EXPLORING THE EXPANDING LANDSCAPE OF PROTEIN PHOSPHORYLATION

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My view of the present state of research on exploring the chemistry-biology interface in protein phosphorylation

Protein phosphorylation is a crucial regulatory mechanism in cell signalling, acting as a molecular switch across the domains of life [1]. In humans, kinases and phosphatases are dedicated to the writing and erasing of protein phosphorylation and make up almost 2.5% of the genome [2]. Following the discovery of serine, threonine, and tyrosine phosphorylation through biochemical approaches, mass spectrometry-based proteomics became the primary method to investigate the function and regulation of Ser/Thr/Tyr phosphorylation in normal physiological and pathological states. As of now, hundreds of thousands phosphorylation sites have been identified - almost entirely by phosphoproteomic approaches [3] – yet many questions about the true size of the phosphoproteome and its site-specific functional relevance remain unknown.

In addition, recent advances have uncovered an even vaster phosphorylation landscape, to include phosphorylation of non-canonical amino acid residues in humans (histidine, arginine, cysteine, aspartate, glutamate, lysine) [1]. Adapting traditional bottom-up phosphoproteomics workflows to allow for high throughput detection of these modifications has proved challenging, as the low pH conditions used in sample preparation typically led to the hydrolysis of the acid-labile phosphoramidate, thiophosphate, and acyl-phosphate moieties [4]. However, as in the case of Ser/Thr/Tyr phosphorylation, the access to chemically synthesized phosphopeptide standards is enabling scientists to develop suitable enrichment and detection methods now [5].

Most recently, more unconventional phosphorylation modes, such as protein pyrophosphorylation (the addition of a phosphoryl group to a pre-existing phosphorylated side chain) and protein polyphosphorylation (the addition of longer polyphosphate chains)

have been proposed to add yet another level of complexity to phosphorylation networks [6,7]. But due to a lack of chemical and analytical tools, these additional signalling entities have remained largely enigmatic.

Our recent research contributions to exploring the chemistry-biology interface in protein phosphorylation

While mass-spectrometry based phosphoproteomics has become routine, identification of protein pyrophosphorylation – a modification where high-energy inositol pyrophosphate messengers are thought to donate their β-phosphoryl group to pre-phosphorylated side-chains, resulting in the formation of a pyrophosphate group (Figure 1a) - has so far exclusively relied on *in vitro* radiolabeling strategies [8,9].

Our group recently disclosed the detection and reliable assignment of protein pyrophosphorylation in two human cell lines using mass spectrometry, providing the first direct evidence of endogenous protein pyrophosphorylation [10]. Such a pyrophosphoproteomics work-flow had been a long-term goal of our lab, and to reach this goal we initially needed to develop chemical methods to synthesize pyrophosphopeptide standards [11-13]. These standards then served to solve the analytical challenge of distinguishing pyrophosphopeptides from bisphosphopeptides using eletron-transfer dissociation mass spectrometry (Figure 1b) [14,15].

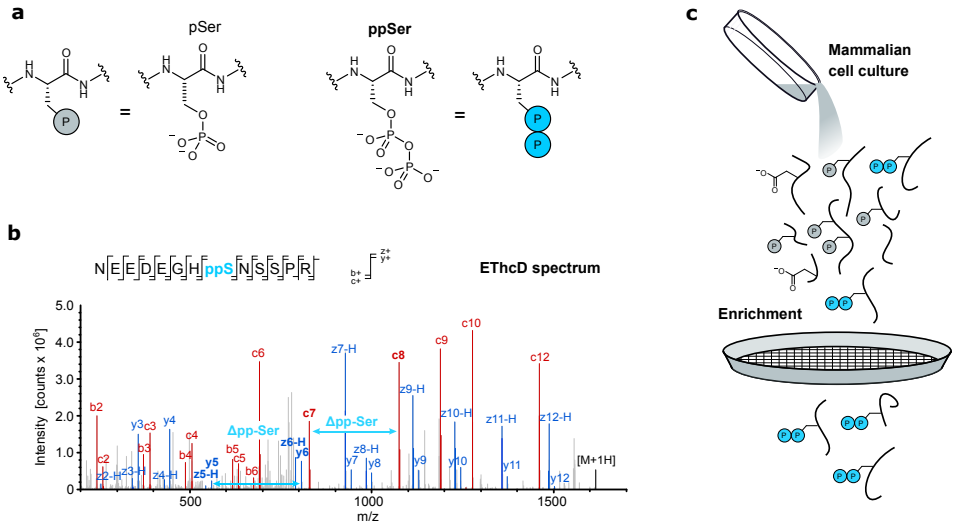

Fig. 1. Detection of protein pyrophosphorylation using mass spectrometry. a) Illustration of serine phosphorylation (pSer) and serine pyrophosphorylation (ppSer). b) Schematic of a work-flow, in which monophosphorylated and non-phosphorylated peptides are depleted by phosphatase treatment and affinity chromatography, to enrich pyrophosphopeptides. c) Fragmentation of a pyrophosphopeptide using electron-transfer dissociation combined with higher energy collision dissociation (EThcD) shows excellent sequence coverage, while leaving the modification intact. Figure adapted from ref [10].

By combining the mass spectrometry method with a tailored enrichment work-flow, we can now detect over 250 pyrophosphosites across more than 100 human proteins (Figure 1c). Our detection was consistent with previous biochemical evidence relating the installation of the modification to inositol pyrophosphates [10].

Protein pyrophosphorylation sites predominantly occurred in acidic serine-rich stretches, and a majority of the identified pyrophosphoproteins localized to the nucleus and/or the nucleolus. In a functional readout for pyrophosphorylation of nucleolar proteins, we observed significantly impaired rDNA transcription in inositol pyrophosphate-depleted cells [10]. In sum, protein pyrophosphorylation can now be added unequivocally to the growing list of endogenous phosphorylation motifs in human cell lines.

Most recently, our lab reported the discovery of yet another mode of phosphorylation – protein oligophosphorylation [16]. Using site-specifically phosphorylated and pyrophosphorylated nucleoside diphosphate kinase A (NME1), the effects of these modifications on enzyme activity were investigated. Nucleoside diphosphate kinase activity was notably reduced by phosphorylation, and even more so pyrophosphorylation, on threonine 94. Nevertheless, both phosphoprotein and pyrophosphoprotein were able to catalyze their own oligophosphorylation using ATP as a co-factor; up to the formation of a hexaphosphate chain. This reaction was critically dependent on the catalytic histidine residue, and cryo-EM analysis of the differently modified proteins suggests an intramolecular phosphoryl transfer. Oligophosphorylation of NME1 in both biochemical samples and in cell lysates was further confirmed using mass spectrometry, relying on chemically synthesized oligophosphorylated peptide standards [17]. Oligophosphorylation also promoted a new set of protein interactions of NME1 [16]. Our results highlight the complex nature of phosphoregulation, and the methods developed by our group now provide the opportunity to investigate the impact of this novel modification in the future.

Outlook to future developments

Protein pyro- and oligophosphorylation may unveil themselves as a central missing links in signal transduction research. For enabling such a paradigm shift though, pyro- and oligophosphorylation have to become more “visible and detectable”, which requires the development and implementation of new chemical and analytical methods for the detection of these unusual signaling entities. Therefore, our group and others should focus on novel tools that can decipher, distinguish, and quantify all the different phosphorylation states of a given peptide or protein. From a mass spectrometry perspective, this will require the further optimization of fragmentation techniques, coupled to isotopic labeling strategies, or parallel reaction monitoring, for quantification. Furthermore, the development of antibodies for the detection of pyrophosphoserine, or triphosphothreonine, for example, would be great assets for the community.

With these tools in hand, we can then further elucidate the functional role of protein pyrophosphorylation, both at the biochemical and cellular level. While the installation of pyrophosphorylation appears to be non-enzymatic in biochemical assays, it is still an open question whether this is also the case in a cellular setting. Following installation, pyrophosphorylation could be detected by specific reader domains. In fact, in a recent study, our group could show how pyrophosphorylation strongly influenced the interactome of a stoichiometrically modified protein [18]. And finally, the removal of pyrophosphorylation sites will need to be explored. Are there dedicated protein pyrophosphatases that convert pyrophosphoserine back to phosphoserine or serine?
The same questions arise for protein oligophosphorylation, and here, there is even more ground to cover. In the next years, it should be characterized which proteins undergo oligophosphorylation, and in response to which stimuli. Adding multiple phosphoryl groups to the same substrate will likely influence the solubility, interactions, and localization of the oligophosphorylated proteins, so the development of functional readouts is also much needed.

A few years ago, nobody anticipated the added complexity of protein pyrophosphorylation, and protein oligophosphorylation, but our scientific community, working at the chemistry-biology interface, is in a great position to reveal how these new phosphorylation modes contribute to signaling cascades and metabolic regulation.

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References

1. T. Houles, S. Yoon, P. P. Roux, *Trends Biochem. Sci*. **49**, 986 (2024).
2. G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* **298**, 1912 (2002).
3. S. J. Humphrey, D. E. James, M. Mann, *Trends Endocrinol. Metab*. **26**, 676 (2015).
4. G. Hardman, S. Perkins, P. J. Brownridge, C. J. Clarke, D. P. Byrne *et al*., *EMBO J*. **38**, 1 (2019)
5. T. Hunter, *Mol. Cell* **82**, 2190 (2022).
6. Bhandari, R., Saiardi, A., Ahmadibeni, Y., Snowman, A.M., Adam, R.C. *et al*., *PNAS* **104**, 15305 (2007).
7. C. Azevedo, T. Livermore, A. Saiardi. *Mol Cell*. **58**, 71 (2015).
8. Z. Szijgyarto, A. Garedew, C. Azevedo, A. Saiardi, *Science* **334**, 802 (2011).

9. P. Lolla, A. Shah, C. P. Unnikannan, V. Oddi, R. Bhandari, *Biochem. J.* **478**, 1647 (2021).
10. J. A. M. Morgan, A. Singh, L. Kurz, M. Nadler-Holly, M. Ruwolt, *et al*., *Nat Chem Biol*. **20**, 1305 (2024).
11. A. M. Marmelstein, L. M. Yates, J. H. Conway, D. Fiedler, *J. Am. Chem. Soc*. **136**, 108 (2014).
12. L. M. Yates, D. Fiedler, *ACS Chem. Biol*. **11**, 1066 (2016).
13. A. M. Marmelstein, J. A. M. Morgan, M. Penkert, D. T. Rogerson, J. W. Chin, *et al*., *Chem Sci*. **9**, 5929 (2018).
14. M. Penkert, L. M. Yates, M. Schümann, D. Perlman, D. Fiedler, *et al*., *Anal Chem*. **89**, 3672 (2017).
15. M. Penkert, A. Hauser, R. Harmel, D. Fiedler, C. P. R. Hackenberger, *et al*., *J Am Soc Mass Spectrom.* **30**, 1578 (2019).
16. A. Celik, F. Schöpf, C. E. Stieger, S. Lampe, B. Hanf, et al., *Nat Chem*. doi: 10.1038/s41557-025-01915-8 (2025).
17. K. Qian, B. Hanf, C. Cummins, D. Fiedler, *Angew Chem Int Ed Engl*. **64**, e202419147 (2025).
18. A. Celik, I. Beyer, D. Fiedler, *J Am Chem Soc*. **146**, 14807 (2024).