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Analysis of regulatory phosphorylation sites in ZAP-70 by capillary high-performance liquid chromatography coupled to electrospray ionization or matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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Abstract

A methodology for the rapid and quantitative analysis of phosphorylation sites in proteins is presented. The coupling of capillary high-performance liquid chromatography (HPLC) to electrospray ionization mass spectrometry (ESI-MS) allowed one to distinguish phosphorylation sites based on retention time and mass difference from complex peptide mixtures. The methodology was first evaluated and validated for a mixture of non-, mono-, and dityrosine-phosphorylated synthetic peptides, corresponding to the tryptic fragment 485–496 (ALGADDSYYTAR) of the human protein tyrosine kinase ZAP-70. The limits of detection for the non-, mono- and diphosphorylated peptides were about 15, 40 and 100 fmol, respectively, when using a 300 μm I.D. column. Application of the method was extended to identify phosphopeptides generated from a trypsin digest of recombinant autophosphorylated ZAP-70, in particular with respect to quantifying the status at the regulatory phosphorylation sites Tyr-492 and Tyr-493. Combination of chromatographic and on-line tandem mass spectrometry data allowed one to ascertain the identity of the detected peptides, a prerequisite to analyses in more complex biological samples. As an extension to the methodology described above, we evaluated the feasibility of interfacing capillary HPLC to matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), using a micromachined piezoelectric flow-through dispenser as the interface. This enabled direct arraying of chromatographically separated components onto a target plate that was precoated with matrix for subsequent analysis by MALDI-TOF-MS without further sample handling.

Keywords: Phosphorylation; ZAP-70

1. Introduction

Protein phosphorylation is recognized to be one of the most ubiquitous cellular regulatory mechanisms [1]. In a typical mammalian cell about one-third of all proteins expressed are thought to be phos-

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phorylated [2] indicating the significance of this post-translational modification. Protein kinases are a general class of substrate-specific phosphotransferase enzymes, catalyzing the transfer of the terminal phosphate moiety of a nucleoside triphosphate (ATP or GTP) to the nucleophilic hydroxyl group of serine (Ser), threonine (Thr) or tyrosine (Tyr) residues of their target proteins. This type of covalent protein modification is used as a means of altering the function of the protein. Protein tyrosine kinases are known to play a central role in mediating cellular signal transduction pathways. Either they catalyze the phosphorylation of tyrosine residues within their own sequences, so called autophosphorylation, or within the sequence of other proteins. Generally, kinases are considered to be low abundance proteins and phosphorylation is often non-stoichiometric. Thus, in order to elucidate the mechanism of regulation it is of vital importance to be able to identify the phosphorylation site(s) of a protein at low concentration levels in a quantitative fashion.

Various analytical techniques for the detection and analysis of protein phosphorylation have been described (reviewed in Ref. [3]). A widely used approach for the detection of phosphorylation sites involves radiolabeling of proteins in cells with γ-32 or -33 ATP followed by one- or two-dimensional polyacrylamide gel electrophoresis. Detection and identification of phosphorylation sites is based on radiography combined with enzymatic digestions of the separated proteins and two-dimensional phosphopeptide mapping. In order to assign specific phosphorylation sites the tryptic peptide fragments are subsequently resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) and finally subjected to Edman degradation, or amino acid analysis. Identified phosphorylation sites are often confirmed by site-directed mutagenesis that also allows addressing the question of their function in a cellular context.

More recently several mass spectrometric techniques have emerged that offer substantial advantages over this protocol, e.g., a more rapid and accurate analysis with less sample consumption, while avoiding hazardous radioactive labeling. Most importantly, collision-induced dissociation (CID) tandem mass spectrometry (MS–MS) can often resolve ambiguities by sequencing the peptide, thus pinpointing to the location of a phosphate group [4]. Coupling HPLC on-line with electrospray ionization mass spectrometry (ESI-MS) is now a powerful tool for the characterization of proteins due to its inherent selectivity, specificity, and sensitivity [5–9]. The striving for higher sensitivity is not only a matter of sophisticated mass spectrometers but also of reducing the chromatographic dilution. The advantages of reducing the column size in HPLC have long been recognized [10–12]. They include increased sensitivity, higher chromatographic resolution, ability to handle minute sample volumes, and easier interfacing to mass spectrometry.

In the present work we describe a methodology that allows one to rapidly analyze phosphorylation of regulatory sites within the protein tyrosine kinase ZAP-70, a critical mediator in the T-cell receptor (TCR) signal transduction pathway [13] down to the low fmol level. As part of this work we studied the autophosphorylation of recombinant ZAP-70, utilizing capillary HPLC coupled to ESI-MS with the possibility to make quantitative analyses.

In addition, we interfaced capillary HPLC to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS utilizing a micromachined piezoelectric flow-through microdispenser as the interface [14]. This allowed the chromatographically separated peptides to be transferred onto a MALDI target plate that was precoated with matrix, as an array of discrete spots without further sample handling. An appraisal of the sensitivity and practicality of each of these two techniques is outlined.

2. Experimental

2.1. Chemicals and reagents

Gradient grade acetonitrile (ACN) and formic acid (HCOOH) were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Fluka (Buchs, Switzerland). α-Cyano-4-cinnamic acid (α-CHCA) was obtained from Aldrich (Steinheim, Germany). Nitrocellulose membranes were obtained from Bio-Rad (Richmond, CA, USA). Acetone and 2-propanol were from Fisher Scientific (Loughborough, UK). Trypsin (sequence grade) was from Promega (Madison, WI, USA). Synthetic pep-

tides corresponding to the nonphosphorylated tryptic fragment 485–496 (–YY–; ALGADDSYYTAR) of ZAP-70 and its corresponding phosphorylated analogs, –pYY– (monotyrosine-phosphorylated at position 492), –YpY– (monotyrosine-phosphorylated at position 493), and –pYpY– (dityrosine-phosphorylated, at positions 492 and 493) were custom synthesized by Sigma (St. Louis, MO, USA). Dithiothreitol (DTT), ethylene glycol-bis(β-aminoethyl ether)-*N′*,*N′*,*N′*,*N′*-tetraacetic acid (EGTA), glycerol, Na_3VO_4, $MnCl_2$, and propionic acid were from Sigma. NaCl and NaH_2PO_4 were obtained from Merck while Tris–HCl was purchased from Esa (Chelmsford, MA, USA). All solutions were prepared using Milli-Q water filtered with a 0.2-μm nylon membrane filter from Millipore (Bedford, MA, USA).

2.2. Autophosphorylation and digestion of recombinant ZAP-70

ZAP-70 was expressed in baculovirus-infected insect cells as an N-terminally 6-His-tagged fusion protein and purified by immobilized metal ion affinity chromatography (IMAC) on Ni^{2+} loaded chelating Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) followed by buffer exchange and anion-exchange chromatography (Q-Sepharose, Amersham Pharmacia Biotech). One aliquot of 250 μl (1 nmol ZAP-70) in 20 m*M* NaH_2PO_4, 150 m*M* NaCl, 1 m*M* DTT, and 30% glycerol was buffer-exchanged using microspin columns packed with Sephadex G-25 (Amersham Pharmacia Biotech) by equilibrating the Sephadex column first with reaction buffer (100 m*M* Tris–HCl, 2 m*M* EGTA, 2 m*M* DTT, 0.25 m*M* Na_3VO_4, and 25 m*M* $MnCl_2$) at 4°C (2×150 μl, 1 min, 735 *g*), whereafter the recombinant ZAP-70 sample was applied and spun for 2 min at 735 *g*, 4°C. The eluate was split into two equal parts (125 μl each) of which one was incubated with ATP at a final concentration of 1.2 m*M*, while the other served as a control (no ATP added). Both samples were reacted at 30°C and 300 rpm for 30 min in a thermomixer (Eppendorf, Hamburg, Germany). Finally, samples were digested with 2.5 μg sequencing grade trypsin (Promega) at 37°C overnight.

2.3. Chromatography

Reversed-phase separations were performed on an integrated capillary HPLC system (Ultimate, LC Packings, Amsterdam, The Netherlands) consisting of a quaternary gradient pump based on flow splitting and a UV detector equipped with a Z-shaped capillary flow cell (45 nl volume, 100 mm path length). A micro autosampler with built-in sample cooling (Famos, LC Packings) was used for sample injection. The separations were carried out on a 15 cm×300 μm I.D. capillary column packed with RP stationary phase (TSK Super ODS, C_{18} modified 2 μm porous Si particles, TosoHaas, Stuttgart, Germany) supplied by Grom Analytik+HPLC (Herrenberg-Kayh, Germany). We also investigated a 15 cm×75 μm I.D. RP column (PepMap, C_{18} modified 3 μm porous Si particles) obtained from LC Packings. The 300 μm I.D. column was operated at a flow-rate of 4 μl min^{-1}, while the 75 μm I.D. column was run at 200 nl min^{-1}. The LC effluent was connected via the fused-silica capillary outlet of the UV detector directly to the nano-LC interface. For all LC–ESI-MS separations eluent A consisted of 0.2% (v/v) HCOOH in ACN–water (5:95, v/v) and eluent B of 0.2% (v/v) HCOOH in ACN–water (90:10, v/v). When using the LC–MALDI-TOF-MS system the formic acid in the eluents was exchanged to 0.1% TFA. The applied gradient was from 2 to 50% B in 45 min. For the tryptic digest separations of recombinant ZAP-70 the initial gradient condition was held for 10 min in order to remove buffer constituents prior to starting the gradient. All separations were performed at ambient temperature and the injection volumes were 5 or 10 μl depending on the sample concentration.

2.4. ESI-MS

Electrospray mass spectra were obtained with a hybrid quadrupole time-of-flight mass spectrometer (Q-Tof, Micromass, Wythenshawe, UK) equipped with an ESI ion source, using MassLynx NT as the data acquisition software. The chromatographic interfacing was made with the nano-LC option interface from Micromass. Essentially it consists of a drilled metal block fitted with a stainless steel

microvolume union (Valco, Schenkon, Switzerland) that joined the column effluent to the fused-silica capillary spray needle (20 μm I.D.×90 μm O.D.). The positioning of the spray needle was controlled using a three-axis positioner-stage. In order to obtain optimal sensitivity the position of the spray was adjusted during infusion of the synthetic peptides dissolved in acidified (0.2% HCOOH) ACN–water (50:50, v/v) at the concentration of 2 pmol μl^{-1}, delivered at a flow-rate of 1.5 $\mu l\ min^{-1}$ with a syringe pump (Model 22, Harvard Apparatus, South Natick, MA, USA). Samples were sprayed at a potential of about +3000 V. A stable constant spray was maintained during gradient separation.

2.5. Tandem mass spectrometry

The hybrid mass spectrometer combines a single quadrupole in series with an orthogonal reflectron time-of-flight mass analyzer and can be used in either MS or MS–MS mode. In MS mode, the quadrupole and the hexapole that follows are used in broadband mode, serving only as ion guides to the time-of-flight analyzer. For MS–MS analysis, the quadrupole resolves the selected precursor ions and the hexapole serves as a collision cell to generate fragment ions. The MS–MS experiments were performed by using the automatic switching function in the acquisition software, i.e., part of the primary total ion current (TIC) signal is diverted to the MS–MS fragment ion spectrum when certain precursor and intensity switch criteria are satisfied [MS to MS–MS switch criteria: intensity must reach the threshold of 2 counts s^{-1} of the selected precursor masses (651.8, 691.8, 731.8)]. At the same time the collision cell is pressurized with argon. The optimal collision energy for the peptides was determined by prior infusion experiments and then programmed into the auto-switching function: nonphosphorylated peptide 23 eV; monophosphorylated peptides 26 eV; diphosphorylated peptide 28 eV. In each case the doubly-charged ion of the peptides was selected as precursor. Autoswitching returned to the MS mode when the MS–MS to MS switch criteria were met (intensity threshold must fall below 2 counts s^{-1} or the set time window of 45 s expired). The obtained fragment ions were assigned using Mascot Search version 1.5 (http://www.matrixscience.com).

2.6. MALDI-TOF-MS

MALDI-TOF-MS was performed on a Voyager DE-PRO (PE-Biosystems, Framingham, MA, USA) with built-in delayed extraction and a linear path of 1.1 m. The mass spectra were acquired in the reflector mode at an accelerating voltage of 20 kV and a delay time of 150 ns. The MALDI target plates were laboratory-made stainless steel plates measuring 47×45 mm mounted in a custom-made holder (PE-Biosystems). The matrix consisted of 2 mg ml^{-1} CHCA and 5 mg ml^{-1} nitrocellulose membrane dissolved in acetone–2-propanol (80:20, v/v) and mixed 1:1. The entire target was homogeneously covered with the matrix solution using an air-brush (Badger, Model 200, IL, USA). Angiotensin I was included in the matrix solution (1 $\mu g\ ml^{-1}$ matrix solution) and used as an internal standard. The outlet of the UV detector consisted of a 50 μm I.D. fused-silica capillary where the end was slid into a 250 μm I.D. PTFE tubing (LC Packings) fitted into a 1/16 in. (0.16 cm) silicon rubber tube at the inlet of the microdispenser with a virtually zero-dead volume connection. The MALDI target plate was placed on a motor-driven *x–y* stage (Vexta, Oriental Motor, Japan) which was computer controlled by using laboratory-developed LabView (National Instruments, Austin, TX, USA) routines. Hereby, the chromatographic separation was transferred to the target plate in an unattended mode. Furthermore, the software allowed one to define the array pattern, adjust the distance between discrete spots, the frequency of the ejected droplets and the duration of the fraction collection time. To enhance evaporation of the dispensed droplets the target holder was heated to 38°C, which resulted in a more efficient sample deposition. Visualization of the sample spotting was obtained by using a microscope (Leitz, Wetzlar, Germany) equipped with a video camera (CCD-72EX, DAGE-MTI, Michigan City, IN, USA). Typical pulse conditions (Pulse generator, Model 8111A, Hewlett-Packard, Palo Alto, CA, USA) were 20–50 Hz, using an applied voltage of 9 V (d.c. power supply, Model E3612A, Hewlett-Packard).

3. Results

In this study, we developed a quantitative method for the analysis of the phosphorylation status of Tyr-492 and Tyr-493 in recombinant ZAP-70 by combining capillary HPLC with ESI-MS using selective ion recording in the positive ion mode and tandem mass spectrometry. Synthetic peptides corresponding to the tryptic fragment 485–496 (ALGADDSYYTAR), containing the Tyr-492 and Tyr-493 phosphorylation sites in the non-, mono-, and diphosphorylated form, respectively, were used to establish the methodology (see Table 1). In RP-HPLC TFA is the most common additive for modifying the mobile phase, possessing good UV transparency, ion-pairing and peptide solubilizing properties, and high volatility. Although TFA is highly useful when using UV detection, it is well known that the combination of TFA and ESI-MS results in signal reduction and spray instability [15,16]. In order to overcome this limitation, acetic acid and formic acid were evaluated as alternative additives at various concentrations. We also investigated post-column infusion of a propionic acid–2-propanol (75:25) mixture prior to the mass spectrometer. This latter method is referred to as the ''TFA fix'' [17] where the mass action of a high concentration of a weaker acid is thought to dissociate the TFA–peptide ion pairs, hereby reducing suppression effects. This approach was abandoned since it diluted the separated analytes and resulted overall in reduced sensitivity. The best alternative to TFA in terms of chromatographic (peak shape, recovery) and electrospray (sensitivity) aspects was the addition of 0.2% formic acid to the mobile phase, almost resulting in baseline separation of the two isomeric monophosphorylated peptides while preserving high sensitivity (Fig. 1). As expected, the most polar diphospho peptide eluted first (11.64 min), followed by the two isomeric monophosphorylated peptides p493 (15.79 min), p492 (16.45 min), and the nonphosphorylated peptide (17.49 min). Since the loss in chromatographic resolution as a result of passing through the UV detector was negligible, it was kept in-line to acquire additional chromatographic information permitting to troubleshoot the chromatographic and MS

Table 1
Synthetic peptides that were used for optimization of LC–MS and MS–MS conditions and for calibration of the LC–MS system (sequence corresponds to residues 485–496 of human ZAP-70; p=PO_3H_2)

Sequence	Acronym	M_r (monoisotopic)
ALGADDSYYTAR	YY	1302.57
ALGADDS(pY)YTAR	pYY	1382.51
ALGADDSY(pY)TAR	YpY	1382.51
ALGADDS(pY)(pY)TAR	pYpY	1462.44

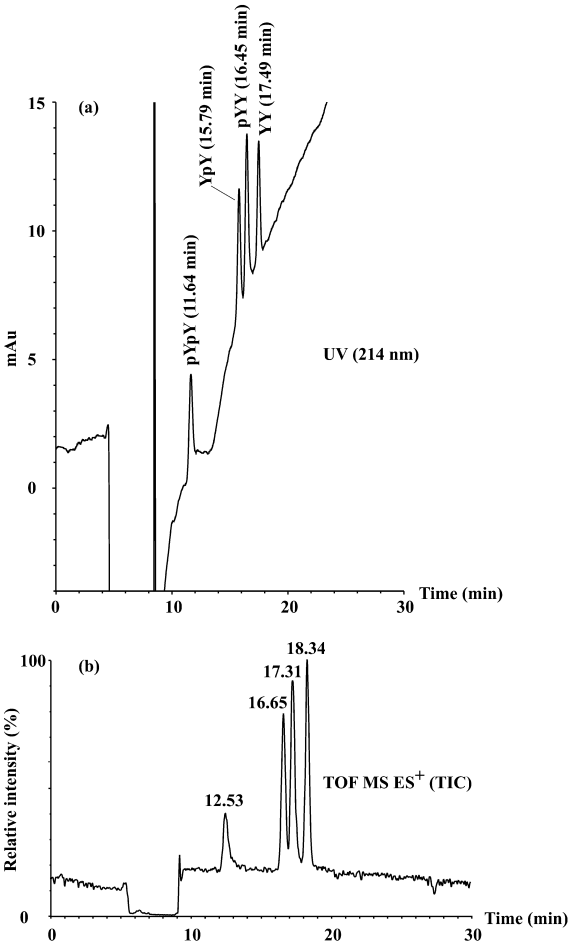

Fig. 1. Comparison of the simultaneously acquired (a) UV (214 nm) and (b) total ion current (TIC) ESI traces from an LC separation (150 mm×300 μm I.D., TSK Super ODS, TosoHaas) of 1 pmol synthetic peptides corresponding to residues 485–496 of human ZAP-70 as shown in Table 1. For chromatographic conditions, see Experimental.

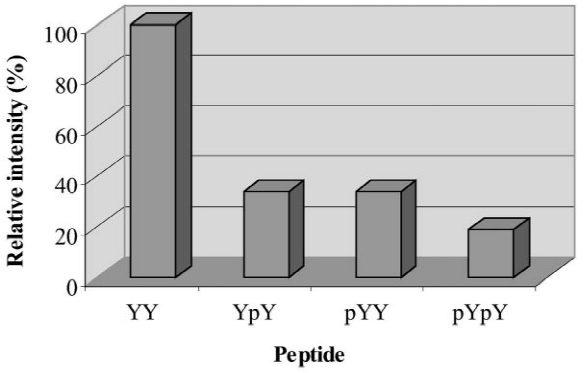

Fig. 2. Comparison of the ionization efficiencies of synthetic peptides corresponding to residues 485–496 of human ZAP-70 in the ESI^+ mode depending on their phosphorylation status. Data were generated by continuous infusion (1.5 pmol μl^{-1}; 1.5 μl min^{-1}; 0.2% aq. HCOOH–ACN, 1:1) and selective ion recording.

part of the LC–MS system separately. The chromatography of the diphosphorylated peptide was problematic since the strong hydrophilic and ionic properties of the peptide resulted in significant losses during RP-HPLC, especially at lower levels. The following data were obtained on a TSK Super ODS column, which gave relatively good recoveries for this peptide.

Part of the reduced signal for the diphosphorylated peptide in ESI-MS was also due to a reduced ionization efficiency (poor ionization of the diphosphopeptide was also experienced by MALDI-TOF-MS analysis) as assessed by infusing the peptide mixture at a concentration of 1.5 pmol μl^{-1}. The signal obtained for the nonphosphorylated peptide was about five-times higher compared to the diphospho form, and about three-times higher than the two monophosphorylated peptides showing that both ionization efficiency and chromatographic recovery need to be considered when performing quantitative phosphorylation studies (Fig. 2).

In order to establish a quantitative method, the linearity of the calibration curve for the synthetic peptide mixture was determined by successive dilutions of a 200 fmol μl^{-1} synthetic peptide mixture dissolved in eluent A, over the range from 15 to 1000 fmol injected on the column. Injections of eluent A were interspersed to serve as blanks. The calibration curves for each peptide were based on selective ion monitoring and showed excellent linearity over the whole range (linear regression data is given in Table 2). The limit of detection (LOD) was defined as the analyte concentration giving a signal equal to the blank signal plus two standard deviations. The nonphosphorylated peptide could be detected down to about 15 fmol while the LODs for the two monophosphorylated peptides were found to be about 40 fmol. The LOD of the diphosphopeptide was 100 fmol. These LOD values can be improved significantly by using capillary columns with smaller I.D. hereby reducing chromatographic dilution even further. In fact, preliminary studies performed on a 75 μm I.D. column at the flow-rate of 200 nl min^{-1} indicated that even the diphosphorylated peptide could be detected below the fmol level. Fig. 3 depicts chromatograms based on selective ion monitoring of the four synthetic peptides with 2.5 fmol injected on the 75 μm column. The S/N ratio obtained for the peptides indicates an LOD below 500 amol.

The methodology described above was subsequently applied to purified recombinant ZAP-70 expressed in insect cells. ZAP-70 was incubated with 1.2 m*M* ATP to induce autophosphorylation in comparison to an identically treated control without ATP. Both the ATP-treated and the control sample were digested with trypsin according to the procedure described in the Experimental section. The resulting selective ion recorded chromatograms of the two reactions are depicted in Fig. 4. While no

Table 2
Linear regression data of the calibration curves obtained for each peptide, the standard deviations of the slope and intercept are given in parentheses

Peptide	Slope	Intercept	Data points	Correlation coefficient
pYpY	0.45 (0.005)	−18.48 (2.92)	4	0.99
YpY	1.72 (0.027)	−19.21 (11.78)	7	0.99
pYY	1.65 (0.023)	−24.07 (10.17)	7	0.99
YY	2.28 (0.013)	11.55 (5.75)	7	0.99

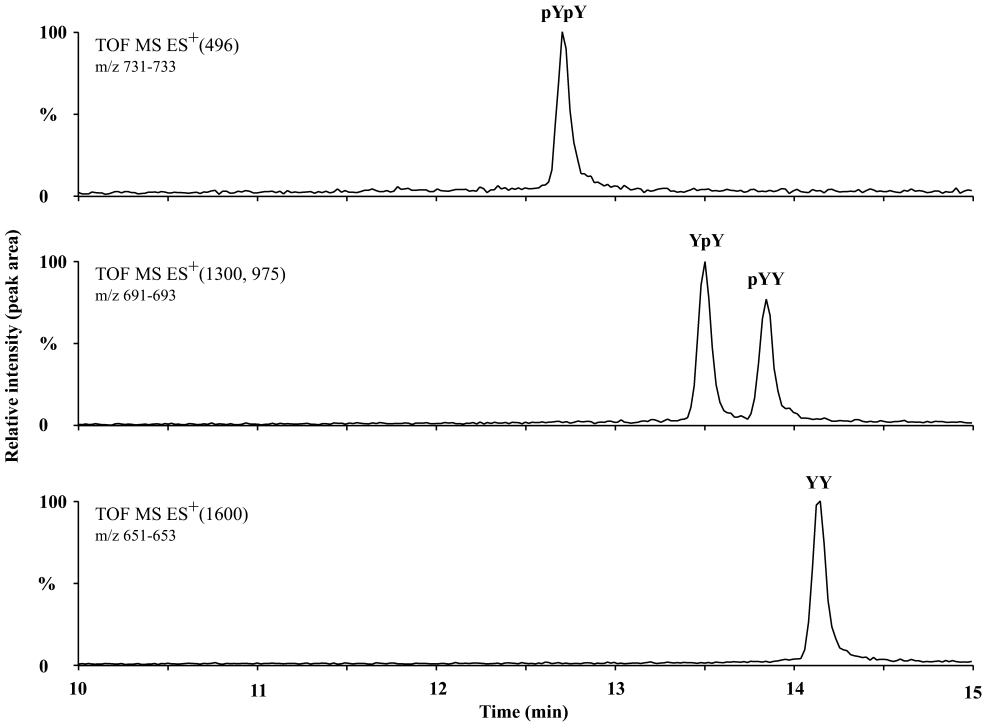

Fig. 3. Separation of synthetic peptides corresponding to residues 485–496 of human ZAP-70 in their different phosphorylation states on a 15 cm×75 μm I.D. capillary column (PepMap, LC Packings) operated at a flow-rate of 0.2 μl min^{-1}. Injected amount was 2.5 fmol and detection made by selective ion recording. Values in parentheses correspond to the peak area of each peptide.

phosphorylation of Tyr-492 or -493 was detectable in the control sample, indicating that insect cells lack the capacity to phosphorylate ZAP-70, incubation with ATP led to phosphorylation of both tyrosines. Furthermore, monitoring the doubly-charged molecular ion for the diphosphorylated peptide ALGADDSpYpYTAR (m/z 731.8) gave a strong signal in the autophosphorylation reaction, indicating that diphosphorylation was favored during incubation with 1.2 m*M* ATP. In order to quantify the results of the autophosphorylation reaction, peak areas were normalized against the calibration curve of the synthetic peptides. The signal obtained in the control reaction corresponded to about 2.5 pmol of nonphosphorylated peptide while the signals obtained for the non-, mono(p493)-, mono(p492)-, and diphosphorylated peptides in the autophosphorylation reaction were equivalent to about 1.0, 0.15, 0.30 and 1 pmol, respectively (Table 3). The phosphorylation of both tyrosines at position 492 and 493 was prevalent in the autophosphorylation reaction with about 40% of ZAP-70 being diphosphorylated. On the contrary, only 6% or 12% of ZAP-70 were monophosphorylated on Tyr-493 or -492, respectively. This result implies that the monophosphorylated products were intermediates on their way to being fully phosphorylated. While final results must await a more detailed kinetic analysis, it is likely that monophosphorylated ZAP-70 is a better substrate for further autophosphorylation on either Tyr-492 or Tyr-493 than its nonphosphorylated counterpart. Finally, 40% of ZAP-70 remained nonphosphorylated under these conditions. Increasing the ATP concentration to 10 m*M* did not reduce the percentage of nonphosphorylated ZAP-70 significantly, indicating that part of the recombinant protein was a poor substrate for autophosphorylation at these tyrosine residues.

Retention time and molecular mass alone are often not sufficient to identify a given peptide within a complex mixture. In order to develop a methodology that would allow ascertaining the identity of the

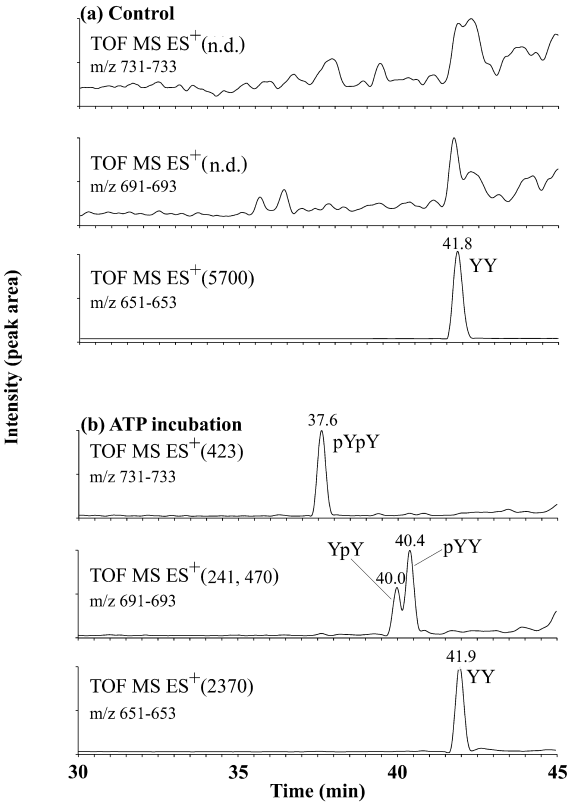

Fig. 4. Autophosphorylation of recombinant human ZAP-70 leads to extensive phosphorylation of both Y-492 and Y-493 as determined by chromatographic analysis of tryptic digests and selective ion recording (see Experimental for conditions). (a) Sample without ATP (control); (b) sample treated with 1.2 m*M* ATP for 30 min at 30°C (see Table 3 for details). Phosphorylation of either Y-492 or Y-493 was not detectable in the control sample down to a level of approximately 1% of the nonphosphorylated peptide (a). n.d.=not detected.

Table 3
Comparison of phosphorylation patterns on recombinant ZAP-70 samples without ATP treatment (control) versus ATP incubation (autophosphorylation)

Peptide	Control	Autophosphorylation
YY	2.5 pmol (100%)	1.0 pmol (42%)
YpY (p493)	n.d.	0.15 pmol (6%)
pYY (p492)	n.d.	0.30 pmol (12%)
pYpY	n.d.	1.0 pmol (40%)

n.d.=Not detected.

detected peptides unambiguously, tandem mass spectrometry was applied to both the autophosphorylated ZAP-70 and the control sample. Fig. 5 illustrates the fragmentation patterns of the different peptides obtained from the autophosphorylation reaction. Individually infused synthetic peptides gave essentially identical patterns. MS–MS was performed on-line in the autoswitching mode by diverting part of the primary TIC to the MS–MS fragment ion spectrum when a set of precursor ion criteria was met. Taken together LC–MS–MS with autoswitching proved to be a powerful method to identify the respective peptides on-line based on partial sequence information with the potential to study these events in more complex biological systems. Furthermore, automated search algorithms were successfully applied to interpret the spectra and interrogate databases.

Since Tyr-292 has been described as the most prominent autophosphorylation site in ZAP-70 [18], we studied the status of phosphorylation at this residue under the described conditions by extracting the selective ion signal for the corresponding doubly-charged tryptic peptide fragment from the acquired data. Fig. 6 shows that our data confirm Tyr-292 to be highly phosphorylated under these conditions while no phosphorylation was detectable in the recombinant starting material again indicating that insect cells did not phosphorylate ZAP-70.

MALDI-TOF-MS is an extremely sensitive technique that is widely available in life science laboratories with the potential for automated, high-throughput analysis. Capillary liquid chromatography has previously been successfully interfaced to MALDI-TOF-MS by our group using a micromachined piezoelectric flow-through microdispenser [19]. This allowed the column effluent to be transferred onto a target plate that was precoated with matrix as an array of discrete spots, while maintaining the chromatographic resolution. Extremely high sensitivities of peptides (100 amol) were obtained making this approach suitable for direct arraying of chromatographically separated components, enabling analysis by MALDI-TOF-MS without further sample handling. Furthermore, the system is fully automated and highly flexible allowing operation in both an ‘‘on-line mode’’ or in a ‘‘static mode’’ [20]. We have previously shown that the above mentioned synthetic peptides can be detected at a concentration of 5 fmol

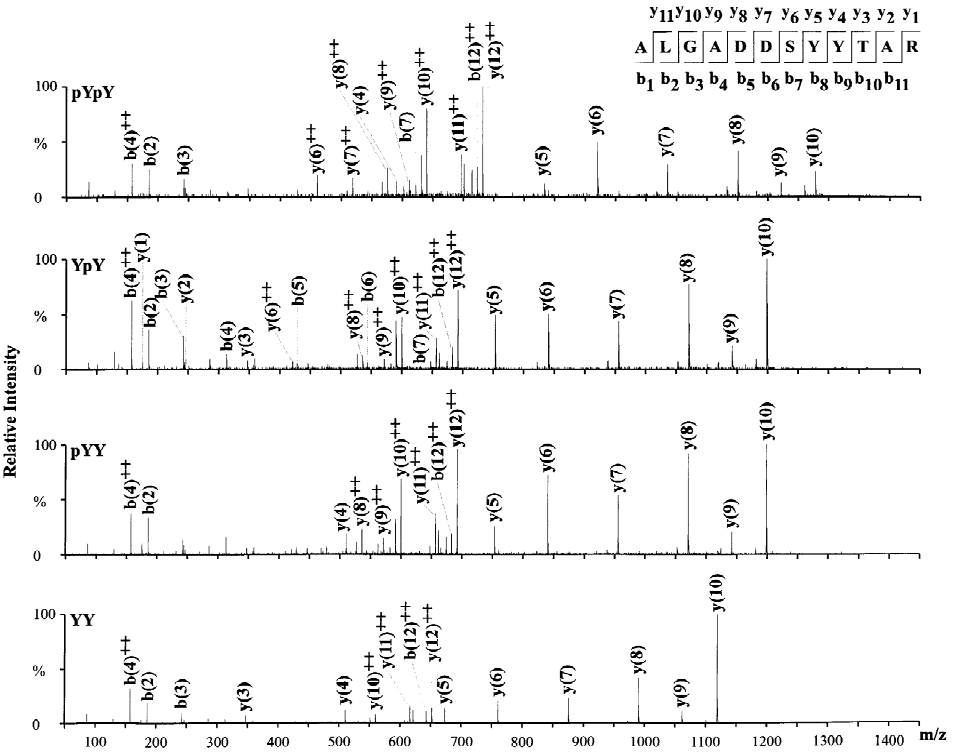

Fig. 5. On-line LC–MS–MS analysis of tryptic fragments corresponding to residues 485–496 of human ZAP-70 in their different phosphorylation states on a 15 cm×300 μm I.D. capillary column (TSK, Super ODS). Analyses were performed using the automatic switching function of the MassLynx software, as described under Experimental, and fragment ions were assigned using Mascot Search version 1.5.

μl^{-1}, corresponding to about 150 amol of each peptide deposited onto the MALDI target plate [20]. In the context of developing more sensitive methods to analyze phosphorylation sites in proteins, LC–MALDI-TOF-MS is an attractive alternative to LC–ESI-MS. Fig. 7a illustrates the system set-up and Fig. 7b the separation of ALGADDSYYTAR and its different phosphorylation states utilizing capillary HPLC coupled to MALDI-TOF-MS. The system set up allowed operation in an unattended mode and the use of target plates that were precoated with matrix made the MALDI analysis simple and straightforward. MALDI spectra with monoisotopic resolution were generated for all four peptides. Angiotensin I was included as an internal standard (m/z 1296.68) in the matrix, hereby increasing the mass accuracy to 50 ppm. The successful separation of the two monophosphorylated isomeric peptides made it possible to discriminate between them. In these initial experiments 1 pmol of each peptide was injected onto the column and the amount of peptides deposited onto each spot corresponded to about 18 fmol, but further increases in sensitivity are likely to be possible. As previously shown, the chromatographic resolution was maintained on the MALDI target plate [19]. This approach will be further optimized and has the potential for high-throughput, automated operation, in particular in combination with modern MALDI instruments that allow to analyze many target plates in an unattended fashion.

4. Discussion

Phosphorylation of tyrosine, serine or threonine residues is undoubtedly one of the most prevalent and important post-translational modification. In order to fully understand the relevance of this

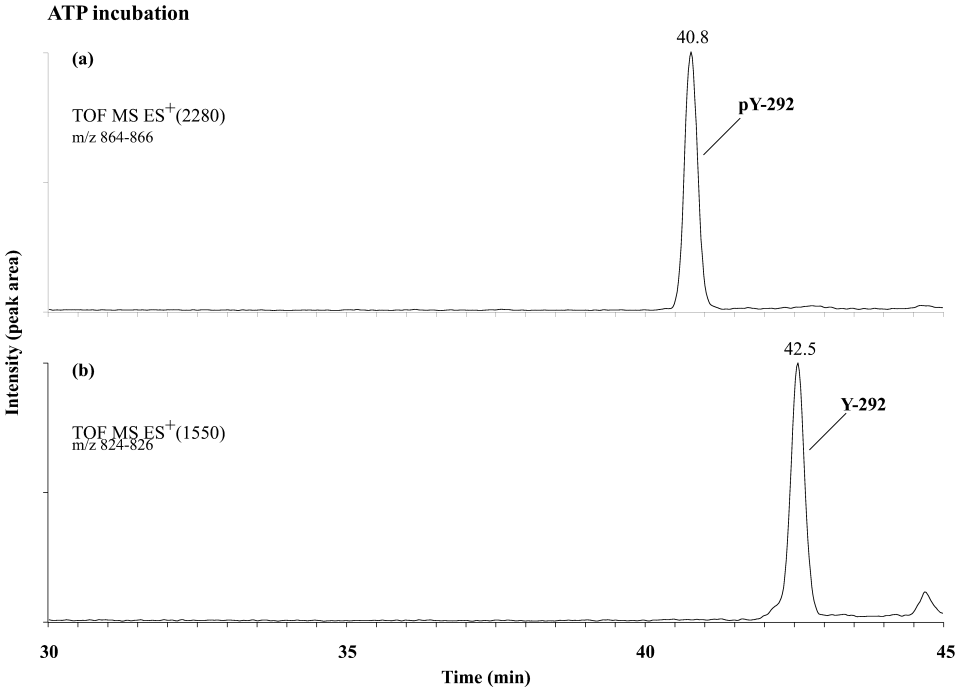

Fig. 6. Autophosphorylation of recombinant human ZAP-70 leads to extensive (a) phosphorylation of Y-292 as determined by chromatographic analysis of tryptic digests and selective ion recording (see Experimental for conditions). Only the non-phosphorylated Y-292 peak (b) could be seen on the sample without ATP incubation (data not shown).

regulatory mechanism, it is crucial to assess not only whether an amino acid residue is phosphorylated but also to measure the extend to which phosphorylation occurs under a given set of conditions. While a number of methods can give a yes/no answer concerning the presence of a phosphorylation site in a given protein (e.g., radiolabeling, phosphoamino acid or phosphopeptide-specific antibodies), they are generally not capable of providing quantitative information. More recently, methodologies are being developed to address this question based on the use of whole-cell stable isotope labeling [21]. In this paper we present an approach that is based on the use of synthetic peptides as external standards corresponding to defined phosphorylation sites combined with high-sensitivity capillary LC–ESI-MS or MS–MS. Excellent linearity of the mass spectrometric response was obtained with LODs ranging from 15 to 100 fmol for the non- and diphosphorylated peptides, respectively. Main factors contributing to decreasing sensitivities for phosphopeptides were determined to be due to a lower ionization efficiency in the positive ionization mode and a decreased recovery of the phosphorylated peptides from the RP stationary phases. In fact, optimal recovery of the diphosphorylated peptide in the sub-picomolar range required careful choice of stationary phase and optimization of mobile phase conditions. Initial results were also obtained using on-line coupling of capillary LC to MALDI-TOF-MS. While this methodology is still in its infancy when compared to LC–ESI-MS, our results show that it is possible to obtain high-quality mass measurements with monoisotopic resolution and a mass accuracy below 50 ppm. Future developments in the area of matrix-assisted laser desorption mass spectrometry will likely render this method more amenable to high-throughput analyses of phosphopeptides based on automated sample handling and enhanced MS–MS capabilities [22]. It should, however, be noted that the diphosphorylated peptide showed a lower ionization efficiency in MALDI comparable to ESI and that this problem needs further investigation.

Our methodology was applied to the protein

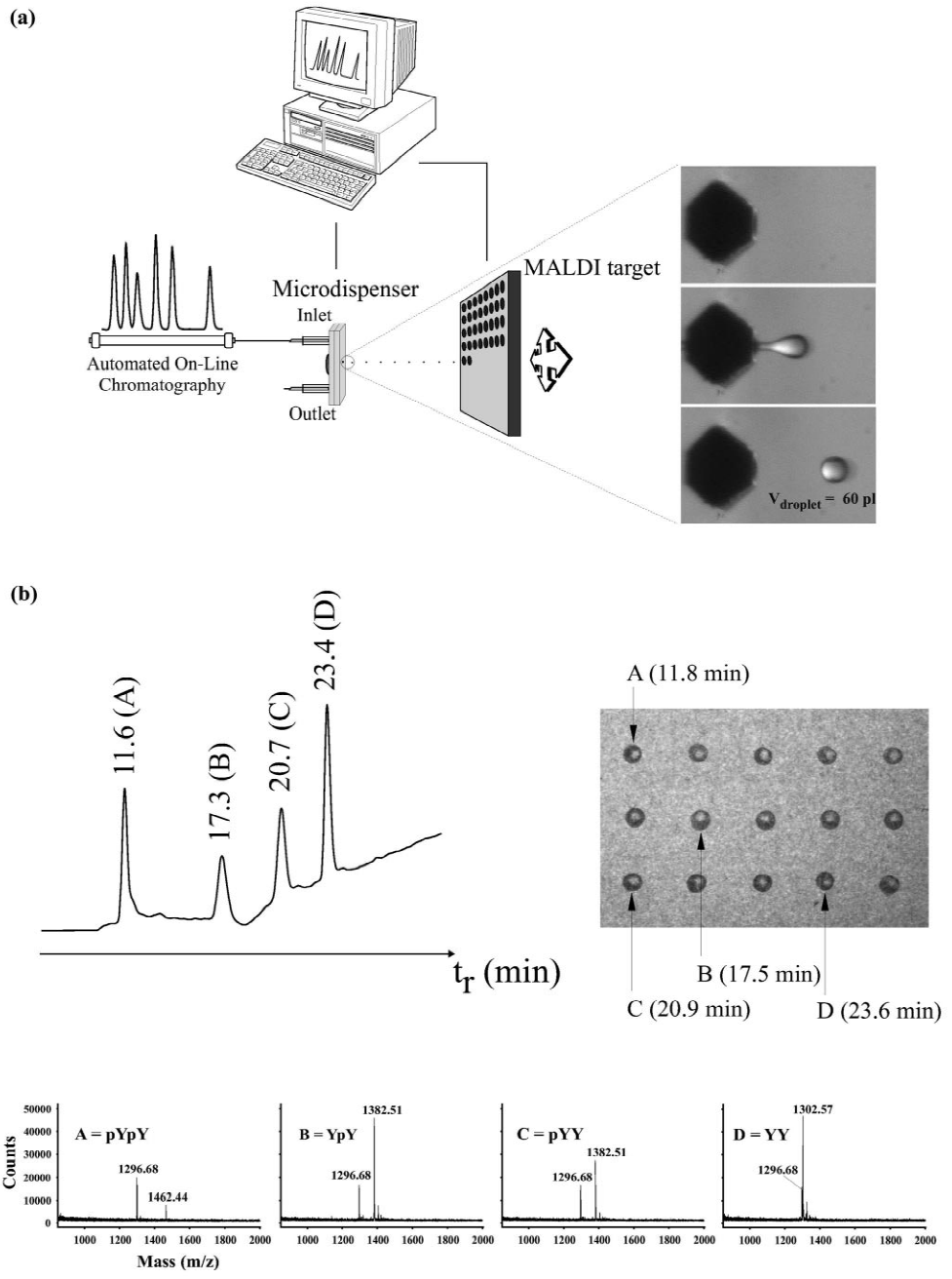

Fig. 7. Capillary LC interfaced to MALDI-TOF-MS via a flow-through piezoelectric microdispenser. (a) System set-up: the capillary column was coupled on-line to the dispenser which transferred separated peptides to a MALDI target plate as an array of discrete spots. The target was precoated with matrix/nitrocellulose using an air-brush device. The enlarged picture of the dispenser nozzle shows a typical stroboscopic droplet formation sequence with images taken at 0, 60 and 120 μs after triggering the voltage pulse. (b) Separation of synthetic peptides (1 pmol injected, 15 cm×300 μm I.D. column, TSK Super ODS) corresponding to residues 485–496 of human ZAP-70 in their different phosphorylation states. UV trace recorded at 214 nm; A=pYpY; B=YpY; C=pYY; D=YY. The positions of the peptides on the spotted array are correlated by their respective retention times. Angiotensin I (m/z 1296.68) was included in the spray-coated matrix as an internal standard. All values correspond to mono-isotopic mass-to-charge ratios of the singly-protonated ions.

tyrosine kinase ZAP-70, a critical mediator of T-cell receptor signaling. Earlier work had shown that phosphorylation of Tyr-493 of ZAP-70 is required for antigen receptor-mediated induction of interleukin-2 (IL-2) secretion in lymphocytes [23] and that Tyr-492 and -493 are the principal phosphorylation sites in ZAP-70 upon T-cell receptor activation [18] while autophosphorylation was shown to occur mainly at Tyr-292 and not at Tyr-492. Our data now indicate that both tyrosine residues are autophosphorylated and that the monophosphorylated species are actually in the minority. This finding may have important implications for the activation status of ZAP-70 in activated T-cells depending on whether it is mainly controlled by autophosphorylation or by phosphorylation mediated through another protein tyrosine kinase p56-lck. At any rate our data indicate that monophosphorylated ZAP-70 has a considerably altered structure around the activation loop occluding the active site in agreement with modeling studies based on the insulin-receptor tyrosine kinase structure [24] making it a better substrate for further autophosphorylation. Future studies will address this question by applying the described methodology to endogenous ZAP-70 isolated by immunoprecipitation from primary human T-cells. Initial data indicate that the high-sensitivity of the capillary LC–MS method allows one to work with as little as $5 \cdot 10^6$ peripheral human blood monocytes, a critical requirement when working with samples of human origin. Utilizing smaller capillary columns (75 μm I.D.) together with a newly designed interface will result in further improvements in sensitivity. Preliminary work showed that the LOD for the diphosphorylated peptide was below 2 fmol, an improvement of at least 50 when compared to the system set up described here. Future studies will explore these possibilities including the methodology of combining capillary HPLC with MALDI-TOF-MS.

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