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Comprehensive phosphopeptide enrichment strategy for analysis of complex biological samples

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Novel Aspect

Combined approach of Immunoprecipitation, SCX, ERLIC and IMAC for comprehensive phosphoproteome analysis of complex biological samples

Introduction

Post-translational modification of proteins plays a critical role in regulating protein activity, protein-protein interaction, localization, and cell signaling. Protein phosphorylation is one of the dynamic post-translational modifications. In mammals almost one third of proteins are phosphorylated. The low stoichiometry of phosphorylation, however, makes the mass spectrometry-based quantitative analysis difficult. Currently, many phosphopeptide enrichment methods are in use, including phosphoproteins/peptides immunoprecipitation with specific antibodies, strong cation exchange chromatography, electrostatic repulsion hydrophilic interaction chromatography, immobilized metal affinity chromatography, zirconium dioxide and titanium dioxide chromatography. Used independently, each of these methods does increase the efficiency of detection for phosphopeptides; each has advantages and limitations. Combining different phosphopeptide enrichment methods is, therefore, crucial for a more comprehensive detection of phosphopeptides in complex biological samples

Methods

Human Corneal Limbal Epithelial cell lines (HCLEs) were grown to confluence, stimulated with EGF, and lysed with 8M urea supplemented with phosphatase and protease inhibitors. For quantitative phosphorylation analyses, stable isotope-labeled cells were used. An average of 10 mg total protein was reduced, alkylated and digested with trypsin. Peptides were desalted with SepPak C18 reversed phase chromatography. Phosphotyrosine peptides were immunoprecipitated using a combination of three anti-phosphotyrosine antibodies. The flow-through was further fractionated with either electrostatic repulsion hydrophilic interaction chromatography (ERLIC) or strong cation exchange (SCX) chromatography. Fractions were further phosphoenriched using immobilized metal affinity chromatography (IMAC) and analyzed by LC/MSn using a nanoAcuity UPLC (Waters) coupled through a TriVersa NanoMate (Advion) to an LTQ-Orbitrap MS (Thermo-Fisher).

Preliminary Data

When employed alone, SCX chromatographic fractionation of samples resulted in low detection efficiency for phosphopeptides. The relatively high amount of non-phosphopeptides in the SCX fractions outcompeted the phosphopeptides for detection. Further phospho-based enrichment of the SCX fractions with either IMAC or TiO2 chromatography eliminated most of the non-phosphopeptides and significantly increased detection efficiency for the phosphopeptides. In a parallel experiment, we fractionated peptides using ERLIC and analyzed the resulting fractions directly by LC/MS/MS. ERLIC alone provided significantly increased detection of phosphopeptides. Given the low stoichiometry of phosphotyrosine compared to phosphoserine or phosphothreonine in biological samples, however, their detection level of remains a challenge. Further reasearch is presently underway to increase the detection efficiency for phosphotyrosine peptides. Our current approach employs immunoprecipitation of phosphotyrosine peptides prior to fractionation by either SCX or ERLIC. The flow-through is then divided in to two and fractionated further - one half is subjected to SCX and the other half to ERLIC. All the immunoprecipitated peptides, the SCX and ERLIC fractions are further phosphoenriched using IMAC before LC/MSn analysis. Preliminary results show, significant increase in phosphopeptide identification.