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TECHNICAL BRIEF

Fast track to a phosphoprotein sketch – MALDI-TOF characterization of TLC-based tryptic phosphopeptide maps at femtomolar detection sensitivity

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Tryptic phosphopeptide mapping by TLC on microcrystalline cellulose has been a convenient method to get a fast and highly reproducible overview of the number of phosphopeptides present in any given ^{32}P-labeled phosphoprotein. This method also provides an immediate presentation of the relative phosphorylation stoichiometry between individual phosphopeptides. However, so far, traditional tryptic phosphopeptide maps have not been useful for phosphoproteomics applications, as the S/N has been very poor, due to the large number of quenching substances and contaminants present on cellulose plates. In this study, we present a rapid and easy method for phosphopeptides identification from 2-D phosphopeptide maps (2-D-PPMs). We obtain improved sensitivity (femtomole levels) upon MALDI-TOF MS analysis of phosphopeptides extracted from 2-D-PPMs. Using this approach we could confidently characterize the major phosphorylation sites of *in vivo* and *in vitro* ^{32}P-labeled proteins.

Keywords:
Edman degradation / Mass spectrometry / Phosphorus-32 labeling of proteins / Protein phosphorylation / Two-dimensional phosphopeptide mapping

Protein phosphorylation has been established as a key regulator of protein functions, and an estimated 1/3 of the proteome includes proteins that may have one to numerous phosphorylation sites that are reversibly phosphorylated [1]. While the characterization of the whole phosphoproteome will be an immensely difficult task, often, the reliable characterization of the dynamic phosphorylation of any given phosphoprotein *in vivo* is a task that imposes multiple challenges. With respect to MS, it has been particularly difficult to obtain sample preparation techniques that would yield an altogether quantitative representation of the complete phosphopeptide mixture. Recent advances in sample preparation techniques have led to many improvements in phosphopeptides purification methods [2, 3]. However problems with adsorptive losses and/or nonspecific binding of nonphosphorylated peptides still remain. This often contributes to reduced sensitivity and lost accuracy when it comes to the presentation of the relative abundance of any given phosphopeptide. Furthermore, phosphorylated peptides are often more difficult to detect and analyze by MALDI-MS than the corresponding unmodified species [4, 5, 6, 15].

Tryptic TLC-based phosphopeptide maps on microcrystalline cellulose have been extensively used to characterize the number of ^{32}P-labeled phosphopeptides in a given phosphoprotein. The advantage of this method is that it provides a rapid, reproducible, and sensitive presentation of the number of phosphopeptides present in a protein, while at the same time, giving an indication of the relative phosphate stoichiometry between different phosphopeptides in the protein. Furthermore, it provides a convenient "fingerprint"

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Abbreviations: 2,5-DHB, 2,5-dihydroxybenzoic acid; **2-D-PPMs**, 2-D phosphopeptide maps; **IF**, intermediate filament; **PA**, phosphoric acid

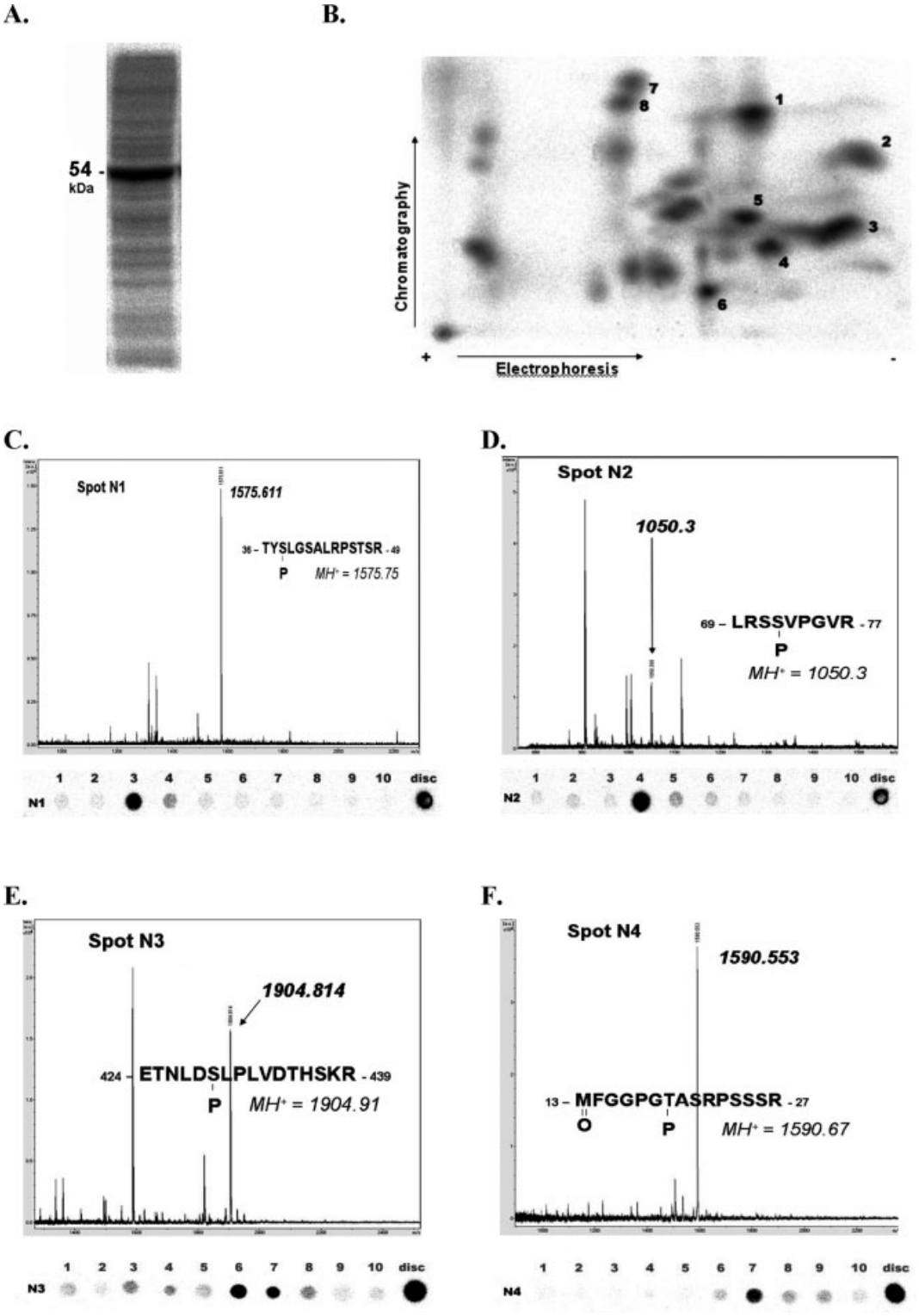
of the phosphorylation pattern of a kinase(s) acting on the protein *in vivo* or *in vitro*. Another advantage of the method is that there is very little or no loss of sample during electrophoretic and TLC separations, and although the yielded spots may contain one or a few other nonphosphorylated peptides migrating at the same point, the ratio between phosphorylated and nonphosphorylated peptides is usually quite favorable. Nevertheless, phosphopeptide maps have not been considered useful as a starting point for mass-spectrometric phosphopeptide characterization, as the S/N has been usually very poor, due to the high number of quenching substances and contaminants present on the TLC plates, and due to the low detection sensitivity of mass spectrometers in the past. Edman degradation of ^{32}P-labeled phosphopeptides extracted from 2-D phosphopeptide maps (2-D-PPMs) is a simple, confident, and sensitive method to determine the exact position of the phosphorylated amino acid in the peptide sequence starting from N-terminus. In contrast, MS/MS fragmentation of the same peptide can often be rather poor and, consequently, of low information value for determination of the precise phosphorylation site. Obviously, TLC-based phosphopeptide mapping has certain limitations, as it involves ^{32}P-labeling. For example, ^{32}P-labeling is cumbersome to perform in tissues, which cannot be simply grown as cell cultures in ^{32}P-containing medium (*in vivo* ^{32}P-labeling), although *in situ* ^{32}P-labeling of proteins in tissues has been demonstrated [14].

Nowadays, there are two most widely used matrices for peptides, analysis by MALDI-MS: CHCA and 2,5-dihydroxybenzoic acid (2,5-DHB). The latter matrix is regarded as a "cool" matrix for MALDI; in other words, it leads to the formation of molecular ions with low internal energy, which remain intact during the MS analysis. This matrix produces rather heterogeneous and large crystalline deposits. In contrast, CHCA matrix generates more homogeneous matrix/analyte deposits known as a "hot" matrix, which leads to significant decomposition of phosphopeptide ions during MS analysis (elimination of the phosphate group −80 Da or of phosphoric acid (PA) −98 Da). CHCA matrix ionizes itself and so do its sodium and potassium adducts. Matrix clusters and metal ion adducts often interfere with peptide ionization and peptide mass spectrum interpretation [4, 7]. The use of special matrix additives (comatrices; such as ammonium salts, organic, and inorganic acids) in MALDI-MS, is a commonly used approach to enhance the phosphopeptide response [4, 7, 8, 15]. Kjellstrom and Jensen [4] found that the addition of 1% PA to 2,5-DHB produced significantly improved phosphopeptide mass spectra upon MALDI. They hypothesized that PA anion has a "salting out" effect on phosphopeptides during the cocrystallization of analyte and matrix molecules. This leads to more efficient incorporation of phosphopeptides into the growing 2,5-DHB crystals. In this way, these matrix/analyte deposits are more susceptible to ionization [4]. We could confirm that the use of 2,5-DHB + 1% PA leads to significantly improved detection sensitivity upon MALDI-MS of a synthetic phosphopeptide as compared with CHCA matrix (Supplementary Fig. 1). In the present study, we wanted to test whether the approach of adding PA as comatrix to 2,5-DHB would be a suitable approach to enhance the phosphopeptide response from TLC-based phosphopeptide maps. By using this approach, we developed a simple concept for sensitive overall phosphorylation site characterization of both *in vitro* and *in vivo* ^{32}P-labeled phosphoproteins.

We first tested the robustness of the approach by analyzing the phosphorylation sites of *in vivo*-derived material, as the analysis of *in vivo*-based proteins is always significantly more challenging than the analysis of purified proteins that have been phosphorylated *in vitro*. As an example, we decided to examine the intermediate filament (IF) protein vimentin immunoprecipitated from HeLa cells, essentially as described previously [5, 14]. Briefly, 60–70% confluency HeLa cells in 100 mm × 20 mm cell culture dishes were preincubated for 2.5 h with 0.3 m*Ci*/mL ^{32}P-ortophosphate (ICN Pharmaceuticals), in phosphate-free minimum essential medium Eagle (MEME; Sigma-Aldrich) with 10% fetal calf serum. This preincubation time saturates the cellular ATP pools with ^{32}P. After that, cells were treated for 30 min with 50 nM of the phosphatase inhibitor calyculin-A (Sigma-Aldrich). The cells were washed with ice-cold PBS and lysed in SDS-buffer, containing: 20 mM Tris-HCl (pH 7.2), 5 mM EGTA, 5 mM EDTA, 0.4% SDS, 10 mM sodium pyrophosphate, 1 mM PMSF, and Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics). The cell lysate was boiled for 5 min and sonicated for 20 s with a probe sonicator. For immunoprecipitation, cell lysate was diluted 1:10 with RIPA-buffer, containing 20 mM HEPES (pH 7.4), 140 mM NaCl, 10 mM sodium pyrophosphate, 5 mM EDTA, 0.4% NP-40, 1 mM PMSF, and Complete Protease Inhibitor Cocktail Tablets [5]. Monoclonal antivimentin antibody V9 (V6630, Sigma-Aldrich) and protein-G beads were used to immunoprecipitate the protein. 1-D electrophoretic separation of the immunoprecipitate was performed with 10% acrylamide SDS-PAGE [9] followed by autoradiography with an imaging plate using Fujifilm BAS-1800 Bioimaging analyzer (Fig. 1A). In-gel tryptic digestion of the ^{32}P-labeled vimentin (54 kDa band), followed by 2-D-PPM on a TLC sheet were carried out as previously detailed [10, 11]. Briefly, the labeled vimentin band (Fig. 1A) was excised from the dried gel and in-gel digested with sequencing grade trypsin (Promega). The tryptic digest was carefully applied on a cellulose TLC sheet (20 cm × 20 cm, Merck KgaA) and separated in two dimensions by electrophoresis and TLC. The first dimension – electrophoresis – was performed in a pH 1.9 buffer (formic acid – 2.3%, acetic acid – 2.9% v/v) at 750 V for 1.5 h using the Hunter Thin Layer Peptide Mapping System, model # HTLE-7000 (C.B.S. Scientific). The sheet was dried and ascending TLC in the second dimension performed for 13–14 h in a chromatography tank saturated with a mobile phase containing 30% water, 37.5% *n*-butanol, 7.5% acetic acid, and 25% pyridine. The sheet was dried and the ^{32}P-phosphopeptides were visualized by autoradiography. As

previously observed [10], phosphorylation of vimentin yielded a relatively high number of different phosphopeptides (Fig. 1B).

The next step was to choose major phosphopeptides from the 2-D-PPM. Based on the autoradiography, several vimentin ^{32}P-phosphopeptides (peptides 1–8; Fig. 1B), were



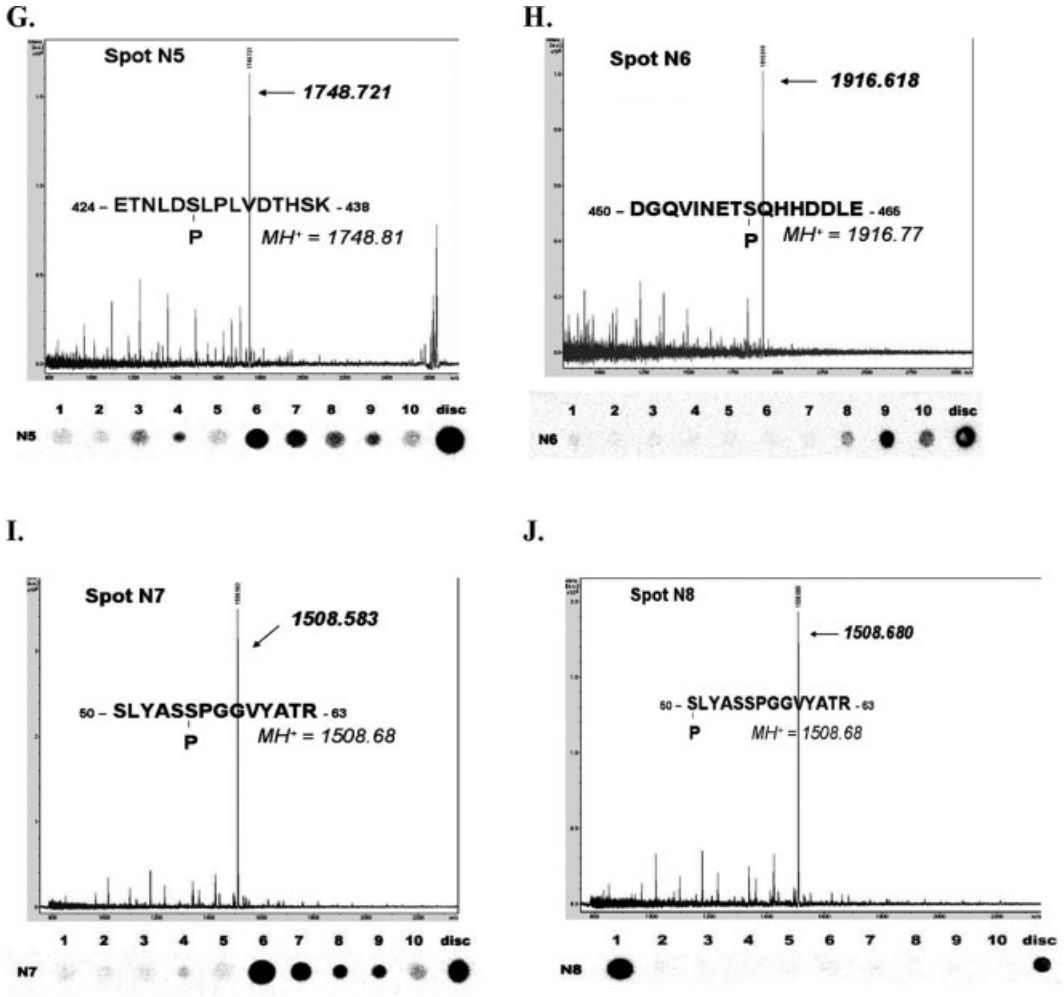


Figure 1. SDS-PAGE and 2-D-PPM of *in vivo* phosphorylated vimentin and identification of the phosphorylation sites on several resulting phosphopeptides. (A) Autoradiography of 10% acrylamide SDS-PAGE shows ^{32}P-labeled vimentin as a 54 kDa band. (B) Tryptic phosphopeptide map of vimentin phosphorylated *in vivo* (directions of electrophoresis and ascending chromatography are indicated). Autoradiography of the TLC plate with the tryptic peptides separated in two dimensions, demonstrates that the *in vivo* ^{32}P-labeled material has several major phosphopeptides. The analyzed spots are marked 1–8. The phosphorylation sites were determined by simple MALDI-TOF MS analysis of the peptides 1–8 eluted from the TLC plate and by manual Edman degradation. Each spectrum shown in (C–J) contains one intense peak, which corresponds to the mass of the phosphorylated form of one of the human vimentin tryptic peptides. Upon MALDI-MS analysis of the phosphopeptide sample eluted from the 2-D-PPM, we usually detect other peaks that correspond to nonphosphorylated peptides (trypsin autodigestion peptides and nonphosphorylated peptides from the analyzed protein that comigrate during 2-D-PPM together with the phosphopeptide of interest). If the concentration and/or the ionization efficacy of a phosphopeptide is very low, then we often detect various polymer peaks, which probably originate from cellulose of a TLC plate. The identity of the phosphopeptides was checked by manual Edman degradation. Ten Edman degradation cycles were performed, the label at each cycle was spotted on a Whatman filter paper, and the paper was measured on a Fuji phosphor imager plate. Numbers on top of the figure indicate cycle (amino acid) number starting from N-terminus of a peptide. A significant release occurs at certain cycle, which corresponds to the position of either Ser or Thr in the peptide being sequenced.

recovered from the TLC plate by carefully removing the microcrystalline cellulose matrix containing the phosphopeptide from the plastic support with a surgical blade. The cellulose powder was scraped off into an Eppendorf tube and then eluted twice with 100 μL of 30% ACN, 0.1% TFA solution. First elution: at 55°C, 50 min with occasional vortexing. The sample was then centrifuged at 10 000 × *g* for 2 min, and the supernatant was collected. Second elution was done by short vortexing with 100 μL of the eluate, followed by centrifugation, after which the supernatants were pooled together. Collected supernatant (about 200 μL) was dried in vacuum centrifuge. The phosphopeptides were dissolved by vortexing with 4–5 μL of 0.1% TFA solution. About 0.5 μL of 20 mg/mL DHB matrix in 50% ACN, 1% PA was mixed with

0.5 μL of sample directly on a MALDI target plate (dried-droplet method) and air-dried. Note, the method we are using does not involve any desalting of peptides extracted from a particular 2-D-PPM spot. In this way we avoid possible sample losses due to an additional purification step after separation of phosphopeptides. MALDI-TOF MS analysis of the chosen phosphopeptides was performed on Bruker Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics) in positive reflector mode. Ions were generated by irradiation of analyte/matrix deposits by a Smartbeam Nd:YAG laser, and, after time-delayed extraction, analyzed with an accelerating voltage of 25 kV. Ion detection range was 600–4000 *m/z*. Results are shown in Fig. 1C–J. In MALDI-MS, a peptide "M" being analyzed forms usually a singly protonated ion $[MH]^+$. If a MALDI mass spectrum peak is 80 Da higher than $[MH]^+$ of a predicted unmodified tryptic peptide, it often indicates phosphorylation. Each spectrum shown in Fig. 1C–J contains one of the *m/z* values, which corresponds to the *m/z* value of the phosphorylated form of one of the human vimentin tryptic peptides (Table 1). Upon MALDI-MS analysis of the phosphopeptide sample eluted from the 2-D-PPM, we usually detect other peaks that correspond to nonphosphorylated peptides (trypsin autodigestion peptides and nonphosphorylated peptides from the analyzed protein that comigrate during 2-D-PPM together with the phosphopeptide of interest). If the concentration and/or the ionization efficacy of a phosphopeptide is very low, then we detect various polymer peaks, which probably originate from cellulose of a TLC plate.

In order to determine the precise site of phosphorylation in a peptide, we used Edman sequencing of the phosphopeptide bearing ^{32}P-phosphogroup. Manual Edman degradation was performed essentially as described previously [5, 10, 14]. Phosphopeptides were immobilized on arylamine membrane disks (Sequelon-AA membranes; Applied Biosystems, Foster City, CA, USA) using water-soluble carbodiimide. The Sequelon-AA membranes consist of a PVDF matrix that has been derivatized with arylamine groups. The C-terminal and side chain carboxyl groups of peptides react with the arylamine groups of the membrane *via* carbodiimide activation. When the immobilized peptides were subjected to manual Edman degradation, release of the ^{32}P-label on the corresponding cycle is consistent with the position of Ser, Thr, or Tyr amino acids on the assigned phosphopeptide (Fig. 1C–J).

Table 1. The amino acid sequences of human vimentin phosphopeptides detected from 2-D-PPM of *in vivo* ^{32}P-labeled vimentin by MALDI-MS (spots of ^{32}P-peptides are shown in Fig. 1B)

Spot number in 2-D-PPM	Peptide sequence	N-terminus–C-terminus	Theoretical MH^+	Observed MH^+
1	TY**pS**LGSALRPSTSR	36–49	1575.75	1575.61
2	LRS**pS**VPGVR	69–77	1050.30	1050.30
3	ETNLD**pS**LPLVDTHSKR	424–439	1904.91	1904.81
4	MoxFGGPG**pT**ASRPSSSR	13–27	1590.67	1590.55
5	ETNLD**pS**LPLVDTHSK	424–438	1748.81	1748.72
6	DGQVINET**pS**QH HDDLE	450–465	1916.77	1916.62
7	SLYAS**pS**PGGVYATR	50–63	1508.68	1508.58
8	**pS**LYASSPGGVYATR	50–63	1508.68	1508.68

In turn, phosphopeptides extracted from 2-D-PPM can also be analyzed by MS/MS sequencing. MS/MS sequencing is a fast and easy way to locate the position of the phosphomoiety in the peptide. However, MS/MS fragmentation depends much on nature of the peptide, and often can be very poor and consequently with low information value. In such a case, and/or when several serine and threonine residues present in the phosphopeptide, it is rather difficult and ambiguous to deduce the exact position of the phosphorylated amino acid (such as in Figs. 1I and J; MS/MS data not shown). On the contrary, Edman degradation unambiguously shows ^{32}P-signal in the corresponding amino acid cycle even when tiny amounts of the ^{32}P-phosphopeptide are being sequenced.

As mentioned above, the abundance, relative quantity, and approximate number of different phosphopeptides present in the digest can be visually estimated from 2-D-PPMs, indicating that the phosphorylated sample has a relatively high number of peptides phosphorylated to variable degrees (Figs. 1B, 2B). However, while phosphopeptides do not undergo further modification during 2-D-PPM [12], a single phosphorylation site can still generate several spots on a 2-D-PPM, often due to miscleavages generating several variants of a given tryptic peptide. Therefore, the true number of phosphorylation sites on the digested protein usually less than the number of spots observed in 2-D-PPM [12].

The third step was to test the sensitivity for the detection of phosphopeptides extracted from 2-D-PPM. To this end, we used *in vitro* phosphorylated material, as it is difficult to reliably quantify *in vivo* phosphorylated material. We performed ^{32}P *in vitro* labeling of vimentin (from purified hamster IF preparation) with cAMP-dependent protein kinase (PKA) exactly according to previously described protocols [5, 14], thereby yielding a highly phosphorylated protein. This *in vitro* ^{32}P-phosphorylation assay was used both for quantitation of phosphopeptides detection level, and as an illustratrative example of how kinase-specific sites could be determined *in vitro*. The phosphopeptide used for quantification represents the main PKA-specific phosphorylation site of vimentin (peptide 1 in Fig. 2B). We were able to confidently detect the phosphopeptide (*m/z* 1575.7; see Fig. 2C) extracted from 2-D-PPM when femtomole levels of it were loaded onto MALDI target plate. The sample loaded on SDS-PAGE (^{32}P-phosphorylated IF preparation) was calculated to contain 500 fmol vimentin (Fig. 2A). This amount of ^{32}P-phosphorylated vimentin was quantified densitometrically using "MCID-M5+" software (Imaging Research), by measuring Coomassie-stained proteins separated on SDS-PAGE (with a standard curve generated by known amounts of BSA). 1-D electrophoretic separation of *in vitro* PKA-phosphorylated IFs

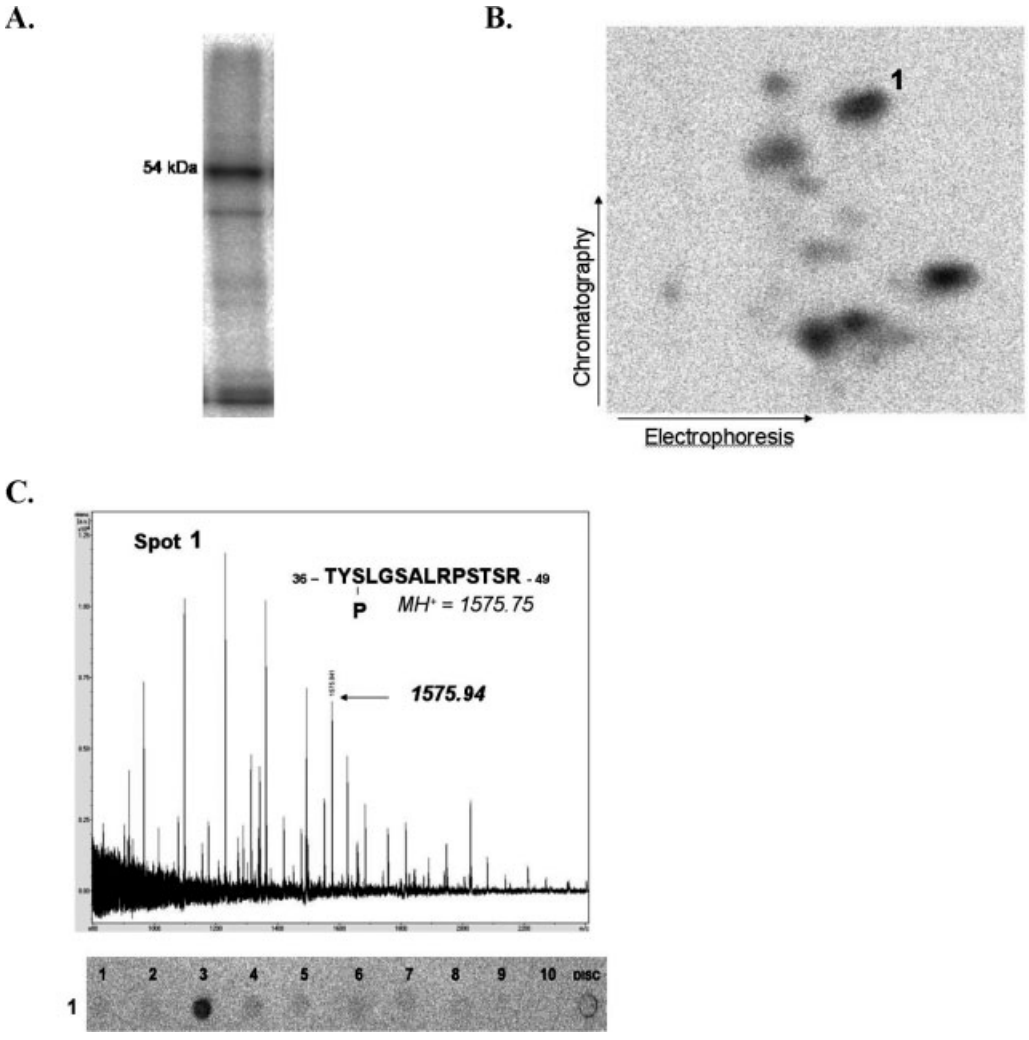


Figure 2. SDS-PAGE and 2-D-PPM of *in vitro* PKA-phosphorylated vimentin. (A) Autoradiography of 10% acrylamide SDS-PAGE shows ^{32}P-labeled vimentin (500 fmol) as a 54 kDa band. (B) Tryptic phosphopeptide map of 500 fmol vimentin phosphorylated *in vitro* with PKA (directions of electrophoresis and ascending chromatography are indicated). Autoradiography of the TLC plate with the tryptic peptides separated in two dimensions, demonstrates that the *in vitro* ^{32}P-labeled material has several major phosphopeptides. Peptide "1" was chosen to show the sensitivity limit of the mass spectrometer. This particular spot gives the best S/N upon MS analysis. Probably, the phosphorylation stoichiometry, and consequently the quantity is the highest for that peptide. Other spots were also considered for MS analysis but due to either low phosphopeptide content, or interference with contaminating TLC polymers were not detected clear enough. (C) MALDI-TOF MS analysis of the peptide 36-TY**pS**LGSALRPSTSR-49 eluted from spot "1": 40 fmol was loaded onto the MALDI target plate (calculations are based on the assumption that all molecules of the vimentin are phosphorylated). Various polymer peaks of relatively high intensity, which probably originate from cellulose of a TLC plate, are seen in the figure.

was performed with 10% acrylamide SDS-PAGE [9] followed by autoradiography with an imaging plate as described above (Fig. 2A).

In-gel tryptic digestion of the ^{32}P-labeled vimentin (54 kDa band), followed by 2-D-PPM on a TLC sheet were carried out as previously detailed (see above) (Fig. 2B). Based on 2-D-PPM autoradiography, one of the major vimentin phosphopeptides (spot 1, Fig. 2B) was recovered from the TLC plate and analyzed by MS and Edman degradation as described above (Fig. 2C). For MALDI-MS analysis, we loaded an aliquote corresponding to approximately 40 fmol of the phosphopeptide onto the MALDI target plate (as calculated on the basis of the original amount of protein loaded on the gel). Note, this loading amount was calculated assuming that *in vitro* phosphorylation level is 100% (*i.e.*, assuming that all vimentin in the reaction mixture is completely phosphorylated by PKA). However, it is known that the real level of *in vitro* phosphorylation is less than 100% and in best assay conditions can reach 50–60%. As we mentioned above, if the concentration and/or the ionization efficacy of extracted

phosphopeptide is very low, we often detect various polymer peaks, which probably originate from cellulose of a TLC plate. These interfering peaks are seen in the spectrum (Fig. 2C).

As the amount of the peptide was calculated to be in the femtomole range, it is worth noting that in a previous study [13], at least a picomole of phosphopeptide in a spot extracted from a 2-D-PPM was required for confident tyrosine phosphorylation characterization using a triple quadrupole LC-ESI-MS/MS instrument.

In summary, we describe a simple method for confident determination of the overall phosphorylation sites on ^{32}P-labeled proteins. On the basis of the methodological concept described above and using the known amino acid sequence of the protein, one can easily deduce the location of the phosphorylated amino acid. By using this method, we have been able to locate major and less prominent *in vivo* phosphorylation sites of both high and low abundance proteins, for example, the caspase-8 inhibitor c-FLIP (both long and short isoforms), the transcriptional regulator PAR-4, and the karyoskeletal proteins lamins A/C (data not shown), and the cytoskeletal protein vimentin. The relative simplicity of the method, the uncomplicated instrumentation required, and last but not the least, the femtomole sensitivity of phosphopeptide detection from a complex phosphopeptide/peptide mixture should make this approach especially attractive to laboratories that do not have access to an array of advanced mass spectrometric techniques.

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References

[1] Hubbard, M. J., Cohen, P., *Trends Biochem. Sci.* 1993, *18*, 172–177.

[2] Larsen, M., Thingholm, T., Jensen, O., Roepstorff, P., Jorgensen, T., *Mol. Cell. Proteomics* 2005, *4*, 873–86.

[3] Kokubu, M., Ishihama, Y., Sato, T., Nagasu, T., Oda, Y., *Anal. Chem.* 2005, *7*, 5144–5154.

[4] Kjellstrom, S., Jensen, O. N., *Anal. Chem.* 2004, *76*, 5109–5117.

[5] Kochin, V., Pallari, H. M., Pant, H., Eriksson, J. E., *Methods Cell Biol.* 2004, *78*, 373–409.

[6] Mann, M., Jensen, O. N., *Nat. Biotechnol.* 2003, *21*, 255–261.

[7] Zhu, X., Papayannopoulos, I. A., *J. Biomol. Tech.* 2003, *14*, 298–307.

[8] Asara, J. M., Allison, J., *J. Am. Soc. Mass Spectrom.* 1999, *10*, 35–44.

[9] Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., Wingfield, P. T., *Current Protocols in Protein Science*, John Wiley & Sons, Inc., Hoboken, USA 1995.

[10] Eriksson, J. E., He, T., Trejo-Skalli, A., Härmälä-Braskén, A. S. *et al.*, *J. Cell Sci.* 2004, *117*, 919–932.

[11] Boyle, W. J., van der Geer, P., Hunter, T., *Methods Enzymol.* 1991, *201*, 110–149.

[12] Affolter, M., Watts, J. D., Krebs, D. L., Aebersold, R., *Anal. Biochem.* 1994, *15*, 74–81.

[13] Goodlett, D. R., Aebersold, R., Watts, J. D., *Rapid. Commun. Mass Spectrom.* 2000, *14*, 344–348.

[14] Eriksson, J. E., Toivola, D. M., Sahlgren, C., Mikhailov, A., Harmala-Brasken, A. S., *Methods Enzymol.* 1998, *298*, 542–569.

[15] Yang, X., Wu, H., Kobayashi, T., Solaro, R., van Breemen, R., *Anal Chem.* 2004, *76*, 1532–1536.

