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Complementary IMAC enrichment methods for HLA-associated phosphopeptide identification by mass spectrometry

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Abstract

Phosphorylation events within cancer cells often become dysregulated, leading to oncogenic signaling and abnormal cell growth. Phosphopeptides derived from aberrantly phosphorylated proteins that are presented on tumors and not on normal tissues by human leukocyte antigen (HLA) class I molecules are promising candidates for future cancer immunotherapies, because they are tumor specific and have been shown to elicit cytotoxic T cell responses. Robust phosphopeptide enrichments that are suitable for low input amounts must be developed to characterize HLA-associated phosphopeptides from clinical samples that are limited by material availability. We present two complementary mass spectrometry-compatible, iron(III)-immobilized metal affinity chromatography (IMAC) methods that use either nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) in-house-fabricated columns. We developed these protocols to enrich for subfemtomole-level phosphopeptides from cell line and human tissue samples containing picograms of starting material, which is an order of magnitude less material than what is commonly used. In addition, we added a peptide esterification step to increase phosphopeptide specificity from these low-input samples. To date, hundreds of phosphopeptides displayed on melanoma, ovarian cancer, leukemia and colorectal cancer have been identified using these highly selective phosphopeptide enrichment protocols in combination with a program called 'CAD Neutral Loss Finder' that identifies all spectra containing the characteristic neutral loss of phosphoric acid from phosphorylated serine and threonine residues. This methodology enables the

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AUTHOR CONTRIBUTIONS J.G.A., J.S. and D.F.H. designed the studies, and J.G.A. and P.D.T. executed them. S.A.P. and M.C. immunopurified HLA-associated peptides from colorectal cancer tumors that were resected by S.T.W. from consenting patients. A.M.P. and W.H.H. transfected FHIOSE cells with sHLA-A*0201 and isolated associated peptides. D.L.B. developed programs that were used during data analysis.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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identification of HLA-associated phosphopeptides presented by human tissue samples containing as little as nanograms of peptide material in 2 d.

INTRODUCTION

Our understanding of cancer progression has deepened over the past 60 years, resulting in the development of more effective treatment options. Immunotherapies, which harness the ability of the immune system to specifically eliminate unhealthy cells, have emerged as a promising new class of cancer therapeutics. Immunotherapeutics are designed to generate antitumor immune responses by stimulating adaptive immune cells^{1–7}. During this response, antigen-presenting cells activate cytotoxic T cells by presenting cancer-specific antigens^{1,2,5,8,9}. Activated T cells are then released to the periphery to survey cells throughout the body. Cytotoxic T cells determine cellular health by binding peptide antigens produced by the MHC class I pathway depicted in **Figure 1**. Briefly, endogenous proteins are degraded by the proteasome and loaded onto HLA class I molecules. HLA–peptide complexes are then shuttled to the cell surface for display to circulating T cells. The majority of HLA-associated antigens are self-derived, and they do not elicit an immune response because T cells are rendered nonreactive to self-peptides before they are allowed to circulate in the periphery⁹. Tolerance toward self-antigens has a key role in preventing autoimmune responses, but it may also be the reason why many current immunotherapeutic candidates, which are derived from overexpressed or cancer-associated self-antigens, are not highly effective⁶.

Unique antigens, such as those resulting from dysregulated cellular signaling involved in cancer progression, are more attractive targets for cancer immunotherapeutics because they are distinct from healthy cells. Dysregulated cell signaling within tumors generates phosphorylated residues on proteins that are unique to the disease^{1,10,11}. The proteolysis of these phosphorylated proteins and the presentation of tumor-specific phosphopeptides to the immune system by the HLA class I antigen–processing pathway distinguishes healthy cells from cancer cells^{1,10,11}. We hypothesize that HLA class I–associated phosphopeptides are candidates for a cancer immunotherapy that harnesses the ability of the immune system to identify and specifically eliminate transformed cells⁵. We have identified HLA-associated phosphopeptides derived from dysregulated cell signaling pathways presented by cancer cells^{1–5} and demonstrated that tumor-specific phosphopeptides elicit responses from healthy donor T cells⁵.

The analysis of HLA class I–associated phosphopeptides is based on an approach that has been used for several years^{1–4}. HLA class I molecules are immunopurified from cancer cells or tissues, and their associated peptides are eluted with acid. The combination of iron(III)-IMAC enrichment and high-performance liquid chromatography coupled to electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS) analysis is then used to identify tumor-specific phosphopeptide antigens that are assessed for immunological activity. Phosphopeptide enrichment is required because abundant unmodified peptides prevent the selection of phosphorylated peptides, which are present at the 1–5% level, for fragmentation during data-dependent HPLC-ESI-MS/MS analyses^{2,12,13}. Therefore, iron(III)-IMAC is

used to enrich for HLA-associated phosphopeptides before HPLC-ESI-MS/MS analysis^{2,12,13}.

There are multiple published protocols that are compatible with mass spectrometry for phosphopeptide enrichment^{14–22}, yet most of these methods were developed for high-throughput applications that require hundreds of micrograms (μg) to milligrams (mg) of protein material^{14–19,22–25}. Analysis of clinical samples may require phosphopeptide enrichment from nanograms (ng) to micrograms (μg) of peptide material, rendering high-throughput phosphopeptide enrichment protocols not applicable. The use of Ti(IV)-IMAC and metal affinity chromatography (MOAC) using TiO_2 for phosphopeptide enrichment is also well established^{21,26,27}. Reports comparing phosphopeptide enrichment procedures have been published, but a consensus about which technique is best for each sample type has not been reached. For example, Zhou *et al.*^{20,28} have reported that TiO_2 and Ti^{4+} enrichments have a higher affinity for phosphopeptides than Fe^{3+} -based IMAC. Matheron *et al.*²⁷ have reported a comparative evaluation of Ti^{4+} -IMAC and TiO_2 enrichment and demonstrated that minimal differences can be found when using either of these phosphopeptide enrichment techniques, but Fe-IMAC was not evaluated in this study. Conversely, Ruprecht *et al.*²² have reported that phosphopeptides enriched by Fe-IMAC columns were 7 and 4 times higher in intensity than peptides enriched using Ti-IMAC tips and TiO_2 batches. In addition, many published phosphopeptide enrichment protocols do not incorporate the use of internal standards to monitor enrichment recovery or checkpoints to monitor peptide losses. Thus, we developed a phosphopeptide enrichment method for low-input samples that incorporates both checkpoints (**Box 1**) and internal standards to monitor peptide recovery. Although we present a low-throughput protocol, the throughput can be increased by chemically labeling samples using isobaric tags before phosphopeptide enrichment, such as iTRAQ (isobaric tags for relative and absolute quantification) or TMT (tandem mass tags), at the cost of absolute abundance estimation. We have previously demonstrated that HLA-associated phosphopeptides can be enriched by iron(III)-IMAC from micrograms of peptide material^{2,12,13}. Despite our past successes, the phosphopeptide enrichment protocol required further optimization so that it could be applied to patient tumor samples. Two areas of improvement included reducing nonspecific binding and increasing peptide recoveries. Some improvement in nonspecific binding can be achieved by esterifying the sample; carboxylic acid residues on amino acid side chains and at the C terminus have an affinity for Fe^{3+} cations, which can be reduced by a Fischer esterification. However, the most abundant nonspecific binding species observed in past studies were highly basic peptides and nitrogen-containing PEG polymer species, both of which negatively affected peptide separation and sequencing². We believe that this type of nonspecific binding was caused by incomplete activation of IDA resin used for iron(III)-IMAC enrichment. In order to eliminate unoccupied diacetic acid sites, changes to the established IMAC protocol were made that included using smaller microcapillary columns, less IDA resin and longer reaction times for Fe^{3+} activation. There is also the problem of peptide adsorption by the plastic interfaces of tubes used during batch-mode enrichments and the pipette tips used to fabricate StageTip devices. This can be reduced if fused silica microcapillary columns are used instead. Ruprecht *et al.*²² have also recently demonstrated similar improvements to phosphopeptide enrichment using an on-column approach instead

of the conventional batch-mode and StageTip protocols by enriching phosphopeptides from sample concentrations ranging from 100 µg to 1 mg. We also used an iron(III)-NTA-IMAC method similar to our IDA-IMAC method because of reports demonstrating that NTA resins provide enhanced phosphopeptide selectivity^{16–19}.

Even with optimized phosphopeptide enrichment, phosphopeptide sequencing using HPLC-ESI-MS/MS is difficult because of the labile nature of the phosphorylation modification^{21–23}. Collision-activated dissociation (CAD) is one of the most commonly used fragmentation methods for HPLC-ESI-MS/MS experiments^{5,12,13}. Higher-energy collisional dissociation (HCD) has also been applied in phosphoproteomic studies^{29,30}. Both CAD and HCD cause the loss of phosphoric acid upon fragmentation, resulting in tandem MS spectra (MS2) that can contain ambiguous sequencing and site localization information. On the other hand, the use of electron transfer dissociation (ETD) preserves labile phosphorylation modifications and produces high-quality phosphopeptide MS2 (refs. 31–35). Although ETD appears to be a simple solution to the labile nature of phosphorylation sites, the mechanism of ETD is charge dependent, and it works best on peptides with a charge of +3 or greater^{31,35}. Heck and colleagues³⁶ have demonstrated that the use of electron transfer–HCD (ETHCD) enhances the detection rate of non-phosphopeptide-enriched HLA-associated peptides that are highly charged (+3 or greater). However, most class I HLA-associated peptides are short peptides of 8–10 amino acid residues in length, with a predominant charge state of +2. These peptide characteristics are not ideal for sequencing using only ETD, and ETHCD acquisition can result in a slower duty cycle that can lead to undersampling. Therefore, we have found that it is beneficial to combine the two fragmentation methods, CAD and ETD, in order to obtain greater sequence information to confidently identify phosphopeptides and site-localize the modification (**Supplementary Fig. 1**).

In our research, both IDA-IMAC and NTA-IMAC enrichment protocols were applied to HLA-associated peptide mixtures isolated from cancer cell lines and human tumor tissue, resulting in the identification of hundreds of phosphopeptides presented to circulating T cells of the immune system^{2,5}. The two iron(III)-IMAC protocols are MS compatible, and they can be used to enrich for subfemtomole-level phosphopeptides from samples containing nanograms to micrograms of peptide material. The protocols described follow the general workflow outlined in our previous studies with modifications to reduce nonspecific binding and improve peptide recoveries from samples with low levels of peptide material. The described protocols enable the identification of cancer-associated HLA-associated phosphopeptides from submicrogram levels of peptide material, something not previously possible. Most important, these protocols could facilitate phosphoproteomic investigations of clinical samples, such as patient tumors and tumor microdissections, potentially directing personalized cancer immunotherapies.

MATERIALS

REAGENTS

- Acetyl chloride, anhydrous (Grace Davison Discovery Science, cat. no. 18095)

- D₀-Methanol (MeOH), anhydrous (Grace Davison Discovery Science, cat. no. 18157) **▲ CRITICAL** Methanol must be anhydrous for complete esterification.
- Acetonitrile, HPLC grade, 99.8% purity (Honeywell, cat. no. LC015-2.5)
- Glacial acetic acid (AcOH), 99.9% purity (Sigma, cat. no. 338826) **CRITICAL** Formic acid (FA) can be substituted for AcOH. FA is a stronger ion-pairing agent, and thus it will result in better offline and online separation. However, FA will cause a loss in sensitivity during electrospray ionization.
- HLA Class I-expressing cell line or tissue (any)
- LC-MS-grade water (Pierce, cat. no. 51140)
- Kasil 1624 potassium silicate solution (PQ Corporation)
- L-Ascorbic acid (Sigma, cat. no. A5960)
- Azulene (Sigma, cat. no. A97203)
- EDTA, analytical grade (Sigma, cat. no. 431788)
- Formamide (Sigma, cat. no. 295876)
- Iron (III) chloride (Sigma, cat. no. 451649) **! CAUTION** Iron (III) chloride is hygroscopic and extremely corrosive. This reagent should be stored in a desiccator.
- ODS-AQ, C18 5-μm spherical silica particles, 120 Å pore size (YMC cat. no. AQ12S05)
- ODS-AQ, C18, 5- to 20-μm spherical silica particles, 120 Å pore size (YMC cat. no. AA12112)
- POROS MC 20 metal-chelating packing material, 20-μm diameter (Applied Biosystems, cat. no. 1542802)
- Qiagen nitrilotriacetic acid silica, 16–24-μm particle size

EQUIPMENT

- High-pressure column packer and sample loader (pressure bomb; see **Supplementary Fig. 2**)
- HPLC (HP 1100; Agilent Technologies) with post-HPLC split for nanoflow³⁷
- CentriVap centrifugal vacuum concentrator (Labconco)
- Amicon Ultra, 3-kDa regenerated cellulose spin filter (Millipore, cat. no. UFC500324)
- Amicon Ultra, 10-kDa regenerated cellulose spin filter (Millipore, cat. no. UFC501024)
- Polyimide-coated fused silica capillary, 360 μm outer diameter (o.d.) × 50 μm inner diameter (i.d.) (PolyMicro Technologies, cat. no. 1068150017)

- Polyimide-coated fused silica capillary, 360 μm o.d. $\times$ 75 μm i.d. (PolyMicro Technologies, cat. no. 1068150019)
- Polyimide-coated fused silica capillary, 360 μm o.d. $\times$ 150 μm i.d. (PolyMicro Technologies, cat. no. 2000024)
- P-2000 microcapillary laser puller with fused silica adapter (Sutter Instrument Co.)
- Mass spectrometer (see Equipment Setup for further information)

Software

- Xcalibur software (Thermo Electron Corporation)
- CAD Neutral Loss Finder (<http://www.huntlab.org/>)
- OMSSA (or similar database search software)

REAGENT SETUP

General comment on sample preparation—The immunopurification of HLA-associated peptides using monoclonal antibodies has been well established^{2,36,38–40}, so we will only briefly describe the peptide isolation from various samples. We focus on the phosphopeptide enrichment from the isolated HLA-associated peptide population in the PROCEDURE section. We suggest using HLA-associated peptides isolated from 10^8 to 10^9 cell equivalents for enrichment.

FHIOSE cell culture and transfection—FHIOSE cells, ovarian surface epithelial cells immortalized with the simian virus 40, were selected because of their transformation with a cancer-related virus. FHIOSE cells are cultured in 1:1 Medium 199 (Life Technologies) and MCDB105 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) FBS and experience 5% CO_2 -humidified environments at 37 °C in a bioreactor with the following conditions. Transfect FHIOSE cells with the soluble HLA (sHLA)-A*0201 construct by electroporation using a Bio-Rad GenePulser Xcell. Note that the sHLA construct lacks a transmembrane domain, and it includes a VLDLr sequence as a downstream purification tag. Cells should be grown to 80–90% confluency and then trypsinized and placed in electroporation cuvettes (750 μl at 5×10^6 cells per ml) along with 30 μg of plasmid DNA. Pulse the cells with a voltage of 250 V and a capacitance at 950 μF . Plate electroporated cells into 96-well plates with complete growth medium, and after 3 d select successfully transfected cells with G418. Transfected FHIOSE cells are further selected by single-cell sorting on an Influx cell sorter (Becton Dickinson) for the best-producing clone before expansion in a bioreactor. The same complete medium used for FHIOSE cell growth should be used for the extracapillary space flow, and the DMEM/F12K basal medium should be used for the intracapillary space flow in the bioreactor. **! CAUTION** Regularly check that the cell lines used in your research match their recognized cell line identity criteria and that they are not infected with mycoplasma.

FHIOSE sHLA-associated peptide isolation—Purify sHLA-A*0201 molecules from FHIOSE by immunoaffinity purification, and extract their associated peptides as previously described^{38,39} and as follows. Pass ~16 liters of bioreactor supernatant (~30 mg sHLA) over

a CNBr-Activated Sepharose 4B Fast-Flow matrix directly coupled to the anti-VLDLr antibody (IgG-6A6, prepared here from ATCC hybridoma line CRL-2197) and wash it with 20 mM sodium phosphate buffer (pH 7.2). Elute the peptides from immunopurified sHLA molecules with 0.2 N acetic acid, and bring the eluate to 10% (vol/vol) acetic acid. Boil it at 76 °C for 10 min, cool it to below 30 °C and pass it through a 3-kDa-MWCO filter. Flash-freeze and lyophilize the peptide; the peptide should be stable in this form at –80 °C for at least 6 months. Approximately 1.5 µg of total peptide, representing <10% of the peptide yield, is sufficient for use in phosphopeptide enrichment.

Colorectal cancer tissue HLA-associated peptide isolation—Collect tissue samples within 1 h of surgical removal. Purify class I HLA molecules by immunoaffinity purification, as previously described^{2,24}. Approximately 1×10^9 cells or 1 g of tissue can be lysed in 10 ml of buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) CHAPS, 1 mM PMSF, aprotinin (5 µg/ml), leupeptin (10 µg/ml), pepstatin A (10 µg/ml), calyculin A (1 µg/ml) and phosphatase inhibitors II and III (Sigma). Homogenize tumor tissues using a mechanical homogenizer (TissueRuptor) after the addition of lysis buffer, and incubate for 2 h at 4 °C. Subject lysed cells and tissues to ultracentrifugation at 100,000g and 4 °C for 1 h, and incubate the resulting supernatant with NHS-sepharose beads preloaded with the pan-anti-HLA class I antibody W6/32. Elute peptides from purified class I HLA molecules with 10% (vol/vol) acetic acid and pass them through a 5-kDa-MWCO spin filter. Flash-freeze and lyophilize the peptide; it should be stable in this form at –80 °C for at least 6 months. ! **CAUTION** If animal material is used, please note that appropriate institutional regulatory board guidelines must be followed; if human tissue is collected, please note that it is important to follow institutional guidelines regarding requesting permission for collection.

HPLC-ESI-MS/MS solvents—Prepare solutions of 0.1 M acetic acid in water (solvent A: 0.1% acetic acid (vol/vol) in water; solvent B: 0.1% acetic acid (vol/vol) and 70% acetonitrile (vol/vol) in water). These solutions can be prepared in advance and stored at 25 °C for 15 d.

EQUIPMENT SETUP

Micropipillary column fabrication—To create a micropipillary column with a Kasil 1624 frit, dip an empty 10–15-cm capillary column into a mixture containing 3:1 Kasil 1624 to formamide for 1–2 s. Cure the Kasil 1624 frit at 60–65 °C for 6 h. After curing, cut the frit to 2 mm and rinse the column with water to remove uncured Kasil 1624. To measure the flow rate of a micropipillary column, collect the flow-through volume in a 1–5-µl glass capillary for a specific amount of time. Calculate the flow rate using the volume captured in the capillary and the time it took to capture this volume.

Mass spectrometer—In our research, we have used the LTQ-FT-ICR hybrid mass spectrometer (Thermo Fisher), which has a linear ion trap with a Fourier transform ion cyclotron resonance (FT-ICR) MS detector, custom-modified with front-end ETD²¹, and the LTQ-Orbitrap mass spectrometer (Thermo Fisher) custom-modified with front-end ETD^{31,34}.

Thermo LTQ Orbitrap Velos mass spectrometers with back-end ETD and Thermo Orbitrap Fusion mass spectrometers³⁴ can also be used; we present data collected on specialized instrumentation because this was what we had available.

HPLC-ESI-MS/MS equipment—The microcapillary HPLC column setup is described in detail by Marto and colleagues³⁷. Acquire mass spectra (MS1) in the high-resolution Fourier transform or Orbitrap mass analyzer and MS2 in the linear ion trap of the instrument using CAD and front-end ETD³⁴. Thermo LTQ Orbitrap Velos mass spectrometers with back-end ETD and Thermo Orbitrap Fusion mass spectrometers can be substituted for similar analyses. Acquire mass spectra using a method consisting of one high-resolution MS1 scan (resolving power of 60,000 at m/z 400), followed by 12 data-dependent low-resolution MS2 scans acquired in the LTQ ion trap that include six CAD scans (3 m/z isolation window, 35% normalized collision energy) and six ETD MS2 scans (3 m/z isolation window, 45-ms reaction time with azulene radical anion) of the top six most abundant precursor ions. Instrument parameters include FTMS automatic gain control (AGC) target for MS1 scans of 2×10^5 (charges), ITMSⁿ AGC target for MS2 scans of 1×10^4 (charges) and azulene ETD reagent target of 2×10^5 (charges). Data-dependent instrument parameters should be set as follows: repeat count of 3, repeat duration of 10 and exclusion list duration of 10. Ions with a charge of +1 can be excluded if polymeric materials with +1 charge states are present in the sample and all scans are collected in centroid mode. However, some HLA-associated peptide ions reside in a +1 charge state, and excluding them from acquisition may result in a lower number of peptide identifications.

PROCEDURE

Peptide desalting ● TIMING 4 h 30 min

- 1| Reconstitute immunopurified HLA-associated peptide samples in 0.1 M acetic acid.
- 2| Pressure-load the peptide sample, using a pressure bomb apparatus similar to the one shown in **Supplementary Figure 2**, into a fused silica microcapillary cleanup column (360 μm o.d. $\times$ 150 μm i.d.) equipped with a 2-mm Kasil 1624 frit and packed with 5 cm of irregular C18 reversed-phase packing material (5–20 μm diameter and 120 Å pore size) at a flow rate of 0.5 $\mu\text{l}/\text{min}$.

▲ **CRITICAL STEP** We suggest using HLA-associated peptides isolated from 1×10^8 to 1×10^9 cell equivalents, and we suggest that the total volume of the sample be 50 μl . We also suggest using irregular C18 packing material for peptide desalting to prevent microcapillary columns from clogging.

- 3| Wash the sample by pressure-loading 0.1 M acetic acid (half volume of sample) at a rate of 0.5 $\mu\text{l}/\text{min}$.
- 4| Rinse the sample with 0.1 M acetic acid for 10 min at a back pressure of 12–15 bar using an HPLC column.

▲ **CRITICAL STEP** This wash removes salt from the sample, and it can be extended if needed. However, extremely hydrophilic peptides can be lost if the wash is extended for >20 min.

5| Elute peptides from the cleanup column at the same back pressure into a microcentrifuge tube using a gradient of 0–80% solvent B over 40 min followed by a 30-min hold of 80% solvent B. The sample is collected with no fractionation during the elution.

6| Dry the peptide-containing eluants in a Centrivap and store them at –35 °C.

■ **PAUSE POINT** Store the desalted HLA-associated peptide sample at –35 °C for 1 week or –80 °C for up to 1 month.

Esterification ● **TIMING 3 h 30 min**

▲ **CRITICAL** Acidic residues (aspartic acid and glutamic acid) and the C terminus will be converted to methyl esters. Each conversion will result in an addition of 14 Da to the acidic residues and the C terminus.

7| Dry the HLA-associated peptides to completion after adding 50 µl of methanol using a CentriVap. Repeat the methanol addition and drying cycle three times to ensure that no water is present, because water will reverse esterification.

▲ **CRITICAL STEP** Use only anhydrous reagents for esterification, because water will reverse the reaction.

8| Prepare esterification reagents by adding 160 µl of anhydrous acetyl chloride dropwise to 1 ml of anhydrous methanol (recommended for samples containing 50 pmol or less of total peptide).

! **CAUTION** Add acetyl chloride dropwise because the reaction is exothermic. Wear appropriate safety equipment when you are handling acetyl chloride.

9| Stir the reaction mixture for 5 min and add 80 µl to the peptide samples.

10| Allow the reaction mixture to sit for 1 h at room temperature (25 °C). Do not incubate the reaction for longer than 1 h because the conversion of aspartic acid and glutamic acid to asparagine and glutamine, respectively, can occur.

11| Dry the sample in the CentriVap for 25 min, add 50 µl of MeOH and dry it again.

12| Repeat Steps 8–11.

■ **PAUSE POINT** Store the esterified sample at –35 or –80 °C for up to 1 week.

Phosphopeptide enrichment

- 13|** Enrich for HLA-associated phosphopeptides using either the IDA-iron(III)-IMAC procedure or the NTA-iron(III)-IMAC procedure below. All steps should be completed at room temperature.

▲ **CRITICAL STEP** 50–60% overlap is observed between IDA-iron(III)-IMAC and NTA-iron(III)-IMAC enrichments, which is probably attributable to the data-dependent nature of the mass spectrometry acquisition. Either enrichment is suitable for sHLA-derived samples and cell line-derived samples. Higher selectivity is observed in NTA-iron(III) enrichments, making them a better choice for more complex tissue samples.

(A) Iron(III)-IDA-IMAC ● TIMING 6 h

- (i) Pack a 360- μ m o.d. $\times$ 75- μ m i.d. fused silica capillary IMAC column equipped with a 1–2-mm Kasil 1624 frit with 5 cm of POROS MC 20 iminodiacetate packing material.

▲ **CRITICAL STEP** The capacity of the column described above is estimated to be 50–100 pmol of phosphopeptide. Construct a new IMAC column before each experiment. Do not reuse IMAC columns to prevent sample contamination.

- (ii) Pressure-rinse the IMAC column with the following steps at a flow rate of 20 μ l/min: a 20-min water rinse, a 5-min 50 mM EDTA rinse and a 10-min water rinse.

- (iii) Activate the column using filtered 100 mM FeCl₃ with three rounds of activation each involving 10 min at a flow rate of 20 μ l/min, followed by 3 min of allowing FeCl₃ to incubate on the column.

▲ **CRITICAL STEP** Make iron solution fresh and filter the particulates before enrichment to prevent microcapillary columns from clogging.

- (iv) Equilibrate the activated column with 25 μ l of 0.01% (vol/vol) acetic acid at a flow rate of 0.5 μ l/min.

▲ **CRITICAL STEP** Step 13A(v–viii) should be completed using flow rates <1 μ l/min to prevent sample losses. We recommend 0.5 μ l/min for these steps.

- (v) Reconstitute the HLA-associated peptide sample in a small volume (we suggest 50 μ l) in 1:1:1 (1:1:1 is defined as a solution made up of equal volumes of MeOH:acetonitrile:0.01% (vol/vol) acetic acid (vol/vol/vol)) directly before phosphopeptide enrichment by iron(III)-IMAC.

- (vi) Load the sample (50 μ l total volume) onto the column at a 1:1:1 ratio (defined in Step 13A(v)) at a flow rate of 0.5 μ l/min or less).

- (vii) Rinse the sample tube with 25 μl of 1:1:1 (defined in 13A(v)) and pressure load this rinse onto the IMAC column at a flow rate of 0.5–1 $\mu\text{l}/\text{min}$.
- (viii) Rinse the column with 15 μl of 0.01% (vol/vol) acetic acid at a flow rate of 0.5–1 $\mu\text{l}/\text{min}$.

(B) Iron(III)-NTA-IMAC ● TIMING 4.5–5.5 h

- (i) Reconstitute the Ni-NTA resin in water (1 mg/ml).
- (ii) Rinse the 10-kDa-MWCO filter (spin filter) twice with 0.01% (vol/vol) acetic acid using a forward and reverse spin for each round.

▲ CRITICAL STEP All forward spins should be completed at 14,000g for 5 min at room temperature, and all reverse spins should be completed at 1,000g for 1 min at room temperature. Mix the resin solution multiple times with a pipette on top of the filter upon the addition of each reagent.
- (iii) Add the 1 mg/ml resin solution and rinse through the spin filter using three forward spins.
- (iv) Wash the resin with water using two forward spins.
- (v) Incubate the resin with 50 mM EDTA using two forward spins, and wash it with water using two forward spins to remove nickel.
- (vi) Activate the resin with 100 mM FeCl_3 using three forward spins.
- (vii) Wash the resin with 0.01% (vol/vol) acetic acid with one forward spin, 15% (vol/vol) acetonitrile in 0.01% (vol/vol) acetic acid with two forward spins and 0.01% (vol/vol) acetic acid with one forward spin.

■ PAUSE POINT Store the Fe-NTA resin in 0.01% (vol/vol) acetic acid at 4 °C. The resin can be used for up to 1 month.
- (viii) Pack a 360- μm o.d. $\times$ 150- μm i.d. fused silica capillary IMAC column equipped with a 1–2-mm Kasil 1624 frit with 2.5 cm of Fe-NTA resin.

▲ CRITICAL STEP The capacity of the column described above is estimated to be 100 pmol of phosphopeptide. Construct a new IMAC column before each experiment. Do not reuse IMAC columns to prevent sample contamination.
- (ix) Activate the sample again with 100 mM FeCl_3 using two rounds of activation that include 5 min at a flow rate of 20 $\mu\text{l}/\text{min}$ followed by 3 min of incubation with no flow.
- (x) Equilibrate with 0.01% (vol/vol) acetic acid at a flow rate of 0.5–1 $\mu\text{l}/\text{min}$.
- (xi) Load the sample onto the column at a 1:1:1 ratio at a flow rate of <1 $\mu\text{l}/\text{min}$.

▲ CRITICAL STEP A flow rate faster than 1 µl/min will markedly reduce peptide recovery. If the flow rate is just under 1 µl/min, it is recommended that the flow-through of the IMAC column be reloaded onto the column to reduce peptide losses. We recommend using a flow rate of 0.5–1 µl/min.

(xii) Rinse the sample tube with 1:1:1 (defined in 13A(v)) (half the volume of the sample), and pressure load the rinse onto the IMAC column at a flow rate <1 µl/min.

(xiii) Wash with 15 µl of 0.1% acetic at a flow rate of 1 µl/min.

- 14| Connect a fused silica microcapillary precolumn (360-µm o.d. × 75-µm i.d.) equipped with a 2-mm Kasil 1624 frit and packed with 7 cm of C18 reversed-phase packing material (5–20 µm diameter, 120 Å pore size) with a Teflon sleeve to the top of the IMAC column.
- 15| Rinse the IMAC-precursor combination with 0.01% (vol/vol) acetic acid for 10 min at a flow rate of 1 µl/min to ensure that no leaks are present.
- 16| Elute the phosphopeptides directly onto the precolumn with a pressure load of 250 mM ascorbic acid in water (pH 2) at a flow rate between 0.5 and 1 µl/min for 35 min.

▲ CRITICAL STEP A flow rate faster than 1 µl/min will markedly reduce peptide recoveries.

- 17| Rinse the IMAC-precursor combination with 0.01% (vol/vol) acetic acid for 10 min at 1 µl/min.
- 18| Disconnect the precolumn from the IMAC column and rinse for 20 min with 0.1 M acetic acid at a flow rate of 60 nl/min on an HPLC column.
- 19| Dry the precolumn and connect it with a Teflon sleeve to a C18 microcapillary analytical column (360 µm o.d. × 50 µm i.d.) fritted with a bottleneck containing irregular C18 packing material (5–20 µm diameter, 120 Å pore size) packed with 7 cm of C18 reverse-phase resin (5 µm diameter, 120 Å pore size) and equipped with a laser-pulled, 2-µm-diameter electrospray emitter tip.

HPLC-ESI-MS/MS analysis ● TIMING 2 h

- 20| Gradient-elute the peptides using 0–60% solvent B in 60 min at a flow rate of 60 nl/min through a microelectrospray tip directly into a high-resolution mass spectrometer equipped with a reverse-phase HPLC microcapillary column. See Equipment Setup for more details about configuration and settings. Data analysis is discussed in ANTICIPATED RESULTS.

▲ CRITICAL STEP Longer gradients can be used to achieve a higher level of on-column separation, but this will result in wider peak widths. In addition, higher flow rates of 200–250 µl/min are suitable on uHPLC systems, such as the Proxeon Easy nLC1000.

? TROUBLESHOOTING

Data analysis ● TIMING Variable: timing depends on the database search program used and the amount of manual validation required. The searches can take a few hours to >24 h (because of the large search space), and manual validation can take days. The neutral loss search takes minutes

- 21| Perform data analysis by using the Xcalibur software (Thermo Electron Corporation). Search data files using OMSSA⁴¹ (version 2.1.1) against the RefSeq database (downloaded June 2009). Searches of CAD and ETD spectra use the following parameters: no enzyme specificity, *E* value cutoff of 1, fixed modifications including methyl ester of aspartic acid, glutamic acid and C terminus, variable modifications including oxidation of methionine, phosphorylation of serine, threonine and tyrosine, and ± 0.01 Da and ± 0.35 Da for precursor and product ion masses, respectively. The data files can also be searched using an in-house program called CAD Neutral Loss Finder that identifies MS2 CAD spectra with the neutral loss of phosphoric acid (98 Da) characteristic of phosphopeptides. Use OMSSA and neutral loss search results to guide the analysis, and determine peptide sequences by accurate mass measurement and manual interpretation of the MS2. Any database search strategy using any Blast-formatted database that enables ‘no enzyme’ searches of both CAD and ETD data can be substituted for OMSSA to help guide manual interpretation.
- 22| During manual validation, extract ion chromatograms for phosphopeptide database hits and precursor peptide ions that experience the loss of phosphoric acid during CAD fragmentation, and manually sequence CAD and ETD MS2 for all phosphopeptides. Often, the peptide ion fragments from both the CAD and ETD MS2 can be used together to determine phosphopeptide sequences. An example of manual validation is shown in **Supplementary Figure 3**. In the case of HLA-associated phosphopeptides, we chose to use manual validation because the population of phosphopeptides that results from unknown genetic mutations, splice variants and aberrant phosphorylations may not be present in human protein databases; therefore, they would not be found using traditional database search methods. This population of phosphopeptides is of high interest because they are likely to be cancer-specific peptides and novel candidates for immunotherapies.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

●TIMING

Steps 1–6, peptide desalting: 4 h 30 min

Steps 7–12, esterification: 3 h 30 min

Steps 13–19, phosphopeptide enrichment

Step 13A(i–viii), IDA-iron(III)-IMAC: 6 h

Step 13B, NTA-iron(III)-IMAC: 4.5–5.5 h

Steps 14–19, 1.5–2 h (depending on elution flow rate)

Step 20, HPLC-ESI-MS/MS analysis: 2 h

Steps 21 and 22, data analysis: variable (>24 h)

ANTICIPATED RESULTS

We applied the IDA-iron(III)-IMAC and the NTA-iron(III)-IMAC protocols to two HLA-associated peptide samples to demonstrate the performance of each approach. **Figure 2** shows a schematic describing workflows for HLA-associated phosphopeptide identification from an sHLA-transfected cell line (**Fig. 2a**) and tissue from a metastatic, human colorectal cancer tumor (**Fig. 2b**). We present data on an immortalized ovarian cell line (FHIOSE) transfected with sHLA, because of the large amount of phosphopeptides present in this sample. We also present data on a colorectal patient tumor to demonstrate that the methods can be used on clinical samples. We suggest that HLA-associated peptides from noncancerous tissues and cell lines be analyzed as negative controls to determine which peptides are specific to cancerous cells. In addition, HLA-peptide isolations from well-characterized cell lines, such as the JY human lymphoblastoid cell line, can be used as positive controls^{2–4,36}.

HLAs with their bound peptides were immunopurified from each sample, and their associated peptides were eluted with acid. The HLA-associated peptides were separated from denatured HLA using MWCO filters, and then they were desalted and esterified. HLA-associated phosphopeptides present at substoichiometric levels were enriched from the samples and analyzed by HPLC-ESI-MS/MS on an LTQ Orbitrap instrument.

Data analysis

All MS/MS spectra were searched with OMSSA (version 2.1.1; ref. 41) against an in-house phosphopeptide library database (built from a list of HLA-associated phosphopeptides previously identified in our laboratory) and the RefSeq human protein database. We observed that the database searches against the RefSeq human protein database (downloaded June 2009) produced a larger number of correct phosphopeptide identifications when it was applied to the data collected from the NTA-iron(III)-IMAC enrichments. We estimate that ~70% of the phosphopeptides present in samples enriched using the NTA-iron(III)-IMAC protocol were found with protein database searches, compared with only 30–40% found when the IDA-iron(III)-IMAC protocol was searched. We made this estimation by comparing the phosphopeptides found with database searches against the RefSeq database to the phosphopeptides found by using our neutral loss search and manual validation. We believe that we observed more phosphopeptides in the database searches of Fe-NTA-IMAC experiments than in those of Fe-IDA-IMAC experiments because of the lower background observed in the NTA-iron(III)-IMAC enrichments. Therefore, MS2 from these experiments probably contained less noise and resulted in more database search matches.

All CAD MS/MS spectra were also searched for the neutral loss of phosphoric acid (98 Da) characteristic of phosphopeptides using an in-house program written in Java called CAD Neutral Loss Finder, which is available at <http://www.huntlab.org>. A description of CAD Neutral Loss Finder can also be found in this website. All MS/MS phosphopeptide spectra were manually validated, and subsets were compared with synthetic peptides for further validation. The mass accuracy of all the identified peptides was observed to be <3 p.p.m.

Data evaluation and interpretation

Chromatograms depicting the separation of enriched phosphopeptides during HPLC-ESI-MS/MS analysis are shown in **Figure 3a,b**. The average recovery of each type of enrichment is shown (**Fig. 3a,b**). Average recoveries were calculated by comparing the peak areas of the extracted chromatograms representing the internal standard phosphopeptides spiked into the sample during checkpoints 2 (DRVYIHPF and RVKsPLFQF) and 3 (RTHsLLLLG) with the peak areas of two internal standard peptides (DRVYIHPFHL and HSDAVFTDNYTR), at 100 fmol each, added before HPLC-ESI-MS/MS analysis. Checkpoints are shown in **Box 1**. Average recoveries represent all peptide losses from Steps 1–20 of the phosphopeptide enrichment protocols. Our calculations represent relative abundances of phosphopeptides detected. We ran technical duplicates (enriching and analyzing the same sample in the mass spectrometer twice) for the FHIOSE phosphopeptide identification experiments using the IDA-iron(III)-IMAC and the NTA-iron(III)-IMAC protocols, shown in **Figure 4**, and for the colorectal cancer phosphopeptide identification using the IDA-iron(III)-IMAC protocol, shown in **Figure 5**. A technical duplicate was not run using the NTA-iron(III)-IMAC protocol (**Fig. 5**) because we had a limited amount of sample.

We observed almost no nonspecific binding in our IDA-iron(III)-IMAC and NTA-iron(III)-IMAC enrichments (<10 unmodified peptides were detected at trace levels in each analysis (>95% specificity)). We also observed reduced background (non-peptidic species such as polymer contaminants) in NTA-iron(III)-IMAC experiments compared with IDA-iron(III)-IMAC experiments (**Fig. 3**). We estimate that each of the IDA-iron(III)-IMAC and the NTA-iron(III)-IMAC enrichments provide a unique set of phosphopeptides that account for 20–30% of the total phosphopeptides identified (**Figs. 4 and 5**). Our comparative analysis also demonstrated >50% overlap of phosphopeptides identified in both types of enrichments (**Figs. 4 and 5**). We believe that both types of enrichment should be completed on a single sample, if possible, to identify the highest number of phosphopeptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 | Checkpoints

We recommend checking the sample at various stages of the PROCEDURE to monitor peptide recoveries. Below is a list of suggested checkpoints. Each analysis can be completed using Step 20 of the PROCEDURE.

1. Analyze 1% of the immunopurified HLA-associated peptide sample using HPLC-ESI-MS/MS to determine how much sample should be used for phosphopeptide enrichment. We recommend using samples isolated from 1×10^8 to 1×10^9 cell equivalents for desalting and enrichment.
2. Analyze 1% of the desalted sample to monitor peptide levels. We recommend adding phosphopeptide standards before desalting to detect peptide losses.
3. Analyze 1% of the esterified sample to ensure reaction completion. We recommend adding additional phosphopeptide standards before esterification to determine whether the esterification is complete and to detect peptide losses.
4. Collect and analyze the flow-through from different steps in the phosphopeptide enrichment to determine peptide losses. We recommend adding additional phosphopeptide standards before enrichment to monitor peptide losses.

Figure 1.

Schematic of the HLA class I pathway. Endogenous proteins are degraded by the proteasome, and the resulting peptides are transported into the endoplasmic reticulum (ER) by a protein called TAP. Inside the ER, peptides are loaded onto HLA class I molecules. Stable HLA–peptide complexes are sent to the Golgi, and then they are shuttled to the cell surface for display. Circulating T cells can survey cellular health by interacting with HLA–peptide complexes. Tumor-specific peptides containing aberrant phosphorylations indicative of malignant transformation, shown in red, can induce T cells to specifically kill transformed cells. Almost all nucleated cells express HLA class I alleles. The immunological activity of cancer-specific HLA-associated peptide antigens makes them strong candidates for future cancer immunotherapeutics.

Figure 2.

Diagram illustrating the phosphopeptide enrichment protocol. **(a,b)** Schematic of the protocol for phosphopeptide enrichment and analysis using sHLA-transfected cells **(a)** and tumor tissue or cultured cells **(b)**. **(a,b)** HLA class I molecules are immunopurified from either the cell medium of sHLA-transfected cells **(a)** or the cell lysate of tumor tissue or cultured cells **(b)**, and their associated peptides are acid-eluted and filtered. The mixture of HLA-associated peptides is then desalted using a C18 column and esterified. Phosphopeptides are enriched from the mixture using iron(III)-IDA and iron(III)-NTA-IMAC and analyzed with HPLC-ESI-MS/MS. The steps in the PROCEDURE are labeled in red.

Figure 3.

Example chromatograms of iron(III)-IDA and iron(III)-NTA-IMAC elutions containing HLA-associated phosphopeptides. **(a)** Chromatograms of iron(III)-IDA (yellow) and iron(III)-NTA-IMAC (red) elutions containing HLA-associated phosphopeptides identified from FHIOSE. **(b)** Chromatograms of iron(III)-IDA (yellow) and iron(III)-NTA-IMAC (red) elutions containing HLA-associated phosphopeptides identified from metastasized colorectal cancer tumor tissue. The total ion current chromatograms are labeled “TIC.” The base peak chromatograms representing the most abundant species eluting throughout the HPLC-ESI-MS/MS analyses are labeled “base peak.” The total peptide recoveries calculated from Steps 1–20 of the PROCEDURE are displayed for each type of phosphopeptide enrichment experiment.

Figure 4.

Pie chart displaying the number of HLA-associated phosphopeptides identified from a sHLA-A*0201-transfected cell line using both the iron(III)-IDA and iron(III)-NTA-IMAC protocols. Shown are the number of HLA-associated phosphopeptides identified using (yellow) iron(III)-IDA-IMAC, (red) iron(III)-NTA-IMAC and (blue) both iron(III)-IDA and iron(III)-NTA-IMAC. 30 phosphopeptides were uniquely identified using iron(III)-IDA-IMAC (yellow). 32 phosphopeptides were uniquely identified using iron(III)-NTA-IMAC (red). 101 phosphopeptides were identified in both enrichment experiments (blue). A total of 163 HLA-associated phosphopeptides were identified from 10% of the sHLA-A*0201-transfected FHIOSE cell line sample.

Figure 5.

Pie chart displaying the number of HLA-associated phosphopeptides identified from metastasized human colorectal cancer tissue using both iron(III)-IDA and iron(III)-NTA-IMAC protocols. Shown are the number of HLA-associated phosphopeptides identified using (yellow) iron(III)-IDA-IMAC, (red) iron(III)-NTA-IMAC and (blue) both iron(III)-IDA and iron(III)-NTA-IMAC. 17 phosphopeptides were uniquely identified using iron(III)-IDA-IMAC (yellow). 23 phosphopeptides were uniquely identified using iron(III)-NTA-IMAC (red). 44 phosphopeptides were identified in both enrichment experiments (blue). A total of 84 HLA-associated phosphopeptides were identified from 0.6 g of metastasized colorectal cancer tissue.

TABLE 1

Troubleshooting table.

Problem	Possible reason	Solution
Peptides larger than 5 kDa are observed in checkpoint 1 in Box 1	5-kDa-MWCO filter failed during isolation	Re-filter immunopurified HLA-associated peptides with a new 5-kDa filter
No peptides are observed in checkpoint 2 in Box 1	Desalting has failed	Analyze the flow-through from desalting
	Sample was loaded too quickly onto the desalting column	If peptides are observed, re-desalt the sample
Esterification is not 100% complete (checkpoint 3 in Box 1)	H ₂ O was present in reagents, reversing the reaction	Open anhydrous esterification reagents and make methanolic HCl immediately before esterification
Phosphopeptides are observed in IMAC enrichment sample load flow-through	Sample pH was too acidic (1:1:1 should be pH ~4–5)	Adjust the pH of the sample to the correct pH
	Sample was loaded too quickly onto the IMAC column	Re-enrich for phosphopeptides from the IMAC flow-through containing peptides
The IMAC column clogs during enrichment	FeCl ₃ was not filtered	Filter FeCl ₃ with a C8 Sep-Pak
	Sample was not desalted	Desalt the sample before enrichment
No peptides are observed in the IMAC elution	Flow rate used during the elution was too fast	Analyze the elution flow-through for phosphopeptides
		Load the elution flow-through slowly (<1 µl/min) onto a C18 precolumn
Nonphosphorylated peptides are observed in IMAC elution	IMAC column was not fully activated with FeCl ₃	Repeat the experiment and add additional FeCl ₃ incubation steps
	0.1% (vol/vol) AcOH wash was not long enough	Repeat the experiment and wash with 0.1% (vol/vol) AcOH for double the suggested time