Combined Enzymatic and Data Mining Approaches for Comprehensive Phosphoproteome Analyses

APPLICATION TO CELL SIGNALING EVENTS OF INTERFERON-γ-STIMULATED MACROPHAGES

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Protein phosphorylation is a central cell signaling event that underlies a broad spectrum of key physiological processes. Advances in affinity chromatography and mass spectrometry are now providing the ability to identify and quantify thousands of phosphorylation sites simultaneously. Comprehensive phosphoproteome analyses present sizable analytical challenges in view of suppression effects of phosphopeptides and the variable quality of MS/MS spectra. This work presents an integrated enzymatic and data mining approach enabling the comprehensive detection of native and putative phosphopeptides following alkaline phosphatase digestion of titanium dioxide (TiO$_2$)-enriched cell extracts. The correlation of retention times of more than 750 phospho- and dephosphopeptide pairs from J774 macrophage cell extracts indicated that removal of the phosphate groups can impart a gain or a loss in hydrophobicity that is partly explained by the formation of a salt bridge with proximal amino groups. Dephosphorylation also led to an average 2-fold increase in MS sensitivity that facilitated peptide sequencing. More importantly, alkaline phosphatase digestion enhanced the overall population of putative phosphopeptides from TiO$_2$-enriched cell extracts providing a unique approach to profile multiphosphorylated cognates that would have remained otherwise undetected. The application of this approach is demonstrated for differential phosphoproteome analyses of mouse macrophages exposed to interferon-γ for 5 min. TiO$_2$ enrichment enabled the identification of 1143 phosphopeptides from 432 different proteins of which 125 phosphopeptides showed a 2-fold change upon interferon-γ exposure. The use of alkaline phosphatase nearly doubled the number of putative phosphopeptides assignments leading to the observation of key interferon-γ signaling events involved in vesicle trafficking, production of reactive oxygen species, and mRNA translation. Molecular & Cellular Proteomics 7: 645–660, 2008.

In biological systems, signal transduction pathways are primarily guided through post-translational modifications (PTMs) of proteins to transmit information from extracellular stimuli into the cytoplasm and nuclei of cells for changes in cytoskeletal structure, vesicle transport, and gene transcription (1). Protein phosphorylation is the most abundant reversible PTM and a major regulator of protein activity or stability. About one-third of proteins encoded by the human genome are assumed to be phosphorylated during their life cycle (2). Phosphorylation can occur on several residues, but it is mostly found on hydroxyamino acids such as serine, threonine, and tyrosine residues with a ratio of about 89:10:1 (3). Because protein phosphorylation holds a central role in signaling networks, considerable effort is attributed to the development of methods for phosphoprotein characterization. Highly sensitive detection techniques are required because phosphoproteins represent only a small proportion of any given cell extracts (typically ~1–2%) (4). Most conventional methods such as two-dimensional gels, $^{32}$P radioactive labeling, and Western blotting have limited features, which do not enable them to comprehensively profile phosphoproteome change or identify the exact phosphorylation site.

To overcome this challenging task, mass spectrometry has emerged as a sensitive, rapid, and effective tool for accurate and specific localization of post-translational modifications such as protein phosphorylation. Nevertheless the negatively charged phosphate group on phosphopeptides renders their MS analysis more difficult than their non-phosphorylated counterpart. For instance, MS/MS spectra of phosphopeptides are less interpretable than their non-phosphorylated counterpart due to the abundant fragment ion corresponding to the loss of the labile phosphate group (80 or 98 Da). Also

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1. The abbreviations used are: PTM, post-translational modification; AP, alkaline phosphatase; DHB, 2,5-dihydroxybenzoic acid; IFN-γ, interferon-γ; ROS, reactive oxygen species; TiO$_2$, titanium dioxide; FA, formic acid; TPCK, l-1-tosylamido-2-phenylethyl chloromethane ketone; IPI, International Protein Index; RT, retention time; cPLA$_2$, cytosolic phospholipase A$_2$; eIF, eukaryotic translation initiation factor; JAK, Janus kinase; STAT, signal transducers and activators of transcription; mTOR, mammalian target of rapamycin; ACN, acetonitrile; BCA, bicinchoninic acid; i.d., inner diameter.
database search engines are better adapted to the correlation of linear sequences compared to peptides with labile functionalities. In positive ion mode, this difficulty is compounded with the under-represented population of phosphopeptide abundance due to suppression effects in an overwhelming large population of non-phosphorylated peptides (2). The coupling of the nano-LC system to the mass spectrometer has been very successful for the separation of phosphopeptides and has led to a decrease in ion suppression (5). However, because multiphosphorylated peptides are very hydrophilic, they may not be retained on the hydrophobic column and will elute in the column flow-through (6). On the other hand, Steen et al. (7) used a small set of synthetic phosphopeptides and their dephosphorylated cognate to indicate that the challenge in phosphopeptide identification is primarily due to the low abundance of this PTM rather than their lower ionization efficiency.

Phosphopeptide enrichment is an indispensable step for the analysis of large scale phosphoproteomics to reduce sample complexity and to enhance their detection. IMAC using Fe(III) or Ga(III) and titanium dioxide (TiO₂) microcolumns are most commonly used as a selective isolation method of phosphopeptides (4). These techniques allow pre-concentrating the sample, retaining phosphopeptides, and removing salts and detergents not compatible with MS (8). Yet one major pitfall is the unspecific binding of acidic residues (glutamic acid and aspartic acid), electron donors (histidine), and hydrophobic peptides (5). To overcome this problem, Ficarro et al. (9) converted all carboxylic groups to the corresponding methyl esters using HCl-saturated, dried methanol before IMAC enrichment. In addition, Larsen et al. (10) demonstrated that 2,5-dihydroxybenzoic acid (DHB) significantly improved phosphopeptide recovery by selectively displacing acidic peptides from the TiO₂ stationary phase. However, depending on the column material, the phosphopeptide populations enriched seem to differ, and the suppression effect of phosphopeptides is still evident in enriched complex samples (11). This raises the question if many more phosphopeptides are still not detected due to their weak ionization efficiency in positive ion mode. Several teams have tried to approach this issue by chemically modifying the phosphate group by β-elimination/Michael addition reactions, which will enhance the level of detected phosphopeptides (12, 13). Yet this method is dependent upon the reaction yield and unwanted side reactions and is only applicable to Ser and Thr residues. The study of tyrosine phosphorylation is mainly achieved by immunoprecipitation with anti-Tyr(P) monoclonal antibodies (14). However, large scale analysis of phosphorytrosine using specific antibodies commercially available can be relatively expensive and would not provide any information on serine and threonine phosphorylation. Another strategy adapted recently identified phosphopeptides in a sample using alkaline phosphatase (AP) treatment applicable to all hydroxyamino acids, even tyrosine residues (15–17). The major drawback from the AP approach is that the information of the phosphorylation site on the peptide is lost.

In the present study, we describe a combined enzymatic and data mining approach to comprehensively identify trace-level phosphopeptides from cytosolic protein extracts of macrophage cells. This strategy is further demonstrated for differential phosphoproteome analyses of macrophages from control and interferon-γ challenge experiments. Several phosphoproteins involved in vesicle trafficking, reactive oxygen species (ROS) production, and mRNA translation were identified as significantly over- or underexpressed upon early macrophage activation. The collection of a large number of phosphopeptides containing even multiphosphorylated species treated with AP enabled a more extensive study on the comparison between phosphopeptides and their dephosphorylated cognate using in-house bioinformatics tools. The higher intensity and the appearance of new putative phosphorylated peptides confirmed the reduced ionization efficiency of phosphopeptides in positive ion mode. The correlation of retention time between phosphopeptides and their dephosphorylated counterparts also provided a meaningful approach to determine changes in hydrophobicity upon dephosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—ACN was purchased from Fisher Scientific. Formic acid (FA), ammonium acetate, and ammonium bicarbonate were obtained from EM Science (Mississauga, Ontario, Canada). Ammonium hydroxide, TFA, acetic anhydride, α-casein, DTT, iodoacetamide, imidazole, sucrose, urea, and acetic acid were purchased from Sigma-Aldrich. [Tyr(PO₄H₂)₄]-Angiotensin II (human) was obtained from Calbiochem. BCA protein assay, immobilized TPCK-treated trypsin, bond breaker tris(2-carboxyethyl)phosphine solution, and the phosphopeptide isolation kit One SwellGel® gallium disc/column were purchased from Pierce. Fetal bovine serum, penicillin-streptomycin, L-glutamine, and high glucose Dulbecco’s modified Eagle’s medium were purchased from HyClone (Perbio, Naigene).

**Preparation of Standard Proteins**—A solution of eight reduced and iodoacetamide-alkylated protein standards (40 fmol/µl each) was prepared from individual digests of bovine serum albumin, rabbit phospholambase b, yeast alcohol dehydrogenase, bovine deoxynibonuclease, horseradish peroxidase C1A, bovine glyceraldehyde-3-phosphate dehydrogenase, Escherichia coli β-galactosidase, and bovine carboxypeptidase A (Michrom Bioresources, Auburn, CA). α-Casein was digested with immobilized trypsin in 50 mM ammonium bicarbonate overnight at 37 °C and then evaporated to dryness.

**Cell Cultures**—J774 (murine macrophage cells) were cultured in Dulbecco’s modified Eagle’s medium, pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin) at 37 °C in 5% CO₂ atmosphere. Cells were plated in 100-mm Petri dishes at a density of 20 million cells/dish corresponding to ~330 µg of cytosolic protein extracts. For interferon-γ (IFN-γ) challenge experiments, J774 cell cultures were incubated for 5 min with mouse interferon-γ (PBL Biomedical Laboratories) (100 units/ml).

**Protein Extraction and Immobilized Trypsin Digestion**—Cells were washed with 1× PBS to remove culture medium. Cells were resuspended in 8.5% sucrose (w/v), 3 mM imidazole and broken with a metal Dounce homogenizer. Nuclei were removed by centrifugation at 860 × g for 15 min. Cell lysates were ultracentrifuged at 150,000 × g for 30 min to discard membrane proteins. Cytosolic proteins in the
supernatant were precipitated by acetone. Proteins were resuspended in 1% SDS, 50 mM ammonium bicarbonate for phosphopeptide TiO₂ enrichment or in 8 M urea, 50 mM ammonium bicarbonate for IMAC Ga⁺ microcolumn phosphopeptide enrichment. Proteins were reduced in 0.5 mM tris(2-carboxyethyl)phosphine for 20 min at 37 °C and then alkylated in 50 mM iodoacetamide for 20 min at 37 °C. 50 mM DTT was added to the protein solution to react with the excess iodoacetamide. Total protein amount was quantitated by BCA protein assay. Proteins were digested in 1 M urea or 0.1% SDS, 50 mM ammonium bicarbonate with immobilized TPCK-treated trypsin (250 μl of trypsin/mg of sample) overnight at 37 °C and high agitation speed. The digest mixture was acidified with TFA and then evaporated to dryness.

**Phosphopeptide Isolation**—Two different affinity-based methods (IMAC Ga⁺ and TiO₂ microcolumns) for phosphopeptide enrichment were used in this study. For the IMAC isolation protocol, J774 cell digests were resuspended in 5% FA and incubated for 20 min at room temperature in a SwellGel gallium disc/column. IMAC beads were washed with 0.1% FA (two times), 30% DHB, 0.07% FA (two times), and finally water. Lastly phosphorylated peptides were eluted with 2 × 25 μl of 0.1% ammonium acetate, pH 9.6. For the TiO₂ procedure, sample loading, washing, and elution were performed by applying gas pressure from a nitrogen tank to the TiO₂ microtots. Each microcolumn was used once to avoid contamination. In-house TiO₂ (5 μm, GL Sciences, Tokyo, Japan) microcolumns were equilibrated with 10 μl of 3% TFA, 70% ACN. The digest mixture was redissolved in 350 mg/ml DHB (Aldrich), 3% TFA, 70% ACN. Unless otherwise indicated, 250 μg (50 μl) of protein digests (equivalent to 15 × 10⁶ cells) were loaded onto a 1.25-mg TiO₂ microcolumn. The column was washed first with 10 μl of 350 mg/ml DHB, 3% TFA, 70% ACN and then two times with 30 μl of 3% TFA, 70% ACN. The bound phosphopeptides were eluted with 30 μl (or 50 μl for the IMAC comparison experiment) of 1% ammonium hydroxide, pH 10. The eluate was acidified with 1 μl of TFA, and 20 μl of the enriched sample was injected on the LTQ-Orbitrap mass spectrometer. Unless otherwise indicated, samples were prepared in triplicate for each condition under study.

**Alkaline Phosphatase Treatment**—The reaction consisted of incubating half of the sample for 2 h at 37 °C at high agitation speed with 20 units of calf intestine AP (New England Biolabs) per 175 μg of protein in basic conditions. For the analysis of standard proteins, 175 μg of digested α-casein and 10 pmol of eight known non-phosphorylated proteins with 0.1 mM PMSF were resuspended in 50 mM ammonium bicarbonate and then treated with 20 units of AP. For the TiO₂-enriched J774 phosphopeptides, half of the eluate (15 μl) was treated with 20 units (2 μl) of AP, and 2 μl of 1% ammonium hydroxide, pH 10, was added to the other half of TiO₂ eluate. After dephosphorylation, treated and non-treated samples were acidified with 1 μl of TFA and injected on the LTQ-Orbitrap mass spectrometer. For N-acetylation of TiO₂-enriched α-casein phosphopeptides, dried samples were initially resuspended in 40 μl of 50 mM ammonium bicarbonate and then derivatized with 100 μl of acetylation reagent (anhydrous MeOH:acetic anhydride, 3:1). After a 30-min derivatization period at room temperature, samples were evaporated on a Speed-Vac concentrator prior to AP digestion.

**LC-MS/MS Analysis**—LC-MS/MS analyses were performed on an LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron, San Jose, CA) coupled to a nano-flow LC system (Eksigent, Dublin, CA) with a Spark-Holland autosampler (Thermo Electron). The analytical column (10-cm length; i.d., 150 μm) and trap column (4-mm length; i.d.; 360 μm) were packed in-house with C18 (3 μm and 300 Å) material (Naigene, Phenomenex, Torrance, CA). Peptides were eluted from the reverse-phase column into the mass spectrometer using a gradient from 2 to 25% B over 83 min followed by a gradient from 25 to 40% over 15 min with a flow rate of 600 nl/min (A: 0.2% FA in water, B: 0.2% FA in ACN). MS spectra were acquired in FTMS positive ion mode for 1 s from m/z 400 to m/z 1600 with a resolution of 60,000. Mass calibration used Calmix (caffeine, MRFA tetrapeptide, and Ultramark) and typically provided mass accuracy within 15 ppm on average for all nano-LC-MS/MS experiments without using a lock mass. The nanoelectrospray voltage and MS/MS fragmentation amplifier voltage were set to 1.6 and 1.3 V, respectively. The mass spectrometer operated in data-dependent mode where a survey scan is initiated in full scan at high mass accuracy in the Orbitrap followed by tandem MS in the linear ion trap for the three most abundant precursor ions. A dynamic exclusion window was applied to prevent MS/MS analysis of previous selected ions 80 s after its acquisition. Only multiply charged ions with an intensity above 10,000 counts were selected for MS/MS sequencing. Tandem MS analysis operated in a total time of 1 s from one-third of precursor ion m/z to m/z 2000.

**Database Searching with Mass Spectrometry Data**—Data were analyzed with Xcalibur (version 2.0 SR1) software, and peak lists were generated using Mascot distiller (version 2.1.1, Matrix Science, London, UK) software and LCQ_plus_zoom script. Database searches were performed against an IPI mouse database containing 52,326 entries (18) (version 3.24, released November 2006) using Mascot (version 2.1, Matrix Science) selecting mouse species for J774 cell extract analysis. Data of α-casein and eight non-phosphorylated proteins were searched against an in-house compiled database where the sequences were taken from the UniProtKB/Swiss-Prot database (release 9.0, October 31, 2006). Parent ion and fragment ion mass tolerances were set at ≤0.05 and 0.5 Da, respectively. One missed cleavage was allowed for tryptin digestion and phosphorylation (STY), oxidation (Met), deamidation (NQ), and carbamidomethylation (Cys) were selected as variable modifications. All protein identifications and IPI entry numbers were reported for peptide sequences matching more than one protein entry. A Mascot search against a concatenated target/decoy database consisting of a combined forward and reverse version of the IPI mouse database was performed to establish a cutoff score threshold for a false-positive rate of less than 2% (p < 0.02) (19). The location of the phosphorylation site was based on the highest ranking assignment with a Mascot score ≥5 to the next leading candidate. All possible phosphorylation sites were reported if the Mascot scores could not provide sufficient discrimination among the leading candidates.

**Peptide Detection and Clustering**—Raw data files (.raw) generated from the LTQ-Orbitrap acquisition software were converted into text files representing all ions according to their corresponding m/z values, retention time, peak widths, intensity, and charge state using in-house peptide detection software.² Intensity values above a user-defined intensity threshold (1,000 counts) were considered. Segmentation analyses were performed across different sample sets using hierarchical clustering with criteria based on their respective m/z, charge, and time within user-defined tolerance (±0.05 m/z and ±1 min). Normalization of retention time is then performed on the initial peptide cluster list using a dynamic and nonlinear correction. A moving-average time-window interpolation scheme is used to compute the time shifts for each peptide across the different data sets. For replicate LC-MS injections, this alignment confines the retention time distribution to less than ±0.1 min (±0.3% relative S.D.) on average. The generated unique list of peptide clusters allowed the direct comparison of peptide abundance between samples in different conditions to identify those showing reproducible and statistically meaningful changes in abundance. Also peptide clusters were aligned with the phosphopeptide identification file from Mascot according to set

² G. Jaitly, E. Bonnell, N. Jaitly, K. Eng, C. Pommès, and P. Thibault, submitted manuscript.
m/z and retention time tolerance (±0.05 m/z and ±1 min). In addition, to compare the retention time and abundance of phosphopeptides and their dephosphorylated counterpart, specific algorithms were developed to cluster peptide ions from control and phosphatase-treated samples that displayed changes in retention time up to 5 min and precise mass shifts for one or multiple phosphate groups (HPO$_4^{2-}$; 79.9663 Da). The Matlab script used to generate the phospho- and dephosphopeptide pairs is included as supplemental material.

RESULTS

Evaluation of Phosphopeptide Enrichment Methods—Large scale proteomics analyses of phosphopeptides have been very difficult due to the strong signal suppression taking place in the presence of a high number of non-phosphorylated peptides (2). Phosphate group affinity media such as TiO$_2$ and metal ions (Ga$^{3+}$ and Fe$^{3+}$) are the most analyzed and optimized enrichment methods for the isolation of phosphopeptides (11, 21). In this study, the phosphopeptide enrichment performance of TiO$_2$ microtips introduced by Larsen et al. (10) was compared with the phosphopeptide isolation kit One SwellGel Ga(III) disc/column from Pierce. In total, 679 peptides were identified with a Mascot score above 25 from the TiO$_2$-enriched sample where 76.9% corresponded to phosphopeptides. On the other hand, the Ga$^{3+}$ metal ion extraction method obtained a much lower phosphopeptide enrichment level (37.0%) from the 451 identified peptides (supplemental Fig. S1, a and b, and supplemental Table S1). A large divergence in selectivity toward phosphopeptides and non-phosphorylated peptides was noted using different affinity media and enrichment protocols. Interestingly 70 and 18% of phosphopeptides isolated by the IMAC and TiO$_2$ technique corresponded to multiphosphorylated peptides, respectively. This comparison suggested that TiO$_2$ microcolumns are less likely to retain peptides with multiple phosphate groups but offer a stronger affinity toward singly phosphopeptides. Because TiO$_2$ microtips offered a much higher number and proportion of phosphopeptides than the SwellGel Ga$^{3+}$ discs from Pierce, further experiments requiring enrichment of phosphopeptides were performed using TiO$_2$ microtips.

Subsequently the overall characteristics of TiO$_2$ beads were evaluated to ensure the most optimal phosphopeptide enrichment of J774 macrophage cell extract. First a column capacity assay was performed in triplicate using five different loading amounts of J774 tryptic digest (63, 125, 250, 500, and 1000 μg). The same level of α-casein (0.4 ng/μg of cell extract) and 1 pmol of [Tyr(PPO$_4^{2-}$)]-Angiotensin II were spiked for different sample loading of cell extracts. Upon LC-MS/MS analysis, more than 80% of assigned peptides corresponded to phosphopeptides except for the 63-μg injection of cell extract. Also the highest number of identified phosphopeptides (Mascot score >25) was obtained when loading 250 μg of cell extract onto TiO$_2$ microtips. Similarly expression profiling analysis indicated that 250 μg of cell extract provided the most abundant level of phosphopeptides. Fig. 1a shows that the recovery level of α-casein phosphopeptides such as VPQLEIVPnPSAEER and TVDMEpSTEVFTK (where bold type indicates the site of modification and pS is phosphoserine) reached a maximum at 250 μg and then decreased for larger amounts of cell extracts. The same trend was also observed for phosphopeptides from the J774 digest (supplemental Fig. S2a). In contrast, non-phosphorylated peptides showed a different behavior on TiO$_2$ beads whereby the number of

![Fig. 1. Loading capacity and linear dynamic range of TiO$_2$ enrichment procedure. a, capacity test of 1.25 mg TiO$_2$ microtips using spiked phosphopeptides from J774 cytosolic cell extract (n = 3). Intensity of α-casein (m/z 830.9^{+} VPQLEIVPnPSAEER) and [Tyr(PPO$_4^{2-}$)]-Angiotensin II (m/z 563.7^{+} DRYpVIHPF) phosphopeptide spiked into J774 protein digest versus various amounts of J774 cell extract (63, 125, 250, 500, and 1000 μg) loaded on the microtips. The optimal loading amount of J774 protein digest was 250 μg, b, plot of (log-log) observed versus calculated fold change of TiO$_2$-enriched α-casein amounts (0, 25, 50, 100, 200, 400, and 800 fmol) spiked into a 250-μg J774 protein digest (n = 3). A linear relation was observed for spiked α-casein phosphopeptides (m/z 830.9^{+} VPQLEIVPnPSAEER, m/z 972.4^{+} DRYpVIHPF) oxidation, m/z 770.3^{+} EQLpSTpSEENKK, m/z 741.8^{+} TVDMEpSTEVFTK oxidation, and m/z 976.5^{+} YKVPQLEIVPnPSAEER) with an average relative S.D. of 34.2%. Average observed fold change for each J774 enriched phosphopeptide sample (m/z 633.8^{+} RAGDVLEDPSPK, m/z 644.3^{+} KAApSPpSPQSVR, m/z 645.8^{+} ApSdpGPLpAEAE, m/z 746.4^{+} AGDVLEDPSPKPK, and m/z 608.8^{+} APTQSpSPPPV) showed no change in intensity with an average relative S.D. of 17.9%. Obs., observed; Calc., calculated; cps, counts/s.](image-url)
unspecific binding significantly increased above 250 μg of cell extract (supplemental Fig. S2b). The second standard, [Tyr(PO₃H₂)]²-Angiotensin II (DRV/pYHPF where pY is phospho-tyrosine), which was equally loaded on each microcol-umn, was lost less retained by TiO₂ beads as more protein digest was loaded onto the microtip (Fig. 1a). At 250 μg of J774 cell extract, only ~35% of [Tyr(PO₃H₂)]²-Angiotensin II was lost, whereas ~65% was not retained by the TiO₂ beads when twice the amounts of cell extract was loaded. Hence all experiments performed in this study used 250 μg of cell extract for 1.25-mg TiO₂ microtips because most phosphopeptides are retained on the column, and a higher enrichment level is obtained.

In a separate study, the sensitivity and linearity response of the TiO₂ enrichment procedure was examined by loading α-casein (0, 25, 50, 100, 200, 400, and 800 fmol) spiked into 250 μg of J774 cell extract onto 1.25-mg TiO₂ microtips (n = 3). The α-casein quantity ratio between different spike levels was determined to obtain the calculated fold change, which was then compared with the observed fold change obtained from intensity ratios of α-casein phosphopeptides of the LC-MS experiments. A graph of the observed and calculated fold change was plotted to determine the correlation between the estimated and experimental ratio. The changes in intensity measurements were also compared with five identified phosphopeptides (Mascot scores >25) of different intensities (RAGDVLEDpSPK, KAαSpSpPQSVR, ApSDPGLPAEPPK, AGDVLEDpSPKRPK, and APQTSpPPPVPR) of spike from J774 cell extract. As expected, no significant change in intensity (average fold change ≤1.05) was noted for phosphopeptides present in the original J774 macrophage cell extracts (Fig. 1b). In contrast, a linear recovery for the five most abundant α-casein phosphopeptides (VPQLEIPNpSA-EER, DIPSpEPStEDQAMEDIK, TVDMEpSTEVFTK, EQLpST-pSSEENKK, and YKVpQLEIPNpSAEER) was observed with an average correlation coefficient of r² of 0.988 over 2 orders of magnitude in spike levels. A log-log plot of calculated and observed ratios is shown in Fig. 1b for all pairwise combination of spike levels. As indicated, a linear correlation between observed and calculated ratios was obtained over the spike range examined. Also the TiO₂ isolation technique provides sensitive enrichment of phosphopeptides because α-casein digests spiked in 250 μg of cell extract were still detected at levels as low as 25 fmol (0.6 ng). Overall the linear recovery of spiked α-casein digest provided evidence that even specific phosphopeptides present in complex cell extracts yield a linear response across different abundance levels. Differential phosphoproteome profiling can be performed to monitor abundance changes of enriched phosphopeptides across different cellular conditions.

**Evaluation of Alkaline Phosphatase Treatment**—Many methods have been used in the past to confirm the presence of phosphorylation on a protein of interest (12, 13, 15–17). Dephosphorylation of the phosphopeptide is the most straightforward and specific approach to confirm phosphorylation of the cognate peptide. For instance, comparison of two-dimensional gels with the native protein and the AP-treated sample can specifically determine whether the protein is phosphorylated (22). In this study, we report the application and analytical potentials of this method for large scale proteomics analyses. The availability of enrichment methods providing high proportion of phosphopeptides (~80%) such as TiO₂ microtips facilitates such differential display analyses by maximizing the number of putative assignments. This strategy consisted of treating half of the TiO₂-enriched J774 phosphopeptides with AP for 2 h at 37 °C in basic conditions and identifying all putative phosphorylated peptides using mass spectrometry and in-house bioinformatics tools (Fig. 2).

The purity of the AP was first established from a silver-stained gel of the enzyme preparation that showed a prominent band at 69 kDa for the expected enzyme with low abundance (<1%) of protein contaminants (data not shown). The approach was then validated using a mixture of eight known non-phosphorylated proteins (glutamate dehydrogenase, al-dolase, lactotransferrin, bovine serum albumin, alcohol dehy-drogenase, catalase, glycero kinase, and lactoperoxidase) as a negative control. The non-phosphorylated protein mixture revealed no significant change in abundance when subjected to AP treatment where 97.5% of ion clusters showed less than 1.5-fold change (Fig. 3, a and b). The negative control confirmed that no proteolytic activity was present in the AP mixture other than dephosphorylation. Hence all changes observed following enzyme incubation are primarily attributed to the loss of the phosphate group.

The specificity of this enzyme was also established using α-casein, which served as positive control. Preliminary experiments performed on native α-casein tryptic digest enabled the identification of six singly and doubly phosphorylated peptides. Consistent with previous investigations, peptides comprising a higher number of phosphorylated amino acids including QMEAEpSpSpSpSP ENPpSVEpK and NTMEH-VpSpSpSSESlpSQTpYK were notably absent or of very low abundances (12, 23). In contrast, the analysis of the same non-enriched α-casein tryptic digest following AP treatment revealed all 10 dephosphorylated tryptic peptides including the pentaphosphorylated peptide QMEAEpSpSpSpSpSEEpNPpSVEpK. From the comparison of peptide abundance across non-treated and AP-treated α-casein, 51.7% of peptide clusters corresponded to non-phosphorylated peptides with changes in intensities lower than 1.5-fold change (Fig. 3d). Also the expression profile shows that 11.6% of peptide clusters representing the phosphopeptide population were only present in the control sample. On the other hand, 36.7% of potential dephosphorylated peptides were either observed with greater than 1.5-fold increase in intensity or uniquely detected in the AP-treated samples. The scatter plot of the corresponding analysis is shown in Fig. 3c and illustrates all peptide clusters with similar intensities along the 45° line,
whereas the peptides on the vertical and horizontal lines correspond to all ions only found in the phosphorylated and dephosphorylated forms. In total, 31 \( \alpha \)-casein phosphopeptides with different charges and a tryptic peptide with a missed cleavage site were matched to their dephosphorylated counterpart by correlating changes in retention time (\( \pm 5 \) min) and mass shifts (79.9663 Da). Examples of two \( \alpha \)-casein phosphopeptides (YKVPQLIEVP\( pS \)AEER and TVDM\( pS \)TEVFTK) correlated to their dephosphorylated counterparts through the loss of one phosphate group are provided as supplemental material. However, another phosphopeptide (NTMEHV\( pSpSpSpS \)EESII\( pS \)QETYK) was only detected upon AP treatment probably because it contained four phosphorylation sites (supplemental Fig. S3, a and b). Correlation of phosphopeptides with their dephosphorylated cognates allowed a direct comparison of their intensity and their elution time in LC-MS. Significant variations of retention time and abundance were noted for different pairs of phosphorylated peptides and their non-phosphorylated counterparts. For instance, peptide pair VPQLEIVP\( pS \)AEER/VPQLEIVPNSAEER had a retention time difference of less than 0.5 min and an intensity variation close to 1-fold. In contrast, the elution time for the doubly phosphorylated peptide, DI\( pS \)pSE\( pS \)TEDQAMEDIK, increased by \( \sim 1.5 \) min upon dephosphorylation, a change that was also accompanied by a 2-fold increase in intensity for the corresponding peptide. Comparison of \( \alpha \)-casein phosphopeptides with the AP-treated pepti
de revealed that dephosphorylation enhances ionization efficiency and confers unexpected changes in retention time that cannot be rationalized with simple structural rules. Rationalization of phosphopeptides behavior in LC-MS thus required a pool of phospho- and dephosphopeptides for more meaningful statistical analyses.

**Phosphopeptide Behavior in LC-MS**—Two major limitations attributed to the presence of the negatively charged phosphate group on the peptide are the suppression in ionization efficiency and the decrease in retention time on reverse-phase chromatography (2, 6). To address this problem, Steen et al. (7) have investigated phosphopeptide behavior in LC-MS by selecting a small set of phosphopeptides. In this study, we present a more comprehensive analysis of the retention time and intensity change performed on the large pool of tryptic TiO\textsubscript{2}-enriched J774 phosphopeptides, including several multiphosphorylated peptides, treated with AP. Initially peptide lists obtained for the control and AP-treated samples were clustered together using in-house bioinformatics tools. A total of 14,147 peptide clusters were obtained where 759 (5%) corresponding to unspecific binding of non-phosphorylated peptides on TiO\textsubscript{2} microtips were found in both conditions. To minimize the rate of false-positive identification, common peptides were removed from the list for subsequent correlation of mass (79.9663 Da) and time (\( \pm 5 \) min) shifts between phosphopeptides and their dephosphorylated counterparts. The list of detected phosphopeptides matched with their dephosphorylated counterparts was aligned with the Mascot identification file (Mascot score \( >20 \)) of phosphopeptides using in-house bioinformatics software (supplemental material). Similarly in cases where
only the dephosphorylated peptide was identified, putative phosphopeptides were determined by matching the detected phosphopeptide-dephosphorylated peptide pairs to the Mascot list (Mascot score $>30$) of assigned dephosphorylated peptides containing at least one Ser, Thr, or Tyr residue. In total, 686 unique peptides (764 peptides when considering sequences with multiple phosphorylation sites) were matched to their dephosphorylated counterpart where 234 were putative phosphopeptides with a higher distribution of single phosphorylation on serine residues (Table I). It is noteworthy that we observed 531, 40, and three phosphopeptides corresponding to Ser, Thr, and Tyr phosphorylation sites, a relative distribution that is consistent with that observed by other groups (3). Fig. 4a shows that non-phosphorylated peptides have very similar intensities before and after AP treatment, whereas the phosphopeptides and putative phosphopeptides showed an average fold change of 1.5 and 2.0, respectively. It is noteworthy that dephosphorylated peptides uniquely found after AP treatment but not observed as phosphopeptides (624 identified peptides of which 210 were already known in the literature) were not considered in this comparison. A higher proportion (>60%) of multiphosphorylated (two, three, and four HPO$_3$ groups) peptides have an intensity increase greater than 2-fold upon dephosphorylation compared with the singly phosphorylated peptides (~33%). These results support the proposal that phosphorylation can suppress MS signal in positive ion mode (2).

Our studies also revealed that a large number of phosphopeptides displayed a more hydrophobic character than their dephosphorylated counterpart. No significant change in

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**Fig. 3. Changes in peptide abundance of positive and negative control following AP treatment.** A scatter plot of ion intensity for eight protein digests non-phosphorylated (200 fmol, 1073 peptide clusters) (a) and α-casein digest (200 fmol, 767 peptide clusters) (c) with and without AP treatment is shown. Vertical and horizontal lines indicate peptide ions observed only in control (phosphopeptides) and after AP treatment (dephosphorylated peptides), respectively. An expression plot showing the change in intensity of peptide ions for eight protein digests (200 fmol) (b) and α-casein digest (200 fmol) (d) following AP treatment is shown.
Interestingly, several multiphosphorylated peptides (28\%) eluted much later (>1 min) than their dephosphorylated counterpart, and ~30\% had a smaller retention time (Fig. 4b). The unexpectedly large population of phosphopeptides of greater hydrophobicity than their dephosphorylated counterparts was intriguing and warranted further investigations.

Several peptide characteristics were evaluated to understand the phosphopeptide behavior in LC-MS analysis. We systematically examined physicochemical properties of phosphopeptides such as peptide length, pI, hydrophobicity, charge, polarity, and amino acid composition to correlate structural changes with corresponding shift in retention times. Regrettably none of these properties could be correlated with the observed retention time shift. We next looked at the location of basic and acidic residues and N terminus from the phosphate group within the singly phosphorylated peptide. Only one distinct distribution examined showed that phosphorylated residues proximal to the N terminus typically eluted later than their dephosphorylated counterpart (Fig. 5). Also phosphate groups closer to the C terminus had an elution time difference >1 min upon dephosphorylation. On the other hand, the location of the phosphorylation site for peptides with unchanged retention time upon dephosphorylation showed no particular trend (Fig. 5). One possible explanation for this effect can be attributed to the salt bridge formation between phosphate groups and the basic residue. In this case, the electrostatic interactions can diminish the overall polarity of the phosphopeptide thus conferring a more hydrophobic character compared with its dephosphorylated counterpart where no salt bridge can be formed. To test this proposal, we enriched an α-casein tryptic digest using TiO$_2$ microcolumns, derivatized free amino groups with acetic anhydride, and compared the shift in retention time of the corresponding peptides following AP treatment. The differences in retention times were also compared with those of non-acetylated peptides (supplemental Fig. S4). Without N-acetylation, α-casein phosphopeptides showed positive and negative retention time shifts when compared with the elution of their dephosphorylated counterparts. It is noteworthy that the non-phosphorylated peptide NANEESYGSSSESAEVEEVK and its N-acetylated derivative showed no significant change in retention time following AP treatment irrespective of the number of N-acetylated groups. However, upon AP treatment a positive shift in retention time was observed for the N-acetylated phosphopeptides indicating an increase of the hydrophobicity of the corresponding dephosphorylated peptides. Two exceptions to this were noted for the phosphopeptides DIGSESpTEDQAMEDIK (where pT is phosphothreonine) and FQpSEEQQTEDELQDK where the acetylated derivative could not be detected in positive ion mode presumably due to their overall negative charge. Interestingly these phosphopeptides were also more hydrophobic than their dephosphorylated counterpart. Although these results are consistent with a salt bridge model, secondary structural changes on the peptide
conformation following AP could also influence the extent of retention time shift. Further structural studies on these aspects are currently underway and will be reported separately.

**Application of Alkaline Phosphatase Treatment for Large Scale Phosphoproteomics Analyses**—The AP approach was further evaluated for a more comprehensive characterization of the differentially expressed phosphoproteome following stimulation of J774 mouse macrophage cells by IFN-γ/H9253. Initially control and macrophage cells incubated with IFN-γ/H9253 for 5 min were digested with trypsin, and phosphopeptides were isolated using TiO$_2$ affinity media prior to their LC-MS/MS analyses on a LTQ-Orbitrap mass spectrometer. The reproducibility of three replicates of control and IFN-γ/H9253-treated samples was initially assessed to validate meaningful expression changes of identified phosphopeptides. More than 95% of peptide clusters showed less than 80% intensity variation indicating that peptides with ≥2-fold change in abundance provided statistically meaningful variation. Following peptide clustering analysis, expression profiling was performed to compare the phosphopeptide abundance in two different conditions. All reproducible changes taking place between control and IFN-γ-treated samples were represented on a volcano plot (Fig. 6a). The comparison of the macrophage phosphoproteome with and without IFN-γ indicated that 68% of all 6170 ion clusters showed less than 2-fold change in abundance across biological replicates and conditions ($n = 3$), whereas 12% (763) and 20% (1218) of ion clusters showed more than 2-fold over- and underexpression, respectively.

Also LC-MS/MS analysis with the LTQ-Orbitrap mass spectrometer resulted in 716 and 917 phosphopeptides (Mascot score ≥20, $n = 3$) in the control and IFN-γ-stimulated samples, respectively (Table II). In total, 1143 unique phosphopeptides from 432 different proteins were detected where 125 phosphopeptides showed a 2-fold change upon IFN-γ administration.

To obtain a higher number of phosphopeptides, half of the TiO$_2$-enriched control and IFN-γ-treated (5 min) J774 cell extract were incubated with AP and analyzed on the LTQ-Orbitrap mass spectrometer. Comparison of both volcano plots in Fig. 6, a and b, showed a ~2-fold increase in putative phosphopeptides after AP treatment. In fact, 61.4% (8493) and 32.9% (4558) of peptide clusters showed a 2-fold increase in the AP-treated and non-treated control J774 cell extract, respectively. Similar results were obtained for the AP-treated IFN-γ sample (63.4%, 8439 peptide clusters and 30.9%, 4111 peptide clusters), further supporting the notion that phosphopeptide ions are under-represented in positive ion peptide maps due to suppression effects (2). Upon enzyme treatment, better MS/MS spectral quality was obtained for the dephosphorylated peptides, and a higher score was assigned by Mascot (Figs. 7, c and d, and 8, b and d). In fact, more than 90% of identified phosphopeptides had a significantly higher score following AP treatment. For instance, 65% of dephosphorylated peptides showed an increase of 20 in their corresponding Mascot score compared with the phosphorylated counterpart. Not only the confidence level of iden-
The number of phosphopeptides identified doubled to 1306 and 1569 for control and IFN-γ treatment, respectively (Table II). All identified phosphopeptides in Table III are highlighted as squares in the volcano plot in Fig. 6, a and b. Among phosphoproteins showing statistically meaningful change in abundance was cytosolic phospholipase A2 (cPLA2) (26) (CS-VpSLSNVEAR, Ser(P)-727) where a 2.6-fold increase in abundance was observed upon IFN-γ treatment in the phosphorylated and dephosphorylated form (Fig. 8, a and c). Also IFN-γ treatment induced dephosphorylation of eukaryotic translation initiation factor 4B (eIF4B) (27) (pSLNENLTNK, Ser(P)-445), which is involved in mRNA translation.

Table III indicates that only a small number of phosphoproteins such as cPLA2 and eIF4B showed stoichiometric change in phosphorylation upon IFN-γ treatment, suggesting...
an under-representation of the phosphopeptide population leading to an incomplete characterization of the corresponding signaling pathways. Hence AP treatment was applied to further characterize differentially regulated phosphoproteins in response to IFN-γ stimulation. A volcano plot representing AP-treated control and IFN-γ-stimulated J774 cells showed

**Fig. 7. Identification of phosphopeptide candidates using alkaline phosphatase.** Contour profiles of m/z versus time for phosphopeptide-enriched extract from J774 without (a) and with AP (b) are shown. MS/MS spectra for m/z 895.88**+** (Mascot score 71) (c) and m/z 855.88**+** (Mascot score 121) (d) corresponding to nucleolar protein Nop56 and m/z 886.38**+** (Mascot score 36) (e) corresponding to Tuftelin-interacting protein 11 are shown.

**TABLE III**

Putative phosphopeptides showing differential abundance in 5-min IFN-γ-stimulated J774 cytosol extracts upon AP treatment

The lowercase p beside S/T indicates a detected phosphorylation site, whereas bold type only (without p) indicates a putative site. PGE₂, prostaglandin E₂.

| Putative phosphopeptides | Proteins | Function | Fold change (Non-treated (p values)) | Fold change (AP-treated (p values)) |
|--------------------------|----------|----------|-------------------------------------|------------------------------------|
| CSVpSLSNVEAR             | Cytosolic phospholipase A<sub>2</sub> | ROS production | 2.6 (0.001) | 2.6 (0.002) |
| GVSPQGAMDRE             | Neutrophil cytosol factor 4 (p40<sup>phox</sup>) | ROS production | 4.9 (0.032) | 2.1 (0.027) |
| FSEMMDHGGDEDVLPEVDG-    | Prostaglandin E synthase 3 | Production of PGE<sub>2</sub> | 2.1 (0.027) | 2.9 (0.004) |
| ADDDSQDSDEDEK            |                      | | | |
| HpSGQDVHVVLK             | NSFL1 cofactor p47 | Vesicle trafficking | -2.1 (0.030) | -2.9 (0.004) |
| RHSSLPTESDEIAPOQR        | AP3 complex subunit δ -1 | Vesicle trafficking | 2.1 (0.038) | 3.0 (0.001) |
| QPSIELPSMAMASTK         | Isoform 2 of lymphocyte-specific protein 1 | Vesicle trafficking | 4.8 (0.001) | 7.2 (0.0001) |
| LSSLRASTSK              | 40 S ribosomal protein S6 | mRNA translation | 2.5 (0.029) | 4.3 (0.016) |
| SEEKAGEI                | Putative RNA-binding protein Luc7-like 1 | mRNA translation | 7.2 (0.0001) | 4.3 (0.016) |
| IDASKNEEDEGHSNSSPR      | Heterogeneous nuclear ribonucleoprotein D0 (AUF1) | mRNA translation | 1.8 (0.044) | 2.6 (0.006) |
| KAAPSLTEDR              | Eukaryotic translation initiation factor 4-γ 1 isoform b (eIF4G1) | mRNA translation | 1.8 (0.044) | 2.6 (0.006) |
| pSLNETLNK               | Eukaryotic translation initiation factor 4B (eIF4B) | mRNA translation | -2.7 (0.0002) | -4.5 (0.003) |
| pTPEFLR                 | Elongation factor 1-γ (eEF-1β) isoform 1 | mRNA translation | -2.3 (0.005) | ND<sup>a</sup> |

<sup>a</sup> Not determined.
that 69.7% of all 10,763 peptide clusters were within 2-fold intensity change, whereas 15.5% (1664) and 14.8% (1594) had a decrease and increase in peptide abundance, respectively (Fig. 6b). The high number of newly detected putative phosphopeptides led to the identification of more early signaling and regulatory events including the enhanced phosphorylation of ROS complex members such as p40phox (GVSPQGAIMDR, Ser-161), which was not identified in the previous analysis, and the phosphosite was never before reported in the literature (Table III). Another interesting finding was AP3 complex protein (RHSSLPTEESDEIAPAQR) involved in vesicle formation and was only detected upon AP treatment with a known phosphosite at Ser-760 (28). In addition, the phosphatase approach enabled a more complete characterization of the initiation of mRNA translation mediated by IFN-γ. Changes in phosphorylation of important phosphoproteins such as putative RNA-binding protein Luc7-like 1 (SEEKAGEI) and 40 S ribosomal protein S6 (LSSLRASTS) were also identified further confirming the importance of mRNA translation upon macrophage activation (27). Fig. 8e shows the change in abundance upon IFN-γ treatment of 40 S ribosomal protein S6 following AP treatment only. It is noteworthy that no phosphopeptide containing one, two, or three phosphate groups was detected in any of the control samples examined. In brief, the AP approach enabled a more comprehensive representation of all phosphoproteins involved in the signal transduction pathways of IFN-γ-stimulated macrophages.

**DISCUSSION**

In summary, we report the analytical potentials of a strategy using AP to detect a higher number of phosphopeptides in complex mixtures. The AP approach was validated with a negative and positive control, which confirmed that the enzyme had high selectivity and enabled specific dephosphorylation of a wide range of substrates. In fact, the mixture of non-phosphorylated peptides showed no change in abundance upon enzyme incubation further supporting the notion that AP has no proteolytic activity other than dephosphorylation. Also the high performance of the AP approach, even for multiphosphorylated peptides, was greatly demonstrated through the analysis of the standard phosphoprotein, α-casein.

For a successful application of the AP treatment on large...
scale experiments, enrichment of phosphopeptides is absolutely required to simplify the mixture and obtain enhanced MS detection. The evaluation of two different phosphopeptide isolation procedures using TiO$_2$ and IMAC (Ga$^{3+}$) microcolumns revealed that TiO$_2$ extraction offered a higher number of phosphopeptides and enrichment level than the IMAC procedure. Also as stated by Bodenmiller et al. (11), we confirmed that complementary phosphopeptide populations are obtained with TiO$_2$ and IMAC (Ga$^{3+}$) isolation methods. TiO$_2$ seemed to have higher affinity toward singly phosphorylated peptides, and DHB loading solution definitely helped to displace most non-phosphorylated acidic residues (11). On the other hand, IMAC retained mostly multiphosphorylated peptides but provided a much lower enrichment level (~37%) than TiO$_2$ microtips (~77%). Thingholm et al. (29) also reported this difference in selectivity of both stationary phases (IMAC and TiO$_2$) toward phosphopeptides and further suggested to combine both enrichment methods for optimal recovery. For our analysis, phosphopeptides were isolated using only TiO$_2$ microtips because this method provided the best overall performance. The optimal loading capacity of the TiO$_2$ enrichment procedure was 250 μg of cell extract loaded onto a 1.25-mg TiO$_2$ microtip offering a linear recovery of spiked α-casein ranging from 25 to 800 fmol.

Enrichment of phosphopeptides has been the most straightforward approach to identify phosphopeptides and their corresponding phosphorylation site. Yet it has been demonstrated that some peptides remain undetected in positive ion mode MS possibly due to their multiple phosphorylation. Hence to obtain the most complete coverage of all phosphopeptides in the sample, several groups have recently shown that treating the enriched mixture with AP reveals the presence of a high number of phosphopeptides (15–17, 30). One major drawback of the AP approach is that no information on the location of the phosphorylated site is revealed, a limitation that is even more evident when more than one potential residue is present. However, this approach will still provide insight on potential phosphopeptides that can be further identified by targeted MS/MS analysis. In-source activation can be used to promote the formation of phosphopeptide fragments barring the dehydroalanine or dehydrobutyryne moieties for confirmation by MS/MS. For large scale experiments, AP treatment is still the preferred strategy over β-elimination to directly confirm the presence of the phosphate group on the protein. In fact, phosphatase treatment on a TiO$_2$-enriched J774 mouse macrophage protein digest showed that a large fraction of phosphopeptides were only detected upon dephosphorylation. These results further strengthen the notion that the mass spectrometry analysis of phosphorylated peptides is limited. The identification level almost doubled upon AP treatment confirming that positive ion detection coupled to the reverse-phase LC method is not fully optimized to detect all phosphopeptides. Further understanding of phosphopeptide behavior in LC-MS is required to better predict phosphopeptide detection in positive ion mode. Our study showed that dephosphorylated peptides can vary significantly in abundance following AP treatment with a large proportion (~40%) showing a 2-fold increase in intensity upon dephosphorylation. The occurrence of new peptides upon AP treatment demonstrates that some phosphopeptides cannot be detected by MS in positive ion mode. Likewise Nielsen et al. (31) provided evidence that post-translational modifications such as phosphorylation on hydrophobic peptides yielded a lower ESI signal response. Steen et al. (7) suggested otherwise by showing that most tested phosphopeptides still had similar ionization efficiency compared with their unphosphorylated cognates. Yet their work reflected the behavior of only a few selected synthetic peptides where a small number comprised doubly phosphorylated peptides.

Another interesting observation mentioned by Steen et al. (7) and Ishihama et al. (16) was that some phosphopeptides actually eluted much later than their dephosphorylated cognate. Similarly our study revealed that a high proportion of dephosphorylated peptides were more hydrophilic than their phosphate counterparts. So far, this study is the first one presenting a large list of detected phosphopeptides to rationalize phosphopeptide behavior in LC-MS. Theoretically removal of a phosphate group should lead to an increase in hydrophobicity of the peptide, yet these results show otherwise. Many different parameters (peptide length, pI, hydrophobicity, charge, polarity, and amino acid composition) were studied to probe the reason behind this surprising change in RT upon dephosphorylation. However, from those characteristics, no coherent reasoning was found to completely explain changes in retention times occurring upon dephosphorylation. Hence we looked more closely at the influence of salt bridges on phosphopeptide conformation. Our data suggested that possible intramolecular electrostatic interactions take place between the N terminus and phosphate group rendering the peptide more hydrophilic upon dephosphorylation. Because phosphopeptides are dissolved in acidic conditions of pH 3.0 for LC-MS analysis, the phosphate group is negatively charged because of its lower pK$_a$ of 1–2. Hence salt bridges can occur between phosphate groups and the positively charged N terminus reducing overall charge and rendering the phosphopeptides more hydrophobic. This proposal was also supported from the retention time behavior of α-casein N-acetylated phosphopeptides where an increase in retention time was consistently observed upon AP treatment in contrast to the non-derivatized phosphopeptides. In a similar fashion, a few studies have shown that introduction or removal of a phosphate group completely destroys the stability and conformation of the peptide because salt bridges are disrupted from basic or N-terminal residues (32, 33). Conformational studies of phosphopeptide secondary interactions are presently in progress to further characterize possible effects that will reveal a more complete reasoning of the model.
Subsequently the analytical potentials of the AP strategy were conveyed through the further understanding of a biological model. Over the past decades, the identification of phosphorylation sites has become a primordial need for the full understanding of cell division, cancer therapy, and even signal transduction in inflammatory response. To survive from viral infections, several mechanisms have evolved to recognize and eliminate invading organisms (34). The inflammatory response is commonly initiated by the secretion of IFN-γ by T-cells that activates important antiviral mechanisms such as the JAK-STAT pathway in macrophages. In the present experiment, we did not observe any phosphopeptides associated with the JAK-STAT pathways possibly due to their relatively low abundance in cytosolic extracts and the incompatibility of their physicochemical characteristics with the present LC-MS systems (i.e., high hydrophilicity and m/z values outside of scanning range). However, we identified several phosphoproteins associated with bactericidal activities of macrophages. Indeed many additional downstream substrates are stimulated through phosphorylation and direct the elimination of the microbe by phagocytosis or the release of ROS and hydrolytic enzymes (27). Hence phosphoproteome analysis in stimulated macrophages is essential for a better understanding of the innate immunity to potential pathogens. Pathogen destruction by phagocytosis is an important step in the inflammatory response (35). Upon IFN-γ stimulation, Hefner et al. (36) demonstrated that cPLA₂ is activated by Ser-727 phosphorylation by mitogen- and stress-activated protein kinase 1 (MSK1) involved in the JAK-STAT pathway. Stimulation cPLA₂ hydrolyzes membrane phospholipids from the sn-2 position to release arachidonic acid, which in turn translocates p47-phox to the cell membrane (37). Concomitantly IFN-γ priming of macrophages triggers phosphorylation of p40-phox, p47-phox, and p67-phox, which are translocated from the cytoplasm to the membrane to associate with cytochrome b₅₅₈ and form the NADPH oxidase enzyme complex (35). Hence the phosphorylation change taking place in both pathways is necessary for the translocation of neutrophil cytosolic factors to the cell membrane. The resulting activated complex catalyzes the electron transfer from NADPH to O₂ to generate a burst of toxic superoxide (O₂⁻), which helps in the killing of phagocytosed microorganisms. In our preliminary phosphoproteome analysis using TiO₂ enrichment only, we observed a limited number of kinase substrates such as cPLA₂ (Ser-727) that showed stoichiometric changes in phosphorylation following IFN-γ stimulation. However, a greater number of differentially phosphorylated proteins involved in the production of ROS including p40-phox were detected upon AP treatment.

The advantages of dephosphorylating phosphopeptides also allowed the characterization of another IFN-γ-stimulated pathway covering the formation of vesicles. Vesiculation is generated by five different adaptor proteins, AP1, AP2, AP3, coat protein I (COP1), and coat protein II (COP2). AP complex originates from the Golgi and endosomal membranes (38). Studies have shown that upon phosphorylation, the AP3 complex is recruited by VAMP-2 to form the coat assembly for pseudopod extension and vesicle formation involved in the pathway to lysosomes (39, 40). In our study, the putative phosphopeptide RHSSLPTESSDEIAQPR from AP3 complex protein had an ~2-fold increase in abundance upon macrophage activation. To our knowledge, no study has yet reported the possible direct linkage of IFN-γ priming to the enhanced phosphorylation of AP3 complex and subsequent vesiculation.

In addition, our study allowed further characterization of the mRNA translation signaling pathway mediated by the action of IFN-γ. Cytokine stimulation has the ability to block cell growth and inhibit viral replication. Yet it will also induce mRNA translation and protein synthesis of selective IFN-sensitive genes (41). The regulatory mechanisms of protein synthesis is known to be mediated very quickly (minutes) through changes of phosphorylation state of translational machinery components reflecting the variations observed upon 5-min incubation of IFN-γ with J774 cells (42). So far, an incomplete description of the protein synthesis signaling pathway has been obtained for cells stimulated by IFN-γ (27). Schep et al. (20) demonstrated that eIF4B binds the 5’-cap structure of the mRNA more tightly in its dephosphorylated form. Likewise our analysis revealed a 4.5-fold decrease in phosphorylation of the eLF4B phosphopeptide (pSLNENTLK) upon IFN-γ treatment. In addition, our study detected an increase in phosphorylation for eLF4G (KAApSLTEDR, Ser-1211) upon IFN-γ induction. Earlier reports proposed the implication of eLF4G1 phosphorylation in the mTOR pathway, but no clear functional effect has yet been recognized (42). Also it has been shown previously that the mTOR pathway required phosphatidylinositol 3-kinase activation induced by type II IFN signaling with p70 S6K/pS6 and 4E-BP1 as putative downstream effectors (27). Upon cytokine induction, the p70 S6 kinase is rapidly activated by phosphorylation, which will in turn phosphorylate 40 S ribosomal protein S6 on Ser-235 and Ser-236. During the initiation step, 40 S ribosomal protein S6 is recruited to the mRNA for the identification of the start codon (42). Until now, only the phosphorylation of 40 S ribosomal protein S6 upon IFN-γ was shown to regulate mRNA translation, but the exact function remained unclear (42). After AP treatment, we were able to identify the 40 S ribosomal protein S6 phosphopeptide, LSSLRATSTSk, with three possible phosphorylation sites already known in the literature (Ser-235, Ser-236, and Ser-240) including those responsible for the regulation of mRNA translation. Upon IFN-γ stimulation we identified significant changes in the phosphorylation state of other proteins involved in the protein synthesis machinery such as elongation factor 1-γ (eEF-1B-γ) isofrom 1 and heterogeneous nuclear ribonucleoprotein D0. The direct implication of these phosphoproteins with the inflammatory response has not been reported in the literature.

The AP strategy enabled a more comprehensive study of...
the change in phosphorylation following IFN-γ signaling highlighting known and novel activation pathways in macrophages. Validation of potential phosphorylation sites can be subsequently obtained using independent techniques such as Western blotting and site mutagenesis. We anticipate that the broader application of AP in quantitative phosphoproteome studies of TiO₂-enriched protein extracts will provide a complementary and meaningful analytical strategy to uncover signaling pathways that remain elusive with current methodology.

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