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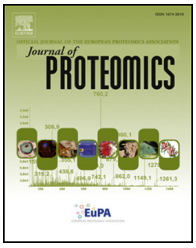

SPECHT — Single-stage phosphopeptide enrichment and stable-isotope chemical tagging: Quantitative phosphoproteomics of insulin action in muscle

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ABSTRACT

The study of cellular signaling remains a significant challenge for translational and clinical research. In particular, robust and accurate methods for quantitative phosphoproteomics in tissues and tumors represent significant hurdles for such efforts. In the present work, we design, implement and validate a method for single-stage phosphopeptide enrichment and stable isotope chemical tagging, or SPECHT, that enables the use of iTRAQ, TMT and/or reductive dimethyl-labeling strategies to be applied to phosphoproteomics experiments performed on primary tissue. We develop and validate our approach using reductive dimethyl-labeling and HeLa cells in culture, and find these results indistinguishable from data generated from more traditional SILAC-labeled HeLa cells mixed at the cell level. We apply the SPECHT approach to the quantitative analysis of insulin signaling in a murine myotube cell line and muscle tissue, identify known as well as new phosphorylation events, and validate these phosphorylation sites using phospho-specific antibodies. Taken together, our work validates chemical tagging post-single-stage phosphoenrichment as a general strategy for studying cellular signaling in primary tissues.

Biological significance
Through the use of a quantitatively reproducible, proteome-wide phosphopeptide enrichment strategy, we demonstrated the feasibility of post-phosphopeptide purification chemical labeling and tagging as an enabling approach for quantitative phosphoproteomics of primary tissues. Using reductive dimethyl labeling as a generalized chemical tagging strategy, we compared the performance of post-phosphopeptide purification chemical tagging to the well established community standard, SILAC, in insulin-stimulated tissue culture cells. We then extended our method to the analysis of low-dose insulin signaling in murine muscle tissue, and report on the analytical and biological significance of our results.

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1. Introduction

Protein phosphorylation is an essential regulatory mechanism that controls most cellular processes including, but not limited to cell division, apoptosis, response to extracellular signals, and growth factor stimulation. Advances in proteomics and mass spectrometry approaches have made the proteome-wide analysis of phosphorylation signaling feasible, and have helped to overcome many obstacles in phosphopeptide detection due to low abundance, signal suppression, and poor ionization efficiency [1]. Furthermore, introduction of stable isotope labeling in culture (SILAC) [2] has made the quantitative comparison of changes in phosphorylation site abundance in cell culture systems possible with high quantitative accuracy and reproducibility by reducing pre-analytical quantitative variability after cell harvesting and during sample manipulation. In SILAC, proteins are metabolically labeled in cell culture by the replacing naturally-occurring "light" amino acids with their "heavy" version (most commonly using arginine and lysine) in the media. After metabolic incorporation of the heavy amino acids for six to eight cellular doublings, cellular proteins are labeled by more than 98% in most cell lines commonly used in biomedical research. Comparison of cellular conditions via protein or phosphorylation site abundance is subsequently accomplished by mixing equal amounts of differentially treated "heavy" and "light" cells and subjecting them to standard proteomics or phosphoproteomics workflows [3]. This approach is widely used and benefits from the early introduction of the isotope labels into the proteomics workflow, which leads to improved robustness of quantification by reducing the impact of experimental errors introduced downstream of label introduction. However, SILAC is limited to cells that can be grown in culture for at least six doublings and incorporate heavy amino acids. Primary human cells are not amenable to this approach, nor are mouse tissues without complicated methods to raise them on expensive and highly specialized diets [4,5]. Other organisms, including many model fungi and bacteria, require additional manipulation to produce auxotrophs that function correctly in the SILAC scheme. While Super-SILAC is emerging as an alternative quantification strategy [6,7], this approach relies on the extent to which the target organism or tissue type can be matched with closely related cell lines in terms of abundance profiles of their protein and post-translational modifications. Alternatively, quantification can be carried out by chemical labeling using iTRAQ [8], TMT [9] reagents or reductive dimethyl-labeling [10,11], each with its own set of advantages and disadvantages. While quantification by iTRAQ or TMT is performed on the MS^2 or MS^3 level [12], quantification by reductive dimethyl-labeling occurs on the MS level in the same manner as SILAC and can be performed on a broader range of mass spectrometers. Of particular note, however, is that the input required in comprehensive phosphoproteomics experiments (~5 mg protein digest per condition [13]) greatly exceeds the capacity of a single iTRAQ/TMT labeling reaction, requiring many aliquots of reagent and thus rendering such experiments very costly and, for many laboratories, impractical to perform on a routine basis. This has led to the development of post-enrichment labeling strategies that focus instead on labeling phosphopeptides after isolation [14,15].

Here, we extend such approaches by combining a rapid, single-stage phosphopeptide enrichment procedure and chemical labeling post-enrichment using reductive dimethyl-labeling and directly compare our results to traditional SILAC. We call our method SPECHT (single stage *p*hosphopeptide *e*nrichment and stable isotope *ch*emical tagging). We begin by testing this approach on complex peptide mixtures from HeLa cells and C2C12 murine myotubes and comparing these results to phosphopeptide quantification by SILAC labeling. We demonstrate that our approach yields highly reproducible phosphopeptide quantification results from whole cell lysates. We then apply this approach to quantify the effects of low-dose insulin stimulation on the mouse muscle phosphoproteome and validate our observations by Western blot analysis.

2. Materials and methods

2.1. Materials

Modified trypsin was from Promega (Madison, WI). Urea, Tris–HCl, $CaCl_2$, ammonium bicarbonate (NH_4HCO_3), sodium fluoride (NaF), potassium chloride (KCl), potassium phosphate (KH_2PO_4), phosphoric acid, sodium ortho-vanadate, sodium molybdate, sodium tartrate, beta-glycerophosphate, DL-dithiothreitol, iodoacetamide, formaldehyde, formaldehyde-^{13}C d_2, sodium cyanoborohydride, triethyl ammonium bicarbonate (TEAB) were from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN), trifluoroacetic acid (TFA) and water were from Honeywell Burdick and Jackson (Morristown, NH). Methanol was from ThermoFisher Scientific (Waltham, MA). High-purity formic acid was from EMD Millipore (Billerica, MA). Sodium cyanoborodeuteride, U-$^{13}C_6$, U-$^{15}N_2$–lysine & U-$^{13}C_6$, U-$^{15}N_4$ arginine were from Cambridge Isotope Laboratories (Tewksbury, MA). SepPak C_{18} solid-phase extraction cartridges and Oasis HLB vacuum extraction plates were from Waters Corporation (Milford, MA). Lactic acid was from Lee BioSolutions, Inc (St. Louis, MO). TiO_2 beads were from GL Sciences (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), PBS, penicillin and streptomycin were from Cellgro Mediatech Inc (Manassas, VA). SILAC DMEM (without arginine and lysine amino acids) was acquired from Invitrogen, Lifetechnologies (Grand Island, NY). Fetal bovine serum (FBS) and dialyzed fetal bovine serum (dFBS) were purchased from Hyclone, ThermoFisher Scientific (Waltham, MA).

2.2. Cells

C2C12 and HeLa cells were grown as adherent cultures in DMEM supplemented with 10% FBS and penicillin and streptomycin or SILAC DMEM supplemented with 10% dFBS, heavy and light arginine and lysine, and penicillin and streptomycin, respectively. For HeLa cells, DMEM was supplemented with 100 mg/l heavy or light arginine and lysine. For C2C12 cells, DMEM was supplemented with 84 mg/l heavy or light arginine and 146 mg/l heavy or light lysine. For SILAC labeling, cells were grown for at least 6 doublings and incorporation of heavy amino acids and arginine to proline conversion was determined. HeLa cells were collected, washed with PBS and frozen in liquid nitrogen. C2C12 myoblasts were differentiated into myotubes