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Niobium(V) Oxide (Nb_2O_5): Application to Phosphoproteomics

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Proteomics-based analysis of signaling cascades relies on a growing suite of affinity resins and methods aimed at efficient enrichment of phosphorylated peptides from complex biological mixtures. Given the heterogeneity of phosphopeptides and the overlap in chemical properties between phospho- and unmodified peptides, it is likely that the use of multiple resins will provide the best combination of specificity, yield, and coverage for large-scale proteomics studies. Recently titanium and zirconium dioxides have been used successfully for enrichment of phosphopeptides. Here we report the first demonstration that niobium pentoxide (Nb_2O_5) provides for efficient enrichment and recovery (~50−100%) of phosphopeptides from simple mixtures and facilitates identification of several hundred putative sites of phosphorylation from cell lysate. Comparison of phosphorylated peptides identified from Nb_2O_5 and TiO_2 with sequences in the PhosphoELM database suggests a useful degree of divergence in the selectivity of these metal oxide resins. Collectively our data indicate that Nb_2O_5 provides efficient enrichment for phosphopeptides and offers a complementary approach for large-scale phosphoproteomics studies.

Reversible protein phosphorylation is a critical mediator of many cellular processes including proliferation, differentiation, and transcription.[1,2] Disruption of these pathways can lead to multiple physiopathological states, and hence there is great interest in techniques that facilitate global and unbiased analysis of phosphorylation cascades. These data can in principle provide insight into normal cell biology, in addition to information relevant to disease onset and progression, new therapeutic entry points, and characterization of existing drugs.

Mass spectrometry-based proteomics, particularly liquid chromatography coupled to electrospray ionization mass spectrometry (LC−ESI-MS), has become the technique of choice for high-throughput, unbiased identification and characterization of proteins in biological systems.[3,4] Moreover multiple approaches now exist to conduct these experiments in a semiquantitative manner to monitor changes in protein expression and post-translational modification status as a function of biological perturbation.[3] Despite these advantages, there is a mismatch between the detection capabilities of mass spectrometry and the concentration dynamic range inherent to most biological systems. As a result, significant effort has been devoted to the development of fractionation and enrichment protocols with the goal of targeted, subproteome analyses (e.g., phosphorylated peptides, membrane proteins, multicomponent protein complexes, etc.).[3,5–7]

Although significant progress has been made in the area of phosphoprofiling by mass spectrometry,[8–10] many challenges remain. For example, phosphoproteins are estimated to account for 30−50% of the eukaryotic proteome, but phosphorylation on a specific protein is typically transient and only present in substoichiometric concentration.[11,12] As a result, numerous strategies have been developed for phosphopeptide enrichment, including immobilized metal affinity chromatography (IMAC),[13,14] phosphoramidate chemistry (PAC), [15] and metal oxides of titanium (TiO_2)[16] and zirconium (ZrO_2).[17] While each of these approaches provides a useful degree of phosphopeptide enrichment, it is also clear that the achievable selectivity is dependent upon very careful control of sample loading and washing buffer compositions for

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each resin.[18,19] Moreover, even under tightly controlled conditions, there is incomplete overlap in phosphopeptide populations enriched by each method.[15] One interpretation of these data and results is that the physio-chemical properties of phosphopeptides compared to peptides in toto do not diverge to an appreciable extent, complicating efforts to achieve high yield and selectivity from a single affinity resin. Hence it is likely that the use of multiple resins, perhaps augmented by chemical-based affinity tags, will provide the best combination of specificity, yield, and coverage for large-scale proteomics studies.

In an effort to discover additional metal oxides that could complement existing techniques for phosphopeptide analysis, we evaluated the potential of niobium pentoxide (Nb_2O_5) for phosphopeptide enrichment. On the basis of the proximity of niobium to titanium and zirconium in the periodic table, we speculated that Nb_2O_5 may display affinity and selectivity for phosphorylated peptides. We utilized Nb_2O_5 resin in both TopTip and fused silica capillary column formats and observed efficient enrichment of phosphopeptides from digests of purified caseins and whole cell lysates. As has been reported for ZrO_2 and TiO_2, the use of lactic acid minimized nonspecific binding of nonphosphorylated peptides to Nb_2O_5. Finally comparison of phosphopeptides enriched by Nb_2O_5 or TiO_2 from cellular lysate indicated approximately 50% overlap in peptide sequence, demonstrating a useful degree of orthogonality between these metal oxide resins. Collectively our data demonstrate that Nb_2O_5 is an effective addition to the repertoire of tools for phosphoproteomics.

MATERIAL AND METHODS

Materials. Titanium dioxide was purchased from GL Sciences (Torrance, CA). Trifluoroacetic acid was obtained from Pierce (Rockford, IL). Sequencing grade, modified trypsin was purchased from Promega (Madison, WI). All other chemicals, including niobium pentoxide (~325 mesh), were purchased from Sigma Aldrich (St. Louis, MO). Fritted TopTip pipet tips were obtained from Glygen (Columbia, MD). Reversed phase C_{18} ZipTips were purchased from Millipore (Billerica, MA). Solid phase extraction cartridges and strong cation exchange embedded 96-well plates were purchased from Waters (Milford, MA) and Supelco (Bellafonte, PA), respectively. Phosphopeptide standards NVPLpYK and VNQIGpTLSESIK were obtained from Waters (Milford, MA). LIEDAEpYTAK and FLApTGDGAR were synthesized using Fast-Moc chemistry on an ABI 431A peptide synthesizer (Applied Biosystems, Framingham, MA) using Fmoc-protected amino acids (Calbiochem-Novabiochem, San Diego, CA). The peptides were cleaved from the resin using 95% trifluoroacetic acid/2.5% triisopropylsilane/2.5% water for 2 h, purified by preparative reversed-phase HPLC, and lyophilized.

Preparation of Casein Standard Protein Digests. α-Casein was dissolved in 50 mM ammonium bicarbonate (pH ~8) and digested 1:20 with trypsin overnight at 37 °C. Aliquots corresponding to 50 pmol/μL were stored at −80 °C.

Cell Culture and Preparation of Digested Lysate. K562 cells were maintained in suspension culture in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were grown at 37 °C in 5% CO_2, and aliquots of ~5×10^7 cells were harvested by centrifugation during log phase. After washing twice with 20 mL of phosphate buffered saline, the pellet was lysed with 3 mL of 8 M urea, 100 mM ammonium bicarbonate, and 30 μL each of Sigma-Aldrich phosphatase inhibitor cocktails I and II. Protein concentration was determined using the Bradford Assay (Biorad Laboratories, Hercules, CA). Dithiothreitol (DTT) was added to a final concentration of 10 mM and incubated for 30 min at 60 °C, followed by addition of iodoacetamide to 20 mM. After 30 min incubation in the dark at room temperature, excess iodoacetamide was quenched by addition of DTT to a final concentration of 20 mM. Reduced and alkylated proteins were diluted to a final volume of 12 mL in 0.1 M ammonium bicarbonate. Trypsin (150 μg, 1:50 enzyme/substrate) was added, and digestion was performed at 37 °C overnight with end-over-end rotation. The resulting peptide solution was acidified with 10% TFA and desalted on a C_{18} solid phase extraction cartridge, followed by final cleanup on strong cation exchange embedded 96-well plates. Eluted peptides (400 μg) were lyophilized by vacuum centrifugation and stored at −80 °C.

Enrichment of Phosphopeptides from Casein Digests with Metal Oxides. Approximately 1−2 mg of metal oxide (Nb_2O_5) or TiO_2) was loaded into TopTips from aqueous slurries using air pressure and conditioned as follows: 2×10 μL with 0.1% trifluoroacetic acid (TFA), 2×10 μL of 70% acetonitrile, 0.1% TFA, 1×10 μL of 100 mM $(NH_4)_2PO_4$ (this step only for Nb_2O_5 to flush fines through the TopTip frit), and 2×10 μL of 0.1% TFA. Digested casein peptides (200 fmol) were diluted with loading buffer consisting of 80% acetonitrile with 0.1% TFA. In some experiments (as noted in the Results and Discussion), the loading buffer and first two wash buffer rinses contained 225 mg/mL 2,5-dihydroxybenzoic acid (DHB) or 100 mg/mL lactic acid (LA) to reduce nonspecific binding of unphosphorylated peptides. TopTips containing conditioned metal oxide were equilibrated with 4×10 μL of 80% acetonitrile, 0.1% TFA. Next, samples were loaded and then washed with 5×10 μL of 80% MeCN with 0.1% TFA, followed by 2×10 μL of 0.1% TFA. Peptides were eluted with 20 μL of 100 mM $(NH_4)_2PO_4$, desalted on C_{18} ZipTips, and deposited in a final volume of 1.5 μL directly onto a MALDI plate. Matrix (1 μL, 10 mg/mL α-hydroxyl cinnamic acid in 70% acetonitrile with 0.1% TFA) was added to each sample spot and they were allowed to air-dry. Samples were analyzed using a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) in reflectron mode averaging 1000 laser shots in a random, uniform pattern (20 subspectra, pass or fail, 50 shots/subspectrum) with a laser intensity of ~3300. Supporting Information Table 1 shows masses of phosphopeptides observed in these experiments and their associated mass errors. MS/MS experiments were performed in reflectron mode averaging 5000 laser shots in a random uniform pattern (100 subspectra, pass or fail, 50 shots/subspectrum) with CID gas on and the precursor mass window set to relative with a value of 200 (fwhm). MALDI MS/MS spectra were centroided, and processed with in-house software to remove peaks <300 Da, within 120 Da of the precursor, or below 100 counts. Processed spectra were searched against the NCBI nr database with possible phosphorylation of S, T, or Y. Mascot search results with embedded MS/MS spectra are shown in Supporting Information Table 2.

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Evaluation of Recovery. A stock solution of phosphopeptide standards (NVPLpYK, VNQIGpTLSESIK, LIEDAEpYTAK, and FLApTGDGAR) was prepared at a concentration of 100 fmol/μL. This solution (10 μL) was loaded onto a Nb_2O_5 packed TopTip and washed 4 × with 10 μL of 80% acetonitrile, 0.1% TFA, then with 2 × 10 μL of 0.1% TFA. Peptides were eluted with 10 μL of 1:20 28% ammonium hydroxide/water, and 2 μL of this solution was spotted on a MALDI plate, followed by the addition of 1 μL of matrix. For the control, 2 μL of the stock solution was spotted followed by addition of 1 μL of matrix solution. Samples were analyzed using a 4800 MALDI-TOF/TOF mass spectrometer in reflectron mode. Recoveries were calculated using the formula: (Nb_2O_5 signal/control signal) × 100%.

Enrichment of Phosphopeptides from K562 Cell Lysate. Lyophilized peptides (400 μg) were resuspended in 150 μL of loading buffer, containing 250 mg/mL LA, and pressure loaded onto self-packed Nb_2O_5 and TiO_2 fused silica columns (530 μm × 4 cm) at a flow rate of 2 μL/min. Columns were sequentially washed with 60 μL of loading buffer (2 μL/min), 60 μL of wash buffer (80% MeCN, 2% TFA), and 60 μL 0.1% TFA (3 μL/min). Peptides were eluted directly to self-packed fused silica reversed-phase precolumns (100 μm × 5 cm, 20 μm C_{18}) using 30 μL of 100 mM $(NH_4)_2PO_4$. Precolumns were washed with 10 column volumes of 0.2 M acetic acid and then connected directly to self-packed fused silica analytical columns (25 μm × 8 cm, 3 μm C_{18}) positioned at the orifice of a hybrid quadrupole time-of-flight mass spectrometer (QSTAR Elite, Applied Biosystems, Foster City, CA). Analytical columns were constructed as described[20] except that bottlenecks were fritted with 7 μm C_{18} particles. An Agilent 1100 series binary HPLC (Agilent, Palo Alto, CA) was used to generate an aqueous−organic gradient (0−3% B in 3 min, 3−30% B in 57 min, 30−50% B in 10 min, 50−70% B in 10 min; A = 0.2 M acetic acid in water, B = 0.2 M acetic acid in acetonitrile) and elute peptides directly into the mass spectrometer at a flow rate of 10 nL/min. LC−MS data were acquired in the information-dependent acquisition (IDA) mode (MS scan, $300 \leq m/z \leq 2000$, top 8 most abundant MS/MS scans using low resolution for precursor isolation and Smart Exit with Enhance All to optimize quality and acquisition speed of the MS/MS spectra, 1.6 kV ESI voltage).

Data Analysis. Mascot Daemon was used to extract and format MS/MS data for subsequent search against human IPI protein database (Mascot version 2.2.00, Matrix Science, Inc., London, U.K.). The search parameters allowed one missed cleavage for trypsin, a variable modification of +80 Da for phosphorylation of serine, threonine, or tyrosine, and a fixed modification of +57 corresponding to carboxyamidation of cysteine. A mass tolerance of 1 Da for precursors and 0.5 Da for fragment ions was specified. Putative phosphopeptide sequence matches with Mascot scores greater than 20, 30, or 40 were extracted and searched against the version 7.0 of the PhosphoELM database.[21] The entire collection of sequences along with Mascot scores, potential site(s) of modification, peak areas, and PhosphoELM entries are reported in Supporting Information Table 3. MS/MS spectra with Mascot assignments are embedded in the table as .png files and can be viewed by mouse-over. False discovery rates (FDR) were evaluated by performing a search with a reverse database and were calculated using the formula: estimated FDR = unique reverse database hits/unique forward database hits.

Frequency of occurrence for amino acids in unique phosphopeptide sequences was calculated by counting the number of each amino acid and dividing by the total number of residues in each data set. The bias in observed amino acid frequency was represented as the log ratio (base 2) of percent composition in TiO_2 sequences versus percent composition in Nb_2O_5 sequences (Supporting Information Table 4). To evaluate the significance of these ratios, a permutation test was performed by combining the Nb_2O_5 and TiO_2 specific sequences and randomly permuting the assignments such that the number of sequences assigned as Nb_2O_5 and TiO_2 remain constant. After each permutation, the amino acid percent compositions and their log ratios were recalculated, for a total of 10 000 permutations. One-sided *P*-values were calculated by summing the frequency (probability) of all log ratios equal to or more extreme than the observed log ratios; two-sided *P*-values were calculated by doubling the one-sided *P*-values (Supporting Information Table 5). We used $\alpha = 0.01$ to determine the significance.

Safety Considerations. Trifluoroacetic acid is corrosive and should be handled in a fume hood with appropriate protective equipment as described in the manufacturer's material safety data sheet.

RESULTS AND DISCUSSION

As a basic test we first used Nb_2O_5 to enrich phosphopeptides from tryptic digests of α-casein. Figure 1A shows that without prior enrichment, only a single α-casein phosphopeptide (inset) is detected and at an abundance less than 5% compared to the nonphosphorylated casein peptides. However Figure 1B demonstrates that use of Nb_2O_5 provides for enrichment of phosphopeptides; signals from casein phosphopeptides dominate the spectrum, with only a single nonphosphorylated peptide (HQGLPQEVLNENLLR) observed with any appreciable intensity. On the basis of this result, we speculated that the use of nonpeptide chemical species with carboxylate groups would minimize binding of acidic-rich peptides to (Nb_2O_5). Similar strategies have been employed to reduce nonspecific binding to titanium and zirconium dioxide resins.[22,23] To test our hypothesis, we added DHB or LA to the loading and wash buffers during enrichment of phosphopeptides from α-casein. Figure 1 shows that both DHB (part C) and LA (part D) significantly reduced retention of (HQGLPQEVLNENLLR) on the Nb_2O_5 resin. Our data in Figure 1 demonstrate that Nb_2O_5 exhibits inherent affinity for phosphopeptides and furthermore that the degree of enrichment may be refined by the use of small molecule chemical competitors. To evaluate the recovery of phosphorylated peptides, we compared the signal intensity for four standard phosphopeptides before and after enrichment on Nb_2O_5. Table 1 shows that recovery efficiency (48−100%) is comparable to that obtained with TiO_2 under similar conditions.[23]

We next sought to compare the performance of Nb_2O_5 versus that for TiO_2, the most commonly used metal oxide for enrichment

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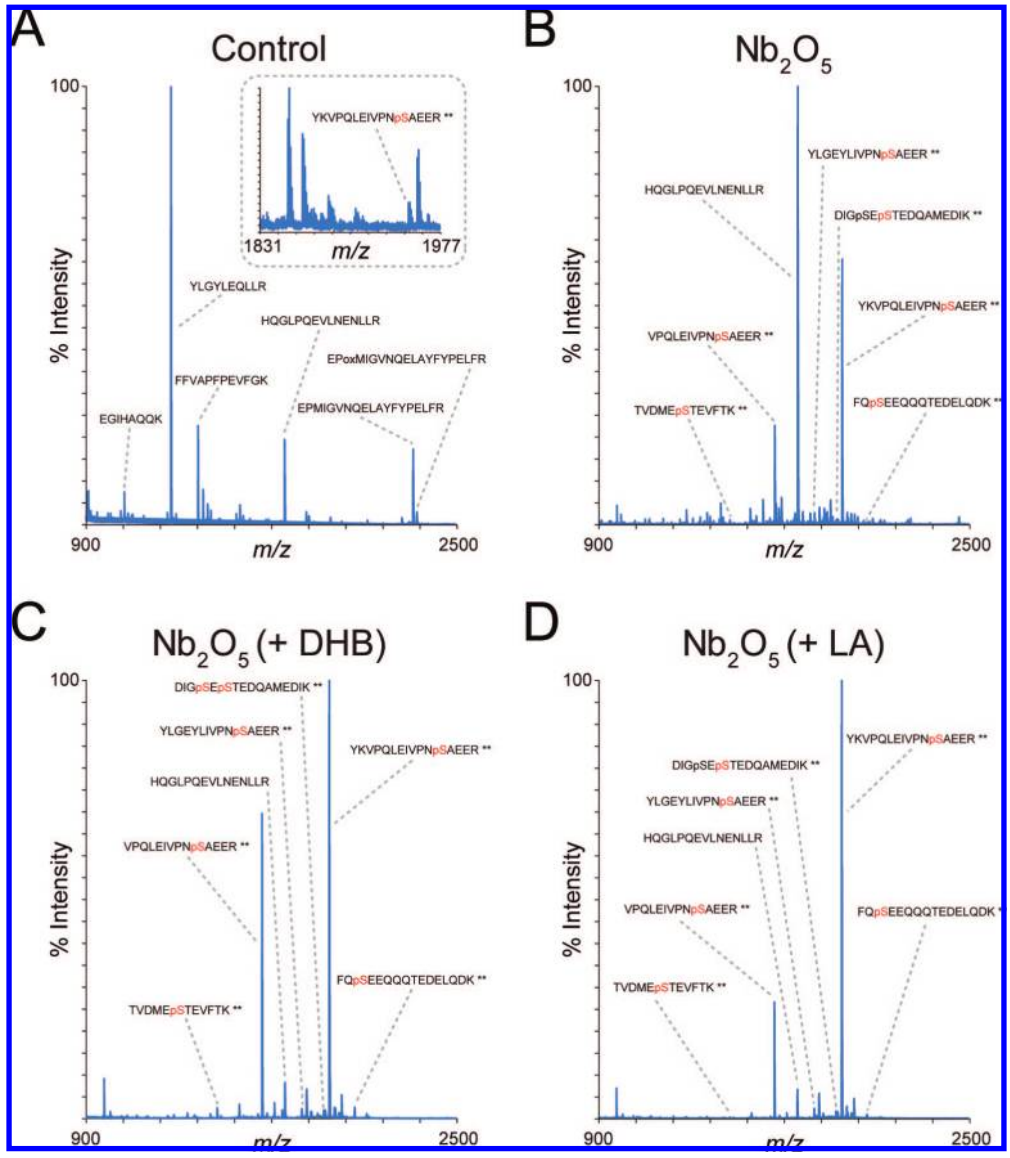

Figure 1. Niobium(V) oxide (Nb_2O_5) provides for enrichment of phosphorylated peptides. MALDI mass spectra of tryptic α-casein peptides (A) before and (B) after enrichment by Nb_2O_5 used in TopTip format. Addition of (C) 2,5-dihydroxybenzoic acid or (D) lactic acid to the loading and rinse buffers improves selectivity of Nb_2O_5 resin.

Table 1. Recoveries of Phosphopeptides from (Nb_2O_5)

peptide sequence	abundance (control)	abundance (Nb_2O_5)	recovery (%)
LIEDAEpYTAK	605.5	292.5	48
FLApTGDGAR	2285.7	1845	81
NVPLpYK	154	76	49
VNQIGpTLSESIK	302	332	~100

of phosphopeptides.[23,16,22,18] Figure 2A shows that TiO_2 provides for enrichment of α-casein phosphopeptides when used in conjunction with lactic acid. Qualitative comparison of Figures 1D or 2A with 1A demonstrates that TiO_2 and Nb_2O_5 perform similarly for enrichment of phosphopeptides from simple peptide mixtures. Interestingly, comparison of Figures 1D and 2A illustrates potential divergence in the selectivity of each resin. For example, Nb_2O_5 exhibits a strong preference for YKVPQLEIVPNpSAEER over VPQLEIVPNpSAEER (Figure 1D), while we and others[23] observe the opposite trend for TiO_2 (Figure 2A). To explore the possibility that the use of both resins in combination may exploit their individual selectivity and provide for more efficient enrichment, we packed a mixed-bed column that contained equal volumes of TiO_2 and Nb_2O_5 resins. Figure 2B shows that YKVPQLEIVPNpSAEER and VPQLEIVPNpSAEER are recovered with nearly equal efficiency, suggesting that the resins perform in an additive manner when used in combination.

While the results depicted in Figures 1 and 2 suggest a divergence in phosphopeptide affinity between TiO_2 and Nb_2O_5 resins, it is unclear whether trends observed for simple standards will apply to analysis of complex biological extracts. For example, previous work indicated that competition between singly- and multiply-phosphorylated peptides present in complex mixtures may introduce significant bias in the distribution of detected peptides.[24,25] To test the suitability of Nb_2O_5 for enrichment of

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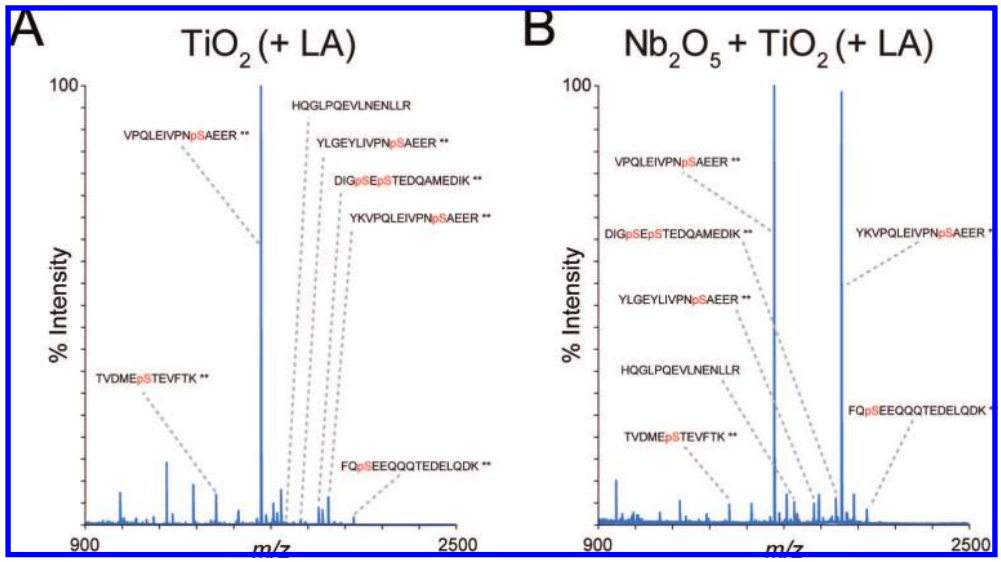

Figure 2. Metal oxides of titanium and niobium exhibit divergent selectivity for α-casein phosphopeptides. MALDI mass spectra of tryptic α-casein peptides enriched by TiO_2 and a mixed bed of TiO_2 and Nb_2O_5, each in TopTip format. (A) Titanium dioxide exhibits a strong preference for VPQLEIVPNpSAEER over YKVPQLEIVPNpSAEER, while niobium pentoxide displays the opposite trend (Figure 1D). (B) A mixed bed column provides uniform enrichment of VPQLEIVPNpSAEER and YKVPQLEIVPNpSAEER.

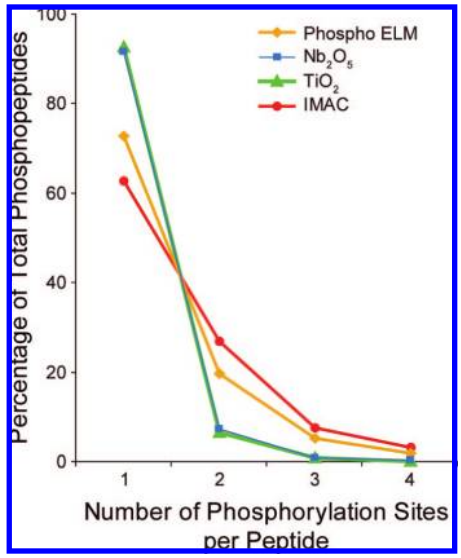

Figure 3. Phosphorylation states of peptides recovered from titanium dioxide (green) or niobium pentoxide (blue) are nearly identical and show good agreement with a distribution derived in silico from the PhosphoELM database (orange).[21] Data from the metal oxide resins exhibit a slight positive bias for singly phosphorylated peptides (compared to PhosphoELM) while previous results[24] obtained from use of an optimized IMAC protocol (red, reprinted from ref 24 Copyright 2006 American Chemical Society) show a slight negative bias.

phosphopeptides from complex mixtures, we prepared tryptic digests of proteins derived from human myeloid K562 cells. Phosphopeptides were enriched on either TiO_2 or Nb_2O_5 microcapillary columns and then analyzed by LC−MS/MS. Figure 3 shows that the percentages of singly- and multiply-phosphorylated peptides recovered from TiO_2 and Nb_2O_5 are nearly identical and agree well when compared to a similar distribution determined in silico from the PhosphoELM database.[21] Interestingly, we note that the metal oxide resins provided a positive bias for detection of singly-phosphorylated peptides versus that predicted by PhosphoELM, while our previous work[24] demonstrated a slight negative bias in detection of singly-phosphorylated peptides using a highly optimized immobilized metal affinity chromatography (IMAC) protocol (Figure 3, red trace. Reprinted from ref 24 Copyright 2006 American Chemical Society).

Given the overlap for TiO_2 and Nb_2O_5 in terms of phosphorylation state distribution, we next asked whether the metal oxide resins exhibited divergence with respect to specific phosphopeptide sequences recovered after enrichment. Figure 4 shows Venn diagrams for (part A) the overlap of total phosphopeptide identifications from TiO_2 and Nb_2O_5 as a function of Mascot score threshold and (part B) the overlap of phosphopeptides identified through use of each metal oxide resin with those sequences that also exist in version 7.0 (July 2007) of the PhosphoELM database (Estimated false discovery rates for the data are reported in Supporting Information Table 6.).[21] Note that for either comparison, the percentage of unique phosphopeptide sequences contributed by each resin is consistent as a function of Mascot score threshold. This suggests that spurious or false positive identifications did not significantly bias our results. Moreover, Nb_2O_5 contributes a larger fraction of unique phosphopeptide sequences as compared to TiO_2 when the total peptide pool is restricted to entries in PhosphoELM (Figure 4B, bottom). We attribute this observation to the fact that IMAC- and TiO_2-based enrichment methods constitute the majority of high-throughput data in PhosphoELM;[21] for example, a report by Olsen et al. that utilized TiO_2 contributed >6000 phosphopeptide sequences to the database.[26] As a result, using PhosphoELM as the basis for comparison further accentuates the divergent selectivity of Nb_2O_5 versus TiO_2.

Given the potential for bias introduced due to inefficient MS/MS sampling of low-level precursors, we next extracted precursor intensities for all putative phosphopeptides identified in TiO_2 and

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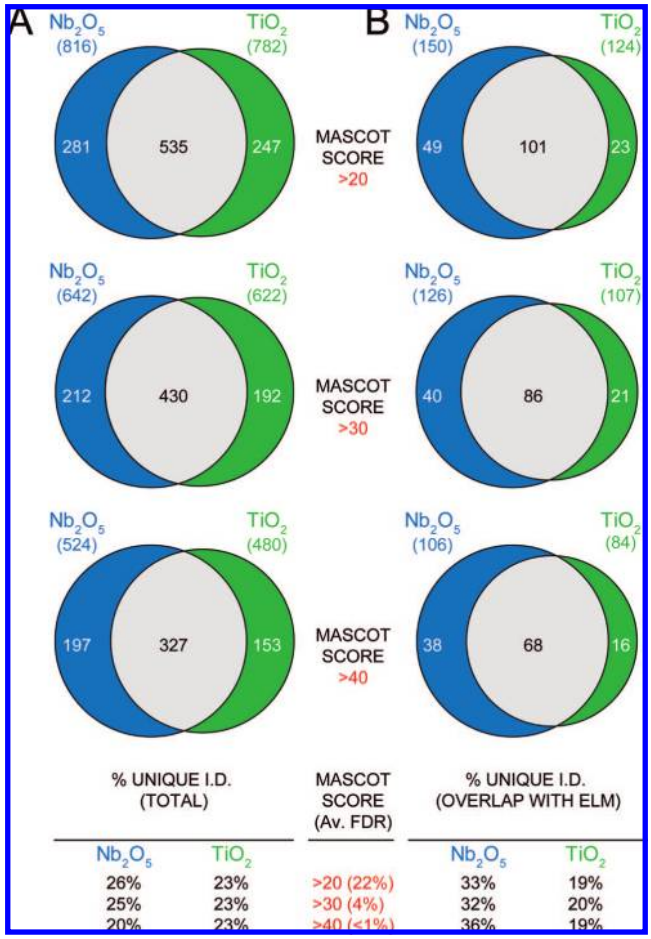

Figure 4. Comparison of phosphopeptide sequences obtained by use of (Nb_2O_5) and (TiO_2) reveals divergent selectivity. Venn diagrams depict the overlap of (A) all phosphopeptide identifications and (B) the subset of phosphopeptide identifications contained in the PhosphoELM database (version 7.0),[21] for each metal oxide as a function of Mascot score threshold. Also shown are the percent contribution (bottom) of unique phosphopeptide sequences from each metal oxide resin to the total pool of identifications (bottom, left) and to the subset of identifications contained in PhosphoELM (bottom, right). The average false discovery rates for different Mascot score thresholds are shown in parentheses.

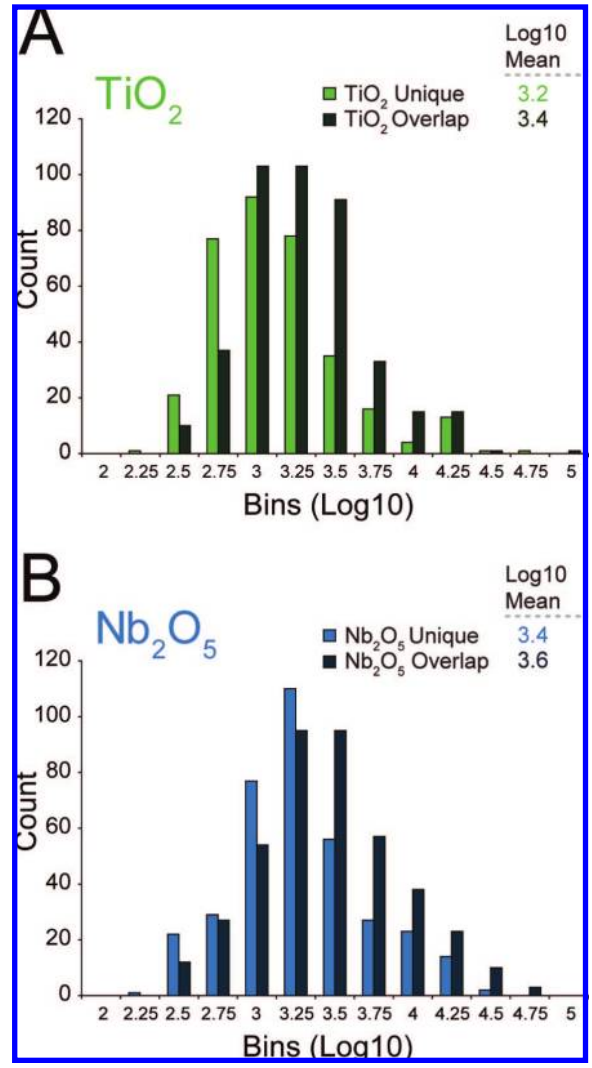

Figure 5. Histogram distributions of peak heights for unique and overlapping phosphopeptides detected in conjunction with (A) TiO_2- and (B) Nb_2O_5-based enrichment.

Nb_2O_5 LC−MS/MS acquisitions and plotted these as histograms. Figure 5 shows that the intensity distributions for phosphopeptides assigned uniquely to either metal oxide do not differ significantly from analogous distributions for phosphopeptides detected in common between TiO_2 and Nb_2O_5. This result confirms that unique peptide sequences identified for each metal oxide are not confined to the low signal-to-noise region and hence cannot be explained simply by the stochastic nature of precursor selection in data-dependent type scanning.

Finally, we compared amino acid content of all putative phosphopeptides as an orthogonal measure of metal oxide selectivity. Figure 6A shows the relative amino acid biases observed in phosphopeptides enriched by TiO_2 (green) and Nb_2O_5 (blue). Application of a permutation test (see Materials and Methods) indicated statistically significant biases (Figure 6B; p-value < 0.01) and suggested that phosphopeptides unique to Nb_2O_5 have an overrepresentation of Arg, Lys, and Pro residues while those unique to TiO_2 are biased toward Asp, Glu, Phe, and Leu. The greater number of Arg and Lys residues observed with Nb_2O_5 correlates with a higher number of missed cleavage peptides found with this oxide (121/381 or 32% from Nb_2O_5 vs 78/347 or 22% with TiO_2). In contrast, the increased frequency of Pro was not associated with missed cleavage phosphopeptides (27 KP or RP observed with Nb_2O_5 vs 25 with TiO_2). Collectively our analyses of phosphopeptide sequence overlap, precursor intensity distribution, and amino acid content provide compelling evidence that TiO_2 and Nb_2O_5 exhibit bona fide divergence in their respective selectivities for phosphopeptides.

CONCLUDING REMARKS

The data and results presented herein demonstrate that niobium pentoxide provides for efficient enrichment of phosphopeptides from simple standards and complex biological extracts. Moreover, comparison of phosphopeptide populations enriched by TiO_2 and Nb_2O_5 reveals a useful degree of divergence in peptide selectivity between the two resins. Given the inherent overlap in physio-chemical properties between phosphopeptides and their unmodified counterparts, it is clear that large-scale efforts to

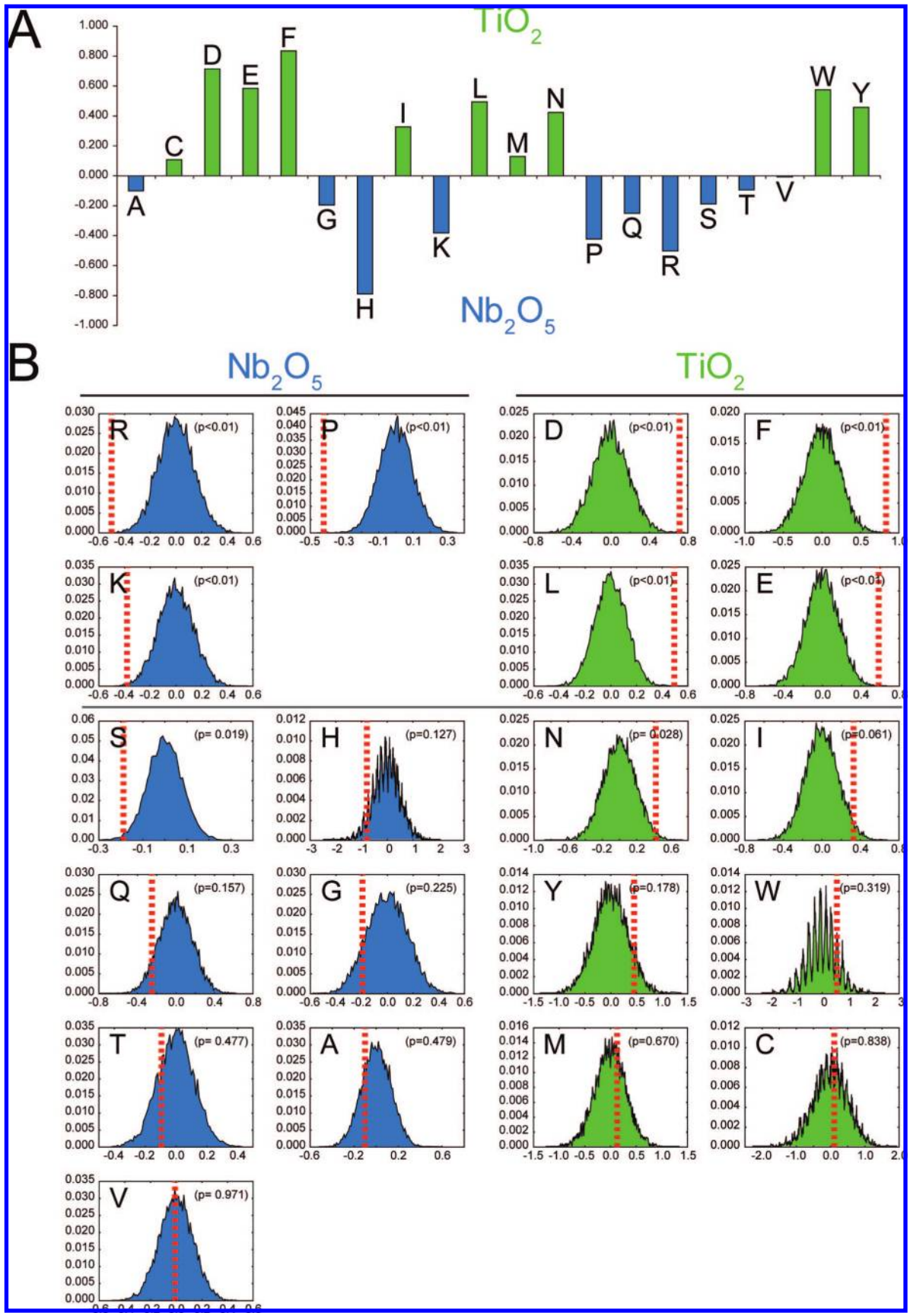

Figure 6. Peptides unique to Nb_2O_5 and TiO_2 have specific amino acid biases. (A) Log ratios (base 2) of % amino acid compositions expressed as $(TiO_2)/(Nb_2O_5)$ show residues that occur with higher frequency in Nb_2O_5 (blue, negative value) or TiO_2 (green, positive value). (B) Histograms derived from permutation tests for each amino acid represent the null distributions. Dashed red line represents the log ratio of the observed amino acid frequency. Residues with *p*-values < 0.01 have a statistically significant bias towards either Nb_2O_5 or TiO_2.

catalog and quantify phosphorylation cascades will benefit from the use of multiple enrichment strategies. Collectively our data demonstrate that Nb_2O_5 is an effective and complementary addition to the phosphoproteomics toolbox.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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