Quantification of Gel-separated Proteins and Their Phosphorylation Sites by LC-MS Using Unlabeled Internal Standards

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Protein phosphorylation plays a critical role in normal cellular function and is often subverted in disease. Although major advances have recently been made in identification and quantitation of protein phosphorylation sites by MS, current methodological limitations still preclude routine, easily usable, and comprehensive quantitative analysis of protein phosphorylation. Here we report a simple LC-MS method to quantify gel-separated proteins and their sites of phosphorylation; in this approach, integrated chromatographic peak areas of peptide analytes from proteins under study are normalized to those of a non-isotopically labeled internal standard protein spiked into the excised gel samples just prior to in-gel digestion. The internal standard intensities correct for differences in enzymatic activities and sample losses that may occur during the processes of in-gel digestion and peptide extraction from the gel pieces. We used this method of peak area measurement with an internal standard to investigate the effects of pervanadate on protein phosphorylation in the WEHI-231 B cell lymphoma cell line and to assess the role of phosphoinositide 3-kinase (PI3K) in these phosphorylation events. Phosphoproteins, isolated from total cell lysates using IMAC or by immunoprecipitation using Tyr(P) antibodies, were analyzed using this method, leading to identification of >400 proteins, several of which were found at higher levels in phosphorylated protein fractions after pervanadate treatment. Pretreatment of cells with the PI3K inhibitor wortmannin reduced the phosphorylation level of certain proteins (e.g. STAT1 and phospholipase Cγ2) while increasing the phosphorylation of several others. Peak area measurement with an internal standard was also used to follow the dynamics of PI3K-dependent and -independent changes in the post-translational modification of both known and novel phospholipase Cγ2 phosphorylation sites. Our results illustrate the capacity of this conceptually simple LC-MS method for quantification of gel-separated proteins and their phosphorylation sites and for quantitative profiling of biological systems. *Molecular & Cellular Proteomics* 4: 1038–1051, 2005.

Reversible covalent modification of proteins is a key molecular mechanism by which membrane receptor activation is converted into intracellular signaling cascades that mediate physiological change. Engagement of ligands with their receptors results in rapid and transient phosphorylation of downstream protein targets, which as a consequence modify their catalytic properties, cellular localization, and/or binding partners. As a consequence, intracellular signaling pathways can control critical aspects of metabolism, cell cycle progression, migration, differentiation, and protein synthesis. Many diseases are a direct consequence of deregulation in these processes (1, 2); these changes may be detectable by quantitative phosphoprotein analysis.

The assessment of the phosphorylation status of specific proteins is commonly performed by immunochemical methods using antisera raised against a peptide containing the site of phosphorylation of the target protein. This approach is restricted to the study of known phosphorylation sites and cannot be used to monitor and discover novel phosphorylated proteins and their sites of phosphorylation. To overcome this problem, quantitative methods to analyze proteins and their sites of modification in a less discriminatory manner are needed.

MS can be used for large scale protein identification (3, 4) and determination of sites of phosphorylation (5–7). Most of these studies, however, cannot be used for quantitative analysis. Although progress in this area is being made, quantitation of proteins and their sites of modification by MS-based methods is still an analytical challenge for the cell biologist.

Quantitative mass spectrometric methods can be classified into those that require labeling of peptides and those that rely on the intrinsic quantitative nature of LC-ESI-MS. For quantitative LC-MS, it is possible to use synthetic isotopically la-
beled phosphorylated and unphosphorylated IS \(^1\) peptides to mimic a peptide derived from the target protein (8). However, this method, termed Aqua, requires the synthesis of internal peptide standards with the same sequence as the target peptide and thus has the same limitations as immunochromatographic methods in that it is a targeted approach. This analytical strategy can also only be used to quantify a small set of proteins at a time.

Another MS-based strategy, involving metabolic labeling of proteins using stable isotopes (the SILAC approach), has been applied to the analysis of proteins phosphorylated on Tyr (9, 10). This method is proving to be of value in unraveling the mechanisms that control signaling pathways in cultured cells. Unfortunately metabolic labeling frequently cannot be applied to primary tissues such as those from patients or animals. SILAC also requires costly and custom based reagents, such as isotopes of at least one common amino acid, and special formulation of cell culture media. An alternative method uses ICAT reagents for the quantification of cysteine-containing proteins extracted from any type of cell type and tissue (11), but this strategy cannot be used as a general method for the quantification of phosphorylated peptides.

To overcome some of the limitations in the approaches discussed above, a novel method (called iTRAQ) has been developed recently in which quantitation is achieved by labeling protein-derived peptides using isobaric reagents. Such labeled peptides produce fragment ions that have four different masses upon fragmentation by collision-induced dissociation, thus allowing the simultaneous analytical comparison of up to four protein samples in a single experiment (12). A potential problem associated with this method is that the labeling reaction is performed after proteolytic digestion of the whole sample without correction for dissimilar protease activities in different reaction vessels.

MS-based quantification approaches that do not depend on protein derivatization or isotopic labeling have also been reported (13–15). These methods have used the information that is inherent in the integrated peak areas that are generated in LC-MS and two-dimensional (2D) LC-MS experiments, which are in principle proportional to protein abundance. Such methods do not require protein labeling are attractive because of their simplicity and affordability and because they do not require the use of chemical reactions that could in principle be a source of experimental variability. However, quantification by LC-MS using peak integration has not been applied to the quantification of proteins separated by gel electrophoresis, one of the most commonly used techniques of protein fractionation. This is most likely due to the fact that sample processing during in-gel digestion and differences in the activities of trypsin in separate reaction vessels can introduce undesirable experimental variation. Here we have addressed such potential shortcomings through the introduction of a protein IS during in-gel digestion and peptide extraction and report that quantification by peak area integration can also be used for the quantification of proteins separated by 1D SDS-PAGE. This is the basis for our strategy to obtain quantitative information by LC-MS that involves first the parallel separation of samples to be analyzed by 1D SDS-PAGE, isolation of gel pieces, and in-gel tryptic digestion during which a protein IS is added. The latter step corrects for differences in enzymatic activities in different reaction vessels and for differences in sample losses and extraction efficiencies that may occur during the in-gel digestion procedure. Thus, by correcting analyte intensity using the IS, our LC-MS method can be used for relative and absolute quantitation of gel-separated proteins and thus to compare protein abundance in related samples.

We used this peak area measurement with an internal standard (PAIS) method in an ongoing study of PI3K signaling and as part of a proteomic approach to investigate the effects of the carcinogenic compound pV on signal transduction through analysis of the phosphoproteome of the WEHI-231 B lymphoma cell line. Vanadium compounds are potent Tyr phosphatase inhibitors and as such activate pathways controlled by Tyr kinases, including the PI3K pathway and its downstream targets such as the Ser/Thr kinases PDK-1 and Akt/PKB (6, 16–21). More than 200 proteins that become phosphorylated upon pV treatment were detected; several of these were found to be sensitive to PI3K inhibition and are therefore candidate proteins in PI3K signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Lysis**—Cell culture reagents were from Invitrogen. The WEHI-231 B cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.05 mM \(\beta\)-mercaptoethanol at 37 °C in a 5% CO\(_2\) atmosphere. For immunoblotting, cells were lysed in Triton X-100 lysis buffer (150 mM NaCl, 1% (w/v) Triton X-100, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4). For IPs, cells were lysed in modified RIPA buffer (150 mM NaCl, 1% (w/v) Nonidet P-40, 0.1% sodium deoxycholate, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4). Both lysis buffers were supplemented with protein and phosphatase inhibitors.

**Measurement of Activation of Akt/PKB**—Cells were stimulated with 500 \(\mu\)M pV for different time points. Wortmannin (WM) was used at 100 nM and added 20 min before the start of pV treatment. Cells were then lysed, and the same amount of protein was loaded in each lane of 1D SDS-polyacrylamide gels. Blots were probed with antibodies (Cell Signaling Technology Inc.) against Akt/PKB-Thr(P)-308, Akt/PKB-Ser(P)-473, or total Akt/PKB and detected by enhanced chemiluminescence.

**Phosphoprotein Enrichment**—Exponentially growing cells (9·10"^7"
cells in 100 ml of culture medium) were either left unstimulated or treated with pV with or without WM as described above followed by lysis and protein quantification. From the time of lysis, all further steps were carried out at 0 or 4 °C. IP using anti-Tyr(P) monoclonal antibodies (clone 4G10, Upstate Biotechnology) covalently coupled to Sepharose was performed essentially as recommended by the manufacturer and as described before (22). Sepharose–protein A beads (Amersham Biosciences) (500 μl of a 50% slurry), prewashed three times in modified RIPA buffer, were added to 14 mg of each protein sample and left tumbling for 6 h. Samples were then centrifuged at 150 × g for 2 min, and the supernatant was transferred to a fresh tube to which 300 μl of a 50% slurry of agarose-conjugated 4G10 anti-Tyr(P) antibodies were added. Samples were left tumbling for another 6 h. Beads were collected by centrifugation as above and washed three times with RIPA buffer, and bound antigens were eluted with 100 μl phenyl phosphate dissolved in RIPA buffer. Phosphoprotein isolation by IMAC was performed in packed phosphoprotein columns as recommended by the manufacturer (Qiagen, Crowne, UK). Eluates from the Tyr(P) IPs or phosphoprotein columns were loaded on a 1D SDS-polyacrylamide gel consisting of a 0.1 × 15 × 3-cm stacking gel over a 10% (0.1 × 15 × 10-cm) separation gel. After separation, proteins were visualized by colloidal Coomassie Brilliant Blue (CBB) staining.

In-gel Digestion—Protein standards and buffer components were from Sigma. In-gel digestion and LC solvents were from Rathbun. Except when indicated, all volumes were adjusted so that solvents covered gel pieces. After excision, gel pieces were washed three times with 50% (v/v) acetonitrile and dried in a SpeedVac following the addition of 10 μl of 25 mM ammonium bicarbonate, pH 8.8. Samples were then left at 50 °C for 45 min after which the DTT solution was aspirated, and 50 μl iodoacetamide in 25 mM ammonium bicarbonate was added. Alkylation was performed for 60 min in the dark. Gel pieces were subsequently washed three times and dried as above. Following addition of reduced and alkylated fetuin (15 μl of a 100 fmol/μl solution), 100 ng of trypsin (dissolved in 25 mM ammonium bicarbonate) was included and incubated at 37 °C for 4 h. Another aliquot of 100 ng of trypsin was added to each sample 4 h later and then incubated overnight at 37 °C. Tryptic peptides were extracted three times from gel pieces using a 5% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile solution. Extracted peptides were dried in a SpeedVac and subsequently dissolved in 15 μl of 0.1% (v/v) formic acid.

Liquid Chromatography-Tandem Mass Spectrometry—LC-MS/MS was performed as described previously (23) by injecting 5 μl of digest in a reversed phase capillary column (dimensions, 75 μm × 150 mm; PepMap, LC Packings) using a nanoflow HPLC system (Ultimate, LC Packings) connected on-line with an ESI Q-TOF mass spectrometer (Micromass). Flow rate was 200 nL/min⁻¹, and separation was performed by gradient elution from 5% solution B to 45% solution B in 90 min followed by an isocratic step at 95% solution B for 10 min. Solution B was 80% (v/v) acetonitrile, 0.1% formic acid; balance solution A was 0.1% formic acid. MS scans were acquired every second, and MS/MS was performed on automatically selected peptide ions, also for 1 s, using the function switching in the software (MassLynx).

Data Analysis—Raw MS/MS data were converted into peak lists using either MassLynx (Waters) or Distiller (Matrix Science). Spectra were smoothed (Savitzky Golay, two channels twice) and centroid at 80% top using the same software. Charge states were calculated by the software, and peaks were deisotoped. Mascot (Version 2.0.02) (24) was used for searching the IP1 mouse data base last updated on November 15, 2004. At the time of searching this data base contained 42,023 sequences. We followed recommended criteria for protein identification when using LC-MS/MS data (25). Proteins were considered to have been identified when at least two peptides matched an entry and the Mascot score was above 40. Correctness of selected identifications (and those derived from one peptide only) was confirmed manually by assigning all the fragment ions in MS/MS spectra to theoretical peptide fragmentations (Protein Prospector (26) was used to obtain theoretical fragment ions). Quantitative data were obtained from protein–derived peptides by inputting their m/z values and retention times into the “Quantify Method” provided with the MassLynx software. This feature of the software automatically obtains extracted ion chromatograms for each of the input m/z values, which together with retention time knowledge are the basis for peak area selection and integration. Integrated peak area values for each protein–derived peptide were divided by the area of the co-eluting IS peptide. In cases where an IS peptide did not co-elute with an analyte peptide, analyte areas were divided by the area of the most proximal eluting IS peptide.

RESULTS

Quantification of Gel-separated Proteins by LC-MS Using a Protein Internal Standard—The first step was to test whether peptide peak areas generated by LC-ESI-MS when normalized to those of a “universal” IS could be used to calculate abundance of proteins separated by SDS-PAGE. Mixtures of BSA and transferrin (TfR), diluted over a 100-fold range, were resolved by 1D SDS-PAGE, visualized by CBB, and subjected to in-gel digestion by trypsin using standard protocols except that just before adding trypsin a protein IS was added to each gel piece. Although in this study we used fetuin (a blood plasma protein) as the IS, any protein not present in the sample to be analyzed could in principle be used. We chose fetuin because we reasoned that being a relatively small protein it may enter pores in the gel pieces in the same way as trypsin during in gel digestion, and it is inexpensive and readily available. Furthermore being a plasma protein it is not expected to be present in cell lysates.

CBB is a commonly used protein stain that is known to produce linear OD signals during scanning with respect to protein amount present in SDS-polyacrylamide gels. Indeed as expected, in this experiment the scanned ODs of the BSA and TfR bands were found to be linear with respect to amount of protein loaded on the gels (Fig. 1a, top and middle panel; only the data for BSA is shown here to illustrate the concept). Similarly, the integrated peak areas of BSA and TfR peptides obtained by LC-ESI-MS (normalized against those of the IS) were also linear with respect to the amounts of BSA and TfR on the gels (Fig. 1A, bottom panel). When the concentrations of the BSA and TfR standards were recalculated using the functions derived from the standard curves, quantitation by our LC-MS approach was found to be comparable to those concentrations obtained using CBB staining (Fig. 1B); indeed they shared similar coefficients of variation (12 and 21%, respectively; Table I). However, LC-MS was found to be more sensitive than CBB in that 13 and 7 ng of BSA loaded on the gel could only be detected by LC-MS with 13 ng of BSA still producing a signal of sufficient intensity to allow accurate quantification.

Signals generated by ESI could possibly be suppressed by...
Fig. 1. Quantitation by LC-MS of proteins separated by 1D SDS-PAGE. 

A, top panel, serial dilutions of BSA and TfR were separated on 10% SDS-polyacrylamide gels and visualized by CBB; middle and bottom panels, standard curves constructed by plotting the amount of BSA in each gel lane against the respective OD reading (middle panel) or against the respective LC-MS intensities of a BSA peptide (sequence, AEFVEVTK) divided by that of a fetuin peptide (sequence, TPIVGQPSIPGGPVR) (bottom panel). 

B, the functions derived from the standard curves in A were used to recalculate the concentration of the protein standards, showing that quantitation by LC-MS correlates to that obtained by CBB. 

C, top panel, aliquots of BSA (500 ng; indicated by arrow) were mixed with the indicated amounts of WEHI-231 cell lysate, separated on a 10% SDS-polyacrylamide gel, and stained by CBB; bottom panel, graph showing that the LC-MS intensities of BSA/IS peptides were not significantly affected by the presence of increasing concentrations of cell lysate. 

D, top panel, the indicated amounts of BSA were mixed with 75 μg of WEHI-231 cell lysate, separated by SDS-PAGE, and stained with CBB; bottom panel, graph showing the correlation of LC-MS intensities of a BSA peptide (sequence, AEFVEVTK) divided by that of a fetuin peptide (sequence, TPIVGQPSIPGGPVR) obtained from the in-gel digestion of the gel slices at which BSA migrated (marked with an arrow).
co-elution of several molecular species from the LC columns, competing at this stage for ionization by protonation before they enter the mass analyzer. To experimentally assess whether this could occur, 500 ng of BSA was mixed with increasing amounts of protein derived from total cell lysates, resolved by SDS-PAGE (Fig. 1C, top panel), and then analyzed by LC-MS. As shown in the results in Fig. 1C (bottom panel), the ionic intensities of BSA/IS were not significantly affected by increasing amounts of added cell lysate. Similarly when standard dilutions of BSA were spiked in a fixed amount of cell lysate (Fig. 1D, top panel), the BSA/IS area ratios still correlated linearly with the amount of BSA loaded in the gel (Fig. 1D, bottom panel). Taken together, these results demonstrate that the quantitative data generated by our LC-MS method are of comparable quality to those obtained from the use of the ODs of CBB-stained gels. Thus, given that signal suppression effects are negligible, we show here that this LC-MS method of quantitation by PAIS can be used to measure the amounts of proteins in complex mixtures.

**Pervanadate Induces Protein Tyr Phosphorylation and the PI3K/Akt Pathway in the WEHI-231 Lymphoma B Cell Line—**pV (oxidized vanadate) is a potent inhibitor of protein Tyr phosphatases, and its addition to the culture medium of the WEHI-231 B cell line resulted in an increase in Tyr phosphorylation in a time- and concentration-dependent manner (Fig. 2A and data not shown). Changes in certain pathways that utilize Tyr(P) signaling can lead to PI3K activation. This was also found to be the case in the WEHI-231 cells as demonstrated by the induction of Thr-308 and Ser-473 phosphorylation of the Ser/Thr protein kinase Akt/PKB, a key downstream target of PI3K, in a manner that was sensitive to the PI3K inhibitor WM (Fig. 2B). These results show that pV, a compound that can indirectly induce Tyr phosphorylation in cells in culture, also induces changes in the phosphorylation on certain Ser and Thr residues; these are the consequence, in part at least, of the activation of the PI3K pathway, which is known to lead to a cascade of phosphorylation events up- and downstream of Akt/PKB by activating several Ser/Thr protein kinases such as PDK-1, p70 S6K, mTOR (mammalian target of rapamycin), and many others (19, 20).

**Analysis of pV-mediated Phosphoprotein Induction by IMAC and LC-MS/MS—**To investigate in a more unbiased manner the protein phosphorylation induced by pV on Tyr, Ser, and Thr residues, we used the PAIS method to quantify proteins present in phosphoprotein fractions isolated from WEHI-231 cells using the relatively nonspecific IMAC reagent in affinity columns. Lysates of untreated or pV-treated cells (with or without the PI3K inhibitor WM) were loaded onto IMAC columns, and the bound proteins were eluted using a low pH washing buffer. The eluted fractions were then analyzed by LC-MS/MS to identify the phosphopeptides present. Table I summarizes the quantitative data obtained by this method, comparing it to that obtained by SDS-PAGE and CBB staining. As shown, the CV values obtained by LC-MS are comparable to those obtained by SDS-PAGE, suggesting that the PAIS method is a reliable tool for quantifying phosphoproteins in complex mixtures.

**TABLE I**

Comparison of quantitative data obtained by CBB and LC-MS methods

| Amount of BSA loaded (ng) | Quantitation by SDS-PAGE (CCB) | Quantitation by LC-MS |
|---------------------------|-------------------------------|----------------------|
|                           | Amount calculated | CV (%) | Amount calculated | CV (%) |
| 6.7                       | ND                | 0.0     | 0.0               | NA     |
| 13.4                      | ND                | 15.7    | 14.5              | 2.2    |
| 33.5                      | 24.6              | 32.8    | 2.2               | 25.9   |
| 67.0                      | 92.7              | 58.5    | 14.4              | 2.2    |
| 134                       | 119               | 150     | 10.9              | 2.2    |
| 201                       | 154               | 159     | 25.9              | 2.2    |
| 268                       | 320               | 313     | 14.4              | 2.2    |
| 536                       | 528               | 520     | 3.0               | 2.2    |
| Average CV (%)            | 20.8              | 12.2    |

**Fig. 2.** Stimulation of Tyr phosphorylation and the PI3K/Akt pathway by pV. Cell extracts from WEHI-231 B cells, treated with 0.5 mM pV for the indicated times, were separated by SDS-PAGE and immunoblotted using anti-Tyr(P) (pTyr) antibodies (A) or antibodies against phosphorylated and unphosphorylated Akt/PKB (B). When indicated, WM (100 nM final concentration) was added 20 min before pV stimulation. pS, Ser(P); pT, Thr(P).
IMAC columns, and the eluted phosphoprotein fractions were resolved by SDS-PAGE (Fig. 3A). Gel lanes were cut into sections of equal molecular weight, and proteins present in the gel pieces were analyzed by PAIS. A total of 371 proteins were identified in the phosphoprotein eluates (Supplemental Table I). Table II shows examples of the quantitative data for a subset of the proteins identified. Treatment with pV did not affect the levels of the majority of these proteins except for a small group of 14, which were 2-fold more abundant in IMAC eluates after pV treatment of cells (Table II). Our interpretation is that these proteins become phosphorylated upon pV treatment.

The CV values for the set of proteins quantified and shown in Table II averaged 19%; in contrast, the average CV for the set of experiments presented in Fig. 1 and Table I was 12%. This difference is most likely due to the fact that to quantify BSA we only used peptides that completely co-eluted with IS peptides, whereas IS peptides did not always co-elute with analyte peptides in the experiments presented in Table II (due to the fact that we used longer gradients for these experiments). It should be noted that our estimated CV values are in the same range as those reported when using the SILAC approach of quantitative MS (9).

Fig. 3 shows an example of the data from this PAIS analysis for gel fractions corresponding to the 140–160-kDa molecular mass range. Eight proteins matching 3 peptide sequences and having Mascot Scores >60 were identified. Elution profiles of peptides derived from some of these proteins are

Fig. 3. Identification of pV-induced phosphorylated proteins in WEHI-231 cells. A, cells were either left unstimulated, stimulated with pV (0.5 mM for 30 min), or treated with WM (100 nM) for 20 min before pV stimulation. Lysates were loaded on IMAC columns, and the phosphoprotein fraction was resolved by the 1D SDS-polyacrylamide gel shown. Gel sections were analyzed by PAIS as described in the text. B, examples of elution profiles of proteins identified in the gel section indicated in A as obtained using extracted ion chromatograms (XIC) of the indicated peptide ion. C, average areas of peptides derived from the proteins identified in the gel section marked in A normalized to those of the IS. dep., dependent; MYB-BP, MYB-binding protein; SMCL, structural maintenance of chromosome 1-like 1 protein.
### TABLE II
Quantification of selected proteins present in phosphoprotein eluates

| IPI accession no. | Protein description | Average level compared with IS | pV | Average(a) | WM | Average(a) | S.D. | n(b) |
|------------------|---------------------|--------------------------------|----|------------|-----|------------|------|------|
| IPI00118899      | α-Actinin 4         | 14.6                           | 5.6| 27.7       | 7.8 | 5          |
| IPI00119663      | MAP kinase 1        | 10.9                           | 2.6| 16.7       | 3.4 | 4          |
| IPI00114965      | STAT1               | 7.9                            | 0.2| 1.7        | 0.2 | 2          |
| IPI00121515      | Activation/proliferation-associated protein 1 | 6.0 | 0.4 | 6.6 | 0.4 | 2 |
| IPI00261239      | Translation initiation factor-2B | 5.7 | ND | 7.7 | ND | 1 |
| IPI00230277      | MAP kinase 3        | 5.7                            | 0.2| 6.0        | 0.5 | 3          |
| IPI00229848      | Phospholipase Cγ2   | 4.0                            | 0.9| 2.5        | 0.7 | 4          |
| IPI00129426      | C-REL proto-oncopogene | 3.4 | 0.4 | 4.3 | 0.7 | 5 |
| IPI00129319      | Riken cDNA          | 3.2                            | 0.4 | 4.3 | 0.7 | 5 |
| IPI00263313      | Translation initiation factor-2B | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00128818      | Pre-mRNA splicing factor helicase | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00169916      | Protein phosphatase regulatory subunit 7 | 1.8 | 0.3 | 2.0 | 0.5 | 5 |
| IPI00229080      | Heat shock protein 84B | 0.9 | 0.2 | 1.1 | 0.1 | 3 |
| IPI00123474      | Nuclear factor-κB-P100 | 1.7 | 0.3 | 2.7 | 0.6 | 4 |
| IPI00137932      | Protein tyrosine kinase, Syk | 1.9 | 0.3 | 2.3 | 0.4 | 3 |
| IPI00122337      | Vigilin             | 1.7                            | 0.5 | 1.5        | 0.2 | 3 |
| IPI00315661      | Heat shock protein 4 | 1.7                            | 0.3 | 2.7        | 0.6 | 4 |
| IPI00123474      | Nuclear factor-κB-P100 | 1.7 | 0.0 | 3.8 | 0.8 | 2 |
| IPI00137932      | Protein tyrosine kinase, Syk | 1.9 | 0.3 | 2.3 | 0.4 | 3 |
| IPI00122337      | Translation initiation factor-2B | 1.8 | 0.8 | 2.7 | 1.3 | 5 |
| IPI00129426      | C-REL proto-oncopogene | 3.4 | 0.4 | 4.3 | 0.7 | 5 |
| IPI00129319      | Riken cDNA          | 3.2                            | 0.4 | 4.3 | 0.7 | 5 |
| IPI00263313      | Translation initiation factor-2B | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00128818      | Pre-mRNA splicing factor helicase | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00169916      | Protein phosphatase regulatory subunit 7 | 1.8 | 0.3 | 2.0 | 0.5 | 5 |
| IPI00229080      | Heat shock protein 84B | 0.9 | 0.2 | 1.1 | 0.1 | 3 |
| IPI00123474      | Nuclear factor-κB-P100 | 1.7 | 0.3 | 2.7 | 0.6 | 4 |
| IPI00137932      | Protein tyrosine kinase, Syk | 1.9 | 0.3 | 2.3 | 0.4 | 3 |
| IPI00122337      | Translation initiation factor-2B | 1.8 | 0.8 | 2.7 | 1.3 | 5 |
| IPI00129426      | C-REL proto-oncopogene | 3.4 | 0.4 | 4.3 | 0.7 | 5 |
| IPI00129319      | Riken cDNA          | 3.2                            | 0.4 | 4.3 | 0.7 | 5 |
| IPI00263313      | Translation initiation factor-2B | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00128818      | Pre-mRNA splicing factor helicase | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00169916      | Protein phosphatase regulatory subunit 7 | 1.8 | 0.3 | 2.0 | 0.5 | 5 |
| IPI00229080      | Heat shock protein 84B | 0.9 | 0.2 | 1.1 | 0.1 | 3 |
| IPI00123474      | Nuclear factor-κB-P100 | 1.7 | 0.3 | 2.7 | 0.6 | 4 |
| IPI00137932      | Protein tyrosine kinase, Syk | 1.9 | 0.3 | 2.3 | 0.4 | 3 |
| IPI00122337      | Translation initiation factor-2B | 1.8 | 0.8 | 2.7 | 1.3 | 5 |
| IPI00129426      | C-REL proto-oncopogene | 3.4 | 0.4 | 4.3 | 0.7 | 5 |
| IPI00129319      | Riken cDNA          | 3.2                            | 0.4 | 4.3 | 0.7 | 5 |
| IPI00263313      | Translation initiation factor-2B | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00128818      | Pre-mRNA splicing factor helicase | 2.7 | 0.7 | 4.4 | 2.3 | 5 |
| IPI00169916      | Protein phosphatase regulatory subunit 7 | 1.8 | 0.3 | 2.0 | 0.5 | 5 |

**Notes:**
- pV: p-value
- WM: Wilcoxon rank sum test
- S.D.: Standard deviation
- n: Number of experiments

**Description:**
- MAP: mitogen-activated protein
- snRNP: small nuclear ribonucleoprotein
- PKC: protein kinase C
- CAD: caspase-activated DNase
- SH3: Src homology 3
- ND: not detectable

**Legend:**
- IS: Internal standard
- IPI: International Protein Index
shown in Fig. 3B. Quantitative data for these eight proteins, obtained from averaging the integrated chromatographic peak areas of all protein-derived peptides normalized to the areas of the internal standard peptides, is shown in Fig. 3C. Whereas the amounts of most proteins in this gel fraction were unaltered by pV treatment, phospholipase C (PLC) and clathrin heavy chain (designated as MKIAA0034 in the IPI data base) were found to be more abundant in the phospho-protein eluates after pV treatment. These observations suggest that these proteins are phosphorylated upon pV treatment, consistent with previously published data on reversible phosphorylation of PLC\textsubscript{2} (27) and clathrin heavy chain (28). The levels of PLC\textsubscript{2} in the phosphoprotein fraction were reduced upon WM treatment, consistent with PLC\textsubscript{2} phosphorylation being, at least partially, dependent on PI3K activation (29).

Several proteins in the other molecular weight fractions were also found to be increased in IMAC eluates upon pV treatment. These data are summarized in Table II, and some detailed examples are shown in Fig. 4. Candidate proteins that become phosphorylated upon pV treatment include STAT1, mitogen-activated protein kinase (MAPK) 1, MAPK3, and inosine-5\textsuperscript{-}monophosphate dehydrogenase, whereas other proteins such as MAPK kinase 2 do not seem to be significantly affected by pV treatment. STAT1 and MAPKs are known to become phosphorylated on Ser, Thr, and Tyr residues (30). Interestingly STAT1 levels in IMAC eluates were sensitive to WM treatment (Fig. 4), suggesting that STAT1 is a candidate protein downstream of PI3K.

Identification of Proteins Phosphorylated at Tyr Residues (and Their Tightly Bound Interacting Partners) upon pV Treatment—The use of IMAC for phosphopeptide enrichment is well documented (5, 31). However, there is only one published report on the use of IMAC application for the enrichment of intact phosphoproteins (32). Therefore, to compare the performance of IMAC with another method for phosphoprotein enrichment and to further investigate the effects of pV on the WEHI-231 B lymphoma cell line, we next immunoprecipitated Tyr\text(P)-containing proteins using anti-Tyr\text(P) antibodies coupled to PAIS analysis of these proteins.

A total of 214 different proteins were identified in anti-Tyr\text(P) IPs, only three of which (heat shock cognate 71-KDa protein, 78-KDa glucose-regulated protein, and chaperone-activity of bc1 complex) were detected in the unstimulated sample. This result is indicative of a low basal level of Tyr\text(P) phosphorylation and an effective induction of Tyr phosphorylation by pV. Comparison of the proteins present in the IMAC phosphoprotein fractions and Tyr\text(P) IPs indicates that in general these two types of affinity chromatography isolate similar classes of proteins with signaling proteins being slightly more represented in the Tyr\text(P) IPs (Fig. 5A). A total of 44 of the proteins enriched in IMAC eluates were also detected in the anti-Tyr\text(P) eluates (Fig. 5B), illustrating the suitability of IMAC chromatography for the isolation of full-length phosphoproteins.

Identification of Phosphorylation Sites—Several phosphorylation sites were detected in members of the B cell receptor signaling pathway (Table III) including novel sites on Bruton’s Ty kinase (Btk), B cell adaptor protein (BCAP), and hematopoietic lineage cell-specific protein (also known as band 3). Btk is known to be phosphorylated on Tyr-223 and Tyr-551 (33, 34). We detected a peptide containing phosphorylated Tyr-551 but did not identify Tyr-223 under our experimental conditions. Instead we sequenced a Btk peptide containing Tyr(P)-315 which is, to the best of our knowledge, a novel site of phosphorylation on Btk. We also found that BCAP is phosphorylated on Tyr-605 and Ser-691, and band 3 protein was found to be phosphorylated on Tyr-103 and Tyr-206 (Table III). Although these proteins are known to be Tyr phosphorylated (35–37), the phosphorylation sites reported here were previously unknown. Examples of MS/MS spectra used for the assignment of these phosphorylation sites are shown in Supplemental Fig. 1.

Other phosphorylation sites that were detected are listed in Table III. For example,\textalpha-actinin was detected in IMAC eluates and Tyr(P) IPs only after pV stimulation in agreement with published work (38, 39) that indicates that this protein is reversibly phosphorylated. Tyr\text(P)\textsubscript{12} phosphorylation of\textalpha-actinin, previously detected upon pV treatment (39), was not identified in our analyses. Instead Tyr-279 was found as a site of phosphorylation in this protein. This Tyr lies in the amino acid sequence context YXXM, the preferred binding motif for the Src homology 2 domains of the regulatory subunit of PI3K (40).

Dynamics of Tyr Phosphorylation in PLC\textsubscript{2}—To assess whether PAIS could be used to monitor the dynamics of protein phosphorylation, PLC\textsubscript{2} was immunoprecipitated at several time points following pV stimulation (with or without prior WM pretreatment), and the phosphorylation at four Tyr sites (Tyr-753, Tyr-759, Tyr-1217, and Tyr-1245) was followed by measuring the appearance of Tyr\text(P)-containing peptides.

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**Table II—continued**

| IPI accession no. | Protein description                        | pV Average level compared with IS\textsuperscript{a} | S.D. | WM Average\textsuperscript{a} | S.D. |
|------------------|-------------------------------------------|-----------------------------------------------------|------|---------------------|------|
| IPI00312116      | Protein tyrosine kinase, BTK               | 0.3                                                  | ND   | 1.0                 | ND   |
| IPI00312101      | SH3-kinase-binding protein 1               | 0.2                                                  | 0.1  | 1.9                 | 0.1  |

\textsuperscript{a} Average level compared with unstimulated sample.

\textsuperscript{b} Number of peptides used for quantitation.
and the disappearance of their unphosphorylated counterparts (Fig. 6). Tyr-753 and Tyr-759 phosphorylation on PLCγ2 have been reported previously to be downstream of PI3K, whereas phosphorylation on Tyr-1217 appears not to depend on PI3K activity (29). We found that only Tyr-753 phosphorylation was slightly sensitive to PI3K inhibition at all time points (Fig. 6), whereas other sites of Tyr phosphorylation were unaltered (Tyr(P)-759 and Tyr(P)-1245) or even enhanced by WM treatment (Tyr(P)-1217). These results are indicative of pV-induced signaling pathways leading to PLCγ2 Tyr phosphorylation in a PI3K-dependent or -independent manner. Our findings on PLCγ2 illustrate an important application for PAIS as an alternative to antibody-based methods for the detection and quantitation of phosphorylated residues. A further advan-

**Fig. 4.** Examples of elution profiles of protein-derived peptides from phosphoprotein eluates. Proteins were identified as described in the legend to Fig. 3. Numbers refer to relative quantitation using peak areas normalized for an IS peptide. MAPKK2, MAPK kinase 2.
tage of LC-MS methods of quantitation, such as PAIS, over immunochemical methods is that the relative amounts of phosphorylated residues are followed by measuring the levels of both phosphorylated and unphosphorylated peptides bearing the site of modification.

**DISCUSSION**

**Quantification of Protein-derived Peptides by LC-MS Using an Internal Protein Standard**—The results presented here indicate that a non-isotopically labeled IS can be used in LC-MS experiments to quantify gel-separated proteins and their phosphorylation sites. This observation is compatible with reports that show that peak areas (without the need for isotopic or any other type of labeling) generated during LC-MS and 2D LC-MS experiments can be used for relative quantitation of proteins present in related cell lysates (15) and subcellular fractions (13). We have also observed previously that mass spectrometric signal intensities from direct LC-MS runs correlate with the quantitative information obtained through the use of the 2D gel electrophoresis and ICAT methods (23).

Here we demonstrate that integrated chromatographic peak areas, when normalized to those of a closely co-eluting IS, can be used for the quantitation of gel-separated proteins. Because quantitation is carried out at the peptide level after proteolytic digestion and because each protein generates several peptides upon proteolysis, the redundancy of quantitative data adds confidence to the differences observed and excludes unlikely artifacts that may occur during LC-MS analysis. Therefore, an important aspect of the PAIS method is that quantitation does not rely on isotopic labeling or derivatization of proteins, thus avoiding potential sources of variability that could in principle occur during the course of these chemical reactions. Another key feature is that the PAIS method can be applied to a broad variety of cell and tissue sources as it does not involve metabolic labeling steps. In addition, it uses off the shelf reagents, is not restricted by custom reagents or culture media, and because of its simplicity can be readily applied in any laboratory with access to LC-MS instrumentation. We have used the PAIS method to identify several proteins phosphorylated upon pV treatment and to assess the role that WM, a PI3K inhibitor, has on some of these phosphorylation events.

**Enrichment and Quantification of Phosphoproteins**—As part of an unbiased strategy to investigate pV-mediated phosphorylation, we used both IMAC and anti-Tyr(P) IPs to obtain phosphoprotein fractions from the WEHI-231 B lymphoma cell line. Several proteins were found in IMAC eluates only after pV stimulation. This illustrates that, in addition to its previously reported use for the isolation of phosphopeptides, IMAC is also an efficient method for enrichment of intact phosphoproteins. Accordingly the relative levels of phosphoproteins in IMAC eluates and anti-Tyr(P) IPs may be proportional to their degree of phosphorylation stoichiometry such that protein amounts in phosphoprotein fractions (e.g. from IMAC or Tyr(P) IPs) can be used to infer their extent of phosphorylation (9). The potential presence of contaminating, non-phosphorylated proteins in IMAC phosphoprotein eluates does not invalidate the method in cases where we define the nature of the phosphorylation and where the levels of specific proteins are increased in the IMAC eluates as a result of pathway activation.

As a result, quantitative MS methods, as the one reported here, can be used for extensive comparative analysis of the relative amounts of proteins in IMAC and anti-Tyr(P) IP phosphoprotein fractions. This is exemplified by our current investigation in which a large number of candidate proteins that become phosphorylated upon pV treatment were detected (Table II, Supplemental Table I, and data not shown). Of those, STAT1 was identified as a protein whose phosphorylation is sensitive to PI3K inhibition (Fig. 4). Although this finding is consistent with published work linking PI3K with STAT signal-
ing pathways (41–43), further studies will be required to investigate the involvement of PI3K on STAT1 regulation in B cells. Other proteins were found at higher levels in IMAC eluates after WM treatment (Table II), indicative of a potential role of PI3K in negative feedback loops similar to that previously shown in dendritic cells treated with WM (44).

A more global analysis of our data indicates that anti-Tyr(P) IPs may be comparatively more effective in enriching for signaling components than IMAC (Fig. 5). IMAC enriches for all phosphoprotein classes, including proteins containing Tyr(P), Thr(P), and Ser(P). The latter two are more common post-translational modifications than Tyr(P), and the abundance of Thr(P)- and Ser(P)-containing proteins may mask the presence of Tyr(P)-containing proteins in IMAC eluates. It is of note that several proteins involved in B cell receptor signaling, such as p85α, BCAP, and B cell linker (known to be phosphorylated on Tyr residues), were only detected in the Tyr(P) IPs. In contrast, MAPKs and other Ser/Thr kinases were detected in IMAC eluates but not in the Tyr(P) IPs, illustrating the complementarity of these two approaches for the isolation of signaling components.

### Table III

| Peptide IPI accession no. | Protein name/description | Position |
|--------------------------|--------------------------|----------|
| DINSLYDVSRI              | Phospholipase Cγ2         | Tyr-753  |
| ROEELNQLFYLTHQNLRI       | Phospholipase Cγ2         | Tyr-753  |
| QEEQNFLFYLTHQNLRI        | Phospholipase Cγ2         | Tyr-753  |
| MYVDPSINIPSMQR           | Enhancer of filamentation 1 | Tyr-117 |
| EFVNVENQLQLYQEK          | Enhancer of filamentation 1 | Tyr-117 |
| LQYPVNSQAQR              | Enhancer of filamentation 1 | Tyr-117 |
| ANPEERGDVYDVLHNPADAK     | Enhancer of filamentation 1 | Tyr-370 |
| RTDAYSQISIHGTLPRI        | P130Cas-binding protein AND34-1 | Tyr-43 |
| LGQFVYSSSESIRI           | BCAP                      | Tyr-605  |
| LRESITR                  | BCAP                      | Ser-691  |
| SAVGHEYAADVEK            | Hematopoietic lineage cell-specific | Tyr-103 |
| GFQGQYGQIK               | Hematopoietic lineage cell-specific | Tyr-206 |
| YTVSVFK                  | Btk                       | Tyr-315  |
| YVLDDEYSSVGSK            | Btk                       | Tyr-551  |
| SATPAPPAEASLPQEPKPR      | Ras-GTPase-activating protein 1 | Ser-225 |
| STSAPADVVARAAGEDLR       | Ras-GTPase-activating BP protein 1 | Ser-229 |
| NTLKENGMQNOQK            | RAN-binding protein 2      | Thr-1108 |
| KSYSNGK                  | Nuclear factor of activated T cells | Ser-257 |
| ARPTTDSDDDYPPRR          | Eukaryotic initiation factor 4B homolog | Tyr-211 |
| ARPTTDSDDDYPPRR          | Eukaryotic initiation factor 4B homolog | Tyr-211 |
| ERPSWR                   | Eukaryotic initiation factor 4B homolog | Ser-406 |
| TGSESSQTGASATSGR         | Eukaryotic initiation factor 4B homolog | Ser-422 |
| DpYDRGYDSR               | Eukaryotic initiation factor 4B homolog | Tyr-266 |
| DpYSRSQDDYR              | Eukaryotic initiation factor 4B homolog | Tyr-316 |
| DKAYSFFGSR               | DEAH box 3, Ddx3           | Ser-229 |
| GRRFDGYGDR               | DEAH box 3, Ddx3           | Tyr-69   |
| GDYDGGGGR                | DEAH box 3, Ddx3           | Tyr-104  |
| SFSEVEEI                 | DEAH box 3, Ddx3           | Tyr-104  |
| YEMFAQTLQQSR             | Valosin-containing protein | Tyr-755  |
| VLAVNQENGLEDYK           | α-Actinin 1                | Tyr-279  |
| YEMFAQTLQQSR             | Transitional ER ATGase     | Tyr-783  |
| FNEENYGK                 | LOC233871 protein          | Tyr-24   |
| LSTPLTDVK                | LOC233871 protein          | Thr-261  |
| LLYEIQNRF                | LOC233871 protein          | Tyr-261  |
| DBGDNYPSVGDY TK          | 8-80295041 Riken cDNA 4930431L18 | Tyr-513 |
| AGCEEDRGLEDDAVEDQSQK     | Similar to KIAA0144 gene product | Tyr-877 |
| SKSMSVDAEDQONR           | Similar to KIAA0144 gene product | Tyr-1471 |
| SOASKTPGYSGAPYWNT        | Similar to KIAA0144 gene product | Tyr-1124 |
| STSTPTSPGPR              | LOC233871 protein          | Ser-439  |
| ESTSWFQDGQAPGVSYQK       | LOC233871 protein          | Tyr-213  |
| KTPQGPSIEYSDQFSLQSTAK    | LOC233871 protein          | Tyr-267  |
| LQDOQYAVLENQK            | LOC233871 protein          | Ser-439  |
| LQDOQYAVLENQK            | LOC233871 protein          | Tyr-267  |
Identification and Quantification of Phosphorylation Sites—
Our studies led to the identification of over 40 phosphorylation
sites, many of which have not been reported before. Using
PLC\textsubscript{γ2} as a paradigm, we further demonstrated the capacity
of PAIS not only for the detection but also for the quantifica-
tion of post-translational modifications (Fig. 6). Watanabe
et al. (27) mapped Tyr(P)-753, Tyr(P)-759, Tyr(P)-1197, and
Tyr(P)-1217 sites on PLC\textsubscript{γ2} by performing an \textit{in vitro} kinase
reaction with Btk using PLC\textsubscript{γ2} as a substrate, whereas Kim
et al. (29) used antibodies against these sites to monitor their
induction quantitatively in cells following stimulation. Like Kim
et al. (29), we detected phosphorylation on all these sites,
apart from Tyr(P)-1197 (Fig. 6), indicating that this Tyr is not
always used as a substrate in intact cells \textit{in vivo} (29). In
addition to the sites mentioned above, we also detected
Tyr(P)-1245 as a PLC\textsubscript{γ2} phosphorylation site. This site has
been reported before (31) but is not as well characterized as
the other PLC\textsubscript{γ2} Tyr(P) sites due to the lack of phosphospec-
sific antibodies. Tyr(P)-1245 was also not detected by Wa-
tanabe et al. (27), indicating that this site is not a substrate of
Btk. In our experiments only Tyr(P)-753 was sensitive to PI3K
inhibition. Thus, this example illustrates that PAIS overcomes
the need for antibodies against phosphorylated residues for
detection and quantitation of their modification. In addition,
PAIS allows the determination of quantitative changes in
phosphorylation stoichiometry twice, namely by measuring
relative amounts of peptides bearing the phosphorylated sites
and the corresponding unphosphorylated species, which is
clearly a further advantage of PAIS over immunochemical
methods for phosphorylation site detection.

It should be noted, however, that not all phosphorylated
residues may be amenable to quantification by mass spec-
trometry. Some residues may be flanked by recognition motifs
for trypsin and other proteases, thus producing peptides of
sizes unsuitable for mass spectrometric detection. More-
over phosphorylated residues may affect protease sub-
strate recognition when this residue is close to a cleavage
site such that the set of proteolytic peptides may be differ-
ent between phosphorylated and unphosphorylated protein
isoforms. These potential problems may be more accentu-
ated when analyzing Ser/Thr sites phosphorylated by ki-
nases such as PKB/Akt whose recognition motif lies close
to Arg/Lys residues. Nevertheless this shortcoming, com-
mon to all quantitative mass spectrometric methods and not
exclusive to our approach, could be addressed by perform-
ing parallel experiments using proteases with different spec-
ificities to obtain complementary sets of protein-derived
peptides.

In conclusion, the PAIS method of quantitative LC-MS al-
lowed identification of several candidate proteins involved in
B cell signaling, illustrating the potential of this approach for
quantitative profiling of signaling pathways. We envisage that
the use of simple and unbiased quantitative mass spectro-
metric methods, as the one presented here, in combination
with affinity techniques for phosphorylated proteins and pep-
tides, such as those used in this study and others performed
at the peptide level reported recently (6, 32), will be funda-
mental to discover new members of signaling pathways, to
follow changes in phosphorylation of signaling proteins upon
agonist stimulation in a quantitative manner, and to assess
the effects that pharmacological inhibitors and gene inactivation may have on signaling pathways.

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