Comprehensive Phosphopeptide Enrichment Strategy for Analysis of Complex Biological Samples

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Overview

Mass spectrometry based analysis of phosphorylated proteins has become a very powerful tool in elucidating how stimulants activate certain downstream signaling networks and its effect on cell function. Given their low stoichiometry, several phosphoenrichment strategies has been employed, yet, each strategy has its advantages and limitations. In this poster, we present a comprehensive phosphoenrichment strategy for analysis of complicated biological samples using a combination of three different but complimentary approaches.

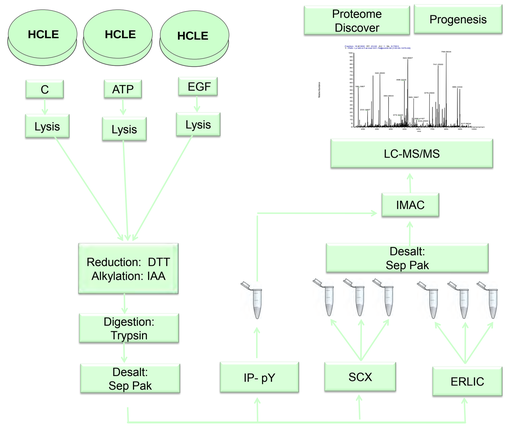
Introduction

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 most common 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, *e.g.*, immunoprecipitation of phosphoproteins/peptides 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 its own advantages and limitations. Combining different phosphopeptide enrichment methods is, therefore, crucial for achieving a truly 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 phospho-enriched using immobilized metal affinity chromatography (IMAC) and analyzed by LC/MS^n using a nanoAcuity UPLC (Waters) coupled through a TriVersa NanoMate (Advion) to an LTQ-Orbitrap MS (Thermo-Fisher).

Work flow



Results

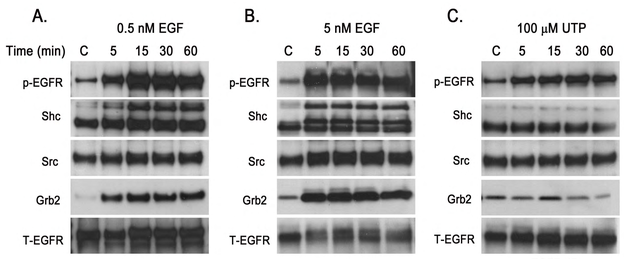


Fig 1. Western blot analysis of time course phosphorylation of Epidermal Growth Factor Receptor (EGFR) and recruitment of adaptor proteins in Human Corneal Epithelial Cells (HCLEs). (A&B) Stimulating HCLEs with EGF resulted in a dose-dependent increase in EGFR phosphorylation and Grb2 association with EGFR. (C) UTP stimulation resulted in reduced EGFR phosphorylation and no increase in Grb2 association.

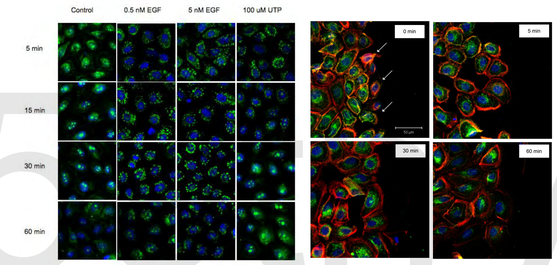


Fig. 2. Internalization of the EGFR in response to Wound, UTP and EGF. HCLEs were (A) cultured to confluence and subjected to scratch wound or (B) stimulated with medium change (control), UTP (100 mmol/L), or EGF (0.5 and 5 nmol/L) over a time course. Cells were fixed and probed with an antibody directed against EGFR (green), counterstained with a nuclear marker, ToPro-3AM (blue). Confocal images represent single optical sections of 4 mm taken in the midregion of the cells. Images represent four independent experiments.

Minimizing loss of phosphopeptides on metal surfaces of UPLC

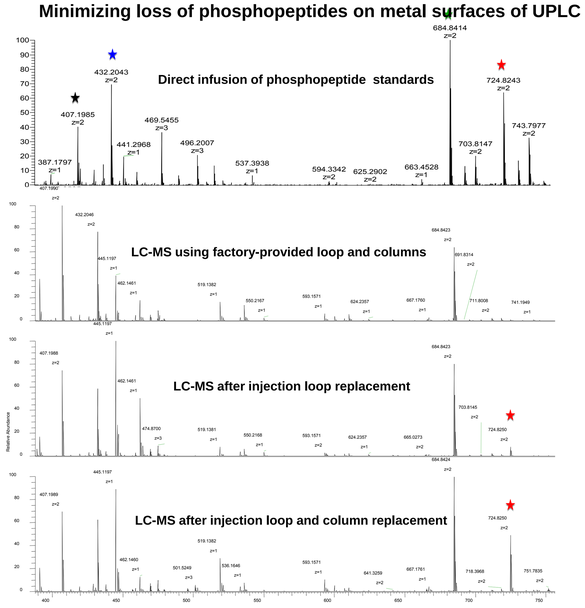


Fig 3. Detection and reproducibility of phosphopeptides was improved after modifications to the UPLC and columns. Replacing the metal injection loop alone improved the detection of multi-phosphorylated peptides. Intensity of these phosphopeptides and reproducibility of detection was further improved by packing home-made columns using silicon-based frits.

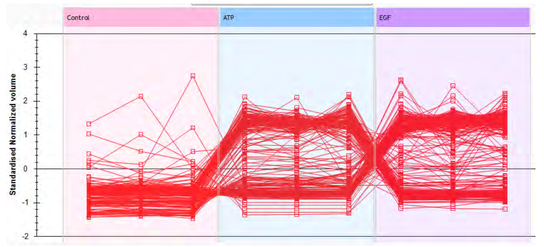


Fig 4. Progenesis™ analysis of Human Corneal Epithelial Cells (HCLEs) phosphoproteomic profile. Stimulating HCLEs with ATP or EGF resulted in a distinct phosphorylation profile. HCLEs were grown to confluence and treated with either media change (control), ATP or EGF. The results from triplicate LC/MS/MS analyses of each condition were analyzed for differential phosphorylation profiles.

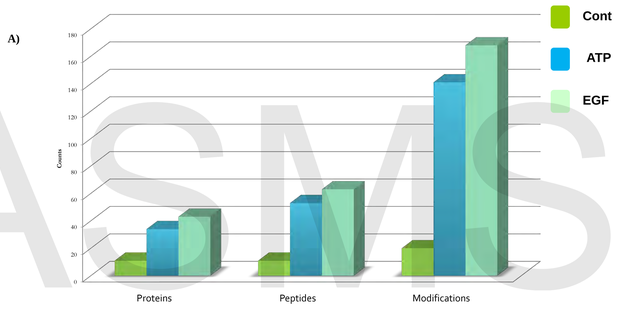


Fig 5 Quantitative comparison of phosphopeptide modification and phosphotyrosine enrichment strategy. (A) EGF treatment results in highest phosphopeptide modification compared to nucleotide treatment and control groups. (B) Immunoprecipitation of phosphotyrosine peptides prior to SCX and ERLIC fractionation, followed by IMAC phosphopeptide enrichment, resulted in the highest phosphotyrosine detection (phosphopeptides ≥ 50 % of the total identified peptides).

Summary of EGFR phosphotyrosine profile following nucleotide and growth factor stimulation

	Confidence	Peptide Sequence	PTM	Charge	m/z	ΔM [ppm]
Cont.	High	GSTAENAEYLR	pY-1173	2	645.7714	-0.42
ATP	Confidence	Peptide Sequence	PTM	Charge	m/z	ΔM [ppm]
	High	RPAGSVQNPVYHNQPLNPAPSR	pY-1086	3	827.0707	-0.59
	High	GSHQISLDNPDYQQDFFPK	pY-1148	2	1158.5015	-0.45
	Medium	MHLPSPTDSNFYR	pY-974	2	830.8446	-0.32
	Low	GSTAENAEYLR	pY-1173	2	645.7714	-0.51
EGF	Confidence	Peptide Sequence	PTM	Charge	m/z	ΔM [ppm]
	High	RPAGSVQNPVYHNQPLNPAPSR	pY-1086	3	827.0707	-0.59
	High	GSHQISLDNPDYQQDFFPK	pY-1148	2	1158.5015	-0.45
	High	MHLPSPTDSNFYR	pY-974	2	830.8446	-0.32
	High	EAKPNGIFKGSTAENAEYLR	pY-1173	3	759.0299	-0.17
	High	DPHYQDPHSTAVGNPEYLNTV QPTCVNSTFDSPAHWAQK	pY-1101 & pY-1114	6	1172.3558	0.46

Table 1: Summary of pY residues of EGFR following nucleotide and growth factor stimulation. Tyrosine residue 1173 (Y1173) of EGFR was constrictively phosphorylated, as shown in control cells. Stimulating cells with ATP resulted in phosphorylation of more tyrosine residues, namely Y974, Y1086, Y1048, and Y1173. EGF stimulation resulted in phosphorylation of EGFR at even more tyrosines and an overall higher level of modification.

Comparison of SCX and ERLIC phosphoenrichment strategies

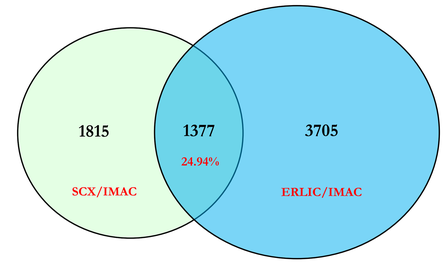


Fig 6. When employed alone, SCX chromatographic fractionation of samples resulted in low detection efficiency for phosphopeptides. Further phospho-enrichment of the SCX fractions with either IMAC eliminated most of the non-phosphopeptides and significantly increased detection efficiency for phosphopeptides. In a parallel experiment, peptides were fractionated using ERLIC and further phospho-enriched using IMAC. ERLIC/IMAC resulted in a significantly increased detection of phosphopeptides. The small overlap (25%) of phosphopeptide detection between SCX/IMAC and ERLIC/IMAC suggests these strategies complement one another.

Conclusions

Phosphopeptides tend to bind nonspecifically to metal surfaces. This affects their detection and the overall reproducibility of phosphopeptide identification. Replacing possibly exposed metal surfaces, for example, the injection loop, T-junction and frits should be considered for efficient and reproducible phosphoproteomic studies.

When employed alone, SCX chromatographic fractionation of samples results in low detection efficiency for phosphopeptides. The relatively high amount of non-phosphopeptides in the SCX fractions outcompete the phosphopeptides for detection. Further phospho-based enrichment of the SCX fractions with either IMAC chromatography eliminated most of the non-phosphopeptides and significantly increased detection efficiency for the phosphopeptides.

ERLIC alone provided significantly increased detection for phosphopeptides. Further phospho-enrichment of ERLIC fractions by IMAC resulted in a still higher level phosphopeptide identification.

Even alllowing for the low stoichiometry of phosphotyrosines compared to phosphoserines or phosphothreonines in biologically derived protein samples, their rate of detection has been very low. Immunoprecipitation of phosphotyrosine peptides, before fractionation of samples by either SCX or ERLIC, significantly increased the detection of pY peptides.

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