In-depth Analyses of Kinase-dependent Tyrosine Phosphoproteomes Based on Metal Ion-functionalized Soluble Nanoparticles*†

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The ability to obtain in-depth understanding of signaling networks in cells is a key objective of systems biology research. Such ability depends largely on unbiased and reproducible analysis of phosphoproteomes. We present here a novel proteomics tool, polymer-based metal ion affinity capture (PolyMAC), for the highly efficient isolation of phosphopeptides to facilitate comprehensive phosphoproteome analyses. This approach uses polyamidoamine dendrimers multifunctionalized with titanium ions and aldehyde groups to allow the chelation and subsequent isolation of phosphopeptides in a homogeneous environment. Compared with current strategies based on solid phase micro- and nanoparticles, PolyMAC demonstrated outstanding reproducibility, exceptional selectivity, fast chelation times, and high phosphopeptide recovery from complex mixtures. Using the PolyMAC method combined with antibody enrichment, we identified 794 unique sites of tyrosine phosphorylation in malignant breast cancer cells, 514 of which are dependent on the expression of Syk, a protein-tyrosine kinase with unusual properties of a tumor suppressor. The superior sensitivity of PolyMAC allowed us to identify novel components in a variety of major signaling networks, including cell migration and apoptosis. PolyMAC offers a powerful and widely applicable tool for phosphoproteomics and molecular signaling. Molecular & Cellular Proteomics 9:2162–2172, 2010.

Reversible phosphorylation of proteins is a major mechanism for the regulation of multiple cellular processes (1, 2). Mass spectrometry-based phosphoproteomics provides a method for the global analysis of protein phosphorylation and molecular signaling in cells (3, 4). Despite the great progress that has been made over the past few years, the isolation of phosphopeptides and their analysis by mass spectrometry are still a considerable challenge because of the typically low stoichiometry of protein phosphorylation and the resulting low abundance of phosphopeptides. An early step in any phosphoproteome analysis is the isolation of phosphopeptides, preferably with high efficiency, selectivity, sensitivity, and reproducibility. Currently, there are three major strategies for the isolation of phosphopeptides: antibody-based affinity capture, chemical derivatization of phosphoamino acids, and metal ion-based affinity capture. Antibody-based methods are used mainly for the selective isolation of phosphotyrosine-containing proteins or peptides (5–8). Chemical derivatization methods begin with the β-elimination of phosphates from phosphoserine and phosphothreonine (9) or the formation of phosphoramidates by reactions with amines (10) to selectively immobilize phosphopeptides. Metal ion-based affinity capture techniques use immobilized metal affinity chromatography (IMAC) with Fe(III) (11, 12) or Ga(III) (13) and, for the past a few years, more successful metal oxide approaches (i.e. TiO₂ (14, 15) and ZrO₂ (16, 17)) for the selective binding of phosphorylated peptides. Almost all of the current isolation methods are based on solid phase extractions, which, due to the nature of the heterogeneous environment and nonlinear binding dynamics when dealing with extremely low abundance phosphopeptides, can yield inconsistent results from one run to the next, even when using the same protocol.

We introduce here a new reagent and a novel chemical strategy termed polymer-based metal ion affinity capture (PolyMAC)† for the isolation of phosphopeptides with exceptionally high reproducibility, selectivity, and sensitivity. This approach is based on a metal ion-functionalized soluble nanopolymer to chelate phosphopeptides in a homogeneous aqueous environment. We present here the preparation and the characterization of PolyMAC reagents and compare these with existing techniques that use IMAC or TiO₂. To illustrate the utility of this approach for the analysis of complex systems, we further demonstrate that the PolyMAC technology greatly facilitates the characterization of the spleen tyrosine kinase (Syk)-dependent phosphoproteome of malignant breast cancer cells.

† The abbreviations used are: PolyMAC, polymer-based metal ion affinity capture; EGFP, enhanced green fluorescent protein; FDR, false discovery rate; GO, gene ontology; PAMAM, polyamidoamine; Syk, spleen tyrosine kinase; Xcorr, cross-correlation factor; IPA, Ingenuity Pathway Analysis; IP, immunoprecipitation; SCRIB, scribble; Tet, tetracycline.
The onset and development of breast tumors takes place through a series of complex processes that include the suppression of oncoprotective genes and activation of oncogenes (18). Among the tumor suppressors identified in breast cancer is Syk, a protein-tyrosine kinase whose expression is reduced in many breast cancer cells and is completely absent in highly tumorigenic cells (19, 20). Moreover, when re-expressed in malignant breast cancer cells, Syk inhibits cell motility, growth, invasiveness, and tumor formation while promoting cell-cell adhesion (19). Although the role of Syk in signaling through antigen receptors is well characterized, little is known about the effectors and the pathways that it regulates in breast epithelial cells. We applied, therefore, the highly efficient PolyMAC approach to profile the Syk-dependent tyrosine phosphoproteome in invasive breast cancer cells by analyzing sites of tyrosine phosphorylation present before and after the induction of its expression. Among a total of nearly 800 sites of tyrosine phosphorylation, we identified over 500 sites that are dependent on the expression of Syk. These phosphorylated sites are present on enzymes participating in signaling networks that are involved in such processes as cell-cell interactions, cell migration, and apoptosis. Overall, PolyMAC represents a remarkable chemical tool for the in-depth study of complex signaling networks.

**EXPERIMENTAL PROCEDURES**

**Preparation of PolyMAC-Ti Reagent**

Polyamidoamine (PAMAM) dendrimer generation 4.0 solution (200 μl; provided as 10% (w/v) in methanol; Sigma) was dried in a microcentrifuge tube, redissolved in 3 ml of 150 mM MES pH 5.5 buffer, and transferred into a 10-ml round bottom flask with a magnetic stir bar. Then, 6.5 mg of 2-carboxyethylphosphonic acid, 6 mg of glyceraldehyde, 10 mg of N-hydroxysuccinimide, and 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added into the flask and stirred overnight. The solution was dialyzed against water to remove any remaining excess reagents. Next, 40 mg of sodium meta-periodate was added to the solution and incubated for 40 min with agitation at the dark at room temperature. The mixture was dialyzed overnight against water. The reagent solution was transferred into a microcentrifuge tube, and 10 μl of titanium oxchloride stock (Sigma) was added and incubated for 1 h with agitation at room temperature. The solution was finally dialyzed overnight to remove any unbound titania, and the final PolyMAC-Ti product was stored at 4 °C.

**Characterization of PolyMAC-Ti Reagents**

**Preparation of Simple Peptide Mixtures and Isotopic Labeling of Angiotensin II Phosphopeptide—Human angiotensin II peptide (molecular mass, 1045 Da) and its phosphorylated form (molecular mass, 1125 Da) were bought directly from Sigma-Aldrich. The model protein β-lactoglobulin was denatured and reduced in 8 M urea containing 5 mM dithiothreitol for 30 min at 37 °C. The protein was further alkylated with 15 mM iodoacetamide for 1 h in the dark at room temperature. The solution was then diluted to 100 μM in 100 mM triethylammonium carbonate and digested overnight with proteomics grade trypsin (Sigma) at a 1:100 ratio at 37 °C. The resulting peptides were dried down using a SpeedVac concentrator and resuspended in water at 100 pmol/μl concentration.

To measure accurate phosphopeptide recovery, the phosphorylated angiotensin peptide was isotopically labeled with “light” and “heavy” amine-specific reagents (ITAG) developed in our laboratory (2) (see supplemental Fig. S1 for the structure of the isotopic tag). Isolation of Phosphopeptides from Simple Peptide Mixture Using PolyMAC-Ti—The peptide mixture (100 pmol each) was resuspended in 300 μl of loading buffer A (150 mM HEPES, pH 6.7) to which 5 nmol of the PolyMAC-Ti reagent was added. The mixture was incubated for 5 min (for the binding kinetics assay, 1 μl of the solution was taken out after the designated incubation time for MALDI experiments) and transferred into a spin column (Boca Scientific) containing the Affi-Gel Hydrazide beads (Bio-Rad). The column was gently agitated for at least 10 min and then centrifuged at 2,300 × g for 30 s to collect the unbound flow-through. The gel was washed once with 200 μl of loading buffer A, twice with the washing buffer (100 mM acetic acid, 1% trifluoroacetic acid, 80% acetonitrile), and once with water. The phosphopeptide was eluted off the dendrimer by incubation of the gel twice with 100 μl of 40 mM ammonium hydroxide solution for 5 min. The eluates were collected and dried down completely under vacuum.

**Isolation of Phosphopeptides from DG-75 Lysate Using PolyMAC-Ti Reagent**—The enrichment of the complex samples using the PolyMAC-Ti reagent was similar to the above described PolyMAC protocol except for minor changes. First, loading buffer A was replaced with loading buffer B (100 mM glycine acid, 1% trifluoroacetic acid, 50% acetonitrile). Second, only 100 μl of loading buffer B was used (instead of 300 μl as described above) to incubate the peptide mixture with the PolyMAC. Lastly, 200 μl of the capturing buffer (300 mM HEPES, pH 7.7) was added to the mixture immediately before transferring the solution into the spin column with Affi-Gel Hydrazide beads adjusted to a final pH of 6.3.

Phosphopeptide Enrichment Using IMAC and TiO2—IMAC phosphopeptide capture was done using the PHOS-Select Iron beads (Sigma) according to the protocol modified from the manufacturer. Briefly, 50 μl of the PHOS-Select resin slurry was transferred into a spin column and washed twice with water. The peptide mixture was resuspended in 200 μl of IMAC loading buffer (250 mM acetic acid, 30% acetonitrile) and added to the resin, and the mixture was incubated for 1 h. For the binding kinetics step, 1 μl of the solution was taken out after the designated incubation time and spotted on a 9262}

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MALDI plate. The flow-through was collected by centrifugation at 2,300 × g for 30 s. The resin was washed once with 200 μl of IMAC loading buffer for 5 min, twice with washing buffer (250 mM acetic acid, 0.1% TFA, 80% acetonitrile), and one last time with water. Phosphopeptides were eluted twice with 100 μl of 400 mM ammonium hydroxide and dried completely under vacuum.

Phosphopeptide enrichment with TiO2 beads (Glygen) was carried out with some modifications according to the latest protocol (22) in batch mode. Briefly, 5 mg of the TiO2 beads was transferred into a spin column and washed twice with water. The peptide mixture was resuspended in 300 μl of TiO2 loading buffer (1 mM glycolic acid, 5% TFA, 80% acetonitrile) and added to the beads, and the mixture was incubated for 1 h. For the binding kinetics step, 1 μl of the solution was taken out after 30 min of incubation and spotted on a MALDI plate. The flow-through was collected by centrifugation at 2,300 × g for 30 s. The resin was washed once with TiO2 loading buffer, twice with washing buffer (1% TFA, 80% acetonitrile), and again with water. Phosphopeptides were eluted twice with 100 μl of 400 mM ammonium hydroxide and dried completely under vacuum. A similar protocol was used with TiO2 nanoparticles from GL Sciences for comparison.

Isolation of Tyrosine Phosphopeptides from MDA-MB-231 Lysate

MDA-MB-231 wild type (Syk-negative) and TRS (tetracycline (Tet) response system) Syk-inducible cells were prepared, grown, and stimulated as described before (23). Briefly, both cell lines were grown to 50% confluence. Syk-inducible cells were incubated with 1 μg/ml tetracycline for 20 h at 37 °C to induce Syk expression. Both sets of cells were then lightly stimulated with 20 μM sodium pervanadate for 15 min at 4 °C. Cells were collected, washed, and lysed as described above. After BCA assay, the samples were normalized to 5 mg each. The samples were denatured, alkylated, and digested as described above. The pH of the samples was adjusted to 7.4 with 100 mM Tris-HCl buffer, pH 8.0, and 200 μl of the PT66 phosphotyrosine antibody bead slurry (Sigma) was added to the peptide samples. The mixture was incubated overnight at 4 °C with agitation (7). The supernatant was carefully removed, and the beads were washed twice with 500 μl of lysis buffer with no phosphatase inhibitors (10 min each) and twice with water (2 min each). Tyrosine phosphopeptides were eluted by incubating the beads three times with 100 μl of 0.1% TFA with 10 min of vigorous agitation, twice with 100 μl of 0.1% TFA in 50% acetonitrile (10 min each), and twice with 50 μl of 100 mM glycine, pH 2.5 (30 min each with vigorous agitation). All eluates were combined and dried completely in the SpeedVac. The peptide mixture was resuspended in 300 μl of loading buffer A to which 5 nmol of the PolyMAC-Ti reagent was added. The mixture was incubated for 5 min and transferred into a spin column containing the Affi-Gel Hydrazide beads. The column was gently agitated for an additional 10 min and then centrifuged at 2,300 × g for 30 s to collect the unbound flow-through. The gel was washed extensively, and tyrosine phosphopeptides were eluted by incubation of the gel twice with 100 μl of 400 mM ammonium hydroxide solution for 5 min. The eluates were collected and dried down completely under vacuum.

Mass Spectrometry Analyses

MALDI-TOF Analyses—MALDI-TOF single MS analyses were performed on simple peptide mixtures using an ABI 4800 MALDI-TOF/TOF mass spectrometer. The dried peptide samples were resuspended in 100 μl of 0.1% TFA, and 0.35 μl of each sample (∼10 fmol per peptide) was mixed with an equal amount of α-hydroxycinnamic acid matrix (10 mg/ml in 0.1% TFA, 80% acetonitrile) and spotted on a MALDI plate. The laser was set at 4,000 intensity, and data were acquired at 500 pulses per spectrum.

LTQ-Orbitrap Analyses—Peptide samples were redissolved in 8 μl of 0.1% formic acid and injected into an Agilent nanoflow 1100 HPLC system. The reverse phase C18 was performed using an in-house C18 capillary column packed with 5-μm C18 Magic bead resin (Michrom; 75-μm inner diameter and 12-cm bed length) on an 1100 Agilent HPLC system (25). The mobile phase buffer consisted of 0.1% HCOOH in ultrapure water with the eluting buffer of 100% CH3CN run over a shallow linear gradient over 60 min with a flow rate of 0.3 μl/min. The electrospray ionization emitter tip was generated on the precolumned column with a laser puller (Model P-2000, Sutter Instrument Co.). The Agilent 1100 HPLC system was coupled on line with a high resolution hybrid linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode in which a full MS scan (from m/z 300 to 1700 with a resolution of 30,000 at m/z 400) was followed by four MS/MS scans of the most abundant ions. Ions with a charge state of 1+ were excluded. The mass exclusion time was 180 s.

Data Acquisition and Analysis

The LTQ-Orbitrap raw files were searched directly against a Homo sapiens database with no redundant entries (67,250 entries; human International Protein Index (IPI) version 3.64) using the SEQUEST algorithm (26) or Mascot on Proteome Discoverer (version 1.0; Thermo Fisher Scientific). Proteome Discoverer created DTA files from the raw data with a minimum ion threshold of 15 and an absolute intensity threshold of 50. Peptide precursor mass tolerance was set at 5 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included a static modification of cysteine residues of +57.0214 Da; a variable modification of +15.9949 Da to include potential oxidation of methionines; and a modification of +79.996 on serine, threonine, or tyrosine for the identification of phosphorylation. Searches were performed with full tryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. False discovery rates (FDRs) were set for 2% for each analysis. Proteome Discoverer generates a reverse “decoy” database from the same protein database, and any peptides passing the initial filtering parameters that were derived from this decoy database are defined as false positive identification. The minimum cross-correlation factor (Xcorr) filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 2% based on the number of random false positive matches from the reverse decoy database. Thus, each data set had its own passing parameters. The numbers of unique phosphopeptides and non-phosphopeptides identified were then manually counted and compared. Phosphorylation site localization from CID mass spectra3 was determined by SEQUEST Xcorr scores, and only one phosphorylation site was counted using the top scored phosphopeptide for any phosphopeptide with potential ambiguous phosphorylation sites (a ΔXcorr score of 0 in supplemental tables refers to the top ranked identification).

Gene Ontology and Ingenuity Pathway Analysis (IPA)

Cellular location, protein category, functions, and processes were listed for identified proteins in the Syk-induced sample. The data were

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3 The tandem mass spectrum galleries are available free of charge at https://proteomecommons.org/tranche/data-downloader. jsf?fileName=bShq34AeE6WnpqA2UrlU3hUmUr%2Bw6R%2FkMx-HOtlfg159dzKzWknAuX%2F%2Fm%2Bya3zh1Jkwi4qhRARae6Look-PYrB8F8WfWypYAAAAAg4ZQ%3D%3D.
Phosphoproteomics by PolyMAC

Fig. 1. Schematic representation of PolyMAC method. The PolyMAC strategy consists of two reaction steps: a homogeneous chelation between phosphopeptides and the PolyMAC-Ti reagent in the solution phase and a heterogeneous reaction between the PolyMAC reagent and the solid phase support.

Immunoprecipitation and Western Blot Experiments

MDA-MB-231 wild-type and Syk-inducible cell cultures were prepared as described above. Cells were collected and lysed in lysis buffer with protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1× phosphatase inhibitor mixture (Sigma), 10 mM sodium fluoride, 1× Mini Complete protease inhibitor mixture (Roche Applied Science)) for 20 min on ice. Samples were normalized based on protein concentration, and 1–2 mg of total lysate was used for immunoprecipitation (IP) experiments. For phosphotyrosine immunoprecipitation, 2 mg of lysates was incubated with 40 μl of PT66 anti-phosphotyrosine antibody immobilized on agarose bead slurry (Sigma) for 2 h at 4 °C. For protein immunoprecipitation, 1 mg of lysates was preincubated with 20 μl of Protein A/G-agarose beads for 20 min at 4 °C to remove nonspecific binding. Then, the lysates were incubated with 4 μg of the selected antibodies (anti-MAPRE1, anti-SCRIB, anti-TRIP4, or anti-dynactin) for 2 h at 4 °C. Finally, the samples were incubated with 40 μl of Protein A/G-agarose beads for 2 h at 4 °C. The beads were washed, and the bound proteins were eluted off by boiling the beads in 2× SDS loading buffer for 5 min. Lysates (50 μg each) and the IP eluents were run on a 12% SDS gel and transferred onto a PVDF membrane. The membranes were blotted against the corresponding proteins (e.g. MAPRE1, dynactin, SCRIB, or TRIP4). Then, the membranes were stripped and rebotted against Tyr(P) using 4G10 anti-phosphotyrosine antibody. Finally, the membranes were stripped again and rebotted against GAPDH and Syk as controls.

RESULTS

Strategy and Design of PolyMAC Reagents—We devised the PolyMAC agents based on a soluble, globular nanopolymer (i.e. dendrimer) that was multifunctionalized with reactive groups for the site-specific chelation of phosphopeptides and with “handle” groups that facilitate the isolation of immobilized phosphopeptides through a highly efficient bioconjugation (Fig. 1). This design takes advantage of not only the multifunctionalized nanoparticles but more importantly the soluble nature of the molecule, allowing for the chelation of a limited sample of phosphopeptides in the solution phase for optimum efficiency and maximum yield. In a second step, the attached phosphopeptides are captured on a solid support (e.g. agarose or magnetic beads) through highly efficient coupling between two bioconjugation groups on the soluble polymer and on the solid phase, respectively. A high concentration ratio of the reactive group to the handle group facilitates the complete recovery of the bound phosphopeptides while eliminating the need for extra steps to remove excess reagents.

In this study, we first modified the PAMAM dendrimer generation 4.0 with phosphonate groups. Subsequently, Ti(IV) ions were immobilized on the surface through chelation to phosphonate groups. In addition, we also functionalized the dendrimer with aldehyde as the handle group (Fig. 1). The density of titanium ions and aldehyde groups can be easily controlled through the synthesis procedure, and the current PolyMAC-Ti reagent has 35 titanium ions and six aldehyde groups per molecule. PolyMAC-Ti agents bound with phosphopeptides are isolated from the solution phase through the formation of hydrazone from the hydrazine and aldehyde reaction (≤5 min) by coupling to the hydrazide-agarose gel. Primary amine groups on peptides may react with aldehyde groups, but the reaction is reversible (27). Non-phosphopeptides are readily washed away, and the phosphopeptides are recovered using ammonia aqueous solution.

PolyMAC Isolates Phosphopeptides from Standard Peptide Mixtures—The ability of PolyMAC-Ti to enrich phosphopeptides was tested first by incubating the reagent with a mixture of angiotensin II (m/z 1046) and tyrosine-phosphorylated angiotensin II (m/z 1126). For comparison, the same peptide mixture also was incubated with IMAC-Fe(III) resin and TiO2 nanoparticles using the most recent published conditions (22, 28, 29). Recoveries of phosphopeptide were analyzed by matrix-assisted laser desorption ionization (MALDI) time-

exported, and InforSense (InforSense Ltd., London, UK) version 4.1 for gene ontology (GO) annotation was used as a part of Proteome Discoverer version 1.0 software to group components, functions, and processes for proteins identified in the aforementioned experiments. The resulting analyses were exported as xml files.

For pathway analyses, high confidence peptides (<2% FDR) from Syk-negative and Syk-induced samples were manually compared, and phosphotyrosine peptides identified only in the Syk-induced sample were selected. The corresponding proteins were assumed to have increased tyrosine phosphorylation on certain sites due to Syk presence. To identify canonical and metabolic pathways and their protein partners, IPA (Ingenuity Systems) was used for categorizing these proteins and grouping them according to processes, localization, and networking. The IPA criteria were set to include only known human cellular proteins and their interactions. The resulting data files were exported as xml files. Top networks identified were further inspected using NCBI and Swiss-Prot web sites as well as the peer-reviewed literature.
of-flight (TOF)/TOF mass spectrometry (Fig. 2 and see supplemental Fig. S2 for the raw files). The phosphopeptide was captured instantly once the PolyMAC-Ti reagent was added to the peptide mixture as shown by the disappearance from the solution of a peak at \( m/z 1126 \), indicating fast binding kinetics. In contrast, the time required to capture the phosphopeptide from solution was much longer for the solid phase reagents (IMAC and TiO\(_2\) beads). In fact, IMAC was unable to completely deplete the phosphopeptide from the solution in 30 min. Phosphopeptides were then eluted from all three supports, and the original starting amount of non-phosphopeptide (\( m/z 1046 \)) was spiked into the eluate as an internal standard to measure the yield of each technique. By comparing the ratio of intensities of the peaks for the two peptides in the eluate with that in the original sample, we quantified the relative recoveries of phosphopeptide for each procedure. The average yields for recovery of phosphopeptide were close to 25% for IMAC resin, 50% for TiO\(_2\) nanoparticles, and >90% for PolyMAC-Ti (Fig. 2).

To evaluate the PolyMAC-Ti reagent using a more complex system, we measured its ability to recover phosphorylated angiotensin II (\( m/z 1046 \)) and phosphoangiotensin II (\( m/z 1126 \)) from a mixture of the phosphopeptide added at a ratio of 1:100 to a tryptic digest of unphosphorylated \( \beta \)-lactoglobulin (supplemental Fig. S3). The phosphopeptide was hardly detectable by MS because of the high background of non-phosphopeptides in the starting mixture. After enrichment using PolyMAC-Ti, the only detectable peak in the spectrum was the phosphopeptide, showing the excellent selectivity of the method. A similar recovery of phosphopeptide of greater than 90% was observed when a non-phosphorylated peptide was included as an internal standard (data not shown).

**PolyMAC Isolates Phosphopeptides from Complex Cell Lysate**—To demonstrate the utility of PolyMAC for biological studies, we analyzed phosphopeptides isolated from extremely complex samples, the whole cell extracts of the human Burkitt lymphoma B cell line DG-75. A total of 100 \( \mu \)g of lysate was digested with trypsin and desalted using a Sep-Pak C\(_{18}\) column. The digest was incubated with 5 nmol of PolyMAC-Ti reagent for 1 min and then for an additional 5 min with 50 \( \mu \)l of hydrazine-agarose. A parallel analysis using TiO\(_2\) beads (Glygen; results with TiO\(_2\) from GL Sciences are included in the supplemental material) under optimal conditions (22, 30) was carried out for comparative purposes. Glycolic acid (100 mM) was added to the binding solution to improve the selectivity for the isolation of phosphopeptides (22, 29). Phosphopeptides were eluted with ammonia, dried under vacuum, reconstituted in 0.1% formic acid, and analyzed by LC-MS/MS. The experiment was repeated three times, each time using a different cell lysate and a different batch of TiO\(_2\) and PolyMAC-Ti reagents. MS/MS data were analyzed using the SEQUEST software by searching against the human protein database and reverse database to estimate the FDR. In single 90-min gradient LC-MS/MS runs, an average of 877 unique phosphopeptides representing 1003 sites of phosphorylation from 100 \( \mu \)g of total lysate were identified using Poly-
MAC-Ti (Fig. 3 and see supplemental Tables S1–S6 for the full lists). In comparison, an average of 420 unique phosphopeptides representing 498 phosphorylation sites were identified from the same sample using the TiO2 method. The number of identified phosphopeptides is consistent with a recent report using titania stage tips in which an average of 580 phosphopeptides were identified from 150 μg of HeLa cell lysate (31). To increase the confidence in our phosphopeptide identification, we also carried out database searches using Mascot. Using the same FDR cutoff, the database search with both SEQUEST and Mascot resulted in over 85% overlap in phosphopeptide identifications, which is consistent with previous studies on the comparison of different database search algorithms (32, 33). Although phosphopeptide identification has remained a challenge, the results indicate that our phosphopeptide identification is reliable. The detailed results with both Mascot and SEQUEST searches are included in the supplemental material. PolyMAC-Ti had an extremely high selectivity for phosphopeptides of 96 ± 0.6% compared with a selectivity of 77 ± 7.5% for TiO2 beads. Furthermore, the PolyMAC-Ti method exhibited lower variability than the TiO2 method in terms of the selectivity and identity of the recovered phosphopeptides. For three parallel analyses using TiO2 beads, 38.4% of phosphopeptides were identified by all three analyses, and there was an ~50% overlap between every two analyses, again consistent with the recent literature report (31). In contrast, 55.2% of phosphopeptides were identified by all three analyses, and there was an ~70% overlap between every two analyses when using PolyMAC-Ti. To further investigate the reproducibility, we enriched one batch of phosphopeptides, then split the sample equally in halves, and analyzed them by LC-MS/MS. We observed an ~70% overlap in phosphopeptides in the analyses, indicating that the limit of ~70% reproducibility in identifying phosphopeptides might attribute to inherent factors in the LC-MS/MS analysis (data not shown) (32, 33). A more detailed comparison of PolyMAC with TiO2 nanoparticles from different commercial sources is included in supplemental Figs. S4–S8 and Tables S1–S6. The experiments, therefore, demonstrated the remarkable reproducibility of PolyMAC-based enrichment method.

Analysis of Syk-dependent Tyrosine Phosphoproteome in Breast Cancer Cells—Next, we applied the PolyMAC strategy to analyze Syk-dependent signaling in breast cancer cells. A line of highly invasive MDA-MB-231 cells, which normally lack Syk, was generated in which the expression of an enhanced green fluorescent protein (EGFP)-tagged form of the kinase could be induced by treatment with Tet (23). Because sites of tyrosine phosphorylation are much less abundant than sites of serine or threonine phosphorylation, we coupled an immuno-affinity step to the PolyMAC method to enrich for phospho-tyrosine-containing peptides. An overall flow chart for the analysis of Syk-dependent tyrosine phosphorylation is illustrated in supplemental Fig. S9. Although there have been reports that tyrosine phosphopeptides can be analyzed with single stage affinity purification based on anti-Tyr(P) antibodies (7, 34), including recent large scale phosphotyrosine profiling (34, 35), tandem affinity approaches combining anti-Tyr(P) antibodies and metal oxide improve the selectivity and confidence in the identification of tyrosine phosphopeptides (5, 8). We carried out parallel experiments to compare three methods using anti-tyrosine phosphopeptide PT66 alone, PT66 + TiO2, and PT66 + PolyMAC purifications. From 750 μg of DG-75 extract in three equal portions (250 μg for each

Fig. 3. Analyses of phosphopeptides from digests of DG-75 B cell lysates using PolyMAC-Ti and TiO2. A, number (average and S.D.) of phosphopeptides identified using PolyMAC-Ti or TiO2 in three separate analyses. B, percentage (average and S.D.) of the total identified peptides that were phosphopeptides in three trials. The error bars indicate the standard deviation of three analyses. C and D, Venn diagram illustrating the overlap in the sets of phosphopeptides identified using PolyMAC-Ti and TiO2.

MAC-Ti (Fig. 3 and see supplemental Tables S1–S6 for the full lists). In comparison, an average of 420 unique phosphopeptides representing 498 phosphorylation sites were identified from the same sample using the TiO2 method. The number of identified phosphopeptides is consistent with a recent report using titania stage tips in which an average of 580 phosphopeptides were identified from 150 μg of HeLa cell lysate (31). To increase the confidence in our phosphopeptide identification, we also carried out database searches using Mascot. Using the same FDR cutoff, the database search with both SEQUEST and Mascot resulted in over 85% overlap in phosphopeptide identifications, which is consistent with previous studies on the comparison of different database search algorithms (32, 33). Although phosphopeptide identification has remained a challenge, the results indicate that our phosphopeptide identification is reliable. The detailed results with both Mascot and SEQUEST searches are included in the supplemental material. PolyMAC-Ti had an extremely high selectivity for phosphopeptides of 96 ± 0.6% compared with a selectivity of 77 ± 7.5% for TiO2 beads. Furthermore, the PolyMAC-Ti method exhibited lower variability than the TiO2 method in terms of the selectivity and identity of the recovered phosphopeptides. For three parallel analyses using TiO2 beads, 38.4% of phosphopeptides were identified by all three analyses, and there was an ~50% overlap between every two analyses, again consistent with the recent literature report (31). In contrast, 55.2% of phosphopeptides were identified by all three analyses, and there was an ~70% overlap between every two analyses when using PolyMAC-Ti. To further investigate the reproducibility, we enriched one batch of phosphopeptides, then split the sample equally in halves, and analyzed them by LC-MS/MS. We observed an ~70% overlap in phosphopeptides in the analyses, indicating that the limit of ~70% reproducibility in identifying phosphopeptides might attribute to inherent factors in the LC-MS/MS analysis (data not shown) (32, 33). A more detailed comparison of PolyMAC with TiO2 nanoparticles from different commercial sources is included in supplemental Figs. S4–S8 and Tables S1–S6. The experiments, therefore, demonstrated the remarkable reproducibility of PolyMAC-based enrichment method.
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experiment), the three methods resulted in the identification of 135, 202, and 315 Tyr(P) sites, respectively (supplementary Fig. S10). Relatively low selectivity of 40% with the PT66 alone method may contribute to the interference from non-phosphopeptide signals that suppress tyrosine phosphopeptide signals, leading to poorer identification.

Syk-negative and Syk-EGFP-expressing MDA-MB-231 cells (- and +Tet) were grown to 50% confluence. Western blotting analyses indicated a marked elevation of tyrosine phosphorylation in cells expressing Syk-EGFP with mild pervanadate treatment (Fig. 4). Cell lysates were digested with trypsin. Phosphotyrosine-containing peptides were immunoprecipitated with anti-phosphotyrosine antibodies linked to agarose beads, eluted under mildly acidic conditions, subjected to PolyMAC-Ti enrichment, and analyzed by nanoflow LC-MS/MS. To evaluate the efficiency of isolation of phosphotyrosine-containing peptides, we labeled phosphoangiotensin II with one of two amine-specific isotope tagging reagents of different molecular mass (see supplemental Fig. S1 for the structures of the tagged phosphopeptides). The light isotope-tagged phosphoangiotensin II (10 fmol) was added to the sample prior to enrichment. After enrichment, 10 fmol of phosphoangiotensin II labeled with the heavy isotope tag was added prior to the LC-MS/MS analysis. As shown in Fig. 5 (see supplemental Fig. S11 for the raw files), greater than 90% of the light isotope-tagged phosphoangiotensin was recovered as measured by the ratio of the light (m/z, MH2+) =

![Fig. 4](image)

**Fig. 4.** Tetracycline-induced Syk expression in MDA-MB-231 breast cancer cells. A, Western blotting indicates elevated tyrosine phosphorylation in response to Syk expression. B, Syk was expressed in transfected MDA-MB-231 cells with Tet treatment as indicated by co-expression of EGFP fused on its C terminus.

![Fig. 5](image)

**Fig. 5.** Comparison of recovery yield of phosphopeptides using PolyMAC-Ti versus TiO2. One phosphopeptide was isotopically labeled to generate identical peptides of two different masses: m/z 620.3 (light, doubly charged) and m/z 623.3 (heavy, doubly charged). The light version was included in the sample for enrichment, and the heavy version was spiked into the final eluate for measurement by LC-MS using an LTQ-Orbitrap spectrometer. Top panels, extracted ion chromatograms of labeled phosphopeptides. Bottom panels, average intensity of doubly charged, labeled phosphopeptide ions.
620.299) to heavy (m/z, MH$^+$ = 623.316) phosphopeptides. In contrast, TiO$_2$ was only able to recover ~10% of the light isotope-labeled phosphopeptide. This measurement of phosphopeptide enrichment and recovery indicates that the PolyMAC method provides an extremely efficient approach for the analysis of sites of tyrosine phosphorylation.

A much larger number of phosphotyrosine-containing peptides were identified in samples from cells induced to express Syk-EGFP as compared with Syk-deficient cells (820 versus 377 unique phosphopeptides), consistent with the Western blotting data. Selectivity for the recovery of phosphopeptides using the PolyMAC-Ti reagent was excellent (only 51 non-phosphopeptides were identified among a total of 871 peptides identified, corresponding to >94% selectivity in the Syk-EGFP-induced sample). Overall, we identified 514 unique sites of tyrosine phosphorylation on 458 different proteins that were present only in samples from Syk-EGFP-expressing cells (Fig. 6A and see supplemental Tables S7 and S8 for the full lists). To complement identification, we also examined the exact ion chromatograms to quantify the relative abundance of phosphopeptides recovered from the two samples. After signal normalization using spiked standard phosphopeptides, we measured the relative abundance of tyrosine phosphopeptides from cells expressing or lacking Syk. The majority of phosphopeptides that were not identified as being present in one sample also were not detectable in the corresponding raw MS data. Only a small fraction of these phosphopeptides were detected with extremely low intensity and poor MS/MS spectra. These data indicated that although lack of identification of a phosphopeptide is not evidence for its absence it is sufficient to reflect a change of phosphorylation within a signaling pathway. For example, cortactin, a cortical actin-associated protein known to play a critical role in stabilizing the adherens junction, was recently discovered as a direct substrate of Syk in breast cancer cells, although the phosphorylation sites were not identified (23). Our analysis identified three phosphorylation sites on cortactin that were modified exclusively in the Syk-expressing MDA-MB-231 cells (supplemental Table S8). Further analysis of these tyrosine phosphoproteomes will provide a unique opportunity for researchers to gain insights into important signaling pathways in breast cancer cells.

GO analyses of proteins with enhanced tyrosine phosphorylation in Syk-induced cells grouped by molecular function, biological process, and subcellular localization indicate roles for the kinase in a variety of cellular locations, functions, and processes (supplemental Fig. S12). Possible cellular signaling pathways influenced by the expression of Syk were further assessed using the induced phosphorylated proteins as input. Our data revealed elevated levels of tyrosine phosphorylation in a number of cancer-related networks, notably cancer cell movement and cell death (Fig. 7 and see supplemental Fig. S13 and Table S9 for the list of components and other examples).

To confirm the identification and novel functions of tyrosine-phosphorylated proteins, we selected several phosphopeptides from the induced samples for further analysis by LC-MS/MS.
phoproteins to explore further, including a microtubule-associated protein, MAPRE1; a nuclear transcriptional coactivator, TRIP4/ASC-1; a cell polarity protein, SCRIB; and a microtubule-based motor protein, dynactin. Tyrosine-phosphorylated proteins were immunoprecipitated from lysates of MDA-MB-231 cells lacking or expressing Syk-EGFP using anti-phosphotyrosine antibodies. The resulting immune complexes were examined by Western blotting with antibodies against MAPRE1, TRIP4, SCRIB, and dynactin. As shown in Fig. 5B, the phosphoproteins were enriched in anti-phosphotyrosine immune complexes prepared from Syk-expressing cells. Similarly, the phosphorylation of each protein on tyrosine was confirmed by the Western blotting of anti-protein immune complexes with anti-phosphotyrosine antibodies.

**DISCUSSION**

Signal transduction pathways modulated by protein-tyrosine kinases are often difficult to dissect because of complexities of interacting networks, a large number of substrates, and low levels of phosphorylation. MS-based phosphoproteomics is presently the most powerful tool for profiling dynamic phosphorylation events on a global scale. Drawbacks of most phosphoproteomics approaches are less than satisfactory reproducibility and selectivity during the phosphopeptide enrichment step. We reasoned that inconsistencies are due largely to the heterogeneity of solid phase extraction-based isolation methods as represented by approaches that use IMAC or metal oxides. Heterogeneous isolation conditions can suffer from nonlinear kinetic behavior, unequal distribution of and/or access to functional groups, and solvation problems. This presents a serious problem for the recovery of low abundance phosphopeptides from complex samples. The PolyMAC approach instead allows the isolation of low abundance phosphopeptides through chelation of Ti(IV) in a homogeneous, aqueous solution. A high concentration of metal ions in solution is achieved through the immobilization of the ions on soluble polymers for fast chelation. Then, in a second step, the PolyMAC-phosphopeptide complexes are gathered from the solution by covalent coupling to a solid support. Besides the hydrazine-aldehyde coupling pair described here, a variety of other highly efficient pairs can be envisioned including azide-alkyne (click chemistry), thiol-iodoacetyl, thiol-maleimide, thioester-cysteine, etc. The preparation of PolyMAC reagents and their use for phosphopeptide isolation could be easily automated for high reproducibility and high throughput. The density of functional groups (Ti(IV) and aldehyde) on the surface of the dendrimer can be adjusted during the synthetic steps. The PolyMAC reagents are much more amenable to analysis using NMR, MS, and other spectroscopic methods than TiO2 or IMAC reagents.

In addition, the dendrimer can be functionalized with different metal ions such as Fe(III) or Ga(III). Our data sets suggest
that PolyMAC-Ti may exhibit selectivity for singly phosphorylated peptides, an observation documented by others using TiO$_2$ or ZrO$_2$ for the isolation of phosphopeptides (36). As a solution, a method that uses IMAC and TiO$_2$ separation strategies in sequence has been proposed for the separation of multiphosphorylated and then monophosphorylated peptides (36). An analogous strategy could be adopted easily by the sequential use of PolyMAC-Fe and PolyMAC-Ti.

We demonstrate in this study that the PolyMAC method offers a rapid process for the recovery of phosphopeptides. The entire isolation procedure, including chelation of phosphopeptides, recovery of PolyMAC-phosphopeptide complexes on agarose beads, washing, and elution, requires less than 30 min while still retaining exceptionally high selectivity, reproducibility, and recovery yields that are superior to the commonly used TiO$_2$ method. PolyMAC is generally applicable for the isolation of phosphopeptides modified on serine, threonine, or tyrosine. By combining PolyMAC with an anti-phosphotyrosine enrichment step, we have developed a strategy for the in-depth analysis of tyrosine phosphoproteomes.

To test this strategy, we probed the phosphotyrosine proteome of invasive breast cancer cells expressing the Syk protein-tyrosine kinase and identified 794 sites of tyrosine phosphorylation, 514 of which were largely dependent on the expression of the kinase. GO annotation of the phosphoproteome identified prominent subclasses of substrates involved in important processes that regulate cell growth, motility, and survival. The large number of tyrosine-phosphorylated cytoskeletal (17%) and plasma membrane (10%) proteins is consistent with the described roles for Syk in modulating cancer cell motility, invasion, and cytoskeletal rearrangements (23). The identification of substrates important in the regulation of apoptosis also is consistent with the reported role for Syk in the enhancement of cell survival (37). In addition, 27% of the phosphotyrosine-containing proteins were annotated to the nucleus, consistent with previous studies suggesting important roles for the kinase in the nucleus (23, 38, 39). The most abundant category for the molecular function of substrates was protein binding (44%), which is consistent with the known role of Syk and tyrosine phosphorylation in regulating protein-protein interactions.

The identification of sites of tyrosine phosphorylation on important components of signaling networks offers exciting new experimental targets in cancer research. Protein-tyrosine kinases play vital roles in human cancer, most often as the products of dominant, transforming genes. In contrast, Syk appears to play an unusual role in a subset of cancers by restricting their metastatic behavior, but the full repertoire of substrates for Syk, especially in epithelial cells, is not known. A previous study identified a number of proteins potentially phosphorylated on tyrosine in Syk-expressing breast cancer cells, but sites of phosphorylation were not determined (21). Interestingly, the majority of the phosphorylation sites identified in this study were characterized by a high abundance of acidic amino acid residues surrounding the phosphorylated tyrosine, which is consistent with the known substrate specificity of Syk (supplemental Figs. S14 and S15). Although it is not yet known which of the substrates are most important for mediating the effects of Syk on epithelial cell function, it is intriguing that known and verified substrates such as MAPRE1 and TRIP4 are involved in activities (i.e. regulation of microtubule structure and activation of NF-$\kappa$B) that are known to be modified as a function of the expression of Syk. Further work will be needed to characterize fully the role of these and other substrates in the Syk-dependent regulation of epithelial cell metabolism.

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$[S]$ This article contains supplemental Figs. S1–S15 and Tables S1–S9.

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