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Phosphorylation of P1, a high mobility group-like protein, catalyzed by casein kinase II, protein kinase C, cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II

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P1, a high mobility group-like nuclear protein, phosphorylated by casein kinase II on multiple sites in situ, has been found to be phosphorylated in vitro by protein kinase C, cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II on multiple and mostly distinct thermolytic peptides. All these enzymes phosphorylated predominantly serine residues, with casein kinase II and protein kinase C also labeling threonine residues. Both casein kinase II and second messenger-regulated protein kinases, particularly protein kinase C, might therefore be involved in the physiological regulation of multisite phosphorylation of P1.

Protein P1; Phosphoprotein; Protein kinase; Peptide mapping

1. INTRODUCTION

Protein P1, an acidic protein of apparent M_r = 48000–53000 which resembles the high mobility group of nuclear proteins and is present in a number of cells and tissues, is highly phosphorylated in situ. During mitosis it seems to be further phosphorylated [1]. Previous studies have indicated that the protein is a substrate for casein kinase II [2], a widely distributed enzyme with broad substrate specificity which appears in nuclear fractions in a variety of tissues [3]. Given the importance of protein phosphorylation in cellular regulation [4], and the finding that in vitro phosphorylation with casein kinase II does not completely convert dephosphorylated P1 to P1 as judged by mobility in acetic acid/urea gel electrophoresis [1], we felt it of importance to examine whether other protein kinases might phosphorylate P1. The present study has analysed whether protein kinases regulated by cyclic AMP, calcium/calmodulin and calcium/diacylglycerol can phosphorylate P1 in vitro.

2. MATERIALS AND METHODS

2.1. *Materials*

P1 from rat liver and calcium/diacylglycerol-dependent protein kinase (protein kinase C) from rat brain were purified as described [1,5]. Calmodulin, the catalytic subunit of cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II (CaM kinase II) were gifts from Dr A.C. Nairn, The Rockefeller University, New York, USA. Casein kinase II was a gift from Dr L.A. Pinna, University of Padova, Italy. Other materials and reagents were from standard commercial suppliers.

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2.2. *Phosphorylation of P1*

For phosphorylation with second messenger-regulated protein kinases, 10 μg protein P1 was incubated in a medium (final vol. 0.1 ml) containing 25 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol. The catalytic subunit of cyclic AMP-dependent protein kinase (25 nM) was added with or without 2 μM Walsh inhibitor, the holoenzyme of CaM kinase II (10 nM) was added with 1.25 mM $CaCl_2$ plus 10 μg/ml calmodulin, and protein kinase C (10 nM) was added with 1.25 mM $CaCl_2$ plus 50 μg/ml phosphatidyl-serine. For casein kinase II-catalyzed incubations, 0.25 μg of enzyme and 150 mM KCl were added to the standard medium. The reactions were initiated by addition of 0.05 mM [γ-^{32}P]ATP (10–30 Ci/mmol) and terminated after 5–30 min, followed by gel electrophoresis in acetic acid/urea- or SDS-containing gels [1,6]. Following Coomassie blue staining and autoradiography, ^{32}P-incorporation into P1 was estimated by Cerenkov counting of gel pieces containing the protein.

2.3. *Phosphopeptide mapping*

Destained and washed gel pieces containing P1 were subjected to limit digestion with thermolysin (0.1 mg/ml), and phosphopeptides were separated by electrophoresis at pH 3.5 and chromatography on cellulose thin-layer plates as described [6].

2.4. *Phosphoamino acid analysis*

Aliquots of thermolytic digests were dried and incubated in 0.2 ml 6 N HCl under N_2 at 110°C for 90 min. Phosphoamino acids were separated and visualized by ninhydrin staining and autoradiography as described [7].

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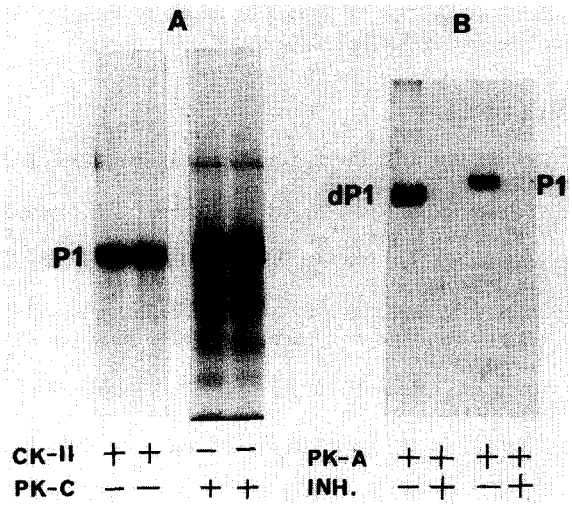

Fig.1. (A) Autoradiogram showing phosphorylation of P1 by casein kinase II (CK-II) and protein kinase C (PK-C). P1 was incubated with [γ-^{32}P]ATP and the indicated enzymes for 15 min, separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (B) Autoradiogram showing phosphorylation of P1 by cyclic AMP-dependent protein kinase. Dephosphorylated P1 (dP1, which shows an increased electrophoretic mobility in these gels) or native P1 were incubated with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase in the absence or presence of Walsh inhibitor (PK-INH) as indicated. The phosphoproteins were separated by urea/acetic acid gel electrophoresis and visualized by autoradiography.

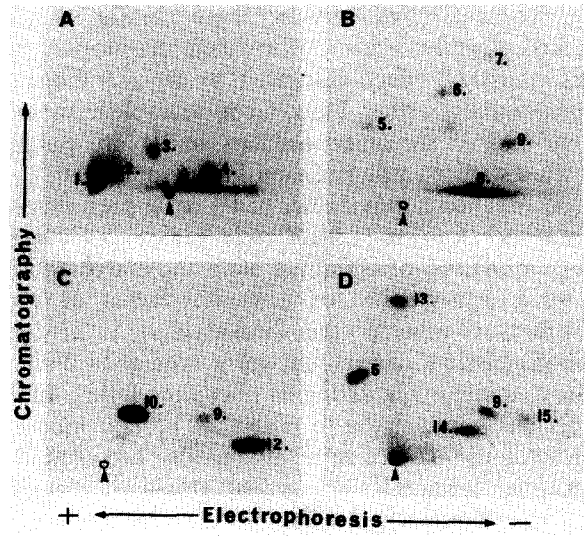

Fig.2. Autoradiogram showing multisite phosphorylation of P1. Casein kinase II (A), protein kinase C (B), cyclic AMP-dependent protein kinase (C) or CaM kinase II (D) were incubated with P1 and [γ-^{32}P]ATP as described. Following limit digestion of P1, the thermolytic phosphopeptides were separated in two dimensions by electrophoresis and chromatography and visualized by autoradiography as described in the text. Numbers indicate major phosphopeptides, and arrowheads indicate application points.

3. RESULTS

3.1. *In vitro phosphorylation of P1*

Protein P1 incorporated considerable amounts of ^{32}P when incubated with [γ-^{32}P]ATP in the presence of either casein kinase II or activated protein kinase C (fig.1A). Under similar conditions, the catalytic subunit of cyclic AMP-dependent protein kinase also induced P1 phosphorylation (fig.1B), although to a somewhat lower extent. The latter phosphorylation could be increased by prior dephosphorylation of P1 with *E. coli* phosphatase [1], and was prevented by the Walsh inhibitor (fig.1B). CaM kinase II could also phosphorylate P1, but induced only 10–20% of P1 phosphorylation when compared to casein kinase II or protein kinase C under comparable conditions (not shown).

3.2. *Peptide mapping of phosphorylated P1*

Limit digestion with thermolysin allowed an examination of the sites in P1 which were phosphorylated by the different kinases. The 4 enzymes tested generated a minimum of 15 phosphopeptides, which presumably represent the domains of the P1 protein that can be phosphorylated by these kinases (fig.2). Most of the casein kinase II-generated phosphopeptides migrated as acidic peptide species during electrophoresis at pH 3.5, while most of both the protein kinase C-generated phosphopeptides and the CaM kinase II-generated phosphopeptides migrated as basic peptides. All phosphopeptides generated by cyclic AMP-dependent protein kinase migrated as basic peptides under the conditions employed. Comparison of the autoradiograms indicated that most of these phosphopeptides were distinct peptide species. Only peptide 5 appeared to be labeled by both protein kinase C and CaM kinase II, while peptide 9 appeared to be labeled both by the two calcium-dependent enzymes and by cyclic AMP-dependent protein kinase (fig.2).

3.3. *Phosphoamino acid analysis of phosphorylated P1*

The 4 protein kinases studied all led to ^{32}P-labeling of serine residues in P1. In addition, casein kinase II and protein kinase C induced slight ^{32}P-labeling of threonine residues (data not shown).

4. DISCUSSION

Protein P1 has been found to be a nuclear protein which is highly phosphorylated in situ [1,2]. Recent analysis, using peptide mapping with *Staphylococcus aureus* V8 protease, has indicated that casein kinase II is responsible for much of this phosphorylation in intact cells [2]. The present report shows that protein kinase C, cyclic AMP-dependent protein kinase and CaM kinase II also can phosphorylate the protein in vitro. Moreover, although the efficiency of P1 phos-

phorylation differed between these kinases, they all induced phosphorylation of multiple thermolytic peptides, most of which appeared to represent distinct phosphorylation sites. Thus, a considerable number of serine and/or threonine residues in P1 are putative substrates for phosphorylation in the intact cell.

The functional importance of P1 or its phosphorylation is not known [1,2]. Casein kinase II-catalyzed phosphorylation of certain protein substrates appears to be a prerequisite for phosphorylation of these substrates by other protein kinases [8,9]. In a similar fashion, casein kinase II may work synergistically with other enzymes in regulating P1 phosphorylation. In this respect it is interesting to note that protein kinase C which can use P1 as a substrate has also been found to be located in the cell nucleus [10]. It will be of importance to examine whether any of the second messenger-regulated protein kinases studied here can catalyze phosphorylation of P1 in intact cells during specific mitotic phases or following extracellular signals which regulate cellular functions through second messengers.

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