVR (747, 756) was identified as belonging to the translated cDNA FLJ61739 with the phosphorylation pattern Ser 749 and 751, but also Ser 749 and 754. At the precursor mass level these two species completely overlap and thus would have the same abundance if quantified by MS XIC. By using the y5 fragment, which has a unique mass for each of the two species, we could determine the relative abundance of these peptides in all three analyzed conditions. It is worthwhile to note that phosphorylation on serine 751 decreases 5 min after EGF stimulation while phosphorylation on serine 754 does not decrease until 20 min after EGF treatment. More examples are illustrated in the Skyline file included as supporting information (for review, available at <https://www.dropbox.com/sh/1tcsvanalqg6cd6/AADhCIPRYcCtaTBoKH4T-jUaa?dl=0>).

Finally, in order to evaluate the accuracy of MS/MS based quantification we considered the variance between technical replicates for those analyzed isobaric species (Figure 2D). The precursor mass ion signal provided the most reproducible observation (average CV 9.66%) as expected, being the most intense detectable ions. We also estimated the variance of the ions used to estimate the relative ratio between isobaric phosphopeptides performing three different comparisons. At first, we just estimated the variance of the intensity of the fragment ions between replicates (product, CV 17.70%); then, we calculated the ratio between the fragment ion of the isobaric species A and isobaric species B, and verified the reproducibility of the measurement between replicates (ratio product, CV 21.14%); finally, we calculated when possible the ratio of two isobaric peptides A and B using different fragment ions, and verified the reproducibility of the resulting ratio (Multiple ratio products, CV 72.46%). Results indicated that precursor and product ion signals had excellent reproducibility in their measurements. However, when calculating the ratio between two isobaric peptides the measurement was more variable depending on which fragment ion was used. This was expected, as very low abundance fragment ions, even if detectable, might have compressed dynamic range and therefore create a bias in the ratio calculation. In this study, if more than one unique fragment was detected, we considered the average ratio between the two isobaric isoforms.

Conclusions

We perform proof of principle experiments to introduce DIA as valuable tool to perform differential quantification of isobaric phosphopeptides in proteomics analyses. The

application of this method is still limited due to the challenge of (i) identifying multiple species from the same MS/MS spectrum, (ii) the sensitivity in detecting unique fragment ions of the same type for the two isobaric isoforms (e.g. y_5 and y_5') and (iii) the mathematical limitations in calculating the relative ratio between more than two isoforms. However, we speculate that the rapidly increasing interest of DIA methods would lead to the development of suitable software for data processing, identification and quantification of positionally isobaric modified peptides, as much is currently missed due to our inability of discriminating the abundance of co-eluting peptides with same precursor mass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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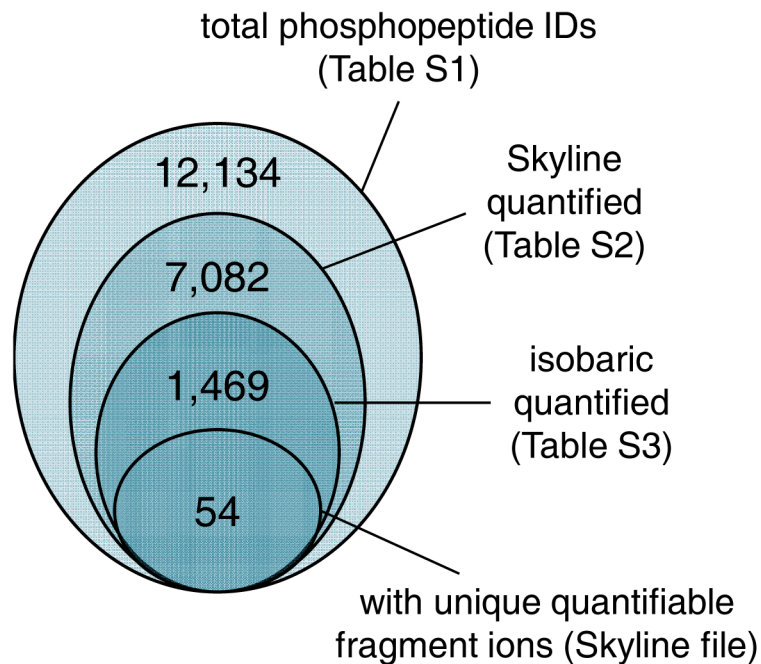


Figure 1. Numbers of characterized phosphopeptides

Large circle, DDA identified phosphopeptides using Proteome Discoverer (Thermo). Second circle, number of quantified using Skyline confidence threshold. Third circle, number of isobaric phosphopeptides present in the Skyline result table. Small circle, differentially quantified isobaric phosphopeptides in this study.

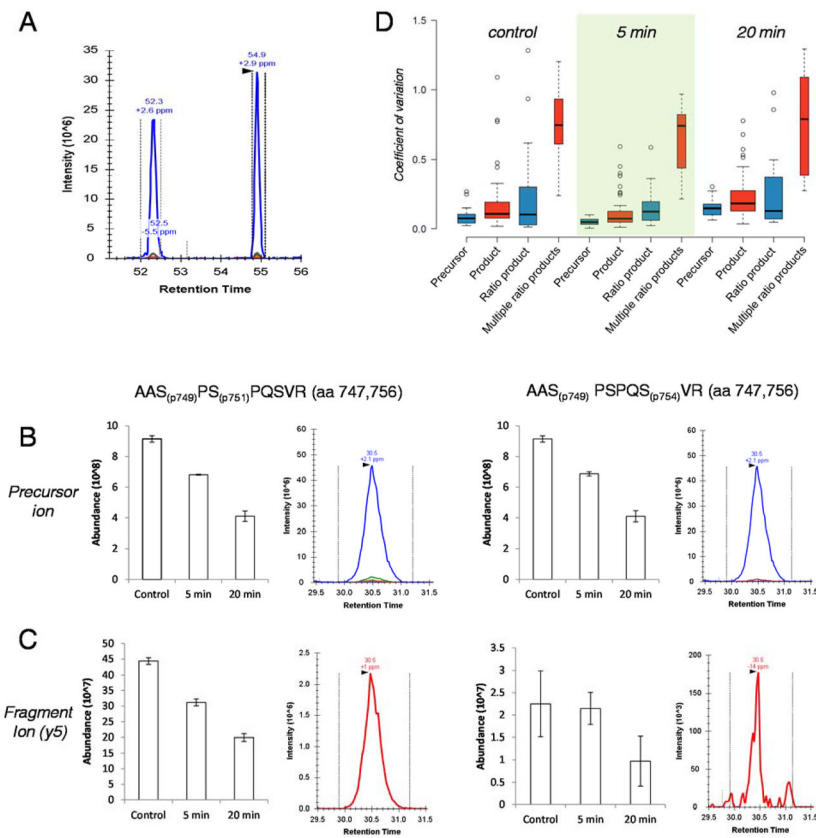


Figure 2. Quantification of isobaric phosphopeptides

(A) LC-MS chromatogram displaying an example of two isobaric phosphopeptides, HNS_pASVENVSLR and HNSAS_pVENVSLR, baseline separated by chromatography. (B) Quantification of two co-eluting isobaric forms of the phosphopeptide AAS_pPSPQSVR, modified as S749/S751 (left) or S749/S754 (right). Bar plot and XIC represent the precursor signal of the given peptide. (C) The two co-eluting peptides were distinguished and quantified using the chromatogram of the unique fragment ion y5. The first showed decreased abundance after 5 min of EGF stimulation, whereas the abundance was unchanged as compared to the control for the S754. (D) Distribution of the coefficient of variation between LC-MS replicates for the analyzed isobaric phosphopeptides. We considered precursor mass signals, product mass signals, calculated ratios and differential ratios calculated using different fragment ions (where possible).