Using PEPscreen™ to Study Protein Phosphorylation and Kinase Activity

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Protein phosphorylation events mediate key cellular signaling pathways, regulating diverse processes such as proliferation, metabolism, and apoptosis. In the human proteome, which numbers up to 23,000 proteins, around 650,000 possible phosphorylation sites have been predicted, and about 100,000 of those sites have been confirmed as actual sites of protein phosphorylation. Protein kinases (PKs) are one of the major classes of proteins that phosphorylate substrates as part of the signaling cascade. PKs occur in substantial numbers in various organisms, from predicted numbers of 518 in humans up to 1,429 in rice. Because of the sheer scale of possible interactions between protein kinases and potential targets (clients), establishing precise interactions between specific PKs and particular sites is crucial to elucidate related biological pathways. On a more technical level, high-throughput assays are needed to establish these valid kinase-client interactions. Past methods have used low-throughput methods such as radiolabeling or 2D-gel electrophoresis. More recent studies have utilized peptide and protein arrays for higher throughput assays. Arrays have the disadvantage, however, that the conformations of the proteins and peptides in the arrays are not native. Thus, interactions between kinases and their substrates are not necessarily reflected accurately. In addition, arrays do not reveal specific individual sites of phosphorylation when a given peptide has multiple amino acids that can be phosphorylated.

Mass spectrometry (MS) has emerged as a highly sensitive and specific technology for probing protein phosphorylation. Professor Jay Thelen of the University of Missouri has used Sigma-Aldrich’s PEPscreen custom peptide libraries technology to develop high-throughput tandem-MS assays for studying kinase phosphorylation of target proteins. His group has developed their Kinase Client (KiC) assay to probe kinase-mediated phosphorylation of multiple target peptides in a single solution-phase reaction. The PEPscreen peptides provide a convenient and cost-efficient source of custom-made peptide libraries for such assays. These peptides are of sufficiently robust purity that parent PEPscreen peptides and subsequently phosphorylated peptides can be readily distinguished in LC-MS/MS analysis.

The group’s earliest report, by Huang et al., created a peptide library consisting of 80 PEPscreen peptides from Sigma-Aldrich. These peptide sequences were principally derived from the serine-containing peptides of three Arabidopsis thaliana proteins: pyruvate dehydrogenase kinase (PDK, At3g06483), and two mitochondrial pyruvate dehydrogenase complex (PDC) subunits, E1α-1 (At1g59900) and E1α -2 (At1g24180). Their initial peptide cocktail contained 46 peptides used as substrates in a single kinase solution reaction. The kinases used in the phosphorylation reaction were A. thaliana PDK and an A. thaliana Ca2+-dependent protein kinase (CPK3, At4g23650). The results showed that PDK phosphorylated a single peptide, YHGH S MSDP GSTYR (underlined Ser, Ser292), under their assay conditions. By contrast, PK3 did not phosphorylate any of the PDK or E1α peptides. This demonstrated the precision of the assay under conformational and thus relevant solution conditions. Focusing on the YHGHSMSDPGSTYR peptide, the authors further studied the effects of sequence variation around the primary site of phosphorylation, Ser292, using an additional set of PEPscreen peptides. Additionally, the second screen included a positive control peptide for CPK3, RASAI KALG SFASN, which contains the phosphorylation site, Ser274, for plasma membrane intrinsic protein 3. CPK3 phosphorylated only the RASA I KALG SFASN peptide at the reported site.

In a study published in 2012, Ahsan et al. further probed the YHGSM SDP GSTYR peptide in terms of phosphorylation of Ser292 in the mitochondrial pyruvate dehydrogenase complex. This study used a library of 59 PEPscreen peptides, where unlike the earlier study, the sequences were all variations on that one peptide, to determine exhaustively the effects of sequence variation around Ser292 on the degree of its phosphorylation. The authors used recombinant PDK for the phosphorylation reaction. In effect, this MS study offered a more economical and faster alternative to a full-scale site-directed mutagenesis study of full proteins with the corresponding sequence variations.
Professor Thelen’s group has indicated up to 100 peptides can be included in a single KiC assay. Using this reaction scale, their most recent use of the PEPscreen technology involves a library of 377 peptide sequences of in vivo phosphorylation sites, to examine phosphorylation by 77 different protein kinases from A. thaliana. Ahsan et al. chose the sequences from past identification of in vivo phosphorylation sites in the developing seed of A. thaliana. Initial screening revealed 23 proteins and 17 PKs of particular interest as candidates for kinase-client pairing. The authors then focused on one full-length protein, protein phosphatase inhibitor-2 (AtPPI-2), as a particular client with potential roles in multiple signaling pathways. As with the prior detailed investigations of the YHGHSMSDPGSTYR peptide, this more recent study demonstrates greater efficiencies in time and resources by focusing initially on target peptides for phosphorylation, rather than trying to work on full-length proteins with each of those 377 sequences.

Tandem MS continues to grow in importance in high-throughput screening as an alternative to the use of arrays, and radiolabeled or fluorescent substrates. Compared to arrays, tandem MS allows the analysis of kinase-mediated phosphorylation in conformationally relevant solution states of both kinase and client. Perhaps the most important advantage of tandem MS over peptide arrays is the ability to indicate phosphorylation site specificity in peptides with multiple Ser, Thr, or Tyr residues. In this context, the PEPscreen custom peptide libraries provide a promising avenue for investigating applications like protein kinase activity and phosphorylation.

References