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193 nm Ultraviolet Photodissociation Mass Spectrometry for Phosphopeptide Characterization in the Positive and Negative Ion Modes

Michelle R. Robinson¹, Juliana M. Taliaferro², Kevin N. Dalby², and Jennifer S. Brodbelt^{1,*}

¹Department of Chemistry, The University of Texas, Austin, TX, USA 78712

²Division of Chemical Biology and Medicinal Chemistry, College of Pharmacy, University of Texas, Austin, TX 78712

Abstract

Advances in liquid chromatography tandem mass spectrometry (LC-MS/MS) have permitted phosphoproteomic analysis on a grand scale, but ongoing challenges specifically associated with confident phosphate localization continue to motivate the development of new fragmentation techniques. In the present study, ultraviolet photodissociation (UVPD) at 193 nm is evaluated for the characterization of phosphopeptides in both positive and negative ion modes. Compared to the more standard higher energy collisional dissociation (HCD), UVPD provided more extensive fragmentation with improved phosphate retention on productions. Negative mode UVPD showed particular merit for detecting and sequencing highly acidic phosphopeptides from alpha and beta casein, but was not as robust for larger scale analysis due to lower ionization efficiencies in the negative mode. HeLa and HCC70 cell lysates were analyzed by both UVPD and HCD. While HCD identified more phosphopeptides and proteins compared to UVPD, the unique matches from UVPD analysis could be combined with the HCD data set to improve the overall depth of coverage compared to either method alone.

Graphical abstract

Corresponding Author: Department of Chemistry, 105 E. 24th St, University of Texas, Austin, TX 78712, jbrodbelt@cm.utexas.edu
Tel: 512-471-0028.

Supporting Information: Additional figures include histograms showing the Xcorr scores and phosphate retention for HCD and UVPD analysis of peptides from casein, Venn diagrams for peptide and protein identifications obtained by HCD and UVPD for HeLa digests, histograms showing the distribution of productions upon HCD and UVPD of HeLa peptides, and a summary of the types of phosphopeptides identified by UVPD versus HCD for HeLa digests.

Keywords

Mass spectrometry; ultraviolet photodissociation; higher energy collision induced dissociation; phosphoproteomics

Introduction

Post-translational modifications (PTMs) direct a variety of biological activities, many of which are critical for sustaining normal cellular function. Phosphorylation is known to modulate enzymatic activity, alter protein folding, and inhibit or promote interactions with other proteins. Abnormalities in phosphorylation pathways have been associated with various diseases, a factor that has stimulated interest in kinases and phosphatases as therapeutic targets and their substrates as potential diagnostic biomarkers.^{1,2} The tremendous impact of phosphorylation has inspired the development of extremely powerful mass spectrometry strategies to map the dynamic phosphoproteome and quantify variations in phosphorylation as a function of cell state. The majority of these strategies are based on bottom-up workflows that rely on the ability to identify phosphorylation sites of proteins via MS/MS characterization of diagnostic proteolytic peptides.³ Due to significant inroads in enrichment methods, chromatographic capabilities, and performance metrics of mass spectrometers, there have been reports of grand scale phosphoproteomics.⁴⁻⁷ For example, identification of thousands of phosphopeptides, corresponding to 18,055 phosphosites and 4,708 phosphoproteins, was achieved using 1 mg of material via a 2D enrichment/fractionation strategy combining immobilized metal affinity chromatography (IMAC), hydrophilic interaction liquid chromatography (HILIC) and reversed phase LC.⁴ Yet even with these advances, there continue to be major efforts to improve depth of coverage, sensitivity of phosphopeptide detection, and confidence in phosphosite localization.

The choice of MS/MS technique can significantly impact the outcome of a PTM mapping experiment, and thus a variety of fragmentation methods have been evaluated for phosphopeptide analysis. Collision induced dissociation (CID) remains popular for peptide sequencing based on the ease of implementation, robust performance, and the multitude of available data analysis tools.⁸ Because CID promotes cleavage at the most labile bonds, phosphorylated peptides readily undergo loss of neutral phosphate groups while more informative backbone fragmentation is suppressed, making it difficult to both sequence the peptide and localize the modification.^{9,10} Higher energy collisional dissociation (HCD) is a beam type variation of CID that results in greater energy deposition. This facilitates cleavage of bonds other than those that are the weakest, and the phosphate neutral loss problem is largely mitigated although not entirely overcome.¹¹ Phosphate neutral loss can be eliminated nearly entirely using electron-based activation methods, including electron transfer dissociation (ETD) and electron capture dissociation (ECD) for which cleavage occurs at the N-C α bond to form *c* and *z* type ions while PTMs are preserved.¹²⁻¹⁶ Both ETD and ECD exhibit a significant charge state dependence, and peptides with insufficient charge density typically do not fragment efficiently. Charge density limitations are particularly problematic for phosphopeptides because the negatively charged phosphate decreases the overall positive charge, thus offsetting the effectiveness of ETD and ECD for phosphopeptide analysis.

Photodissociation has been explored as an alternative to collision- or electron-based dissociation methods. Infrared multiphoton dissociation (IRMPD) has been the most widely adopted, but because fragmentation occurs through the slow heating of vibrational modes, many of the same limitations that are observed for CID of phosphopeptides also apply for IRMPD.¹⁷ At the same time, IRMPD can provide an effective means for screening complex peptide mixtures for phosphorylation based on the superior IR absorbance of phosphopeptides compared to nonphosphorylated peptides, causing the former to dissociate while the latter are left intact.^{18–21}

In the near IR regime, femtosecond laser-induced ionization/dissociation (fs-LID) uses ultrafast laser pulses to induce electron loss through tunneling followed by peptide bond cleavage to form *a*, *b*, *c*, *x*, *y*, and *z* ions.^{22,23} Because dissociation is initiated by electron abstraction, fs-LID is most effective for singly protonated peptides based on their lower ionization potentials compared to more highly charged ions, a trend that is opposite to CID/HCD and ETD/ECD fragmentation. Along with superior sequence coverage, singly charged phosphopeptides exhibited the greatest phosphate retention on productions. No phosphate neutral loss was detected from *c/z* or *a/x* ions with the exception of potentially diagnostic *a*+1–98 ions observed C-terminal to the site of phosphorylation. FsLID has also been applied in negative ion mode for the analysis of multiply deprotonated phosphopeptide anions, where phosphate retaining *a/x* ions facilitate sequencing and modification localization.²⁴ Long activation times and narrow charge state restrictions ultimately have limited the adoption of fsLID for large-scale analysis.

Ultraviolet photodissociation has also been applied for the analysis of phosphopeptides. For phospho-serine containing peptides, 220 nm UVPD resulted in the dominant neutral loss of tyrosine side-chains via homolytic bond cleavage while other fragmentation channels including peptide backbone cleavage and phosphate loss were suppressed.²⁵ In contrast, 266 nm UVPD yielded mostly *b* and *y* ions with only minor *a* and *x* ions.²⁶ Likewise, phosphate neutral loss patterns were similar between 266 nm UVPD and CID even for phospho-tyrosine peptides for which the site of phosphorylation coincides with the site of photon absorption.²⁶ Photodissociation using 157 and 193 nm photons is also widely applicable for proteomics applications because photons are absorbed efficiently by the peptide backbone. High energy fragmentation channels are accessible upon 157 and 193 nm photoexcitation to yield *a/x*, *c/z*, and *d/v/w* side chain loss ions in addition to more conventional *b/y* ions.^{17,27,28} Both 157 nm and 193 nm UVPD have been used for various proteomics applications^{27,29–32}, ranging from single protein analysis to more complex whole cell lysates in both positive and negative modes.^{33–36} To date, PTM analysis has been primarily conducted in negative mode based on the improved retention of labile modifications including sulfation, O-glycosylation, and phosphorylation; however, larger scale analysis in the negative mode has been significantly hindered by poor ionization efficiency.^{35,37–40} Both 157 nm UVPD and 193 nm UVPD have been used to characterize phosphopeptides.^{35,41–44} For example, the fragmentation behavior of singly protonated phosphopeptides with C-terminal arginine residues was explored using 157 nm UVPD, showing that the loss of the phosphate group was time-dependent and varied based on whether the phosphate group was appended to tyrosine, serine, or threonine.⁴² 193 nm UVPD of singly protonated phosphopeptides that had N-terminal basic residues resulted in prominent *a*+1–98 ions

observed C-terminal to sites of phosphorylation, thus providing a signature for the site of phosphorylation.⁴¹ Productions that are diagnostic for phosphorylation have also been observed in the 193 nm UVPD spectra of phosphopeptide anions where formation of γ - $\text{H}_3\text{PO}_4\text{-NH}_3$ ions was observed N-terminal to the site of phosphorylation in addition to an array of phosphate retaining sequence ions.³⁵ Unique phosphorylation sites in the protein TrpM7 were identified using ET-UVPD in which hydrogen rich radical peptide ions generated from electron transfer without dissociation (ETnoD) were activated and dissociated using 193 nm UVPD, resulting in a rich array of productions for which z ions were most abundant.⁴³ The most recent study compared 193 nm UVPD and HCD for the analysis of phosphopeptides from a HeLa digest and found improved modification retention using UVPD.⁴⁴

The present study expands upon UVPD-based phosphoproteomics via analysis of phosphopeptides enriched from casein proteins in addition to HeLa and HCC70 primary ductal carcinoma cell lysates. UVPD is implemented on an Orbitrap Fusion Tribrid mass spectrometer, and both polarities are evaluated for phosphopeptide characterization. The overall depth of coverage is compared between UVPD and HCD as well as differences in the identified phosphopeptides such as length, charge state, and number of phosphorylations. The extent of phosphate neutral loss upon UVPD is also explored.

Experimental

Cell Culture and Sample Preparation

HeLa and HCC70 stable shRNA scramble cells were maintained at 37 °C and 5% CO_2 and cultured in accordance with ATCC guidelines. HeLa was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and HCC70 was grown in RPMI media supplemented with 10% FBS, 2 mM glutamine, 1% antibiotic/antimycotic (10 U/mL penicillin, 10 $\mu\text{g/mL}$ streptomycin, 0.025 $\mu\text{g/mL}$ amphotericin B), and 1 $\mu\text{g/mL}$ puromycin. Cells were lysed in buffer containing 50 mM ammonium bicarbonate (pH 8), 8 M urea, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 μM microcystin-LR, 100 nM calyculin A, complete EDTA-free protease inhibitor mix (Roche), and phosSTOP phosphatase inhibitor mix (Roche). Protein concentration was measured by Bradford assay and lysates were stored at -80°C prior to digestion.

Protein disulfide bonds were reduced by 30 minute incubation with 5 mM dithiothreitol at 56 °C then alkylated by 30 minute incubation with 15 mM iodoacetamide at room temperature in the dark. Lysates were then digested with Lys-C (Promega, Madison, WI) in a 1:200 enzyme to protein ratio for 2–4 hours at 37 °C. The urea concentration was then diluted to 1.5 M by addition of 50 mM Tris HCl, 5 mM CaCl_2 and trypsin (Thermo Fisher Scientific, Grand Island, NY) was added in a 1:50 enzyme to protein ratio for overnight digestion at 37 °C. Digests were desalted using Waters tC18 Sep-Pak cartridges and dried by speed vac. Immobilized metal affinity chromatography (IMAC) was used for phosphopeptide enrichment.⁴⁵ Briefly, Ni-NTA magnetic agarose beads (Qiagen, Germantown, MD) were prepared for IMAC by shaking in 40 mM EDTA (pH 7.5) for 1 hour followed by thorough washing with water and shaking in 100 mM FeCl_3 for 1 hour.

Protein digests were resuspended in IMAC loading buffer composed of 80% ACN containing 0.15% TFA and added to the IMAC beads following the removal of FeCl_3 solution. Phosphopeptide binding was carried out over 1 hour of shaking followed by three washes in loading buffer to remove non-phosphorylated peptides. Phosphopeptides were recovered from the beads by vortexing for 1 min and 15 sec in 100 μL of 50% ACN, 0.7% NH_4OH (pH ~11) and the eluent was immediately neutralized in 50 μL of 4% formic acid and dried completely. IMAC enriched HeLa samples from 500 μg of starting material were resuspended in 20 μL of 0.2% formic acid prior to LCMS analysis.

An equimolar mixture of alpha s1 casein, alpha s2 casein, and beta casein from bovine were digested in 50 mM Tris HCl (pH 7.5) with trypsin in a 1:50 enzyme to protein ratio overnight at 37°C. Digested peptides were directly added to a 5x excess (w/w) of ceramic hydroxyapatite type I, 20 μm beads (Bio Rad, Hercules, Ca) suspended in 50 mM Tris HCl. Phosphopeptide binding to HAP occurred for 1 hour at room temperature with shaking. Unmodified peptides were washed away using 50 mM Tris HCl containing 20% ACN. Bound phosphopeptides were recovered from the HAP resin by 15 minutes of shaking in 1 M KH_2PO_4 (pH 7.8). The eluent was split and desalted using both C18 and graphite solid phase extraction (SPE) to avoid the loss of very hydrophilic phosphopeptides. Desalted samples were dried and resuspended in water for LCMS analysis.

LC-MS/MS Analysis

Cell lysate separations were carried out on a Dionex Ultimate 3000 nano LC equipped with water as mobile phase A and acetonitrile (ACN) as mobile phase B, each containing 0.1% formic acid. For LCMS analysis 4 μL of IMAC-enriched HeLa phosphopeptides was directly injected onto a 30 cm $\times$ 75 μm UPLC column with integrated emitter (New Objective, Woburn, MA), packed in house using 1.8 μm , 120 Å UChrom C18 (nanoLCMS Solutions, Gold River, CA). The column was heated to 60 °C inside a custom built column oven that was fabricated similarly to that described by Coon and coworkers.⁴⁶ For HeLa phosphopeptides, separation occurred over an 80 minute gradient in which the percentage of mobile phase B was increased from 2–25% B during the first 65 minutes and further increased from 25–40% B during the final 15 minutes. For HCC phosphopeptides, the same gradient steps were used, but the increase from 2–25% B was carried out more gradually over 118 minutes. Eluting peptides were analyzed using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific Instruments, Bremen, Germany) equipped with a 193 nm Coherent ExciStar XS excimer laser (Santa Clara, CA) for ultraviolet photodissociation.⁴⁷ Orbitrap detection was used for both MS1 and MS2 measurements at resolving powers of 60K and 15K (at m/z 200), respectively. Data dependent MS/MS analysis was performed in top speed mode with a 3 s cycle time during which precursors detected within the range of m/z 400–1500 were selected for activation in order of abundance. Quadrupole isolation with a 1.6 m/z isolation window was used and dynamic exclusion was enabled for 45 s. AGC targets were 2E5 for MS1 and 1E5 for MS2, with 50 ms and 100 ms maximum injection times, respectively. The signal intensity threshold for MS2 was 5E4. HCD was performed using 30% normalized collision energy and for UVPD, 2 pulses at 2.5 mJ were used. For triplicate HCD and triplicate UVPD LCMS runs, one microscan was used for MS¹ and two microscans were used for MS2. The number of MS2 microscans was decreased to one for

the HCC lysate in order to maximize the depth of coverage. Alternating activation experiments were also performed for HeLa in which the same precursor ion was selected for consecutive activation using UVPD and HCD. For these experiments, the MS/MS parameters listed above were used for run one, the MS2 signal intensity threshold was increased to 5E6 for run two, and the maximum MS2 injection time was increased to 300 ms for run three.

Negative mode LCMS analysis of phosphopeptides from alpha and beta casein proteins was carried out on a Thermo Scientific Instruments Velos Pro dual linear ion trap mass spectrometer equipped with a 193 nm Coherent ExciStar XS excimer laser (Santa Clara, CA) as previously described for UVPD.^{27,48} A Dionex ultimate 3000 nano LC was used for separations in which mobile phase A was 5 mM ammonium acetate in water and mobile phase B was 5 mM ammonium acetate in 90% methanol. Peptides were loaded for 4 minutes at 5 μ L/min onto a 3 cm $\times$ 100 μ m trap column (New Objective, Woburn, MA) packed with 5 μ m Michrom magic C18. The analytical column was 20 cm long $\times$ 75 μ m OD with an integrated emitter tip (New Objective, Woburn, MA) packed with 3.5 μ m Waters Xbridge BEH C18 (Milford, MA). Separation occurred over a 45 minute linear gradient from 2–45% B. MS1 survey scans were acquired from m/z 400–2000 and data dependant UVPD using a single 2 mJ pulse was carried out on the top five most abundant precursors.

Data Analysis

Proteome Discoverer 1.3 was used for positive mode UVPD and HCD analysis of the IMAC enriched cell lysates. For UVPD, a non-fragment filter was applied to MS2 spectra to remove precursor peaks within a 1 Da mass window offset prior to database search using Sequest and a human database obtained from Uniprot. All productions including a , b , c , x , y , and z were used for spectrum matching for UVPD while only a , b , and y ions were used for HCD. Neutral losses from a , b , and y ions were considered for both UVPD and HCD. The following additional database search parameters were consistent between UVPD and HCD experiments: 10 ppm precursor mass tolerance; 0.02 Da fragment mass tolerance; N-terminal acetylation, methionine oxidation, and serine/threonine/tyrosine phosphorylation dynamic modifications; carbamidomethyl cysteine static modifications. Percolator was used for PSM validation and phosphoRS 3.0 was used for phosphosite localization. Only rank 1, high confidence PSMs were considered and an isoform probability of 75% was required for phosphosite localization.

Negative mode UVPD results for alpha and beta casein phosphopeptides were interpreted using MassMatrix Xtreme 3.0.10.16, which is programmed to search for a , x , c , z , and y type productions and has been optimized for negative mode UVPD analysis. Peptide mass and fragment mass tolerances were ± 1.00 Da and ± 0.80 Da, respectively, and the minimum pp score was 4 while the minimum pp_{tag} score was 1.

Results and Discussion

UVPD and HCD analysis of alpha and beta casein phosphopeptides

UVPD and HCD were initially evaluated for phosphopeptide analysis in the positive mode using phosphopeptides generated from trypsin digestion of bovine phosphoproteins alpha S1 casein, alpha S2 casein, and beta casein. Eight singly phosphorylated peptides including DIGpSESTEDQAMDIK, EKVNELpSK, EQLSTpSEENSK, FQpSEEQQTEDELQD, NMAINPpSK, TVDMEpSTEVFTK, VPQLEIVPNpSAEER, and YKVPQLEIVPNpSAEER as well as three doubly phosphorylated peptides including DIGpSEpSTEDQAMEDIK, EQLpSTpSEENSK, and EQLpSTpSEENSKK were identified in LC-MS runs by both UVPD and HCD. All peptides were detected in the 2+ charge state and three peptides, FQpSEEQQTEDELQDK, VPQLEIVPNpSAEER, and YKVPQLEIVPNpSAEER, were detected in both 2+ and 3+ charge states. HCD and UVPD spectra for TVDMEpSTEVFTK are displayed in Figure 1A–B. For both activation methods, the dominant productions series are *b* and *y* ions; however, UVPD also yields numerous *a*, *x*, *c*, and *z* ions, which contribute to an overall richer MS/MS spectrum. The additional productions generated by UVPD have the potential to facilitate more confident peptide identifications when using database search programs as previously demonstrated for unmodified peptides.²⁷ Significantly higher Sequest Xcorr scores for UVPD compared to HCD for seven out of the eleven peptides examined (Figure S1 A–B) are a direct indication of more confident sequence assignments. Even small increases in Xcorr can help to distinguish the correct peptide sequence from false matches as demonstrated by the commonly used ΔC_n threshold of just 0.1, where ΔC_n represents the difference in Xcorr between the highest scoring candidate and each lower scoring candidate.⁴⁹ One drawback of using UVPD is the lower fragmentation efficiency compared to HCD as shown by the abundant undissociated precursor ion in Figure 1B. The low fragmentation efficiency of UVPD is largely related to achieving a compromise between using a photon flux that offers adequate overlap with the ion population and yet does not promote excessive multi-photon absorption that results in decomposition of peptides and primary productions into extremely small, uninformative fragments and internal ions. A non-fragment filter may be applied prior to the database search to remove the surviving precursor ion from the MS/MS spectrum and enhance the relative abundances of productions to improve search results. For abundant precursor ions, the fragment ions remain above the noise level of the mass spectrometer, but for less abundant precursor ions the lower fragmentation efficiency can preclude accurate peptide sequencing. The implications of UVPD fragmentation efficiency are discussed in more detail in a subsequent section: *Alternating UVPD and HCD analysis of IMAC enriched HeLa*.

For peptides that carry PTMs, retention of modifications on productions is critical in order to facilitate accurate localization within the peptide sequence. For TVDMEpSTEVFTK, phosphate neutral losses from *y*7, *y*8, *y*9, and *y*10 are among the most abundant productions in the HCD spectrum (Figure 1A), and the abundances of these ions exceed those of their phosphate-retaining counterparts. In contrast, the abundances of the phosphate-retaining *y*7, *y*8, *y*9, and *y*10 ions produced upon UVPD of TVDMEpSTEVFTK are greater compared to the same *y*-ions, which undergo phosphate neutral loss (Figure 1B). Greater phosphate

retention using UVPD is consistent with recent reports and is believed to be a result of the fast and high energy deposition upon absorption of 193 nm photons (6.4 eV per photon) compared to lower energy, step-wise collisional activation methods such as CID and HCD.⁴⁴ When the abundances of phosphate-retaining productions were compared to the abundances of phosphate neutral loss productions, all eleven casein phosphopeptides exhibited improved phosphate retention when subjected to UVPD compared to HCD. In Figure S2A and S2B, the abundance of phosphate-retaining productions is plotted for each peptide as a percentage of the total abundance of productions that include the site of phosphorylation. All ions series were considered for UVPD, while *a*, *b*, and *y* ions were considered for HCD. An average increase in phosphate retention of 20% for doubly charged peptides and 25% for triply charged peptides was observed using UVPD.

Photodissociation of deprotonated phosphopeptides yielded significantly different fragmentation patterns compared to the UVPD spectra of protonated phosphopeptides. For UVPD of doubly deprotonated TVDMEpSTEVFTK, *a* and *x* ions accounted for full sequence coverage (Figure 1C). The phosphate loss behavior also differed, with very few productions exhibiting phosphate loss for the deprotonated phosphopeptides. This is in stark contrast to both UVPD and HCD of doubly protonated TVDMEpSTEVFTK (Figure 1A–B) for which nearly every product ion that included the phosphorylated residue experienced some degree of phosphate loss. Despite superior phosphate retention of deprotonated peptides, the ionization efficiency in the negative mode is lower than the positive mode for phosphopeptides and most classes of peptides, making it challenging to adopt negative polarity UVPD-MS for large scale analysis.⁵⁰

Only the most highly phosphorylated peptides with the greatest net negative charge, including QMEAEpSIpSpSpSEEIVPNpSVEQK, NTMEHVpSpSpSEESIIPpSQETYK, ELEELNVPGEIVEpSLpSpSpSEESITR, and RELEELNVPGEIVEpSLpSpSpSEESITR, ionized more efficiently in negative mode compared to positive mode. To aid in the detection of these peptides, hydroxyapatite (HAP) enrichment was used based on the previously demonstrated propensity for isolation of phosphopeptides with higher phosphorylation states as well as acidic sequence motifs.^{51,52} Following HAP enrichment, the recovered phosphopeptide isolate was split and desalted using either graphite SPE or C18 SPE to ensure recovery of both hydrophilic and hydrophobic peptides. In Figure 2A and 2B, LC-MS base peak chromatograms are displayed for positive mode and negative mode ESI-MS analysis of the same HAP-enriched casein protein digest. Chromatographic peaks shaded in blue represent peptides recovered from C18 SPE and peaks shaded in purple represent peptides recovered using graphite SPE. The most hydrophilic peptides, QMEAEpSIpSpSpSEEIVPNpSVEQK and NTMEHVpSpSpSEESIIPpSQETYK, were only detected following desalting on graphite while all other peptides were effectively recovered after desalting on C18. In positive mode (Figure 2A), singly and doubly phosphorylated peptides were preferentially detected while the most highly phosphorylated peptides from alpha and beta casein were not detected. These peptides, each modified with at least four and up to five negatively charged phosphates, ionized more efficiently in negative mode while at the same time ionization was suppressed for peptides in lower phosphorylation states (Figure 2B). UVPD was applied for characterization of these multiply phosphorylated peptides. Regardless of the peptide length and number of phosphorylations, the sequence coverage

afforded by UVPD remained high with no significant phosphate neutral losses observed as demonstrated for UVPD of quadruply deprotonated QMEAEpSlpSpSEEIVPNpSVEQK shown in Figure 2C. While UVPD in the negative ion mode has shown merit for phosphopeptide characterization, especially for peptides in high phosphorylation states, improvements in ionization efficiency are required before negative UVPD can be routinely implemented on a larger scale. Thus, UVPD analysis in the positive mode will be the focus of the remainder of the study.

UVPD and HCD analysis of IMAC-enriched phosphopeptides from HeLa digests

To evaluate the performance of UVPD for phosphopeptide analysis on a more global scale, phosphopeptides were enriched from a HeLa cell lysate using Fe(III)-IMAC. UVPD and HCD analyses of the IMAC-enriched HeLa digests were carried out in triplicate using an Orbitrap Fusion mass spectrometer using high resolution/high accuracy analysis for both precursor ions and fragment ions. This dual high accuracy mode improves the accuracy of peptide identifications, especially for UVPD which uses a greater search space to account for all possible product ion series. Greater depth of coverage was achieved using HCD compared to UVPD, which is consistent with recent results reported in another study.⁴⁴ At 1% FDR, HCD identified an average of 4,033 phosphopeptides from 4,280 total peptides per LC-MS run, whereas UVPD identified an average of 2,897 phosphopeptides from 3,065 total peptides accounting for 94% enrichment efficiency for both activation methods (Tables S1 and S2). When searched collectively the replicate LC-MS runs yielded 6,565 phosphopeptides and 2,027 phosphoproteins for HCD compared to 3,945 phosphopeptides and 1,500 phosphoproteins for UVPD (Figure 3A–B). Electron transfer dissociation (ETD) has also been well established for the analysis of phosphopeptides. In a similar study comparing ETD to HCD and EThcD for phosphopeptides, a 47% decrease in phospho-PSM identifications was observed using ETD relative to HCD.⁵³ A smaller decrease of 33% in the number of phospho-PSM identifications was observed between UVPD and HCD, suggesting that UVPD would likely provide greater phosphoproteome coverage than ETD.

Not all UVPD identifications were encompassed by the larger HCD dataset as shown in the Venn diagrams in Figure 3A; 231 phosphopeptides, corresponding to 49 phosphoproteins, were uniquely identified using UVPD. When the overlap between replicate runs acquired using the same activation mode was evaluated, a significant amount of variation was observed as well. On average 63% of the peptides from each individual run were also identified in the other two replicate runs (Figure S3A). The overlap in protein identifications was greater with 77% of proteins from each run detected in all three replicates (Figure S3B). The variations in peptide and protein identifications between replicate runs are a reflection of the sample complexity, which results in selection of different precursor ions for MS/MS analysis in each LC-MS run. Comparing UVPD and HCD based on the collective results from triplicate analysis improves the likelihood that differences in observed peptide and protein identifications are specifically related to the activation method and not due to random sampling or under-sampling in the MS/MS workflow.

In addition to the number of peptide and protein identifications, other properties of the phosphopeptides identified by UVPD and HCD were explored and compared. Consistent

with the results for alpha and beta casein, the average Xcorr score of UVPD-identified peptides (4.3) was greater than the average Xcorr score of HCD-identified peptides (3.6) (Figure 3B). Despite higher Xcorr scores that are indicative of more extensive fragmentation, phosphate localization using the phosphoRS⁵⁴ algorithm developed by Taus *et al.* was slightly less successful for UVPD (localization rate of 74%) compared to HCD (81%) based on the lower number of peptides with a phosphoRS isoform probability greater than 75% (Figure 3B). This result was unexpected given the trend of greater phosphate retention on UVPD-generated productions that was observed for phosphopeptides from alpha and beta casein. Thus, the percent phosphate retention on UVPD versus HCD productions was also evaluated for the HeLa dataset. For all peptides in the same charge state and considering only the productions that contained the site of phosphorylation and therefore could undergo neutral loss, the ion abundances for phosphate-retaining ions and the corresponding phosphate loss ions were summed. Using these values, the percent phosphate retention was calculated by the following equation:

$$\% \text{ Phosphate retention} = \frac{\sum i(\text{phosphate retaining ions})}{\sum i(\text{phosphate retaining ions}) + \sum i(\text{phosphate loss ions})} \times 100$$

The results for peptides in the 2+, 3+, and 4+ charge states are plotted in Figure 3C. For all charge states, UVPD productions exhibited greater phosphate retention compared to HCD. The improvement using UVPD compared to HCD is greatest for peptides in lower charge states for which the lower overall proton mobility leads to increased phosphate rearrangement and loss upon collisional activation.^{55,56} For doubly charged peptides an 18% increase in phosphate retention was achieved using UVPD relative to HCD, and a 22% increase was achieved for triply charged peptides. These results suggest that UVPD should perform as well if not better than HCD for phosphorylation site assignment. Consequently, lower UVPD localization rates are likely the result of using non-optimal localization parameters associated with the phosphoRS algorithm. With the current photodissociation set-up on the Orbitrap Fusion, UVPD spectra are designated as CID events at zero normalized collision energy. Thus UVPD data is processed by phosphoRS localization software analogous to CID data, and only singly and doubly charged *b* and *y* ions are considered for localizat on.⁵⁴ While *b* and *y* ions do constitute the majority of the UVPD product on current, other ion types also contribute as shown in the bar graphs in Figure S4A–B which display the distribution of *a*, *b*, *c*, *x*, *y*, and *z* ions based on the total number of ions (S4A) as well as the abundances of each ion type (S4B). Further, the UVPD product ion distributions appears to be charge state dependent with the ratio of *y* ions to other product ion types decreasing as the peptide charge increases, indicating a shift toward more uniform product ion distribution for peptides in higher charge states. When these and other characteristic UVPD fragmentation trends are incorporated into phosphorylation localization software, the accuracy of phosphorylation site assignment will likely improve and better reflect the improvements in phosphate retention and extent of fragmentation that is observed for UVPD.

The photodissociation behavior of tyrosine-phosphorylated peptides was also explored separately from the serine- and threonine-phosphorylated peptides that make up the majority

of phosphopeptides in HeLa cells. The side chains of aromatic amino acids including tryptophan, phenylalanine, and tyrosine may absorb 193 nm photons, and several studies have demonstrated enhanced UVPD efficiency for peptides containing a greater number of aromatic residues²⁷ or following the attachment of aromatic chromophores.⁵⁷ Because the aromatic side chain of tyrosine may enhance or modulate absorption of 193 nm photons, the phosphate retention on productions from tyrosine-containing phosphopeptides was of special interest. For peptides with phosphorylation confidently localized on tyrosine, the average percent phosphate retention was $86 \pm 3\%$ and $85 \pm 5\%$ for HCD and UVPD, respectively, in the three replicate runs, indicating that UVPD does not promote increased phosphate loss for phospho-tyrosine peptides. A commercial phospho-tyrosine peptide, TSTEPQpYQPGENL, was also analyzed by direct infusion with UVPD-MS and HCD-MS to confirm the phosphate retention trend. As shown in Figure S5, both HCD and UVPD spectra are comprised primarily of phosphate-retaining product ions.

In addition to scoring and localization results, several more general characteristics of HeLa phosphopeptides were compared to further distinguish the performance metrics of UVPD and HCD. In Figure 3D, the distributions of 2+, 3+, and more highly charged (>3+) peptides identified by UVPD and HCD are displayed. Singly charged phosphopeptides are not included in this comparison because only a handful of singly protonated phosphopeptides were identified, thus prohibiting adequate statistical comparisons. While the charge state distributions for UVPD and HCD were similar, a larger percentage of phosphopeptides specifically identified by UVPD were doubly charged compared to HCD, the latter, which identified a larger percentage of more highly charged phosphopeptides. Although subtle, this trend reinforces previous results demonstrating successful UVPD even for peptides with low proton mobility.^{33,34} Peptide length distributions were also compared between those phosphopeptides identified by UVPD versus HCD (Figure 3E), and UVPD appeared to outperform HCD for the detection of short peptides containing fewer than 12 amino acids. Because shorter peptides are likely to carry fewer charges, the UVPD length distribution correlates well with the UVPD charge state distribution. Finally, differences in the overall phosphorylation state as well as differences in the individual frequencies of phospho-serine, phospho-threonine, and phospho-tyrosine were explored for UVPD and HCD phosphopeptides (Table S3). Singly phosphorylated peptides were most commonly observed using both UVPD and HCD for which only 5% and 10% of peptides were detected with more than one phosphorylation, respectively. The distributions of phosphorylation sites found on serine, threonine, and tyrosine residues were almost identical between the two activation methods, in addition to closely agreeing with previously reported distributions for human cell lines, lending additional validity to both datasets.⁵⁸

Alternating UVPD and HCD analysis of IMAC enriched HeLa

To this point, no significant differences related to UV photoactivation could account for the disparity in the number of peptide and protein identifications compared to HCD. A series of experiments in which HCD and UVPD were implemented in back-to-back alternating scans were carried out to further investigate the basis for the lower number of UVPD identifications. First separate UVPD-only and HCD-only analyses were performed to provide a baseline for comparison (Figure 4A). The number of scans averaged was reduced

from two to one for this experiment to minimize the time penalty for UVPD which requires 2 ms per laser pulse used for activation in contrast to the faster sub-millisecond HCD activation period. In subsequent LC-MS runs, UVPD and HCD were carried out within the same analysis as consecutive, alternating activation events, ensuring identical precursor selection and number of MS/MS acquisitions for each activation method. Based on the data sets acquired using the alternating MS/MS scan mode, the difference in the number of UVPD and HCD peptide identifications dramatically decreased; however, there were still 1022 unique HCD peptides (Figure 4B) indicating an additional handicap associated with UVPD.

Sensitivity is also a concern for UVPD due to lower fragmentation efficiency and because UVPD results in a greater array of fragmentations, thus sub-dividing ion current into more channels. The overall lower ion load for a phosphoproteomics sample relative to a complete proteome exacerbates this problem.⁴⁴ To evaluate differences in MS/MS sensitivity between UVPD and HCD, two additional alternating LC-MS/MS experiments were conducted in which low abundance precursor ions were either excluded from activation by raising the threshold for MS2 selection, or their abundances were boosted by increasing the maximum ion injection time. When the maximum ion injection time was increased from 100 ms to 300 ms (Figure 4C), the overlap in peptide identifications substantially improved while the number of unique peptides was reduced to 71 for UVPD and 319 for HCD. The best overlap between activation techniques was achieved when MS/MS activation was restricted to precursor ions with abundances greater than or equal to 5E6 (Figure 4D), suggesting that UVPD can approach the same depth of protein identifications when existing sensitivity and speed limitations are further improved.

HCC70 lysate analysis

The HCC70 cell line is a primary ductal carcinoma that is classified as triple negative which means that cells are negative for expression of estrogen receptor and progesterone receptor and negative for overexpression of human epidermal growth factor receptor 2.^{59–61} Because these receptors are the primary targets of current chemotherapies, triple negative breast cancers are more difficult to treat and have a poorer prognosis, thus representing an important area of research.⁶² Two HCC70 lysates, one maintained in full serum and the other serum-starved for 6 hrs prior to lysis, were analyzed in triplicate by UVPD and HCD. Consistent with the HeLa results, HCD identified more phosphopeptides and phosphoproteins than UVPD, but similar trends between serum-fed and serum-starved cells were observed using both activation methods (Figure 5). For both HCD and UVPD analyses, a greater number of phosphopeptides and phosphoproteins were observed in the serum-starved sample (Figure 5A–B) for which phosphorylation is expected to be more homogenous based on cell synchronization in G0 phase. Collectively, for both serum-fed and serum-starved cells using UVPD and HCD, 9324 total phosphorylation sites were identified from 13417 phosphopeptides which accounted for 3134 phosphoproteins. Compared to HCD alone, integration of the UVPD and HCD data sets resulted in 207 additional phosphopeptides and 24 additional phosphoproteins (Figure 5C), thus showing the gains in using UVPD and HCD as complementary methods to extend phosphoprotein analysis.

Conclusion

Photodissociation at 193 nm offers several promising benefits compared to other activation methods for the analysis of phosphopeptides. The functionality of UVPD for sequencing both peptide cations and anions enabled phosphopeptide characterization across a wider range of phosphorylation states spanning from monophosphorylated peptides up to pentaphosphorylated peptides. The most highly phosphorylated peptides required negative mode analysis for detection, while peptides in lower phosphorylation states were effectively detected in the positive ion mode. Compared to HCD, UVPD facilitated more confident phosphosite determination based on both the greater array of productions that were generated and the improved phosphate retention on those productions. For the analysis of phosphopeptides from a HeLa cell lysate, no significant differences in the phosphopeptide populations were observed between UVPD and HCD data sets, but the overall number of identifications was lower for UVPD based on lower speed and sensitivity compared to HCD. While the speed of UVPD is ultimately limited by the repetition rate of the laser, improvements in sensitivity are anticipated with further development and optimization of UVPD-MS instrumentation aimed at increasing the UVPD fragmentation efficiency. In its current state, UVPD remains a complementary technique to HCD for phosphopeptide characterization and can be used strategically to increase the overall depth of coverage for phosphoproteomics experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Harsha HC, Pandey A. Phosphoproteomics in cancer. *Themat. Issue Oncoproteomics*. 2010; 4(6): 482–495.
2. Fang B, Haura EB, Smalley KS, Eschrich SA, Koomen JM. Methods for investigation of targeted kinase inhibitor therapy using chemical proteomics and phosphorylation profiling. *Target Cancer Ther*. 2010; 80(5):739–747.
3. Riley NM, Coon JJ. Phosphoproteomics in the Age of Rapid and Deep Proteome Profiling. *Anal Chem*. 2016; 88(1):74–94. [PubMed: 26539879]
4. Zhou H, Di Palma S, Preisinger C, Peng M, Polat AN, Heck AJR, Mohammed S. Toward a Comprehensive Characterization of a Human Cancer Cell Phosphoproteome. *J Proteome Res*. 2012; 12(1):260–271. [PubMed: 23186163]
5. de Graaf EL, Giansanti P, Altelaar AFM, Heck AJR. Single-step Enrichment by Ti4+-IMAC and Label-free Quantitation Enables In-depth Monitoring of Phosphorylation Dynamics with High Reproducibility and Temporal Resolution. *Mol Cell Proteomics*. 2014; 13(9):2426–2434. [PubMed: 24850871]

6. Kelstrup CD, Jersie-Christensen RR, Batth TS, Arrey TN, Kuehn A, Kellmann M, Olsen JV. Rapid and Deep Proteomes by Faster Sequencing on a Benchtop Quadrupole Ultra-High-Field Orbitrap Mass Spectrometer. *J Proteome Res.* 2014; 13(12):6187–6195. [PubMed: 25349961]
7. Batth TS, Francavilla C, Olsen JV. Off-Line High-pH Reversed-Phase Fractionation for In-Depth Phosphoproteomics. *J Proteome Res.* 2014; 13(12):6176–6186. [PubMed: 25338131]
8. Brodbelt JS. Ion Activation Methods for Peptides and Proteins. *Anal Chem.* 2016; 88(1):30–51. [PubMed: 26630359]
9. Thingholm TE, Jensen ON, Larsen MR. Analytical strategies for phosphoproteomics. *PROTEOMICS.* 2009; 9(6):1451–1468. [PubMed: 19235172]
10. Boersema PJ, Mohammed S, Heck AJR. Phosphopeptide fragmentation and analysis by mass spectrometry. *J Mass Spectrom.* 2009; 44(6):861–878. [PubMed: 19504542]
11. Nagaraj N, D'Souza RCJ, Cox J, Olsen JV, Mann M. Feasibility of Large-Scale Phosphoproteomics with Higher Energy Collisional Dissociation Fragmentation. *J Proteome Res.* 2010; 9(12):6786–6794. [PubMed: 20873877]
12. Wiesner J, Premisler T, Sickmann A. Application of electron transfer dissociation (ETD) for the analysis of posttranslational modifications. *PROTEOMICS.* 2008; 8(21):4466–4483. [PubMed: 18972526]
13. Stensballe A, Jensen ON, Olsen JV, Haselmann KF, Zubarev RA. Electron capture dissociation of singly and multiply phosphorylated peptides. *Rapid Commun Mass Spectrom.* 2000; 14(19):1793–1800. [PubMed: 11006587]
14. Molina H, Horn DM, Tang N, Mathivanan S, Pandey A. Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry. *Proc Natl Acad Sci.* 2007; 104(7):2199–2204. [PubMed: 17287340]
15. Swaney DL, Wenger CD, Thomson JA, Coon JJ. Human embryonic stem cell phosphoproteome revealed by electron transfer dissociation tandem mass spectrometry. *Proc Natl Acad Sci.* 2009; 106(4):995–1000. [PubMed: 19144917]
16. Sweet SMM, Bailey CM, Cunningham DL, Heath JK, Cooper HJ. Large Scale Localization of Protein Phosphorylation by Use of Electron Capture Dissociation Mass Spectrometry. *Mol Cell Proteomics.* 2009; 8(5):904–912. [PubMed: 19131326]
17. Brodbelt JS. Photodissociation mass spectrometry: new tools for characterization of biological molecules. *Chem Soc Rev.* 2014; 43(8):2757–2783. [PubMed: 24481009]
18. Flora JW, Muddiman DC. Gas-Phase Ion Unimolecular Dissociation for Rapid Phosphopeptide Mapping by IRMPD in a Penning Ion Trap: An Energetically Favored Process. *J Am Chem Soc.* 2002; 124(23):6546–6547. [PubMed: 12047170]
19. Flora JW, Muddiman DC. Selective, Sensitive, and Rapid Phosphopeptide Identification in Enzymatic Digests Using ESI-FTICR-MS with Infrared Multiphoton Dissociation. *Anal Chem.* 2001; 73(14):3305–3311. [PubMed: 11476230]
20. Crowe M, Brodbelt J. Infrared multiphoton dissociation (IRMPD) and collisionally activated dissociation of peptides in a quadrupole ion trap with selective IRMPD of phosphopeptides. *J Am Soc Mass Spectrom.* 2004; 15(11):1581–1592. [PubMed: 15519225]
21. Crowe MC, Brodbelt JS. Differentiation of Phosphorylated and Unphosphorylated Peptides by High-Performance Liquid Chromatography-Electrospray Ionization-Infrared Multiphoton Dissociation in a Quadrupole Ion Trap. *Anal Chem.* 2005; 77(17):5726–5734. [PubMed: 16131088]
22. Kalcic CL, Gunaratne TC, Jones AD, Dantus M, Reid GE. Femtosecond Laser-Induced Ionization/Dissociation of Protonated Peptides. *J Am Chem Soc.* 2009; 131(3):940–942. [PubMed: 19128059]
23. Smith SA, Kalcic CL, Safran KA, Stemmer PM, Dantus M, Reid GE. Enhanced Characterization of Singly Protonated Phosphopeptide Ions by Femtosecond Laser-induced Ionization/Dissociation Tandem Mass Spectrometry (fs-LID-MS/MS). *J Am Soc Mass Spectrom.* 2010; 21(12):2031–2040. [PubMed: 20888783]
24. Smith SA, Kalcic CL, Cui L, Reid GE. Femtosecond laser-induced ionization/dissociation tandem mass spectrometry (fsLID-MS/MS) of deprotonated phosphopeptide anions. *Rapid Commun Mass Spectrom.* 2013; 27(24):2807–2817. [PubMed: 24214867]

25. Lemoine J, Tabarin T, Antoine R, Broyer M, Dugourd P. UV photodissociation of phospho-seryl-containing peptides: laser stabilization of the phospho-seryl bond with multistage mass spectrometry. *Rapid Commun Mass Spectrom.* 2006; 20(3):507–511. [PubMed: 16402344]
26. Park S, Ahn W-K, Lee S, Han SY, Rhee BK, Oh HB. Ultraviolet photodissociation at 266 nm of phosphorylated peptide cations. *Rapid Commun Mass Spectrom.* 2009; 23(23):3609–3620. [PubMed: 19890956]
27. Madsen JA, Boutz DR, Brodbelt JS. Ultrafast Ultraviolet Photodissociation at 193 nm and its Applicability to Proteomic Workflows. *J Proteome Res.* 2010; 9(8):4205–4214. [PubMed: 20578723]
28. Cui, Weidong, Thompson, MS., Reilly, JP. Pathways of Peptide Ion Fragmentation Induced by Vacuum Ultraviolet Light. *J Am Soc Mass Spectrom.* 2005; 16(8):1384–1398. [PubMed: 15979330]
29. Zhang L, Reilly JP. De Novo Sequencing of Tryptic Peptides Derived from *Deinococcus radiodurans* Ribosomal Proteins Using 157 nm Photodissociation MALDI TOF/TOF Mass Spectrometry. *J Proteome Res.* 2010; 9(6):3025–3034. [PubMed: 20377247]
30. Madsen JA, Xu H, Robinson MR, Horton AP, Shaw JB, Giles DK, Kaoud TS, Dalby KN, Trent MS, Brodbelt JS. High-Throughput Database Search and Large-Scale Negative Polarity LC-MS/MS with Ultraviolet Photodissociation for Complex Proteomic Samples. *Mol Cell Proteomics.* 2013; 12(9):2604–2614. [PubMed: 23695934]
31. Greer SM, Cannon JR, Brodbelt JS. Improvement of Shotgun Proteomics in the Negative Mode by Carbamylation of Peptides and Ultraviolet Photodissociation Mass Spectrometry. *Anal Chem.* 2014; 86(24):12285–12290. [PubMed: 25420043]
32. Greer SM, Parker WR, Brodbelt JS. Impact of Protease on Ultraviolet Photodissociation Mass Spectrometry for Bottom-up Proteomics. *J Proteome Res.* 2015; 14(6):2626–2632. [PubMed: 25950415]
33. Robinson MR, Madsen JA, Brodbelt JS. 193 nm Ultraviolet Photodissociation of Imidazolinylated Lys-N Peptides for De Novo Sequencing. *Anal Chem.* 2012; 84(5):2433–2439. [PubMed: 22283738]
34. Holden DD, Pruet JM, Brodbelt JS. Ultraviolet photodissociation of protonated, fixed charge, and charge-reduced peptides. *Int J Mass Spectrom.* 2015; 390:81–90.
35. Madsen JA, Kaoud TS, Dalby KN, Brodbelt JS. 193-nm photodissociation of singly and multiply charged peptide anions for acidic proteome characterization. *PROTEOMICS.* 2011; 11(7):1329–1334. [PubMed: 21365762]
36. Shaw J, Madsen J, Xu H, Brodbelt J. Systematic Comparison of Ultraviolet Photodissociation and Electron Transfer Dissociation for Peptide Anion Characterization. *J Am Soc Mass Spectrom.* 2012; 23(10):1707–1715. [PubMed: 22895858]
37. Luo Y, Yogesha SD, Cannon JR, Yan W, Ellington AD, Brodbelt JS, Zhang Y. Novel Modifications on C-terminal Domain of RNA Polymerase II Can Fine-tune the Phosphatase Activity of Ssu72. *ACS Chem Biol.* 2013; 8(9):2042–2052. [PubMed: 23844594]
38. Robinson M, Moore K, Brodbelt J. Direct Identification of Tyrosine Sulfation by using Ultraviolet Photodissociation Mass Spectrometry. *J Am Soc Mass Spectrom.* 2014; 25(8):1461–1471. [PubMed: 24845354]
39. Madsen JA, Ko BJ, Robotham SA, Xu H, Horton AP, Iwashkiw JA, Shaw JB, Feldman MF, Brodbelt JS. Concurrent Automated Sequencing of the Glycan and Peptide Portions of O-Linked Glycopeptide Anions. *Anal Chem.* 2013; 85(19):9253–9261. [PubMed: 24006841]
40. Han S-W, Lee S-W, Bahar O, Schwessinger B, Robinson MR, Shaw JB, Madsen JA, Brodbelt JS, Ronald PC. Tyrosine sulfation in a Gram-negative bacterium. *Nat Commun.* 2012; 3:1153. [PubMed: 23093190]
41. Shin Y, Moon J, Kim M. Observation of phosphorylation site-specific dissociation of singly protonated phosphopeptides. *J Am Soc Mass Spectrom.* 2010; 21(1):53–59. [PubMed: 19836972]
42. Kim T-Y, Reilly J. Time-resolved observation of productions generated by 157 nm photodissociation of singly protonated phosphopeptides. *J Am Soc Mass Spectrom.* 2009; 20(12):2334–2341. [PubMed: 19819166]

43. Madsen JA, Cheng RR, Kaoud TS, Dalby KN, Makarov DE, Brodbelt JS. Charge-Site-Dependent Dissociation of Hydrogen-Rich Radical Peptide Cations upon Vacuum UV Photoexcitation. *Chem — Eur J*. 2012; 18(17):5374–5383. [PubMed: 22431222]
44. Fort KL, Dyachenko A, Potel CM, Corradini E, Marino F, Barendregt A, Makarov AA, Scheltema RA, Heck AJR. Implementation of Ultraviolet Photodissociation on a Benchtop Q Exactive Mass Spectrometer and Its Application to Phosphoproteomics. *Anal Chem*. 2016; 88(4):2303–2310. [PubMed: 26760441]
45. Jackson SS, Coughlin EE, Coon JJ, Miyamoto S. Identifying post-translational modifications of NEMO by tandem mass spectrometry after high affinity purification. *Protein Expr Purif*. 2013; 92(1):48–53. [PubMed: 24012789]
46. Richards AL, Hebert AS, Ulbrich A, Bailey DJ, Coughlin EE, Westphall MS, Coon JJ. One-hour proteome analysis in yeast. *Nat Protoc*. 2015; 10(5):701–714. [PubMed: 25855955]
47. Klein DR, Holden DD, Brodbelt JS. Shotgun Analysis of Rough-Type Lipopolysaccharides Using Ultraviolet Photodissociation Mass Spectrometry. *Anal Chem*. 2016; 88(1):1044–1051. [PubMed: 26616388]
48. Gardner MW, Vasicek LA, Shabbir S, Anslyn EV, Brodbelt JS. Chromogenic Cross-Linker for the Characterization of Protein Structure by Infrared Multiphoton Dissociation Mass Spectrometry. *Anal Chem*. 2008; 80(13):4807–4819. [PubMed: 18517224]
49. Eng JK, McCormack AL, Yates JR. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom*. 1994; 5(11):976–989. [PubMed: 24226387]
50. McAlister GC, Russell JD, Rumachik NG, Hebert AS, Syka JEP, Geer LY, Westphall MS, Pagliarini DJ, Coon JJ. Analysis of the Acidic Proteome with Negative Electron-Transfer Dissociation Mass Spectrometry. *Anal Chem*. 2012; 84(6):2875–2882. [PubMed: 22335612]
51. Mamone G, Picariello G, Ferranti P, Addeo F. Hydroxyapatite affinity chromatography for the highly selective enrichment of mono- and multi-phosphorylated peptides in phosphoproteome analysis. *PROTEOMICS*. 2010; 10(3):380–393. [PubMed: 19953538]
52. Fonslow BR, Niessen SM, Singh M, Wong CCL, Xu T, Carvalho PC, Choi J, Park SK, Yates JR. Single-Step Inline Hydroxyapatite Enrichment Facilitates Identification and Quantitation of Phosphopeptides from Mass-Limited Proteomes with MudPIT. *J Proteome Res*. 2012; 11(5):2697–2709. [PubMed: 22509746]
53. Frese CK, Zhou H, Taus T, Altelaar AFM, Mechtler K, Heck AJR, Mohammed S. Unambiguous Phosphosite Localization using Electron-Transfer/Higher-Energy Collision Dissociation (EThcD). *J Proteome Res*. 2013; 12(3):1520–1525. [PubMed: 23347405]
54. Taus T, Köcher T, Pichler P, Paschke C, Schmidt A, Henrich C, Mechtler K. Universal and Confident Phosphorylation Site Localization Using phosphoRS. *J Proteome Res*. 2011; 10(12):5354–5362. [PubMed: 22073976]
55. Cui L, Reid GE. Examining factors that influence erroneous phosphorylation site localization via competing fragmentation and rearrangement reactions during ion trap CID-MS/MS and -MS3. *PROTEOMICS*. 2013; 13(6):964–973. [PubMed: 23335301]
56. Lanucara F, Chi Hoo Lee D, Evers CE. Unblocking the Sink: Improved CID-Based Analysis of Phosphorylated Peptides by Enzymatic Removal of the Basic C-Terminal Residue. *J Am Soc Mass Spectrom*. 2014; 25(2):214–225. [PubMed: 24297471]
57. Vasicek L, Brodbelt JS. Enhancement of Ultraviolet Photodissociation Efficiencies through Attachment of Aromatic Chromophores. *Anal Chem*. 2010; 82(22):9441–9446. [PubMed: 20961088]
58. Olsen JV, Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P, Mann M. Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks. *Cell*. 2006; 127(3):635–648. [PubMed: 17081983]
59. Gazdar AF, Kurvari V, Virmani A, Gollahon L, Sakaguchi M, Westerfield M, Kodagoda D, Stasny V, Cunningham HT, Wistuba II, et al. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. *Int J Cancer*. 1998; 78(6):766–774. [PubMed: 9833771]

60. Kim MS, Kim T, Kong S-Y, Kwon S, Bae CY, Choi J, Kim CH, Lee ES, Park J-K. Breast Cancer Diagnosis Using a Microfluidic Multiplexed Immunohistochemistry Platform. PLoS ONE. 2010; 5(5):e10441. [PubMed: 20454672]
61. Kim MS, Kwon S, Kim T, Lee ES, Park J-K. Quantitative proteomic profiling of breast cancers using a multiplexed microfluidic platform for immunohistochemistry and immunocytochemistry. Biomaterials. 2011; 32(5):1396–1403. [PubMed: 21093044]
62. J CK, V GS, Stanley L. Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. Breast Dis. 2010; 1(2):35–48.

Figure 1.

MS/MS analysis of doubly charged TVDMEpSTEVFTK (m/z 733.8 in positive mode and m/z 731.8 in negative mode) from alpha S2 casein using (A) positive mode HCD, (B) positive mode UVPD, and (C) negative UVPD. Two laser pulses of 2.5 mJ were used for UVPD, and 30 NCE was applied for HCD. Each spectrum is the average of five scans.

Figure 2.

Based peak MS1 chromatograms for (A) positive mode and (B) negative LC-MS analysis of alpha S1, alpha S2, and beta casein phosphopeptides enriched on hydroxyapatite beads.

Chromatographic peaks shaded in blue are recovered following C18 SPE and those shaded in purple are recovered using graphite SPE. (C) UVPD mass spectrum for phosphopeptide, QMEAEpSlpSpSpSEEIVPNpSVEQK (4-, m/z 679.61), from alpha S1 casein using two 2 mJ pulses. Five UVPD spectra were averaged over retention time 10.98–11.96 min.

Figure 3.

Comparison of UVPD and HCD for HeLa phosphoproteome analysis: (A) overlap between UVPD-identified and HCD-identified phosphopeptides and phosphoproteins, (B) phosphorylation site localization performance, (C) phosphate retention on productions, (D) phosphopeptide charge state distribution, and (E) phosphopeptide length distribution. All results are based on three replicate UVPD and HCD LC-MS runs.

Figure 4.

Overlap in peptide identifications from IMAC-enriched HeLa analyzed using UVPD and HCD applied in (A) separate LCMS runs or (B–D) alternated for each precursor selected within the same LC-MS run. For alternating UVPD/HCD runs, 100 ms and 300 ms maximum ion injection times were applied with a 5E4 MS2 threshold in B and C, respectively. The threshold for MS2 selection was increased to 5E6 with a 100 ms maximum injection time in D.

Figure 5. HCC70 phosphoproteomics results from UVPD and HCD analysis. (A) Overlap in peptide for cells maintained in full serum (fed) compared to cells that were serum-starved prior to lysis. (B) Number of phosphoproteins and unique phosphosites identified in serum-fed and serum-starved samples using UVPD and HCD. (C) Overlap in total UVPD and HCD peptides and proteins from both serum-fed and serum-starved cells.