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# Robust Phosphoproteomic Profiling of Tyrosine Phosphorylation Sites from Human T Cells Using Immobilized Metal Affinity Chromatography and Tandem Mass Spectrometry

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**Protein tyrosine phosphorylation cascades are difficult to analyze and are critical for cell signaling in higher eukaryotes. Methodology for profiling tyrosine phosphorylation, considered herein as the assignment of multiple protein tyrosine phosphorylation sites in single analyses, was reported recently (Salomon, A. R.; Ficarro, S. B.; Brill, L. M.; Brinker, A.; Phung, Q. T.; Ericson, C.; Sauer, K.; Brock, A.; Horn, D. M.; Schultz, P. G.; Peters, E. C. *Proc. Natl. Acad. Sci. U.S.A.* 2003, *100,* 443−448). The technology platform included the use of immunoprecipitation, immobilized metal affinity chromatography (IMAC), liquid chromatography, and tandem mass spectrometry. In the present report, we show that when using complex mixtures of peptides from human cells, methylation improved the selectivity of IMAC for phosphopeptides and eliminated the acidic bias that occurred with unmethylated peptides. The IMAC procedure was significantly improved by desalting methylated peptides, followed by gradient elution of the peptides to a larger IMAC column. These improvements resulted in assignment of ∼3-fold more tyrosine phosphorylation sites, from human cell lysates, than the previous methodology. Nearly 70 tyrosine-phosphorylated peptides from proteins in human T cells were assigned in single analyses. These proteins had unknown functions or were associated with a plethora of fundamental cellular processes. This robust technology platform should be broadly applicable to profiling the dynamics of tyrosine phosphorylation.**

Protein phosphorylation, catalyzed by enzymes termed protein kinases, is a common and important posttranslational modification.[1] The human genome encodes an estimated 518 kinases, including 58 receptor tyrosine kinases and 32 nonreceptor tyrosine kinases.[2] Tyrosine phosphorylation is important in the control of cell signaling in higher eukaryotes.[3] Stimulated receptor tyrosine kinases are rapidly activated by phosphorylation on tyrosine residues, which act as docking sites for assembly of multiprotein signaling complexes, leading to activation of downstream cellular signaling cascades.[3−6] Remarkable advances have been realized by characterizing signaling proteins individually or in small groups;[7] however, comprehensive analyses of the dynamics of tyrosine phosphorylation cascades will be required to improve our understanding of cell signaling.[1,6]

For a variety of reasons, including the low abundance of phosphorylated proteins, low stoichiometry of phosphorylation, and inefficient ionization of phosphopeptides, analysis of protein phosphorylation is challenging.[1,8] Medium- to low-abundance proteins often are not detected using two-dimensional (2D) gel electrophoresis.[9] In addition, if phosphorylated proteins are detected by 2D gel electrophoresis, assigning phosphorylation sites requires use of additional methods, such as 2D phosphopeptide mapping,[10] Edman degradation,[10,11] and mass spectrometry (MS).[12] However, protein phosphorylation can be assessed via immobilized metal affinity chromatography (IMAC), followed by liquid chromatography (LC) coupled to MS.[6,8,13−18] Because estimates suggest that only ∼0.05% of protein phosphorylation

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Table 1. Selectivity of IMAC for Phosphopeptides, Improved by Methylation[a]

| | ratio, phosphopeptides to non-phosphopeptides[b] | total percentage of acidic amino acid residues[c] |
|---|---|---|
| TP, unmethylated, $Ga^{3+}$-IMAC | 0.18 | 32.2 |
| TP, methylated, $Ga^{3+}$-IMAC | 2.67 (15[d]) | 14.0 |
| IP, unmethylated, $Ga^{3+}$-IMAC | 0.4 | 27.3 |
| IP, methylated, $Ga^{3+}$-IMAC | 8 (20) | 7.8 |
| TP, unmethylated, $Fe^{3+}$-IMAC | 0.08 | 29.7 |
| TP, methylated, $Fe^{3+}$-IMAC | 13.0 (163) | 10.3 |
| IP, unmethylated, $Fe^{3+}$-IMAC | 0.13 | 26.5 |
| IP, methylated, $Fe^{3+}$-IMAC | 45.0 (346) | 12.0 |

[a] T cells were treated with pervanadate and lysed. Total protein (TP) was prepared from the lysate, or immunoprecipitation (IP) of the lysate was performed with anti-phosphotyrosine antibodies. Proteins were digested with trypsin and peptides were desalted. One-half were methylated using established methods (see Experimental Section), dried, and resuspended in 0.1% HOAc. The other half (unmethylated) were directly resuspended in 0.1% HOAc. Peptides were enriched with IMAC method D, eluted to a RP precolumn, and subjected to nano-LC/ESI-MS/MS using a top 4 data-dependent, MS/MS method. MS/MS spectra were matched to peptide sequences using SEQUEST. The search parameters included no enzyme specificity for peptide sequences and differential phosphorylation of Y, S, and T residues. Peptides were considered to be correct when Xcorr was >2.5 for triply, >2.0 for doubly, and >1.5 for singly charged peptides (equal or greater stringency than previously applied; Link et al., *Nat. Biotechnol.* **1999**, *17*, 676) and when peptides were fully tryptic. Most of these predicted phosphopeptides were manually verified in other experiments (individual peptides are shown in Tables S1 and S2; all other peptides reported in this work were manually verified). [b] Number of phosphorylated peptides ÷ number of nonphosphorylated peptides. [c] [(number of aspartic acid residues + number of glutamic acid residues) ÷ total number of amino acid residues] × 100; the predicted average of acidic residues for the human genome = 10.7%. [d] Fold increase in the ratio of phosphorylated to nonphosphorylated peptides because of methylation.

occurs on tyrosine residues in higher eukaryotes,[1,19] profiling tyrosine phosphorylation is very challenging without enrichment of proteins containing phosphotyrosine residues.[6,20] Thus, anti-phosphotyrosine immunoprecipitation (IP[20]), methylation of peptides[8,15,21] in conjunction with IMAC, nanoflow reversed-phase (RP) HPLC (nano-LC)/electrospray ionization (ESI), and tandem MS (MS/MS) were combined previously in a powerful technology platform capable of identifying ~20 tyrosine phosphorylation sites per analysis.[6] In the present paper, the effect of peptide methylation on enrichment of peptides by IMAC is quantified, and significant improvements to IMAC methodology are presented. The improvements resulted in increased robustness and provided greater coverage of protein phosphorylation. Dozens of tyrosine phosphorylation sites per analysis were assigned to proteins (from human cell lysates), considered herein as profiling of tyrosine phosphorylation. Many of these proteins are known or proposed to be highly relevant to a plethora of fundamental biological processes, and numerous sites of phosphorylation were assigned on proteins with unknown functions.

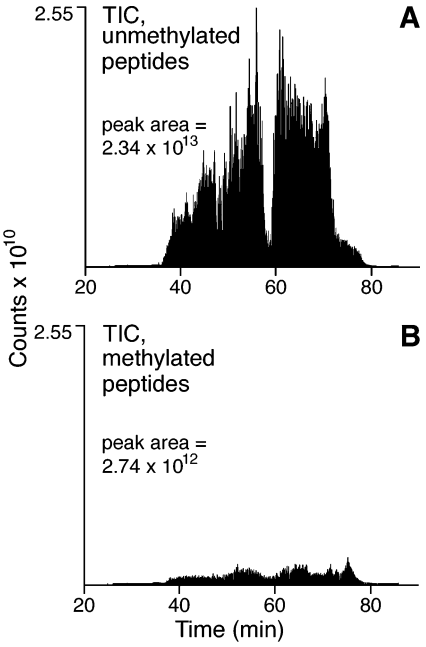


Figure 1. The total ion current (TIC) was dramatically reduced by methylation of a complex mixture of peptides that were subsequently enriched by IMAC. Jurkat T cell lysates were immunoprecipitated (IP) using anti-phosphotyrosine antibodies and digested with trypsin. Peptides were either directly enriched with $Fe^{3+}$-IMAC, method D (A), or methylated using a standard procedure, then enriched with $Fe^{3+}$-IMAC, method D (B), and analyzed using nano-LC/ESI-MS/MS. A top four data-dependent, MS/MS method was used (see Experimental Section). The TIC of combined ESI-MS and MS/MS scans and the cumulative peak area of ESI-MS scans is shown (A, B).

## EXPERIMENTAL SECTION

**Protein Preparation, Digestion, Cell Culture, and IP.** To prepare a standard peptide mixture containing tyrosine phosphorylated peptide standards, bovine serum albumin, bovine heart cytochrome *c*, ovalbumin, and phosvitin (Sigma Chemical Co., St. Louis, MO) were solubilized at 100 pmol/$\mu$L in 100 mM $NH_4HCO_3$ buffer, pH 8.3. Twelve microliters of each protein and 1 $\mu$L (1 nmol) of the synthetic standard LIEDNEpYTAR were added to 500 $\mu$L of 100 mM $NH_4HCO_3$ buffer, pH 8.3 containing 8.8 M urea, and placed at 96 °C for 4 min. Water (540 $\mu$L) and 9 $\mu$L (4.5 $\mu$g) of modified trypsin (Promega Inc., Madison, WI) were added, the mixture was incubated at 37 °C overnight, and 100-$\mu$L aliquots were stored at −80 °C. Immediately before methylation, synthetic DRVpYIHPF (angiotensin II phosphate) was added to the mixture at a final concentration of 500 fmol/$\mu$L.

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Table 2. Comparison of Methylation Efficiency between Two Methylation Procedures[a]

| | Fully Methylated LIEDAEpYTAK[b,c] | Trimethylated LIEDAEpYTAK[b] | Fully Methylated/ Trimethylated |
|---|---|---|---|
| established | $2.59 \times 10^{10}$ | $8.10 \times 10^{8}$ | 32.0:1 |
| modified | $6.16 \times 10^{9}$ | $4.12 \times 10^{9}$ | 1.50:1 |
| | **Fully Methylated DRVpYIHPF[b,d]** | **Hemimethylated DRVpYIHPF[b]** | **Fully Methylated/ Hemimethylated** |
| established | $2.81 \times 10^{10}$ | $4.53 \times 10^{8}$ | 62.0:1 |
| modified | $1.72 \times 10^{10}$ | $5.43 \times 10^{9}$ | 3.17:1 |

[a] A two-peptide mixture containing LIEDAEpYTAK and DRVpYIHPF was methylated either as described in the Experimental Section (established) or with another procedure (modified) as described elsewhere (see ref 16). An aliquot (2.5 pmol of each peptide) was loaded onto a RP precolumn (no IMAC) and subjected to nano-LC/ESI-MS, and SIC peak areas were quantified using Xcalibur version 1.3 software (Thermo Finnigan, Inc., San Jose, CA). [b] Counts in SIC peak area. [c] This peptide contains four carboxyl groups. [d] This peptide contains two carboxyl groups.

Table 3. Ratio of Fully to Partially Methylated Phosphopeptides Recovered from IMAC, Improved by Desalting[a]

| | Fully Methylated LIEDNEpYTAR[b] | Trimethylated LIEDNEpYTAR[b] | Fully Methylated/ Trimethylated |
|---|---|---|---|
| RP only (no IMAC) | $1.47 \times 10^{10}$ | $2.27 \times 10^{9}$ | 6.48:1 |
| IMAC, RP (method D) | $6.27 \times 10^{9}$ | $7.67 \times 10^{9}$ | 0.82:1 |
| desalt, IMAC, RP (method C) | $5.72 \times 10^{9}$ | $1.99 \times 10^{9}$ | 2.87:1 |
| | **Fully Methylated DRVpYIHPF[b]** | **Hemimethylated DRVpYIHPF[c]** | **Fully Methylated/ Hemimethylated** |
| RP only (no IMAC) | $6.56 \times 10^{9}$ | $1.49 \times 10^{9}$ | 4.40:1 |
| IMAC, RP (method D) | $2.06 \times 10^{9}$ | $1.72 \times 10^{9}$ | 1.20:1 |
| desalt, IMAC, RP (method C) | $2.60 \times 10^{9}$ | $8.07 \times 10^{8}$ | 3.22:1 |

[a] A four-protein mixture containing LIEDNEpYTAR was digested with trypsin; DRVpYIHPF was added; and the peptides were methylated (established procedure), dried, and resuspended. An aliquot was loaded directly onto a RP precolumn and subjected to nano-LC/ESI-MS/MS (no IMAC). An equal aliquot was loaded onto an IMAC column (method D), and another equal aliquot was loaded onto a RP desalting column, rinsed with 0.1% HOAc, and eluted to the IMAC column using 70% ACN/0.1% HOAc (method C). Peptides were eluted from each IMAC column to a RP precolumn and detected using a top 1 data-dependent, targeted MS/MS method; and SIC peak areas were quantified using Xcalibur version 1.3 software. [b] Counts in SIC peak area (similar to peaks in Figure 2B, C, and E). [c] Counts in SIC peak areas (similar to peaks in Figure 2F); summarized from peaks 1 and 2.

To assign endogenous cellular protein phosphorylation sites, Jurkat (T cell leukemia) clone E6-1 was obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 $\mu$g/mL streptomycin sulfate, and 100 U/mL penicillin G (all from Sigma) in a 5.0% $CO_2$ incubator at 37 °C. Cells were treated with pervanadate to inhibit tyrosine phosphatases and mimic the effect of T cell antigen receptor complex (TCR) ligation.[22] A pervanadate stock was freshly prepared before each use: 100 mM $Na_3VO_4$ was added to an equal volume of 10 mM $H_2O_2$ (Sigma), incubated at ~22 °C for 20 min, then stored on ice until use. Jurkat cells were washed with RPMI lacking FBS and resuspended at $\sim 1 \times 10^7$ cells/mL in RPMI containing 500 $\mu$M pervanadate and lacking FBS, for 20 min at 37 °C, 5% $CO_2$. Cells ($\sim 1 \times 10^9$) were washed with RPMI lacking FBS at 4 °C, then lysed for 20 min with rotation at 4 °C in 25 mL of lysis buffer [20 $\mu$g/mL aprotinin, 20 $\mu$g/mL leupeptin, 50 mM Tris pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Perfabloc, 2 mM $Na_3VO_4$ (tyrosine phosphatase inhibitor), 10 mM $\beta$-glycerophosphate (serine/threonine phosphatase inhibitor), and 1 mM EDTA (Sigma)].

To prepare a total protein digest, the NP-40 lysate was dialyzed against 50 mM $NH_4HCO_3$ buffer, pH 8.3, containing 4 M Urea. Proteins were isolated using Trizol (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions. Protein pellets were resuspended in 100 mM $NH_4HCO_3$ buffer, pH 8.3 containing 8 M urea, then diluted with an equal volume of water. Modified trypsin was added, 1:20 (w/w) trypsin/total protein, and the mixture was incubated overnight at 37 °C. Aliquots of tryptic digests (from ~225 $\mu$g protein, in 100 $\mu$L) were either desalted and methylated (established procedure, below) or desalted for direct analysis by IMAC method D/nano-LC/ESI-MS/MS (top 4, data-dependent method; below).

For IP, lysates from T cells were centrifuged at 12000*g* for 20 min at 4 °C. Ten picomoles of LIEDNEpYTAR and anti-phosphotyrosine agarose (Sigma; clone PT66; 150 $\mu$L resin/$\sim 1 \times 10^9$ cell equivalents) was added to the supernatant for 4 h at 4 °C with rotation. Beads were washed once with 50 mL of lysis buffer and once with 50 mL of 20 mM Tris buffer, pH 7.4, 120 mM NaCl. Proteins were recovered from the beads with 100 mM $NH_4HCO_3$ buffer, pH 8.3 containing 8 M urea for 5 min at 96 °C, and the supernatant was filtered using PVDF membranes with a pore size of 0.2 $\mu$m (Millipore Inc., Bedford, MA). The mixture was diluted with an equal volume of water and proteins were digested overnight with 5 $\mu$g of modified trypsin at 37 °C.

**Peptide Methylation.** Peptide methylation (established protocol) was essentially as described.[6,8,15,21] Briefly, 10 pmol of DRVpYIHPF was added to tryptic digests from $\sim 1 \times 10^9$ cell equiv

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Table 4. Recovery of Phosphopeptides, Increased by Increased IMAC Capacity[a]

| | Fully Methylated LIEDNEpYTAR[b] | Trimethylated LIEDNEpYTAR[b] | Fully Methylated/ Trimethylated |
|---|---|---|---|
| smaller IMAC (method C) | $9.01 \times 10^9$, 1.00× yield[c] | $5.31 \times 10^9$, 1.00× yield | 1.70:1 |
| larger IMAC (method B) | $2.27 \times 10^{10}$, 2.52× yield | $1.40 \times 10^{10}$, 2.64× yield | 1.62:1 |
| | Fully Methylated DRVpYIHPF[b] | Hemimethylated DRVpYIHPF[d] | Fully Methylated/ Hemimethylated |
| smaller IMAC (method C) | $4.19 \times 10^{10}$, 1.00× yield | $5.41 \times 10^{10}$, 1.00× yield | 0.77:1 |
| larger IMAC (method B) | $1.07 \times 10^{11}$, 2.55× yield | $1.34 \times 10^{11}$, 2.48× yield | 0.80:1 |

| | No. of Endogenous Phosphopeptides |
|---|---|
| smaller IMAC (method C) | 2 |
| larger IMAC (method B) | 7 |

[a] Jurkat T cells were treated with pervanadate and lysed; LIEDNEpYTAR was added; IP of the lysate was performed with anti-phosphotyrosine antibodies; proteins recovered from IP were digested with trypsin; DRVpYIHPF was added; peptides were desalted, methylated, dried, and resuspended. Half (method C) were loaded onto a RP desalting column, rinsed, and eluted, to a IMAC column, as in Table 3. The other half (method B) were processed identically, except that the IMAC column had a larger capacity (see the text). Elution from IMAC to a RP precolumn, MS analysis and peak area quantification were as in Table 3. [b] Counts in SIC peak area (similar to peaks in Figure 2B, C, and E). [c] The number of counts recovered using the smaller IMAC column was considered 1.00× yield to clarify the increase due to the larger IMAC column. [d] Counts summarized from peaks 1 and 2 (similar to peaks in Figure 2F).

Table 5. Increased Phosphopeptide Recovery from Gradient Elution from the Desalting Column to the IMAC Column[a]

| | Fully Methylated LIEDNEpYTAR[b] | Trimethylated LIEDNEpYTAR[b] | Fully Methylated/ Trimethylated |
|---|---|---|---|
| 70% ACN elution (method B) | $2.91 \times 10^{10}$, 1.00× yield[c] | $2.24 \times 10^{10}$, 1.00× yield | 1.30:1 |
| gradient elution (method A) | $3.08 \times 10^{10}$, 1.06× yield | $2.74 \times 10^{10}$, 1.22× yield | 1.12:1 |
| | Fully Methylated DRVpYIHPF[b] | Hemimethylated DRVpYIHPF[d] | Fully Methylated/ Hemimethylated |
| 70% ACN elution (method B) | $6.86 \times 10^{10}$, 1.00× yield | $1.55 \times 10^{11}$, 1.00× yield | 0.44:1 |
| gradient elution (method A) | $1.93 \times 10^{11}$, 2.81× yield | $1.08 \times 10^{11}$, 0.70× yield | 1.79:1 |

| | No. of Endogenous Phosphopeptides |
|---|---|
| 70% ACN elution (method B) | 2 |
| gradient elution (method A) | 7 |

[a] Peptides from Jurkat T cells and standard peptides were prepared as in Table 4. One-half as many cell equivalents per analysis were used, as compared to Table 4. One-half of the methylated peptides were loaded onto a RP desalting column, washed, and eluted to a larger IMAC column using 70% ACN, as in Table 4 (method B). The other half was processed in the same manner, except that elution from the desalting to the IMAC column was performed using a 0−70% ACN gradient (method A). Phosphopeptide analysis was as in Table 4. [b] Counts in SIC peak area (similar to peaks in Figure 2B, C, E). [c] The number of counts recovered using 70% ACN elution is considered to be 1.00× yield. [d] Counts summarized from peaks 1 and 2 (similar to peaks in Figure 2F).

(above), and the mixture was loaded onto C18 RP peptide macrotrap cartridges (Michrom Bioresources, Auburn, CA) and washed with 1 mL of 0.1% acetic acid (HOAc) to remove urea and salts from the digest. Peptides were eluted with 500 $\mu$L of 70% acetonitrile (ACN)/0.1% HOAc and dried in a speed vac (Thermo Savant, Holbrook, NY). Peptide methylation was at room temperature for 2 h with 2 N d3-methanolic DCl under nitrogen. Methanolic DCl was removed in a speed vac, and the sample was reconstituted in 0.1% HOAc and stored at −80 °C until phosphopeptides were enriched by IMAC. Aliquots of standard protein digests were methylated using the same procedures.

To compare methylation protocols, two synthetic peptide standards, LIEDAEpYTAK and DRVpYIHPF (100 pmol each) were desalted with macrotrap cartridges and dried as described above. Two aliquots were methylated in 100 $\mu$L of methanolic DCl for 2 h. One was dried as described above, and the other (modified procedure) was processed as described elsewhere.[16] The modified procedure was used in only one series of experiments (specified in the Results and Discussion).

**IMAC.** Different IMAC methods were explored, starting with method D and culminating with method A. Method D was as described.[6] Briefly, peptides were directly loaded onto $Ga^{3+}$- or $Fe^{3+}$-activated IMAC columns (360-$\mu$m o.d. × 100-$\mu$m i.d. fused silica (Polymicro Technologies, Inc., Phoenix, AZ) packed with 8 cm of POROS 20 MC resin (Perceptive Biosystems, Inc., Framingham, MA) at a flow rate of ∼1 $\mu$L/min. The IMAC column was washed at ∼5 $\mu$L/min with 20 $\mu$L of 25/74/1 ACN/water/HOAc containing 100 mM NaCl, followed by 10 $\mu$L of 0.1% HOAc at ∼5 $\mu$L/min. Peptides were eluted from IMAC columns to micro capillary RP precolumns using 5 $\mu$L of 50 mM $Na_2HPO_4$ buffer, pH 9.0 at ∼2 $\mu$L/min. The precolumn was immediately rinsed with 10 $\mu$L of 0.1 M HOAc at ∼5 $\mu$L/min.

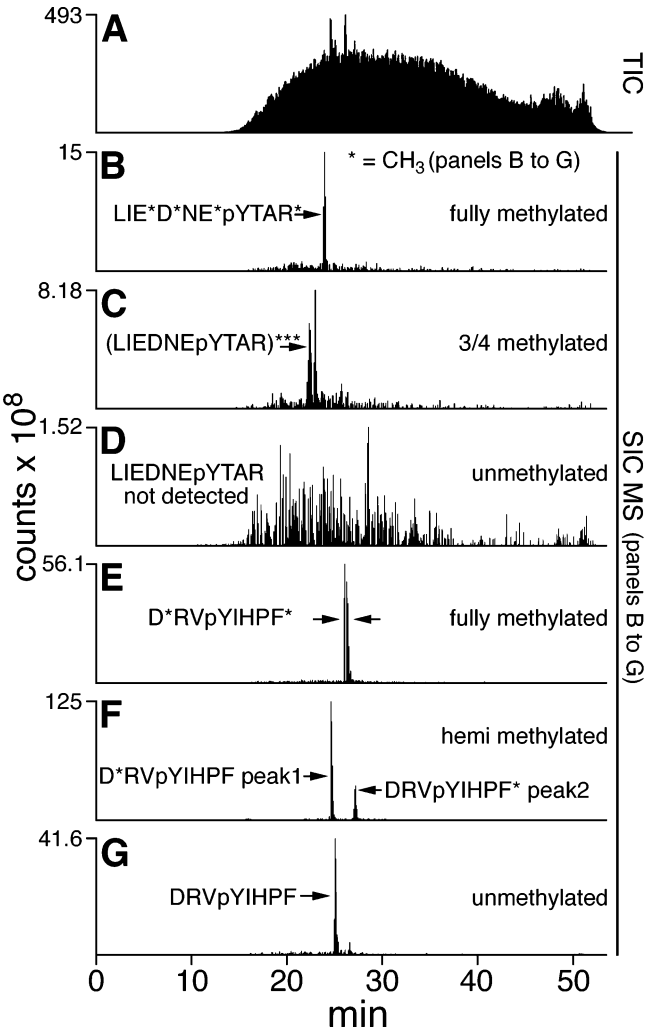


Figure 2. Nano-LC elution profiles of standard peptides enriched from a complex mixture of peptides using IMAC. Jurkat T cell lysates and the standard LIEDNEpYTAR were IP using anti-phosphotyrosine antibodies and digested with trypsin; a second standard (DRVpYIHPF) was added; and peptides were methylated, subjected to IMAC, and analyzed using nano-LC/ESI-MS/MS. A top 1 data-dependent targeted MS/MS method was used (see Experimental Section). Asterisks (*) represent $d_3$-methylated carboxylic acid groups (panels B−G). (A) TIC of MS and MS/MS scans. (B−G) Selected ion current (SIC) of ESI mass spectra detecting precursor ions from fully methylated LIEDNEpYTAR (B) and trimethylated LIEDNEpYTAR (C). The identity of the second peak is unknown, but similar peaks were usually not detected (C). (D) Unmethylated LIEDNEpYTAR was not detected. Precursor ions from fully methylated (E), hemimethylated (F; two peaks), and unmethylated (G) DRVpYIHPF were also detected in SICs. The entire peak in panel E (split, due to absence of signal in one full scan mass spectrum) was D*RVpYIHPF*. The identities and methylation status of the precursor ions (B, C, and E−G) were confirmed using MS/MS scans (Figures 3 and 4).

Methods C−A used only $Fe^{3+}$-IMAC. Method C was the same as method D with two exceptions: First, evaporative removal of methanolic DCl was from 1.5-mL microtubes (Sarstedt, Inc., Newton, NC) with a hole in the cap made with a 26-gauge needle to reduce peptide losses by controlling bumping, rather than from siliconized 0.6-mL tubes (Fisher Scientific) with the caps open. Second, methylated peptides were loaded onto a RP desalting column (360-$\mu$m o.d. × 200-$\mu$m i.d., fused silica; Polymicro Technologies) packed with 12 cm of YMC 5-15 $\mu$m C18 resin (Waters Inc., Milford, MA), desalted for 10 min with 0.1% HOAc at ~7 $\mu$L/min and eluted to the IMAC column with 70% ACN/0.1% HOAc at ~1 $\mu$L/min for 20 min, then at ~3 $\mu$L/min for 20 min.

Method B was the same as method C with the exception that a larger IMAC column (360-$\mu$m o.d. × 200-$\mu$m i.d. fused silica, packed with 15 cm of Poros 20 MC resin) was used. Following elution of the peptide methyl esters from the RP desalting to the IMAC column, the IMAC column was washed with 25/74/1 ACN/water/HOAc containing 100 mM NaCl at ~15 $\mu$L/min for 5 min, followed by 0.1% HOAc at ~20 $\mu$L/min for 2.5 min. Enriched phosphopeptides were eluted from IMAC to a RP precolumn using 12 $\mu$L of 50 mM $Na_2HPO_4$ buffer, pH 9.0, at ~2 $\mu$L/min.

Method A was the same as method B with the exception that elution of peptides from the desalting column to the IMAC column was with a 0−70% solvent B gradient in 17 min, then 70−95% B in 1 min, at a flow rate of 1.8 $\mu$L/min (solvent A = 0.1 M HOAc in water; solvent B = 0.1 M HOAc in ACN).

**Nano-LC/ESI-MS/MS.** Peptides were analyzed by a nano-LC/ESI system modified from that described previously,[23] coupled to an LCQ DECA quadrupole ion trap mass spectrometer (Thermo Finnigan, Inc., San Jose, CA) operating in the positive ion mode. Micro capillary RP precolumns containing peptides were connected to RP analytical columns [360-$\mu$m o.d. × 50-$\mu$m i.d. fused silica packed with 8 cm of 5-$\mu$m C18 particles with integrated ESI emitter tips (~5 $\mu$m i.d.)]. Peptides were eluted into the mass spectrometer with an HPLC gradient (0−70% B in 30 min; solvents A and B as above). Initially, the flow rate at the emitter tip was set to ~100 nl/min. When the sample started to elute, the flow rate was lowered to ~25 nL/min by lowering the HPLC flow rate ("peak parking"). The ESI voltage (1.8 kV) was applied to the HPLC mobile phase before flow splitting. When analyzing LIEDAEpYTAK and DRVpYIHPF in samples comparing methylation procedures, the mass spectrometer recorded ESI mass spectra only. When targeting LIEDNEpYTAR and DRVpYIHPF in samples originating from standard proteins or from pervanadate-stimulated T cells, the mass spectrometer recorded continuous cycles of one ESI mass spectrum, followed by an MS/MS scan of the most abundant ion in each ESI mass spectrum and then MS/MS scans of precursor ions at $m/z$ 686.7 and 581.1 corresponding to fully methylated LIEDNEpYTAR and DRVpYIHPF, respectively. Dynamic exclusion, applied only to the most abundant ion from each MS scan, specified a repeat count of two and exclusion duration of 1.0 min (top 1 data-dependent, targeted MS/MS). When detecting synthetic standards and assigning the maximum possible number of phosphorylation sites from pervanadate-stimulated T cells, the mass spectrometer recorded continuous cycles composed of one ESI mass spectrum followed by MS/MS scans of the four most abundant ions in each ESI mass spectrum. Dynamic exclusion specified a repeat count of one, and exclusion duration of 1.5 min (top 4 data-dependent MS/MS).

**Database Analysis.** Tandem mass spectra were matched to amino acid sequences in the NCBI nonredundant human protein database using SEQUEST (Thermo Finnigan[24]). Search parameters specified a differential modification of +80 Da to serine,

(23) Martin, S. E.; Shabanowitz, J.; Hunt, D. F.; Marto, J. A. *Anal. Chem.* **2000**, *72*, 4266−4274.
(24) Eng, J.; McCormack, A.; Yates, J. R. *J. Am. Soc. Mass Spec.* **1994**, *5*, 976−989.

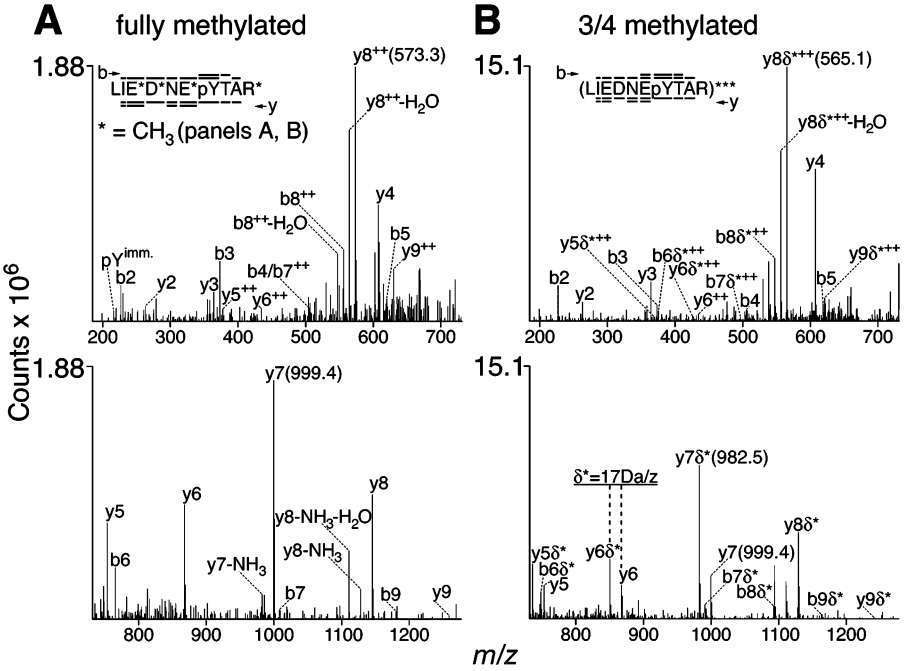


Figure 3. The identity of LIEDNEpYTAR was confirmed, and its methylation state was analyzed using MS/MS scans. Spectra are displayed on two axes to show spectral detail (A and B). Asterisks (*) represent $d_3$-methyl groups (17.0 Da). The *m*/*z* of the y7 and $y8^{2+}$ ions is shown for comparison (A and B), demonstrating that an extra methyl group increases the *m*/*z* of product ions. (A) MS/MS spectrum of fully methylated LIEDNEpYTAR ions (from the precursor peak shown in Figure 2B; doubly charged; *m*/*z* = 686.7). Assigned product ions are indicated. The b ions (singly and doubly charged) are shown as over-lines on the peptide sequence, and the y ions are shown as underlines. (B) MS/MS spectrum of trimethylated LIEDNEpYTAR ions (from the precursor peak shown in Figure 2C; *m*/*z* = 678.2; doubly charged). Product ions with a free carboxyl group are followed by "$\delta$*". Product ion identities, overlines and underlines are as in (A); only $\delta$* product ions are shown on the peptide sequence. Fully methylated and $\delta$* product ion pairs (e.g., y5 and y5$\delta$*) spanned the peptide sequence (B only).

threonine, and tyrosine residues (phosphorylation) plus a static modification of +17 Da to glutamic acid and aspartic acid residues and the C terminus of each peptide (deuterated methylation). The reported SEQUEST assignments were verified by manual interpretation of MS/MS spectra except in one experiment where specified.

## RESULTS AND DISCUSSION

**Improvement of an IMAC/Nano-LC/ESI-MS/MS Platform for Assignment of Tyrosine Phosphorylation Sites.** Because protein tyrosine phosphorylation sites are important for cell signaling and a challenge to assign,[1,6,8] our goal was an improved IMAC/nano-LC/ESI-MS/MS platform for robust assignment of a more comprehensive profile of tyrosine phosphorylation. Methylation of peptides was reported to enhance selective enrichment of phosphopeptides by IMAC in some systems,[6,8,15] but not necessarily in others.[14,16−18] One group suggested that methylation frequently did not increase the selectivity of IMAC for phosphopeptides from a recombinant eukaryotic protein purified from bacteria via the use of a histidine tag.[16] However, the peptide methylation procedures that were used and the inclusion of reduction and alkylation procedures[16] differed from the procedures in other studies.[6,8,15] In other systems, phosphopeptides from pools of purified class I major histocompatibility complex molecules[14] and tryptic digests of the inner surfaces of purified *Arabidopsis* plasma membranes[17] were enriched by IMAC in the absence of peptide methylation. The success of these studies may have been influenced by the specific nature of the peptides analyzed. In contrast, methylation was an integral component of IMAC analyses of phosphopeptides from whole yeast cell lysates,[8] capacitated human sperm,[15] and phosphopeptides from IP of human T cell lysates with anti-phosphotyrosine antibodies.[6]

Thus, to better understand the effect of peptide methylation on IMAC enrichment, we compared the selectivity of IMAC for methylated or unmethylated peptides prepared from either total protein or IP of lysates from pervanadate-treated human T cells. Pervanadate inhibits tyrosine phosphatases and mimics the effect of TCR ligation.[22] Peptides from these complex mixtures were enriched by IMAC method D, eluted to a RP precolumn and analyzed using nano-LC/ESI-MS/MS. Without methylation, $Ga^{3+}$- and $Fe^{3+}$-IMAC enriched predominantly unphosphorylated, acidic peptides (Tables 1, S1). For example, in the $Fe^{3+}$-IMAC analysis of unmethylated peptides derived from IP, the 50 SEQUEST-derived peptide sequences with the highest Xcorr values corresponded to nonphosphorylated peptides. In striking contrast, peptide methylation resulted in dramatic improvement in selective recovery of phosphopeptides without a significant bias toward acidic sequences (Tables 1, S2). The 43 methylated peptides with the highest Xcorr values were phosphorylated, and only one peptide in the collection lacked a phosphorylation site. Furthermore, the cumulative area of the TIC following peptide recovery from IMAC was reduced by methylation (Figure 1). Taken together, these results demonstrate that in the absence of methylation, predominantly nonphosphorylated, acidic peptides from human T cells were recovered from IMAC and that methylation increased selective recovery of phosphopeptides by IMAC.

Because methylation increased the selectivity of IMAC for phosphopeptides (Tables 1, S1, S2), it is important to use

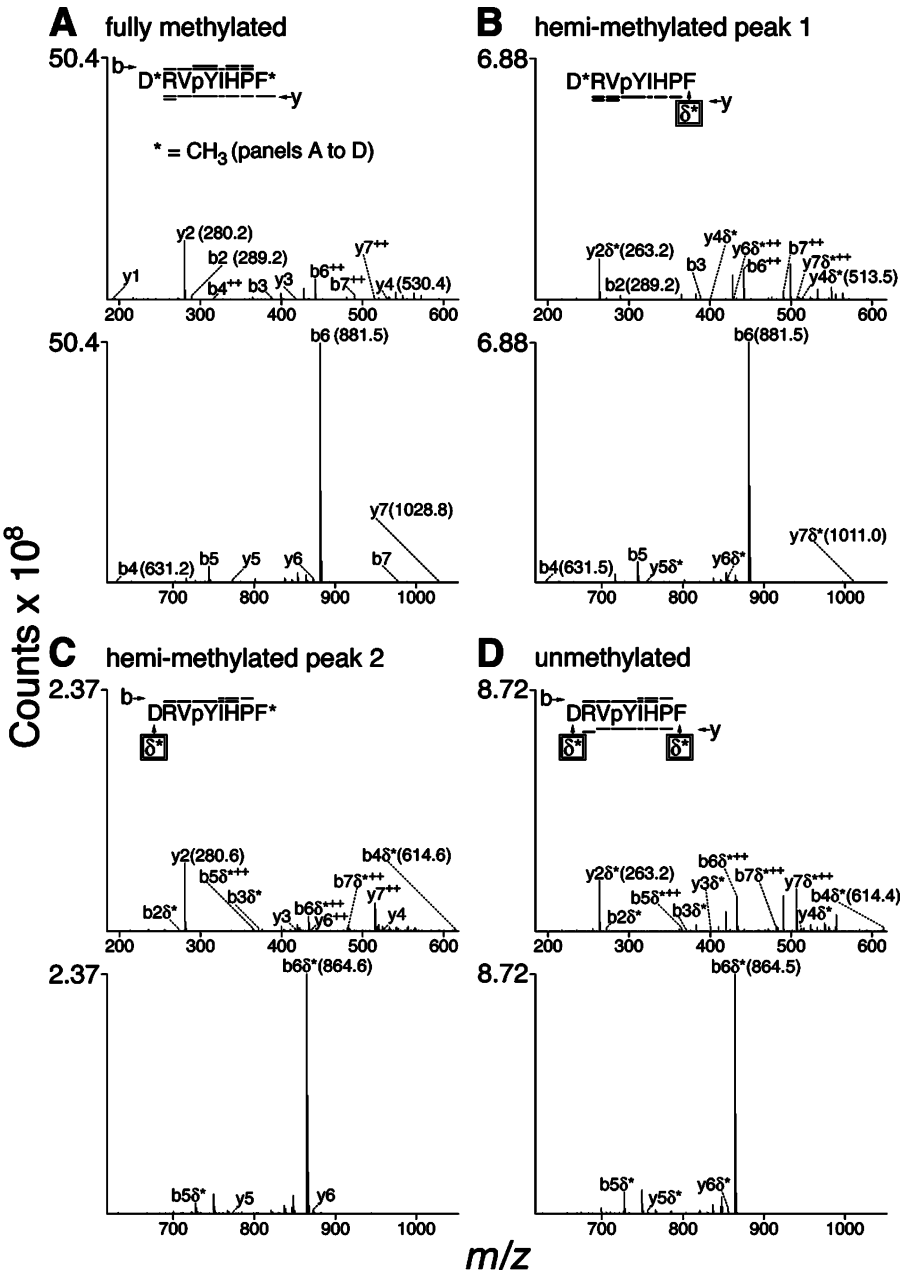


Figure 4. The identity of DRVpYIHPF (Figure 2E−G) was confirmed, and its methylation state was analyzed using MS/MS scans. Spectra are displayed on two axes to show spectral detail (A−D). Asterisks (*) represent deuterated methyl groups. The *m*/*z* of selected product ions is shown for comparison of methylation sites of the peptides (A−D). Identities of the assigned product ions and designation of the b and the y ions is as in Figure 3. (A) MS/MS spectrum of fully methylated DRVpYIHPF ions (from the precursor ion peak shown in Figure 2E; doubly charged; *m*/*z* = 581.1). (B) MS/MS spectrum of hemimethylated DRVpYIHPF ions, peak 1 (from the precursor ion peak 1, Figure 2F; doubly charged; *m*/*z* = 572.6). Only product ions containing a free carboxyl group are shown in the sequence (B−D). (C) MS/MS spectrum of hemimethylated DRVpYIHPF ions, peak 2 (from the precursor ion peak 2, Figure 2F; doubly charged; *m*/*z* = 572.6). (D) MS/MS spectrum of unmethylated DRVpYIHPF ions (from the precursor ion peak in Figure 2G; doubly charged; *m*/*z* = 564.1).

conditions that drive the methylation reaction to the maximum possible degree of completion. We therefore compared a modified methylation procedure[16] to an established procedure[21] for converting phosphopeptide standards to their methyl esters. Following the reactions, phosphopeptide standards were analyzed by RP-HPLC/MS. The established procedure resulted in an ~20-fold higher ratio of fully to partially methylated, tyrosine-phosphorylated synthetic peptides (Table 2). The modified procedure also resulted in a greater abundance of peptides with more than one unmethylated carboxyl group (not shown). Therefore, the established procedure was used for all additional experiments.

To further improve detection of tyrosine-phosphorylated peptides, we performed detailed studies of IMAC recovery of methylated standards using $Fe^{3+}$-IMAC. To ensure that IMAC recovery of methylated, standard, tyrosine phosphorylated peptides from complex mixtures could be monitored, the T cells were treated with pervanadate and lysed. The standard peptide LIEDNEpYTAR was added to cell lysates, anti-phosphotyrosine IP was performed on the lysate, proteins were recovered and digested with trypsin, and the standard peptide DRVpYIHPF was added. The resulting complex mixture was methylated, and peptides were enriched by IMAC, eluted to a RP precolumn, and analyzed using nano-LC/

Table 6. Recovery of Fully Methylated Phosphopeptide Standards and Assignment of Tyrosine Phosphorylation Sites on Endogenous Cellular Proteins, Highest with IMAC Method A[a]

| IMAC Method, Analysis No. | Fully Methylated LIEDNEpYTAR (A)[b] | Trimethylated LIEDNEpYTAR (B)[b] |
|---|---|---|
| A, 1 (desalt, larger IMAC, gradient elution) | $2.76 \times 10^9$ | $5.80 \times 10^8$ (4.76:1[c]) |
| A, 2 | $3.33 \times 10^9$ | $8.42 \times 10^8$ (3.95:1) |
| B, 1 (desalt, larger IMAC, 70% ACN elution) | $9.97 \times 10^8$ | $2.95 \times 10^8$ (3.38:1) |
| B, 2 | $4.21 \times 10^8$ | $3.31 \times 10^8$ (1.27:1) |
| C, 1 (desalt, smaller IMAC, 70% ACN elution) | $3.52 \times 10^8$ | $1.87 \times 10^8$ (1.88:1) |
| C, 2 | $2.25 \times 10^8$ | $1.47 \times 10^8$ (1.53:1) |
| D, 1 (smaller IMAC) | $1.69 \times 10^8$ | N/D (N/A)[d] |
| D, 2 | $2.16 \times 10^8$ | $9.41 \times 10^7$ (2.30:1) |

| | Fully Methylated DRVpYIHPF (C)[b] | Hemimethylated DRVpYIHPF(D)[e] |
|---|---|---|
| A, 1 (desalt, larger IMAC, gradient elution) | $1.20 \times 10^{10}$ | $3.83 \times 10^8$ (31.33:1[c]) |
| A, 2 | $1.40 \times 10^{10}$ | $2.95 \times 10^8$ (47.46:1) |
| B, 1 (desalt, larger IMAC, 70% ACN elution) | $2.71 \times 10^9$ | $3.62 \times 10^8$ (7.49:1) |
| B, 2 | $2.53 \times 10^8$ | $1.95 \times 10^8$ (1.30:1) |
| C, 1 (desalt, smaller IMAC, 70% ACN elution) | $6.78 \times 10^8$ | $2.70 \times 10^8$ (2.51:1) |
| C, 2 | $4.04 \times 10^8$ | $2.71 \times 10^8$ (1.49:1) |
| D, 1 (smaller IMAC) | $1.05 \times 10^9$ | $3.20 \times 10^8$ (3.28:1) |
| D, 2 | $9.31 \times 10^8$ | $3.34 \times 10^8$ (2.79:1) |

| | No. of Endogenous Phosphopeptides (E) |
|---|---|
| A, 1 (desalt, larger IMAC, gradient elution) | 113[f] (69[g]) |
| A, 2 | 105 (61) |
| B, 1 (desalt, larger IMAC, 70% ACN elution) | 58 (36) |
| B, 2 | 55 (30) |
| C, 1 (desalt, smaller IMAC, 70% ACN elution) | 56 (30) |
| C, 2 | 50 (22) |
| D, 1 (smaller IMAC) | 32 (21) |
| D, 2 | 38 (26) |

[a] Peptides from Jurkat T cells ($1 \times 10^8$ cell equiv/analysis) and standard peptides were prepared as in Table 4, then subjected to the IMAC procedures summarized in parentheses. Replicate analyses and different methods were all executed back-to-back. Enriched phosphopeptides were eluted to a RP precolumn, and nano-LC/ESI-MS/MS was performed using a top 4 data-dependent MS/MS method (see Experimental Section). Peak areas of the standard peptides were quantified as described in Table 3. All accepted SEQUEST assignments were manually verified. [b] Counts in SIC peak area (similar to peaks in Figure 2B, C, E). [c] Ratio, fully methylated/tri- or hemimethylated. [d] Not detected (not applicable). [e] Counts summarized from peaks 1 and 2 (similar to peaks in Figure 2F). [f] Total number of peptides from the cells with assigned phosphorylation sites on any residue, that is, pY, pS, pT; see Table S3. [g] This is the number of phosphotyrosine-containing peptides from the cells with assigned phosphorylation sites. Some of these phosphopeptides also contained pS or pT sites, whereas others contained only pY sites; each peptide is shown in Table S3.

ESI-MS/MS. Peaks corresponding to LIEDNEpYTAR and DRVpYIHPF were detected in ESI mass spectra (Figure 2). Fully and trimethylated LIEDNEpYTAR were confirmed using MS/MS scans, whereas unmethylated LIEDNEpYTAR was not detected (Figure 2B−D; Figure 3). Singly and doubly methylated LIEDNEpYTAR were sporadically detected (data not shown), so their analysis was not further pursued. As expected, fully methylated LIEDNEpYTAR yielded only fully methylated product ions (Figure 3A). In contrast, trimethylated LIEDNEpYTAR yielded product ions that were fully or partially methylated, and the unmethylated carboxyl group was apparently distributed among the four positions (Figure 3B). Similarly, fully, hemi-, and unmethylated DRVpYIHPF (Figure 2E−G) were identified by MS/MS (Figure 4). Hemimethylated DRVpYIHPF peak 1 (Figure 2F) contained an unmethylated carboxyl group at its C terminus (Figure 4B) and hemimethylated DRVpYIHPF peak 2 (Figure 2F) contained an unmethylated carboxyl group on the aspartic acid side chain (Figure 4C), showing that when methylated at different positions, this hemimethylated standard was resolved chromatographically. Thus, having identified the methylation states of IMAC-enriched, phosphotyrosine-containing standards in a complex mixture (Figures 2−4), we reasoned that signals from these peptides could be quantified to measure the recovery of tyrosine phosphorylated peptides as IMAC conditions were varied.

Initially, improvement in recovery from IMAC was monitored in a less complex mixture. The standards were added to a four-protein digest, the mixture was methylated, IMAC was performed, and the standards were quantified from their signals in ESI mass spectra. The mass spectrometer selected the most abundant precursor ion for MS/MS and then targeted the standard peptides for MS/MS during each MS plus MS/MS cycle (top 1 data-dependent, targeted MS/MS; see the Experimental Section). Direct loading of methylated peptides on the IMAC column (IMAC method D) resulted in selective recovery of partially methylated standards, but desalting methylated peptides before IMAC (IMAC method C) improved the ratio of recovery of fully to partially methylated standards as compared to method D (Table 3). Desalting exchanged methylated peptides from a highly acidic environment (pH <1) to less acidic conditions (pH ~3.5; data not shown) for subsequent presentation to the IMAC column. Consistent with these data, decreased IMAC performance was observed at pH <1 with $Ga^{3+}$-IMAC.[13] Desalting may also have

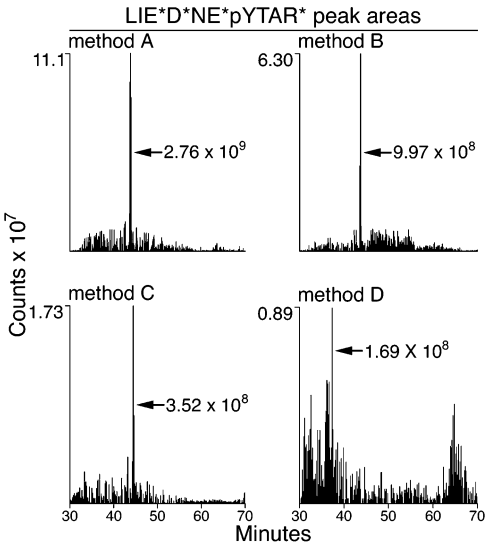


Figure 5. Fully methylated LIEDNEpYTAR in a complex mixture of peptides yielded the largest, most distinct SIC peak area using IMAC method A/nano-LC/ESI-MS/MS. A given preparation of peptides from human T cells containing synthetic standards was subjected to each of the IMAC methods (Table 6 and the Experimental Section). Each IMAC column was eluted to a RP precolumn, and the peptides were subjected to nano-LC/ESI-MS/MS using "peak parking" and a top 4 data-dependent MS/MS method.

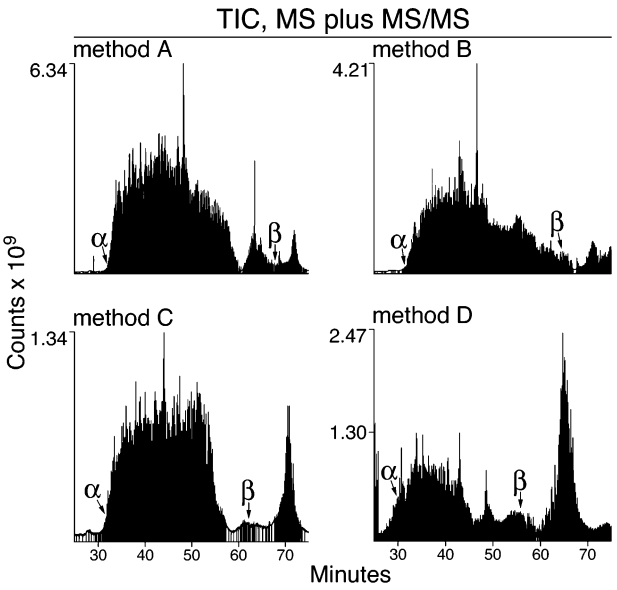


Figure 6. TIC areas were largest for IMAC method A. The sample and analysis were as in Figure 5. Phosphopeptides with assigned phosphorylation sites eluted between the arrows labeled "α" and "*β*". Verified MS/MS spectra were examined to determine the time span when phosphopeptides containing assigned phosphorylation sites were detected.

removed hydrophilic contaminants that interfered with phosphopeptide binding to, or subsequent recovery from, the IMAC column.

Next, we investigated the effect of increasing IMAC capacity on recovery of the standards. Because our overall goal was assignment of an increased number of tyrosine phosphorylation sites on proteins from living systems, methylated peptides prepared from a human T cell line were used to monitor recovery of phosphopeptides from a larger IMAC column. Recovery of the standards and assignment of endogenous cellular protein phosphorylation sites increased with the use of the larger IMAC column (method B; bed volume ~4.1 $\mu$L) compared to the smaller IMAC column (method C; bed volume ~0.63 $\mu$L) (Table 4).

After further improving recovery of phosphopeptides using method B, we also reasoned that gradient elution of peptides to the IMAC column could increase recovery of the peptide standards. To test this, we eluted peptides from the desalting column to the IMAC column using a 0−70% ACN gradient (IMAC method A), in comparison to a constant level of 70% ACN (IMAC method B). Gradient elution yielded increased recovery of fully methylated DRVpYIHPF and assignment of increased numbers of phosphorylation sites on endogenous cellular proteins (Table 5). Thus, on the basis of Tables 3 to 5, we conclude that when testing one variable at a time and targeting the standards, RP desalting of methylated peptides prior to IMAC and gradient elution to a larger IMAC column (IMAC method A) yielded the largest recovery of standard phosphopeptides and assignment of phosphorylation sites on endogenous cellular proteins.

**Comparison of IMAC Methods: Emphasis on Assignment of Protein Tyrosine Phosphorylation Sites in Human T Cells.** For a comparison of a uniform, complex peptide mixture, peptides were prepared from a human T cell line as described above and enriched with each of the four IMAC methods. Data-dependent nano-LC/ESI-MS/MS was used to fragment the most abundant peptide ions from IMAC without targeting the standards. In addition, duplicate samples were analyzed back-to-back to evaluate reproducibility and robustness. Recovery of fully methylated LIEDNEpYTAR and DRVpYIHPF and their signal-to-noise (S/N) ratios were highest using method A (Table 6, columns A−D; Figure 5; data not shown). Thus, method A also resulted in the largest recovery of fully methylated, tyrosine phosphorylated standards when a uniform sample containing a complex mixture of peptides was analyzed with a MS plus MS/MS method that did not target the standards.

However, increased coverage of phosphorylation sites comprising the tyrosine phosphoproteome was our overall goal. Method A resulted in the largest total ion current during phosphopeptide elution (Figure 6) and in assignment of phosphorylation sites on the largest number of endogenous proteins from human T cells (Table 6, column E). The methodology was robust, because dozens of tyrosine phosphorylation sites from endogenous cellular proteins were consistently assigned in these and numerous, additional analyses. The assignment of most phosphorylation sites was unambiguous, the others were localized to two or three residues, and most sites were assigned more than once (Table S3). Approximately 60% of the peptides were tyrosine-phosphorylated (Tables 6, S3), consistent with previous findings that the IP step is important for analyzing tyrosine phosphorylation, due to the low abundance of these sites.[6,20] The rest of the phosphopeptides contained serine and threonine phosphorylation only. Furthermore, the most serine and threonine phosphorylation sites were assigned using IMAC method A (Table 6, column E; Table S3). Taken together, our results support the proposal that desalting methylated peptides followed by gradient elution to a larger IMAC column resulted in assignment of the greatest number of cellular protein phosphorylation sites, consistent with the results of analyses targeting standards.

Some phosphorylation sites were assigned using all four IMAC/nano-LC/ESI-MS/MS methods (Table S3). Although method A resulted in exclusive assignment of the largest number of phosphorylation sites, some that were assigned using methods B, C, and/or D were not assigned using method A. Peptides not detected via MS/MS scans using method A generally produced weak signals when their phosphorylation sites were assigned by nano-LC/ESI-MS/MS analyses in conjunction with other IMAC methods. Low-intensity peaks containing candidate ions at the correct elution time with the correct $m/z$ were often detected by ESI mass spectra of the complex peptide mixtures that had been enriched by method A, but were not selected for data-dependent MS/MS analyses due to the stochastic nature of the selection of low S/N precursor ions.

Collectively, phosphorylation sites were assigned on 138 different proteins from the T cell line that was used (Table S4). Receptors and additional signaling proteins known to function in T cell activation, proliferation, differentiation, and survival; cytokine expression; TCR endocytosis; and cell−cell interaction involving helper T cells were the most common proteins with assigned phosphorylation sites. Phosphorylation sites were also identified on proteins that are involved in a plethora of general cellular processes, on proteins associated with predisposition to various diseases, and on proteins of unknown function (Table S4).

## CONCLUSIONS

Although methylation was not absolutely required for detection of phosphopeptides from complex mixtures derived from human T cells, methylation dramatically improved the selectivity of IMAC for phosphopeptides and the total number of phosphopeptides with assigned phosphorylation sites. When using anti-phosphotyrosine IP to prepare samples from cell lysates, combined with peptide methylation, IMAC method A/nano-LC/ESI-MS/MS consistently resulted in assignment of the most sites of protein phosphorylation among the methods that we tested, most notably in dozens of tyrosine phosphorylation sites from single analyses using Jurkat and a variety of other (unpublished results) human cell lines. Method A consistently yielded ∼3 times more sites of tyrosine phosphorylation on endogenous cellular proteins than the previous method D. The improved profiling of tyrosine phosphorylation events by method A, as compared to method D, was demonstrated by sequential improvements. Further analysis of the tyrosine phosphorylaton sites that were assigned should improve our understanding of cell signaling.

**Abbreviations.** IMAC, immobilized metal affinity chromatography; LC, liquid chromatography; nano-LC, nano-flow LC; ESI, electrospray ionization; MS; mass spectrometry; MS/MS, tandem mass spectrometry; pY, phosphotyrosine residue; pS, phosphoserine residue; pT, phosphothreonine residue; ACN, acetonitrile; HOAc, acetic acid; DRVpYIHPF (angiotensin II phosphate); RP, reversed phase; o.d., outside diameter; i.d., inside diameter; TCR, T cell antigen receptor complex; IP, immunoprecipitation; S/N, signal-to-noise ratio.

## ACKNOWLEDGMENT

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## SUPPORTING INFORMATION AVAILABLE

A listing of Tables S1−S4 and Figure S1 is available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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