Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Sensitive measurement of total protein phosphorylation level in complex protein samples

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1. Materials

All of the reagents for pIMAGO synthesis (except IRDye® 680 NHS ester, obtained from LI-COR), protein dephosphorylation, kinase assay, cell lysis reagents, trypsin, and all standard proteins were obtained from Sigma-Aldrich. Anti-phospho-threonine antibody was obtained from Cell Signaling Technology. Recombinant Syk kinase was obtained from ProQinase. SnakeSkin dialysis tubing and Universal Nuclease for Cell Lysis were obtained from Pierce. Sep-Pak C18 column was obtained from Waters. All polyacrylamide gels, protein ladders, PVDF membranes, and other gel-running supplies were obtained from Invitrogen. The 96-well microplates were obtained from Corning. Cell culture reagents were obtained from Invitrogen. Jurkat cell lysates (control and treated) were obtained from Cell Signaling Technology. HeLa cell lysates (control and treated) were obtained from Abcam. *E. coli* BL-21 strain and MCF7 cell line were generously provided by Dr. Robert Geahlen (Purdue). Yeast cells were generously provided by Dr. Mark Hall (Purdue). Arabidopsis samples were generously provided by Dr. Jian-Kang Zhu (Purdue). Purified Band 3 protein was generously provided by Dr. Phillip Low (Purdue).

2. Experimental

2.1 Synthesis of pIMAGO

IRDye® 680 NHS ester (LI-COR, Lincoln, NE), 1.5 mg (1 μmol) was first dissolved in 100 μL of DMSO and then supplemented with 400 μL of water and 400 μL of 300mM HEPES (pH 7.7). The prepared dye solution was mixed with 70 μL of polyamidoamine dendrimer generation 4 (PAMAM G4; Sigma-Aldrich) solution (~28 μmol amino groups in 0.44 μmol dendrimer) provided as 10% (w/v) in methanol, and stirred overnight at room temperature in the dark to functionalize the dendrimer with the dye (~2 dyes per dendrimer). The solution was further mixed with 0.65 mg of 2-carboxyethyl-phosphonic acid, 3 mg of N-hydroxysuccinimide (dissolved in 30 μl of water), and 30 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and stirred overnight at room temperature in the dark to functionalize the dendrimer with phosphonic acid. The reaction solution was dialyzed against water using SnakeSkin® pleated dialysis tubing (3,500 MWCO, 22 mm dry diameter; Pierce). The reagent solution was transferred to a dark tube and 11 μL of 10 M titanium oxychloride stock in 30% HCl was added. The mixture was incubated for 1 hour with agitation at room temperature to chelate titanium with phosphonic acid groups on the dendrimer. The final solution was again dialyzed overnight against 0.05% HCl to remove any unbound titanium and stored at 4°C for further use.

2.2 Preparations of Cell Lysates

E. coli cells (strain: BL21) were grown in 250 mL of Luria-Bertani (LB) medium under shaking. At OD600 = 1.0, the cells were precipitated by centrifugation (10 min at 5,000 × g), washed, and stored at -80 °C. Cells were lysed in lysis solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1× protease inhibitor cocktail) for 30 min on ice. The cell debris was cleared at 14,000 × g for 10 minutes and the supernatant containing soluble proteins was collected.

Yeast cells were grown in 1 L of Yeast Extract-Peptone-Dextrose (YPD) medium under shaking. At OD600 = 1.0, the cells were precipitated by centrifugation (5 min at 1,000 × g), washed, and stored at -80 °C. Cells were lysed in lysis solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1× protease inhibitor cocktail, 1 mM sodium orthovanadate, 1× phosphatase inhibitor cocktail, 10 mM sodium fluoride) with glass beads under vigorous shaking for 30 min at 4 ^{o}C. The cell debris was cleared at 14,000 × g for 10 minutes and the supernatant containing soluble proteins was collected.

DG-75 cells were cultured in RPMI 1410 medium containing 10% fetal calf serum, 1% sodium pyruvate, 0.5% streptomycin/penicillin, and 0.05% β-mercaptoethanol. MCF7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. For PMA (phorbol-12-myristate-13-acetate) treatment, MCF7 cells were treated with 200 nM PMA for 30 min after overnight serum starvation. Untreated cells were cultured under the same conditions but treated instead with DMSO (dimethyl sulfoxide). DG-75 and MCF7 cells were precipitated by centrifugation (5 min at 2,000 × g), washed, and stored at -80 °C. Cells were lysed in lysis solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1× protease inhibitor cocktail, 1 mM sodium orthovanadate, 1× phosphatase inhibitor cocktail, 10 mM sodium fluoride) for 30 min on ice. The cell debris was cleared at 14,000 × g for 10 minutes and the supernatant containing soluble proteins was collected. For nuclease treatment, DG-75 cell lysates were further incubated with Universal Nuclease for Cell Lysis (Pierce) at room temperature for 30 minutes and cleared again by centrifugation. For protein precipitation, DG-75 cell lysates (150 μL) were mixed with cold acetone (600 μL) and incubated for 1 h at -20 °C. After centrifugation for 10 min at 14,000 × g, the supernatant was discarded and the protein pellet was air dried and then resuspended with 6 M urea and briefly sonicated.

The concentration of cell lysate was measured by the bicinchoninic acid (BCA) assay. For mass spectrometry (MS)-based phosphoproteomic analysis, cell lysates were denatured and reduced in 50 mM trimethylammonium bicarbonate containing 6 M urea and 5 mM dithiothreitol for 30 min at 37 °C. The proteins were further alkylated in 15 mM iodoacetamide for 1 h in the dark at room temperature. The pH was adjusted to 8.0 and the samples were diluted 8 fold and digested with trypsin (at 1:100) overnight at 37 °C. Next day, the samples were desalted with a 100-mg Sep-Pak C18 column (Waters) and vacuum dried. The resulting peptides were then enriched by PolyMAC method to isolate phosphopeptides as described before.[1]

2.3 Preparations of Plant Samples

Arabidopsis thaliana seeds were cultured in liquid medium (half-strength murashige and skoog salts and 1.5 % sucrose) with 24-h light at room temperature with shaking at approximately 30 rpm for 12-day-old (seedling samples) or 28-day-old (mature samples). Seedlings and mature samples were ground in lysis buffer (4% SDS, 0.1 M DTT in 0.1 M Tris-HCl, pH 7.5) and boiled at 95°C for 10 min. After centrifugation at 13,000 × g for 10 min, the supernatant was used for Filter Aided Sample Preparation (FASP) with some modifications.[2] Samples (400 µg) were loaded into a 30,000 MWCO FASP filter and mixed with 200 µl of 8 M urea and then centrifuged at 13,000 × g for 15 min to remove the flow-through. Proteins were alkylated in 30 mM IAA at 37°C for 1 h and centrifuged again at 13,000 × g for 15 min to remove the flow-through. Proteins were washed with 200 µl of 8 M urea in 50 mM ammonium bicarbonate for three times, and digested with trypsin in 50 mM ammonium bicarbonate (at 1:100) overnight at 37°C. Digested peptides were eluted from the filter by 100 µl of 50 mM ammonium bicarbonate and 50 µl of 0.5 M NaCl, sequentially. The filtrate was collected by centrifugation at 13,000 × g for 15 min, and combined, and desalted with a 100-mg Sep-Pak C18 column (Waters) and vacuum dried. The resulting peptides were enriched by PolyMAC method to isolate phosphopeptides as described before.[1]

2.4 LC-MS/MS Analysis

The eluted phosphopeptides were dried and resuspended in 10 µL of 0.5% formic acid and injected into an Eksigent 2D Ultra nanoflow HPLC system. The reverse phase C18 was performed using an in-house C18 capillary column packed with 5 µm C18 Magic beads resin (Michrom; 75 µm i.d. and 30 cm of bed length). The mobile phase buffer consisted of 0.1% formic acid in ultra-pure water with the eluting buffer of 100% CH_3CN run over a shallow linear gradient over 90 min with a flow rate of 300 nL/min. The electrospray ionization emitter tip was generated on the prepacked column with a laser puller (Model P-2000, Sutter Instrument Co.). The Eksigent Ultra HPLC system was coupled online with a high resolution 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* 300-2000 with the resolution of 30,000) was followed by 20 MS/MS scans of the most abundant ions. Ions with charge state of +1 were excluded. The mass exclusion time was 90 s. The LTQ-Orbitrap raw files were searched directly against the corresponding database using a combination of SEQUEST algorithm and MASCOT on Proteome Discoverer (Version 1.4; Thermo Fisher). Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 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 below 1% for each analysis. Proteome Discoverer generates a reverse "decoy" database from the same protein database, and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identification. The minimum cross-correlation factor (Xcorr) filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 1% based on the number of random false positive matches from the reversed "decoy" database. Thus, each data set had its own passing parameters.

2.5 Absolute Quantification of Phosphorus Amount by ICP-MS

Samples were prepared for element analysis by inductively coupled plasma mass spectrometry (ICP-MS) as instructed. Briefly, 10 mg of α-casein was weighed out and dissolved in 9 mL of concentrated nitric acid overnight for complete digestion. The digested sample was serially diluted using nanopure water until the final concentration of nitric acid reached 2% w/v. A series of two-fold dilutions were further prepared using nitric acid (2% w/v). ICP-MS analyses of samples were then performed at the Campus-wide Mass Spectrometry Center (CWMSC), Purdue University, West Lafayette, IN.

3. References

1) A. B. Iliuk, V. A. Martin, B. M. Alicie, R. L. Geahlen and W. A. Tao, *Mol Cell Proteomics*, 2010, **9**, 2162-2172.
2) J. R. Wisniewski, A. Zougman, N. Nagaraj and M. Mann, *Nat Methods*, 2009, **6**, 359-362.

Supplementary Figures

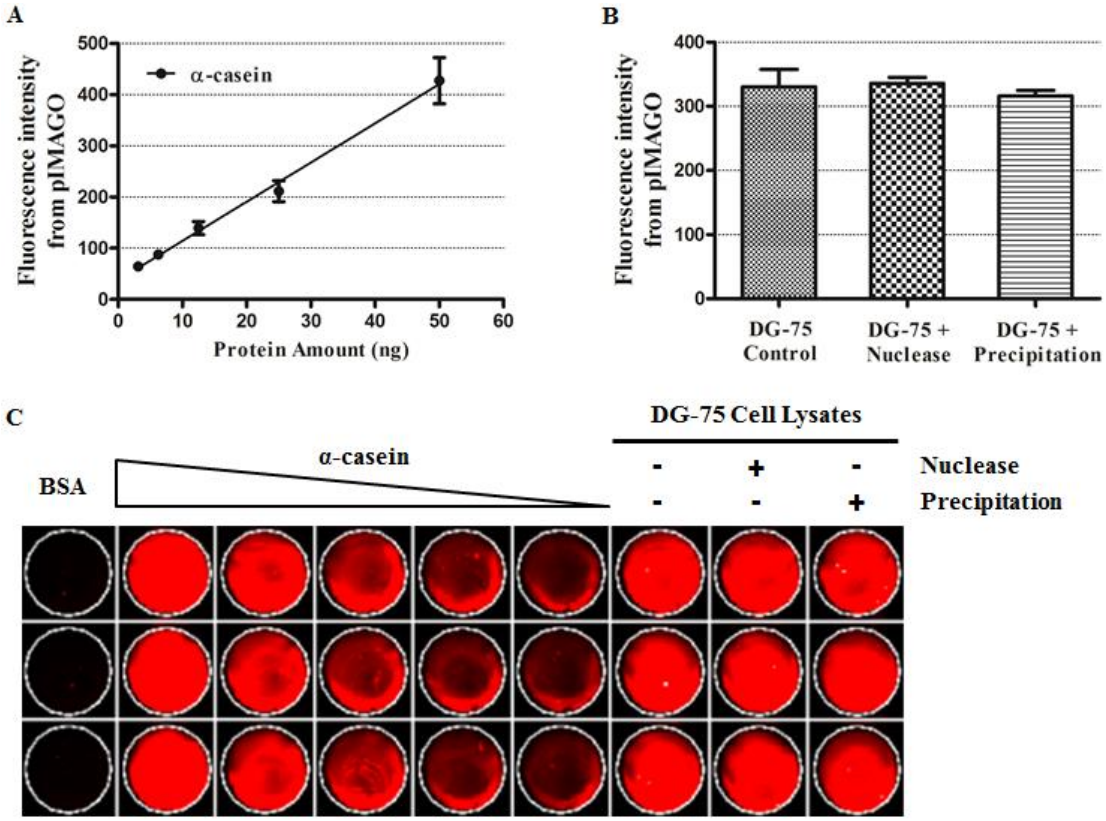

Figure S1. Investigation of potential interferences from other phosphate-containing molecules such as nucleic acids and phospholipids that may remain in cell lysates. Cell lysates of human cell DG-75 were subjected to either nuclease treatment to degrade all forms of DNA and RNA or protein precipitation using acetone to remove both phospholipids and nucleic acids. A) Quantitation of fluorescent signals from pIMAGO-based detection of the phosphoprotein standard α-casein (shown as Mean ± SD). B) Measurement of fluorescent signals from pIMAGO-based detection of DG-75 cell lysates with or without treatments (shown as Mean + SD). C) The corresponding fluorescence image scanned at 700 nm.

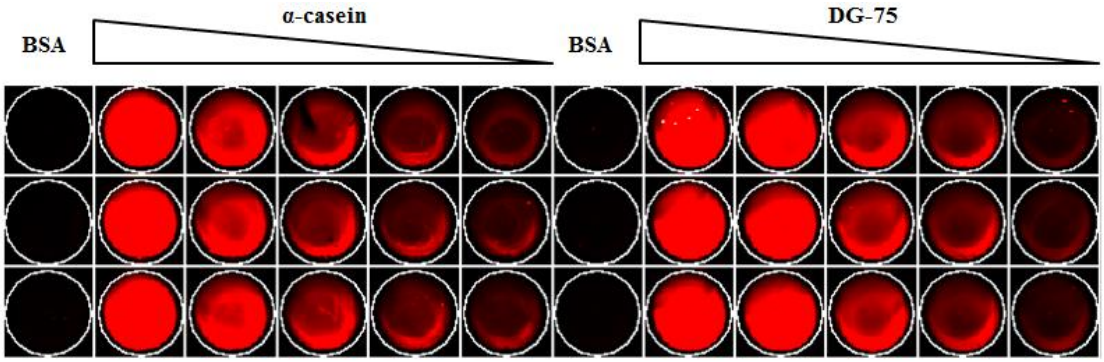

Figure S2. Quantitation of fluorescent signals from pIMAGO-based detection of α-casein and DG-75 cell lysates. Different amounts of α-casein with two-fold serial dilutions ranging from 3 ng to 50 ng and DG-75 cell lysates ranging from 30 ng to 500 ng were immobilized and measured by the pIMAGO phosphorylation assay. The fluorescence image was scanned at 700 nm.

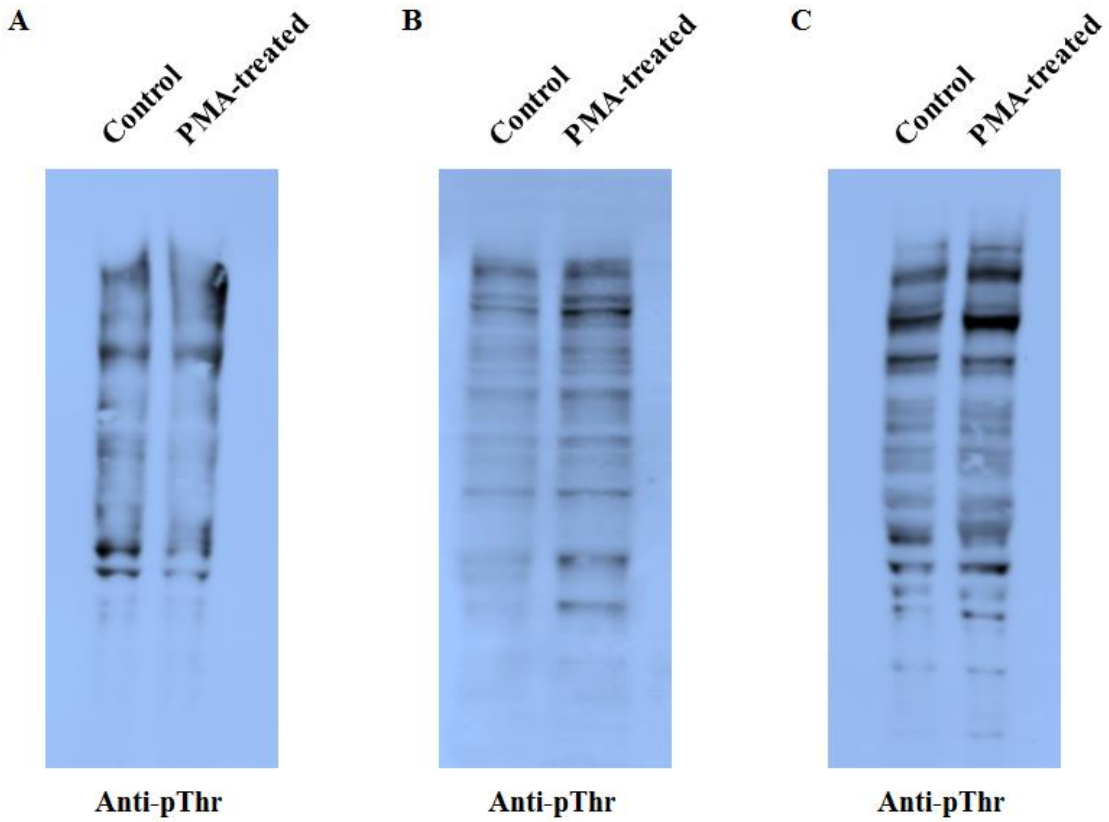

Figure S3. Detection of threonine phosphorylation of control and PMA-treated cells. A) Jurkat cell lysates (control and treated, 15 µL each) from Cell Signaling Technology, B) MCF7 cell lysates (control and treated, 20 µg each), and C) HeLa cell lysates (control and treated, 15 µL each) from Abcam were separated by SDS-PAGE, transferred onto a PVDF membrane, and detected using an anti-phospho-threonine antibody (Cell Signaling Technology).