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The phosphorylation of nucleoplasmin by casein kinase-2 is resistant to heparin inhibition

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Highly purified preparations of casein kinase-2 from the nuclei of *Xenopus laevis* oocytes and from calf thymus can phosphorylate in vitro purified nucleoplasmin from *X. laevis* oocytes and eggs. The phosphorylation of nucleoplasmin by both kinase preparations is quite insensitive to heparin in contrast with casein phosphorylation which is completely abolished by heparin concentrations above 10 μg/ml. However, the phosphorylation of nucleoplasmin and casein are inhibited in a very similar fashion by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a well characterized specific inhibitor of casein kinase-2. Similarly, nucleoplasmin phosphorylation by the oocyte enzyme can be stimulated several-fold by spermine, another characteristic of this enzyme. These findings indicate that the phosphorylation of nucleoplasmin by purified casein kinase-2, while showing typical response to DRB and spermine, exhibits anomalous behavior in its resistance to heparin inhibition. It is possible that the large clusters of acidic amino acids in nucleoplasmin permit this substrate to interact with the enzyme more efficiently than other protein substrates. Heparin is generally considered a potent and specific inhibitor of casein kinase-2. This study, however, questions the validity of utilizing heparin inhibition as a criterion for casein kinase-2 involvement.

Protein phosphorylation; Casein kinase-2; Heparin; Nucleoplasmin; Spermine; (Oocyte nucleus)

1. INTRODUCTION

A widely distributed cyclic AMP-independent protein kinase that phosphorylates preferentially acidic proteins has been called casein kinase-2 (also casein G or protein kinase NII). This protein kinase is present in the cell nucleus and in cytoplasm and is activated by polyamines and inhibited by low concentrations of heparin [1-4].

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Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DTT, dithiothreitol

The inhibition by heparin concentrations below 10 μg/ml has been used as a distinctive characteristic of casein kinase-2 because most of the other protein kinases require much higher concentrations of this polyanion to show any inhibition [4,5].

The nucleotide analog DRB is also a potent and specific inhibitor of casein kinase-2 [6]. Casein kinase-2 has been implicated in the phosphorylation of several important proteins such as: protein synthesis initiation factor 2 (eIF_2) [7]; the regulatory subunit of cAMP-dependent protein kinase-2 [8]; high mobility group protein 17 [9]; RNA polymerase II [10]. Evidence has also been presented for the involvement of casein kinase-2 in the regulation of transcription by RNA polymerase II [11].

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Nucleoplasmin is an acidic protein that is abundant in the nucleus of amphibian oocytes, and it apparently plays an important role in the assembly of nucleosomes by binding histones and transferring them to DNA [12]. Recent work has indicated that the nucleosome assembly activity of nucleoplasmin may be regulated by phosphorylation-dephosphorylation of this protein. Further, it has been shown that hormonal induction of oocyte maturation causes a large increase in the phosphorylation of nucleoplasmin with a concomitant increase in its nucleosome assembly activity [13,14].

We have reported that casein kinase-2 is a major protein phosphorylating activity present in the isolated nuclei of *Xenopus laevis* oocytes [15]. In the present report data are presented to show that highly purified casein kinase-2 from *X. laevis* oocytes or from calf thymus is able to phosphorylate purified preparations of nucleoplasmin. Of special interest is the demonstration that the phosphorylation of nucleoplasmin by casein kinase-2 is anomalous in that it is resistant to heparin inhibition.

2. MATERIALS AND METHODS

Large female *X. laevis* were purchased from the South African Snake Farm, Cape Province, Republic of South Africa.

Nuclei were isolated from stage 5 and 6 oocytes by the technique described by Burzio and Koide [16].

Casein kinase-2 was purified from *X. laevis* oocyte nuclei using chromatography on DEAE-Sephadex as described in [15] and subsequent fractionation on phosphocellulose as described by Hathaway and Traugh [17]. The enzyme was highly purified, showing subunits of 44 and 26 kDa. Casein kinase-2 of calf thymus was purified as described [11], the resulting enzyme being at least 95% pure.

Nucleoplasmin was purified from *X. laevis* oocytes or eggs according to the method of Dingwall et al. [18] and migrated as a single polypeptide of 31 kDa on SDS gels.

2.1. *Protein kinase assay*

Assays were carried out in a total volume of 30 μl with the following components: 50 mM Hepes, pH 7.9; 100 mM KCl; 7 mM $MgCl_2$; 10 mM DTT; 10 or 100 μM [γ^{32}-P]ATP with a specific activity of 500–4000 cpm/pmol; 1 mg/ml of bovine serum albumin; 15 mg/ml of casein prepared as described by Traugh and Traut [19]; and enzyme as indicated in the legends to the figures. Incubations were for 15 min (casein substrate) or 30 min (nucleoplasmin substrate) at 30°C. The reaction was stopped by applying a 25 μl aliquot to a 1 × 2 cm phosphocellulose strip (Whatman P 81) and immersing it in 75 mM phosphoric acid. The papers were washed extensively in the same acid, dried and radioactively measured as described [20]. Values were corrected for background levels obtained with boiled enzyme. Phosphorylation of endogenous protein was negligible (less than 2% of the activity observed with casein). Reactions were carried out in duplicate and results averaged. When inhibition by DRB was measured, controls were carried out with an equivalent concentration of the vehicle ethanol. Ethanol concentrations did not exceed 0.05% and did not alter enzyme activity.

The unit of enzyme activity is defined as the amount which catalyzes the incorporation of 1 pmol of $^{32}P_i$ per min under standard conditions.

2.2. *Polyacrylamide gel electrophoresis and autoradiography*

Fractionation of phosphorylated proteins was carried out on SDS-polyacrylamide gel electrophoresis using 13% acrylamide according to Laemmli [21]. Samples were heated for 3 min at 100°C in electrophoresis sample buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 1% β-mercaptoethanol and 4 M urea, as described by Cotten et al. [14]. Molecular mass marker proteins used were: phosphorylase *b*, 94 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21 kDa; and α-lactalbumin, 14.4 kDa.

Autoradiographs of the dried gels were carried out using Kodak Royal X-OMAT film [α^{32}-P]ATP was prepared and purified by the method of Walseth and Johnson [22] using ^{32}P from Amersham.

Protein concentration was estimated by the method of Bradford [23] with bovine serum albumin as standard.

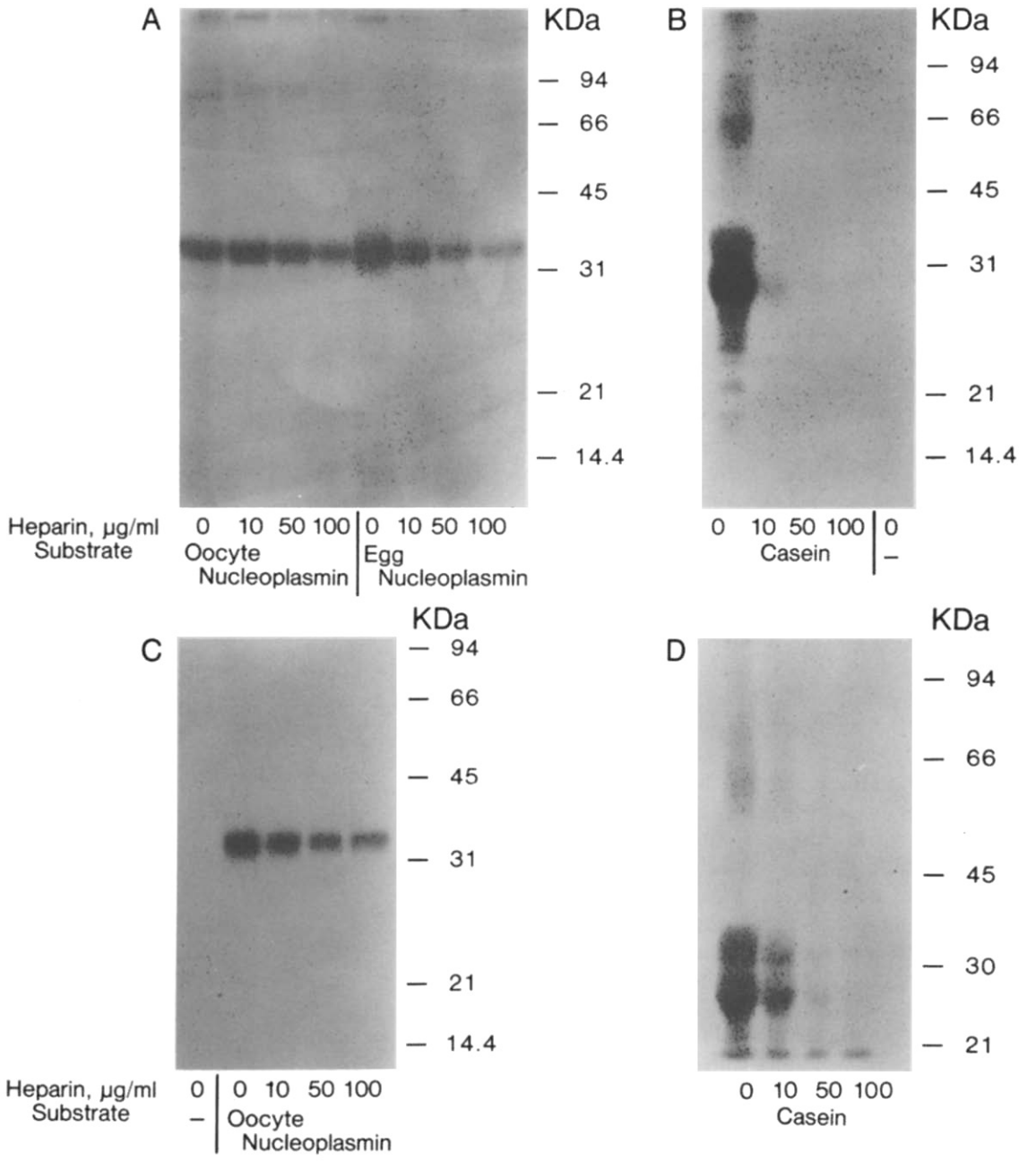

Fig.1. Effect of heparin on the phosphorylation of nucleoplasmin and casein by purified casein kinase-2 preparations from amphibian oocytes and calf thymus. Oocyte casein kinase-2 (90 units) was assayed in the absence or the presence of different concentrations of heparin and nucleoplasmin (NP) (A) from oocytes (23 μg/ml) or eggs (6.5 μg/ml) or with casein (B), 15 mg/ml. A control for phosphorylation of endogenous protein is shown in (B). Calf thymus casein kinase-2 (60 ng) was assayed in the absence or presence of different concentrations of heparin and 23 μg/ml oocyte nucleoplasmin (C) or with 15 mg/ml casein (D). A control for phosphorylation of endogenous protein is shown in (C). ^{32}P incorporation was evaluated by autoradiography after SDS-polyacrylamide (13%) gel electrophoresis as given in section 2. Numbers to the right of each panel are molecular masses given in kDa.

3. RESULTS

Incubation of nucleoplasmin with a highly purified preparation of casein kinase-2 from the nuclei of *X. laevis* oocytes and [γ-^{32}P]ATP results in a clear phosphorylation of this protein which migrates as a 31 kDa polypeptide (its true molecular mass is 22024 Da [24]). As can be observed in the autoradiography of fig. 1A, the phosphorylation of nucleoplasmin is only slightly inhibited by heparin at a concentration of 10 μg/ml and, even at a concentration of 100 μg/ml of the polyanion, phosphorylation of nucleoplasmin is clearly observed. In this experiment, nucleoplasmin obtained from both ovarian oocytes or ovulated eggs was tested, giving qualitatively similar results. The amount of egg nucleoplasmin employed was one third the amount of oocyte protein. This observation can be contrasted with the phosphorylation of a commercial casein preparation by the same enzyme, as shown in fig.1B. In this case heparin at 10 μg/ml inhibits about 90% of the phosphorylation of casein and the higher concentrations of heparin completely block the reaction.

In order to confirm the observation that the nucleoplasmin phosphorylating activity was indeed catalyzed by the oocyte casein kinase-2 not due to a contaminating kinase activity, a similar experiment was performed with a highly purified preparation of casein kinase-2 obtained from calf thymus [11]. The results presented in fig.1C and D again show that with the thymus casein kinase-2, nucleoplasmin is phosphorylated and that this phosphorylation is highly resistant to heparin inhibition as compared to casein phosphorylation.

The nucleotide analog, DRB, has also been shown to be a specific inhibitor of casein kinase-2 [6,11]. The phosphorylation of nucleoplasmin and casein by the oocyte casein kinase-2 was tested in the presence of increasing concentrations of DRB (fig.2). It can be observed that the percent inhibition of the phosphorylation of both proteins at each DRB concentration is very similar if not identical.

Another characteristic of casein kinase-2 is its capacity to be stimulated by polyamines. The results presented in fig.3 show the effect of spermine on the phosphorylation of nucleoplasmin and casein by the casein kinase-2 of *X. laevis* oocytes.

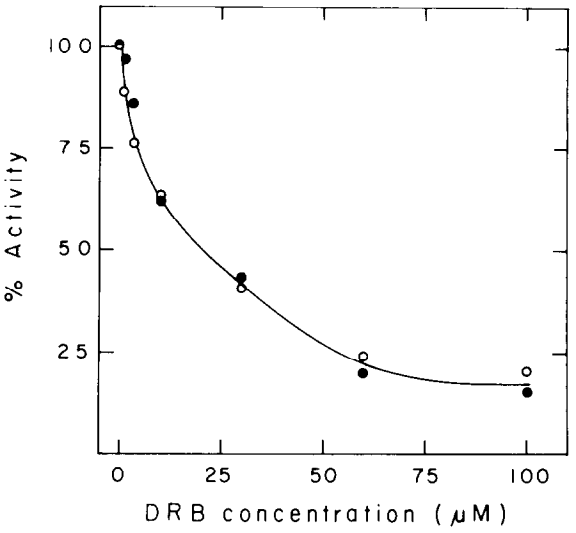

Fig.2. DRB inhibition of nucleoplasmin and casein phosphorylation by oocyte casein kinase-2. Purified casein kinase-2 (90 units) was assayed in the presence of different concentrations of DRB and nucleoplasmin (●, 23 μg/ml) or casein (○, 15 mg/ml) as described in section 2. Activity is given as % of control.

With both protein substrates the stimulatory effect of spermine is very similar, showing an optimum at about 2 mM spermine. In both cases the stimulation was about 5-fold.

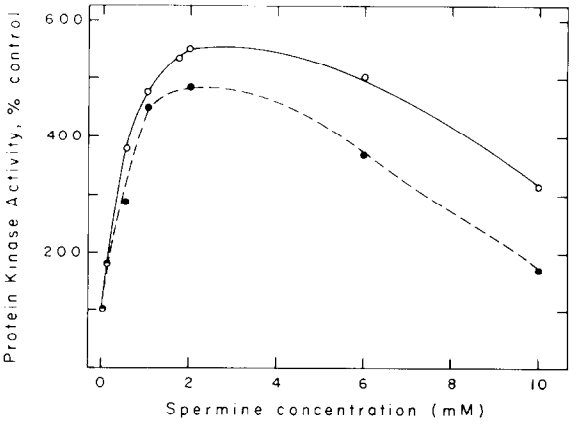

Fig.3. Phosphorylation of nucleoplasmin and casein by oocyte casein kinase in the presence of spermine. 90 units of oocyte casein kinase-2 were assayed in the presence of different concentrations of spermine with substrate nucleoplasmin (●, 23 μg/ml) or casein (○, 15 mg/ml) as given in section 2. Activity is given as % of control.

4. DISCUSSION

The work presented above demonstrates that highly purified casein kinase-2 preparations isolated from two different species are able to phosphorylate nucleoplasmin purified from *X. laevis* oocytes and eggs. However, the phosphorylation of nucleoplasmin by casein kinase-2 does not conform to the test of sensitivity of this enzyme to inhibition by low concentrations of heparin, a widely used criterion for the involvement of casein kinase-2 in protein phosphorylation reactions [1,3,4]. There are at least two possible explanations for this anomalous observation: (i) the phosphorylation of nucleoplasmin is catalyzed by a different protein kinase that contaminates both casein kinase-2 preparations; (ii) the phosphorylation of nucleoplasmin by casein kinase-2 is unusually resistant to heparin inhibition. The second explanation seems more plausible because the casein kinases from both tissues were extensively purified and no evidence the presence of a contaminating activity was observed. A stronger argument for the involvement of casein kinase-2 in nucleoplasmin phosphorylation is provided by the fact that the reaction is inhibited by DRB, a specific inhibitor of this enzyme, and activated by spermine.

The question then arises as to why the phosphorylation of nucleoplasmin by casein kinase-2 is much more resistant to heparin inhibition than that of casein or of other known protein substrates. An explanation may be found in the amino acid sequence of nucleoplasmin which has been recently deduced from the nucleotide sequence of its gene [24,25]. This protein contains large clusters of glutamic and aspartic acid which in some instances are preceded by serines or threonines. The sequence from amino acids 128 to 146 contains 17 acidic amino acids out of a total of 19 residues. Pinna and his co-workers [3] have established that the enzyme preferentially phosphorylates a serine and a threonine that is followed by a cluster of two or more acidic amino acids at the carboxyl end. This same group has shown that polyglutamic acid (averaging 70 residues) is a strong competitive inhibitor of casein phosphorylation with a K_i of 0.1 μM [26]. Shorter polyglutamine peptides (10 residues) have lower affinities for the enzyme.

This evidence supports the proposal that the active site of casein kinase-2 has high affinity for polyanionic peptides or other acidic polymers and that heparin inhibition is due to its similarity to these compounds. The unusually long stretch of polyglutamic acids in nucleoplasmin may allow this substrate to compete advantageously with heparin, raising the concentration of the compound required for inhibition. This finding questions the validity of using the sensitivity to heparin as a criterion in defining the participation of casein kinase-2. In addition, it demonstrates that nucleoplasmin and other nuclear proteins rich in acidic residues may be the preferred substrates of casein kinase-2.

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