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Acidic pentapeptide phosphorylated in vitro by calf thymus protein kinase NII binds to DNA in the presence of Mg^{2+} cations

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The pentapeptide pyroGlu-Ala-Glu-Ser-Asn has been synthetized and phosphorylated in vitro at level of serine by protein kinase NII isolated from calf thymus chromatin. It is noteworthy that the calf thymus kinase NII shows a remarkable affinity for this peptide. The [^{32}P]peptide is able to bind to several DNAs in the presence of Mg^{2+} (λ phage, calf thymus, pBR540 plasmid). This binding appears not specific with regard to the type of DNA and its base sequence. These data support the hypothesis that phosphorylated acidic domains of nuclear nonhistone proteins could bind directly to DNA in the presence of Mg^{2+} cations

Protein kinase NII; DNA-binding peptide; Phosphopeptide

1. INTRODUCTION

It has long been known that nonhistone nuclear proteins are unusually rich in aspartic and glutamic acids [1–3]. Kuehl et al. [4] demonstrated that nonhistone chromosomal proteins are rich in highly charged, acidic oligopeptides up to 12 residues in length, and more rarely contain very long peptides consisting exclusively of acidic residues such as are found in the nonhistone chromosomal proteins HMG1 and HMG2. In addition several authors have reported that the acidic domains, largely distributed in the nuclear nonhistone proteins, frequently contain additional negative charges as phosphoserine or phosphothreonine [5–10]. The enzyme responsible for the phosphorylation of serine or threonine within acidic sequences is the protein kinase NII [11]. The phosphorylation of these acidic protein domains by protein kinase NII appears to be involved in the modulation of protein activity and in the DNA–protein interaction.

We have isolated a family of DNA-binding acidic phosphorylated oligopeptides controlling transcription 'in vitro' and in several cell cultures [12–14]. The biochemical characterization of these peptides shows the presence of a blocked N-terminus which is probably represented by pyroglutamic acid [15,16].

On the basis of the available biochemical information we have designed and synthetized a pentapeptide containing pyroGlu-Ala-Glu-Ser-Asn. In this work we have phosphorylated this pentapeptide by protein kinase NII, isolated from calf thymus chromatin, in order to obtain a peptide model to study the possible interactions between acidic protein domains and DNA.

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2. MATERIALS AND METHODS

2.1. *Purification of nuclear NII kinase*

Nuclear NII kinase was purified from calf thymus. Fresh thymus was quick-frozen and stored in liquid nitrogen. Samples of 250 g were homogenized with a Potter homogenizer in 5 vols of solution consisting of 20 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride (PMSF). After centrifugation at 4000 × *g* for 15 min, the pelleted chromatin was suspended in 1 l of the homogenization buffer containing 1.4 M sucrose and centrifuged at 8000 × *g* for 1.5 h. The pellet was washed once with homogenization buffer and was subsequently extracted with 10 mM Tris-HCl (pH 8.0), 0.35 M NaCl, 0.5 mM PMSF. The extract was centrifuged overnight at 100 000 × *g* and the supernatant was fractionated with ammonium sulfate. The 30–50% saturated fraction was solubilized in 30 ml of 50 mM Tris-HCl (pH 8.0), 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM PMSF and subjected to DEAE-phosvitin-Sepharose- and phosphocellulose chromatography as described by Inoue et al. [11].

2.2. *Analytical methods*

Protein electrophoretic analysis of the enzyme preparation was performed in sodium dodecyl sulfate (SDS) polyacrylamide gels (10%) as described by Laemmli [17]. The gel, silver-stained with a kit from Bio-Rad, shows polypeptide bands at 44 kDa, 36 kDa and 27 kDa. Autophosphorylation of the 27 kDa subunit was observed following incubation of the purified kinase with [γ^{32}P]ATP under normal conditions of phosphorylation. The activity of the kinase NII isolated from calf thymus chromatin is strongly inhibited by heparin at concentrations below 1 μg/ml.

The assay mixture for kinase activity contained 50 mM Tris-HCl

(pH 8), 10 mM $MgCl_2$, 50 mM NaCl, 10–20 μg of casein (or phosvitin or other substrate), 5 μCi of [γ-^{32}P]ATP, and enzyme in a final volume of 0.1 ml. The samples were incubated at 37°C for 20 min, and the reaction was stopped by the addition of 1 ml 10% trichloroacetic acid plus 1.5% (w/v) pyrophosphate. The precipitates were collected on nitrocellulose filters and washed twice with 2 ml portions of trichloroacetic acid-pyrophosphate. ^{32}P-radioactivity was determined by Cerenkov counting.

The phosphorylation of synthetic peptide pyroGlu-Ala-Glu-Ser-Asn and other peptide analogues was performed with the same phosphorylation mixture; following incubation at 37°C for 20 min, aliquots were applied directly onto electrophoretic cellulose plates in order to isolate the phosphorylated compound.

In control experiments the synthetic basic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) (Serva, Heidelberg) was phosphorylated in vitro by 3'-5'-cAMP-dependent protein kinase (Sigma). The phosphorylation procedure and the isolation of phosphorylated peptide were performed according to Kemp et al. [21].

2.3. *Synthesis and purification of peptides*

The peptides, reported in Table I, were synthesized by solution methods using a stepwise procedure. The benzyloxycarbonyl group was selected for temporary protection of the α-amino group, while the *t*-butyl ester was reserved for permanent protection of the carboxy groups. In the coupling reaction, the pentafluorophenyl esters of the protected amino acid were employed. The *Z* group was removed by catalytic hydrogenolysis. All protected intermediates were purified by crystallization until homogeneous in thin-layer chromatography. The final removal of protecting groups was performed with trifluoroacetic acid for 30 min at room temperature. The free peptides were purified by preparative reverse phase HPLC in 0.1% trifluoroacetic acid with 2-propanol gradient.

3. RESULTS AND DISCUSSION

After incubation of synthesized pentapeptide (pyroGlu-Ala-Glu-Ser-Asn) with the protein kinase NII, about 80 μl of the phosphorylation mixture containing 20 μg of peptide were subjected to thin layer electrophoresis, and subsequently the plate was analyzed by autoradiography. The result shows that the protein kinase isolated from calf thymus chromatin has remarkable affinity for the pentapeptide (Table I); it is evident that

Table I

Phosphorylation of synthetic peptides by calf thymus protein kinase NII

Substrate	Relative phosphorylation rate
pyroGlu-Ala-Glu-Ser-Asn	100
Glu-Ala-Glu-Ser-Asn	66
pyroGlu-Ala-Gln-Ser-Asn	55
pyroGlu-Ala-Glu-Ser-Leu	18
pyroGlu-Ala-Glu-Ser	ND
Leu-Arg-Arg-Ala-Ser-Leu-Gly	ND
AcSer-NH_2	ND

The concentration of all substrates was 2 mM. The incubation time was 20 min. the phosphorylation rates are relative to that of the peptide pyroGlu-Ala-Glu-Ser-Asn (100%) (K_m 4 mM, V_{max} 173 nmol·mg·min^{-1}). The phosphorylation of the peptides was performed and estimated as described under Materials and Methods. ND, not detectable.

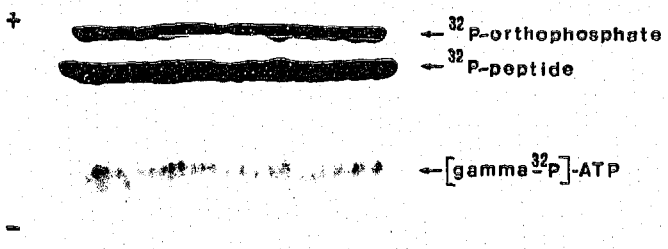

Fig. 1. Autoradiogram of high-voltage preparative electrophoresis of the pentapeptide pyroGlu-Ala-Glu-Ser-Asn phosphorylated in vitro by kinase NII. The electrophoresis was performed on cellulose thin layer plates (20 × 20 cm) for 2 h at 600 V, in acetic acid/formic acid/water/acetone (8:2:75:15 by vol., pH 1.9).

almost all the radioactive phosphate is rapidly transferred (Fig. 1). The kinetic data of the pentapeptide phosphorylation (Table I) are comparable with other synthetic peptides phosphorylated by protein kinase NII as reported by Meggio et al. [18] and Litchfield et al. [19]. The specificity of phosphorylation efficiency has been demonstrated by utilizing a series of peptide analogues; the data reported in Table I show clearly that 3 requirements are important for the peptide phosphorylation; (i) the asparagine on the C-terminal position of serine residue; (ii) the glutamic acid (negative charge) on the N-terminal position of serine residue; and (iii) the blocked peptide N-terminus (absence of positive charge). The synthetic pentapeptide and its

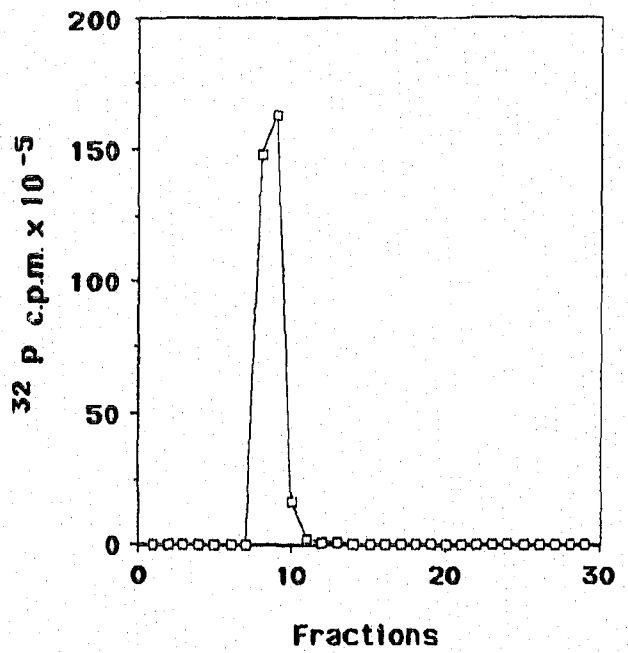

Fig. 2. Chromatography on Sephadex G10 of the in vitro phosphorylated pentapeptide extrcted from electrophoretic plate. The column (1 × 30 cm) was equilibrated and eluted with double-distilled water. The pattern of the ^{32}P-radioactivity elution is reported; the peak corresponds to column void volume.

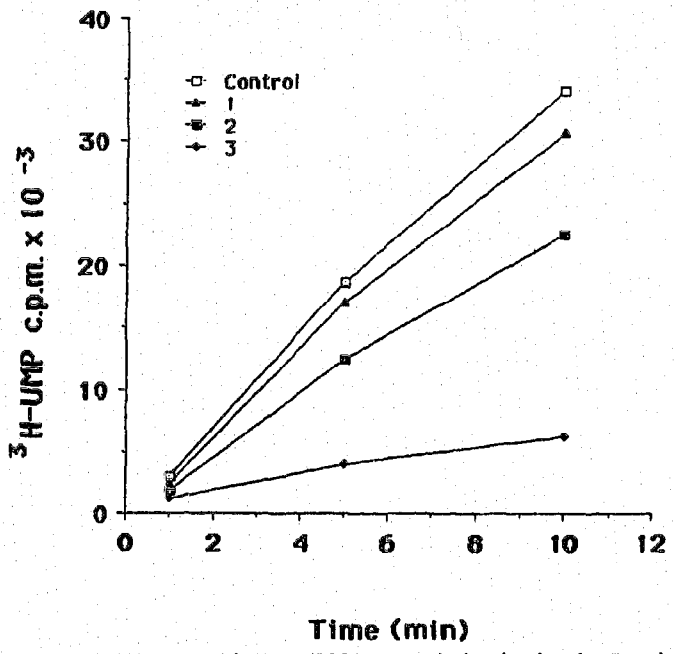

Fig. 3. Time-course of λ phage DNA transcription in vitro by *E. coli* RNA polymerase utilizing a nucleotides/salt conventional mixture [12]: incorporation of [^{3}H]UMP [^{3}H]UTP, Amersham, 12.3 Ci/mmol). Determination of radioactivity incorporated into the acid-soluble fraction was carried out as reported in [12]. Control, no peptide; samples treated with [^{32}P]pentapeptide corresponding to 0.32 pmol (1), 0.96 pmol (2) and 4.8 pmol (3), respectively.

analogues are not phosphorylated by 3'-5'-cAMP-dependent protein kinase (data not shown).

The phosphorylated peptide has been extracted from the cellulose by 50 mM ammonium bicarbonate. After lyophilization (twice) the ^{32}P-labeled peptide was purified by gel filtration on Sephadex G10 (Fig. 2). The radioactivity of the in vitro phosphorylated peptide eluted at void volume was measured by Cerenkov counting: 2.8×10^7 cpm. To confirm that the phosphorylation by protein kinase NII occurs at the level of serine, an aliquot of the phosphorylated peptide has been partially hydrolyzed in 6 N HCl for 3 h at 110°C. The hydrolysate was mixed with phosphoserine standard, applied onto a cellulose sheet and subjected to ascendent thin layer chromatography in *n*-butanol/pyridine/acetic acid/ water (6:2:3:3, by volume). Unlabeled phosphoserine was stained with ninhydrin and ^{32}P-radioactivity was detected by autoradiography. The results confirm the presence of radioactive phosphoserine in the phosphopeptide (data not shown).

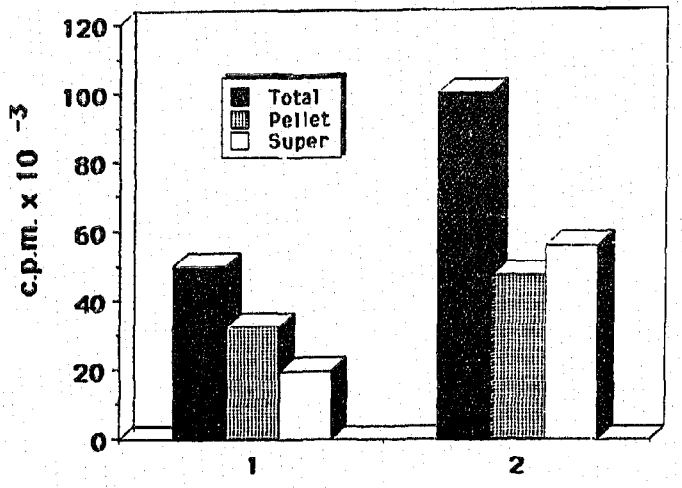

Fig. 4. Binding of ^{32}P-peptide to pBR540 DNA in presence of 10 mM $MgCl_2$. Following incubation with phosphorylated peptide ((1) 1.6 pmols or (2) 3.2 pmol) in 10 mM Tris-HCl, pH 7.5, (30 μl) with or without 10 mM $MgCl_2$, the pBR540 was precipitated by addition of 0.1 M sodium acetate and 2 vols of cold ethanol. The pellet was washed once with 70% ethanol/30% water and subsequently the ^{32}P-radioactivity was measured in the supernatant and in the pellet.

The phosphorylated peptide causes a sharp dose-dependent inhibition of λ phage DNA transcription in vitro by *E. coli* RNA polymerase (Fig. 3). It is noteworthy that unphosphorylated peptide does not impair the transcription rate even at high concentrations (up to 5 μg of peptide/μg DNA).

In order to investigate if the transcription is inhibited through binding of the ^{32}P-peptide to the DNA, the in vitro phosphorylated peptide was incubated with λ phage and calf thymus DNA in the presence or absence of $MgCl_2$; considering that the transcription mixture contains Mg^{2+} cations. Subsequently the DNA was precipitated by the addition of 0.1 M sodium acetate and 2 vols of ethanol and the ^{32}P-radioactivity measured in the pellet and in the supernatant.

Table II

Binding of in vitro phosphorylated peptide to calf thymus and λ phage DNA

DNA 2 μg	^{32}P-peptide (cpm)	$MgCl_2$ (mM)	Precipitation by ethanol: Pellet (cpm)	Precipitation by ethanol: Supernatant (cpm)
calf thymus	50 000	10	41 265	13 685
	100 000	10	68 018	27 580
	200 000	10	117 891	90 536
	50 000	0	2 516.	49 861
	100 000	0	2 243	111 540
	200 000	0	3 746	186 332
λ phage	50 000	10	47 135	9 501
	100 000	10	87 068	16 293
	400 000	10	188 675	235 927
	800 000	10	362 727	452 162
	50 000	0	1 324	46 419
	100 000	0	1 480	101 110
	400 000	0	4 213	390 819
	800 000	0	3 724	803 631

Following incubation with phosphorylated peptide in 10 mM Tris-HCl, pH 7.5, (30 μl) in presence (or absence) of 10 mM $MgCl_2$ the DNA was precipitated by addition of 0.1 M sodium acetate and 2 vols of cold ethanol. The pellet was washed once with 70% ethanol/30% water and subsequently the ^{32}P-radioactivity was measured in the supernatant and in the pellet. Considering the Cerenkov counting efficiency, 100 000 cpm correspond to 3.2 pmol of ^{32}P-peptide.

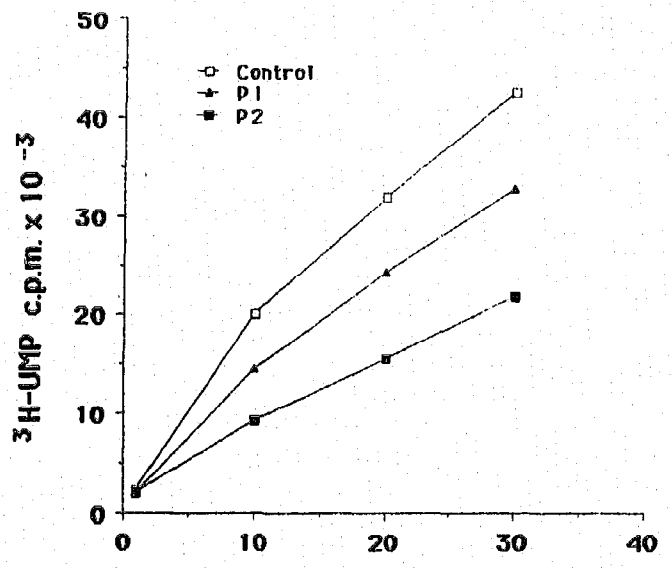

Fig. 5. Time-course of PBR540 DNA transcription in vitro by *E. coli* RNA polymerase utilizing a nucleotide/salt conventional mixture [12]: incorporation of [^{3}H]UMP. Determination of radioactivity incorporated into the acidic-insoluble fraction was carried out as reported in [12]. pBR540 plasmid DNA (2 μg) was incubated with phosphorylated peptide in presence of $MgCl_2$ as described in the legend of Table II and the DNA-peptide complexes precipitated with ethanol, resolubilized and transcribed. Control DNA (□); DNA in presence of ^{32}P-peptide, 1.6 pmols (▲) and 3.2 pmols (■).

The results reported in Table II show that the phosphorylated peptide is able to bind to DNA and the presence of Mg^{2+} cations is necessary for the binding. Furthermore we have demonstrated that the unphosphorylated pentapeptide is not able to compete with the phosphorylated form for binding to DNA. The specificity of Mg^{2+}-mediated binding between DNA and phosphorylated acidic peptides is supported by experiments demonstrating that the basic phosphorylated heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly is not able to bind to DNA in the absence or presence of Mg^{2+} cations (data not shown).

The DNA binding ability of the [^{32}P]pentapeptide (pyroGlu-Ala-Glu-Ser-Asn) has in addition been confirmed with the pBR540 DNA plasmid (Fig. 4). The [^{32}P]- peptide–pBR540 DNA complex was subsequently tested as template in the transcription reaction performed in vitro with *E. coli* RNA polymerase. The binding with the peptide strongly decreased the template capacity of DNA (Fig. 5). This binding appears not to be specific with regard to the type of DNA and its base sequence, and it probably occurs through a bridge formed by Mg^{2+} cations.

The results presented in this work support the hypothesis that phosphorylated acidic domains of nuclear proteins can bind directly to DNA in the presence of Mg^{2+} cations. This possibility appears interesting because the most general idea is that acidic proteins bind to chromatin through electrostatic interactions with the histones [20]. Another interesting point is that the phosphorylated pentapeptide shows some functional characteristics very similar to those of the acidic peptides we isolated, thus representing a molecular model which may be developed and improved to obtain synthetic peptides able to reproduce the biological activity shown by the peptide fraction controlling DNA transcription in vitro and RNA synthesis in several cell systems [12–16].

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