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Selective Isolation at the Femtomole Level of Phosphopeptides from Proteolytic Digests Using 2D-NanoLC-ESI-MS/MS and Titanium Oxide Precolumns

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Selective detection of phosphopeptides from proteolytic digests is a challenging and highly relevant task in many proteomics applications. Often phosphopeptides are present in small amounts and need selective isolation or enrichment before identification. Here we report a novel automated method for the enrichment of phosphopeptides from complex mixtures. The method employs a two-dimensional column setup, with titanium oxide-based solid-phase material (Titansphere) as the first dimension and reversed-phase material as the second dimension. Phosphopeptides are separated from nonphosphorylated peptides by trapping them under acidic conditions on a TiO_2 precolumn. Nonphosphorylated peptides break through and are trapped on a reversed-phase precolumn after which they are analyzed by nanoflow LC-ESI-MS/MS. Subsequently, phosphopeptides are desorbed from the TiO_2 column under alkaline conditions, reconcentrated onto the reversed-phase precolumn, and analyzed by nanoflow LC-ESI-MS/MS. The selectivity and practicality of using TiO_2 precolumns for trapping phosphopeptides are demonstrated via the analysis of a model peptide RKISASEF, in a 1:1 mixture of a non- and a monophosphorylated form. A sample of 125 fmol of the phosphorylated peptide could easily be isolated from the nonphosphorylated peptide with a recovery above 90%. In addition, proteolytic digests of three different autophosphorylation forms of the 153-kDa homodimeric cGMP-dependent protein kinase are analyzed. From proteolytic digests of the fully autophosphorylated protein at least eight phosphorylation sites are identified, including two previously uncharacterized sites, namely, Ser-26 and Ser-44. Ser-26 is characterized as a minor phosphorylation site in purified PKG samples, while Ser-44 is identified as a novel in vitro autophosphorylation target. These results clearly show that TiO_2 has strong affinity for phosphorylated peptides, and thus, we conclude that this material has a high potential in the field of phosphoproteomics.

Dynamic posttranslational modification is a general mechanism for fine-tuning protein structure and function. In particular, protein phosphorylation plays a key role in the regulation of virtually all cellular events. Many crucial biological processes such as cell cycle, cell growth, cell differentiation, and metabolism are orchestered and tightly controlled by reversible protein phosphorylation, modulating protein activity, stability, interaction, and localization.[1,2] To gain further insight into the regulation of these processes by reversible phosphorylation, it is often necessary to characterize the phosphorylation state of specific proteins under certain conditions. Several analytical techniques exist for the analysis of protein phosphorylation, such as (i) ^{32}P-labeling, (ii) Edman sequencing, and (iii) mass spectrometric methods, of which most are based either upon the mass increment of 80 amu of a single phosphorylation event or upon specific fragmentation patterns of the incorporated phosphate moiety in for instance neutral loss scans.[3−5] Mass spectrometric methods for identifying and characterizing phosphoproteins are inherently faster than Edman sequencing or radiolabeling. Nevertheless the identification and detailed investigation of phosphoproteins including the localization of phosphorylation sites by mass spectrometry remains challenging. Often specific isolation of phosphoproteins or phosphopeptides is a prerequisite for mass spectrometric analysis. Commonly used enrichment techniques are IMAC,[6] immunoprecipitation using phosphoprotein specific antibodies,[7] or specific chemical modification strategies, targeted for phosphorylated amino acids.[8,9] Immobilized metal affinity chromatography combined with elec-

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(1) Graves, J. D.; Krebs, E. G. *Pharmacol. Ther.* **1999**, *82*, 111−121.
(2) Hunter, T. *Cell* **2000**, *100*, 113−127.
(3) Huddleston, M. J.; Annan, R. S.; Bean, M. F.; Carr, S. A. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 710−717.
(4) Carr, S. A.; Huddleston, M. J.; Annan, R. S. *Anal. Biochem.* **1996**, *239*, 180−192.
(5) Zappacosta, F.; Huddleston, M. J.; Karcher, R. L.; Gelfand, V. I.; Carr, S. A.; Annan, R. S. *Anal. Chem.* **2002**, *74*, 3221−3231.
(6) Posewitz, M. C.; Tempst, P. *Anal. Chem.* **1999**, *71*, 2883−2892.
(7) Pandey, A.; Podtelejnikov, A. V.; Blagoev, B.; Bustelo, X. R.; Mann, M.; Lodish, H. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 179−184.
(8) Oda, Y.; Nagasu, T.; Chait, B. T. *Nat. Biotechnol.* **2001**, *19*, 379−382.
(9) Zhou, H.; Watts, J. D.; Aebersold, R. *Nat. Biotechnol.* **2001**, *19*, 375−378.

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trospray ionization mass spectrometry has been successfully used in off-line[10] and on-line[11−13] applications and probably offers so far the highest potency in sample recovery and sample throughput. However, enrichment and recovery strongly depends on the type of metal ion, column material, and loading/eluting procedures that are used.[6,14,15] Furthermore, IMAC requires additional metal ion loading and washing steps, increasing total sample analysis time, and tedious to configure in on-line applications. Here we report a novel automated method for the selective enrichment of phosphopeptides from complex mixtures using a two-dimensional column switching setup, with titanium oxide-based solid-phase material (Titansphere). This relatively new base material for HPLC columns consists of porous titania microspheres with a smooth and alkaline surface and unique amphotheric ion-exchange properties.[16−20] The property of titanium oxide to selectively absorb water-soluble organic phosphates has been previously demonstrated.[21−24] The objective of this study is to critically evaluate the potential applicability of TiO_2 as chromatographic medium capable of selectively isolating and enriching phosphorylated peptides from complex mixtures in a fast and automated manner. For this purpose, an automated on-line two-dimensional liquid chromatography system was developed, which allows isolation and mass spectrometric identification of phosphorylated peptides from proteolytic digests. Selectivity and sensitivity of the developed method are demonstrated first using model peptides. At least 125 fmol of the synthetic phosphopeptide RKIpSASEF was selectively isolated and detected from its nonphosphorylated form. Furthermore, using this method the autophosphorylation status of the cGMP-dependent protein kinase was characterized. PKG is a 153-kDa homodimeric protein that acts directly downstream in the nitric oxide/cGMP-mediated signaling pathway in control of a variety of cellular responses, including smooth muscle relaxation.[25] In vitro, PKG autophosphorylates in the presence of Mg^{2+}-ATP and cAMP or cGMP.[26] The major sites of autophosphorylation have been previously identified as Ser-50, Thr-58, Ser-72, and Thr-84. Minor phosphorylation was observed on Ser-1 and Ser-64.[27] Using this model protein and the developed phosphopeptide enrichment method, we were able to retrieve several phosphopeptides from highly autophosphorylated PKG, including di- and trivalent phosphopeptides, covering almost all known autophosphorylated residues. Additionally, we were able to identify a known in vivo phosphorylation site and to postulate two novel target residues, one for in vivo phosphorylation and one for in vitro autophosphorylation.

EXPERIMENTAL SECTION

Material and Reagents. Synthetic peptides RKISASEF and RKIpSASEF were obtained from, respectively, Promega (Madison, WI) and Neosystem Laboratoire (Strasbourgh, France). Human [Glu[1]]-fibrinopeptide B was obtained from Sigma (St. Louis, MO). Bovine lung PKG type Iα was purified to homogeneity essentially as described by Francis et al.[28] Aqua C_{18} (5 μm, 200 Å) reversed-phase material was purchased from Phenomenex (Torrance, CA). Titanium oxide (Titansphere, 5 μm) was a gift from GL Sciences (GL Sciences Inc., Tokyo, Japan). Sequencing grade trypsin, chymotrypsin, and Glu-C were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Preparation of Frits and Columns. Porous ceramic frits were prepared in 50−100-μm-i.d. undeactivated, fused-silica capillaries as described by Meiring et al.[29] Columns were packed using a 50 bar pressure vessel as described by van der Heeft et al.[30] In short, 50-μm-i.d. analytical reversed-phase columns were prepared by back flushing a 100-μm-i.d. prepacked fused-silica capillary, filled with ∼5−6 cm of a Aqua C_{18} stationary phase, directly into a 50-μm fritted fused-silica capillary, thereby avoiding long packing time. Titanium oxide and reversed-phase precolumns were made by directly packing 100-μm-i.d. fritted fused-silica capillaries to column lengths of 10−15 mm of either Titansphere or Aqua C_{18} stationary phase.

2D-NanoLC-MS/MS. The two-dimensional nanoflow LC system consisted of a LC-Packings Ultimate quaternary solvent delivery system, a Famos autosampler, and a Switchos six-port valve switching module (LC-Packings, Amsterdam, The Netherlands) operating in a nanoflow HPLC setup as described by Meiring et al.[29] Double-stage precolumns consisted of a 100 μm i.d. × 10 mm L titanium oxide precolumn butt-connected to a 100 μm i.d. × 10 mm L Aqua C_{18} precolumn by means of a True ZDV MicroTight union (Upchurch Scientific, Oak Harbor, WA). A schematic representation of the nano-HPLC-MS setup is given in Figure 1. Two-dimensional separation of phosphopeptides from non-phosphopeptides is achieved by loading the analytes onto the double precolumn in 100% solvent A (i.e., water and 0.1 M acetic acid) using an unsplit flow of 3 μL/min for 20 min (resulting precolumn pressure ∼80−100 bar). Phosphopeptides are retained on the titanium oxide, while nonphosphorylated peptides flow through the titanium oxide precolumn and are concentrated on the C_{18} precolumn. After sample loading, the six-port valve is switched and the flow is increased to 400 μL/min. The restrictor (50 μm i.d. × 30 cm L fused silica) provides a column back pressure of ∼130−150 bar, and a resulting column flow of ∼100−150 nL/min is obtained. A linear gradient to 100% solvent B (i.e.,

(10) Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. *Nat. Biotechnol.* **2002**, *20*, 301−305.
(11) Cao, P.; Stults, J. T. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1600−1606.
(12) Cao, P.; Stults, J. T. *J. Chromatogr., A* **1999**, *853*, 225−235.
(13) Nuwaysir, L. M.; Stults, J. T. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 662−669.
(14) Haydon, C. E.; Eyers, P. A.; Aveline-Wolf, L. D.; Resing, K. A.; Maller, J. L.; Ahn, N. G. *Mol. Cell. Proteomics* **2003**, *2*, 1055−1067.
(15) Nuhse, T. S.; Stensballe, A.; Jensen, O. N.; Peck, S. C. *Mol. Cell. Proteomics* **2003**, *2*, 1234−1243.
(16) Kawahara, M.; Nakamura, H.; Nakajima, T. *J. Chromatogr., A* **1990**, *515*, 149−158.
(17) Tani, K.; Suzuki, Y. *Chromatographia* **1994**, *38*, 291−294.
(18) Tani, K.; Suzuki, Y. *Chromatographia* **1997**, *46*, 623−627.
(19) Tani, K.; Sumizawa, T.; Watanabe, M.; Tachibana, M.; Koizumi, H.; Kiba, T. *Chromatographia* **2002**, *55*, 33−37.
(20) Jiang, Z. T.; Zuo, Y. M. *Anal. Chem.* **2001**, *73*, 686−688.
(21) Matsuda, H.; Nakamura, H.; Nakajima, T. *Anal. Sci.* **1990**, *6*, 911−912.
(22) Ikeguchi, Y.; Nakamura, H. *Anal. Sci.* **2000**, *16*, 541−543.
(23) Ikeguchi, Y.; Nakamura, H. *Anal. Sci.* **1997**, *13*, 479−483.
(24) Kawahara, M.; Nakamura, H.; Nakajima, T. *Anal. Sci.* **1989**, *5*, 763−764.
(25) Hofmann, F.; Dostmann, W.; Keilbach, A.; Landgraf, W.; Ruth, P. *Biochim. Biophys. Acta* **1992**, *1135*, 51−60.
(26) Hofmann, F.; Flockerzi, V. *Eur. J. Biochem.* **1983**, *130*, 599−603.
(27) Aitken, A.; Hemmings, B. A.; Hofmann, F. *Biochim. Biophys. Acta* **1984**, *790*, 219−225.
(28) Francis, S. H.; Wolfe, L.; Corbin, J. D. *Methods Enzymol.* **1991**, *200*, 332−341.
(29) Meiring, H. D.; van der Heeft, E.; ten Hove, G. J.; de Jong, A. P. J. M. *J. Sep. Sci.* **2002**, *25*, 557−568.
(30) van der Heeft, E.; ten Hove, G. J.; Herberts, C. A.; Meiring, H. D.; van Els, C. A.; de Jong, A. P. *Anal. Chem.* **1998**, *70*, 3742−3751.

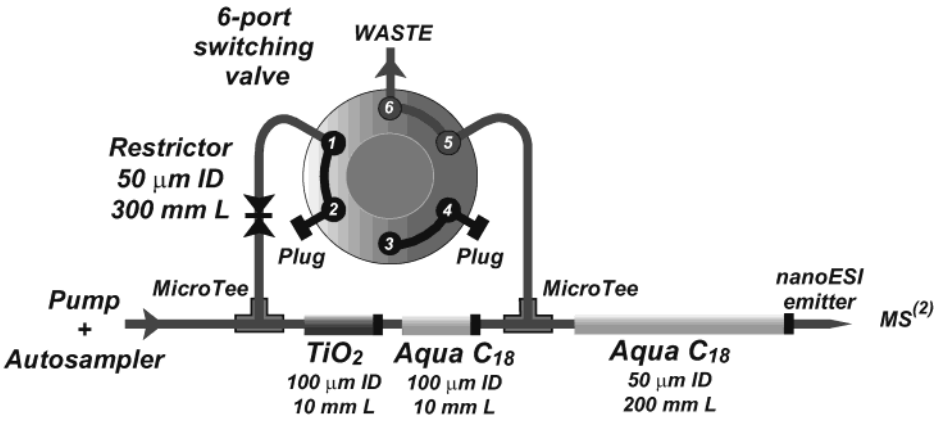

Figure 1. Schematic representation of the two-dimensional LC-MS setup. This setup comprises a six-port switching valve, a dual TiO_2/C_{18} precolumn, and a C_{18} analytical column. During sample loading from the injection loop of the autosampler the flow is 3 μL/min and the restrictor is closed. In this situation, the content of the sample loop is transported directly over the double precolumn, where phosphopeptides are trapped on the TiO_2 particles and non-phosphopeptides are trapped on the C_{18} particles. After sample loading, the six-port valve switches the restrictor to the waste line and simultaneously the flow is increased to 400 μL/min. This results in a pressure increase to ~150 bar and a column flow over the analytical column of ~100 nL/min is obtained. The gradient pump delivers a linear H_2O/acetonitrile gradient, which analytically separates the content of the C_{18} precolumn. After this first analysis, the content of the TiO_2 precolumn is loaded onto the C_{18} precolumn using a strong base. A second H_2O/acetonitrile gradient is used to analytically separate the trapped phosphopeptides.

80% acetonitrile and 0.1 M acetic acid) is used to analytically separate the content of the C_{18} precolumn. After this first reversed-phase gradient, trapped phosphopeptides are eluted and reconcentrated on the C_{18} precolumn by injecting 30 μL of ammonium bicarbonate, pH 9.0. A second H_2O/acetonitrile gradient was used to analytically separate the content of the C_{18} precolumn. The flow of the analytical column was directly infused into a Micromass Q-TOF 1 mass spectrometer (Micromass UK Ltd., Manchester, U.K.) using homemade nano-ESI spray tips (~7-μm i.d.), prepared as described by Meiring et al.[29] Proteolytic digests of PKG were analyzed in both continuous mode and data-dependent mode. Scans were acquired in positive ion continuous mode from m/z 300 to 1200 amu s^{-1}. During data-dependent analysis, three precursors/scan were allowed at a threshold of 50 counts. TOF-MS/MS spectra were acquired for 2 s, using an interscan time of 0.1 s. Data were analyzed using the MassLynx 3.5 software (Micromass) and using the MASCOT software (www.matrixscience.com). MASCOT searches were performed in the SWISS-PROT database, and a mammalian taxonomy restriction was used. The mass tolerance of both precursor ion and fragment ions was set to ±0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, while serine, threonine or tyrosine phosphorylation was set as variable modification. All phosphopeptides identified during MASCOT searches were confirmed by manual interpretation of the spectra.

Sample Preparation. Autophosphorylated PKG Iα was prepared by incubating PKG with 5 mM $MgCl_2$, 10 mM cAMP and 100 μM ATP. For partial and high levels of phosphate incorporation, the protein was incubated at 30 °C for 10 min and 3 h, respectively. Nonautophosphorylated enzyme was not incubated with $MgCl_2$ and ATP. The autophosphorylation reactions were stopped with the addition of a molar excess of EDTA. After treatment with dithiothreitol and iodoacetamide, protein samples were buffer exchanged to 25 mM ammonium hydrogen carbonate, pH 8.0, using Ultrafree-0.5 Centrifugal Filter Units (5000 NMWL) (Millipore, Bedford, MA). Protein samples were digested with trypsin, chymotrypsin, or GluC at a concentration of 1 pmol/μL in 25 mM ammonium hydrogen carbonate and 10% acetonitrile at 37 °C for 16 h. The protein/trypsin ratio used was 1:50 by weight, while protein/chymotrypsin and protein/GluC ratios were 1:20 by weight. Prior to injection, the sample was diluted 40 times in MilliQ water containing 0.6% acetic acid.

RESULTS AND DISCUSSION

2D-LC-MS Setup. To explore the general applicability of Titansphere for phosphopeptide trapping, a nanoflow-LC setup (See Figure 1), comprising a six-port switching valve, a 100-μm-i.d. precolumn, and a 50-μm-i.d. analytical column was employed. A test sample, consisting of 125 fmol of a stoichiometric mixture of the monophosphorylated peptide, RKIpSASEF, and its non-phosphorylated counterpart, RKISASEF, was used. A normal reversed-phase analysis was obtained by loading the two peptides onto the C_{18} precolumn in 100% solvent A. A linear H_2O/acetonitrile gradient was used to analytically separate the content of the C_{18} precolumn at a flow rate of 100−150 nL/min. In Figure 2A are shown from top to bottom the BPI chromatogram and the SIC of, respectively, the $[M + 2H]^{2+}$ of RKISASEF (at m/z 469.2) and the $[M + 2H]^{2+}$ of RKIpSASEF (at m/z 509.2). At ~28 min, both RKISASEF and RKIpSASEF eluted with similar mass spectrometric responses. To demonstrate the capability of TiO_2 to selectively retain phosphorylated peptides, the same peptide mixture was loaded onto a double precolumn consisting of a TiO_2 precolumn placed in front of the C_{18} precolumn. After sample loading, a linear H_2O/acetonitrile gradient was started. Figure 2B shows the acquired BPI and selected ion chromatograms for both peptides of this first reversed-phase run. The peak that elutes at ~29 min is the nonphosphorylated peptide, indicated by the selected ion chromatogram of the nonphosphorylated peptide (SIC of m/z 469.2). A small amount of the doubly protonated species of the phosphorylated peptide was also observed; however, compared to Figure 2A, the intensity is ~100 times lower. This initial result directly indicates that TiO_2 is capable of trapping specifically the phosphorylated peptide under acidic conditions. The basis for this affinity must be ascribed to the phosphate

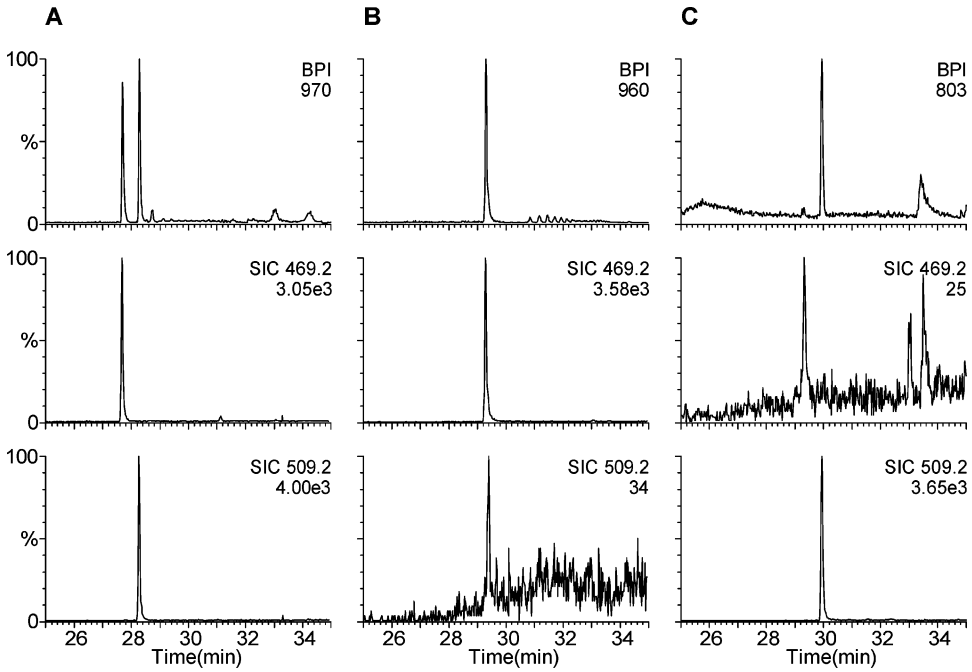

Figure 2. Selective isolation of 125 fmol of the synthetic phosphopeptide RKIpSASEF from its nonphosphorylated form. Panel A shows the following, from top to bottom; BPI chromatogram, the selected ion chromatogram for the doubly charged molecular ions of RKISASEF and of RKIpSASEF, respectively, of a normal reversed-phase analysis (i.e., without using a TiO_2 precolumn). Panel B shows the following, from top to bottom; BPI chromatogram, the selected ion chromatograms for the doubly charged molecular ions of RKISASEF and of RKIpSASEF, respectively, from the analysis of the first gradient, which contains the breakthrough of the TiO_2 precolumn. Panel C shows the following, from top to bottom; BPI chromatogram, the selected ion chromatograms for the doubly charged molecular ions of RKISASEF and of RKIpSASEF, respectively, from the analysis of the second gradient, which contains the eluate of the TiO_2 precolumn. Values given for the base peak intensity and for the selected ion counts, listed in the upper right corner of each chromatogram, are in arbitrary units.

moiety, since the nonphosphorylated peptide was not retained. To elute the phosphorylated peptide from the TiO_2 column, 30 μL of 250 mM ammonium bicarbonate, pH 9.0, was directed over the double TiO_2/C_{18} precolumn at a flow rate of 3 μL/min. After 20 min, a second linear H_2O/acetonitrile gradient was used to analytically separate the content of the C_{18} precolumn. Figure 2C shows the acquired BPI and selected ion chromatograms for both peptides of this second reversed-phase run. Figure 2C clearly demonstrates that under these conditions the phosphorylated peptide was recovered from the TiO_2 precolumn. Furthermore, it illustrates that the phosphopeptide was retained on the TiO_2 precolumn even under hydrophobic conditions, since the first H_2O/acetonitrile gradient was directed over the TiO_2 column.

PKG Autophosphorylation. To further explore the general applicability of TiO_2 for the selective enrichment of phosphopeptides from more complex mixtures, the homodimeric 153-kDa PKG was analyzed. PKG is a serine/threonine-specific kinase that is allosterically regulated by the second messenger molecule cGMP. Upon activation, PKG undergoes autophosphorylation and this process affects the kinetic properties of PKG.[31] Proteolytic digests of differentially autophosphorylated PKG (see Experimental Section) were subjected to analysis using the developed 2D-LC setup in order to determine residues involved in the autophosphorylation reaction and to determine the basal phosphorylation state of purified PKG. For this purpose, proteolytic digests of differentially autophosphorylated PKG were analyzed in two separate experiments. In the first analysis, mass spectrometric data were acquired in the data-dependent acquisition mode in order to obtain primary sequence information of the proteolytic fragments. A second analysis was repeated, and this time, only LC-ESI-MS spectra were acquired. From this latter analysis, extracted ion chromatograms were generated for each phosphopeptide that was identified from the MS/MS spectra obtained in the first data-dependent analysis. This allowed semiquantitative comparison of each individual phosphopeptide between the nonphosphorylated and partially and highly autophosphorylated states of PKG. Figure 3A shows the BPI chromatogram of the first analytical gradient of ~250 fmol of the tryptic digest of nonautophosphorylated PKG. Approximately 80 peptides were recovered, among which no phosphopeptides were identified. Panels B-D of Figure 3 show the BPI chromatograms obtained after elution of the TiO_2 precolumn at high pH for nonphosphorylated and partially and highly autophosphorylated PKG, respectively. All phosphopeptides that were identified in these analyses are listed in Table 1 and labeled in Figure 3. The tryptic digest of nonautophosphorylated PKG contained three phosphopeptides, which were identified as residues 24−37, 514−532, and 513−532, carrying a single phosphate moiety. The deconvoluted low-energy CID spectrum of residues 24−37 + 1P is given in Figure 4A. The phosphorylation site was unambiguously assigned to Ser-26, which is a previously uncharacterized phosphorylation site of PKG. In a similar fashion, phosphopeptides 514−532 and 513−532 were found to be both phosphorylated on Thr-516. Nonphosphorylated PKG is not stimulated to autophosphorylate in vitro, and all phosphorylations in this state of the protein are likely due to in vivo autophosphorylation or due to in vivo activity of other protein

(31) Chu, D. M.; Francis, S. H.; Thomas, J. W.; Maksymovitch, E. A.; Fosler, M.; Corbin, J. D. *J. Biol. Chem.* **1998**, *273*, 14649−14656.

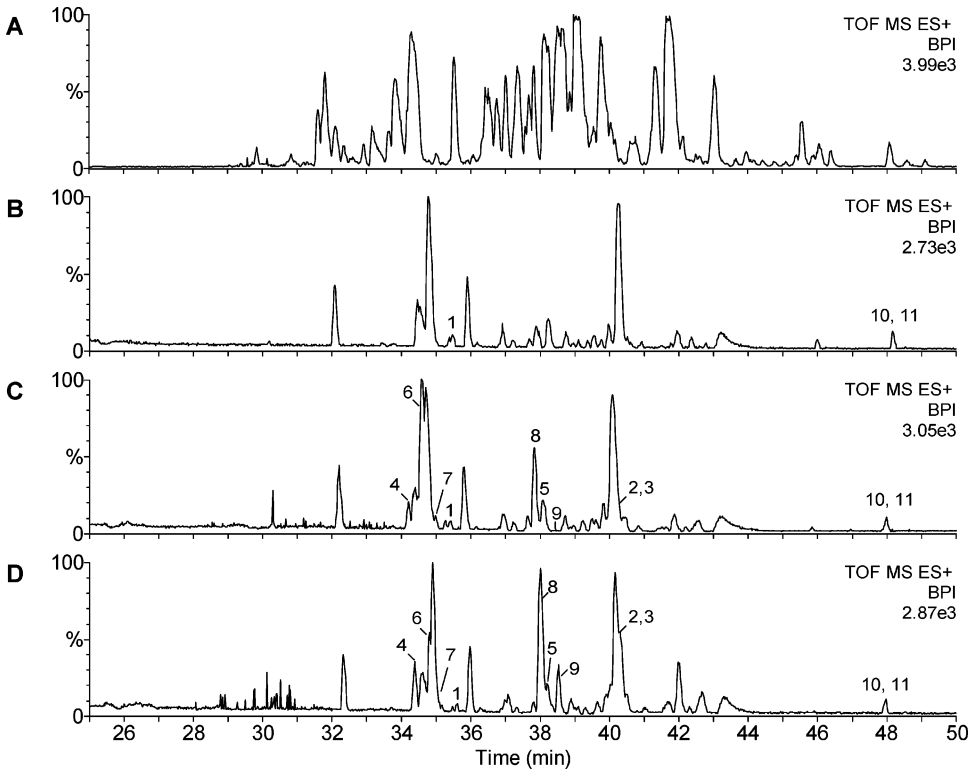

Figure 3. Nano-2D-LC-MS analysis of 250 fmol of tryptic PKG, phosphorylated to different degrees. Shown are the BPI elution profiles of (A) the first gradient of native (nonautophosphorylated) PKG and the second gradients of (B) native PKG, (C) partially autophosphorylated PKG, and (D) highly autophosphorylated PKG. The phosphopeptides, which were identified from low-energy CID spectra in a separate 2D-LC-MS/MS analysis, are labeled and listed in Table 1. Values given for the base peak intensity, listed in the upper right corner of each chromatogram, are in arbitrary units.

Table 1. Identified Tryptic Phosphopeptides from PKG

			mass [b]		peptide observed in[c]		
no.	residues	sequence [a]	$M_{r\ found}$	$M_{r\ calc}$	non	partial	high
1	24−37	(K)-RL**pS**EKEEEIQELKR-(K)	1865.91	1865.92	+	+	+
2	42−56	(K)-C*QSVLPVP**pS**THIGPR-(T)	1726.87	1726.82	−	++	++
3	42−56	(K)-C*Q**pS**VLPVP**pS**THIGPR-(T)	1806.86	1806.78	−	+	++
4	72−77	(R)-**pS**FHDLR-(Q)	853.33	853.35	−	+	++
5	60−77	(R)-AQGISASEPQTYR**pS**FHDLR-(Q)	2155.08	2154.99	−	+	−
6	57−71	(R)-T**pT**RAQGISAEPQTYR-(S)	1757.84	1757.81	−	++	+
7	57−71	(R)-T**pT**RAQGI**pS**AEPQTYR-(S)	1837.82	1837.78	−	+	+
8	57−77	(R)-T**pT**RAQGISAEPQTYR**pS**FHDLR-(Q)	2593.18	2593.15	−	+	++
9	57−77	(R)-T**pT**RAQGI**pS**AEPQTYR**pS**FHDLR-(Q)	2673.21	2673.11	−	+	++
10	514−532	(K)-TW**pT**FC*GTPEYVAPEIILNK-(G)	2318.21	2318.07	+	+	+
11	513−532	(K)-KTW**pT**FC*GTPEYVAPEIILNK-(G)	2446.31	2446.16	+	+	+

[a] Amino acid sequence of phosphorylated peptides identified from tryptic digests on the basis of their low-energy CID spectrum. C* denotes carbamidomethyl cysteine, pS denotes phosphoserine, and pT denotes phosphothreonine. [b] All mass values are listed as monoisotopic mass. [c] Semiquantitative information about the presence of the identified phosphopeptide derived from its elution profiles (extracted ion chromatogram): (−) not present; (+) low abundant; (++) highly abundant.

kinases. Purified fractions of PKG are known to contain 1.1−1.4 mol of phosphate/mol of subunit.[27] The peptide containing pThr-516 originates from the activation loop within the catalytic core of the protein kinase. Previously, mutation studies showed that this phosphothreonine is essential for protein kinase activity.[32] However, this is the first direct evidence that this residue is phosphorylated in PKG isolated from bovine lung tissue. Additionally, the nonphosphorylated form of this peptide was not observed, and therefore, it is postulated that this site is almost completely phosphorylated in PKG isolated from bovine lung tissue. The tryptic digest of partially autophosphorylated PKG contained besides the three phosphopeptides, which were also identified in the nonautophosphorylated PKG sample, a series of phosphopeptides that originate from the N-terminal part of the protein. PKG is known to autophosphorylate within minutes on residues Ser-50, Thr-58, Ser-72, and Thr-84.[26,27] The most abundant peak in the second gradient of partial autophosphorylated PKG (peak 6 in Figure 3C) originates from the monophosphorylated peptide 57−71. The low-energy CID spectrum of this phosphopeptide con-

(32) Feil, R.; Kellermann, J.; Hofmann, F. *Biochemistry* **1995**, *34*, 13152−13158.

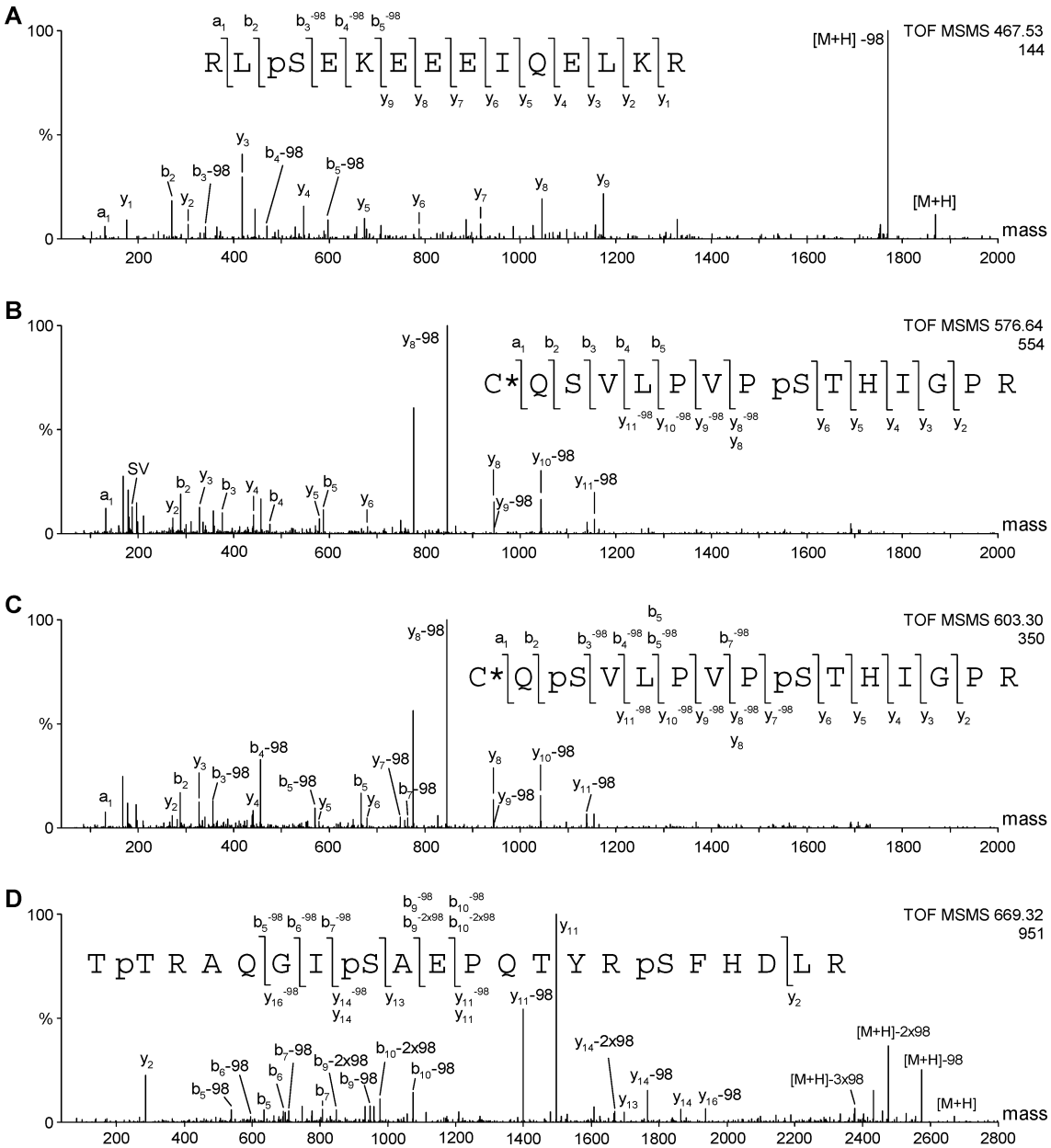

Figure 4. Singly charged or deconvoluted (MaxEnt3) low-energy CID spectra of (A) the $[M + 4H]^{4+}$ of residues 24−37 carrying one phosphate situated at Ser-26, (B) the $[M + 3H]^{3+}$ of residues 42−56 carrying one phosphate at Ser-50, (C) the $[M + 3H]^{3+}$ of residues 42−56 carrying two phosphates at Ser-44 and Ser-50, and (D) the $[M + 4H]^{4+}$ of residues 57−77 carrying three phosphate moieties at Thr-58, Ser-64, and Ser-72. The a, b, and y ions are labeled in each spectrum, as well as b or y ions corresponding to the neutral loss of phosphoric acid (−98). Additionally, an internal acyl ion (annotated by SV) is highlighted in (B). This internal acyl ion is absent in the low-energy CID spectrum of the doubly phosphorylated peptide of residues 42−56 (C), suggesting it is this serine that is phosphorylated in this doubly phosphorylated peptide.

firmed that Thr-58 is the phosphorylated residue. It appears that phosphorylation of Thr-58 is nearly complete after 10 min of incubation, since only low-abundant nonphosphorylated peptides containing Thr-58 were detected in the first gradient (data not shown). Apparently, partial phosphorylation of Ser-64 also occurred after 10 min, as indicated by the presence of the doubly phosphorylated peptide 57−71, of which the MS/MS spectra confirmed phosphorylation of both Thr-58 and Ser-64. Simultaneously, partial phosphorylation of Ser-72 occurred as indicated by the presence of phosphopeptides 72−77 and 60−77 and the doubly phosphorylated peptide 57−77. The doubly phosphorylated peptide 57−77 is among the most abundant peaks in the chromatogram of highly autophosphorylated PKG. The triply phosphorylated peptide of residues 57−77 is also observed, and its deconvoluted low-energy CID spectrum confirming phosphorylation of Thr-58, Ser-64, and Ser-72 is given in Figure 4D. Phosphorylation of Ser-50 also occurred within the first 10 min of autophosphorylation. The deconvoluted low-energy CID spectrum of the monophosphorylated peptide 42−56 is given in Figure 4B. Interestingly, a doubly phosphorylated peptide of residues 42−56 was also identified in both partially and highly autophosphorylated PKG. The deconvoluted low-energy CID spectrum of this peptide (Figure 4C) confirmed phosphorylation of both Ser-50 and Ser-44. This latter serine is a previously uncharacterized phosphorylation site of PKG. Chymotryptic and GluC digests were prepared and analyzed in the same way as the tryptic digests. All

Table 2. Identified GluC and Chymotryptic Phosphopeptides from PKG

no.	residues	sequence[a]	mass[b] $M_{r\ found}$	mass[b] $M_{r\ calc}$	peptide observed in[c] non	partial	high
12	71−80	(Y)-R**pS**FHDLRQAF-(R)	1355.64	1355.61	−	+	++
13	69−76	(Q)-TYR**pS**FHDL-(R)	1117.53	1117.45	−	+	++
14	53−70	(H)-IGPRT**pT**RAQGISAEPQTY-(R)	2025.09	2024.97	−	+	+
15	81−92	(F)-RKF**pT**KSERSKDL-(I)	1573.88	1573.79	−	−	+
16	516−530	(W)-**pT**FC*GTPEYVAPEIIL-(N)	1788.88	1788.80	+	+	+
17	76−87	(D)-LRQAFRKF**pT**KSE-(R)	1589.94	1589.81	−	−	+
18	502−522	(D)-FGFAKKIGFGKKTW**pT**FC*GTPE-(Y)	2486.30	2486.19	+	+	+

[a] Amino acid sequence of phosphorylated peptides identified from GluC or chymotryptic digests on basis of their low-energy CID spectrum. C* denotes carbamidomethyl cysteine, pS denotes phosphoserine, and pT denotes phosphothreonine. [b] All mass values are listed as monoisotopic mass. [c] Semiquantitative information about the presence of the identified phosphopeptide derived from its elution profiles (extracted ion chromatogram): (−) not present; (+) low abundant; (++) highly abundant.

phosphopeptides that were identified in these analyses are listed in Table 2. The overall amount of phosphopeptides identified from these two digests is lower compared to the tryptic digests. However, it turned out that both proteases are complementary to trypsin. Both chymotrypsin and GluC digests of highly autophosphorylated PKG contained peptides phosphorylated on Thr-84. This phosphorylation site could not be identified from the tryptic digests. Thr-84 is located in a region with a relative high amount of arginine and lysine residues. For now it is assumed that proteolytic digestion with trypsin generates too small fragments, containing threonine-84 (phosphorylated or nonphosphorylated) to be detected by the current method.

Semiquantitation of PKG Phosphorylation. After several phosphopeptides were identified from the proteolytic digests, selected ion chromatograms were generated for the parent mass of each phosphopeptide from the ion chromatograms of the separate LC−MS runs. From these selected ion chromatograms (see Figure 5) it is possible to obtain a semiquantitative indication about the kinetics of each phosphorylation site along the autophosphorylation reaction. For instance, by comparing extracted ion chromatograms of the $[M + 4H]^{4+}$ at m/z 467.53 corresponding to residues 24−37 + 1P (Figure 5A), it appears that the amount of this phosphopeptide is not influenced by the autophosphorylation process. Furthermore, by comparing the mass spectrometric response of this phosphopeptide with the nonphosphorylated form, it appears that the phosphorylation level of Ser-26 is rather low (data not shown). This could indicate that Ser-26 is not an in vitro autophosphorylation site of PKG and its phosphorylation is probably due to the action of an other protein kinase in vivo. The intensities of the extracted mass chromatograms for residues 42−56 with one and two phosphate moieties are given in Figure 5B and C, respectively. From these extracted mass chromatograms it seems that both these sites are in fact autophosphorylation sites of PKG in vitro, since both peaks show an increase along the incubation time of the autophosphorylation reaction. The extracted mass chromatograms of the triply phosphorylated peptide of residues 57−77 (Figure 5D) show that after 10 min of autophosphorylation a small amount of PKG is already phosphorylated on Ser-64, while this increases further with longer incubation. Autophosphorylation of Ser-64 was previously associated with activation of type Iα PKG in the absence of cyclic nucleotides.[33] Our results suggest that after 10 min a substantial amount of PKG is phosphorylated on Ser-64. In summary, using this strategy, we confirmed complete phosphorylation of Thr-516 and minor phosphorylation of Ser-26 and both are most likely phosphorylated in vivo. In vitro stimulated autophosphorylation results in rapid phosphorylation of Ser-50, Thr-58 and Ser-72 and slower phosphorylation of Ser-44, Ser-64 and Thr-84.

Nonspecific Absorption. The LC−MS/MS data of the second gradients of nonphosphorylated and partially and highly autophosphorylated PKG contained besides the previously mentioned phosphopeptides several nonphosphorylated peptides that had generally in common that they were relatively acidic (i.e., the peptides contained several aspartic and glutamic acids) (sequence information not shown). Apparently they also have some affinity for the alkaline surface of TiO_2, although it should be noted that most of these peptides were also present (often in much higher abundance) in the first linear gradient, suggesting they are not that well retained on the TiO_2. Although phosphopeptides are easily discriminated from nonphosphorylated peptides on the basis of the parent mass and fragment ion spectra, the occurrence of these nonphosphorylated peptides in the second gradient could be considered as a negative side effect. Nonspecific absorption of nonphosphorylated peptides with an acidic nature is something that is also commonly observed in IMAC experiments.[10,15] Ficarro et al.[10] showed that the conversion of carboxylic acids into their methyl ester derivatives decreased the nonspecific binding of acidic peptides in IMAC. To determine whether esterification of carboxyl groups could also reduce the absorption of acidic peptides on TiO_2, the relatively acidic peptide [Glu[1]]-fibrinopeptide B (EGVNDNEEGFFSAR) was used. This peptide has been shown to have a relatively high affinity for TiO_2. Methylated [Glu[1]]-fibrinopeptide was obtained with acetyl chloride and methanol, essentially as described by Ficarro et al.[10] A mixture of 100 fmol of [Glu[1]]-fibrinopeptide and methylated-[Glu[1]]-fibrinopeptide was loaded onto the double TiO_2/C_{18} precolumn. Figure 6 shows the BPI chromatogram and selected ion chromatograms for both peptides of the first (A) and second gradient (B) of this analysis. Figure 6A shows that methylated [Glu[1]]-fibrinopeptide has no affinity for the titanium oxide, while Figure 6B shows that [Glu[1]]-Fibrinopeptide has an affinity for titanium oxide and could be recovered in the same way the phosphopeptides were recovered (i.e., desorbtion from TiO_2 under alkaline conditions). This

(33) Busch, J. L.; Bessay, E. P.; Francis, S. H.; Corbin, J. D. *J. Biol. Chem.* **2002**, *277*, 34048−34054.

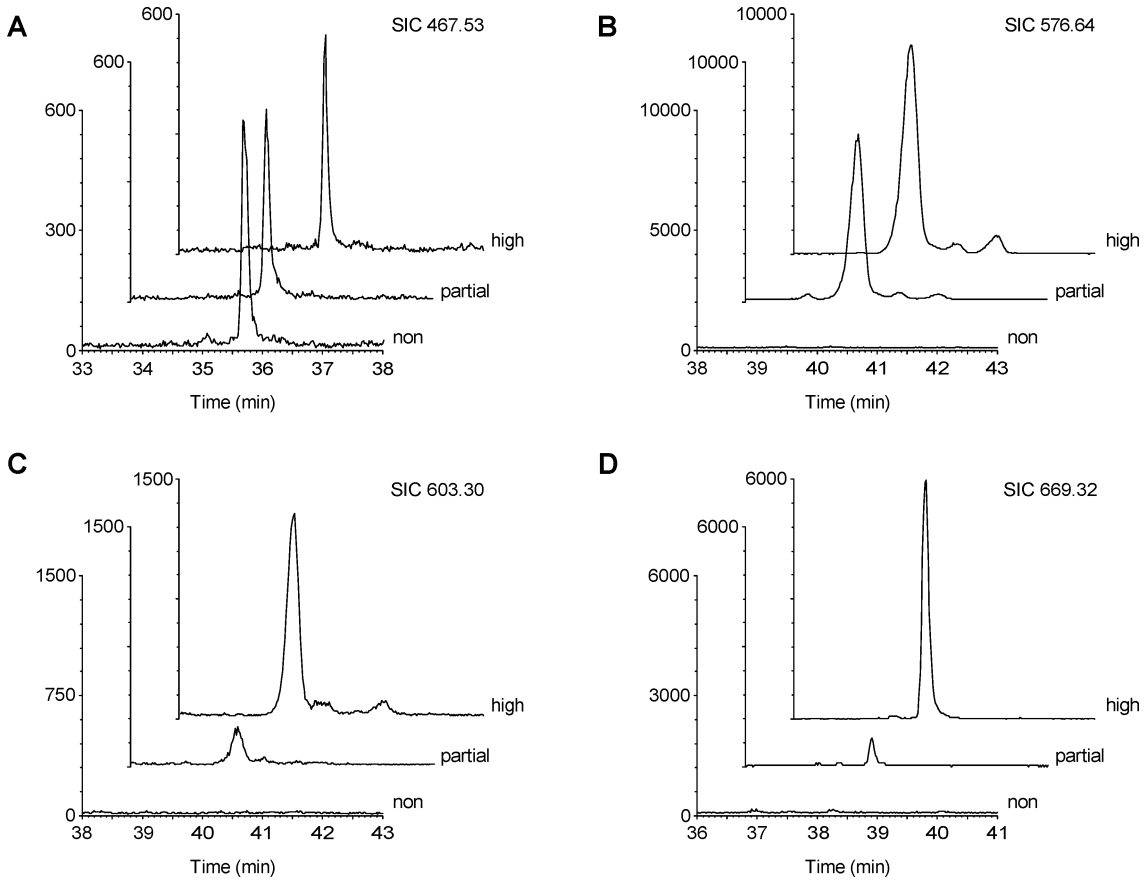

Figure 5. Selected ion chromatograms generated for the parent mass of several identified phosphopeptides from nonphosphorylated and partially and highly autophosphorylated PKG. Panel A shows that the amount of the phosphopeptide of residues 24−37 does not change during autophosphorylation. Panels B and C show that the mono- and diphosphorylated peptides of residues 42−56 increase upon autophosphorylation. Panel D shows that the triphosphorylated peptide of residues 57−77 increases during autophosphorylation. Values given for the selected ion counts are in arbitrary units.

illustrates that the absorption of acidic nonphosphorylated peptides by TiO_2 can be decreased by esterification of the carboxylic acids.

CONCLUSIONS

Due to the biological importance of reversible protein phosphorylation, a continuing development of techniques for the identification and localization of phosphorylation sites in proteins is highly desirable. In particular, the availability of robust enrichment techniques seems to be especially promising for attempting global analysis of protein phosphorylation events in the future.[10,34,35] Here we report a novel analytical procedure for phosphopeptide enrichment prior to mass spectrometric analysis. The procedure relies on the unique ion-exchange properties of Titansphere, a new type of column material that consists of spherical particles of titanium oxide. Titania has attracted interest as an alternative support to silica for column packing material in high-performance liquid chromatography because it exhibits high mechanical, chemical, and thermal stability. Additionally, Titansphere possesses unique surface chemistry, which displays amphoteric ion-exchange properties.[16,20] Previously it was shown that titanium oxide can effectively retain organic phosphates.[21−23] In this work, the objective was to use TiO_2 as chromatographic medium for the selective isolation and enrichment of phosphorylated peptides from complex mixtures in a fast, robust, and automated manner. The developed nanoflow 2D-LC-MS/MS setup allows mass spectrometric characterization of both nonphosphorylated and phosphorylated peptides in two separate measurements. The prepared TiO_2 precolumns could be used for over 200 runs without showing signs of reduced performance (data not shown). Configuration of an autosampler that injects both the sample and the buffer for the elution of the TiO_2 precolumn allows simple automation and abolishes the need for an additional third pump. In comparison with IMAC, this chromatographic approach has the advantage that lesser column handling steps are required, and therefore, it seems to be a more robust enrichment strategy for the selective enrichment and characterization of phosphopeptides from complex mixtures. Using the developed setup, the phosphorylated peptide RKIpSASEF could be easily separated from its nonphosphorylated counterpart at the low-femtomole level with a yield above 90%. In addition, the analysis of highly autophosphorylated PKG showed that even di- and triphosphorylated peptides could be retained and recovered. Comparison of the elution profiles of identified phosphopeptides between the three different autophosphorylated PKG samples allowed the discrimination between in vitro autophosphorylation and in vivo phosphorylation events. In the case of the previously uncharacterized phosphorylation of Ser-26, elution profiles revealed that this phosphopeptide does not increase in abundance during

(34) Resing, K. A.; Ahn, N. G. *Methods Enzymol.* **1997**, *283*, 29−44.
(35) McLachlin, D. T.; Chait, B. T. *Curr. Opin. Chem. Biol.* **2001**, *5*, 591−602.

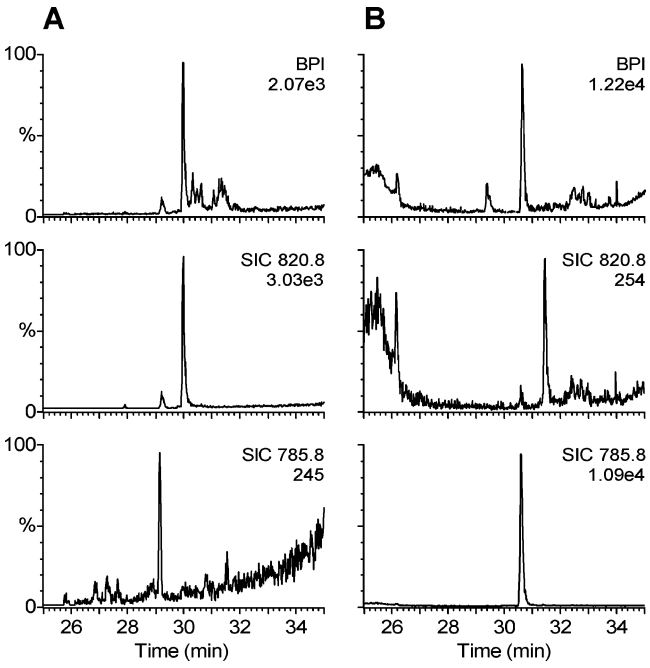

Figure 6. Selective isolation of 100 fmol of [Glu[1]]-fibrinopeptide from fully methylated [Glu[1]]-fibrinopeptide. Panel A shows the following, from top to bottom; BPI chromatogram, the selected ion chromatograms for the doubly charged molecular ion of methylated-[Glu[1]]-fibrinopeptide (at *m*/*z* 820.8) and of [Glu[1]]-fibrinopeptide (at *m*/*z* 785.8), respectively, from the analysis of the first gradient. Panel B shows the following, from top to bottom; BPI chromatogram, the selected ion chromatograms for the doubly charged molecular ion of methylated-[Glu[1]]-fibrinopeptide (at *m*/*z* 820.8) and of [Glu[1]]-fibrinopeptide (at *m*/*z* 785.8), respectively, from the analysis of the second gradient, which contains the eluate of the TiO_2 precolumn. Values given for the BPI and the selected ion counts, listed in the upper right corner of each chromatogram, are in arbitrary units.

incubation with cAMP and Mg^{2+}/ATP, indicating it is not an autophosphorylation site in vitro. In fact, purified PKG from bovine lung already contained this phosphorylated residue. These observations strongly support the idea that this residue is in fact phosphorylated in PKG isolated from bovine tissue, and its phosphorylation is most likely due to the action of another protein kinase. In contrast, the level of phosphorylation of another previously uncharacterized phosphorylation site, Ser-44, increased during autophosphorylation. This strongly suggests that this residue is in fact a newly characterized autophosphorylation site of PKG.

The nature of the tight interaction between phosphoamino acids and the Titansphere is not fully understood. Relative acidic peptides appear to have some affinity for Titansphere as well, although it is less pronounced than the affinity of phosphopeptides. This is a negative side effect and could lead to false positives in the search for phosphorylation sites of unknown samples. Ficarro et al.[10] have overcome this problem in IMAC by treatment of all peptides with a methyl esterification reaction that actually reduced nonspecific binding of acidic peptides. Using the relatively acidic [Glu[1]]-fibrinopeptide B, which showed an affinity for TiO_2, and the esterification protocol described by Ficarro et al.[10] we show that the absorption of acidic peptides to TiO_2 can be minimized. These results clearly show that TiO_2 has a high potential in the field of phosphoproteomics.

Abbreviations: 2D-LC, two-dimensional liquid chromatography; amu, atomic mass unit; ATP, adenosine 5′-triphosphate; BPI, base peak intensity; cAMP, adenosine 3′,5′-cyclic monophosphate; CID, collision-induced dissociation; cGMP, guanosine 3′,5′-cyclic monophosphate; ESI, electrospray ionization; IMAC, immobilized metal affinity chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PKG, cGMP-dependent protein kinase; SIC, selected ion chromatogram.

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