Identification of the sites in myelin basic protein that are phosphorylated by meiosis-activated protein kinase p44^{mpk}

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Myelin basic protein serves as a convenient substrate for detection of a 44 kDa protein-serine/threonine kinase (p44^{mpk}) that is activated near the time of germinal vesicle breakdown in maturing echinoderm and amphibian oocytes. In vitro phosphorylation by purified p44^{mpk} from sea star oocytes was primarily on threonine residues on a single tryptic peptide of bovine brain myelin basic protein. Amino acid composition analysis of the isolated posphopeptide revealed that it was rich in proline residues. Automated solid-phase sequencing by Edman degradation identified the major site as Thr-97 in the sequence NIVTPRTPPPSQGK, which corresponds to residues 91–104 in bovine brain myelin basic protein. Thr-94 was also phosphorylated by p44^{mpk} to a very minor extent.

Myelin basic protein kinase; MAP-2 kinase; Meiosis

1. INTRODUCTION

Abundant cellular proteins can be useful probes for the detection of protein kinases for which the physiological substrates are unknown (see [1] for review). We have found that bovine brain myelin basic protein (MBP) serves as an efficient substrate for a 44 kDa protein-serine/threonine kinase that is activated near the time of nuclear envelope breakdown in 1-methyladenine-treated sea star oocytes [2] as well as *Xenopus laevis* oocytes induced to mature with progesterone [3]. This sea star kinase, provisionally designated p44^{mpk}, has been purified to homogeneity from maturing oocytes and shown to have a restricted specificity [4]. Microprotein sequencing of tryptic peptides derived from the purified kinase has indicated that it is a novel protein kinase (R.A., J.S. and S.P., unpublished). However, p44^{mpk} shares many characteristics with a murine 42 kDa mitogen-activated protein kinase (p42^{MAP}) that phosphorylates microtubule-associated protein MAP-2 [5,6].

The protein kinases p44^{mpk} and p42^{MAP} both appear to operate in protein kinase cascades, albeit at different points in the cell cycle; p44^{mpk} is active at M-phase and p42^{MAP} during the G_0 to G_1 transition. The activations of the two kinases are correlated to their phosphorylation by unknown protein-tyrosine kinases, and both kinases, in turn, can phosphorylate homologous 90–92 kDa ribosomal protein S6 kinases [7,8] (J.S. and S.P., unpublished). It has been proposed that p42^{MAP} is the 42 kDa phosphoprotein that undergoes tyrosine phosphorylation in a variety of mammalian and avian cells in response to polypeptide mitogens and phorbol ester tumor promoters, based upon their co-elution from different types of chromatography columns [9]. Recently, we have observed that rabbit polyclonal antibodies generated against p44^{mpk} purified from sea star oocytes immunoreacts with murine p42^{MAP}, further supporting the relatedness of these kinases (T. Sturgill, J.S. and S.P., unpublished). To begin to delineate the consensus phosphorylation site sequences in substrates that are recognized by p44^{mpk}, we have characterized the sites that are phosphorylated by this kinase in MBP using high sensitivity solid-phase sequence analysis.

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Abbreviations: cdc-2 kinase, protein kinase encoded by homologs of the *S. pombe* cell division control-2 gene; MBP, myelin basic protein; p42^{MAP}, mitogen-activated microtubule-associated protein-2 kinase; p44^{mpk}, meiosis-activated MBP kinase; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Materials*

The protein kinase p44^{mpk} was isolated from maturing *Pisaster ochraceus* oocytes [4]. Bovine brain MBP, phosphoamino acid standards, and trypsin (type IX, porcine pancreas) were purchased from Sigma. [γ-^{32}P]ATP was from ICN.

2.2. *Phosphorylation of MBP*

The phosphorylation of MBP by p44^{mpk} was performed at 30°C for 30 min. The reaction mixture contained in a final volume of 50 μl: 1 mg/ml MBP, 2 μg/ml p44^{mpk}, 50 μM [γ-^{32}P]ATP (2000 cpm/pmol), 30 mM β-glycerophosphate, 20 mM Mops (pH 7.2), 20 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol and 0.5 mM sodium vanadate. The reaction was terminated by addition of 10 volumes of cold acetone

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(−20°C) and incubation on ice for 30 min. The phosphorylated MBP was pelleted by centrifugation in a microfuge for 5 min.

2.3. *Trypsin digestion and fractionation of peptides*

Phosphorylated MBP was resuspended in 10 μl of 0.5 M $NaHCO_3$, 8 M urea (pH 8.2). The concentration of urea was lowered to 2 M by the addition of 30 μl H_2O. Trypsin (2 μl of 1 mg/ml) was added and the incubation performed at 37°C overnight. The resulting peptides were separated by narrow-bore reverse phase high performance liquid chromatography (column, Vydac C-4, 2.1 × 150 mm) using a Waters peptide analyzer. Prior to application to the column, 10 μl of trifluoroacetic acid (TFA):H_2O (10:90, v/v) were added to the mixture of peptides. Chromatography solvents were: solvent A, 0.01% TFA in H_2O; and solvent B, 0.08–0.095% TFA in H_2O:acetonitrile (30:70, v/v). UV absorption of the solvents was matched at 215 nm by titrating the TFA concentration in solvent B. Peptides were eluted with a 0–70% acetonitrile gradient, monitored by UV absorption at 215 nm, and collected manually for further analysis. Peptides co-eluting from this initial separation were further resolved using conditions of different chromatographic selectivity (column, Vydac C-18, 2.1 × 150 mm; solvent A, 150 mM NaCl in H_2O; solvent B, acetonitrile:H_2O (30:30, v/v).

Two-dimensional peptide mapping of ^{32}P-labelled peptides was performed with trypsin-digested MBP and HPLC-isolated peptides. The samples were vacuum dried in a Speedy Vac system, resuspended in 5 μl electrophoresis buffer (1% ammonium bicarbonate, pH 8.9) and spotted on to a cellulose TLC plate (Merck). Electrophoresis in the first dimension was at 600 V for 40 min. The plate was dried and developed in the second dimension in butanol:pyridine:acetic acid:H_2O (32.5:25:5:20, v/v/v/v). The TLC plate was dried and exposed to film (Kodak, XAR-5) for autoradiography.

2.4. *Amino acid composition analysis, phosphoamino acid analysis and amino acid sequencing*

Phosphoamino acid analysis was performed by hydrolyzing a small amount of prelabelled peptide with 200 μl of constant boiling HCl (Pierce) for 1 h at 105°C. The HCl was vacuum dried in a Speedy Vac system, and the amino acids resuspended in 100 μl of H_2O. The sample was dried again and resuspended in 5 μl of electrophoresis buffer (H_2O:acetic acid:pyridine (945:50:5, v/v/v)) containing 1 mg/ml each of phosphoserine, phosphothreonine and phosphotyrosine. After spotting the samples on to silica TCL plates, electrophoresis was performed for 60 min at 1000 V with a constant cooling flatbed electrophoresis apparatus. Standards were visualized by ninhydrin and autoradiography was performed on the TLC plate.

Amino acid composition analysis of totally hydrolyzed HPLC purified peptides was performed using standard PITC precolumn derivatization protocols and analytical instrumentation (Applied Biosystems, Foster City, CA, USA).

Protein sequence analysis of phosphopeptides was performed on a Milligen Model 6600 solid-phase sequenator (Milligen/Biosearch) using standard protocols or an ABI Model 477 instrument (Applied Biosystems) using degradation cycles modified for the efficient extraction of β-eliminated phosphate [10]. Peptides were covalently attached by water soluble carbodiimide to arylamine modified glass fibre membranes or arylamine modified polyvinylidene difluoride membranes (Sequelon AA, Milligen/Biosearch) as described [11,12]. The site of phosphorylation was determined by correlating the PTH signal with β-eliminated [^{32}P]phosphate in a given cycle.

3. RESULTS

MBP was cleaved with trypsin following in vitro phosphorylation of the protein to a stoichiometry of 0.8 mol P/mol MBP by purified $p44^{mpk}$ with [γ-^{32}P]ATP. HPLC analysis of tryptic peptides from MBP revealed the presence of at least three partially resolved peptide peaks (nos 7, 8 and 9) that contained radio-label (Fig. 1A). The early elution (12–13% acetonitrile) of these peptides from the C4 reverse phase column implied that they were relatively hydrophilic. Autoradiography following two-dimensional separation of the phosphopeptides in HPLC peaks nos 7, 8 and 9 showed an identical pattern of one dominant phosphopeptide (data not shown). The appearance of the same major spot on autoradiograms of phosphopeptide maps was also seen when the unfractionated preparation of trypsinized radio-labelled MBP was subjected to the same procedure (Fig. 2).

To establish the nature of the amino acid residue in MBP that was phosphorylated by $p44^{mpk}$, a small amount of the phosphopeptide collected from HPLC (peak no. 8) was hydrolyzed with 6 M HCl. The resulting phosphoamino acids were analyzed by electrophoresis on silica TLC plates (Fig. 3). Over 90% of the phosphorus was incorporated on threonine. There

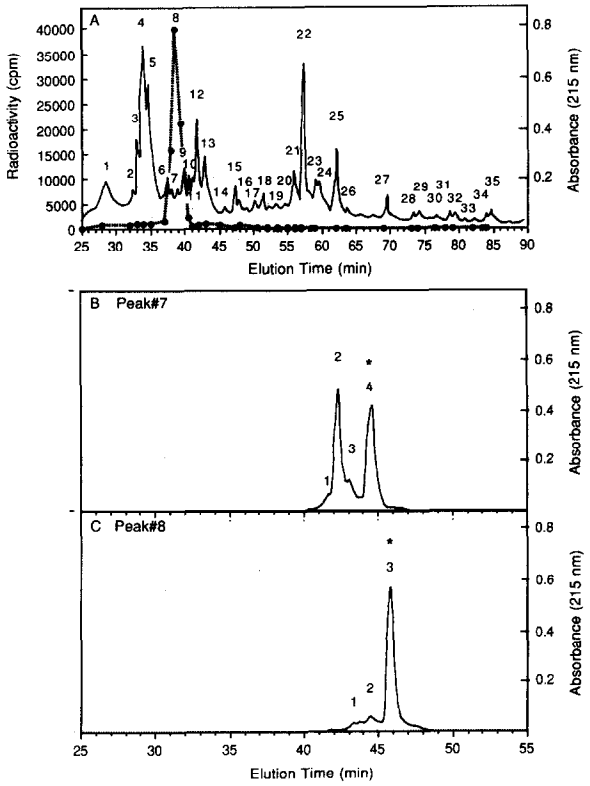

Fig. 1. HPLC profile of tryptic peptides derived from $p44^{mpk}$-phosphorylated MBP. (A) The peptides were resolved on a C-4 reverse phase column (unbroken line) and the corresponding radioactivity in each fraction was quantitated by liquid scintillation counting (•). The recovery of radio-label in the major peptide peaks was 38%. (B) Peak no. 7 from panel A was rechromatographed on a C-18 reverse phase column. The radioactivity in peak nos 1, 2, 3 and 4 was 145, 345, 225 and 4650, respectively. The recovery of radio-label in peak no. 4 was 38%. (C) Peak no. 8 from panel A was rechromatographed on a C-18 reverse phase column. The radioactivity in peak nos 1, 2 and 3, was 615, 1235, and 9935, respectively. The recovery of radiolabel in peak no. 3 was 28%. The peptide peaks were monitored at wavelengths 215 and 280 nm, but only the data from 215 nm is shown (unbroken line).

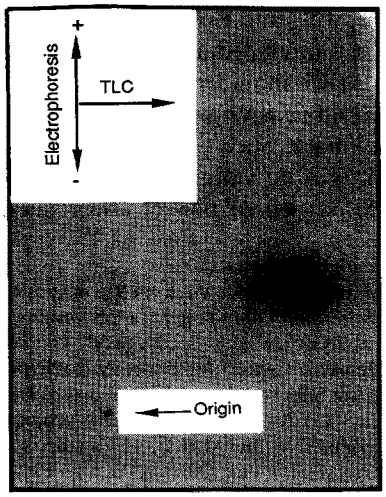

Fig. 2. Autoradiogram of two-dimensional map of tryptic phosphopeptides derived from MBP phosphorylated in vitro by $p44^{mpk}$. MBP (50 μg) was incubated with $p44^{mpk}$ (0.1 μg) in the presence of [γ-^{32}P]ATP, and subsequently tryptic phosphopeptides were prepared and subjected to electrophoresis and TLC as described in section 2.

was little serine phosphorylation. No tyrosine phosphorylation was detected.

Amino acid composition analysis of the phosphopeptide containing the site phosphorylated by $p44^{mpk}$ revealed that it had a high proline content (data not shown). As judged by the cleavage specificity of trypsin for peptide bonds C-terminal to basic amino acids, only a peptide spanning residues 91–104 of MBP featured the determined amino acid composition. This preliminary assignment of this peptide as containing the in vitro phosphorylation site of MBP by $p44^{mpk}$ was

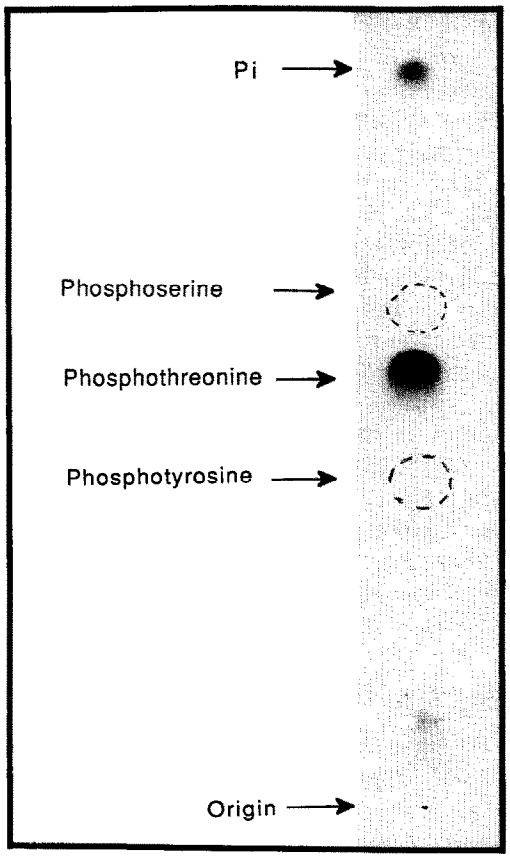

Fig. 3. Phosphoamino acid analysis of MBP phosphorylated by $p44^{mpk}$, HPLC peak no. 8 from phosphorylated and trypsinized MBP was hydrolyzed with constant boiling HCl and subjected to phosphoamino acid analysis as described in section 2. The phosphoamino acid standards were visualized with ninhydrin and their migration positions are indicated on the autoradiogram.

Table I

Sequence of radio-labelled tryptic peptide from $p44^{mpk}$ phosphorylated MBP

Cycle	Deduced amino acid	Position	Radioactivity (cpm)	
			Peak (no. 7)	Peak (no. 8)
1	Asn	91	20	126
2	Ile	92	10	43
3	Val	93	11	25
4	Thr	94	29	16
5	Pro	95	12	14
6	Arg	96	10	13
7	Thr	97	63	108
8	Pro	98	25	26
9	Pro	99	19	17
10	Pro	100	18	23
11	Ser	101	17	12
12	Gln	102	13	9
13	Gly	103	12	21
14	Lys	104	12	18

The radio-labelled peptides in HPLC peaks (no. 7) and (no. 8) were further separated from co-eluting, non-radio-labelled peptides by HPLC, and then sequenced using solid-phase Edman degradation. The deduced amino acid residue and the amount of radioactivity released in each cycle is shown. Cycle 1 radioactivity arose from the removal of [^{32}P]phosphate by β-elimination during sequencing.

confirmed by subjecting peptides contained in HPLC peak no. 8 to peptide sequence analysis. Rechromatography of peak no. 8 using different separation conditions indicated that it contained 3 different peptide species (Fig. 1C). The major protein peak, which contained most of the ^{32}P-label, corresponded to peptides 91–104. The determined sequence of this phosphopeptide is shown in Table I.

The phosphopeptide contained threonine residues at positions 94 and 97. Therefore, it was important to establish the sites phosphorylated by p44^{mpk}. Quantitation of the radioactivity (β-eliminated phosphate) extracted with each cycle during solid-phase Edman degradation of the phosphopeptide demonstrated that Thr-97 was the major site of phosphorylation (Table I). A minor fraction of phosphopeptide, mainly eluting in peak no. 7 from the first HPLC column (Fig. 1A) and peak no. 4 from the second HPLC column (Fig. 1B), contained a smaller amount of ^{32}P-label incorporated into Thr-94, in addition to Thr-97.

4. DISCUSSION

Thr-97 and Thr-94 of MBP represent the first p44^{mpk} phosphorylation sites that have been identified. Protein kinase C has been shown to initially phosphorylate Ser-114 in the corrected bovine MBP primary sequence [13]. However, a synthetic peptide patterned after residues 96–106 of MBP was not phosphorylated by protein kinase C [13]. The presence of proline residues surrounding Thr-97 may participate in recognition of this site by p44^{mpk}. The *cdc*-2-encoded protein kinase appears to phosphorylate serine and threonine residues that are bordered by a proline on the C-terminal side of the phosphorylation site [14], although this has yet to be established with synthetic peptide substrates. However, preliminary studies with synthetic peptide substrates of p44^{mpk} imply that a proline residue on the N-terminal side of the phosphorylated amino acid may be a positive determinant for recognition by this protein kinase (I. Clark-Lewis, J.S. and S.P., unpublished data). Additional peptides modelled after the p44^{mpk} phosphorylation site in MBP should permit a clearer definition of the critical residues involved in substrate recognition by the protein kinase. Such phosphorylation site sequence information will be valuable for the identification of physiological targets of p44^{mpk} and related homologs.

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