Global Phosphoproteomics of Activated B cells Using Complementary Metal Ion Functionalized Soluble Nanopolymers

Keerthi B. Jayasundera[1], Anton B. Iliuk[2], Andrew Nguyen[3], Renee Higgins[4], Robert L. Geahlen[4,5], and W. Andy Tao[1, 2, 4, 5 *]

LIST OF ABBREVIATIONS

AGPAT6: 1-acylglycerol-3-phosphate O-acyltransferase 6

AKT: Serine/threonine protein kinase Akt

ALDOA: Aldolase A fructose-bisphosphate

ATXN3: Ataxin 3

BCL10: B-cell lymphoma/leukemia protein

BLK: B lymphocyte kinase

BLNK: B-cell linker protein

BTK: Butron's tyrosine kinase

BUB1B: Mitotic checkpoint serine/threonine-protein kinase BUB1 beta

CARMA1: CARD-containing binding partner of Bcl10

CDK1: Cyclin-dependent kinase 1

CHD1L: Chromodomain helicase DNA binding protein 1-like

DDX39A: DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A

ERK: Extracellular signal-regulated kinases

GNAS: GNAS complex locus

HILIC: Hydrophilic interaction chromatography

HNRNPH3: Heterogeneous nuclear ribonucleoprotein H3 (2H9)

HNRNPK: Heterogeneous nuclear ribonucleoprotein K

HNRNPM: Heterogeneous nuclear ribonucleoprotein M

HSP90AA1: Heat shock protein 90kDa alpha (cytosolic), class A member 1

IMAC: Immobilized metal affinity chromatography

ITAM: Immunoreceptor tyrosine-based activation motif

LYN: v-yes-1 Yamaguchi sarcoma viral related oncogene homolog.

MALT: Mucosa-associated lymphoid tissue lymphoma translocation protein

MOAC: Metal oxide affinity chromatography

NF-κB: Nuclear Factor-Kappa B

NFAT: Nuclear factor of activated T-cells

NIMA: NIMA (Never in Mitosis Gene A)-Related Kinase (NEK)

NPM1: Nucleophosmin

NUDC: Nuclear migration protein nudC

PAMAM: Poly(amidoamine) dendrimer

PI3K: Phosphoinositide-3 kinase

PLCγ: Phospholipase C gamma

POLR2A: Polymerase (RNA) II (DNA directed) polypeptide A, 220kDa

PolyMAC: Polymer based metal affinity capture

PRPF8: Pre-mRNA processing factor 8

PSMC1: Proteasome (prosome, macropain) 26S subunit, ATPase, 1

PSMC2: Proteasome (prosome, macropain) 26S subunit, ATPase, 2

PSMC5: Proteasome (prosome, macropain) 26S subunit, ATPase, 5

PTEN: Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase

RBM39: RNA binding motif protein 39

RPL11: Ribosomal protein L11

RPLC: Reversed phase liquid chromatography

RPS19: Ribosomal protein S19

RPS7: Ribosomal protein S7

SH2: Src homology domain 2

SHIP1: Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1

SILAC: Stable isotope labeled amino acids in culture

SNRPB2: Small nuclear ribonucleoprotein polypeptide B

TARDBP: TAR DNA binding protein

TYMS: Thymidylate synthetase

VAV: Guanine nucleotide exchange factor

VCP: Valosin containing protein

WDR43: WD repeat domain 43

METHODS

Synthesis of PolyMAC-Zr reagent

Polyamidoamine dendrimer generation 4 (PAMAM G4) solution (200 µl; provided as 10% wt/vol in methanol; Sigma-Aldrich) was dried in a microcentrifuge tube and redissolved in 1 ml of dimethyl sulfoxide. Then 6 mg of Boc-aminooxy acetic acid, 15 mg of N-Hydroxybenzotriazole (HOBt) and 10 µl of N,N'-Diisopropylcarbodiimide (DIC) were dissolved in 1 ml of DMSO in a microcentrifuge tube and reacted for 30 min at room temperature with shaking. The above two solutions were transferred into a 10 ml round-bottom flask and stirred overnight at room temperature. Then 3 ml of water was added to the reaction mixture and dialyzed against water to remove excess reagents and concentrated using a 3500 molecular weight cut-off centrifugal filter. The mixture was transferred to a 10 ml round-bottom flask and an equal volume of 250 mM MES (2-(N-morpholino) ethyanesulfonic acid; pH 5.5) buffer was added to it. Next 16 mg of carboxyethyl phosphonic acid, 16 mg of N-hydroxysuccinimide and 160 mg EDC (1-ethyl-3-(3-dimethylaminopropyl)carbidiimide hydrochloride) were added to the mixture and stirred overnight at room temperature. The solution was dialyzed against water and one-third of it was reacted with 0.1 M solution of $ZrOCl_2$ for 1 1/2 hours at room temperature. The mixture was dialyzed against water and transferred to a glass tube and evaporated to complete dryness. Then the solid was redissolved in 80% trifluoroacetic acid, reacted for 1 1/2 hours and evaporated under N_2 gas. The mixture was dialyzed successively in 1:4 DMSO: water mixture and water. The final PolyMAC-Zr product was stored at 4 ^{o}C.

Isolation of phosphopeptides from DG75 cell lysate

DG-75 cell culture was grown to 80% confluency in RPMI-1640 media (Gibco) substituted with 10% inactivated FBS, 1% sodium pyruvate, 0.5% streptomycin/penicillin, and 0.05% β-mercaptoethanol. Then the cells were washed with PBS, collected, and frozen at -80 °C. Cells were lysed in 1 mL of lysis solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1x phosphatase inhibitor cocktail (Sigma-Aldrich), 10 mM sodium fluoride) for 20 minutes on ice. The resulting mixture was centrifuged at 16,100 × g for 10 minutes, and supernatant containing soluble proteins was collected. The protein concentration was measured using the BCA assay. DG-75 protein lysate was denatured and reduced in 50 mM trimethyl ammounium bicarbonate containing 0.1% RapiGest and 5 mM dithiothreitol for 30 minutes at 50 °C. The proteins were further alkylated in 15 mM iodoacetamide for 1 hour in the dark at room temperature, and digested with proteomics grade trypsin at 1:100 ratio overnight at 37 °C. The pH was adjusted below 3 with hydrochloric acid and the sample was incubated for 40 minutes at 37 °C. The sample was centrifuged at 16,100 × g and supernatant collected. The resulting peptides were desalted with a Sep-Pak C18 column. The enrichment of phosphopeptides was carried out using 100 µg samples, according to the protocol described below.

Phosphopeptide enrichment

The peptide mixture was dissolved in 100 µl of the loading buffer (200 mM glycolic acid, 1% trifluoroacetic acid and 50% acetonitrile), 5 nmol of PolyMAC-Zr was added to it and incubated for 10 minutes at room temperature. Then 250 µl of capture buffer (300 mM HEPES buffer at pH 7.7) was added to increase the pH above 6.3 and transferred to a spin column (Boca Scientific) containing 50 µl of Carbolink coupling agarose gel (Thermo Scientific). The samples

were incubated with beads for 10 minutes and centrifuged at 2,300 x g for 30 seconds to remove the flowthrough. The gel was washed with 200 µl portions of loading buffer, twice with washing buffer (100 mM acetic acid, 1% trifluoro acetic acid, 80% acetonitrile) and once with water. Then the phosphopeptides were eluted off the gel-bound PolyMAC-Zr by incubating twice with 100 µl portions of 400 mM ammonium hydroxide solution. During washing and elution, each incubation step was carried out for 5 minutes with shaking. PolyMAC-Ti enrichment was carried out in a similar fashion, as described before[23].

Growing DG75 cell lysates in SILAC "heavy" and "light" media

For SILAC experiments, cells were grown to 50% confluency in SILAC RPMI-1640 media (Gibco) substituted with 10% dialized inactivated FBS (Sigma-Aldrich), 1% sodium pyruvate, 0.5% streptomycin/penicillin, 0.05% β-mercaptoethanol, and either L-Lysine and L-Arginine for "light" samples, or $^{13}C_6$-Arginine and $^{13}C_6$-Lysine (Isotec) for "heavy" samples in 5% CO_2 at 37ºC. Cells were grown for at least 6 divisions to ensure complete incorporation of the "heavy" amino acids (confirmed by mass spectrometry analysis; data not shown). Before each set of experiments, "light" and "heavy" cells were normalized on both cellular and protein levels.

Piceatannol treatment and IgM pathway stimulation

The cell cultures were collected separately, washed with PBS and resuspended in PBS to obtain a cell density of 2 x 10^7 cells per milliliter, followed by treatment with piceatannol at the concentration of 50 µg per milliliter. Then the cells were incubated at 37^{o}C for 30 minutes. The B cell receptor signaling pathway was stimulated by treating the cells with the anti-IgM antibody

at a concentration of 10 µL per milliliter and incubated at 4^{o}C for 15 minutes. Finally, the cells were washed with PBS, collected, and frozen at -80 °C.

Sample fractionation and phosphopeptide enrichment

Cells were lysed, protein content extracted, and the concentration was determined using the BCA assay as described above. Then, 2.5 mg samples of "heavy" and "light" labeled protein preparations were normalized mixed and digested with trypsin. The peptide sample was injected into Agilent 1100 HPLC system and separated either using 4.6 mm x 150 mm XBridge BEH C_{18}, 3.5 µm particle Reverse Phase Liquid Chromatography (RPLC) column (Waters) or 4.6 mm x 200 mm Polyhydroxyethyl A, 5µm particle Hydrophilic Interaction Chromatography (HILIC) column (PolyLC Inc.). For the HILIC fractionation, the 5 mg peptide sample was dissolved in 2 ml of solvent B (0.1% formic acid in acetonitrile) and the sample was injected in 90% solvent B at a flow rate of 0.1 mL/min. Solvent A consisted of 0.1% formic acid in water. After loading the sample onto the column, it was washed with 90% solvent B for 15 minutes at 0.5 ml/min flow rate. Peptides were eluted in 85% B to 65% B gradient in 40 minutes followed by 65% B to 20% B gradient in 20 minutes at the same flow rate. For the RPLC fractionation, the 5 mg peptide sample was dissolved in 4 mL of solvent A (10mM TMAB in water, pH 8) and the sample was injected in 98% solvent A at a flow rate of 0.5 ml/min. After loading the sample onto the column, it was washed with 98% solvent A for 10 minutes at 1 ml/min flow rate. Peptides were eluted over 98% A to 60% A gradient in 60 minutes at 0.5 ml/min flow rate (solvent B used for elution was 10mM TMAB in acetonitrile, pH 8). For each separation, twenty fractions were collected. For the two sets of RPLC fractions, phosphopeptide enrichment was carried out with

PolyMAC-Zr and PolyMAC-Ti, whereas the phosphopeptides in HILIC fractions were enriched only with PolyMAC-Zr.

LTQ-orbitrap analysis

Peptide samples were redissolved in 10 µl of 0.25% formic acid and injected into the Eksigent nano LC Ultra 2D system. The reverse phase C18 was performed using an in-house C-18 capillary column packed with 5 µm C18 Magic bead resin (Michrom; 75 µm i.d. and 12 cm of bed length). The mobile phase buffer consisted of 0.1% formic acid in ultra-pure water with the elution buffer of 0.1% formic acid in 100% CH_3CN run over a shallow linear gradient (from 2% CH_3CN to 35% CH_3CN) over 90 min with a flow rate of 300 nl/min. The electrospray ionization emitter tip was generated on the packed column with a laser puller (Model P-2000, Sutter Instrument Co.). The Eksigent Ultra 2D HPLC system was coupled with a hybrid linear ion trap Orbitrap mass spectrometer (LTQ-Orbitrap Velos; Thermo Fisher). The mass spectrometer was operated in the data-dependent mode in which a full scan MS (from m/z 300 – 1700 with the resolution of 30,000 at m/z 400) was followed by 20 MS/MS scans (for SILAC samples - 7 MS/MS scans were used) of the most abundant ions. Ions with charge state of +1 or undetermined charge states were excluded. The mass exclusion time was 90 s.

Database search

The LTQ-Orbitrap raw files were searched directly against human database using SEQUEST or MASCOT on Proteome Discoverer (Version 1.3, Thermo Fisher). Proteome Discoverer created DTA files from raw data files with minimum ion threshold 15 and absolute intensity threshold 50. Peptide precursor mass tolerance was set to 10 ppm, and MS/MS tolerance was set to 0.8 Da.

Search criteria included a static modification of cysteine residues of +57.0214 Da and a variable modification of +15.9949 Da to include potential oxidation of methionine and a modification of +79.996 Da on serine, threonine or tyrosine for the identification of phosphorylation. Searches were performed with full tryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. False discovery rates (FDR) were set to 1% for each analysis. Proteome discoverer generates a reverse "decoy" database from the same protein database and any peptide passing the initial filtering parameters that were derived from the decoy database is defined as false positive identification. The minimum cross-correlation factor (Xcorr) filter was readjusted for each individual charge state separately in order to optimally meet predominant target FDR of 1% based on the number of random false-positive matches from the reverse "decoy" database. Thus, each dataset had its own passing parameters. The number of unique phosphopeptides and non-phosphopeptides identified were then counted using in-house software and compared. Phosphorylation site localization from CID spectra was determined by PhosphoRS on Proteome Discoverer 1.3. For SILAC experiments, in addition to the above parameters, a dynamic modification of +6.020 Da was added on arginine and lysine. The quantification method was set to SILAC 2plex (Arg6, Lys6) and Light/Heavy ratios were reported.

Data Analysis

A list of all phosphopeptides identified in different fractions was prepared and unique peptides were extracted based on the m/z value and the charge state using in-house software. The phosphosite localizations were obtained from PhosphoRS assignments. If the same peptide sequence appears more than once in this list with different light/heavy ratios, the average ratio

was calculated and assigned for that particular sequence. The mean and the standard deviation were calculated for the non-phosphorylated peptides and the cut off values for phosphopeptides were determined by considering the 2x the standard deviation from this mean. In order to predict upstream kinases for identified phosphosites, an in-house software utilizing the kinase motifs listed on human protein reference database was used.

Pathway Analysis

A list of proteins corresponding to peptides showing significantly increased or decreased phosphorylation was extracted. This list with decreased and increased phosphorylation and the corresponding SILAC ratios were submitted to Ingenuity Pathway Analysis (IPA) (Ingenuity Systems). The IPA criteria were set to include only known human cellular proteins and their direct interactions. In addition, protein functions were predicted using DAVID bioinformatics tool.

Cell Culture and biological reagents for ubiquitination study

DT40 B cell lines were grown to a density of 0.4 x 10^6 cells/mL in RPMI-1640 medium containing 7.5% FBS, 1% chicken serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 50μM 2-ME. For cell stimulation, phorbol 12-myristate-13-acetate (PMA) was used (Sigma-Aldrich). For immunoblotting, anti-Syk (N-19) and anti-ubiquitin (P4D1) were purchased from Santa Cruz Biotechnology. Anti-GAPDH was purchased from Ambion.

Ubiquitination Assay

DG75 cells in RPMI 1640 media were treated with 5 μM MG132 for 3 hours in an incubator at 37^{o}C, washed and resuspended in PBS. The cells were divided into four groups; two were treated with piceatannol at a concentration of 50 μg/mL and the other two were treated with DMSO control by incubating in a 37 ^{o}C water bath for 30 minutes. One set from each treatment group was stimulated with anti-IgM antibody for 15 minutes on ice. Cells were washed with PBS and pellets were prepared. Cell pellets were lysed using a solution containing 1% Noindent P-40, 50mM Tris-Cl pH 8.0, 100mM NaCl, 5mM EDTA, 1mM $NaVO_3$, 10mM NaF, 1x phosphatase inhibitor cocktail (Sigma-Aldrich), and 1x mammalian protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated using SDS-PAGE and protein ubiquitination was measured via Western blot.

Flag-tagged ubiquitin was over-expressed in Syk-EGFP-expressing or Syk-negative DT40 chicken B cells. Cells were then treated with 100ng/mL PMA or DMSO for the indicated times. Cells were lysed using a solution containing 1% Noindent P-40, 50mM Tris-Cl pH 8.0, 100mM NaCl, 5mM EDTA, 1mM $NaVO_3$, 10mM NaF, 20 mM N-ethylmaleimide, 1x phosphatase inhibitor cocktail (Sigma-Aldrich) , and 1x mammalian protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated using SDS-PAGE and protein ubiquitination was measured via Western blot.

Immunoblotting

For immunoblotting, membranes were blocked in 5% goat serum for 1hr. All primary antibodies were incubated for 1 hour at room temperature and visualized using an HRP-conjugated secondary antibody and ECL reagents.

LC-MS analysis of ubiquitination

DG75 cells were cultured, treated with MG132 and divided into two groups. One group was treated with piceatannol as detailed above and the other group was treated with DMSO control. The protein content was extracted and 40 mg portions were digested as detailed before; chloroacetamide was used in place of ioadoacetamide. Ubiquitinated peptides were enriched using UbiScan ubiquitin remnant antibody kit (Cell Signaling Technology) according to manufacturer's protocol, and the LC-MS analysis was carried out as before.