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Confident Site Localization Using a Simulated Phosphopeptide Spectral Library

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Supporting Information

ABSTRACT: We have investigated if phosphopeptide identification and simultaneous site localization can be achieved by spectral library searching. This allows taking advantage of comparison of specific spectral features, which would lead to improved discrimination of differential localizations. For building a library, we propose a spectral simulation strategy where all possible single phosphorylations can be simply and accurately (re)constructed on enzymatically dephosphorylated peptides, by predicting the diagnostic fragmentation events produced in beam-type CID. To demonstrate the performance of our approach, enriched HeLa phosphopeptides were dephosphorylated with alkaline phosphatase and analyzed with higher energy collisional dissociation (HCD), which were then used for creating a spectral library of simulated phosphopeptides. Spectral library searching using SpectraST was performed on data sets of synthetic phosphopeptides and the HeLa phosphopeptides, and subsequently compared to Mascot and Sequest database searching followed by phosphoRS and Ascore afforded localization, respectively. Our approach successfully led to accurate localization, and it outperformed other methods, when phosphopeptides were covered by the library. These results suggest that the searching with simulated spectral libraries serves as a crucial approach for both supplementing and validating the phosphorylation sites obtained by database searching and localization tools. For future development, simulation of multiply phosphorylated peptides remains to be implemented.

KEYWORDS: beam-type CID, LC−MS/MS, phosphoproteomics, phosphorylation site localization, spectral library searching, spectral simulation

INTRODUCTION

For two decades now sequence database searching has been the method of choice for semiautomated peptide and protein identification in tandem mass spectrometry (MS/MS) based proteomics (Figure 1A).1−3 Recently, a new type of search algorithms based on spectral library matching has become available.4−6 Unlike sequence database search engines that compare MS/MS spectra to theoretical information based on a sequence database, spectral library methods match experimental MS/MS spectra to a library of MS/MS spectra derived from previous identifications. By comparing two experimental data, the spectral library matching approach takes advantage of comparison of specific spectral features that are often not used or neglected in database searches, including actual peak intensities, neutral losses from fragments, and various uncommon or even unknown fragments characteristic to specific peptides, thus improving and expediting the identification of the best match.7 The method has also been shown to be effective for the analysis of phosphopeptides, as exemplified in PhosphoPep8,9 and ProMEX10 (Figure 1A), because the similarity scores of spectral searching algorithms are more precise than classical database search approaches. Nevertheless, while these phosphopeptide databases are extensive and some can be downloaded (e.g., PhosphoPep from PeptideAtlas11) and then searched by SpectraST,4 all phosphopeptide libraries at this time need to be considered incomplete and are not yet cross platform compatible.

Supporting Information

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To achieve high confidence spectral searching, a library of high-quality MS/MS spectra with accurately identified peptide data must be created. This can be time-consuming and costly, particularly for phosphopeptide data, as the precise localization of phosphorylation sites remains a difficult task even for most but a few expert mass spectrometrists. Recently, a concept for creating a spectral library of computationally simulated phosphopeptides has been reported by Hu and Lam.\textsuperscript{12,13} For the simulation of known phosphorylation sites, they used MS/MS spectra of nonphosphorylated peptides generated by ion trap collision-induced dissociation (CID), in which masses of fragment ions were shifted by +80 Da to predict fragment ions retaining a phosphate group. SpectraST searching against their spectral library supplemented with simulated spectra was proven to be highly sensitive for the identification of phosphopeptides compared to database searching.\textsuperscript{13} When this approach (called semiempirical prediction) was applied to simulation of unmodified peptides with amino acid substitutions, it also outperformed computational modeling of CID spectra using Mass Analyzer,\textsuperscript{14,15} in spectral similarity and identification sensitivity.\textsuperscript{12} However, the accuracy of the phosphorylation simulation and the reliability in the phosphorylation site localization have not been investigated yet.

Previously we have demonstrated that similar, but not identical, fragmentation patterns are observed when phosphopeptides and their enzymatically dephosphorylated counterparts (i.e., nonphosphorylated peptides) are compared. Specifically, these observations include the presence of fragment ions in spectra acquired in a quadrupole time-of-flight (Q-TOF) instrument, and their intensity distribution.\textsuperscript{16}
Fragment ions containing the phosphorylated serine and threonine residues show a loss of 18 Da in comparison to the corresponding fragment ions derived from the dephosphorylated peptide (Figure 1B). This loss occurs with maintained intensity because, along with a prominent neutral loss of phosphoric acid from those residues, a beam-type quadrupole collision cell of the Q-TOF instrument induces accompanying fragmentation of the peptide backbone; therefore, the loss from fragment ions can be used to confidently localize the phosphorylation sites (reference-facilitated phosphoproteomics).\(^1\)

Here, we propose a strategy for simple, but accurate, simulation of phosphopeptides by resembling the diagnostic fragmentation events in beam-type CID (Figure 1A,B). In this strategy, all possible single phosphorylations are computationally simulated on dephosphorylated peptides. By matching to accurately simulated spectra, SpectraST is enabled to localize phosphorylation sites on actual phosphopeptides (Figure 1C). To demonstrate the performance of this method, we have obtained dephosphorylated peptides derived from HeLa cells and acquired beam-type CID spectra for those peptides, which were then used for creating a spectral library after the phosphorylation simulation. SpectraST searching using the simulated spectral library was tested on data sets of synthetic phosphopeptides and HeLa phosphopeptides, and evaluated in comparison to other existing methods including sequence database searching followed by phosphorylation site localization.

### EXPERIMENTAL SECTION

**Sample Preparation**

HeLa cells were grown in DMEM (GIBCO) supplemented with 10% fetal calf serum (Sigma), at 37 °C in 5% CO\(_2\). Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed directly on 15 cm plates with 400 \(\mu\)L of buffer containing 100 mM ammonium bicarbonate (AMBIC), pH 8, 5 mM EDTA, a phosphatase inhibitor mix (Roche) and 8 M urea. The cells were scraped from the dish surface and the solution transferred in Eppendorf tube and sonicated with 3 cycles of 30 s each on ice. The protein extract was centrifuged at 16 000 \(\times\) g for 30 min. The supernatant was collected and protein concentration measured with BCA assay (Invitrogen). The proteins were reduced by incubation with tris(2-carboxyethyl)-phosphine (TCEP) at a final concentration of 10 mM at room temperature for 45 min. The produced free thiolos were alkylated with 20 mM iodoacetamide at room temperature for 45 min in the dark. The solution was diluted with 50 mM AMBIC pH 8, to a final concentration of 1 M urea and digested with sequencing grade-modified trypsin (Promega) overnight at 37 °C. Peptides were desalted on a C18 Sep-Pak cartridge (Waters) and dried in a Speedvac. For phosphopeptide enrichment experiment, 10 mg of total peptide was used. Dried peptides were reconstituted in a solution consisting of 80% acetonitrile (ACN), 3.5% TFA saturated with phosphoric acid. About 3 mg of TiO\(_2\) resin (GL Sciences) was placed into a 1.5 mL Eppendorf tube and was subsequently washed with 300 \(\mu\)L of 2-propanol and equilibrated with 280 \(\mu\)L of the saturated phosphoric acid solution for 30 min. The resin is successively transferred to the peptide solution and incubated on rotation for 45 min at room temperature. After incubation step the resin was centrifuged at 100g for 1 min and the peptide solution was discarded. The resin was subsequently thoroughly washed two times each with 280 \(\mu\)L of the phosphoric acid solution and two times with 80% ACN, 0.1% TFA solution, and finally two times 50% ACN, 0.1% TFA. In the final step, phosphopeptides were eluted from the TiO\(_2\) resin using two times 150 \(\mu\)L of a 0.3-M NH\(_4\)OH solution (pH 10.5). After elution, the pH of the pooled eluates was rapidly adjusted to 2.7 using 50% TFA, and phosphopeptides were purified using an appropriate reverse phase column suitable for up to 100 \(\mu\)g of total peptide (C18Micro Spin Column, Nest Group). The peptides were desalted as before but in addition that the last two column washes were performed with water and the peptides eluted with 300 \(\mu\)L of 80% ACN in water, without any acid to consent to easily adjust the pH to 7.9 for successive enzymatic reaction. The eluted sample was divided in two and dried in SpeedVac. To dephosphorylate peptides, one of those was reconstituted in 150 \(\mu\)L of 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol pH 7.9 and incubated with 5 \(\mu\)L of alkaline phosphatase (New England Biolabs) for 3 h at 37 °C. The solution was successively acidified using 50% TFA and again desalted. The dried peptides were reconstituted in 2% ACN, 0.5% formic acid (FA) and analyzed by LC−MS/MS.

**Mass Spectrometry**

LC−MS/MS analyses for the HeLa phosphopeptide and dephosphorylated peptide samples were performed in duplicate by submitting 1 \(\mu\)L of the peptides (estimated concentration 1−2 \(\mu\)g/\(\mu\)L). An EASY-nLC II nanoflow LC instrument was coupled to an electrospray ionization (ESI) mass spectrometer LTQ Orbitrap Velos (Thermo Fisher Scientific). A 100 \(\mu\)m \(\times\) 3 cm trap column and a 75 \(\mu\)m \(\times\) 15 cm analytical column were in-house packed with Magic C18AQ resin (200 Å, 5 \(\mu\)m; Michrom Bioresources). The mobile phases were 2% ACN, 0.2% FA (A) and 95% ACN, 0.2% FA (B). LC gradient elution condition was initially 2% B to 20% B (70 min), 40% B (100 min), and 100% B (105−110 min), with a flow rate of 300 nL/min. Data dependent acquisition was performed in positive ion mode. MS spectra were acquired from \(m/z\) 300 to \(m/z\) 2000 in the Orbitrap set to a resolution of 30 000 at \(m/z\) 400 with a target value of 1 000 000 ions and a maximal injection time of 100 ms in profile mode. The 10 most abundant ions of which charge states were 2+ or higher were selected for subsequent fragmentation, and MS/MS spectra were acquired in the Orbitrap with a resolution of 7500 at \(m/z\) 400, a target value of 50 000 ions, a maximal injection time of 200 ms, and the lowest mass fixed at \(m/z\) 100, in centroid mode. Four fragmentation techniques were used; CID with normalized collision energy of 35 and activation time of 10 ms, multistage activation (MSA) with normalized collision energy of 35 and activation time of 10 ms for the precursors and precursor −98/z,18 higher energy collisional dissociation (HCD) with normalized collision energy of 40 and activation time of 0.1 ms, and electron-transfer dissociation (ETD) with supplemental activation and activation time of 100 ms (charge state dependent). Note, the instrument control software LTQ Tune Plus version 2.6.0.1050 was used in this study, and the HCD energy of 40 on that version would be equivalent to 35 on updated versions, e.g., 2.6.0.1065 SP3. Dynamic exclusion duration was 60 s. The lockmass option was used (\(m/z\) 445.120025).

For analysis of synthetic phosphopeptides (PEPotec, Thermo Fisher Scientific), LC−MS/MS was performed using the Orbitrap system and also an EASY-nLC 1000 nanoflow LC instrument coupled to a Q Exactive ESI-quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific). Twenty
Table 1. LC–MS/MS Data Sets Used in This Study

<table>
<thead>
<tr>
<th>data set</th>
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<th>description of sample</th>
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<tr>
<td>HeLa phosphopeptide data sets</td>
<td>HCD, ETD, CID, MSA</td>
<td>Phosphopeptides were enriched from a HeLa tryptic digest</td>
<td>HeLa</td>
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<td>HeLa dephosphorylated peptide data sets</td>
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<td>Marx data set”</td>
<td>HCD</td>
<td>&gt;100 000 of singly phosphorylated peptides and nonphosphorylated counterparts were synthesized</td>
<td>Marx et al., 2013 (ref25)</td>
</tr>
</tbody>
</table>

“Downloaded from the PRIDE database.

synthetic singly phosphorylated peptides were mixed into 2 groups (10 peptides each), where phosphopeptide isoforms were separated from each other (see Supporting Information Table 1 for the peptide selection). Both the mixtures (1 pmol peptide each, based on the concentration given by the manufacturer) were analyzed in triplicates. The columns and the mobile phases mentioned above were used. LC gradient elution condition was initially 5% B to 35% B (10 min), and 100% B (12–20 min), with a flow rate of 300 nL/min. Data dependent acquisition was performed as described above, with some modifications. Dynamic exclusion was disabled. In the Orbitrap system, MS spectra were acquired in the Orbitrap, and then MS/MS spectra were acquired in the LTQ ion trap with a target value of 5000 ions and a maximal injection time of 50 ms, in centroid mode. In the Q Exactive system, MS spectra were acquired from m/z 300 to m/z 2000 with a resolution of 70 000 at m/z 200, a target value of 1 000 000 ions, and a maximal injection time of 120 ms, in profile mode. The 10 most abundant ions were selected for subsequent fragmentation (HCD) with normalized collision energy of 30. MS/MS spectra were acquired with a resolution of 17 500 at m/z 200, a target value of 50 000 ions, a maximal injection time of 250 ms, and the lowest mass fixed at m/z 100, in profile mode. All LC–MS/MS data sets used in this study are listed in Table 1.

Database Search

Orbitrap and Q Exactive data files were converted to mzXML files using Trans Proteomic Pipeline (TPP) version 4.4 VUVUZELA revision 1, and then to mgf files using MzXML2Search, with the default parameters, except for centroiding the fragment ions acquired by Q Exactive in profile mode. The HeLa phosphopeptide and dephosphorylated peptide data acquired with the four fragmentation methods (see Table 1) were searched with Mascot v2.4.1 (Matrix Science) against a concatenated forward–reverse SwissProt database (release date March 2010, Homo sapiens, total 40530 sequences). But, search results of the phosphopeptide data HCD data used for creating a simulated HeLa spectral library (see below) were obtained also with an older version of Mascot (v2.2.6) before the software update. The following Mascot search condition was used for both the phosphopeptide and dephosphorylated peptide data: carbamidomethylation (C) as a fixed modification, and oxidation (M), phosphorylation (S, T, and Y) and acetylation (protein N-terminus) as variable modifications. Trypsin was specified as an enzyme and two missed cleavage sites were allowed. Peptide mass tolerance and MS/MS ion tolerance were set to 50 ppm and 0.05 Da, respectively. Sequest searches of the HeLa phosphopeptide HCD data were done using the SORCERER platform v4.2.0 (Sage-N Research) with corresponding search parameters. To reduce a bias at the identification step prior to the investigation of phosphorylation site localization, the same identification condition was applied to different programs, i.e., a false discovery rate (FDR) of 1% was estimated using the target-decoy strategy by PeptideProphet. For each peptide spectral match, a discriminant score is calculated which is in turn used to assign probability values. Minimum probabilities corresponding to 1% FDR were applied as cutoffs, e.g., 0.80 and 0.90 for the Mascot and Sequest search results of the HeLa phosphopeptide HCD data, respectively. For phosphorylation site localization, Ascore v2008.04.16f BETA (Sage-N Research) was used on the Sequest results. Another localization tool phosphoRS v3.0 was used on the Mascot search results, by submitting the mgf files of the HeLa phosphopeptide HCD data to Proteome Discoverer v1.4.0.288 (Thermo Fisher Scientific) after adjusting mgf file formats. The phosphoRS option for considering H3PO4 neutral loss ions was disabled (refer to the Results and Discussion for the evaluation of that option). Also, to comparatively evaluate Mascot delta scores of nonphosphorylated peptides with those obtained from a large synthetic peptide data set (see below), Mascot searches of the HeLa dephosphorylated peptide HCD data were repeated via Proteome Discoverer v1.4.

For the 20 synthetic phosphopeptide HCD data obtained using the Q Exactive (see Table 1), Ascore v2011.01.12a BETA was used on Sequest in the same condition. PhosphoRS (the initial version implemented in the Proteome Discoverer v1.3.0.339) and phosphoRS v3.0 (the neutral loss option enabled or disabled) were used on Mascot v2.4.0 in the same condition, except for submitting the Q Exactive raw data to Proteome Discoverer without the file conversion. Also, to evaluate PTM score, MaxQuant v1.3.0.5 (Andromeda searching) was used by submitting the raw data with corresponding search parameters, but the data were deconvoluted before extraction of the ten most abundant peaks per 100 Th as described by Marx et al. Since the sequences and phosphorylation sites are known and the data complexity was low, all statistical filters were disabled. In this study, a large and complex synthetic peptide data set was also used, which required a different database search condition (described below).

Phosphorylation Simulation Algorithm

HCD MS/MS spectra of the HeLa dephosphorylated peptides identified by Mascot were modified to resemble spectra of singly phosphorylated peptides (Figure 1B). For that, peaks of main ions (a, b, y, and remaining precursor ions; neutral loss of water; monoisotopic and 3 isotopic ions; singly and multiply charged up to precursor charge but not more than 3) were
shifted by −18.0106 Da according to the site on which phosphorylation was being simulated. The shifted peaks correspond to ions with a neutral loss of phosphoric acid (−H3PO4, −97.9769 Da) that are expected to be present in MS/MS spectra of serine and threonine phosphorylated peptides. The main ions maintaining a phosphate group (+HPO3) were formed by creating peaks with masses 79.9663 Da greater than the ions in dephosphorylated peptide with 10% of their original intensity. When tyrosine phosphorylation was simulated, a different shift was performed resulting in the main ions maintaining a phosphate group (+HPO3) but not the neutral loss. In addition, the tyrosine immonium ion was shifted as well.

The peaks to be shifted were searched by their theoretical masses in the peak lists of the original MS/MS spectra within a mass window of ±0.05 m/z. Fragment ion m/z values were calculated according to Roepstorff–Fohlman–Bienemann nomenclature. A modification at residue i means that there is a mass shift in the b series of ions \( b_i \) to \( b_{i\pm} \), a series of ions \( a_i \) to \( a_{i\pm} \), and in the y series of \( y_{n+i} \) to \( y_{n\pm} \), where \( n \) is the number of residues in the peptide (see Figure 1B). The precursor mass increase by 79.9663 Da was taken into account.

The algorithm was implemented using Proteowizard, which is an external set of TPP software libraries and tools. The code of the converter msconvert was modified to add simulation functionality. The program SimPhosho and an Excel macro (for calculating deltaDot score, described below) will be available from our Web site (http://www.btk.fi/proteomics/) and a source code can be obtained from the authors upon request. The detailed workflow is summarized in Supporting Information Figure 1.

**Spectral Library of Simulated HeLa Phosphopeptides**

Spectral libraries of the simulated HeLa phosphopeptides were built using functionalities of SpectraST (version 4.0, released beta). The detailed workflow is summarized in Supporting Information Figure 1. Raw spectral libraries were generated from the two replicate HCD runs of the dephosphorylated HeLa sample, after applying the phosphorylation simulation to PeptideProphet validated Mascot search results with minimum probability of 0.90. These raw libraries were joined together to form a library, where spectral replicates of the same peptide ion were combined into a representative consensus spectrum. Since peptides having protein N-terminal acetylation were not recognized by the SpectraST used in this study, those peptides were not taken into account for the library (also in all search results obtained in this study). Finally, to allow FDR estimation of spectral search results, decoy entries were added to the consensus library using built-in function on SpectraST in the following way. Each peptide sequence is shuffled randomly to form a decoy sequence. After that the fragment ion peaks are repositioned according to that decoy sequence. The resulting spectral library (SimHeLa library) consisted of total 23,126 spectra: 11,563 consensus spectra of simulated phosphopeptides (4729 and 5410 spectra of doubly and triply charged phosphopeptides, respectively, and 567 spectra of phosphopeptides with methionine oxidation) and the other half 11,563 was artificially generated spectra of decoy entries. Only 2495 spectra (22% of the total) originated from single spectral replicates, 8327 (72%) had 2–3 replicates, 699 (6%) had 4–9 replicates, 33 had 10–19 replicates, and 9 spectra had 20 or more replicates based on which the consensus spectra were built. Also, to comparatively evaluate SimHeLa library with and without adding large sets of spectra, ion trap CID spectral libraries of mouse and yeast phosphopeptides downloaded from PeptideAtlas (51,420 and 18,412 spectra, respectively; release date 2013.07.15) were supplemented with decoy entries and then joined to SimHeLa library using SpectraST (SimHeLa-mouse-yeast library). All spectral libraries used in this study are listed in Table 2.

### Spectral Library Search for HeLa Data

The HeLa phosphopeptide HCD data (mzXML) were searched against SimHeLa and SimHeLa-mouse-yeast libraries by SpectraST with the default parameters. The results were validated by PeptideProphet, where an FDR of 1% required minimum probability of 0.70 and 0.80 for the results obtained with SimHeLa and SimHeLa-mouse-yeast libraries, respectively. A customized deltaDot score cutoff was applied to the spectral search results. DeltaDot score contributes to the total discriminant score (F-value) for every spectral match and reflects how much the first matching peptide spectrum differs from the second best match in dot product scores. When the DeltaDot score is equal to 0, it means that those spectral matches are not discriminated due to the identical dot score. DeltaDot score used in SpectraST considers a match to a nonhomologous peptide as the second best hit, therefore it is not applicable for assessing a difference in the quality of a match of phosphopeptides with alternative phosphorylation sites. Due to that, we recalculated DeltaDot score by comparing first and second best hits even if they represent the same peptide sequence with only one difference in positions of phosphorylation sites. Occasionally, due to a lack of the second best hits in SpectraST search results exported with the default.

<table>
<thead>
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<th>description of source data</th>
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<tr>
<td>SimMarx library</td>
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<td>Phosphopeptide HCD spectra simulated based on synthetic nonphosphorylated peptides of Marx data set</td>
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<tr>
<td>SimHeLa-mouse-yeast library</td>
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<td>SimHeLa library merged with CID spectral libraries of mouse and yeast phosphopeptidesb</td>
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</tr>
<tr>
<td>Hu&amp;Lam library</td>
<td>106 330a</td>
<td>Libraries of experimentally obtained and simulated CID spectra of phosphopeptidesb</td>
<td>Hu and Lam, 2013 (ref13)</td>
</tr>
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</table>

*a*Decoy entries included. b*Downloaded from the PeptideAtlas database.*
parameters, the recalculated score would be higher than the original. In this case, we used the original deltaDot score instead of the recalculated. A recalculated deltaDot score was selected as a prerequisite cutoff used for phosphorylation site localization. The detail of the whole workflow is presented in Supporting Information Figure 1. For sharing viewable annotated spectral matches, the SpectraST search of the HeLa phosphopeptide HCD data was repeated using SimHeLa annotated spectral matches, the SpectraST search of the Supporting Information Figure 1. For sharing viewable of phosphopeptide isoforms described above was manually peptide sequences and data simplicity. The cross-contamination libraries). No FDR cuto ...searches using two spectral libraries (Hu&Lam and SimHeLa-simHeLa-mouse-yeast libraries). Also, the CID data of the 20 synthetic phosphopeptides (converted to mzXML as well) were searched using four spectral libraries (synthetic phosphopeptide, Hu&Lam, SimHeLa, and Mass Spectrometry Database Searches for a Large Synthetic Peptide Data Set A large synthetic peptide data set generated by Marx et al. was also used for evaluating the performance of SpectraST and phosphoRS. The 96 Orbitrap Velos HCD raw data were downloaded from PRIDE, which contain both the singly phosphorylated peptides and the nonphosphorylated counterparts (Marx data set, see also Table 1). A simulated phosphopeptidic spectral library was created as described above, with some modifications. The raw data were converted to mzXML and then mgf files. The data were searched with Mascot v2.4.1, against the provided sequence database containing concatenated synthetic peptide sequences as artificial proteins and human IPI protein sequences as decoy entries. The following Mascot search condition was used: oxidation (M) and phosphorylation (S, T, and Y) as variable modifications, maximum four missed cleavages by trypsin, and MS and MS/MS mass tolerances of 50 ppm and 0.05 Da, respectively. The mass tolerance was significantly increased from the condition reported by Marx et al. (5 ppm), since we found that there seemed to be mass calibration fluctuation (+10–20 ppm) in those data apparently due to the lock-mass calibration failure (this was also seen in our Orbitrap Velos data). PeptideProphet validated nonphosphorylated peptides with minimum probability of 0.95 were used for the phosphorylation simulation, after applying Mascot delta score of 10 as an additional cutoff. On the basis of 29 944 peptide sequences, a consensus spectral library of the simulated phosphopeptides was created with decoy entries (SimMarx library, total 285 252 spectra) and used for SpectraST searching as described above. The results were filtered based on the following Mascot/phosphoRS search result, where only phosphopeptide spectral matches sharing the same sequences between two searches were taken into consideration (also refer to Results and Discussion section).

The Mascot search of the Marx data set was repeated for evaluating phosphoRS v3 with and without the neutral loss considering, via Proteome Discoverer v1.4 after adjusting mgf file formats. An FDR of 1% was estimated using the target-decoy strategy separately for phosphorylated and nonphosphorylated peptides, as described by Marx et al. The phosphopeptides identified with the synthetic peptide sequences were used for the phosphoRS evaluation. The nonphosphorylated peptides were used for evaluating Mascot delta scores at a peptide sequence level.

Data Sets Availability
Raw mass spectrometry data, protein sequence database, spectral libraries, and search results have been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD000474. For viewing the annotated spectra in the mz result file, a free Proteome Discoverer viewer is available from the Thermo Omics Software Portal (http://portal.thermo-brims.com)

RESULTS AND DISCUSSION

LC–MS/MS Analysis of Phosphorylated and Dephosphorylated Peptides Derived from HeLa Cells
The experimental protocol used here is similar to the one described previously (Figure 1A). Digested human HeLa cell proteins were enriched for phosphopeptides using TiO₂, and half of the sample was dephosphorylated by calf intestinal alkaline phosphatase treatment. Both samples were analyzed by LC–MS/MS (Table 1). An LTQ Orbitrap Velos instrument equipped with an octopole collision cell was used for acquiring MS/MS spectra generated by HCD (beam-type CID). While the Orbitrap has several fragmentation options including ion
trap CID, MSA (also ion trap CID), and ETD, the most striking similarity of phosphorylated and dephosphorylated peptides could be seen in HCD spectra, and also in ETD spectra (see representative spectra in Supporting Information Figure 2). Mascot database searches revealed that, at an FDR of 1% estimated by PeptideProphet, HCD outperformed all other fragmentation methods in the number of identifications, both in the analysis of phosphorylated and dephosphorylated peptides (Supporting Information Figure 3), a result that agrees with previous reports.25,31 Given the high spectral similarity and the superior number of identifications, HCD was expected to be the most suitable fragmentation method for the spectral simulation of phosphorylation (refer to the Introduction and Figure 1 for the simulation strategy). The majority of phosphopeptides identified by HCD were singly phosphorylated (84%) (Supporting Information Figure 4). Eighty-six percent of the singly phosphorylated peptides had more than one possible phosphorylation site (S, T or Y), which require validation of the precise phosphorylation site location. With high efficiency dephosphorylation in vitro (99%), we observed HCD spectra for 81% of the singly phosphorylated peptides in their dephosphorylated form (Supporting Information Figure 4). This provided a data set that allowed for development of the phosphorylation simulation approach and for evaluation of its performance in spectral library searching.

Creating a Spectral Library of Simulated Phosphopeptides

On the basis of the HCD spectra obtained from the HeLa dephosphorylated peptides (Table 1), spectra of singly phosphorylated peptides were computationally simulated. The principle of the simulation is illustrated in Figure 1B and further details are described in Experimental Section. For building a simulated spectral library, stringent identification criteria were used to ensure high quality HCD spectra of the HeLa dephosphorylated peptides (PeptideProphet minimum probability of 0.90 on Mascot search results), resulting in identification of 2654 unique peptides at 0.35% FDR. On the basis of these HCD spectra, 11 563 consensus spectra simulated for singly phosphorylated peptides were generated to cover all the possible phosphorylation sites on the peptide sequences. The simulated spectra and 11 563 decoy entries were used to compile a spectral library (SimHeLa library, see Table 2).

To evaluate SimHeLa library, 20 singly phosphorylated peptides were synthesized and HCD spectra were acquired using a Q-Exactive instrument (Table 1). The synthetic peptides were selected from differentially localized/identified phosphopeptides in preliminary analysis of the HeLa data set to clearly observe the difference in the performance of tested programs (see details in Supporting Information Table 1). LC-MS/MS analysis was performed in triplicates without using the dynamic exclusion function, resulting in acquisition of many spectral replicates for each phosphopeptide (>2000 spectra for 20 phosphopeptides, refer to Supporting Information Table 2). SpectraST searching of these synthetic phosphopeptide HCD data was performed with SimHeLa library. Representative spectral matches demonstrated well-predicted fragment ions in simulated spectra (Figure 1C and Supporting Information Figure 5). To investigate the spectral similarity, SimHeLa library was compared to a spectral library built from the same synthetic phosphopeptides (Table 2), and consequently SimHeLa library gave somewhat lower dot product scores in the searches (17% lower, see Supporting Information Figure 6A). Since phosphorylation sites on the synthetic phosphopeptides were known, performance in site localization could be investigated. At 1% FLR, SimHeLa library showed 17% lower sensitivity than the synthetic phosphopeptide spectral library did (Supporting Information Figure 6B). In this data analysis, 2 of the 20 phosphopeptides, which lacked alternative phosphorylation sites in the synthetic phosphopeptide spectral library (see Supporting Information Table 1), were excluded since the FLR calculation requires observation of false localization matches.

Furthermore, SimHeLa library was compared with the library previously reported by Hu and Lam, which consisted of experimentally obtained phosphopeptide spectra and those simulated for known phosphorylation sites based on non-phosphopeptide spectra (Hu&Lam library, see Table 2).13 As Hu&Lam library was developed based on ion trap CID spectra, the 20 synthetic phosphopeptides were analyzed also by ion trap CID without using the dynamic exclusion function (Table 1). Out of the 20 phosphopeptides, 6 were excluded from data analysis as their sequences were not covered by Hu&Lam library. In SpectraST searching of these limited data sets, SimHeLa library outperformed Hu&Lam library, of which the localization sensitivity at 1% FLR was only 2.4% (on the CID data) as opposed to the method reported here which was 61% (on the HCD data) (Supporting Information Figure 7). This localization failure of Hu&Lam library was presumably due to the combination of false localization on experimental spectra stored in the library, inaccurate simulation on CID spectra (see representative spectral matches in Supporting Information Figure 8), and also incomplete coverage of phosphorylation sites on stored peptides. Due to the high spectral similarity of phosphopeptide isoforms (i.e., the same sequence with different phosphorylation sites), a missing phosphorylation site in a spectral library may induce false localization to the alternative sites stored in the library.

Taken together, these results suggest that HCD spectra of dephosphorylated peptides served as a basis for the accurate simulation of phosphorylation. And also, in contrast to the previous method, the simulated spectral library covering all possible singly phosphorylated forms enabled the confident site localization by SpectraST searching. The proposed approach, spectral simulation and SpectraST (SimSpectraST), was further evaluated in comparison to different localization tools.

Performance Evaluation of SimSpectraST, Mascot/phosphoRS, Sequest/Ascore, and Andromeda/PTM Score, On Synthetic Phosphopeptide Data Sets

Before comparing localization tools, scores used in SpectraST were optimized for our purpose. Since SpectraST considers the deltaDot score only between nonhomologous alternative peptides, that score was recalculated to discriminate alternative phosphorylation sites even when those sites are on the same peptide sequence. In SimSpectraST searching of the 20 synthetic phosphopeptide HCD data using SimHeLa library (see Tables 1 and 2), a recalculated deltaDot score ≥0.005 maximized the localization sensitivity to 67% at 1% FLR (Supporting Information Figure 9). Therefore, that score cutoff was selected as a prerequisite for the site localization, and used in combination with the total discriminant score F-value.

SimSpectraST searching was evaluated by comparison to other tools developed for phosphorylation site localization, including phosphoRS22 on Mascot searching, Ascore21 on Sequest searching, and PTM score23 on Andromeda searching. At 1% FLR on the 20 synthetic phosphopeptide HCD data,
SimSpectraST searching using SimHeLa library resulted in 34% more spectral matches than other tested programs, including Sequest with and without Ascore, Mascot with and without phosphoRS v3, and Andromeda with PTM score. The SimSpectraST result obtained using SimHeLa library (see Table 2) was sorted by F-value, after applying a recalculated deltaDot score ≥0.005 (see Supporting Information Figure 9). The neutral loss option of phosphoRS v3 was disabled (refer to panel D). SimSpectraST outperformed the other methods in the number of correct spectrum matches at 0−5% FLRs. (B−E) Score thresholds for 1% FLR were examined on these search results. (B) FLRs across SimSpectraST F-value are shown after applying the recalculated deltaDot cutoff. An FLR of 1% required F-value of 0.49. (C) FLR analysis as a function of Ascore on Sequest identifications showed Ascore of 3 as a 1% FLR cutoff. (D) FLRs as a function of phosphoRS probability on Mascot identifications are shown. PhosphoRS and its version 3 were examined. PhosphoRS v3 with the H3PO4 neutral loss option enabled was also tested with and without excluding peptides not containing C-terminal Lys/Arg, which generate the predominant H2O loss (see Supporting Information Figure 5B). PhosphoRS v3 probability of 99.3% was taken as a 1% FLR cutoff. (E) FLR analysis as a function of PTM score on Andromeda identifications showed PTM score of 0.992 as a 1% FLR cutoff.

Figure 2. Phosphorylation site localization on synthetic phosphopeptides using different methods. (A) In the site localization on the 20 synthetic phosphopeptide HCD data (see Table 1), SimSpectraST was compared with 5 other tested programs/combinations, including Sequest with and without Ascore, Mascot with and without phosphoRS v3, and Andromeda with PTM score. The SimSpectraST result obtained using SimHeLa library (see Table 2) was sorted by F-value, after applying a recalculated deltaDot score ≥0.005 (see Supporting Information Figure 9). The neutral loss option of phosphoRS v3 was disabled (refer to panel D). SimSpectraST outperformed the other methods in the number of correct spectrum matches at 0−5% FLRs. (B−E) Score thresholds for 1% FLR were examined on these search results. (B) FLRs across SimSpectraST F-value are shown after applying the recalculated deltaDot cutoff. An FLR of 1% required F-value of 0.49. (C) FLR analysis as a function of Ascore on Sequest identifications showed Ascore of 3 as a 1% FLR cutoff. (D) FLRs as a function of phosphoRS probability on Mascot identifications are shown. PhosphoRS and its version 3 were examined. PhosphoRS v3 with the H3PO4 neutral loss option enabled was also tested with and without excluding peptides not containing C-terminal Lys/Arg, which generate the predominant H2O loss (see Supporting Information Figure 5B). PhosphoRS v3 probability of 99.3% was taken as a 1% FLR cutoff. (E) FLR analysis as a function of PTM score on Andromeda identifications showed PTM score of 0.992 as a 1% FLR cutoff.
phosphoRS (v2) and Andromeda/PTM score,25 we also tested this large HCD data set (Marx data set, see Table 1) with Mascot/phosphoRS v3 and subsequently with SimSpectraST. On Mascot identifications at a target-decoy FDR of 1%, phosphoRS v3 required probability of 99.6% for 1% FLR, while considering the neutral loss did not reach 1% FLR also on this data set (Figure 3A). This probability threshold of 99.6% was identical to the one obtained with phosphoRS v2 on the same data set previously.25 However, it was higher than the above-mentioned threshold obtained on the 20 synthetic phosphopeptide HCD data (99.3%, see Figure 2D), presumably due to the lower reliability of sequence identifications from the Marx data set. This data set is complex, with the exceptionally large number of homologous sequence peptides (isomeric, isobaric, and similar mass), which are difficult to be discriminated. Indeed, in the identifications of the nonphosphorylated counterparts from the Marx data set at 1% FDR, 29.1% of the spectral matches were observed with Mascot delta score of 0 (i.e., ambiguous identifications), and 60.4% with the delta score ≤10 (Figure 3B). This was significantly different from those of our HCD data sets (Table 1), as the HeLa dephosphorylated peptides and the 20 synthetic phosphopeptides (site isoforms not considered) showed 0.2% and 0.1% of the spectral matches with the delta score ≤10, respectively (Figure 3B). Nevertheless, the data set was tested with SimSpectraST. A simulated phosphopeptide spectral library was built from the nonphosphorylated peptides of the Marx data set by using stringent criteria, the delta score ≥10 and PeptideProphet probability ≥0.95 (SimMarx library, see Table 2). In SimSpectraST searching of the phosphopeptides from the Marx data set, the sequence identification was rather challenging probably due to the use of wide fragment mass tolerance by SpectraST. Therefore, only phosphopeptide spectral matches sharing the same sequences with the Mascot identifications at 1% FDR were taken into account (31 037 phosphopeptide spectral matches). SimSpectraST required F-value of 0.556 for 1% FLR, after applying recalculated deltaDot score ≥0.005.

Figure 3. Evaluation of Mascot/phosphoRS and SimSpectraST on a large HCD data set of synthetic phosphopeptides. An HCD data set of >100 000 synthetic phosphopeptides and their nonphosphorylated forms generated by Marx et al.25 (Marx data set, see Table 1) was used. (A) Mascot/PhosphoRS v3 was evaluated on the phosphopeptides of the Marx data set. On Mascot identifications at 1% FDR (target-decoy, Mascot score ≥28.2, 97 253 phosphopeptide spectral matches), phosphoRS v3 required probability of 99.6% for 1% FLR. (B) Mascot delta score at a sequence identification level was investigated. In the identifications of the nonphosphorylated counterparts from the Marx data set at 1% FDR (target-decoy, Mascot score ≥36.6), 29.1% of the spectral matches were observed with Mascot delta score of 0, and 60.4% with the delta score ≤10. In contrast, HCD data of HeLa dephosphorylated peptides and the 20 synthetic phosphopeptides (see Table 1) showed 0.2% and 0.1% of spectral matches with the delta score ≤10 in the same condition (Mascot score ≥36.6), respectively. These indicate the sequence identification ambiguity for the Marx data set. (C) SimSpectraST was evaluated on the Marx data set using SimMarx library (see Table 2). Only SimSpectraST identifications sharing sequences with the Mascot identifications at 1% FDR were taken into account (31 037 phosphopeptide spectral matches). SimSpectraST required F-value of 0.556 for 1% FLR, after applying recalculated deltaDot score ≥0.005.

Performance Comparison of SimSpectraST with Mascot/phosphoRS and Sequest/Ascore, on HeLa Phosphopeptide Data

SimSpectraST searching was evaluated also on singly phosphorylated peptides in the HeLa phosphopeptide HCD data using SimHeLa library (see Tables 1 and 2), at an FDR of 1% estimated by PeptideProphet (Supporting Information Figure 11). The result was compared to that obtained with Mascot/phosphoRS v3 and Sequest/Ascore. Although the three identification methods (i.e., SimSpectraST, Mascot, and Sequest) agreed only on 78.4% of phosphorylation sites on shared sequence identifications, it was clearly improved to 97.3% after applying the respective 1% FLR cutoffs of the localization methods (SimSpectraST, phosphoRS v3, and Ascore) (Figure 4A, and Supporting Information Figure 12).
The FLR cutoffs were applied only to peptides having >1 Ser/Thr/Tyr residues in the sequences. The SimSpectraST results at 1% FDR and 1% FLR are listed in Supporting Information Table 3, and also all the results of the three methods at 1% FDR are summarized in Supporting Information Table 4. To estimate actual FLRs in those results, all the site disagreement spectra (56 spectra) were manually interpreted. When we assume that all the agreed sites were correctly localized, estimated FLRs for SimSpectraST, phosphoRS v3, and Ascore were calculated as 1.5%, 0.63%, and 0.92%, respectively (Supporting Information Figure 12). These are well approximated to the expected FLR of 1%, supporting the validity of the cutoff scores obtained on the 20 synthetic phosphopeptide HCD data (refer to Figure 2). While the numbers of spectral matches at that condition (1% FDR, 1% FLR) were similar among the three methods (0.26% difference), SimSpectraST showed >16% more spectral matches than the other methods when compared only for the peptide sequences covered by SimHeLa library (Figure 4A,B). Those spectral matches by SimSpectraST resulted in 1198 unique phosphopeptides, which was >6.8% more than those by the other two methods (Figure 4C). These results support that SimSpectraST searching was sensitive in the phosphopeptide identification with simultaneous confident localization when compared in the same condition, while it has the limitation in the peptide coverage.

In that condition, the distribution (between peptide N- and C-termini) and composition (Ser, Thr, and Tyr) of the localized HeLa phosphorylation sites were similar among the three methods (Figure 4D,E). SimSpectraST localized phosphorylation sites to the N-terminal positions of tryptic peptides somewhat less frequently than phosphoRS v3 and Ascore; however, no significant difference in the site distribution was observed among the three methods (Figure 4D). In other words, the result of SimSpectraST would be as biased as those of phosphoRS v3 and Ascore. Most of tryptic peptides have the C-terminal lysine/arginine, therefore the peptide C-terminal phosphorylation sites are observed less frequently than other sites within peptide sequences. Also, tryptic cleavage tends to be inhibited by neighboring phosphorylation, which may reduce the occurrence of the peptide N-terminal phosphorylation sites. From the analytical point of view, localizing the N-terminal sites by HCD is sometimes difficult since a1 and b1 fragment ions are usually not observed (unless the peptides are N-terminally modified or contain N-terminal lysine/arginine) and furthermore the predominant H2O loss on a- and b-ions may mimic the
H₃PO₄ loss from phosphorylated serine/threonine (see representative MS/MS spectra in Supporting Information Figure 5B). Regarding the composition of the phosphorylation sites, the three methods localized: Ser, 92.9–94.2%; Thr, 5.6–6.6%; and Tyr, 0.2–0.5% (Figure 4E).

The effect of the size of spectral libraries was investigated in SimSpectraST searching. SimHeLa library was supplemented with publically available ion trap CID spectral libraries of mouse and yeast phosphopeptides, resulting in a 7-fold larger library (SimHeLa-mouse-yeast library, see Table 2). In SimSpectraST searching of the 20 synthetic phosphopeptide HCD data (see Table 1) at 1% FLR, SimHeLa-mouse-yeast library required the same F-value threshold (0.49) and showed 2.1% less spectral matches than SimHeLa library (Supporting Information Figure 13A,B). In the searching of the HeLa phosphopeptide HCD data, SimHeLa-mouse-yeast library showed 95 less spectral matches to human phosphopeptides (2.5% less) than SimHeLa library. Instead, additional 205 spectral matches to mouse/yeast phosphopeptides were observed (Supporting Information Figure 13C), even though the site localization by CID-HCD spectral matching was not so reliable (see Supporting Information Figure 7B). These results indicate that, the increased size of the spectral library did not lead to significant change in the score threshold and the sensitivity.

■ CONCLUSION

This study shows that simulated MS/MS spectra of singly phosphorylated peptides in combination with SpectraST spectral library searching successfully leads to accurate phosphorylation localization outperforming all other available and tested programs. For preventing the false localization, a spectral library of phosphopeptides needs to contain all possible isoforms, which was readily accomplished by the simulation algorithm. The results obtained from the HeLa sample and the synthetic peptides indicate that SimSpectraST is a sensitive method for the site-specific identification of phosphopeptides. While the identification capability is currently limited due to the incomplete peptide coverage of the library, our results suggest that SimSpectraST serves as a promising orthogonal method for supplementing and/or validating the phosphorylation sites obtained by database searching and localization tools. Creating a spectral library is indeed laborious; however, that library can be used in many (10s, 100s, 1000s, ... ) searches, e.g., for large-scale label-free quantifications. The simulation was developed for the HCD spectra but should also work on other beam-type MS instruments such as Q-TOF's. With some modifications, we expect the method to be applicable to ETD fragmentation. Finally, simulated phosphopeptide spectra could also be used for targeted quantification by selected reaction monitoring (SRM) in creation of transition lists and for data-independent analysis in creation of spectral libraries, in cases when particular phosphopeptides have not been observed by MS/MS before, cutting the costs of synthetic peptides. For further development, simulation of multiply phosphorylated peptides remains to be implemented.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information Tables 1 and 2 contain the list and data of 20 synthetic phosphopeptides, respectively. Supporting Information Tables 3 and 4 contain HeLa phosphopeptide data. Supporting Information Figures contain various detailed results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AMBIC, ammonium bicarbonate; ETD, electron-transfer dissociation; FA, formic acid; FDR, false discovery rate; FLR, false localization rate; HCD, higher energy collisional dissociation; MSA, multistage activation; MS/MS, tandem mass spectrometry; SimSpectraST, spectral simulation and validation method for peptide identification from MS/MS; SRM, selected reaction monitoring; TCEP, tris(2-carboxyethyl)phosphine; TPP, Trans Proteomic Pipeline

■ REFERENCES


