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Measurement of relative amounts of phospho- and dephospho-B-50(GAP-43) peptides by fast atom bombardment-mass spectrometry

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The biological role of phosphoproteins depends upon their degree of phosphorylation in vivo. Methods currently available to measure the degree of phosphorylation of a protein involve indirect procedures to detect the ^{32}P-phosphate incorporation. We report here a direct method to measure relative amounts of phospho- and dephospho-forms of peptides based upon a mass spectrometric technique. The intensities of the molecular ions corresponding to the two forms of the peptides are proportional to their relative amounts. This is demonstrated for a peptide fragment of the protein B-50(GAP-43) and for kemptide, respectively substrates for protein kinases C and A, and demonstrates the applicability of fast atom bombardment-mass spectrometry to quantitate peptides bearing post-translational modifications.

Phosphoprotein; B-50(GAP-43); Kemptide; Fast atom bombardment-mass spectrometry

1. INTRODUCTION

Protein phosphorylation is a dynamic process regulated by protein kinases which are able to transfer a phosphate group from an ATP molecule to a specific amino acid residue, and by protein phosphatases which are able to shut off the signal by restoring the native form of the protein.

Since the biological properties of a phosphorylatable protein depend upon its degree of phosphorylation, measurement of relative amounts of phospho- and dephospho-forms of specific proteins is of great importance in establishing their physiological role in normal and pathological conditions.

The methods currently available for measuring the degree of phosphorylation of proteins are largely based upon radioenzymatic procedures involving back-titration to measure the number of in vitro phosphorylatable sites of a protein by means of ^{32}P-phosphate incorporated into the residual dephospho-form. Frequently ^{32}P incorporation is measured after ortho-phosphate labeling followed by two-dimensional gel electrophoresis [1]. A more direct approach is the immunoprecipitation procedure to separate and identify the ^{32}P-labeled protein of interest (see for instance [2]). For phosphotyrosine residues an immunological method is also available, but this is inherently non-specific, since the antibody recognizes a consensus sequence common to different proteins [3].

Fast atom bombardment (FAB)-mass spectrometry (MS) has become an important analytical technique for investigating biologically important molecules such as carbohydrates, nucleotides, peptides and proteins [4]. Recently Shroeder et al. [5] and Labdon et al. [6] described the application of mass spectrometry to localize the phosphorylation sites in the known primary structures of polypeptides: these studies led us to evaluate the possibility of applying this technique to the quantitative measurement of the relative amounts of dephospho- and phospho-forms of peptides in the same sample.

We report here that it is possible to quantitate by FAB-MS the relative amounts of phospho- and dephospho-forms of a peptide corresponding to the 39–51 amino acid sequence of the nervous tissue-specific protein, B-50(GAP-43). This protein seems to play a key role in axonal growth cones, the polyphosphoinositide cycle, transmitter release and long-term potentiation, and in some of these processes at least its degree of phosphorylation seems to be crucial [7–12]. The synthetic peptide of B-50 used in this study has the advantage of having a single and well-defined phosphorylation site at serine in position 41, specifically phosphorylated by the Ca^{2+}/phospholipid-dependent protein kinase (PKC) [13–15], and the two forms can be separated by gel electrophoresis, thus allowing for an

Abbreviations: FAB, fast atom bombardment; MS, mass spectrometry; PKC, Protein kinase C.

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alternative procedure to validate the results obtainable by FAB-MS.

To further evaluate the general applicability of the method, FAB-MS has been utilized to follow the kinetics of the phosphorylation of kemptide, a substrate for the cAMP-dependent protein kinase (PKA), which has already been used as a test molecule in the qualitative application of this technique [5].

2. MATERIALS AND METHODS

The peptide AcetylGln-Ala-Ser-Phe-Arg-Gly-His-Ile-Thr-Arg-Lys-Lys-Leu, (subsequently termed B-50/39-51) was kindly provided by Organon (Oss, The Netherlands); kemptide was purchased from Sigma (St. Louis, USA).

2.1. *B-50/39-51 phosphorylation*

Purified PKC, prepared according to Kikkawa et al. [16], was utilized and the reaction was performed according to Dekker et al. [17] with minor modifications. Briefly, B-50/39-51 was phosphorylated with purified PKC (0.012 μg PKC 10 μg peptide, 100 μM ATP) in a reaction mixture containing (final concentrations): Tris 10 mM (pH 7.4) $MgCl_2$ 10 mM, $CaCl_2$ 1 mM and phosphatidylserine (1 μg/100 μl). The incubation was carried out at 30°C for 60 min to reach the maximum extent of phosphorylation and the reaction was stopped in liquid nitrogen. In some experiments γ[^{32}P]ATP (Amersham, UK; 3,000 Ci/mmol) was added (100 μM/10 μCi/tube).

To remove salts, which could interfere with the ionization of the peptide, samples were applied to a C18 Sep-Pak cartridge (Millipore) and eluted, after washing with distilled H_2O, with H_2O/CH_3CN (50%/50%) in the presence of trifluoroacetic acid. Samples were then dried under nitrogen and resuspended in thioglycerol used as matrix for FAB-MS analysis.

Samples containing variable amounts of dephospho- vs. phospho-form of the peptide were prepared by adding, to 10 μl (600 ng/μl) aliquots of the de-salted phosphorylation mixture, 10 μl of a thioglycerol solution containing increasing amounts (0–1000 ng/μl) of the dephospho-form of the peptide and thorough mixing. 2 μl of this final solution was applied to a stainless steel FAB target (1.7 × 6 mm) for the analysis.

A VG 70-250 SEQ apparatus equipped with a FAB ion source (VG Analytical, UK) was utilized to collect mass spectra of positive ions (8 kV accelerating voltage). The instrument was calibrated with a CsI/glycerol mixture, which generates a set of ions with well-defined mass-to-charge (m/z) values to be used as reference in the mass range utilized. Mass spectra were recorded in a mass range of m/z 1,300–1,700 at 2,000 resolution and operating the data system in Multi Channel Acquisition mode. Spectra were accumulated in a fixed time range (1.6 min) to compensate for the bias towards either the phosphorylated or non-phosphorylated form of the peptide.

Area of peaks corresponding to phospho- and dephospho-forms were measured by the data system and peak area ratios were then obtained (dephospho/phospho). The ratio calculated for the sample obtained after phosphorylation with no additional dephospho-peptide was considered as the basal value. All the samples were run in triplicate and data so obtained were averaged.

Nine experiments of phosphorylation, de-salting and addition of increasing amounts of the dephospho-peptide were performed independently.

Separation of phospho- and dephospho-peptide with polyacrylamide gel electrophoresis was accomplished according to Anderson et al. [18] with minor modifications; gels were stained with Coomassie blue, de-stained and autoradiographed. Densitometric analysis of stained gels was performed on a Ultroscan XL (LKB, USA) densitometer coupled with a M24 Olivetti computer system.

2.2. *Kemptide phosphorylation*

The reaction was carried out in the presence of the catalytic subunit of PKA (Sigma, St. Louis, USA; ratio of kemptide:kinase = 150:1) in buffer (Tris 20 mM, $MgCl_2$ 10 mM, DTT 10 mM at pH 7.5) according to Di Luca et al. [19]. The phosphorylation rate of the peptide was analyzed at different times (0, 0.15, 10, 20, 40, 60 min). Samples – de-salted and dried as described above – were analysed by FAB-MS in a mass range of m/z 700–950 at 1,000 resolution.

3. RESULTS AND DISCUSSION

Fig. 1A shows the mass spectrum of 600 ng of unphosphorylated peptide. In this spectrum a major peak at m/z 1,583.9 is present and corresponds to the protonated molecular ion of B-50/39-51, as expected from the calculated mass of the peptide (theoretical monoisotopic mass 1,583.91). This peak represents the predominant ion of the mass spectrum and only minor fragment ions can be detected; these correspond to losses of methyl- and acetyl groups from the *N*-acetyl-terminal of the molecule (m/z 1,568.9 and 1,539.9, respectively). No other ions due to peptide fragmentation are present in the region of the mass spectrum below m/z 1,300 (data not shown), as expected from a soft ionization procedure such as FAB.

A qualitatively similar mass spectrum was obtained with lower amounts of B-50/39-51 and the protonated molecular ion was observed with as low as 50 ng (30 pmol) of the peptide, with a signal-to-noise ratio of more than 3:1 (inset Fig. 1A).

The B-50/39-51 fragment was subjected to in vitro phosphorylation with purified PKC. Even when the phosphorylation reaction was carried out to exhaustion of PKC activity in our experimental conditions, both phospho- and dephospho-forms were present in the same sample mixture.

Fig. 1B shows the mass spectrum of the de-salted mixture; the most abundant peaks correspond to the protonated molecular ions of dephospho- (m/z 1,583.9) and phospho-B-50/39-51 (m/z 1,663.9), indicating the addition of a single phosphate group to the peptide; moreover it is well known that PKC is able to phosphorylate only the serine residue in this particular B-50 sequence [13–15]. Minor peaks, as previously discussed, are present also in this case, together with additional peaks at m/z 1,605.9 and 1,685.9, corresponding to the adduct between the molecular ions with Na^+, as expected when sodium salts are not completely removed from the reaction mixture by the de-salting procedure [20].

After 1 h of incubation only about one third of the peptide was converted to its phospho-form as can be deduced from the mass spectrum. To verify that the intensity of molecular ions reflects actual amounts of the two forms in the reaction mixture we have subjected aliquots of the same sample, from a different and independent experiment in which γ-[^{32}P]ATP was added for phosphorylation, to a gel electrophoresis system able to

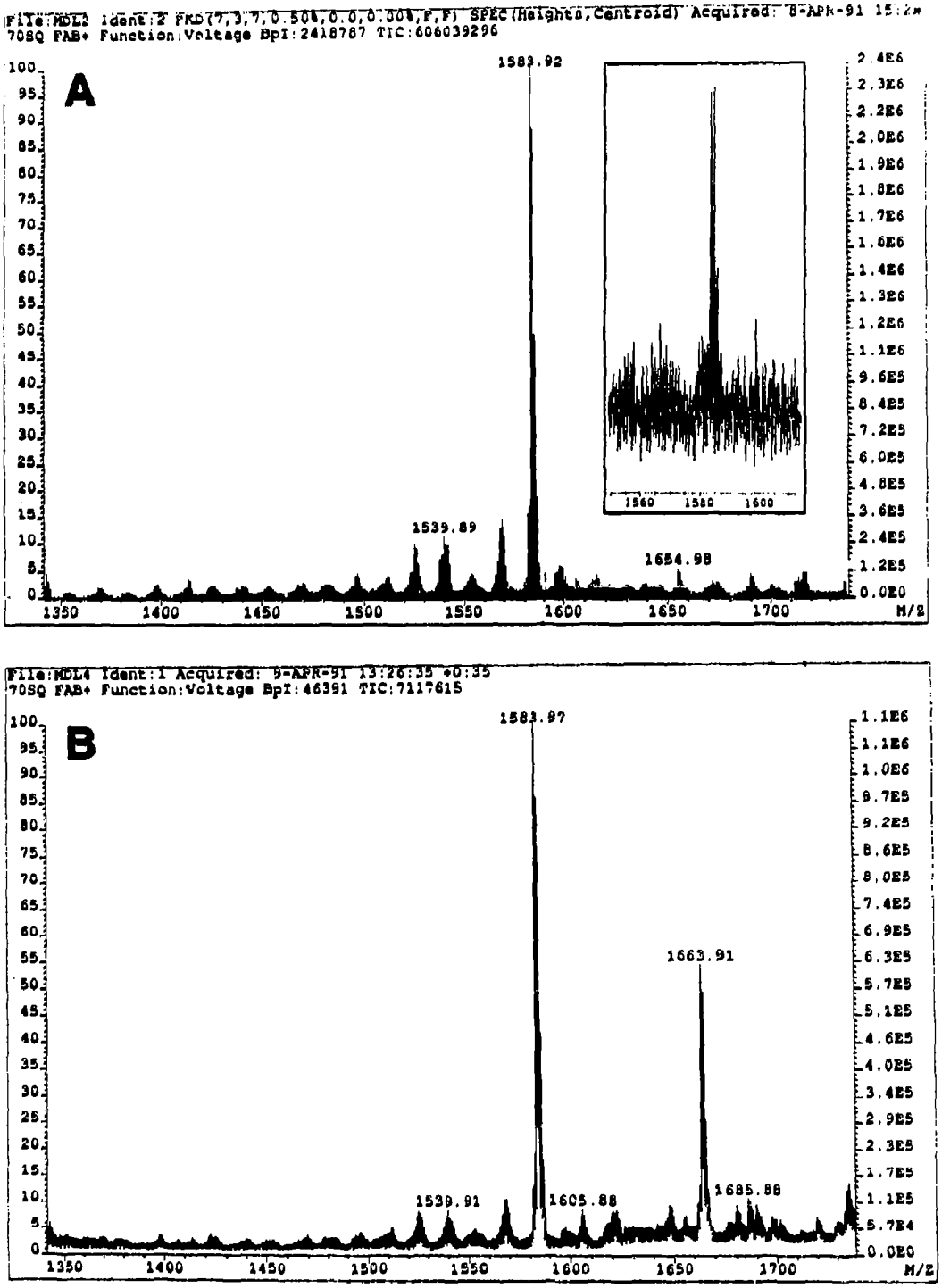

Fig. 1. (A) Typical example of FAB mass spectrum of 600 ng of B-50/39-51; the peak at m/z 1,583.9 corresponds to the protonated molecular ion of the peptide. The inset shows the signal recorded with 50 ng of the peptide. (B) Mass spectra of B-50/39-51 after exhaustive in vitro phosphorylation, the two major peaks at m/z 1,583.9 and 1,663.9 correspond to the molecular ions of dephospho- and phospho-peptide, respectively.

separate phospho- and dephospho-B-50 peptides, and autoradiographs were obtained to locate the phosphoform in the gel (Fig. 2). Densitometric analysis of the gel shows that the ratio of the optical densities of the two protein bands is identical to that obtained by mass spectrometry (ratio dephospho–phospho peptides, mean ± S.D. in the experiment shown: mass spectrometry 3.35 ± 0.31; gel electrophoresis 3.15 ± 0.12).

These results clearly indicate that no molecular rearrangements, such as dephosphorylation processes,

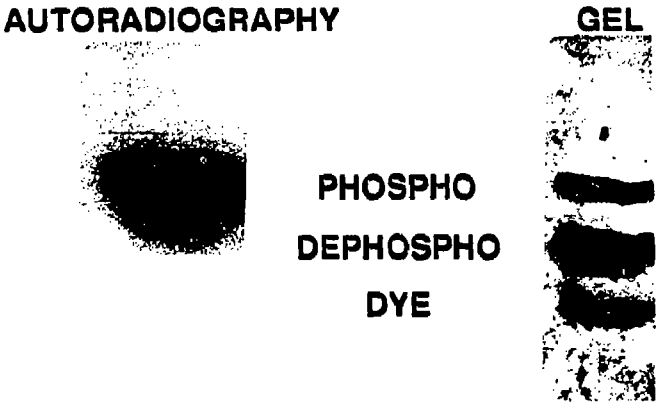

Fig. 2. Gel electrophoresis analysis of phospho- and dephospho-B-50/39-51. Autoradiography was used to identify the phospho-form in the gel.

occur in the source of the mass spectrometer and that the two forms of the peptide are equally sensitive to ionization. Therefore measurement of the ratio of the peak intensities corresponding to the molecular ions of the phospho- and dephospho-peptide seems to be a reliable parameter for their relative amounts in a given sample.

To validate this assumption, we have added to aliquots of the same reaction mixture increasing amounts (50–1,000 ng) of dephospho-peptide and determined if the change in their relative amounts was paralleled by a change in the ratio of the intensities of the respective molecular ions.

Fig. 3 shows that plotting changes in ratios of peak intensities of ions at m/z 1,583.9 and 1,663.9, against amounts of dephospho-peptide added results in a linear relationship at least up to 1,000 ng of dephospho-form added. This shows that FAB-MS can be utilized to detect changes in the ratios of the two forms of the peptide.

To verify the applicability of this method to other phosphopeptides, we measured the relative intensities of the molecular ions of phospho- and dephospho-kemptide, generated by analyzing with FAB-MS the reaction

Table I

Changes, as a function of time, of the peak heights of the molecular ions corresponding to the dephospho (m/z 772.5) and phospho (m/z 852.5) forms of kemptide during in vitro phosphorylation by the catalytic subunit of PKA

Time (min)	Peak height (%)	
	Dephospho	Phospho
0	100	0
0.15	86.9 ± 1.0	13.1 ± 3.8
10	49.9 ± 1.9	50.1 ± 4.9
20	6.2 ± 1.5	93.8 ± 1.0
40	4.2 ± 1.1	95.8 ± 0.9
60	3.7 ± 1.2	96.2 ± 1.1

Mean ± S.E.M. of three experiments.

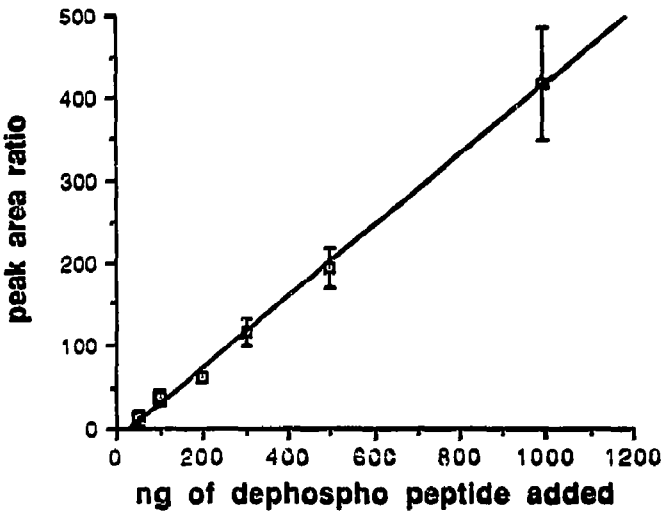

Fig. 3. Linear relationship (r^2=0.998) obtained by plotting changes in the ratio of 1,583.9/1,663.9 peak areas against increasing amounts of dephospho-peptide. Mean ± S.D. (n = 9).

mixture where the PKA-dependent phosphorylation was occurring. Table I shows that the peak height corresponding to the dephospho-form decreases as a function of time after addition to the reaction mixture of the catalytic subunit of PKA. The peak height corresponding to the phospho-form increased progressively, reaching its maximum value between 20 and 40 min.

These data demonstrate therefore, that FAB-MS can be utilized to detect changes in the relative amounts of phospho- and dephospho-peptides in a sample where these amounts are progressively changed through an enzymatic process, better mimicking a biological situation.

In conclusion, we report here results showing that FAB-MS can be applied to evaluate relative amounts of phospho- and dephospho-forms of peptides and that changes of these relative amounts can be monitored by evaluating changes in the intensities of peaks corresponding to their molecular ions. The advantage of this over other methods already available is that both forms of the peptide can be quantitatively measured in the same experimental conditions, in the same sample and are unambiguously identified by mass spectrometry. Experiments are now in progress to show its applicability to fragments obtained by proteolytic digestion of B-50 immunoprecipitated from rat brain tissue.

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