SIMAC (Sequential Elution from IMAC), a Phosphoproteomics Strategy for the Rapid Separation of Monophosphorylated from Multiply Phosphorylated Peptides

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The complete analysis of phosphoproteomes has been hampered by the lack of methods for efficient purification, detection, and characterization of phosphorylated peptides from complex biological samples. Despite several strategies for affinity enrichment of phosphorylated peptides prior to mass spectrometric analysis, such as immobilized metal affinity chromatography or titanium dioxide, the coverage of the phosphoproteome of a given sample is limited. Here we report a simple and rapid strategy, SIMAC (sequential elution from IMAC), for sequential separation of monophosphorylated peptides and multiply phosphorylated peptides from highly complex biological samples. This allows individual analysis of the two pools of phosphorylated peptides using mass spectrometric parameters differentially optimized for their unique properties. We compared the phosphoproteome identified from 120 μg of human mesenchymal stem cells using SIMAC and an optimized titanium dioxide chromatographic method. More than double the total number of identified phosphorylation sites was obtained with SIMAC, primarily from a 3-fold increase in recovery of multiply phosphorylated peptides. Molecular & Cellular Proteomics 7:661–671, 2008.

Reversible protein phosphorylation is an important post-translational modification in most intracellular biological processes (1) because it can increase or decrease a regulatory response to external stimulation or an affinity toward other proteins or nucleic acids. Often multiphosphorylation on adjacent amino acids can have a large impact on the activity of regulatory proteins (2–4). One of the challenges in large scale phosphoproteomics is the analysis of multiply phosphorylated peptides. The presence of mono- or non-phosphorylated peptides in samples for MS suppresses the ionization of multiple phosphorylated peptides and thereby decreases the chance to detect them. Therefore new phosphoproteomics tools are required to study multiple phosphorylation of proteins.

Phosphopeptide enrichment prior to MS analysis is essential for large scale phosphoproteomics studies because phosphorylated peptides are rarely detected in “shotgun” MS analysis. A widely used enrichment technique for phosphorylated peptides is the use of metal ions for the binding of the negatively charged phosphopeptides, i.e. IMAC. IMAC was introduced to the characterization of phosphorylated proteins by Andersson and Porath (5) and was later extensively adapted for enrichment of phosphorylated peptides prior to mass spectrometric analysis (6–12). The IMAC technique improves identification of phosphopeptides from complex biological mixtures (6, 8, 11). However, non-phosphorylated peptides containing multiple acidic amino acid residues co-purify with the phosphorylated peptides in IMAC. O-Methyl esterification of the acidic residues has been suggested as a means to prevent this, but this step may introduce unwanted side reactions and loss of peptides due to extensive lyophilization (13).

IMAC appears to have a stronger selectivity for multiply phosphorylated peptides in biological buffers (6, 14). To counter this, buffer exchange using reversed phase chromatography prior to IMAC has been used in many studies (8, 11) with a high risk of losing phosphorylated peptides (15). Titanium dioxide (TiO₂)¹ chromatography has proven to be an efficient alternative to IMAC (16, 17). It has a higher selectivity for phosphorylated peptides than IMAC, and unspecific binding from non-phosphorylated peptides can be significantly reduced by including 2,5-dihydroxybenzoic acid (DHB), phthalic acid, or glycolic acid and high concentrations of TFA in the loading buffer (14, 17, 18). In addition, TiO₂ chromatography of phosphorylated peptides is extremely tolerant of most biological buffers (14). A comparison of different phosphopeptide enrichment methods including phosphoramidate chemistry (19), IMAC, and TiO₂ chromatography has recently

¹ The abbreviations used are: TiO₂, titanium dioxide; SIMAC, sequential elution from IMAC; pdMS, phosphorylation-directed multi-stage tandem MS; hMSC, human mesenchymal stem cell; SRRM1, serine/arginine repetitive matrix protein 1; DHB, 2,5-dihydroxybenzoic acid; ECD, electron capture dissociation; ETD, electron transfer dissociation; LTQ, linear trap quadrupole.

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shown that each method isolated different, partially overlapping segments of a phosphoproteome, indicating that no single method is capable of providing a whole phosphoproteome (20).

In contrast to IMAC a bias toward monophosphorylated peptides has been observed for TiO$_2$ chromatography. We propose that TiO$_2$ is able to bind multiply phosphorylated peptides as well as monophosphorylated peptides but that the multiply phosphorylated peptides are difficult to elute from the TiO$_2$ material due to a high binding affinity. In addition, multiply phosphorylated peptides are suppressed in the ionization process of MS in the presence of monophosphorylated peptides and non-modified peptides, making them less detectable. Thus monophosphorylated peptides are predominantly identified in large scale phosphoproteomics experiments using TiO$_2$ chromatography. Furthermore most mass spectrometers are only able to perform a limited number of tandem MS experiments (MSMS) in a given time period and therefore in most cases will only fragment the abundant monophosphorylated peptides in a complex mixture, leaving behind the multiply phosphorylated peptides. Finally the fragmentation of phosphorylated peptides in MS is more difficult than that of non-phosphorylated peptides because the loss of the phosphate group(s) is the predominant fragmentation pathway. This results in low yield of peptide backbone fragments that are the basis of subsequent identification and phosphorylation site assignment. The fragmentation of phosphorylated peptides can be improved significantly by using phosphorylation-directed multistage tandem MS (pdMS$^3$) (21) where an ion originating from a neutral loss signal detected in the MS$^2$ is selected for a second round of CID (MS$^3$) to obtain more peptide sequence information. Unfortunately this strategy favors the analysis of monophosphorylated peptides because multiply phosphorylated peptides will subsequently lose more phosphoric acid. Electron capture/transfer dissociation (ECD/ETD) is an emerging alternate technology that can be applied to multiply phosphorylated peptides because this method primarily results in peptide backbone fragmentation without concomitant loss of the phosphate groups (22, 23). However, ECD/ETD is not yet readily available in most research laboratories.

Here we present a rapid and simple strategy that results in greatly improved recovery of larger numbers of phosphorylated peptides from low amounts of complex biological samples. The method does not require new or specialized equipment and has three components. The primary basis of the method is sequential elution from IMAC (which we call “SIMAC”) and is based on our observation that acidic conditions primarily elute monophosphorylated peptides from IMAC material, whereas subsequent basic elution recovers the multiply phosphorylated peptides that are normally not readily detected. A second component of the method uses TiO$_2$ following IMAC chromatography to remove most of the non-phosphorylated peptides from the pool of monophosphorylated peptides in a complex mixture. Finally the two distinct phosphopeptide pools can now be analyzed separately using tandem mass spectrometry parameters (pdMS$^3$) that are optimized for each type of sample. The SIMAC method was applied to a phosphoproteomics study of human mesenchymal stem cells (hMSCs) in which only 120 µg of total protein was used as the starting material. Overall the SIMAC method more than doubled the total number of phosphorylation sites sequenced from a small amount of starting material in comparison with TiO$_2$ chromatography using simple and inexpensive approaches and without the need for multidimensional LC.

**EXPERIMENTAL PROCEDURES**

**Materials**

Modified trypsin was from Promega (Madison, WI). Endoproteinase Lys-C was from Wako (lysyl endopeptidase). Poros Oligo R3 reversed phase material was from PerSeptive Biosystems (Framingham, MA). GE Loader tips were from Eppendorf (Eppendorf, Hamburg, Germany) and Alpha Laboratories (Hampshire, UK). Orthophosphoric acid (85%, v/v) was from J. T. Baker Inc. Ammonia solution (25%) was from Merck. 3M Empore C$_8$ disk was from 3M Bioanalytical Technologies (St. Paul, MN). All reagents used in the experiments were sequence grade, and the water was from a Milli-Q system (Millipore, Bedford, MA).

**Model Proteins and Peptide Mixture**

The development and optimization of the presented method were performed using a peptide mixture originating from tryptic digestions of 12 standard proteins. Transferrin (human) was a gift from ACE Biosciences A/S. Serum albumin (bovine), β-lactoglobulin (bovine), carbonic anhydrase (bovine), β-casein (bovine), α-casein (bovine), ovalbumin (chicken), ribonuclease B (bovine pancreas), alcohol dehydrogenase (bakers’ yeast), myoglobin (whale skeletal muscle), lysozyme (chicken), and α-amylase (Bacillus sp.) were from Sigma. Each protein was dissolved in 50 mM ammonium bicarbonate, pH 7.8, 10 mM DTT (Sigma) and incubated at 37 °C for 1 h. After reduction, 20 mM iodoacetamide (Sigma) was added, and the sample was incubated at room temperature for 1 h. The reaction was quenched with 10 mM DTT, and the proteins were subsequently digested with trypsin (1–2% (w/w), modified trypsin, Promega) at 37 °C for 12 h.

**Total Protein from Human Mesenchymal Stem Cells**

Human mesenchymal stem cells (hMSC-TERT20) were grown in T75 flasks in minimum Eagle’s medium (Earle’s) without phenol red, with Glutamax-I (Invitrogen), containing 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen) at 37 °C until they reached 90% confluence. The confluent cells were washed once with PBS buffer (37 °C), and 5 ml of medium (37 °C) was added to cover the cells. Phosphatase inhibitor mixtures 1 and 2 (P2850 and P5726, Sigma) (50 µl of each) were added to the medium. The cells were incubated with the phosphatase inhibitors for 30 min at 37 °C. After washing with ice-cold PBS buffer, the cells were harvested using Cell Dissociation Buffer (Invitrogen). After harvesting the cells, the cell pellet was resuspended in 1.5 µl of lysis buffer (7 M urea (Sigma), 2 M thiourea (Merck), 1% N-octyl glycoside (Sigma), 40 mM Tris (Sigma), 300 units of Benzonase (Merck)). The cells were then sonicated at 40% output with intervals of 3 x 15 s on ice to disrupt the cells and incubated at −80 °C for 30 min. After incubation, 20 mM DTT was added. The sample was incubated at room temperature for 35 min. After incubation 40 mM iodoacetamide was added followed by incu-
bation for 35 min at room temperature in the dark. For protein precipitation 14 ml of ice-cold acetone was added to the solution followed by incubation at −20 °C for 20 min. The proteins were pelleted by centrifugation at 6000 × g for 10 min at −4 °C, and the pellet was lyophilized and stored at −20 °C until further use.

**Proteolytic Digestion of Proteins from Human Mesenchymal Stem Cells**

A total of 1 mg of the precipitated and lyophilized protein from human mesenchymal stem cells (weighed out) was redissolved in 6 ml urea, 2 ml thiourea to a concentration of 50 µg/ml. The proteins were subsequently incubated with 1 µg of endoproteinase Lys-C (lysel endopeptidase, Wako)/50 µg of protein at room temperature for 3 h. The endoproteinase Lys-C-digested sample was diluted five times with 50 ml NH₄HCO₃ and 1 µg of chemically modified trypsin/50 µg of protein was added. The sample was incubated at room temperature for 18 h.

**SIMAC Procedure**

**IMAC**—The procedure is here described for the optimization using 1 pmol of peptide mixture. The numbers in the parentheses illustrate the volumes used for 120 µg of hMSC-TERT20 tryptic digest. For each experiment 7 µl (50 µl) of iron-coated PHOS-select™ metal chelate beads (Sigma) was used. The beads were washed twice in loading buffer (0.1% TFA, 50% acetonitrile) as described previously (24). The beads were incubated with 30 µl (150 µl) of loading buffer and 1 pmol of peptide mixture (120 µg of hMCS-TERT20 tryptic digest). The beads were shaken in a Thermonixer (Eppendorf) for 30 min at 20 °C. After incubation, the beads were packed in the constricted end of a 200-µl GELoader tip by application of air pressure forming an IMAC microcolumn. For the complex hMCS-TERT20 tryptic digest, the IMAC beads were packed in 200-µl GELoader tips (Alpha Laboratories). The IMAC flow-through was collected in an Eppendorf tube for further analysis by TiO₂ chromatography (see below). The IMAC column was washed using 20 µl (40 µl) of loading buffer, which was pooled with the IMAC flow-through. The mono-phosphorylated peptides and contaminating non-phosphorylated peptides were eluted from the IMAC column using 10 µl (50 µl) of 1% TFA, 20% acetonitrile, and the multiply phosphorylated peptides were subsequently eluted from the same IMAC microcolumn using 40 µl (70 µl) of ammonia water, pH 11.3 (10 µl of 25% ammonia solution (Merck) in 490 ml of ultra-high quality water). The IMAC flow-through and the IMAC eluents were dried by lyophilization.

**Titanium Dioxide (TiO₂) Chromatography—**After lyophilization, the pooled flow-through and wash from the IMAC microcolumn was enriched for phosphopeptides using TiO₂ chromatography. For the complex mixture the monophosphorylated peptide fraction (1% TFA) was also subjected to TiO₂ chromatography as described below. A TiO₂ microcolumn was prepared by stamping out a small plug of C₈ material from a 3M Empore™ C₈ extraction disk (3M Bioanalytical Technologies) and placing the plug in the constricted end of a P10 tip (17, 18). The TiO₂ beads (suspended in 100% acetonitrile) were packed in the P10 tip where the C₈ material prevented the beads from leaking. The TiO₂ microcolumn was packed by the application of air pressure. Buffers used for loading or washing of the microcolumn contained 80% acetonitrile to prevent nonspecific binding to the C₈ membrane and the TiO₂ beads. The lyophilized sample was resuspended in 2 µl of 4 M urea and 3 µl of 1% SDS (14) and diluted five times in loading buffer (1 M glycolic acid (Fluka) in 80% acetonitrile, 5% TFA) and loaded onto a TiO₂ microcolumn of −5 mm. (For more complex samples two TiO₂ microcolumns were used per sample.) The TiO₂ microcolumn was washed with 5 µl of loading buffer and subsequently with 30 µl of wash buffer (80% acetonitrile, 5% TFA). The phosphopeptides bound to the TiO₂ microcolumns were eluted using 50 µl of ammonium water (pH 11.3) followed by elution using −0.5 µl of 30% acetonitrile to elute phosphopeptide bound to the C₈ disk. The eluent was acidified by adding 5 µl of 100% formic acid prior to the desalting step.

**Desalting the TiO₂ Eluates Using R3 Microcolumns Prior to MALDI Mass Spectrometry**

The Poros Oligo R3 reversed phase resin (PerSeptive Biosystems) was dissolved in 70% acetonitrile. The R3 beads were loaded onto constricted GELoader tips, and gentle air pressure was used to pack the beads to obtain R3 microcolumns of ~3 mm (25). Each acidified sample was loaded onto a R3 microcolumn. The R3 microcolumns were subsequently washed with 30 µl of 0.1% TFA, and the phosphopeptides were eluted directly onto the MALDI target using 0.5 µl of 20 µg/µl DHB (Fluka), 50% acetonitrile, 1% phosphoric acid. For LC-ESI MSMS analysis of the monophosphorylated peptides originating from the complex sample, the phosphopeptides were desalted in a similar way; however, the phosphorylated peptides were eluted from the Poros R3 column using 30 µl of 70% acetonitrile, 0.1% TFA followed by lyophilization. The phosphopeptides were subsequently resuspended in 0.5 µl of 100% formic acid and 10 µl of Buffer A (0.5% acetic acid) prior to LC-ESI MSMS analysis.

**MALDI MS**

MALDI MS was performed on a Bruker Ultraflex (Bruker Daltonics, Bremen, Germany). All spectra were obtained in positive reflector ion mode. The matrix used was 20 mg/ml DHB in 50% acetonitrile, 0.1% TFA, 1% phosphoric acid (26). The spectra were processed using the flexAnalysis software (Bruker Daltonics).

**Nano-LC-MS**

The nano-LC-MS experiments were performed using a 7-tesla LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany). The sample was applied onto an EASY nano-LC system (Proxeon Biosystems, Odense, Denmark). The peptides were concentrated on a 1.0-cm precolumn (75-µm inner diameter, 360-µm outer diameter, ReproSil-Pur C₁₈ AQ 3 µm (Dr. Maisch, Ammerbuch-Entringen, Germany)). The peptides were eluted from the precolumn using a gradient of 100% phase A (0.5% acetic acid aqueous solution) to 40% phase B (0.5% acetic acid, 80% acetonitrile) in 100 min at 200 nl min⁻¹ directly onto an 8-cm analytical column (50-µm inner diameter, 360-µm outer diameter, ReproSil-Pur C₁₈ AQ 3 µm).

The instrument was operated in a data-dependent mode automatically switching between MS, MS², and pdMS³ (21). The pdMS³ acquisition was set to automatically select and further fragment the fragment ion originating from the loss of phosphoric acid from the parent ions (standard pdMS³ settings) when analyzing the monophosphorylated fractions from the SIMAC experiments or the phosphorylated peptides purified by TiO₂ chromatography. For the analysis of multiply phosphorylated peptides from the SIMAC experiments the pdMS³ acquisition was set to automatically select and fragment the fragment ion originating from the loss of a minimum of two phosphate groups from the parent ion (optimized pdMS³ settings).

**Database Searching Using an In-house MASCOT Server**

The MS² data were processed (smoothing, background subtraction, and centroiding) using the program DTASuperCharge (Source-Forge, Inc.). The processed files were subsequently searched against the human sequence library in the International Protein Index (IPI) protein sequence database (IPI human 20061202, 70,878 sequences).
using an in-house Mascot server (version 2.1) (Matrix Science Ltd., London, UK). The search was performed choosing trypsin as the enzyme with one miss cleavage allowed. Carbamidomethyl (Cys) was chosen as the fixed modification. As variable modifications, oxidation (Met), N-acetylation (protein), phosphorylation (STY), and intact phosphorylation (STY) were chosen. The data were searched with a peptide mass tolerance of ±30 ppm and a fragment mass tolerance of ±0.6 Da. A concatenated decoy database search was performed in a concatenated decoy human database (IPIhuman+decoy; total number of sequences, 136,764) derived from the IPI human database listed above for each of the conditions.

Phosphopeptide Evaluation

A peptide identified by Mascot was accepted if it had a peptide score above 20 in all the experiments performed.

RESULTS

Development of the SIMAC Strategy—The selectivity of IMAC is biased toward capture of multiply phosphorylated peptides (6, 8, 14). Purification of monophosphorylated peptides using IMAC is directly affected by the buffers used, whereas multiply phosphorylated peptides seem to have a higher affinity toward the IMAC material in such buffers (14). Thus a desalting step is required prior to phosphopeptide enrichment with IMAC. This suggests that multiply phosphorylated peptides may bind more strongly to the IMAC resin than do monophosphorylated peptides. Presumably this is due to multipoint binding that is much more difficult to dissociate. We speculated that monophosphorylated peptides might be eluted from the IMAC resin under conditions where multiply phosphorylated peptides remain bound, allowing separation of phosphopeptides into two pools. This was tested and optimized using a mixture of peptides originating from tryptic digests of 12 different standard proteins, including three phosphoproteins. A list of the theoretical tryptic phosphorylated peptides derived from the 12 standard proteins and their molecular masses is shown in Table I.

First phosphorylated peptides were eluted from the IMAC resin using a gradient of increasing pH (data not shown). This did not result in a separation of the monophosphorylated from the multiply phosphorylated peptides but eluted the same phosphopeptides over several fractions. Next we used a decreasing pH gradient. This was based on our observations that the use of 0.1% TFA in the loading buffer (24) resulted in an excess of monophosphorylated peptides but not multiply phosphorylated peptides in the flow-through from an IMAC column. This suggested a weaker binding of monophosphorylated peptides to the IMAC resin in an acidic environment compared with the multiply phosphorylated peptides, providing an opportunity to separate the two populations by low pH. An aliquot of the tryptic peptide mixture (1 pmol) was batch-incubated with 7 μl of iron-coated PHOS-select IMAC beads in 30 μl of 0.1% TFA, 50% acetonitrile for 30 min. After incubation, the IMAC beads were packed in the constricted end of a GELoader tip. The IMAC microcolumn was washed using the loading buffer. The phosphorylated peptides were eluted stepwise from the IMAC microcolumn using 20% acetonitrile and increasing concentrations of TFA: 0.2% (pH 3.5), 0.3% (pH 4.0), 0.4% (pH 4.5), 0.5% (pH 5.0), and 0.6% (pH 5.5) TFA. The detected phosphorylated peptides from the standard protein digest were calculated by General Protein/Mass Analysis for Windows (GPMAW) 6.0. The phosphorylated peptides were derived by tryptic digestion of ovalbumin (Ov.), α-casein S1 (α-S1.) and S2 (α-S2.), and β-casein (β-C.). The phosphorylation sites are bold and underlined.

| Peptide sequence | No. of phosphate groups | (M + H)⁺ |
|------------------|-------------------------|----------|
| EQLSTSEENK (α-S2. 141–151) | 2 | 1411.50 |
| TVDMESTEVFKT ( α-S2. 153–164) | 1 | 1466.61 |
| TVDMESTEVFKT (α-S2. 153–165) | 1 | 1594.70 |
| VPOLEIPNSAEER (α-S1. 121–134) | 1 | 1660.79 |
| D1GESETEGDAMEDIK (α-S1. 58–73) | 2 | 1927.69 |
| YKVPOLEIPNSAEER (α-S1. 119–134) | 1 | 1951.95 |
| FSESEQEQUDELOQDK (β-C. 33–48) | 1 | 2061.83 |
| EVVGSAEAGVDAASVSEEFR (Ov. 340–359) | 1 | 2088.89 |
| NVPGEIVSLLSEESITR (β-C. 22–40) | 4 | 2352.87 |
| IEKFQSEEQQTEDELOQDK (β-C. 30–48) | 1 | 2432.10 |
| LPGGFDIEAQGTSVNHSVSLR (Ov. 62–84) | 1 | 2511.13 |
| FSESEQEQUDELOQDKHIPF (β-C. 33–52) | 1 | 2556.20 |
| VN EldSIADEGDAMEDIK (α-S1. 52–73) | 3 | 2678.01 |
| QMEAEVSISSEENVPNSVEQK (α-S1. 74–94) | 5 | 2703.10 |
| QMEAEVSISSEENVPNSVEQK (α-S1. 74–94) | 5 | 2720.91 |
| KNTMEHVSISSEENVPNSVEQK (α-S2. 16–36) | 4 | 2745.99 |
| EKVELDSEGDAMEDIK (α-S1. 52–73) | 3 | 2935.15 |
| NASEEYGVSSEAEVATEEVK (α-S2. 61–85) | 4 | 3008.01 |
| REELNVPGEIVSLLSEESITR (β-C. 16–40) | 4 | 3122.27 |

a Chymotryptic cleavage (sequence was verified by MALDI tandem MS).

b Variant of the sequence QMEAE (verified by MALDI MSMS).

Phosphopeptide Enrichment by Sequential Elution from IMAC

| TABLE I |

List of phosphorylated peptides identified from the standard peptide mixture using MALDI MS

The detected phosphorylated peptides from the standard protein digest were calculated by General Protein/Mass Analysis for Windows (GPMAW) 6.0. The phosphorylated peptides were derived by tryptic digestion of ovalbumin (Ov.), α-casein S1 (α-S1.) and S2 (α-S2.), and β-casein (β-C.). The phosphorylation sites are bold and underlined.
Each IMAC eluent was desalted and concentrated on reversed phase microcolumns and eluted directly onto a MALDI MS target using DHB matrix solution. The resulting MALDI MS mass maps are shown in a–e, respectively. f, MALDI MS mass map of phosphorylated peptides subsequently eluted with ammonia water (pH 11.30). An asterisk indicates the loss of phosphoric acid. The triangle at m/z 1561.64 indicates a doubly charged ion from m/z 3122.27. P, phosphate group; ox, oxidation.

The SIMAC strategy was first tested using 1 pmol of the standard peptide mixture. After incubation of the sample with the IMAC resin the mixture was packed in a GELoader tip and washed. Phosphorylated peptides in the flow-through and wash were pooled and purified by TiO$_2$ chromatography, and the MALDI MS peptide mass map revealed the presence of only monophosphorylated peptides (Fig. 3a). The monophosphorylated peptides were eluted from the IMAC microcolumn using acidic conditions (1% TFA, 20% acetonitrile), and the MALDI MS peptide mass map was dominated by monophosphorylated peptides (Fig. 3b). The multiply phosphorylated peptides were subsequently eluted using basic conditions (ammonia water, pH 11.30), and the MALDI MS peptide mass map was dominated by multiply phosphorylated peptides (Fig. 3c). This clearly shows that the sequential elution from the IMAC resin with acid followed by base results in a more complete coverage of the phosphorylated peptides.
A Modified pdMS\textsuperscript{3} Method Improves Identification of Multiply Phosphorylated Peptides—Prior to applying the method to a whole cellular phosphoproteome we explored the importance of introducing a third step to SIMAC. Due to the limitations of CID for fragmentation of phosphorylated peptides in MS resulting in low fragmentation of the peptide backbones, pdMS\textsuperscript{3} can be used to obtain sequence information on the phosphorylated peptides. Hereby the fragment ion originating from the loss of phosphoric acid is selected for a second round of CID. This strategy has recently been applied to large scale phosphoproteomics studies (8, 21). However, for phosphorylated peptides containing more than one phosphate group pdMS\textsuperscript{3} often results in a loss of a second phosphoric acid and will not provide adequate sequence information to identify the peptide sequence in many cases. To increase the number of identified multiply phosphorylated peptides from the high pH fraction from the SIMAC strategy we improved the pdMS\textsuperscript{3} settings so that the first loss of phosphoric acid was ignored, and instead the subsequent MS\textsuperscript{3} was performed on the ion corresponding to the second loss of phosphoric acid.

Multiply phosphorylated peptides isolated from 120 μg of digested hMSC proteins, using the SIMAC strategy, were split into two samples, and one-half was analyzed using the traditional pdMS\textsuperscript{3} settings on an LTQ-FTICR MS instrument, and the other half was analyzed using the optimized pdMS\textsuperscript{3} settings (ignoring the first loss of phosphoric acid) for phosphorylated peptides with more than one phosphate group. The results of the database search after the two pdMS\textsuperscript{3} experiments are shown in Fig. 4a. When using the traditional pdMS\textsuperscript{3} we identified 52 multiply phosphorylated peptides from the sample, whereas we identified 84 multiply phosphorylated peptides when using the optimized pdMS\textsuperscript{3} settings. Despite that the new pdMS\textsuperscript{3} setting favors doubly phosphorylated peptides we also observed an increase in the identification of peptides with three or four phosphate groups using this method compared with the normal settings (data not shown). Overall this suggests that different pdMS\textsuperscript{3} settings are appropriate for the different SIMAC eluents.

SIMAC Improves Large Scale Phosphoproteome Analysis—The SIMAC strategy (Fig. 2) was applied to a whole protein lysate from hMSCs and was compared with a recently optimized TiO\textsubscript{2} strategy (14, 18). The cells were incubated with two phosphatase inhibitor mixtures for 30 min prior to cell lysis. This treatment was performed to preserve the phos-
Phosphopeptide enrichment by sequential elution from IMAC

Fig. 3. MALDI MS mass map of 1 pmol of peptide mixture originating from tryptic digestion of 12 proteins, including three phosphoproteins, enriched for phosphorylated peptides using the SIMAC strategy. a, MALDI MS mass map of phosphorylated peptides from the IMAC flow-through subsequently enriched by TiO₂ chromatography. b, MALDI MS mass map of phosphorylated peptides eluted from the IMAC microcolumn using 1% TFA. c, MALDI MS mass map of phosphorylated peptides subsequently eluted from the IMAC microcolumn using ammonia water (pH 11.30). An asterisk indicates the loss of phosphoric acid. P, phosphate group; ox, oxidation.

Phosphate groups present on proteins from hMSCs. Because of secondary effects of the phosphatase inhibition in cells, an increase in the basal phosphorylation is expected by this treatment to an extent similar to when cells are stimulated with external biological stimuli. The total protein complement was precipitated with ice-cold acetone.

One milligram of protein was subjected to proteolysis using lysyl endopeptidase and trypsin. In the traditional method peptides originating from a total of 120 µg of the precipitated protein mixture were subjected to TiO₂ chromatography using an optimized protocol (14, 18), and the sample of enriched phosphorylated peptides was analyzed by LC-LTQ-FTICR MS using the traditional pdMS³ settings. A phosphorylated peptide was accepted as present in a given sample if it had been assigned in the subsequent Mascot database search with a Mascot score value of at least 20. This value was chosen based on our experience and that from other research groups (27) that phosphorylated peptides are generally scored lower by Mascot than non-modified peptides. A total of 232 mono-phosphorylated peptides, 54 multiply phosphorylated peptides, and 39 non-phosphorylated peptides were identified by the TiO₂ strategy (Fig. 4b). Thus 88.0% of the peptides identified were phosphorylated.

A concatenated decoy database search identified 15 peptides with a score above 20 (Mascot scores of 21–36), indicating a false positive rate of 4.6%. However, we believe that this value is an overestimate as many of the sequences identified in the decoy database search are either small (<5 amino acids) or non-phosphorylated. The latter will have a higher Mascot score than phosphorylated peptides. Lists of the identified phosphorylated peptides are shown in Supplemental Table 1, A and B. The lists include only non-redundant peptides. If peptide sequences are shared by more than one protein entry only one of the protein entries is included in the list.

Another 120 µg of hMSC tryptic digest was subjected to the SIMAC phosphopeptide enrichment method. The IMAC flow-through and 1% TFA eluent were subjected to TiO₂ chromatography and analyzed by the LC-LTQ-FTICR MS instrument using the traditional pdMS³, whereas the base-eluted multiply phosphorylated peptides were analyzed using the optimized pdMS³ settings for multiply phosphorylated peptides (Fig. 4c). The number of monophosphorylated peptides identified in the IMAC flow-through, 1% TFA, and pH 11.30 fractions were 179, 232, and 80, respectively. After accounting for overlaps, 306 unique monophosphorylated peptides were observed overall (Fig. 4d). Among these, 55 monophosphorylated peptides were identified only from the IMAC flow-through, stressing the importance of analyzing the flow-through of the initial IMAC. The data suggest an uneven selectivity or binding capacity of the IMAC resin for various monophosphorylated peptides. No clear trend in the amino acid composition was observed among the monophosphorylated peptides identified in the different fractions that might account for this. The
number of multiply phosphorylated peptides identified in the IMAC flow-through, 1% TFA, and pH 11.30 fractions were 8, 11, and 179, respectively. The numbers of non-phosphorylated peptides identified in the three fractions were 57, 2, and 4, respectively. Thus 89.6% of the peptides identified were phosphorylated. A concatenated decoy database search was performed for the SIMAC flow-through, 1% TFA, and pH 11 fractions, resulting in a false positive rate of 6.5, 6.4, and 6%, respectively. For the same reasons as above we believe that this rate is overestimated.

More than 96% of the multiply phosphorylated peptides identified using SIMAC were identified in the pH 11.30 fraction (Fig. 4e), emphasizing the efficiency of analyzing multiply phosphorylated peptides after removal of monophosphorylated peptides from the sample. These contained 186 unique phosphorylated peptides. Lists of the phosphorylated peptides identified using the SIMAC strategy are shown in Supplemental Table 1, A and B. The lists include only non-redundant peptides. If peptide sequences are shared by more than one protein entry only one of the protein entries is included in the list.

In total from 120 μg of proteins, the optimized TiO₂ method identified 286 phosphorylated peptides of which 54 (18.8%) were multiply phosphorylated. In contrast, the SIMAC strategy identified 492 phosphorylated peptides of which 186 (37.8%) were multiply phosphorylated (Fig. 5a). Among all 362 monophosphorylated peptides identified by either method, 176 were identified by both techniques, whereas 56 were only identified by TiO₂, and 130 were only identified by SIMAC (Fig. 5b). Among all 197 multiply phosphorylated peptides identified by either method, 43 were identified by both techniques, 43 were identified by both methods, and 130 were only identified by SIMAC (Fig. 5c).

Overall there were 716 unique phosphorylation sites identified using the SIMAC strategy compared with 350 phosphorylation sites identified using the optimized TiO₂ method (Fig. 5d). We compared the distribution of these sites between the two methods (Fig. 5, b and c). Of the 286 phosphorylated peptides identified using TiO₂ chromatography, 232 were monophosphorylated, 45 were doubly phosphorylated, eight were triply phosphorylated, and one was carrying four phos-
phosphorylated peptides. In comparison, the SIMAC strategy identified 306 monophosphorylated, 151 doubly phosphorylated, 32 triply phosphorylated, and three quadruply phosphorylated peptides. Therefore the main difference between the two methods is the greatly increased number of multiply phosphorylated peptides identified. Using the SIMAC strategy three times as many multiply phosphorylated peptides were identified.

Serine/Arginine Repetitive Matrix Protein 1—To illustrate the strength of the SIMAC method on a well characterized phosphoprotein we manually validated the phosphorylated peptides identified from the nuclear serine/arginine repetitive matrix protein 1 (SRRM1) within the 120 μg of hMSC total cell lysate. A previous study identified 35 phosphorylation sites in this protein using 8 mg of HeLa cell nuclear preparation as starting material (21). We compared the performance of SIMAC with an optimized TiO$_2$ strategy in parallel experiments. The phosphorylated peptides identified from SRRM1 are listed in Supplemental Table 2. Using the TiO$_2$ approach 17 phosphorylation sites were identified in five monophosphorylated and six multiply phosphorylated peptides. Using SIMAC we identified 48 phosphorylation sites from nine monophosphorylated and 20 multiply phosphorylated peptides. All the monophosphorylated peptides identified by SIMAC were found in the IMAC flow-through or the 1% TFA fractions, and all the multiply phosphorylated were identified in the pH 11.30 fraction except for two peptides that were identified in both fractions. Seven of the identified SRRM1 phosphorylation sites were not listed in the Swiss-Prot database, and two more had been reported previously only as “potential” sites. These data clearly illustrate the strength of the SIMAC strategy in improving coverage of the phosphorylation sites from a single protein from limited amounts of starting material.

**DISCUSSION**

SIMAC is a rapid and simple method that greatly improves large scale phosphoproteomics. The new strategy is based on sequential elution of monophosphorylated versus multiply phosphorylated peptides with acid or base (respectively) prior to MS analysis and combines the advantages of IMAC and...
The overlap between the monophosphorylated peptides observed in the different SIMAC fractions revealed that most of the monophosphorylated peptides were found in the pH 1.0 eluent. However, many unique monophosphorylated peptides were exclusively identified from the IMAC flow-through, illustrating the importance of also analyzing this normally discarded fraction when working with highly complex mixtures. The overlap between the multiply phosphorylated peptides observed in the different SIMAC fractions showed that more than 96% of the multiply phosphorylated peptides identified using SIMAC were identified in the pH 11.30 fraction. Furthermore, the advantage of analyzing the multiply phosphorylated peptides separately with optimized neutral loss settings was demonstrated as almost 24% more multiply phosphorylated peptides were identified using optimized neutral loss settings. The optimized neutral loss settings used in this study favor the identification of diphosphorylated peptides, which also proved to constitute more than 80% of the multiply phosphorylated peptides identified. The use of optimized neutral loss settings where the first two losses of phosphoric acid would be ignored would favor triphosphorylated peptides and would be expected to shift the distribution of multiply phosphorylated peptides. Replacing CID with ECD/ETD for fragmentation would evade this problem because this method primarily results in peptide backbone fragmentation without concomitant loss of the phosphate groups (22, 23).

The SIMAC strategy is easily adaptable to fit other phosphoproteomics study designs. It could be combined with prefractonation either at the protein or peptide level or alternative proteolysis using enzymes with different cleavage specificities to increase the number of identified phosphorylation sites from complex samples. Presently manual inspection showed that only a small fraction of the phosphorylated peptide ions detected by LC-MSMS is identified using the Mascot database search software (<30%). To increase the number of identified phosphopeptides, the SIMAC strategy could be combined with multistage activation (28) or ETD (23), which would provide a better coverage of the peptide sequence.

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