Journal of Chromatography B, 878 (2010) 515–524

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A new acid mix enhances phosphopeptide enrichment on titanium- and zirconium dioxide for mapping of phosphorylation sites on protein complexes

Michael Mazanek[a], Elisabeth Roitinger[b], Otto Hudecz[b], James R.A. Hutchins[b], Björn Hegemann[b], Goran Mitulović[b], Thomas Taus[b], Christoph Stingl[a], Jan-Michael Peters[b], Karl Mechtler[a,b,*]

[a] Institute of Molecular Biotechnology (IMBA), Dr. Bohr-Gasse 3-5, A-1030 Vienna, Austria
[b] Research Institute of Molecular Pathology (IMP),[1] Dr. Bohr-Gasse 7, A-1030 Vienna, Austria

ARTICLE INFO

Article history:
Received 4 September 2009
Accepted 10 December 2009
Available online 22 December 2009

Keywords:
Phosphopeptide enrichment
Titanium dioxide
Zirconium dioxide
Spectrum database
APC/C
Condensin-I

ABSTRACT

The selective enrichment of phosphorylated peptides prior to reversed-phase separation and mass spectrometric detection significantly improves the analytical results in terms of higher number of detected phosphorylation sites and spectra of higher quality. Metal oxide chromatography (MOC) has been recently described for selective phosphopeptide enrichment (Pinkse et al., 2004 [1]; Larsen et al., 2005 [2]; Kweon and Hakansson, 2006 [3]; Cantin et al., 2007 [4]; Collins et al., 2007 [5]). In the present work we have tested the effect of a modified loading solvent containing a novel acid mix and optimized wash conditions on the efficiency of TiO_2-based phosphopeptide enrichment in order to improve our previously published method (Mazanek et al., 2007 [6]). Applied to a test mixture of synthetic and BSA-derived peptides, the new method showed improved selectivity for phosphopeptides, whilst retaining a high recovery rate. Application of the new enrichment method to digested purified protein complexes resulted in the identification of a significantly higher number of phosphopeptides as compared to the previous method. Additionally, we have compared the performance of TiO_2 and ZrO_2 columns for the isolation and identification of phosphopeptides from purified protein complexes and found that for our test set, both media performed comparably well. In summary, our improved method is highly effective for the enrichment of phosphopeptides from purified protein complexes prior to mass spectrometry, and is suitable for large-scale phosphoproteomic projects that aim to elucidate phosphorylation-dependent cellular processes.

1. Introduction

The phosphorylation of proteins on Tyr, Thr, and Ser residues is an essential and frequently occurring mechanism for controlling biochemical processes. The high importance of protein phosphorylation makes the comprehensive identification of phosphorylation sites an important task. Mass spectrometry has been proven to be a suitable and reliable technique for the detection and measurement of peptides in proteomic samples. However, detection of phosphopeptides in complex proteomics samples is still challenging despite the recent major improvements in mass spectrometry, due to their low abundance relative to other proteins in a biological sample. The signals of phosphorylated peptides are often suppressed by signals of higher-abundant non-phosphorylated peptides. Further, the ionization efficiency for phosphorylated peptides by electrospray is lower compared to other peptides. Therefore, to detect phosphorylated peptides it is necessary to reduce the complexity of a sample prior to its introduction into the mass spectrometer for analysis. This can be achieved by performing an additional separation step on the protein level, e.g. affinity purification of a protein complex of interest. Another important technique which can boost the detection of phosphosites is the selective enrichment of phosphopeptides from peptide mixtures prior to analysis.

Several strategies to achieve this aim exist [5]; these can be grouped into chemical derivatization and affinity-based methods. The chemical methods use β-elimination and Michael addition [7,8] or phosphoramidate chemistry [9,10]. Affinity-based methods for phosphopeptide enrichment include immobilized metal affinity chromatography (IMAC) [11–17] and metal oxide chromatography (MOC) [1], both of which are based on the affinity of the negatively

Abbreviations: AA, Acetic acid; ABC, Ammonium bicarbonate; APC/C, Anaphase promoting complex/cyclosome; BPC, Base peak chromatogram; DHB, 2,5-Dihydroxybenozoic acid; DMEM, Dulbecco's Modified Eagle Medium; FA, Formic acid; FBS, Fetal bovine serum; HFBA, Heptafluorobutyric acid; MOC, Metal oxide chromatography; MSA, Multi-stage activation; OSA, Octanesulfonic acid; RP, Reversed-phase (chromatography); SCX, Strong cation exchange (chromatography); XIC, Extracted ion chromatogram.

* Corresponding author at: Research Institute of Molecular Pathology (IMP), Dr. Bohr-Gasse 7, A-1030 Vienna, Austria. Tel.: +43 1 79044 4280; fax: +43 1 7987153.
E-mail address: mechtler@imp.univie.ac.at (K. Mechtler).

[1] http://www.imp.ac.at/research/protein-chemistry.

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.12.017

charged phosphate groups for positively charged metal ions. IMAC uses Fe^{3+}, Ga^{3+} or other metal ions immobilized via a linker to a solid support, whereas MOC uses solid metal beads or beads coated with titanium dioxide [1,4], zirconium dioxide [3] or aluminium oxide [18]. In another chromatographic approach, phosphopeptides are enriched by employing a strong cation exchange (SCX) column, where phosphopeptides tend to elute in the early fractions [19]. SCX is effectively used in combination with prior separation by SDS-PAGE [19,20] or with subsequent application of IMAC [21] or MOC [22]. The phosphopeptide-enriched fractions are then subjected to RP-chromatography coupled to an appropriate mass spectrometer. One limitation of both IMAC and MOC is that non-phosphorylated peptides containing residues with acidic side chains copurify with the phosphorylated peptides. To prevent this unwanted binding all peptides can be derivatized in a methyl esterification reaction, which converts acidic side chains to methyl esters [16], although this procedure can lead to increased sample complexity due and to substantial sample loss. Therefore, methyl esterification is only applicable if sufficient sample material is available.

Protein complexes isolated from cells are typically of low concentration and give low (silver-stainable) yields. For such samples, phosphopeptide enrichment techniques which require methyl esterification are not suitable. Recently, an improved method for phosphopeptide enrichment using TiO_2 microcolumns was published [2], which does not involve any prior chemical modification step. Here it was shown that addition of 2,5-dihydroxybenzoic acid (DHB) or phthalic acid at high concentration during sample loading led to a reduction of the binding of unphosphorylated peptides to TiO_2, and thus to an improved selectivity for phosphopeptides. The major limitation of this protocol is that it is not directly applicable to LC–MS/MS analysis, because residual amounts of DHB in the sample contaminate both the LC system and the mass spectrometer. Therefore, we have searched for alternative reagents for the exclusion of non-phosphorylated peptides, and have developed and described an offline TiO_2 chromatography approach which is directly compatible with subsequent analysis by online nano-LC–MS/MS [6]. In this method we used a loading buffer consisting of a combination of high concentrations of 1-octanesulfonic acid (OSA) and low concentrations of DHB. This mixture helped to reduce the binding of non-phosphorylated peptides, thereby increasing the trapping selectivity of phosphopeptides, but at the same time did not cause any noticeable contamination of the system. Recently, the successful use of other reagents as non-phosphopeptide excluders such as lactic acid and β-hydroxypropanoic acid [23], glutamic acid [24] and glycolic acid [25] were reported.

We wished to further improve the MOC technique in order to successfully apply it to affinity-purified protein complexes and enhance our ability to identify phosphosites. In the present study we have investigated the effect of an additional ion-pairing agent, namely heptafluorobutyric acid (HFBA) on the phosphopeptide trapping selectivity, in order to improve our phosphopeptide enrichment method. Using HFBA, we have optimized our loading and wash procedure, with the aim of further reducing the retention of unphosphorylated peptides by MOC. Finally, we have compared TiO_2 chromatography with ZrO_2 chromatography with regard to the enrichment efficiency and selectivity for phosphopeptides. The effect of the different composition in the loading and washing buffer and the column material on the phosphopeptide trapping efficiency and selectivity was tested with a set of 12 synthetic peptides (10 phosphorylated and 2 unphosphorylated) mixed with a tryptic digest of BSA. The optimized enrichment protocol using either TiO_2 or ZrO_2 chromatography was then applied to the analysis of two affinity-purified protein complexes, which are known to be phosphorylated during mitosis. Both chromatographic media performed similarly well and their application to the samples resulted in the detection and identification of a considerably higher number of phosphopeptides as compared to the previous enrichment protocol, or the analysis of an unenriched sample.

Table 1
Synthetic peptides used for method development and optimization.

Name	Mr	Sequence
PP1	2093.8613	TASDTDSS**Y**#AIPTAGMSPSR
PP2	1758.7938	SVENLPEAGI**T**#HEQR
PP3	1137.5292	N**S**#VEQGRRL
PP4	1343.5871	APPDNLPSPGG**S**#R
PP5	1302.5493	LIEDNE**Y**#TAR
NP6	1263.6208	APPDNLPSPGGSR
PP7	1313.599	R**S**#DGGHTVLHR
PP8	1931.8296	ENIMR**S**#ENSESQLTSK
PP9	2229.9807	QLGEPEK**S**#QDSSPVL**S**#ELK
PP10	2309.9471	QLGEPEK**S**#QD**S**#SPVL**S**#ELK
PP11	963.3587	SV**S**#DYEGK
GluFib	1569.6696	EGVNDNEEGFFSAR

The bold letters followed by # indicate a phosphorylated amino acid.

2. Materials and methods

2.1. General reagents

All reagents were used as purchased without further purification. Acetic acid (AA) 100% Suprapur, formic acid (FA) 98–100% Suprapur, ACN HPLC grade and ammonia 25% p.A. were purchased from VWR International (Vienna, Austria). Ammonium bicarbonate Ultra (ABC) and HFBA GC grade >99.0% were purchased from Fluka (Buchs, Switzerland). 1-Octanesulfonic acid p.A. (OSA), iodoacetamide (IAA), ammonium dihydrogen phosphate 99.99%, 2,5-dihydroxybenzoic acid (DHB), and bovine serum albumin (minimum 99%) were purchased from Sigma–Aldrich (Steinheim, Germany). DTT was purchased from Roche Applied Science (Vienna, Austria). Trypsin gold was purchased from Promega (Madison, WI, USA). All phosphorylated and non-phosphorylated peptides (Table 1) were synthesized by Fmoc chemistry in-house using phosphorylated amino acids and non-phosphorylated amino acids purchased from Novabiochem (Laeufelfingen, Switzerland).

2.2. Preparation of peptides, standard proteins and biological samples

Preparation of digestion buffers and enzyme stocks was as previously described [6]. BSA was dissolved in 50 mM ABC pH 8 to give a 1 mg/ml solution. The protein was reduced at 56 °C for 30 min in 5 mM DTT, then alkylated in the dark in 25 mM IAA at room temperature for 30 min, then digested with trypsin (1:20, w/w) at 37 °C overnight. Lyophilized synthetic peptides (10 phosphorylated and 2 unphosphorylated, for sequences see Table 1) were each dissolved in water, then mixed together to give a final concentration of 1 μM each. For the experiments the mix was further diluted to a final concentration of 500 fmol of each peptide, supplemented with 500 fmol of trypsinized BSA in 40 μl of loading buffer of the final desired concentration (see later); this mixture was loaded onto the column.

The anaphase promoting complex (APC/C) was affinity purified from nocodazole-arrested HeLa cells and tryptically digested exactly as described [6]. For the purification of the Condensin-I complex a HeLa-'Kyoto' cell line expressing Kleisin-γ-GFP-FLAG [26] was cultured on monolayers in ten 25-cm × 25-cm tissue culture trays in DMEM plus 10% FBS, 0.2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G-418 (Gibco/Invitrogen). At approximately 75% confluence, cells were treated with 0.1 μg/ml nocodozole for 18 h, then harvested and pelleted. The cell pellet was resuspended in Lysis Buffer (50 mM HEPES–KOH, pH 7.5, 5 mM EDTA, 150 mM KCl, 10% (v/v) glycerol,

1% (v/v) Triton X-100, 20 mM β-glycerophosphate, 10 mM NaF, 10 mM Na-pyrophosphate, 1 μM okadaic acid, 10 μg/ml each of leupeptin, pepstatin and chymostatin, 0.1 mM PMSF, 1 mM Na_3VO_4, 1 mM DTT) to generate a 10 ml suspension. Lysis was performed by forcing the cell suspension through a 20 G needle 10 times, then the lysate was clarified by centrifugation at 48,000 × *g* for 15 min, followed by transfer of the supernatant into a fresh tube. Anti-FLAG M2 beads (100 μl bed volume, Sigma–Aldrich) were washed three times with 0.1 M glycine, pH 2.0, then three times with Lysis Buffer. The clarified cell extract was then mixed with the beads with rotation for 1 h at 4 °C, then the beads were pelleted and the supernatant removed. The beads were washed six times with Wash Buffer (identical to Lysis Buffer except that 0.5% NP40 was used instead of 1% Triton), then three times with 50 mM HEPES–KOH, pH 7.5, 5 mM EDTA, 150 mM KCl, 10% (v/v) glycerol, then three times with 150 mM KCl. Bound protein complexes were eluted from the beads by incubation with 100 μl 0.1 M glycine, pH 2.0; this was repeated once, then both eluates were neutralised by addition of Tris–HCl, pH 9.2. 10 μl of each neutralised eluate was analysed by SDS-PAGE and silver staining to assess the yield and purity of the preparation. The tryptic in-solution digest was performed as described for the APC/C.

2.3. Purification of phosphorylated peptides using TiO_2 and ZrO_2 microcolumns

Tips filled with titanium dioxide or zirconium dioxide (10 μl bed volume, sphere size 10 μm diameter) produced by Glygen (USA) (TopTips), were purchased from SunChrom (Germany).

In the optimized procedure for phosphopeptide enrichment the test sample containing 500 fmol of trypsinized BSA and of 12 synthetic peptides or digested biological samples were diluted in 40 μl loading solvent (20% acetic acid, 420 mM 1-OSA, 50 mg/ml DHB and 0.1% HFBA). Metal oxide columns were first washed with 40 μl of 80% ACN followed by an equilibration step in 40 μl of loading solvent. Each step was performed within approximately 2 min using a Pipettor (Glygen). Subsequently, the samples were loaded very slowly to the columns, applying gentle pressure on the Pipettor to produce a flow of approximately 3 μl/min. After the loading of the samples the tips were washed with 40 μl of loading solvent followed by 40 μl of 80% ACN in 0.1% TFA, each step performed within 2 min. Bound peptides were eluted within 10 min using 40 μl of 50 mM ammonium dihydrogenphosphate, pH 10.5 (adjusted with ammonia solution). Eluted peptides were acidified by addition of 10 μl of concentrated TFA. The refined method was compared to our previous enrichment method described in detail previously [6], which differs from the above described method in the following steps: (i) the loading solvent consisted of 20% acetic acid, 300 mM 1-OSA and 20 mg/ml DHB; (ii) after the loading of the samples the columns were washed with 2× 25 μl of 30% ACN; (iii) bound peptides were eluted with 40 μl (3× 13.3 μl) of elution buffer (125 mM ABC, phosphoric acid to produce 50 mM ammonium phosphate, adjusted with ammonia solution to pH 10.5).

2.4. Nano-reversed-phase HPLC

All chromatographic separations for method development, method tests and biological samples were performed on an UltiMate Plus Nano-LC system (consisting of Famos, Switchos, UltiMate pumping system and UV detector) from Dionex Benelux (Amsterdam, The Netherlands), which was coupled online to the mass spectrometer. The samples were applied to a reversed-phase trap column (PepMap C18, 300 μm ID × 5 mm length, particle size 5 μm, pore size 100 Å) using 0.1% TFA at a flow rate of 20 μl/min. After 40 min the trap column was switched online with an analytical separation column (PepMap C18, 75 μm ID × 150 mm length, particle size 3 μm, pore size 100 Å). Bound peptides were eluted at a flow rate of 275 nl/min by applying different gradients of ACN in 0.1% FA in different time frames depending on the complexity of the sample. For simple test samples a linear gradient of 100% phase A (0.1% aqueous FA, 5% ACN) to 50% phase B (0.1% aqueous FA, 80% ACN) in 30 min was used. After applying a high organic wash step (95% B), the trap column was switched back to the offline mode and equilibrated with the loading mobile phase. The mass spectrometric data were recorded only for the time when trap and separation columns were online. For the analysis of the protein complexes the following two-step linear gradient was used: firstly a gradient with a very shallow increase from 0 to 35% B in 85 min was applied, followed by a steep gradient from 35 to 60% B in 5 min. The subsequent high organic wash and equilibration steps were performed as described above.

2.5. Mass spectrometry

The peptides eluting from the nano-RP-HPLC were analyzed on an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a nano-electrospray ion source (Proxeon). The mass spectrometer was operated in data-dependent mode: 1 full scan (m/z 400–1800) was acquired in the Orbitrap (Resolution 60,000 at m/z = 400 after accumulation to a target value of 500,000) followed by MS/MS scans of the five most abundant ions in the LTQ using a multistage activation (MSA) fragmentation and detection protocol [27]. MSA was performed on the neutral loss masses −32.6, −49 and −98 Da. The chosen ions were excluded from further selection for 180 s. Additionally, singly charged parent ions were excluded from selection for MS/MS experiments and the monoisotopic precursor selection feature was enabled. The general settings of the LTQ were: ion transfer tube temperature, 200 °C; normalized collision energy, 35%; activation value q, 0.25; activation time, 30 ms; isolation width ±2 Da.

2.6. Peptide identification, validation and quantification

For peptide identification all MS/MS spectra were searched against a customised protein sequence database using both the Bioworks Browser 3.3.1 SP1 (Thermo Fisher Scientific, San Jose, CA, USA) and Mascot 2.2.0 (Matrix Science, London, UK). The database contained 184,316 entries, comprising the complete human sequences from Swiss-Prot, TrEMBL, PIR, GenBank, EMBL, DDBJ, RefSeq and Celera (hKBMS). The generation of dta-files for both Sequest and Mascot was performed using extract_MSn version 4.0 (pReferences minimum mass, 450; maximum mass, 7000; grouping tolerance, 0; intermediate scans, 0; precursor charge, AUTO; minimum peaks, 15). The following search parameters were used: carbamidomethylation on cysteine was set as a fixed modification, oxidation on methionine, phosphorylation on serine, threonine and tyrosine was set as variable modifications. Monoisotopic masses were searched within unrestricted protein masses for tryptic peptides. The peptide mass tolerance was set to ±3 ppm and the fragment mass tolerance to ±0.5 Da. The maximal number of missed cleavages was set to 2. For better visualization, improving sensitivity, and confidence enhancing of results obtained through database searching with different search engines, Scaffold 1.7 (Proteome Software Inc., Portland, OR, USA; www.proteomesoftware.com) was used. Scaffold verifies peptide identifications assigned by Sequest and Mascot using the X!Tandem database searching program [28]. Scaffold then probabilistically validates these peptide identifications using PeptideProphet [29] and derives corresponding protein probabilities using ProteinProphet [30]. The Scaffold validation was performed in 'MudPit' mode; the results from all three search engines were then com-

bined into a single output result file. As minimum acceptance thresholds for the database search results the Mascot Ions Score cut-off was defined as 15 and the significance threshold set to $p < 0.05$. For Sequest results, the cross-correlation (X_c) score was set to 2 for 2+ charged, 2.2 for 3+ charged, and 3 for 4+ charged precursors. Each phosphospectrum complying with these criteria was additionally validated by manual inspection. Factors such as the presence of neutral losses of the phosphate group from the precursor and the fragment ions, the appearance of continuous ion series, the number of unexplained high-abundance peaks and the overall presence of background noise peaks were used as criteria for manual validation. Phosphopeptide spectra were accepted as correct only if they were positively identified by one or more of the three algorithms, and passed the manual spectrum validation. If a phosphopeptide was positively identified only in one sample, its corresponding mass trace was searched for in the other three samples. If a clear mass trace was detected in the other samples, showing a similar elution profile and elution time, it was further investigated. In some cases a low quality spectrum was recorded from such a precursor that did not pass the acceptance thresholds of the different algorithms, but matched the spectrum of the positively identified phosphopeptide; in these cases the phosphopeptides were included in the results. A label-free quantification approach was used to determine the recovery rate of the different peptides from the test mixture and from the affinity-purified protein complexes, by means of peak area integration for every charge state. For the peak area integration the mass traces for each charge state of every peptide were extracted from the base peak chromatogram with ±5 ppm mass tolerance and the sum of the calculated areas of all observed charge states was used for the quantification. To obtain a sufficient number of full-scan data points for each peptide, each full MS scan was followed by only one MSA scan in the test experiments. All MS/MS spectra relevant for this publication are shown in the Mascot result view as supplemental data (supplementary Figs. S1–S8). Additionally, they are accessible via the following webpage: http://www.imp.ac.at/research/proteinchemistry/labhomepage/spectrumdb/?expname=ti09. This webpage is the front-end to a database, SpectrumDB, developed in-house, containing all the data regarding phosphopeptides and their associated MS/MS spectra, such as peak list, precursor mass, mass modifications and sequence. Data are submitted to SpectrumDB using several PHP scripts, which automatically generate links from a PostgreSQL databank, where all spectrum data are stored. The PHP scripts also provide a convenient mechanism for browsing the database. From the website front-end, the user can browse and search information on phosphoproteins and phosphopeptides. By clicking on the relevant entry in the scanID column, a Java applet, SpecVisualizer, is launched, providing an interactive display of the corresponding MS/MS spectrum and fragment-ion lists.

3. Results and discussion

We have previously described a reliable protocol for the selective enrichment of phosphopeptides using TiO_2-packed tips [6]. Although reliable, this method clearly had significant room for improvement in terms of phosphopeptide selectivity and recovery. Thus, we wished to further improve this procedure by assessing the contributions of two key variables, the mobile and the stationary phases. Firstly, by trying innovative combinations of acids in the loading and washing steps, we aimed to enhance the specific trapping of phosphopeptides onto the solid phase. Secondly, we performed a comparison between two metal dioxide phases, TiO_2 and ZrO_2, to assess their performances in terms of selectivity and recovery.

The starting material (a mixture of synthetic peptides, or digested purified protein complexes affinity purified from cells) was diluted in different loading buffers and treated by MOC under the desired conditions (workflow diagram: supplementary Fig. S11). Eluted peptides were analysed by nano-RP-HPLC and mass spectrometry. Database searching and quantitative analysis enabled the peptides and phosphopeptides obtained under the different conditions to be identified and compared.

3.1. An improved procedure for selective enrichment of phosphopeptides

In our previous work [6] we observed that addition of OSA and DHB to the loading buffer led to a more selective binding of phosphorylated peptides and to lower binding rates of acidic unphosphorylated peptides on TiO_2 columns as compared to DHB alone. One important parameter that can determine the selectivity in MOC enrichment is the pH of the loading buffer, which should be kept in a range whereby carboxylic acid groups are protonated and therefore neutral in charge, whereas phosphate groups are deprotonated and therefore negatively charged. HFBA has been previously used as an efficient ion-pairing agent in RP-chromatography [31], and is a stronger acid than TFA or acetic acid. For these reasons we wanted to test the effect of including HFBA in the loading buffer on the selectivity of our MOC procedure.

In addition to having 0.1% HFBA as a new component in the loading solvent we increased the amount of DHB to 50 mg/ml and OSA to 420 mM as compared to 20 mg/ml and 300 mM in our previous protocol, because we anticipated that a higher concentration would further lower the binding of acidic, unphosphorylated peptides. For the same reasons we included a wash step using the loading solvent, as reported previously [32,33]. To avoid contamination of the LC–MS system by DHB we included an extra wash step with 80% ACN before the elution step. For the same reason, we already introduced in the previous protocol a washing step of 40 min on the RP-trap column, before switching it online with the analytical separation column. The effect of different wash times on the signal intensities in the UV chromatogram is demonstrated in supplementary Fig. S9.

We wished to test our modified MOC procedure using two types of analytical sample: a mixture of trypsinized BSA and synthetic phosphopeptides and non-phosphopeptides of defined composition and concentration; and digests of phosphoprotein complexes, affinity purified from cultured human cells. In both cases we wished to compare the selectivity of the new procedure relative to that previously published, and to compare the selectivities of TiO_2 and ZrO_2 stationary phases.

3.2. Evaluation of the new procedure using a mix of defined peptides

In a first experiment we investigated the effect of our modified MOC protocol on the efficiency of phosphopeptide enrichment by TiO_2 using a standardized mix containing 500 fmol trypsinized BSA plus 500 fmol each of 10 phosphopeptides plus two unphosphorylated peptides. The peptide mix was either directly separated and analysed by nano-RP-HPLC–MS/MS (Fig. 1a) or was subjected to TiO_2 enrichment before the analysis (Fig. 1b). In the untreated sample several BSA-derived (BSA1–7) and synthetic peptides were detected with high intensities. In contrast, following treatment of the mix with the modified MOC procedure, the levels of the two unphosphorylated synthetic peptides (NP6, GluFib) and the BSA-derived peptides were strongly reduced or below the detection limit, whereas the five phosphopeptides which could be detected in the untreated sample were seen with similarly high intensities in the enriched sample. Additionally, the remaining five synthetic

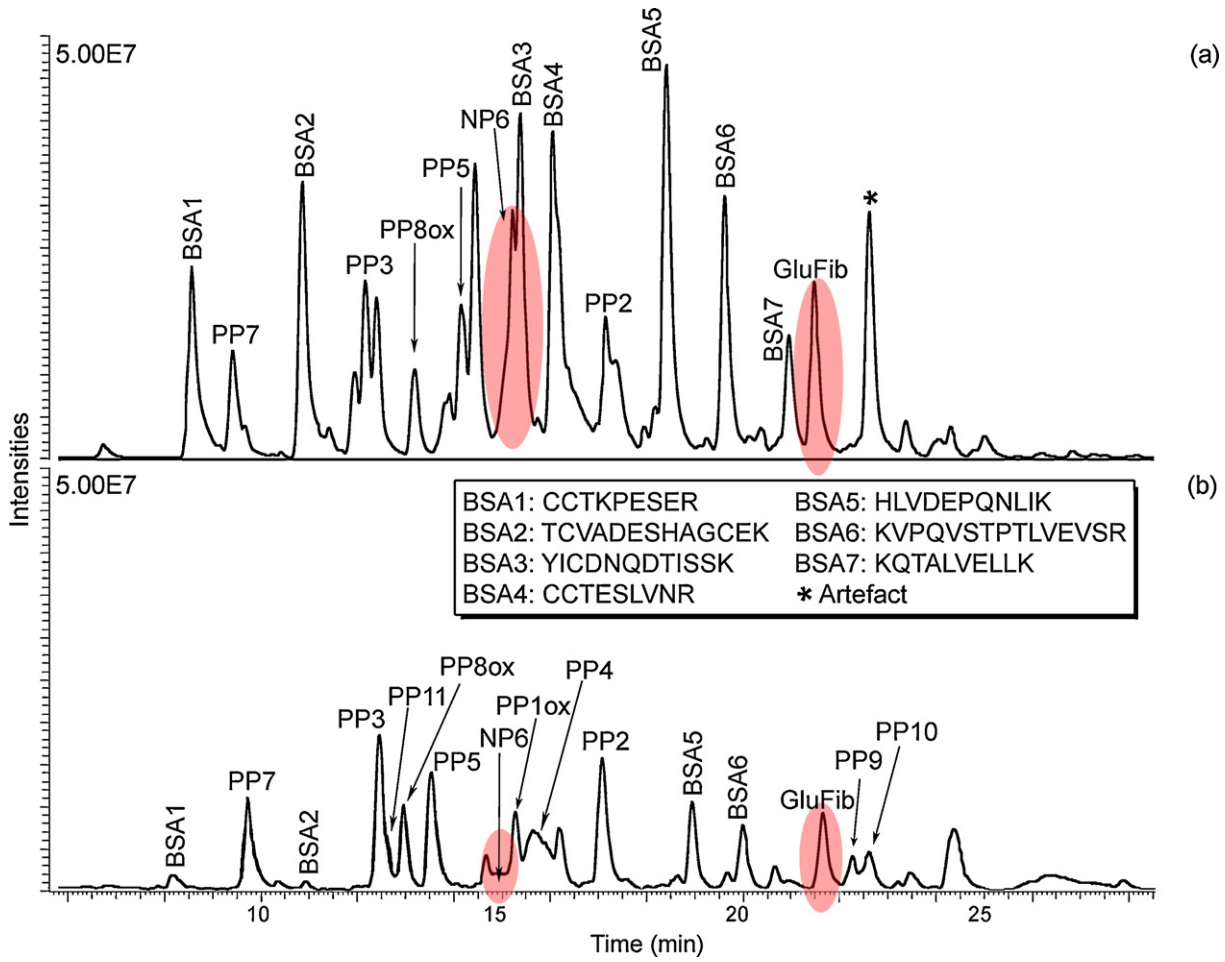

Fig. 1. Specific enrichment of phosphopeptides by metal oxide chromatography. MS base peak chromatogram showing the separation of a mixture containing 500 fmol trypsinized BSA and 500 fmol each of 10 phosphorylated and 2 unphosphorylated peptides by nano-RP-HPLC coupled to an LTQ Orbitrap mass spectrometer (a) without and (b) after being subjected to a TiO_2 purification step using the modified protocol. Note that following the use of TiO_2 tips (b), the signals of the unphosphorylated synthetic peptides (shaded areas) and the BSA-derived peptides (BSA1 to BSA7) were greatly reduced, whereas the recovery of phosphorylated peptides was very efficient.

phosphopeptides from the mix could only be detected in the enriched sample, suggesting that this protocol has the potential to selectively enrich phosphopeptides.

In a second experiment we compared the efficiency of this new method with our previously published protocol [6]. 500 fmol of the test sample were either directly analysed by nano-RP-HPLC–MS/MS or were subjected to TiO_2 enrichment prior to analysis, applying either the old or the new protocol. Additionally, the new protocol was applied to phosphopeptide enrichment using ZrO_2 tips. The experiment was repeated five times and the mean value of the recovery rate of each of the peptides was calculated. As shown in Fig. 2, application of the new protocol to phosphopeptide purification on TiO_2 resulted in a slightly higher recovery rate for phosphopeptides (in five out of seven cases where there was a difference), and to a clear reduction in the binding of the two unphosphorylated synthetic peptides and the BSA-derived peptides, when compared to the old protocol. Four BSA-derived peptides were chosen that illustrate the range of the recovery rates seen for all BSA peptides. Phosphopeptide enrichment on ZrO_2 using the new protocol showed a lower recovery rate for 7 out of 10 phosphopeptides when compared to the signal intensity after TiO_2 treatment. With respect to the binding of unphosphorylated peptides, ZrO_2 was found to be greatly superior in selectivity to TiO_2: both unphosphorylated synthetic peptides and the four BSA-derived peptides were almost completely excluded from binding to ZrO_2.

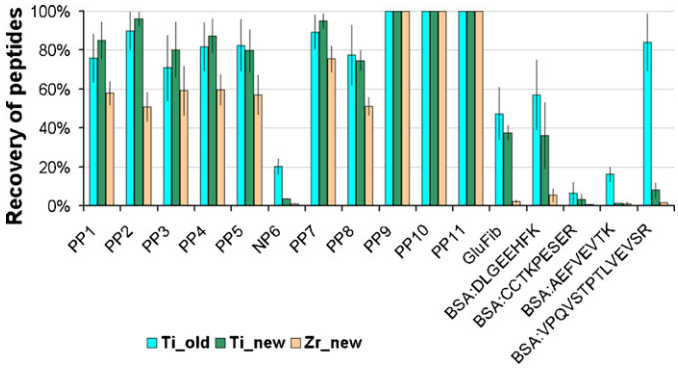

Fig. 2. Comparison of the previous and the new enrichment protocol with respect to the recovery of peptides following TiO_2 and ZrO_2 chromatography. A mix containing 500 fmol trypsinized BSA plus 500 fmol of the 12 peptides were either directly analysed by nano-RP-HPLC–MS/MS or were subjected to TiO_2 enrichment prior to the analysis applying either the old (Ti_old) or the newly modified protocol (Ti_new). Additionally, the new protocol was applied to phosphopeptide enrichment using ZrO_2 tips (Zr_new). The experiment was repeated five times and the mean values of the peak areas for each peptide were calculated. The recovery rate was determined by using the untreated sample as a reference, set to 100%. The error bars show the standard deviation of the five measurements.

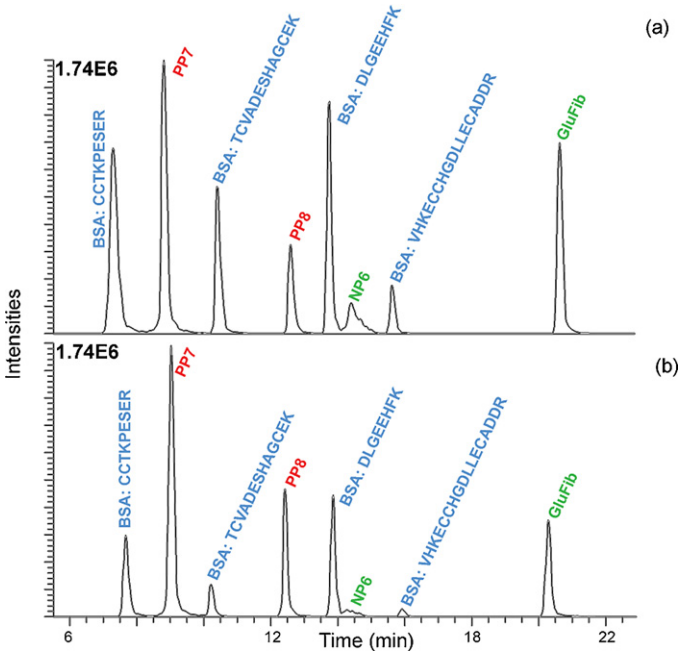

Fig. 3. Effect of the addition of loading buffer to the wash solvent on the retention of acidic unphosphorylated peptides by TiO_2. A mix containing 500 fmol trypsinized BSA plus 500 fmol of the 12 peptide mix was subjected to MOC using TiO_2 tips, using the new enrichment protocol (a) excluding, and (b) including a wash step using the loading buffer. Shown here are the extracted ion chromatograms (XIC) of different BSA-derived and synthetic peptides which eluted from TiO_2 under the two different wash conditions. All 10 phosphopeptides showed similarly high signal intensities after the additional wash step. To make the chromatogram easily comprehensible 8 of the 10 phosphopeptide signals were removed. The reduction in peak heights for BSA-derived peptides and unphosphorylated peptides in (b) compared to (a) indicates that the addition of loading buffer significantly increased the efficiency of the wash step.

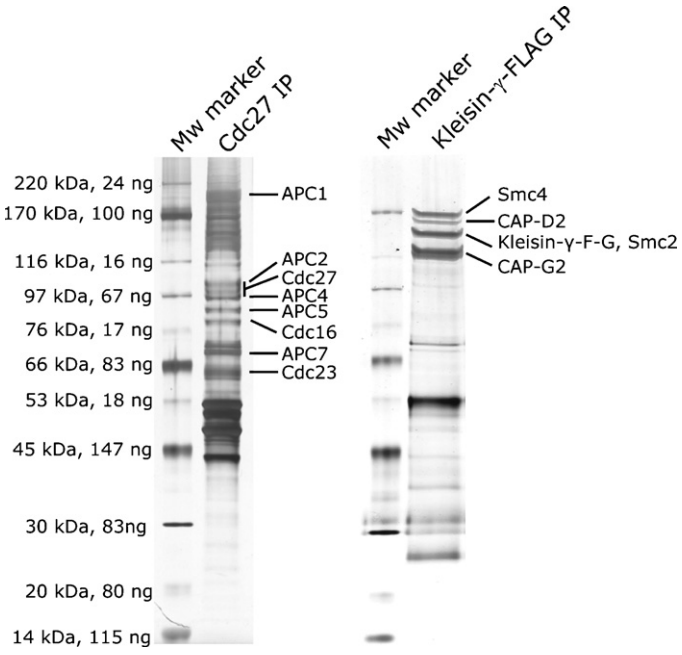

Fig. 4. Protein complexes used for method testing. The anaphase promoting complex (APC/C) was immunopurified from an extract of nocodazole-arrested HeLa cells using anti-Cdc27 antibodies, and the Condensin-I complex was immunopurified from an extract of nocodazole-arrested HeLa-'Kyoto' cells expressing Kleisin-γ-GFP-FLAG (Kleisin-γ-G-F), using anti-FLAG-antibodies. 10% of each sample was analysed by SDS-PAGE followed by silver staining.

Table 2
Phosphopeptides and phosphosites identified for two mitotic protein complexes. Shown are the numbers of phosphopeptides and phosphosites identified for the APC/C and Condensin-I complexes by application of the three different MOC enrichment procedures, and without enrichment (untreated). In the fifth column the total number of unique peptides and sites obtained combining all four measurements is listed.

	Ti_old	Ti_new	Zr_new	Untreated	Total
Protein complex: APC					
Phosphorylation sites	44	71	64	43	75
Phosphopeptides	47	86	73	49	92
Protein complex: Condensin-I					
Phosphorylation sites	26	37	45	21	48
Phosphopeptides	42	59	71	26	78

One crucial step during the MOC procedure is the washing of the tips with bound peptides before elution. Washing under too stringent conditions can be a reason for sample loss, and washing under too mild conditions can lead to a high background of unphosphorylated peptides, which bind with lower affinity, or of residual amounts of chemicals, causing problems in subsequent analysis steps. We therefore tested a new wash procedure as part of our MOC-based enrichment protocol, including an additional wash step using the loading buffer, and increasing the concentration of ACN in the second wash step from 30% to 80% plus addition of 0.1% TFA. We then tested the possible effects of the additional wash step on the retention of unphosphorylated acidic peptides by a TiO_2

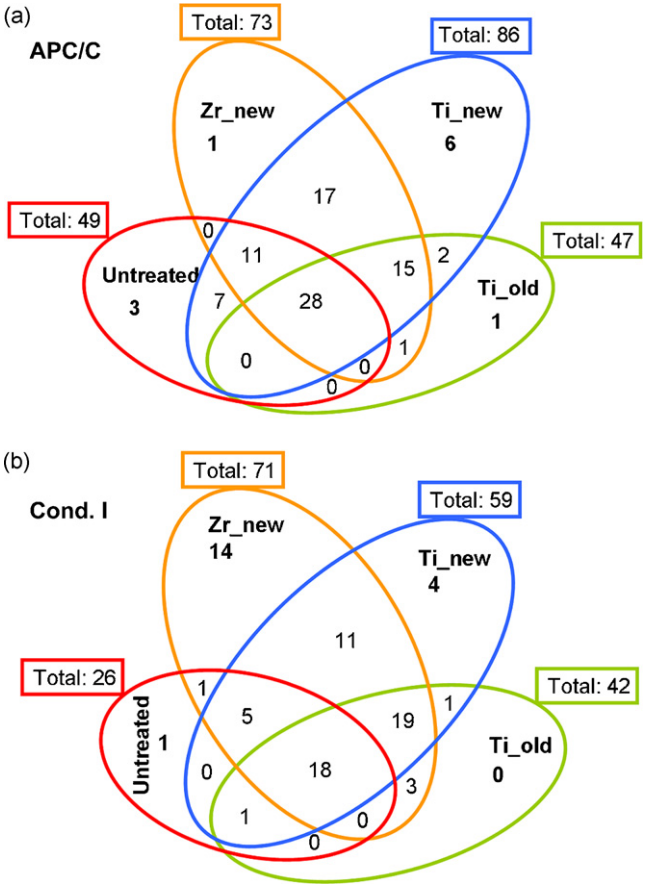

Fig. 5. Overlap between the phosphopeptides identified under the four different conditions. The Venn diagram shows the number of phosphorylated peptides identified in (a) the APC/C; (b) the Condensin-I complex after application of the three different MOC procedures and in the untreated sample. This figure illustrates the overlap between the sets of phosphopeptides identified following the different enrichment techniques.

Table 3
Quantification of the phosphopeptide enrichment ratios and the depletion efficiencies of unphosphorylated peptides from the APC/C.

Peptide	Protein	(a) Ratio: phosphorylated:unphosphorylated			
		Untreated	Ti_old	Ti_new	Zr_new
Protein complex: APC					
SPSISNMAALSR S%PS%ISNMAALSR	APC1	0.2	0.8	12.7	23.3
ISTITPQIQAFNLQK ISTIT#PQIQAFNLQK	APC3	0.2	1.3	19.4	14.0
LDSSIISEGK LDSSIIS#EGK	APC3	0.3	1.8	23.3	30.0
SVFSQSGNSR SVFSQSGNS#R	APC3	0.3	0.5	9.3	22.8
GGITQPNINDSLEITK GGITQPNINDS#LEITK	APC3	0.3	0.9	6.1	24.4
QPETVLTETPQDTIELNR QPETVLTET#PQDTIELNR	APC3	0.4	1.0	4.2	15.6
NIISPPWDFR NIIS#PPWDFR	APC6	1.3	2.1	186.7	55.8

Peptide	Protein	(b) Quantitation of unphosphorylated peptides			
		Untreated	Ti_old	Ti_new	Zr_new
Protein complex: APC					
SPSISNMAALSR	APC1	100%	16%	2%	<1%
DVALSVLSK	APC3	100%	24%	2%	<1%
QPETVLTETPQDTIELNR	APC3	100%	14%	5%	<1%
DAVFLAER	APC3	100%	20%	5%	<1%
ISTITPQIQAFNLQK	APC3	100%	5%	<1%	<1%
LDSSIISEGK	APC3	100%	14%	1%	<1%
SALQELEELK	APC3	100%	23%	<1%	<1%
SVFSQSGNSR	APC3	100%	25%	2%	<1%
GGITQPNINDSLEITK	APC3	100%	15%	3%	<1%
HSLSELIDISIAQK	APC5	100%	26%	4%	3%
FGHYQQAELALQEAIR	APC5	100%	<1%	2%	5%
NIISPPWDFR	APC6	100%	21%	<1%	<1%

(a) The ratios of the peak areas of the phosphorylated peptides to the corresponding unphosphorylated peptides from the APC/C were calculated for the untreated sample and the three different enrichment procedures. Phosphorylated residues are marked with #. Where the phosphorylation sites could not be clearly assigned, possible candidate residues are marked with %. (b) The peak areas of 12 unphosphorylated peptides from the APC/C were calculated for the untreated and the enriched samples. The recovery rate was determined by using the untreated sample as a reference, set to 100%.

tip. The test sample containing a mixture of 500 fmol BSA-derived tryptic peptides plus 500 fmol of the standard mix of 12 peptides was loaded onto the TiO_2 tip, then peptides that did not bind were washed out by either addition of loading buffer before flushing with 80% ACN (Fig. 3b), or with a single 80% ACN washing step (Fig. 3a). The supplementary wash step using the loading buffer led to significantly lower signals in the extracted ion chromatogram (XIC) for the BSA-derived peptides and unphosphorylated peptides NP6 and GluFib (Fig. 3b). At the same time, the intensities of the signals corresponding to the phosphopeptides stayed unchanged in the two washing procedures, indicating that the additional wash step using loading buffer efficiently and selectively displaced bound unphosphorylated peptides.

3.3. Evaluation of the new procedure using biological samples

The purpose of further improving phosphopeptide enrichment techniques was to identify in vivo phosphorylation sites from biological samples of low concentration, such as affinity-purified protein complexes. To test the improvement of our new MOC method compared to the previous method for this purpose, we chose two protein complexes affinity purified from mitotic human cells. In our previous work [6] we analysed the phosphorylation sites of the anaphase promoting complex (APC/C), immunopurified from extracts of HeLa cells arrested in mitosis by nocodazole treatment. Here we have again used the APC/C, and additionally a second mitotic complex, Condensin-I. The APC/C is an E3 protein ubiquitin ligase that plays essential roles in the progression of the cell cycle through mitosis [34]. It consists of more than a dozen subunits, several of which have been reported to undergo mitotic phosphorylation [6,35–37]. Condensin-I associates with chromatin and contributes to its condensation during early mitosis [38] and is required for chromosome segregation in anaphase [39,40]. The complex consists of five subunits, several of which have been reported to be phosphorylated in mitosis [41–43].

Both the APC/C and Condensin-I were affinity purified from human cells under conditions that preserve the phosphorylation state of the proteins. To assess the yield and purity of the isolated complexes, 10% of each sample was analysed by SDS-PAGE and silver staining (Fig. 4), from which the amount of each sample was estimated to be a few micrograms. The remaining 90% of each sample was digested with trypsin, then split into four equal parts, which were subjected to the following phosphopeptide enrichment methods: The first part, as a control, was analysed without prior enrichment. Two parts were subjected to TiO_2 enrichment prior to analysis, applying either the old or the newly modified enrichment protocol. The fourth part was treated using ZrO_2 tips using the new protocol. The peptides which were eluted from the different MOC tips, and the control sample, were separated by nano-RP-HPLC applying a shallow ACN gradient to improve the separation, and analysed by mass spectrometry as described. The recorded base peak chromatograms of the different MOC-treated samples showed fewer and less intense

peaks compared to the untreated samples (supplementary Fig. S10 and data not shown), indicating that MOC-treatment efficiently reduces the complexity and overall amount of the peptides in the mixture. All phosphopeptide MS/MS spectra and associated information are presented in supplementary Tables S1–S3 and supplementary Figs. S1–S8. Additionally, they are accessible via the following link: http://www.imp.ac.at/research/proteinchemistry/labhomepage/spectrumdb/?expname=ti09.

The results obtained for the three different enrichment procedures and the untreated sample of the two protein complexes are shown in Table 2 and supplementary Table S2. For both the APC/C and the Condensin-I complex, the phosphopeptide enrichment on TiO_2 using the old protocol gave similar results as the untreated sample. In contrast, the isolation of phosphopeptides using the new protocol on both the TiO_2 and ZrO_2 material led to a significantly increased number of identified phosphopeptides. Summing up the number of distinct phosphopeptides identified in all four analyses, we found 92 phosphopeptides (containing 75 phosphosites) corresponding to 9 APC/C subunits and 3 associated proteins (see Table 2) and 78 phosphopeptides (48 phosphosites) corresponding to the 5 subunits of the Condensin-I complex. In total, we found 170 phosphopeptides (123 phosphosites) in both complexes. The identity of these 123 phosphosites in the different proteins is shown in supplementary Table S3.

The number of phosphopeptides identified with the four different procedures are shown in the Venn diagrams in Fig. 5. For both complexes, most of the phosphopeptides found in the untreated sample could also be found in the samples enriched on TiO_2 or ZrO_2 using the optimized protocol, indicating that not many phosphopeptides were lost during the new MOC procedure. In contrast, many fewer peptides from the untreated sample were found in the sample enriched on TiO_2 according to the previously published protocol. This can be explained in part by the less efficient removal of unphosphorylated peptides as compared to the samples treated with the optimized protocol (see Tables 3 and 4a). As a consequence, fewer phosphopeptide precursors of low intensity were chosen for fragmentation.

We wished to establish whether the increased numbers of phosphopeptides identified following enrichment using our new protocol on TiO_2- and ZrO_2-packed tips reflected largely overlapping or distinct populations. Combining data from both purified complexes, 124 of 165 phosphopeptides were identified in common to both TiO_2 and ZrO_2. Of the remaining phosphopeptides, about 50% were found only in the TiO_2-treated samples and 50% only in the ZrO_2-treated samples, indicating that these two media may exhibit different selectivities. Analysing the mass, p*I* and hydrophobicity of these phosphopeptides, no correlation could be found between these

Table 4
Quantification of the phosphopeptide enrichment ratios and the depletion efficiencies of unphosphorylated peptides from the Condensin-I complex.

Peptide	Protein	(a) Ratio: phosphorylated:unphosphorylated			
		Untreated	Ti_old	Ti_new	Zr_new
Protein complex: Condensin-I					
ESTGNMVTGQTVCK ES%T%GNMVTGQTVCK	CAP-D2	0.03	<0.01	4.84	4.64
YQPLASTASDNDFVTPEPR YQPLASTASDNDFVT#PEPR	CAP-D2	0.09	1.49	8.89	6.82
LNLAQFLNEDLS LNLAQFLNEDLS#	CAP-G	0.02	0.19	0.11	0.99
TSQDYQALTVHDNLAMK T%S%QDYQALTVHDNLAMK	CAP-G	0.02	0.39	4.14	22.40
DAPSLEEVEGHVADGSATEMGTTK DAPSLEEVEGHVADGS#ATEMGTTK	Kleisin-γ	0.03	0.19	0.37	3.35
KAPLNIPGTPVLEDFPQNDDEKER KAPLNIPGT#PVLEDFPQNDDEKER	Kleisin-γ	0.13	0.29	0.57	0.70
TESPATAAETASEELDNR T%ES%PATAAETASEELDNR	Smc4	0.91	6.01	6.79	49.69
RREEGPPPPSPDGASSDAEPEPPSGR RREEGPPPPS#PDGAS%S%DAEPEPPSGR	Smc4	1.61	20.52	35.89	213.96

Peptide	Protein	(b) Quantitation of unphosphorylated peptides			
		Untreated	Ti_old	Ti_new	Zr_new
Protein complex: Condensin-I					
GNAIYNLLPDIISR	CAP-D2	100%	35%	8%	2%
GFVHPDPLWIPFK	CAP-D2	100%	48%	2%	3%
LLENPTINHQK	CAP-D2	100%	7%	1%	<1%
TSQDYQALTVHDNLAMK	CAP-D2	100%	8%	1%	<1%
YQPLASTASDNDFVTPEPR	CAP-D2	100%	5%	2%	<1%
EAITHLLGVALTR	CAP-D2	100%	49%	23%	6%
ELEIGQAGSQR	CAP-D2	100%	4%	2%	3%
FPNLVDPWTPHLYAR	CAP-D2	100%	19%	2%	2%
FQAVVALAVGR	CAP-D2	100%	9%	<1%	<1%
ALSSLELSSHLAK	CAP-G	100%	14%	1%	<1%
APIVTVGVNNDPADVR	CAP-G	100%	4%	6%	<1%
FSEGNILELLHR	CAP-G	100%	38%	17%	3%

(a) The ratio of the peak areas of the phosphorylated peptides to the corresponding unphosphorylated peptides from the Condensin-I complex were calculated for the untreated sample and the three different enrichment procedures. Phosphorylated residues are marked with #. Where the phosphorylation sites could not be clearly assigned, possible candidate residues are marked with %. (b) The peak areas of 12 unphosphorylated peptides from the Condensin-I complex were calculated for the untreated and the enriched samples. The recovery rate was determined by using the untreated sample as a reference, set to 100%.

values and their binding properties to TiO_2 or ZrO_2 (data not shown).

In summary, phosphopeptide fractions identified with either TiO_2 or ZrO_2 using the optimized protocol showed a high overlap, but there still remains a significant fraction of unique peptides on each side, suggesting that the application of both materials to the same sample in parallel would lead to a more comprehensive result.

To compare the selectivity of phosphopeptide enrichment under the three conditions, we performed a label-free quantification to determine the ratios of the peak areas of the phosphorylated peptides to those of their unphosphorylated counterparts (Tables 3 and 4a). Using the previous protocol the ratios were elevated by a factor of 2–6, relative to the untreated sample. For our new TiO_2 or ZrO_2 enrichment methods, these ratios showed a more prominent increase of from 10-fold to over 100-fold relative to the untreated sample, demonstrating a strong selective enrichment of phosphopeptides. To further demonstrate the selectivity of phosphopeptide enrichment under the three conditions, we quantified the recovery rates of unphosphorylated peptides that were found in all samples (Tables 3 and 4b). 12 unphosphorylated peptides were chosen from each complex. The binding of unphosphorylated peptides to TiO_2 or ZrO_2 was strongly reduced after application of the optimized enrichment protocol. In agreement with the data presented in Fig. 2 we found that unphosphorylated peptides were more efficiently excluded from binding to ZrO_2 than to TiO_2: treated with ZrO_2 the analysed unphosphorylated peptides showed a mean recovery rate of 1.5%, whereas with TiO_2 the binding rate averaged 3.8%. These lower amounts of unphosphorylated peptides found with ZrO_2 explain the elevated phosphopeptide enrichment ratios for this medium relative to TiO_2; 11 of the 15 ratios were higher for ZrO_2 (Tables 3 and 4a). In contrast, when applying the previous protocol the analysed unphosphorylated peptides were recovered with a mean value of 18.6%. These data clearly demonstrate the superiority of the optimized protocol compared to the previous one in terms of the exclusion of unphosphorylated peptides. In summary, the application of our optimized protocol containing the new acid mix to TiO_2- and ZrO_2-based MOC enrichment led to a more efficient and selective enrichment of phosphopeptides from digested purified protein complexes.

To establish how many of the phosphosites identified from APC/C and Condensin-I have not previously been reported, we compared our complement of phosphosites with that present in three publicly available phosphosite databases, Phospho.ELM (http://phospho.elm.eu.org) [44], PHOSIDA (http://www.phosida.com) [22,45] and PhosphoSitePlus (http://www.phosphosite.org) [46]. This analysis showed that using our improved phosphopeptide enrichment technique, we could identify 21 sites from APC/C (and associated proteins) and 16 sites from Condensin-I that had not been previously reported, highlighting the potential of this technique for novel phosphosite discovery.

Many regulatory proteins are phosphorylated in the cell on multiple residues, which may be in close proximity to each other [47]. It is therefore important to be able to isolate peptides containing more than one phosphate group. One issue with phosphopeptide enrichment techniques is the efficiency with which multiply-phosphorylated peptides can be isolated. From our phosphopeptide dataset, 116 peptides were singly phosphorylated, 41 doubly phosphorylated, 10 triply phosphorylated and 3 were phosphorylated on four sites. These data indicate that our MOC procedure can indeed be used to isolate multiply-phosphorylated peptides from proteins of biological origin.

As an example of an identified phosphopeptide, Fig. 6 shows the MS/MS spectrum of a peptide derived from the CAP-D2 subunit of the Condensin-I complex, using TiO_2 tips, in three different phosphorylation states. The quality of the spectrum is good, displaying

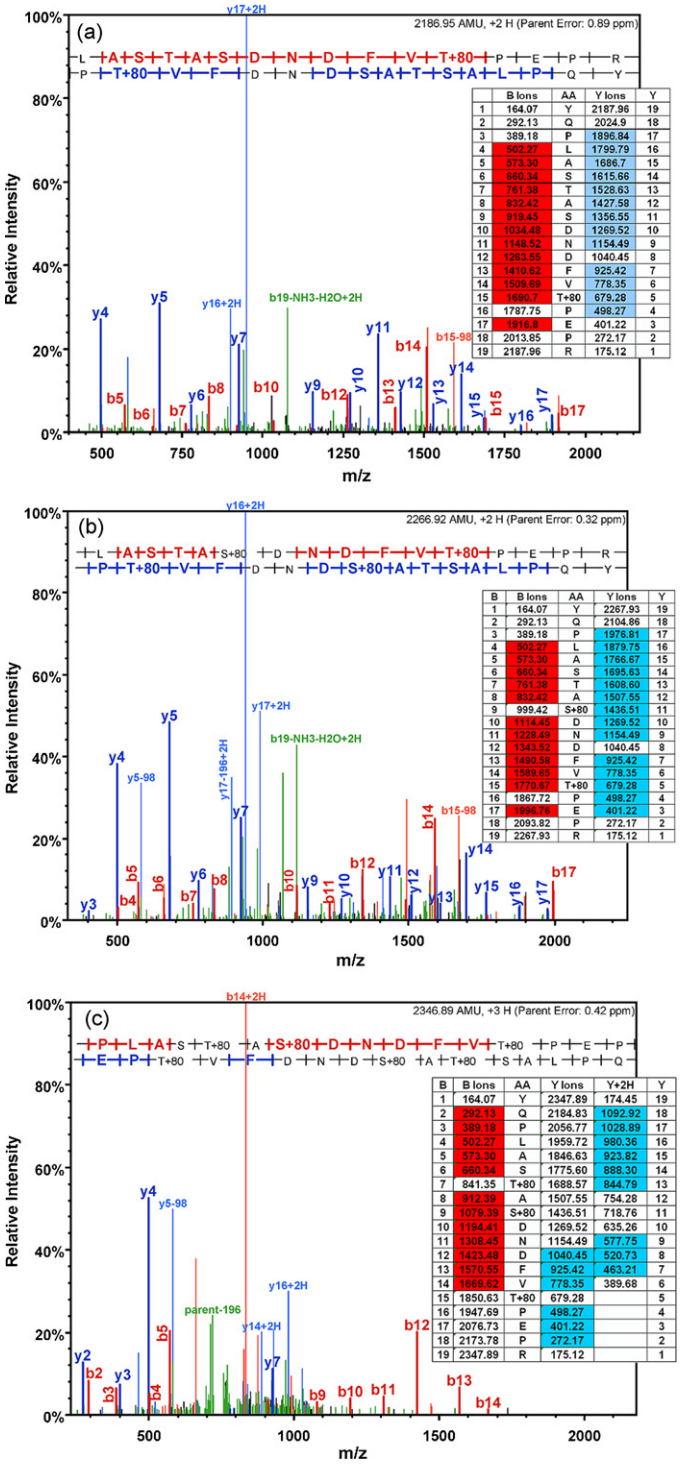

Fig. 6. Peptides isolated in three different phosphorylation states. The MS/MS spectra show the fragmentation patterns corresponding to the peptide YQPLASTASDNDFVTPEPR originating from the Condensin-I subunit CAP-D2, isolated by TiO_2 chromatography in three phosphorylation states: (a) singly, (b) doubly and (c) triply phosphorylated.

an almost continuous ladder of b- and y-ions and a low number of uninterpretable fragments, even for the triply phosphorylated peptide. The high mass accuracy of the identified peptides (parent mass error less than 1 ppm) further supports the correct interpretation of the MS/MS spectra. The doubly- and triply phosphorylated peptides were only found following enrichment.

To further characterize the protein complex-derived phosphopeptides enriched using the MOC procedures, they were grouped according to their molecular weights (supplementary Fig. S12a) and p*I* values (supplementary Fig. 12b). We found that there was no difference in the distribution of the molecular weight of the peptides between the untreated sample and the MOC-enriched peptides. Regarding the p*I* value of the peptides, approximately 90% of them display p*I* values below 5 and the residual 10% have p*I* values in the range between 5 and 7. Interestingly, the phosphopeptides of the untreated sample show a similar distribution to that of the MOC-enriched peptides, which argues against a preferential selection of acidic peptides by TiO_2 or ZrO_2.

We have developed and further improved a technique for the selective enrichment of phosphopeptides from a mixture of phosphorylated and unphosphorylated peptides, such as that produced from a protein digest. The method is particularly suited to the enrichment of phosphopeptides from digests of protein complexes isolated from cells, for the identification of in vivo phosphorylation sites. Using our optimized method containing the novel acid mix, we have for the first time performed a detailed comparison of TiO_2 or ZrO_2 stationary phases for the selective enrichment of phosphopeptides from digested protein complexes. As TiO_2 and ZrO_2 were shown to display distinct but overlapping selectivities, where possible it would be advantageous for the proteomic investigator to perform MOC using both phases in parallel. As this method is applicable to large-scale phosphoproteomic studies, we anticipate that it will be used to broaden the knowledge of the role of protein phosphorylation in the regulation of diverse cellular processes.

Acknowledgements

We are grateful to our colleagues from IMP-IMBA Vienna: Ines Steinmacher for support in carrying out mass spectrometry measurements; Mathias Madalinski and Gabriela Krssakova for peptide synthesis; and Andreas Schmidt and Thomas Köcher for useful discussions and a critical reading of the manuscript. This work is supported by the Austrian Proteomics Platform (APP) within the Austrian Genome Research Programme (GEN-AU) and by the MitoCheck project within the Sixth Framework Programme of the European Commission.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.12.017.

References

[1] M.W. Pinkse, P.M. Uitto, M.J. Hilhorst, B. Ooms, A.J. Heck, Anal. Chem. 76 (2004) 3935.
[2] M.R. Larsen, T.E. Thingholm, O.N. Jensen, P. Roepstorff, T.J. Jorgensen, Mol. Cell Proteomics 4 (2005) 873.
[3] H.K. Kweon, K. Hakansson, Anal. Chem. 78 (2006) 1743.
[4] G.T. Cantin, T.R. Shock, S.K. Park, H.D. Madhani, J.R. Yates 3rd, Anal. Chem. 79 (2007) 4666.
[5] M.O. Collins, L. Yu, J.S. Choudhary, Proteomics 7 (2007) 2751.
[6] M. Mazanek, G. Mituloviae, F. Herzog, C. Stingl, J.R. Hutchins, J.M. Peters, K. Mechtler, Nat. Protoc. 2 (2007) 1059.
[7] M.B. Goshe, T.P. Conrads, E.A. Panisko, N.H. Angell, T.D. Veenstra, R.D. Smith, Anal. Chem. 73 (2001) 2578.
[8] Y. Oda, T. Nagasu, B.T. Chait, Nat. Biotechnol. 19 (2001) 379.
[9] H. Zhou, J.D. Watts, R. Aebersold, Nat. Biotechnol. 19 (2001) 375.
[10] W.A. Tao, B. Wollscheid, R. O'Brien, J.K. Eng, X.J. Li, B. Bodenmiller, J.D. Watts, L. Hood, R. Aebersold, Nat. Methods 2 (2005) 591.
[11] L. Andersson, J. Porath, Anal. Biochem. 154 (1986) 250.
[12] D.C. Neville, C.R. Rozanas, E.M. Price, D.B. Gruis, A.S. Verkman, R.R. Townsend, Protein Sci. 6 (1997) 2436.
[13] D. Figeys, S.P. Gygi, Y. Zhang, J. Watts, M. Gu, R. Aebersold, Electrophoresis 19 (1998) 1811.
[14] S. Li, C. Dass, Anal. Biochem. 270 (1999) 9.
[15] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[16] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Nat. Biotechnol. 20 (2002) 301.
[17] T.S. Nuhse, A. Stensballe, O.N. Jensen, S.C. Peck, Mol. Cell Proteomics 2 (2003) 1234.
[18] F. Wolschin, S. Wienkoop, W. Weckwerth, Proteomics 5 (2005) 4389.
[19] S.A. Beausoleil, M. Jedrychowski, D. Schwartz, J.E. Elias, J. Villen, J. Li, M.A. Cohn, L.C. Cantley, S.P. Gygi, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12130.
[20] B.A. Ballif, J. Villen, S.A. Beausoleil, D. Schwartz, S.P. Gygi, Mol. Cell Proteomics 3 (2004) 1093.
[21] A. Gruhler, J.V. Olsen, S. Mohammed, P. Mortensen, N.J. Faergeman, M. Mann, O.N. Jensen, Mol. Cell Proteomics 4 (2005) 310.
[22] J.V. Olsen, B. Blagoev, F. Gnad, B. Macek, C. Kumar, P. Mortensen, M. Mann, Cell 127 (2006) 635.
[23] N. Sugiyama, T. Masuda, K. Shinoda, A. Nakamura, M. Tomita, Y. Ishihama, Mol. Cell Proteomics 6 (2007) 1103.
[24] J. Wu, Q. Shakey, W. Liu, A. Schuller, M.T. Follettie, J. Proteome Res. 6 (2007) 4684.
[25] S.S. Jensen, M.R. Larsen, Rapid Commun. Mass Spectrom. 21 (2007) 3635.
[26] T. Hirota, D. Gerlich, B. Koch, J. Ellenberg, J.M. Peters, J. Cell Sci. 117 (2004) 6435.
[27] M.J. Schroeder, J. Shabanowitz, J.C. Schwartz, D.F. Hunt, J.J. Coon, Anal. Chem. 76 (2004) 3590.
[28] R. Craig, R.C. Beavis, Rapid Commun. Mass Spectrom. 17 (2003) 2310.
[29] A. Keller, A.I. Nesvizhskii, E. Kolker, R. Aebersold, Anal. Chem. 74 (2002) 5383.
[30] A.I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, Anal. Chem. 75 (2003) 4646.
[31] M. Shibue, C.T. Mant, R.S. Hodges, J. Chromatogr. A 1080 (2005) 58.
[32] T.E. Thingholm, T.J. Jorgensen, O.N. Jensen, M.R. Larsen, Nat. Protoc. 1 (2006) 1929.
[33] B. Bodenmiller, L.N. Mueller, M. Mueller, B. Domon, R. Aebersold, Nat. Methods 4 (2007) 231.
[34] J.M. Peters, Nat. Rev. Mol. Cell Biol. 7 (2006) 644.
[35] C. Kraft, F. Herzog, C. Gieffers, K. Mechtler, A. Hagting, J. Pines, J.M. Peters, EMBO J. 22 (2003) 6598.
[36] F. Herzog, K. Mechtler, J.M. Peters, Methods Enzymol. 398 (2005) 231.
[37] J.A. Steen, H. Steen, A. Georgi, K. Parker, M. Springer, M. Kirchner, F. Hamprecht, M.W. Kirschner, Proc. Natl. Acad. Sci. U.S.A. 17 (2008) 17.
[38] T. Hirano, Curr. Biol. 15 (2005) R265.
[39] D. Gerlich, T. Hirota, B. Koch, J.M. Peters, J. Ellenberg, Curr. Biol. 16 (2006) 333.
[40] J.J. Lipp, T. Hirota, I. Poser, J.M. Peters, J. Cell Sci. 120 (2007) 1245.
[41] K. Kimura, M. Hirano, R. Kobayashi, T. Hirano, Science 282 (1998) 487.
[42] M. Nousiainen, H.H. Sillje, G. Sauer, E.A. Nigg, R. Korner, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 5391.
[43] S.A. Beausoleil, J. Villen, S.A. Gerber, J. Rush, S.P. Gygi, Nat. Biotechnol. 24 (2006) 1285.
[44] F. Diella, S. Cameron, C. Gemund, R. Linding, A. Via, B. Kuster, T. Sicheritz-Ponten, N. Blom, T.J. Gibson, BMC Bioinform. 5 (2004) 79.
[45] F. Gnad, S. Ren, J. Cox, J.V. Olsen, B. Macek, M. Oroshi, M. Mann, Genome Biol. 8 (2007) R250.
[46] P.V. Hornbeck, I. Chabra, J.M. Kornhauser, E. Skrzypek, B. Zhang, Proteomics 4 (2004) 1551.
[47] P. Cohen, Trends Biochem. Sci. 25 (2000) 596.