Sequence-Dependent Enrichment of a Model Phosphopeptide: A Combined MALDI-TOF and NMR Study

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ABSTRACT: The goal of this study was to detect and quantify by MALDI-TOF MS the phosphorylation of a peptide containing the recognition motif of the Protein Kinase C (PKC). Such model peptide can be used as a phosphorylation probe to follow intracellular kinase/phosphatase activities. This study allowed us to establish relationships between sequence specificities and affinity for TiO$_2$ or IMAC media. The peptide has the sequence biotin-GGGGCFRTSPFLK-NH$_2$ in which the serine residue can be phosphorylated. Enrichment of the corresponding phosphopeptide, by the dedicated IMAC and TiO$_2$ affinity chromatography methods, proved inefficient. By combining MALDI-TOF and NMR data, we first showed that the lack of affinity of the phosphopeptide for TiO$_2$ was partly related to the basic property of its peptide sequence. Furthermore, the peptide shows local structure around the P$^9$-S$^{10}$ segment, with formation of a salt bridge between the guanidinium group of the R$^7$ side chain and the phosphate moiety. In conjunction with an inadequate position of the biotin-G$_4$ N-terminal tag, this local structure could shield the phosphate group, preventing interaction with TiO$_2$. To improve TiO$_2$ affinity, the peptide sequence was modified accordingly. The new sequences retained the biological properties while their enrichment by IMAC or TiO$_2$ became possible.

The goal of this study was to detect and quantify by MALDI-TOF MS the phosphorylation of a peptide containing the recognition motif of the protein kinase C (PKC). This type of post-translational modification is known to be among the most biologically relevant, affecting the properties of many proteins and regulating essential cell functions. Studies aiming at the identification and characterization of phosphoproteins by mass spectrometry (MS) are therefore taking more and more importance. However, although MS-based techniques have the advantage of a high resolution, specificity and sensitivity, phosphopeptides and phosphoproteins still remain difficult to analyze because of their low abundance in vivo. In matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) MS, the detection of phosphorylated peptides often suffers from signal suppression effects.$^{1-3}$ Another parameter reducing the intensity of a phosphopeptide ion signal in reflector mode is the often abundant neutral loss of the labile phosphate group ($-$H$_3$PO$_4$ and $-$HPO$_4^{2-}$) by post source decay (PSD) [$[(M + H)^+ - 98$ and $(M + H)^+ - 80$, respectively].$^{4-7}$ In addition, phosphorylated species are difficult to detect in positive ion MS mode$^8$ essentially because of an unfavorable negative net charge of the phosphate group at acidic pH ($<$2).

Selective phosphopeptide enrichment techniques are therefore needed, most of them based on affinity chromatography.$^9$ Metal ions such as Fe$^{3+}$ and Ga$^{3+}$ are immobilized on a chelating support in the Immobilized Metal Affinity Chromatography (IMAC) enrichment procedure. At acidic pH, electrostatic interactions between the negatively charged phosphate groups and the positively charged metal cations cause the adsorption of the phosphopeptides on the support. An elution at basic pH allows the recovery of the bound species. On the basis of the same principle, the metal oxide chromatography (MOC) uses metal oxides (TiO$_2$, ZrO$_2$) as affinity agents$^{10,11}$ thanks to their amphoteric nature ($-$MOH$_3^+ \leftrightarrow -$MOH $\leftrightarrow -$MO$^-$).$^{11}$ Electrostatic interactions with negatively charged species can thus occur at pH lower than the zero point of charge (ZPC), pH at which the surface is neutralized.$^{11,12}$ In addition to these electrostatic processes, the metal itself constitutes hard Lewis acid sites at low pH and coordinates with Lewis bases, like phosphate moieties.$^{9,13}$ Several groups have studied the pH dependent adsorption of acidic molecules, amino acids and proteins on metal oxide surfaces, and reported that they were strong enough, at acidic pH, to be irreversible.$^{14-17}$ More specifically, the strong adsorption of phosphorylated species on TiO$_2$ has been extensively shown.$^{17,18,19}$

IMAC and MOC methods are widely used in phosphoproteomics because of the strong affinity of the chromatographic media for phosphorylated peptides.$^{10}$ The two methods seem to

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have different specificity and should therefore be used complementarily. Although they both suffer from unspecific binding of acidic residues, analytical solutions now exist that reduce the impact on the enrichment results. TiO₂ enrichment is less time-consuming than IMAC thanks to a reduced adsorption time, and also more robust. Another pitfall of both techniques, especially TiO₂ enrichment, so far less emphasized, is their poor affinity for phosphopeptides containing several basic residues. Phosphopeptides with more than two basic residues are generally underrepresented in TiO₂ data sets. Even though it is expected that the samples arising from phosphoproteomics studies contain less peptides with multiple basic residues, because those experiments usually include a trypsin digestion step, it has been shown that such peptides are underrepresented after a TiO₂ enrichment. Considering that many kinase substrates have highly basic recognition patterns, this can largely impair their detection in phosphoproteomics studies. The lack of enrichment of this class of molecules remains unclear and difficult to rationalize because these techniques are still essentially empirical.

The peptide presented here (biot-Add) is derived from the protein adducin whose C-terminal region has been reported to be a substrate of phorbol ester-activated PKC isoforms (classical α, βI, βII, γ and novel δ, ε, η isoforms), allowing regulation of the protein adducin by PKC phosphorylation. The goal of this study was to target endogenous PKC in CHO cells with a short peptide that mimicks the PKC phosphorylation site at Ser 726 of the adducin protein after intracellular delivery by a carrier peptide. This is achieved by coupling this substrate peptide to a cell penetrating peptide (CPP) that is able to cross cell membrane and deliver its cargo inside the cells. After biot-Add internalization into cells, PKC phosphorylation can occur in the cytosol to give the biot-pAdd peptide. Both phosphorylated and nonphosphorylated forms will be tracked by MALDI-TOF MS. It is important to point out that the in vivo experiments we are aiming at are not performed in classical biological concentrations. The extracellular concentration of the conjugates cargo/CPPs is typically of 1–10 μM (for 10⁶ cells) and the amount of detected internalized peptides ranged from about 0.5–50 pmol. The biot-Add peptide used in this study might also be evaluated as a phosphorylation probe in order to study, for example, (i) the kinase activity in an intracellular medium, (ii) the modulation of PKC phosphorylation can occur in the cytosol to give the biot-pAdd peptide. Both phosphorylated and nonphosphorylated forms will be tracked by MALDI-TOF MS. It is important to point out that the in vivo experiments we are aiming at are not performed in classical biological concentrations.

The experimental section

Materials. α-Cyano-4-hydroxycinnamic acid (CHCA) was from Sigma, 2,5-dihydroxybenzoic acid (DHB) was from Fluka. Acetonitrile (ACN) HPLC-grade was from VWR Prolabo, deionized water (18.4 MΩ cm) was obtained from an Elga PURELAB Classic. Poros R2 and Poros Oligo R3 reversed-phase material were from PerSeptive Biosystems. GELoader Tips, and all the plastic tubes were from Eppendorf. The 3M Empore C₁₈ disk was from 3M Bioanalytical Technologies. Titanium dioxide beads (5 μm) were obtained from a Titansphere HPLC Column (GL Sciences Inc.), IMAC (Phos-Select) was from Sigma. Rat brain Protein Kinase C (PKC) (primarily α, βI, βII and γ isoforms with lesser amounts of δ and ζ isoforms) and phosphatase inhibitor cocktail were from Calbiochem, protease inhibitor from Roche, calf intestinal alkaline phosphatase from Promega, streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin C₁) from Invitrogen, and all other reagents from Sigma.

Fmoc Synthesis of the Peptides. The peptides used in the study (biot-Add: biotin-GGGGCFRTPSFLKK-NH₂, biot-pAdd: biotin-GGGGCFRTPSFLKK-NH₂, Add-biot: Ac-CFRTPSFLKKGGGG-NH-(CH₂)₂-NH-biotin, pAdd-biot: Ac-CFRTPSFLKKGGGG-NH-(CH₂)₂-NH-biotin, biot-(O₂)E₂-Add: biotin(O₂)-EGGGECEFRTPSFLKK-NH₂ and biotE₂-pAdd: biotin-EGGGECEFRTPSFLKKNH₂) were synthesized by Dr. C. Piesse, (Plateforme d’Ingénierie des protéines, IFR 83, UPMC, France), as described previously. For Add-biot and pAdd-biot, the resin used was a N-Biotin-N’-Fmoc-ethylendiamine MBP-AM resin (Biotin NovaTagresin), substituted at 0.46 mmol/g. Purity of the peptides was assessed by analytical RP-HPLC and MALDI-TOF MS and was found >95%.

In Vitro PKC Phosphorylation. The peptides (final concentration 32.5 μM) were phosphorylated by incubation with 0.1 μg of PKC in PKC buffer (0.1 mM ATP, 10 mM MgCl₂, 1 mM EDTA, 1 mM CaCl₂, 40 mM HEPES, pH 6.0) at 30 °C for 1 h. The reaction was terminated by adding 1% TFA and cooling the solution on ice prior to the desalting step.

Cell Culture. Chinese hamster ovary (CHO) K₁ cells were cultured and grown to confluence (10⁶ cells per dish) as described previously.
Phosphorylation in a Cell Lysate. To activate the classic ($\alpha$, $\beta_{II}$ and $\gamma$) and novel (C, E and O) isoforms of the cytosolic PKC, phorbol 12-myristate 13-acetate (PMA) was added at 100 nM per dish in the complete medium for 15 min at 37 °C. The incubation medium was then removed and adherent cells were washed once with 5 mL of PKC buffer. After addition of 1 mL of PKC buffer, cells were scraped off from culture plates with a rubber policeman, transferred to a 1.5-mL Eppendorf tube (per dish) and centrifuged for 2 min at 1000 rpm (Sanyo MSE Micro Centaur centrifuge). The supernatant was removed by aspiration and 50 μL of lysis buffer (1:100 phosphatase inhibitor cocktail Set II stock, protease inhibitor cocktail (complete mini, Roche, used according to the supplier recommendation), 50 μM phosphatidylserine in PKC buffer) were added to the pellet before sonication for 15 min at 4 °C. After centrifugation at 10 000 rpm for 5 min, the supernatants were recovered and split into two different tubes. The cell lysates were then incubated with 50 pmol of the desired peptide in the presence of $2.5 \times 10^{-9}$ mol of ATP, first for 1 h at 30 °C, then for 15 min at 100 °C. Samples were then diluted into 1 mL of a 50 mM Tris pH 7.4, 0.1 mg·mL$^{-1}$ BSA buffer and incubated with 5 μL (per sample) of streptavidin-coated magnetic beads for 60 min at room temperature, under gentle agitation. All the washing steps of the magnetic beads were performed as described previously.32

Phosphopeptides Enrichment using TiO2 Microcolumns. TiO$_2$ microcolumns were prepared as described previously.37 The indicated quantity of phosphorylated peptides (either synthetic phosphopeptides or phosphopeptides resulting from the in vivo PKC phosphorylation in the case of the peptide biotE$_2$-pAdd) was diluted in 50 μL of an acidic loading solution (LS). Different LS were used: DHB (300 mg·mL$^{-1}$) in 80% ACN, 50% H$_2$O containing 2% TFA (LS1); DHB (25 mg·mL$^{-1}$) in 50% ACN, 50% H$_2$O containing 2% phosphoric acid (PA) (LS2); TFA 0.1% (LS3), 2% (LS4), 10% (LS5) or 50% (LS6); formic acid (FA) 0.02% (LS7) or 0.2% (LS8); lactic acid 1 (LS9) or 3 mol·L$^{-1}$ (LS10) in H$_2$O containing 0.1% TFA. The loading, washing, elution, and desalting steps were performed as described previously.37

Phosphopeptides Enrichment Using IMAC Microcolumns. Fe$^{3+}$ IMAC microcolumns were prepared by placing a small plug of Cu$_2$O material into a GEloader Tip and then packing the columns with 2 μL of the Phos-select solution in a loading/washing (L/W) solution: 80% ACN, 20% H$_2$O containing 10% TFA (2% TFA as final concentration). The columns were then washed with 20 μL of the L/W solution. The indicated quantities of phosphorylated peptides were diluted in 30 μL of the L/W solution and loaded into the column. The bound peptides were washed with twice 20 μL of the L/W solution and 10 μL of H$_2$O prior to the elution with 25 μL of the elution solution (1.3% NH$_4$OH), followed by 1 μL of ACN 30% before pooling those eluates and performing the desalting step.

Phosphopeptides Enrichment Using ZrO$_2$ Microcolumns. The ZrO$_2$ material was obtained from Sigma. Microcolumns were prepared by packing the columns into a GEloader with 2 μL of a suspension of ZrO$_2$ in ACN. The peptides were loaded in 50 μL TFA 2%. Washing, elution, and desalting steps were performed as for TiO$_2$ microcolumns.37

Phosphopeptides Enrichment Using SiO$_2$ Microcolumns. The SiO$_2$ material was obtained from Sigma. Microcolumns were prepared by packing the columns into a GEloader with 5 μL of a suspension of SiO$_2$ in ACN. The peptides were loaded in 50 μL TFA 2%. Washing, elution, and desalting steps were performed as for TiO$_2$ microcolumns.37

The validity of all the purification techniques has been checked on $\alpha$- and $\beta$-casein trypsic digests as well as other test phosphopeptides such as FLTEYVApSIR (Supporting Information, Figure S-2).

In Vitro Dephosphorylation by Alkaline Phosphatase Treatment. The phosphorylated peptides (final concentration 3 μM) were incubated with alkaline phosphatase (final concentration 0.1 U μL$^{-1}$) in 100 mM NH$_4$HCO$_3$ pH 8 at 37 °C for 30 min. The solution was acidified using 0.1% TFA prior to the desalting step.

Desalting and Concentration of the Samples. Microcolumns packed with Poros R3 resin were prepared as described previously.38 The bound peptides were eluted directly onto the MALDI target using 0.6 μL of the desired matrix solution.

Matrix Preparation for MALDI MS. CHCA (5 mg·mL$^{-1}$) was dissolved in 50% ACN, 50% H$_2$O containing 0.1% TFA. DHB (25 mg·mL$^{-1}$) was dissolved in 50% ACN, 50% H$_2$O containing 2% PA. The dried droplet method for target preparation was chosen.39

Mass Spectrometry. Positive and negative ions MALDI-TOF as well as positive ions MALDI-TOF-TOF mass spectra were recorded on the Applied Biosystems 4700 Proteomics Analyzer instrument. MALDI-TOF MS was performed in reflector mode (focus mass at 1800 u) near the threshold of laser fluence. Calibration was performed using external standards (Proteomix 4 LaserBio Laboratories). MALDI-TOF-TOF experiments were carried out in CID mode with gas (N$_2$, $\sim$2 × 10$^{-7}$ Torr) with collision energy of 1 keV. Typically the precursor ion (M + H)$^+$ was selected in a window (−5u, 5u) centered on the first isotope. Data Explorer version 4.6 software was used to analyze the spectra.

NMR Spectroscopy. The NMR experiments were recorded on a Bruker Avance III spectrometer operating at a 1H frequency of 500 MHz and equipped with a TCI cryoprobe. Spectra were recorded at 25 °C. NMR experiments were processed with Bruker TOPSPIN 2.0 program and 2D NMR spectra were analyzed with SPARKY.

NMR samples used for conformational studies contained 1 mM peptide in 550 μL of H$_2$O/D$_2$O (90:10 v/v) and 2% TFA, in 5 mm tubes. Sodium 2,2-dimethylsilapentane-5-sulfonate (Isotec, Sigma Aldrich) was used as an internal reference (0.1 mM) for chemical shift calibration. Homonuclear 2D TOCSY (Total Correlation Spectroscopy) and 2D ROESY (Rotating Overhauser Effect Spectroscopy) experiments were recorded with mixing times of 66 and 300 ms, respectively. The solvent signal was suppressed with a WATERGATE sequence. Natural abundance 2D 1H–13C HSQC (Heteronuclear Single Quantum Coherence Spectroscopy) spectra were recorded using gradient pulses for coherent selection. Structure calculations were performed with InsightII/Discover programs (Accelrys) using cff91 forcefield.

NMR Monitoring of the Peptide Affinities for TiO$_2$. Samples used to record 1D 1H NMR spectra contained 0.1 mM peptide in 170 μL of H$_2$O/D$_2$O (90:10 v/v), in 3 mm tubes. 1D WATERGATE spectra were recorded with a relaxation delay of 1 s, an acquisition time of 2.3 and 256 scans. 1D reference spectra of the desired peptides in 2% TFA were recorded. The peptide solution was then mixed with TiO$_2$ beads (~100 mg), and after mixing and gentle centrifugation, a 1D NMR spectrum of the supernatant.
was recorded. The supernatant was then mixed again with the same beads while NaOH was added up to pH > 10.5. A third NMR spectrum of the supernatant was recorded.

## RESULTS AND DISCUSSION

In this study, we report that the enrichment of a specific phosphopeptide is hindered by (i) the basic nature of its sequence, (ii) its folding because of specific amino acid residues and (iii) the presence of a biotin tag at the N-terminus.

**Description of the Initial Peptide.** The initial peptide (biot-Add) was designed to be used as a reporter of cytosolic localization by tracking and quantifying its phosphorylation in cells. Biot-Add peptide biotin-GGGGCFRTPSFLKK-NH2 is derived from the C-terminal sequence of the protein adducin, whose phosphorylation is regulated by phosphorylation of the S10 in a highly basic PKC recognition domain located between F6 and K14.40 Its PKC substrate properties were checked in vitro by incubation with purified PKC (Supporting Information, Figure S-3A) and in vivo in CHO-K1 cell lysates (Supporting Information, Figure S-4A).

**Behavior of biot-Add during Phosphopeptide Enrichment Techniques.** The ability to separate biot-pAdd from biot-Add in a mixture of different peptides was tested using several chromatography techniques.

The direct MALDI-TOF analysis of an equimolar mixture of three peptides, biot-pAdd, biot-Add and a control phosphopeptide FLTEYVARpSIR, was acquired before TiO2 enrichment in the positive mode (Figure 1A). In addition to the three corresponding protonated species (M + H)+ at m/z 1759.83, m/z 1679.86 and FLTEYVARpSIR (M + H)+ at m/z 1278.62 (internal control of TiO2 enrichment). (A) Direct analysis of the mixture and (B) after a TiO2 enrichment procedure. The peptide at m/z 1244.66 and those marked with * are deletion peptides arising from the solid-phase synthesis of biot-pAdd. G4 stands for GGGG. The experiments were repeated several times and the relative intensities of both ions (at m/z 1244.66 and 1278.62) varied. Figure 1B constitutes a representative spectrum highlighting the enrichment of the deletion peptide in opposition to biot-pAdd.

As expected, biot-Add was not recovered, since it is not phosphorylated. However, biot-pAdd was not enriched either, contrarily to the phosphopeptide (FLTEYVARpSIR) at m/z 1278.6 and the deletion phosphopeptide of biot-pAdd (Ac-FRTPpSFLKK-NH2). The total amount of the deletion peptide (m/z 1244.66) in the peptide mixture should be less than 1 pmol according to the purity of the corresponding synthetic phosphopeptide used (>95%). Even though the corresponding signal after TiO2 enrichment is weak, partly because of the low quantity, this deletion peptide is enriched while biot-pAdd is not.

As these results were unexpected for biot-pAdd, we tried several optimizations of the TiO2 protocol by modifying (i) the acid used in the loading solution, (ii) the loading pH (0.5, 1, 2.5 and 3), (iii) the loading time (5 and 30 min), (iv) the loading temperature (4 °C, room temperature and 60 °C) (see details in the Experimental Section, in the paragraph phosphopeptides enrichment using TiO2 microcolumns). These tests are summarized in Table S-1, Supporting Information. None of these modifications allowed the recovery of biot-pAdd and similar spectra to the one in Figure 1B were obtained (data not shown). An IMAC procedure proved also unsuccessful (data not shown). Enrichment with ZrO2, another common oxide with amphoteric properties because it was reported in the literature that basic peptides showed a binding affinity to SiO2.41 These tests were unsuccessful as with TiO2 or IMAC (data not shown).

To understand these results, we used 1D 1H NMR spectroscopy to identify which step (loading or elution) of the TiO2 chromatography was responsible for the poor recovery of biot-pAdd.

**1H NMR Study of the Affinity of biot-pAdd for the TiO2.** This protocol is performed in three steps: (i) A reference spectrum of biot-pAdd (10−4 mol·L−1) is recorded in TFA 2% (LS4). (ii) This solution is then mixed with TiO2 beads (~100 mg). The TiO2 beads are pellet, and the supernatant subjected to 1H NMR analysis. If the peptide is retained by TiO2, a significant decrease of the signal compared to the reference spectrum should be observed. (iii) This solution is mixed again...
with the same TiO₂ beads, and brought to the basic pH used for the elution (>10.5) with NaOH. After beads removal, the supernatant is analyzed by ¹H NMR. If the peptide elutes from TiO₂, the NMR peptide signal should reappear (with possible chemical shifts arising from pH modification). This protocol is designed to monitor the efficiency of the phosphopeptide enrichment with TiO₂ or IMAC. It enables (i) the identification of the step of the enrichment that fails (loading or elution) and (ii) the quantification and comparison of the affinities of different phosphopeptide sequences for the TiO₂ medium (under the same experimental conditions), which is not possible in a direct fashion with MS analysis. Moreover it allowed us to observe that the affinity for TiO₂ was much higher in TFA 2% than it was in the classical TFA 0.1%. Therefore apart in the case of Figure 1, we chose the loading solution LS4 for all sample preparations.

This protocol was first set up with the phosphopeptide FLTEYVA₅SIR which was correctly loaded and eluted from the TiO₂ microcolumns as shown with the MS data (Figure 1A, B). The peptide signal in the load spectrum (Figure 2B) has indeed disappeared compared to the reference spectrum (Figure 2A). ¹H NMR spectra of biot-pAdd were acquired under the same conditions as those described above (Figure 2C and D; for the sake of simplicity, only the reference and load spectra are shown). Since no significant difference was observed between the reference and load spectra, it can be concluded that biot-pAdd has a very low affinity for TiO₂ beads.

Understanding the Lack of Affinity of biot-pAdd for TiO₂
Biot-pAdd contains three basic residues (R⁺, K¹³, K¹⁴) and its calculated isoelectric point is 10.6 (GPMAW 6.1 software).

IMAC and TiO₂ chromatography methods, however, are biased in favor of acidic peptides. Therefore, the number of positive charges in biot-pAdd might be too high under the acidic conditions prevailing in the loading step, leading to charge repulsions with the positively charged TiO₂. Strong "covalent-like" interactions between R residues and phosphate groups have been reported, which likely exist in the biot-pAdd peptide, thus explaining the low availability of the phosphate group for TiO₂ interaction. We have tested this hypothesis by synthesizing an analog of biot-pAdd with two additional acidic E residues (biotE₂-Add) to decrease the net number of positive charges in the peptide.

Since we observed that a deletion phosphopeptide lacking the N-terminal [biotin-G₄] tag and C⁻ residue was enriched with TiO₂ (Figure 1B), we assumed that either the tag or the C residue might hinder the interaction of the phosphate group with TiO₂.

Therefore, we also synthesized an analog of biot-pAdd in which the [biotin-G₄] tag was added to the C-terminal end (pAdd-biot).

To define which of these two parameters (high basicity of the sequence and position of the biotin tag) was the most detrimental to TiO₂ enrichment, we also compared the TiO₂ affinities of these two analogs biotE₂-pAdd and pAdd-biot.

Influence of the Net Charge of the Peptide on TiO₂ Enrichment
BiotE₂-Add (biotin(O₂)₅EGGGGECFRTPSFLKK-NH₂) was designed taking two major constraints into account: (i) the two E residues should be kept apart in the peptide sequence because of the strong tendency of this amino acid to cyclization during synthesis and (ii) the PKC recognition motif should be retained. This restriction limits the design of new peptide substrates to modifications at both termini. We first checked that BiotE₂-Add was still a PKC substrate with CHO-K₁ cell lysates. After a direct MALDI-TOF analysis (Figure 3A), a major ion at m/z 1969.94 is observed, corresponding to the protonated form of biotE₂-Add, as well as a weak intensity ion at m/z 2049.92 corresponding to the phosphorylated peptide. This latter could be highly enriched after a TiO₂ procedure (Figure 3B). As expected, the non phosphorylated form is also retained on TiO₂, but to a lesser extent, through the acidic residues. Adding two acidic residues to the biot-pAdd sequence has decreased its net charge from +2 to 0 at acidic pH (loading pH) and drastically improved its affinity for TiO₂. This is consistent with the under-representation of highly basic and/or positively charged phosphopeptides already reported in phosphoproteomics studies. However, the total lack of affinity of biot-pAdd for TiO₂ cannot be explained only by the presence of the basic recognition motif of the protein kinase C. These types of basic phosphopeptides are in fact quite frequently identified in the data sets of phosphoproteomics studies, even if they are less represented than the three other kinds of kinase substrates (acidophilic, proline-directed, and others).

Effect of the Position of the [Biotin-G₄] Tag on TiO₂ Enrichment
pAdd-biot (Ac-CFRTP₅SFLKKGGGG-NH-(CH₂)₂-NH-biotin) was synthesized with a biotinylated resin (see Experimental Section) that adds a supplementary flexible linker (NH-(CH₂)₂-NH) between the biotin and the first coupled G residue. It was first checked that Add-biot was still a PKC substrate in vitro by incubation with purified PKC (Supporting Information, Figure S-3B) and in vivo in CHO-K₁ cell lysates (Supporting Information, Figure S-4B). The phosphorylated pAdd-biot peptide is successfully purified and characterized using either TiO₂, IMAC (Supporting Information, Figure S-5B), ZrO₂ (Supporting Information, Figure S-SC) or SiO₂ (Supporting Information, Figure S-1C).
S-SD) microcolumns in conjunction with MALDI-TOF detection, although the recovery was not total as shown by NMR studies. Indeed, after incubation of pAdd-biot with TiO2 in the loading conditions (Figure 2F), a significant but incomplete decrease of the peptide signal compared to the reference spectrum (Figure 2E) was observed. Based on the 1H NMR data, we conclude that the affinity of pAdd-biot for TiO2 is greater than that of biot-pAdd but remains lower than that of FLTEYVApSIR under the same conditions (Figure 2A and B), presumably because of the basic character of the peptide, as discussed previously. Interestingly, the position of the [biotin-G4] tag had a strong influence on the affinity of the peptide for TiO2.

To explain the difference of affinity between biot-pAdd and pAdd-biot peptides, we assumed that the N-terminal [biotin-G4] tag could also restrain the accessibility of the phosphate group. Two results support this hypothesis: (i) phosphorylation by PKC in cell lysates is more efficient for Add-biot than for biot-Add (Supporting Information, Figure S-4A and B), suggesting that the serine residue is less accessible already in the non phosphorylated form of biot-Add (data not shown). (ii) In vitro dephosphorylation of biot-pAdd and pAdd-biot using an alkaline phosphatase is incomplete for both peptides, although the enzyme was able to completely dephosphorylate other phosphopeptides in the same conditions (data not shown). Therefore, the phosphate group is still not fully accessible in pAdd-biot, which could explain why the affinity of the peptide for TiO2 is still low.

NMR Conformational Studies. To determine whether conformational effects could contribute to the shielding of the phosphate group, we analyzed the conformations of biot-Add, biot-pAdd, and pAdd-biot peptides by NMR spectroscopy, using similar conditions to those used in the loading steps (aqueous solution at very acidic pH, TFA 2%). Sequence-specific resonance assignments (provided as Supporting Information Tables S-2 to S-4) were obtained by the analysis of homonuclear 2D TOCSY (through bond) and 2D ROESY (through space) correlation experiments. The conformation of the backbone of the three peptides (biot-Add, biot-pAdd, and pAdd-biot) was analyzed using the following NMR parameters: the chemical shift deviations (CSDs) of Hα and Cα resonances with respect to random coil values, the comparison of intraresidual and sequential Hα-HN ROE correlation intensities and the values of 3JHN-Hα coupling constants. The CSDs show near zero values throughout the peptide sequence indicating that the peptides do not adopt stable regular secondary structures in highly acidic aqueous solution. This is also confirmed by the distribution of 3JHN-Hα coupling constant values (≈ 6–7.5 Hz) and the pattern of intraresidual and sequential ROEs between HN and Hα protons. However, a medium-range ROE correlation was observed between the Hβ proton of T8 and the amide proton of S10 in the three peptides, indicating a local structuration in segment 8–10. It can thus be inferred that the presence of the p9 residue, known to promote the formation of turns, restrains the peptide conformational space in the vicinity of the S10 residue.

Furthermore, the comparison of R7 chemical shifts between phosphorylated and non phosphorylated peptides also provides valuable information. Indeed, whereas the two methylenic CH2 δ proton of R7 are almost equivalent in biot-Add peptide, they show significant chemical shift differences in both biot-pAdd and pAdd-biot peptides. Thus the phosphorylation of S10 residue induces a chemical environment difference that is sensed by the...
R side chain, supporting the formation of a salt bridge between R and pS. In contrast, no significant chemical shift differences were observed for the CH2 protons of the basic K and K residues in the three peptides. Conformations of biot-pAdd peptide were calculated by restrained molecular dynamics. The analysis of NMR structures shows that β-turn or γ-turn conformations involving P9 residue can account for the observation of H2-T8-HN S ROE and lead to a close proximity of pS and R7 side chains (Figure 4).

Strong "covalent-like" interactions between R residues and phosphate groups have been reported and may contribute to further decrease the availability of the phosphate group in biot-pAdd peptide for TiO2 interaction. Such electrostatic interaction between guanidinium and phosphate groups is expected to be extremely strong as reported in the examples of a covalent-like interaction between a basic epitope containing adjacent arginine residues and an acidic epitope containing a phosphorylated serine or a phosphoserine-arginine interaction stabilizing a leucine zipper coiled coil. Moreover, the role of phosphorylated residues in peptide—peptide non covalent complexes formation was also reported. In our case, the restrained conformational space caused by the P residue might promote this guanidinium/phospho-sphere interaction. This could explain why a local structure can be observed even in very acidic conditions (TFA 2%, pH < 1), which do not favor any folding especially in the case of such a short peptide. This type of non covalent interaction (salt bridge) in solution can be maintained in the gas phase. The deleterious effect of the phosphorylation has been previously described in the Electron Capture Dissociation (ECD) of peptide ions. Improved sequence coverage was only achieved by increasing the ECD electron energy. In the case of the initial peptide, biot-pAdd, the local folding might bring the N-terminus in close proximity of the phosphate group and when the biotin-G4 tag is present, the phosphate group would be shielded and no longer accessible to TiO2. It is usually assumed that the incorporation of a biotin tag, whose steric hindrance is similar to that of an amino acid, should have no or little effect on the peptide properties. This is especially true if this biotin is separated from the peptide sequence by a sufficiently long and flexible linker such as for example an aminopentanoic acid or four G residues. In this case, however, the localization of the tag had unforeseen consequences.

We identified two origins for the lack of affinity of the studied phosphopeptide biot-pAdd for the TiO2: the steric bulk of the N-terminus and the high basicity of the sequence. In order to rank the influence of those two parameters, we compared the TiO2 affinity of the two phosphorylated analogs, pAdd-biot (acetylated at the N-terminus and highly basic) and biotE2-pAdd (with the biotin tag at the N-terminus and a reduced basicity). The direct MALDI-TOF analysis of an equimolar mixture of these two peptides was acquired in the positive mode (Figure 5A). In addition to the two corresponding protonated species (M + H) at m/z 1844.92 and m/z 2017.96, the deletion peptide at m/z 1244.68 (Ac-FRTPpSFLKK-NH2), as previously quoted in the text, was also detected. It also results from the synthesis of biotE2-pAdd. The classical TiO2 enrichment was performed (40 pmol of the total peptide mixture) in the loading solution LS4 (Figure 5B). BiotE2-pAdd is much more enriched than pAdd-biot and the deletion peptide is now hardly detected. We also analyzed the flow through of the microcolumn, which contains molecules that did not adsorb on TiO2 (figure 5C). In this flow through, mostly the deletion peptide and pAdd-biot are detected. By comparison with Figure 5A we observe that the deletion peptide has an affinity for TiO2 inferior or comparable to pAdd-biot. The TiO2 affinities rank as follow: biotE2-pAdd > pAdd-biot > deletion peptide > biot-pAdd. We can thus conclude that the crucial parameter that hampered the TiO2 enrichment of those model peptides is first the high basicity of the sequence, followed by the steric bulk of the N-terminal.

**CONCLUSIONS**

In the goal of developing a methodology to follow intracellular PKC phosphorylation after delivery by a cell penetrating peptide, we have observed an inefficient enrichment using classical techniques. Phosphopeptides with more than two basic residues, such as the PKC products analyzed in this study, have a lower affinity for TiO2 or IMAC and are therefore difficult to detect by MS analysis. Similar phosphopeptides can easily be found in tryptic digests for phosphoproteins in which the phosphorylated residue is located close or even next to a K or R residue. The local environment of these residues, which should be recognized by the digestion enzyme, is therefore acidified and the hydrolysis can be impaired, leading to missed cleavages. Similar problems can be found when using digestion enzymes with other specificities or CNBr chemical cleavage for the identification and characterization of phosphoproteins. These results have relevance to phosphoproteomic studies because they can partly explain the under-representation of basic phosphopeptides in phosphoproteomics data sets. It should be noted that the lack of enrichment described in this study is partly dependent on the presence of the Pro residue next to the pSer. This residue at the p - 1 position is not classical for PKC substrates; therefore the findings might be different for other basophilic kinase substrates. However, since several basic residues are common for all PKC substrates and the high basicity of the sequence was found herein the most deleterious for accurate TiO2 enrichment of the phosphopeptides, we expect that such problems would arise with other PKC substrates and more generally in the identification of PKC substrates.
of phosphopeptides in phosphoproteomics studies using IMAC or MOC chromatography.

**ASSOCIATED CONTENT**

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

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**REFERENCES**


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