Quantitative Comparison of IMAC and TiO₂ Surfaces Used in the Study of Regulated, Dynamic Protein Phosphorylation

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Protein phosphorylation regulates many aspects of cellular function, including cell proliferation, migration, and signal transduction. An efficient strategy to isolate phosphopeptides from a pool of unphosphorylated peptides is essential to global characterization using mass spectrometry. We describe an approach employing isotope tagging reagents for relative and absolute quantification (iTRAQ) labeling to compare quantitatively commercial and prototypal immobilized metal affinity chelate (IMAC) and metal oxide resins. Results indicate a prototype iron chelate resin coupled to magnetic beads outperforms either the Ga³⁺-coupled analog, Fe³⁺, or Ga³⁺-loaded, iminodiacetic acid (IDA)-coated magnetic particles, Ga³⁺-loaded Captivate beads, Fe³⁺-loaded Poros 20MC, or zirconium-coated ProteoExtract magnetic beads. For example, compared with Poros 20MC, the magnetic metal chelate (MMC) studied here improved phosphopeptide recovery by 20% and exhibited 60% less contamination from unphosphorylated peptides. With respect to efficiency and contamination, MMC performed as well as prototypal magnetic metal oxide-coated (TiO₂) beads (MMO) or TiO₂ chromatographic spheres, even if the latter were used with 2,5-dihydroxybenzoic acid (DHB) procedures. Thus far, the sensitivity of the new prototypes reaches 50 fmol, which is comparable to TiO₂ spheres. In an exploration of natural proteomes, tryptic (phospho)peptides captured from stable isotopic labeling with amino acids in cell culture (SILAC)-labeled immunocomplexes following EGF-treatment of 5×10^7 HeLa cells were sufficient to quantify stimulated response of over 60 proteins and identify 20 specific phosphorylation sites. (J Am Soc Mass Spectrom 2007, 18, 1932–1944) © 2007 American Society for Mass Spectrometry

Characterization of protein phosphorylation status, especially following stimulated transduction events, can provide mechanistic insights into the biological basis of signaling, cell cycle progression, adhesion, migration, and numerous other functions. Nowadays, the identification of phosphorylation sites in a complex milieu is carried out mainly by mass spectrometry. However, the sensitivity of analysis is largely hindered by low stoichiometry of phosphorylation, the reversible nature of the modification, and relatively weak ionization of phosphopeptides. It has been noted that enrichment of phosphopeptides from a pool of unphosphorylated peptides dramatically improves the success frequency for characterization.

Several methods for enriching phosphorylated peptides have been reported, including chemical derivatization of phospho-residues [1–5], antibody-based capture, immobilized metal affinity chromatography (IMAC) [6–10], enrichment on metal oxide surfaces

[11–13], strong cation exchange chromatography [14, 15], and phosphoramidate chemistry [16, 17]. For example, anti-phosphotyrosine antibodies have been used successfully to enrich tyrosine phosphorylated proteins and peptides [18–22]. Some authors report hundreds of phosphopeptides are identified in yeast using IMAC strategies [7]. More recently, TiO₂ chromatographic spheres were utilized to isolate phosphopeptides with high specificity in the presence of 2,5-dihydroxybenzoic acid (DHB) in the loading buffer [11, 12].

Although ³²P-radiolabeled phosphopeptides have been used to quantify capture efficiency of a few IMAC materials in MS studies, most evaluations of capture materials have been performed in a qualitative fashion [9, 23]. New techniques make it possible to evaluate phosphoprotein and phosphopeptide capture efficiencies without using radioactive labels. Among them, iTRAQ is a straightforward multiplexed strategy for protein and peptide quantification [24–27]. Briefly, iTRAQ reagents are isobaric peptide reactive labels prepared with stable isotopes substituted at select atomic positions. They allow quantitative comparison of up to four samples each labeled with an equivalent

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total mass but different isotopic structure. The samples are mixed, and during collision induced dissociation, the iTRAQ tags fragment to yield predictable but distinct reporter ions.

SILAC is a method used to label proteins *in vivo* with stable isotopes of atomic nuclei. Two cultures of cells are grown, one using heavy isotopically labeled amino acids and the other using their light congeners. The cultures may be treated differently, for example one may be exposed to a drug or other potential modifier, and the resulting protein abundances deduced directly by comparison of the MS signal intensity of peptides from known proteins [28].

In the present study, we first used iTRAQ labeling of a standard mixture to quantitatively evaluate a variety of metal chelate (MC) and metal oxide (MO) affinity materials with respect to their selectivity in phosphopeptide isolations. We then characterized the performance of the most selective resins using peptides from immunoprecipitated phosphoproteins derived from EGF-stimulated cell culture. Our results indicate that, when adapted to magnetic Dynabeads, the Fe³⁺ chelator of this study (MMC) performs as well as TiO₂ coated Dynabeads (prototype, MMO) or chromatographic Titansphere (TiO₂ spheres) and better than several other alternative materials for phosphopeptide enrichment. This conclusion was borne out with both standard mixtures and with phosphopeptides isolated from cultured cells. Using a SILAC strategy on 5×10^7 HeLa cells deprived of serum overnight, we identified over 60 proteins that respond specifically to 5 min EGF stimulation and elucidated 25 uniquely affected phosphorylation sites, several of which are novel.

Materials and Methods

Materials

Magnetic beads coated with zirconium oxide were purchased from Calbiochem (ProteoExtract phosphopeptide capture kit, cat. no. 525250, San Diego, CA). Titansphere, 5 μ m TiO₂ chromatography-grade spheres (TiO₂ spheres) were purchased from GL Sciences Inc. (Torrance, CA). A phosphopeptide standard mixture [angiotensin II (DRVYIHPF, 1046.54), angiotensin I (DRVYIHPFHL, 1296.88), myelin basic protein fragment 104–118 (GKGRGLSLRFSWGA, 1578.85), pTpY peptide (MAP kinase fragment 177–189, DHTGFLpTEpYVATR, 1669.67), pY peptide (insulin receptor fragment 1142–1153 (TRDlpYETDYRK, 1702.75), pT peptide (VPIPGRFDRRpTVE, 1720.89), pS peptide (RII phosphopeptide fragment 81–99, DLDVPIPGRFDRRpSVAAE, 2192.08)] was purchased from Invitrogen (cat. no. P33357).

HeLa cells were purchased from ATCC. iTRAQ, NuPAGE gel, NuPAGE sample buffer, SimplyBlue SafeStain, NuPAGE transfer buffer, WesternBreeze, Captivate beads, TiO₂ coated Dynabeads (prototype, MMO), Dynabeads-MyOne-tosylactivated beads (cat.

no. 655-01), DMEM medium, SILAC/DMEM (cat. no. SP10001 that contains dialyzed FBS, [U-¹³C₆] L-lysine and lysis buffers), [U-¹³C₆, ¹⁵N₄] L-arginine (cat. no. MS10009), recombinant human EGF, and monoclonal anti-phosphotyrosine antibodies (PY-Plus, a monoclonal cocktail containing clones PY-7E1 and PY20; cat. no. 13-6600) were from Invitrogen.

The Nexus tetradentate metal chelator (Valen Biotech, Inc., Atlanta, GA) was coupled to Dynabeads-MyOne tosylactivated beads (MMC) according to G. Pevow, U.S. patent application 20020019496. Immobilization of anti-phosphotyrosine antibody (PY-Plus) on Dynabeads-MyOne tosylactivated beads was performed according to the manufacturer's instruction. Monoclonal 4G10 anti-phosphotyrosine antibody and agarose-conjugated 4G10 anti-phosphotyrosine antibody were purchased from Upstate Biotechnology Inc. (cat. no. 16-199, Lake Placid, NY). α -Cyano-4-hydroxy cinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), GaCl₃ and FeCl₃ were purchased from Sigma (St. Louis, MO). Trypsin was obtained from Promega (Fitchburg, WI). Poros 20 MC and Poros R2 resin were purchased from Applied Biosystems (Foster City, CA). Geloader tips (10 μ L size) were purchased from Eppendorf (Westbury, NY).

iTRAQ Labeling and Isolation of Phosphopeptides for MS Analysis

Phosphopeptide standard mixtures were isotopically labeled with iTRAQ 114, 115, 116, or 117 according to manufacturer's instructions. Magnetic beads or resins derivatized with different functional ligands were preloaded with Fe³⁺ or Ga³⁺ by the respective manufacturers. Isolation of phosphopeptides using traditional metal chelate resins followed the basic procedure described in the Captivate phosphopeptide isolation kit (Invitrogen). Briefly, the capture medium was equilibrated with binding/washing buffer A (50 mM sodium acetate in 20% ethanol, pH 4.0). Upon phosphopeptide binding, the capture medium was washed five times with buffer A, followed by elution with 10 μ L of buffer B (50 mM sodium phenylphosphate in 0.3 N ammonium hydroxide). To isolate phosphopeptides using MMO or TiO₂ spheres, 0.1% trifluoroacetic acid (TFA) or 300 mg/mL DHB/80% acetonitrile/0.1% TFA was used as binding buffer and 80% acetonitrile in 0.1% TFA was used as washing buffer [12].

Aliquots of iTRAQ labeled peptides (typically 5 μ L of 2 pmol/ μ L stock) were acidified with 2 μ L of 50% acetic acid, diluted with 36 μ L binding buffer, and then incubated with 8 μ L of 50 mg/mL magnetic beads for 10 min with repetitive pipetting, or they were passed through an Eppendorf GeLoader tip prepacked with Poros 20MC or TiO₂ spheres (~3 mm packing length). After extensive washing, phosphopeptides were eluted with 10 μ L of elution buffer as described above.

To quantitatively compare capture efficiency, phos-

Figure 1. Experimental strategy for quantitative comparison of a variety of metal resins using iTRAQ. Peptide standard mixtures were isotopically labeled with iTRAQ reagents. Aliquots of labeled peptides were then incubated with a variety of IMAC or metal oxide resins to isolate phosphorylated peptides. Peptides eluted from the beads were mixed accordingly. Reverse capture of phosphopeptides could be performed to reduce bias. Phosphopeptide mixture was desalted using a C₁₈ tip column, followed by the analysis of MALDI-TOF-TOF. Quantification was conducted manually in the MS/MS spectra.

phopeptides isolated by two different affinity chemistries were mixed and acidified with 4 μ L of 50% acetic acid, followed by desalting with a self-packed Poros R2 tip column. Phosphopeptides were eluted directly onto a MALDI plate and mixed with CHCA matrix at 1:1 ratio, followed by analysis using a 4700 proteomics analyzer (Applied Biosystems).

Before sample analysis, the CID gas cell was purged to obtain stable isotopic ratios within the reporter ion. A total of 14,000 laser shots were used to average the signals of fragmenting ion set. Rather than invoking automated iTRAQ quantification in the GPS Explorer software, we manually evaluated the relative abundance of reporter ions in the tandem mass spectrometry (MS/MS) spectra by integration in Data Explorer software (PerSeptive Biosystems, Inc., Framingham, MA) wherein the most intense reporter ion was set at 100% in intensity.

SILAC Labeling and Isolation of Phosphopeptides for LC-MS Analysis

HeLa cells were maintained in DMEM medium containing 10% FBS. For SILAC labeling, aliquots of HeLa cells were propagated for at least six doublings (~10 days) in SILAC DMEM media containing 10% dialyzed FBS and supplemented with light L-lysine and light L-arginine (light medium) or heavy [U -¹³C₆] L-lysine and heavy [U -¹³C₆, ¹⁵N₄] L-arginine (heavy medium). After overnight starvation with the corresponding serum-free light or heavy medium, HeLa cells labeled in heavy medium (50×10^6 cells) were stimulated with 150

ng/mL EGF for 5 min while HeLa cells labeled with light medium (50×10^6 cells) remained untreated. Upon stimulation, heavy-labeled cells and light-labeled cells were lysed immediately in NP-40 lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM Na₃VO₄, 10 mM NaF, and protease inhibitor cocktail. In some cases, crossover stimulation was also performed to verify the identity of phosphopeptide as well as the isotopic ratio.

Cell lysates were clarified by centrifugation at $100,000 \times g$ for 15 min and the mixed cell lysates were immunoprecipitated with ~50 μ g of either phosphotyrosine antibody (PY-Plus) conjugated to Dynabeads or 4G10 phosphotyrosine antibody conjugated to agarose beads. After mixing by gentle rotation for 2 h at 4 °C, the Dynabeads were captured with a magnet, whereas the agarose beads were harvested by centrifugation. The beads were washed five times with lysis buffer and then eluted with 50 μ L of 100 mM glycine (pH 2.5). The eluates were neutralized with 1 M Tris, reduced with 10 mM DTT and alkylated with 30 mM iodoacetamide in 1 $\times$ SDS sample buffer, and then separated on SDS-PAGE. Alternatively, the immunocomplexes were directly eluted with 50 μ L of SDS sample buffer containing 50 mM DTT, followed by the analysis of SDS-PAGE. Protein bands were excised from gels and subjected to in-gel tryptic digestion as described [29]. Peptide extracts were dried using a Speed Vac.

Approximately one-third of the peptide extract was used for protein identification using nanoelectrospray LC-MS (Q-TOF premier; Waters Corporation, Milford, MA) or MALDI-TOF-TOF (4700 Proteomics Analyzer; Applied Biosystems) [29]. The rest of the sample was incubated with 250 μ g of MMC-Fe³⁺ or MMO (TiO₂) for 10 min in 24 μ L of binding buffer to isolate phosphopeptides. In either case the resins were washed five times with washing buffer and then eluted with 10 μ L of elution buffer. The eluted phosphopeptides were acidified with 2 μ L of 50% acetic acid and then desalted with a self-packed Poros R2 tip column, followed by analysis using nanoelectrospray LC-MS or MALDI-TOF-TOF. The Mascot protein score obtained from isolated phosphopeptides was generally low as it is based on one or two peptide hits. To improve the relevance of phospho-

Table 1. Relative efficiency of phosphopeptide capture

Capture media	Relative efficiency
MMC-Fe ³⁺	1
MMC-Ga ³⁺	0.1 $\pm$ 0.05
Prototype IDA coated Dynabeads-(Fe ³⁺)	0.2 $\pm$ 0.08
Prototype NTA coated Dynabeads-(Fe ³⁺)	0.85 $\pm$ 0.05
Poros 20MC-IDA-Fe ³⁺	0.82 $\pm$ 0.05
Captivate-Ga ³⁺	0.1 $\pm$ 0.05
Calbiochem ZrO ₂	0.1 $\pm$ 0.05
Titansphere® TiO ₂ (GL Sciences)	0.95 $\pm$ 0.05
Prototype TiO ₂ -coated Dynabeads (MMO)	0.95 $\pm$ 0.05

Figure 2. Quantitative comparison of a variety of IMAC and metal oxide resins on the recovery of phosphopeptides. Magnetic beads (8 μ L of 50 mg/mL suspension) or resins with different functional ligands were preloaded with the indicated metal ions. Poros 20MC or chromatographic TiO₂ spheres were packed into a tip column $\sim$ 3 mm long. Labeled peptides were incubated with magnetic beads or passed through the tip columns. After extensively washing, phosphopeptides were eluted and mixed accordingly. Reverse capture of phosphopeptides was performed to minimize unequal sample preparation. Phosphopeptides were desalted and analyzed by MALDI-TOF-TOF. To simplify the graphic, only recovery of phosphopeptide TRDlpYETDYYRK is shown.

protein identification, we supplemented the analysis of enriched phosphopeptides with LC-MS analysis of all peptides extracted from in gel digests. Because the proteins loaded on the gel were immunoprecipitated with anti pTyr, these sum of these two targeted approaches conducted under SILAC labeling gives high confidence in distinguishing response to EGRF stimulus.

For LC-MS analysis, an Atlantis dC18, 3 μ m, 100 μ m $\times$ 100 mm column (Waters Corporation) was used for peptide separation. An elution gradient of 5% to 45% (vol/vol) acetonitrile in 0.1% formic acid over 45 min, and then 45% to 95% acetonitrile in 0.1% formic acid over 5 min was used. In the data-dependent mode, four component trigger functions were used to acquire MS/MS data with 1.9 s scan time. For MALDI-TOF-TOF analysis, the desalted phosphopeptides were directly spotted on MALDI plates, and mixed with CHCA matrix at 1:1 ratio. A total of 14,000 shots were used to average each MS/MS spectrum.

Raw data files from Q-TOF instrument were processed without smoothing or baseline subtraction using

Mascot Daemon (version 2.2; Matrix Science, London) and then searched against NCBI database (April 15, 2006) using the Mascot search algorithm version 2.2. The searches were constrained to tryptic peptides with one missed cleavage allowed. Oxidation of methionine residues, cysteine carbamidomethylation, phosphorylation, heavy Lys and heavy Arg with δ mass of 6 and 10 Da, respectively, were selected as variable modifications. The mass tolerance of the precursor peptide ion was set at 50 ppm and mass tolerance for the MS/MS fragment ions was set at 0.2 Da. The Mascot output showed peptides labeled with either light or heavy Lys and/or Arg. Quantification of peptide pair was performed and validated manually by examining both MS and MS/MS spectra.

Raw data files obtained from the 4700 Proteomics Analyzer were processed using GPS Explorer (version 3.0, Applied Biosystems) and then searched against NCBI database. Oxidation of methionine residues, cysteine carbamidomethylation, phosphorylation, heavy Lys and heavy Arg with δ mass of 6 and 10 Da, respectively, were selected for variable modification.

Figure 3. An example of quantitative comparison of unphosphorylated peptides carried over during phosphopeptide enrichment. Phosphopeptides were isolated using either Poros 20MC or MMC (see the Experimental section) as described above. Unphosphorylated peptides carried over were analyzed by MALDI-TOF-TOF.

One missing cleavage was allowed. Mass tolerances for precursor and MS/MS fragment were set at 150 ppm and 0.5 Da, respectively. The MS/MS spectra of phosphopeptides were examined manually to confirm the neutral loss of phosphoric acid.

Results and Discussion

Experimental Strategy

In Figure 1, we describe an iTRAQ-based strategy to quantitatively compare the phosphopeptide capture efficiency of different selective capture media, two at a time. Peptide standard mixtures containing serine, threonine, or tyrosine-phosphorylated peptides and unphosphorylated peptides were chemically labeled with iTRAQ-114, 115, 116, or 117, respectively. Aliquots of the labeled peptides were then incubated with the same amount of capture media. For comparison of Medium A to Medium B, phosphopeptides eluted from Medium A were mixed with phosphopeptides eluted from Medium B. To eliminate bias stemming from unequal sample preparation, we performed crossover experiments; that is, phosphopeptides with a given iTRAQ label were isolated on reciprocal media and mixed again. The eluted phosphopeptide mixture was desalted using a tip column prepacked with Poros R2 resin and then analyzed using a MALDI-TOF-TOF instrument. Quantification was based on the relative intensity ratio of reporter ions in the MS/MS spectrum.

No single chromatographic material has proven adequate to bind all phosphopeptides [30]. For example, it has been shown that Poros R2 resin retains only certain

subsets of phosphopeptides, depending primarily on sequence and hydrophobicity [31]. In these cases, the sequential use of multiple reverse phase materials, such as Poros R1, R2, R3, and graphite powder, improves the retention of phosphopeptides [31, 32]. However, failure to incorporate all of these resins does not affect the interpretation of our results because the protocol is designed to measure the relative effectiveness of resins used upstream in the capture of phosphopeptides. At the point the recovered peptides are desalted on Porous R2, they exist as a mixture of heavy- and light-labeled, sequence-matched phosphopeptides. Hence, losses on the Porous R2 resin will not introduce bias into the relative measurement.

Quantitative Comparison of Phosphopeptide Capture Media Using Standard Peptide Mixtures

Metal chelate resins preloaded with either Fe^{3+} or Ga^{3+} metal ions and other media were used to isolate iTRAQ labeled phosphopeptides, as described above. Phosphopeptides isolated from Medium A and Medium B were mixed, desalted, and analyzed by MALDI-TOF-TOF. The relative efficiency of phosphopeptide capture was determined as the intensity ratio of the reporter ions. The capture efficiency of MMC-Fe^{3+} was assigned an arbitrary-ranking value of 1. The values for all other capture media were calculated relative to the capture efficiency of MMC-Fe^{3+} .

Figure 4. Sensitivity of phosphopeptide enrichment using MMC. One μL of 50 mg/mL bead suspension was incubated with either 50 fmol or 100 fmol of peptide mixtures. After extensively washing, phosphopeptides were eluted directly onto a MALDI plate and mixed with α -cyano-4-hydroxy cinnamic acid matrix, followed by the analysis using MALDI-TOF. (a) Peptide mixture; (b) and (c) phosphopeptides isolated from 100 fmol or 50 fmol of peptide mixture, respectively.

reporter fragment ions isolated on magnetic beads functionalized with IDA-Fe³⁺ (Figure 2b), suggesting that the performance of MMC was better than that of magnetic beads coupled with IDA. MMC charged with Fe³⁺ also performed better than Captivate beads charged with Ga³⁺, since their isotope ratio was ~20:1 (Figure 2c). Recent reports describe zirconium oxide particles in microtips as effective for phosphopeptide enrichment [13]. But based on the observed isotope signal ratio of 20:1 (Figure 2d), MMC charged with Fe³⁺ performed significantly better than magnetic particles functionalized with zirconium oxides.

From the earliest reports of the Tempst laboratory, Poros 20MC has become one of the most commonly used IMAC resins [6]. Poros 20MC has an iminodiacetic acid functional surface. When compared to Poros 20MC-Fe³⁺, the MMC-Fe³⁺ recovered slightly more phosphopeptide (Figure 2e), however, the specificities vary and the relative recovery will also depend on the nature of the peptides (see below).

This iTRAQ labeling strategy can also be employed to compare the relative specificity of peptide isolation since peptides with acidic residues may also be captured during phosphopeptide enrichment. As depicted in [Figure 3](#), the MMC-Fe³⁺ showed 50% to 70% less contamination of unphosphorylated peptides DRVYIHPF and DRVYIHPFHL compared with Poros 20MC-Fe³⁺.

Chromatographic TiO₂ spheres from GL Sciences have been used successfully to isolate phosphopeptides with high specificity by including 2,5-dihydroxybenzoic acid (DHB) in the loading buffer [12]. For this particular test set of peptides, the 1:1 ratio of reporter fragment ion signals, shows that MMC-Fe³⁺ (used without DHB) performed as well as TiO₂ spheres using DHB protocols (Figure 2f).

Sensitivity of Phosphopeptide Enrichment Using MMC

The sensitivity of phosphopeptide enrichment is the key element for transduction studies as phosphorylation is primarily a transient, reversible event and targeted phosphoresidues represent relatively low abundant species. To test the sensitivity of phosphopeptide enrichment, peptide standard mixtures containing either 50 or 100 fmol of phosphopeptides were incubated with 50 μ g of MMC in a small PCR tube for 10 min using repetitive aspirations. After extensive washing, phosphopeptides were eluted with 5 μ L of buffer. After desalting with a self-packed Poros R2 tip column, phosphopeptides were eluted directly onto a MALDI plate and mixed with the matrix at 1:1 ratio, followed by MALDI-TOF analysis. As depicted in Figure 4c, definitive, well resolved signals were detected using 50 fmol of phosphopeptides with good selectivity and specificity. Based on

the signal intensity, 5 fmol of phosphopeptides would probably be the lower limit of detection.

Quantitative Comparison of Dynabeads-TiO₂ to TiO₂ Spheres

It has been reported that the addition of DHB in the loading buffer significantly improves the specificity of phosphopeptide enrichment when using TiO₂ spheres from GL Sciences (Tokyo, Japan) [12]. However, a high concentration of DHB in the loading buffer can reduce the flow rate of microtip column. DHB also crystallizes readily in the loading buffer composition, making it difficult to operate a TiO₂ sphere microcolumn in fully automated fashion or couple to reverse phase guard cartridges on-line. Potentially, magnetic particles functionalized with TiO₂ would minimize these problems. Hence, an isolation procedure employing MMO was used to isolate phosphopeptides from a standard peptide mixture with either 0.1% TFA or 300 mg/mL DHB/80% acetonitrile/0.1% TFA as binding buffer. Based on the resultant MALDI-TOF spectrum, the DHB protocol provided only a minor improvement in the specificity of phosphopeptide enrichment on MMO for this particular set of peptides [Figure 5a left (1)]. However, for nonmagnetic, chromatographic TiO₂ spheres, the DHB protocol did significantly improve the specificity of enrichment [Figure 5a right (2)]. These results suggest that the commercial and prototype resins may support alternative binding interaction between TiO₂ and phosphopeptides and that the solid-phase support might play an important role in the specificity of enrichment [12].

To compare MMO directly to TiO₂ spheres, the iTRAQ labeled phosphopeptides isolated with MMO or TiO₂ spheres using the DHB protocol were mixed, desalted, and analyzed by MALDI-TOF-TOF. As depicted in Figure 5b, the performance of MMO is comparable to TiO₂ spheres based on the recovery of four different phosphopeptides.

Isolation of Phosphopeptide(s) from a Biological Sample Using MMC or Dynabeads-TiO₂

Next we evaluated the biological relevance of MMC and MMO performance using a cell culture experiments. There is increasing evidence in the literature that SILAC is an excellent approach to study differential protein expression and posttranslational modification, especially phosphorylation [29, 33–37]. In this study, we combined SILAC with MMC and MMO to quantify the dynamic changes of tyrosine phosphorylation in response to EGF stimulation in HeLa cells.

HeLa cells were propagated in SILAC DMEM media containing either light or heavy Lys and Arg. After overnight starvation, cells labeled with heavy Lys and Arg were stimulated with EGF for 5 min while cells labeled with light Lys and Arg remained untreated. Cells labeled with light or heavy amino acids (50×10^6 cells each) were lysed immediately and mixed at 1:1 ratio based on cell number. Because phosphorylated Tyr residues represent only 0.05% of the phosphoamino acids within a cell [38], we followed a strategy to enrich tyrosine phosphorylated proteins in the initial steps. Cell lysate was incubated for 2 h with 50 μ g of either phosphotyrosine antibody (PY-Plus) conjugated to Dynabeads or 4G10 phosphotyrosine antibody conjugated to agarose. The magnetic or agarose beads were washed five times with lysis buffer, followed by elution of phosphorylated proteins with 100 mM glycine at pH 2.5 or SDS sample buffer. Samples were reduced and alkylated, followed by separation on SDS-PAGE. Based on Coomassie staining, more proteins were captured by PY-Plus antibody than by 4G10 antibody (Figure 6a). The protein bands were excised from the gel and then subjected to in-gel tryptic digestion. The peptide extracts were dried using a speed vac.

The tryptic peptide extract from a gel band which contains EGF receptor was analyzed by MS (Figure 6b, Panel a). Because this mixture contains both phosphorylated and nonphosphorylated peptides, interpretation of phosphorylation would be difficult. Isolation of phosphorylated peptides from the extracted digest mixture using metal chelate or metal oxide affinity media

or light cell lysate, respectively. (a) Cell lysate mixture derived from unstimulated light-labeled cells and EGF-stimulated heavy-labeled cells were immunoprecipitated with phosphotyrosine antibody (PY-Plus) conjugated to Dynabeads or 4G10 phosphotyrosine antibody conjugated to agarose beads, followed by separation on SDS-PAGE. Protein bands were excised and subjected to in-gel tryptic digest [(b), Panel a]. Peptide extracts were dried using a Speed Vac and resuspended in binding buffer, followed by isolation of phosphorylated peptides using the MMC [(b), Panel b] or MMO [(b), Panel c] resins. The isolated peptides were analyzed by MALDI-TOF-TOF. The identity of phosphopeptide was confirmed by MS/MS analysis [(b), Panels d and e]. In separate experiments, the isolated phosphopeptides were analyzed by electrospray nano LC/MS (c). Cell lysates were prepared by mixing EGF-stimulated light-labeled cells and unstimulated heavy-labeled cells [(c), Panel a] or mixing unstimulated light-labeled cells and EGF-stimulated heavy-labeled cells [(c), Panel b]. The identities of light- or heavy-labeled phosphopeptides were confirmed by MS/MS analysis [(c), Panels c and d]. Quantification of phosphorylation in response to EGF stimulation was performed manually by examining the MS spectrum.

Table 2. Proteins and phosphopeptides upregulated upon EGF stimulation in HeLa cells

Protein name	NCBI GI number	Mascot score	Phosphopeptides	SILAC ratio (H/L)	Site observed	Cited ^a already?	Recovered by ^b
Annexin I	442631	213		4 ± 0.5			
ARF GTPase-activating protein GIT1	4691726	575		3.1 ± 0.3			
Ankyrin repeat and sterile alpha motif domain containing 1	38683797	367		4.2 ± 0.5			
Ankyrin repeat domain 13	34190139	181		4 ± 0.5			
Adaptor-related protein complex 2, beta 1 subunit isoform b	4557469	286		2.9 ± 0.4			
cbl-b	862407	953		3.5 ± 0.4			
Crk-associated substrate p130Cas	6740102	237		1.8 ± 0.2			
Cas-Br-M ecotropic retroviral transforming sequence	52426745	848		4.2 ± 0.5			
C3orf6 protein	30089162	154		9.9 ± 1.2 ±			
C20orf18	12697475	129		8.2 ± 0.8			
Centaurin delta 2 isoform b	16975484	706	HpYSVVLPTVSHSGFLYK	6.1 ± 0.7	Y737	N	c
Centaurin, delta 3	21264337	361		4.3 ± 0.5			
CENTD2 protein	33991628	116		8.3 ± 0.9			
Cortactin isoform a	20357552	315	LPSSPVpYEDAASFK	2.6 ± 0.3	Y421	Y	a, b, c
Dopamine receptor interacting protein 4	33339157	172		4.1 ± 0.5			
DOCK4	29335973	219		2.7 ± 0.3			
DOC-2	1297330	299		4.1 ± 0.5			
1-8D	23396	127		4.5 ± 0.6			
EGFR protein	757924	1851	YSSDPTGALTEDSIDDTFLPVPEpYINQSVPK YpSSDPTGALTEDSIDDTFLPVPEpYINQpSVPK ALMDEEDMDVDVDADEpYLIPQQGFSSPSTSR GSHQISLDNPDpYQQDFFPK ELVEPLpTPSGEAPNQALLR MHLPSPTDSNFpYR GSTAENAEpYLR	>20 ± 3	Y1092 S1070 Y1018 Y1172 T693 Y998 Y1197	Y Y Y Y Y Y Y	c c c a, b, c a, b, c a, b, c a, b, c
Epidermal growth factor receptor substrate	466260	213		7.2 ± 0.8			
Epidermal growth factor receptor pathway substrate 15-like 1	10864047	177		3.3 ± 0.4			
Endofin	16904136	451		18 ± 2			
E3 ubiquitin-protein ligase CBL	115855	407		11.6 ± 1.5			
Epsin 2a	3894395	186	GSSQPNLSTSHSEQepYGK	6.1 ± 0.7	Y186	N	a, b
ELMO2 protein	33111868	161		9 ± 1.1			
Focal adhesion kinase	182395	267		1.7 ± 0.2			
G protein-coupled receptor kinase-interactor 2 isoform 1	17149830	543		3.7 ± 0.4			
G protein coupled receptor kinase-interactor 2, isoform 4	12655059	214		2.4 ± 0.3			
GRB2-associated binding protein 1, isoform a	40674494	445	VDpYVVVDQKQ	3.2 ± 0.4	Y659	Y	c
Guanine nucleotide-releasing factor 2	57208999	107		4.9 ± 0.6			
Hepatocyte growth factor-regulated tyrosine kinase substrate HRS isoform 2	9022389	156		11.4 ± 1.5			
HER2 receptor	306840	712	GTPTAENPEpYGLDVPV pSGGGDLTLGLEPSEEEAPR TLSEVDpYAPAGPAR	16 ± 3 14 ± 2.5	Y1248 S1054 Y1135	Y N Y	c c a, b, c
Inositol polyphosphate 5-phosphatase	2653424	1595		14 ± 2.5			
ISGF3G protein	15082338	381		5.9 ± 0.7			
Junction plakoglobin (Desmoplakin-3)	130257	152		4.1 ± 0.5			
Mitogen-activated protein kinase 1	66932916	202		1.8 ± 0.2			
Myosin X	7108753	1341		3.9 ± 0.5			
NCK interacting protein with SH3 domain isoform 1	7705266	175		2.1 ± 0.3			
PI-3 kinase	3954946	706	LLGSVDpYDGINDAITR	3.5 ± 0.4	Y228	Y	a, b
Phosphatidylinositol-binding clathrin assembly protein	25090897	209		4.9 ± 0.6			
Plcg1	3336979	932	pYQQPFEDFR IGTAEPDpYGALYEGR FIHQQQSSSPVpYGSSAK VGEEEHVpYSFPNK	8.5 ± 1 1.7 ± 0.2	Y1253 Y771 Y88 Y118	Y Y Y Y	a, b c c c
Paxillin	4506345	409		1.7 ± 0.2			
Polyubiquitin	2627129	402		10.5 ± 1.5			
Protein-tyrosine phosphatase	4519425	303		3.7 ± 0.4			
Rho guanine nucleotide exchange factor (GEF) 7	55959541	918	MpSGFIYQKQ	4.1 ± 0.5	S340	Y	a, b, c
Rho guanine nucleotide exchange factor 5 isoform 1	50959191	274	pSTAALEEDAQILK LESLSSETPGSPSPR DpYSTVSASPTALSTLK	3.3 ± 0.4	S525 S1180 Y710	Y N N	c a, b, c c
60% similar to AB002297	2588635	157		3 ± 0.4			

Table 2. Continued

Protein name	NCBI GI number	Mascot score	Phosphopeptides	SILAC ratio (H/L)	Site observed	Cited ^a already?	Recovered by ^b
Signal transducing adaptor molecule 1	4507249	654		6 ± 0.8			
Signal transducing adaptor molecule 2	20381123	710		7.1 ± 0.9			
Signal transducer and activator of transcription Stat5B	1330324	132	AVDGPYVKPQIK	5.4 ± 0.6	Y699	Y	c
SHC1 protein	17380299	216	ELFDDPSpYVNVQNLDK	3.5 ± 0.4	Y427	Y	a, b, c
Spermatogenesis associated PD1	4730927	149		10.5 ± 1.2			
Tight junction protein ZO-1	292938	402		2.8 ± 0.3			
Tyrosine kinase, non-receptor, 2 isoform 1	56549666	599	pYATPQVIQAPGPR	2.2 ± 0.3	Y826	Y	c
Target of myb1-like protein 2	18698597	114		5.9 ± 0.8			
Vav 2 oncogene	40549448	275		5.0 ± 0.6			
VAV-3 protein	3928847	759		3.1 ± 0.3			
Valosin containing protein	29144989	131		2.7 ± 0.3			
Unnamed protein product	34530097	434		9.3 ± 1.2			
Unknown	14249875	173		17 ± 3			
KIAA0849 protein	14133221	587		6.2 ± 0.8			
Phosphoprotein controls							
Ephrin receptor EphA2	22713539	574	QSPEDVpYFSK VLEDDPEATpYTTSGGK	1	Y575 Y772	N Y	c c
TRAP150	4530441	404	ASAVSELpSPR IDlpSPSTFR	1	S243 S6812	Y Y	c c
BCL2-associated transcription factor 1	7661958	125	IDlpSPSTLR	1	S658	Y	c

^aAccording to peer-reviewed work as referenced on PhosphoSite® URL: <http://www.phosphosite.org>.

^bThe phosphopeptides were recovered using MMC-Fe (a) or MMO-TiO₂ (b) and without treatment (c).

could dramatically improve the situation. The phosphopeptides enriched by MMC (Figure 6b, Panel b) and MMO (Figure 6b, Panel c) looked similar, except for minor variances in ion abundance. Clearly, the spectrum is greatly simplified and, in either case, phosphorylated peptides may be identified more easily.

Figure 6 illustrates a major *m/z* 2322.1 peptide detected upon enrichment using either MMC or MMO (Figure 6b, Panel b or c). In addition, some minor peaks at *m/z* 2499.1 and 1670.6 were also observed. With increased laser intensity, peptides at *m/z* 2242.0, 2419.2, and 1590.7 were also detected. These arise, presumably, due to the neutral loss of phosphate from their corresponding parental ions at *m/z* 2322.1, 2499.1, and 1670.6, respectively.

The MS/MS analysis of peptides at *m/z* 2499.1 and 1670.6 suggested that they were probably phosphorylated, as abundant neutral loss ions were notable at 80 or 98 Da below their respective precursors (data not shown). Unfortunately, the specific phosphoresidues in these peptides could not be assigned precisely due to the low intensity of internal fragments in the MS/MS spectra. On the other hand, the peptide at *m/z* 2322.1 was identified as the heavy, [¹³C₆]-Lys-labeled phosphopeptide GSHQISLDNPDpYQQDFFPK derived from EGFR (Figure 6b, Panel d). The fragmentation ions that occurred on the sequence of the phosphopeptide were indicated in Figure 6b, Panel e. A very tiny peak at *m/z* 2316.1 corresponding to the light [¹²C₆]-Lys-labeled phosphopeptide was also detected in the MALDI-TOF spectrum (see inset in Figure 6b, Panel c). Based on the relative signal intensity, the tyrosine phosphorylation of EGFR increased more than 20-fold upon EGF stimulation for 5 min.

The same heavy [¹³C₆]-Lys-labeled phosphopeptide GSHQISLDNPDpYQQDFFPK was identified and quantified in separate nanoelectrospray LC-MS experiments (Figure 6c, Panels a and b). In Panel a, cells labeled with light Lys and Arg were stimulated with EGF and then mixed with equal amount of unstimulated cells previously labeled with heavy Lys and Arg (forward stimulation, F). Whereas in Panel b, cell lysate mixture was prepared from unstimulated light-labeled cells and EGF-stimulated heavy-labeled cells (reverse stimulation, R). Detection of the immonium ion at *m/z* 216.02 is specific for tyrosine-phosphorylated peptide. The identities of the light [¹²C₆]-Lys-labeled phosphopeptide (Figure 6c, Panel c) and the heavy [¹³C₆]-Lys-labeled phosphopeptide (Figure 6c, Panel d) were confirmed by MS/MS scans. The b ion series derived from light or heavy labeled peptides are identical, but the y ion series derived from heavy labeled peptide are 6 Da larger than that of light labeled peptide because a lysine residue is present at C-terminus of the phosphopeptide (data not shown). In the forward stimulation experiment, a very tiny peak with 3 Da mass units higher than the precursor ion in the MS scan represented the heavy [¹³C₆]-Lys-labeled phosphopeptide (Figure 6c, Panel a), whereas a very small peak with 3 Da mass units lower than the precursor ion in the MS scan represented the light [¹²C₆]-Lys-labeled phosphopeptide in the reverse stimulation experiment (Figure 6c, Panel b). An additional peak at *m/z* 1164.5 (Figure 6c, Panel b) was due to the metabolic conversion of arginine to proline [37]. The signal intensity ratio of EGF-stimulated phosphopeptide to unstimulated phosphopeptide was greater than 20:1, which is consistent with the result obtained by MALDI-TOF-TOF analysis. These results indicated that

the MMC or MMO effectively isolated phosphopeptides from in-gel tryptic digest with good selectivity and specificity.

In this study, 62 gene products were up-regulated in response to EGF stimulation (Table 2). Results reported are the culmination of three biological replicates. The majority of the proteins have been previously characterized by Mann's or White's research groups in the proteomic analysis of EGF signaling network [20, 39]. However, additional proteins, such as ankyrin repeat, annexin I, adaptor-related protein complex 2, C3orf6 protein, centaurin, DOCK4, spermatogenesis associated PD1, and endofin, have not been reported. Ankyrin plays a structural role by binding to both integral membrane proteins and spectrin/fodrin proteins of the cytoskeleton. It has been shown that the cytoskeletal association of ankyrin is down-regulated by phosphorylation via its cooperative interaction with spectrin and protein 3 [40–42]. Growth hormone induces tyrosine phosphorylation of annexin I in rat osteosarcoma cells and EGF stimulates annexin I-dependent inward vesiculation in a multivesicular endosome subpopulation. During the processing of epidermal growth factor receptors, annexin I is phosphorylated in the multivesicular body [43–45].

C3orf6 protein (Ymer) is phosphorylated at tyrosine 145 and 146 upon EGF stimulation. Phosphorylated Ymer is also associated with EGF receptor and functions in its endocytosis and degradation [46]. Centaurins are a family of proteins that contain GTPase activating protein domains. It has been reported that centaurin- α 1 contributes to ERK activation in growth factor signaling, linking the PI3K pathway to the ERK mitogen-activated protein kinase pathway through its ability to interact with PIP3 [47].

Spermatogenesis associated PD1 (SPATA2) is highly hydrophilic, serine-rich, and contains 17 cysteine residues that are located predominantly in the C-terminal domain. It has been proposed that SPATA2 contains several potential phosphorylation sites [48].

With the aid of MMC or MMO resins, 22 distinct tyrosine phosphorylation sites and nine serine or threonine phosphorylation sites were characterized. With the exception of six residues, all of the phosphorylation sites have been previously reported by White's research group [39]. However, several novel phosphorylation sites on HER2 receptor, centaurin, epsin 2a, and EphA2 were identified (Table 2). Investigators studying HEK293 cells noted that, in response to fibronectin attachment, centaurin-3 (ARAP3) undergoes rapid and robust phosphorylation and that down regulation of centaurin-3 phosphorylation controls RhoGTPase and cell spreading [49]. The rat epsin and Eps15 are mitotic phosphoproteins and their mitotic phosphorylation inhibits binding to the clathrin adaptor AP-2, regulating clathrin-mediated endocytosis [50].

Some signaling molecules, such as MAP kinase, are phosphorylated on both tyrosine and threonine/serine residues. By using anti-phosphotyrosine antibodies to

immunoprecipitate protein and protein complexes rather than tryptic peptides, additional Ser or Thr phosphorylation sites may be identified on tyrosine phosphorylated proteins and their binding partners. For example, in addition to tyrosine phosphorylation, threonine and serine phosphorylation of EGFR were also observed. Threonine phosphorylation of EGFR has been shown to play an important role in regulating the tyrosine kinase activity of EGFR [51]. These results indicate that a combined approach of SILAC with IMAC or TiO₂ enables quantification of phosphorylation networks, even dynamic change at specific residues. Ubiquitination of the phosphorylated EGF receptor (EGFR) occurs upon binding Cbl, and this plays an important role in regulating both EGFR localization and degradation. It is therefore not unusual to find that tryptic peptides of ubiquitin recovered in the EGFR gel band respond to EGF stimulation with a 10-fold abundance change [52].

Conclusion

Characterization of metal chelate resins and metal oxide surfaces is most informative if performed in a quantitative fashion. In this study, we demonstrate that iTRAQ is an excellent approach to evaluate the performance of IMAC and metal oxide resins quantitatively. In general, the chelating resins in this study performed better when loaded with Fe³⁺ than those loaded with Ga³⁺. The MMC-Fe³⁺ described herein tends to have less nonspecific binding than the Poros 20MC-Fe³⁺ resins. Moreover, the specificity and sensitivity of MMC are comparable to MMO or TiO₂ spheres when the present protocol is employed. The enrichment of phosphopeptides from MMC or MMO virtually eliminated the problem of suppression by unphosphorylated peptides, although absolute purity of the eluates was not achieved.

To further evaluate the performance of MMC and MMO, we devised a combined approach of SILAC, immunoaffinity isolation of phosphoproteins and MMC or MMO capture of phosphopeptides to quantify the dynamic changes of phosphorylation in response to EGF stimulation. Previously, Mann et al. used SILAC approach to monitor tyrosine phosphorylation at the protein level without site specification [20]. White et al. combined iTRAQ and IMAC to study the systems biological response of EGF signaling cascades, generating time course studies of phosphorylation profiles for 78 tyrosine phosphorylation sites on 58 proteins [39].

In this study, we identified more than 62 proteins and 36 phosphopeptides up-regulated upon EGF stimulation, but we did not detect all expected phosphorylation sites. This could be partially explained by the fact that certain peptides were not amenable to MALDI-TOF-TOF or LC/MS analysis because of low abundance, unsuitable size, or poor ionization efficiency. Moreover, the neutral loss of phosphoric acid derived was observed as the most intense fragment ions in the

MS/MS spectra of many phosphopeptides, leaving the majority of the spectrum difficult to interpret. White et al. utilized an optimized IMAC protocol to enrich phosphorylated peptides from a tryptic digest of proteins isolated from liver lysate, reporting the identification of more than 300 phosphorylation sites when including Mascot Score 15 or above [53]. Although we have identified numerous putative phosphopeptides with Mascot Score 15 or above, we have not included those alignments in our report because the confidence of protein identification is insufficient. One-peptide hits within this combined dataset rank below 40% confidence. Although this is not inconsistent with other larger studies, Oda and colleagues demonstrate that MS/MS/MS analysis on peptides with neutral loss could dramatically improve the identification of phosphopeptides [54].

Taken together, immunoprecipitation by anti-phosphotyrosine antibody followed by IMAC or metal oxides has proven to be a successful approach to identify tyrosine phosphorylated proteins/peptides. Combining SILAC and IMAC, one could study the dynamic regulation of phosphorylation at specific sites, upon stimulation with growth factors or drug treatment.

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