Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 456-463

A NUCLEAR CASEIN TYPE II KINASE FROM MAIZE ENDOSPERM PHOSPHORYLATING HMG PROTEINS

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Received May 22, 1989

Summary: A casein kinase of the type II was isolated and enriched from nuclear lysates of maize endosperm tissue. The kinase activity requires 10 mM Mg^{2+} for maximal activity, can utilize either ATP or GTP as phosphate donors and is inhibited by polyamines, heparin and monovalent cations. A substrate specificity of the kinase activity towards specific nuclear proteins is indicated by its phosphorylation of high mobility group (HMG) proteins isolated from endosperm and its lack of accepting histones as protein substrates.

Protein kinases exist in great variability in many organisms and are often used as efficient mediators for a fast response in regulatory reactions and signal transduction pathways (1). They act mostly in membranes and in the cytoplasm, but more recently their essential role has also been recognized for regulatory mechanisms located in the cell nucleus. In this respect phosphorylations of specific DNA binding proteins, as well as growth associated phosphorylations of chromosomal histone and non-histone proteins have been observed indicating the importance of phosphorylation in chromatin structure (2,3,4). Among the nuclear kinases of animal cells, the casein type kinases, which preferentially phosphorylate acidic proteins and are independent of cyclic nucleotides and calcium, have obtained particular interest. The type I casein kinases consist of one small subunit and utilize ATP as phosphate donor, while those of the type II represent a complex tetramer structure and can utilize either ATP or GTP (5). Recently, nuclear casein type II kinases have been characterized from dicotyledonous plants (6,7), but little is known about this type of enzymes in monocotyledonous plants. Since substantial progress has also recently been made in the analysis of the regulation of nuclear plant genes, a better knowledge of nuclear kinases and their involvement in regulatory features has become highly desirable. Towards this goal, we have isolated and enriched a nuclear kinase from maize endosperm by ATP-agarose chromatography. This enzyme activity was identyfied as a casein type II kinase and could be successfully used for the specific phosphorylation of non-histone chromosomal HMG proteins (4,8) from endosperm.

Abbrevations: HMG proteins, high mobility group proteins; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride.

0006-291X/89 $1.50

Materials And Methods

Materials

Dephosphorylated casein from bovine milk, alkaline phosphatase from calf intestinal mucosa, spermine, heparin, heparin-agarose and ATP-agarose were obtained from Sigma, while histone mix from calf thymus was obtained from Boehringer. [γ-^{32}P]ATP (5000 Ci/mmol) and [γ-^{32}P]GTP (10 Ci/mmol) were purchased from Amersham. Ultrogel AcA 44 was obtained from Pharmacia.

Purification of casein kinase

Nuclear extracts from maize endosperm tissue (8 days after pollination) were prepared as described previously by Maier et al. (9,10). The collected extracts from 320 g frozen material were applied to a 2 ml heparin-agarose column and eluted with a linear gradient (10 ml) from 0 M to 1 M KCl in a buffer containing 50 mM Tris/HCl pH 7.9, 1 mM EDTA, 12.5 mM $MgCl_2$, 20% glycerol, 1 mM DTT and 0.5 mM PMSF. The pooled fractions displaying casein kinase activity were applied to a 1 ml affinity chromatography column of ATP-agarose, which was eluted with a linear gradient (10 ml) from 500 mM to 1 M NaCl in a 10 mM potassium phosphate buffer containing 7.5 mM $MgCl_2$, 5 mM EDTA, 10 mM 2-mercaptoethanol and 0.5 mM PMSF. The collected fractions were stored at -70°C for later use. To determine the molecular weight of the casein kinase, a high activity fraction was applied to an Ultrogel AcA 44 gel filtration column in a buffer containing 20 mM Tris/HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA, 0.02% NaN_3, 10% glycerol, 1 mM DTT and 0.5 mM PMSF. All procedures were performed in the cold room.

Phosphorylation assay

Protein kinase activity was assayed routinely in a standard incubation mixture of 50 μl containing 2.5 mg/ml dephosphorylated casein, 0.6 μg enzyme preparation, 50 mM Hepes/NaOH (pH 7.5), 10mM $MgCl_2$, 1 mM EGTA, 3 μCi [γ-^{32}P]ATP. The reaction, started by the addition of radioactive ATP, was conducted at 25°C for 30 minutes and terminated by the addition of 10 μl SDS loading buffer (11), followed by heating at 95°C for 5 minutes. The assay mixture was subjected to SDS-PAGE according to Laemmli (11). The incorporation of radioactive phosphate was determined by autoradiography (Fuji x-ray film RX) and subsequent scanning with a Uvikon densitometer, or by Cerenkov counting of radioactive gel bands in a Beckman scintillation counter.

Isolation of HMG proteins

The purification procedure was performed with modifications according to Spiker (12). Nuclear extracts from 320 g frozen endosperm tissue were prepared and applied to a 2 ml heparin-agarose chromatography column as specified above. To the proteins eluted at approximately 700 mM KCl trichloroacetic acid was added to a final concentration of 2% (w/v), followed by a incubation on ice for 1 h before the solution was centrifuged at 12000 g for 10 minutes. After adding 2 volumes of acetone to the supernatant and allowing it to stand on ice for 2 h, the precipitated HMG proteins were pelleted by centrifugation at 25000 g for 20 minutes. The pellet was washed with acetone, vacuum dried and resuspended in the buffer used for heparin-agarose chromatography.

Results

Enrichment of nuclear kinase activity from endosperm by ATP-agarose chromatography

Nuclear lysates from 320 g frozen maize endosperm tissue were isolated as described previously (9,10) and enriched for kinase activity by heparin-agarose. The column fractions were assayed for kinase activity with casein, which had previously been found to be an efficient substrate for the kinase activity. The phosphoproteins formed by this enzymatic reaction were analyzed by a polyacrylamide gel electrophoresis as shown in Figure 1 for a kinetic analysis of the kinase reaction. To quantify the radioactive phosphate incorporated into protein, the autoradiograms of separation gels were scanned or radioactive bands were cut from the gel and counted in a

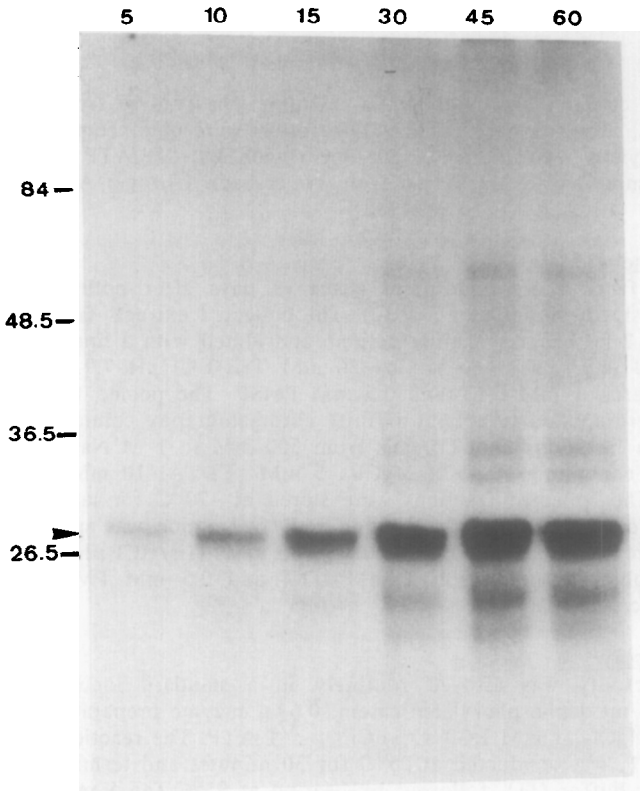

Figure 1
Kinetic of the phosphorylation reaction.
Autoradiogram of a kinetic analysis of the phosphorylation reaction in standard assays with casein as protein substrate. The casein band is indicated by an arrow. The numbers on the side indicate molecular weight markers in KDa and the numbers on the top indicate reaction time in minutes..

scintillation counter. The slightly enriched kinase activity contained in the pooled heparin-agarose fractions was further enriched by ATP-agarose chromatography leading to a total enrichment of approximately 100 fold. In addition the kinase preparation was now free of phosphatases, as demonstrated by an enzyme test with p-nitrophenyl-phosphate.

The isolated kinase activity is similar to casein II type enzymes

The efficient acceptance of casein as protein substrate by the kinase acivity, as well as the results of the following experiments characterized the kinase activity as belonging to the casein type II (5). As shown in Figure 2a, the radioactive labelling of casein by the kinase activity with [γ-^{32}P]ATP is inhibited by addition of an excess of nonradioactive GTP to the reaction mixture, suggesting that GTP can also serve as a phosphate donor. This was demonstrated more directly by working with [γ-^{32}P]GTP as phosphate donor (Fig. 3).
The kinase reaction displays a Mg^{2+} optimum of 10 mM (Fig. 2b) and is inhibited by addition of increasing concentrations of NaCl or KCl, leading to 50% inhibition at about 5 mM (Fig. 2c).

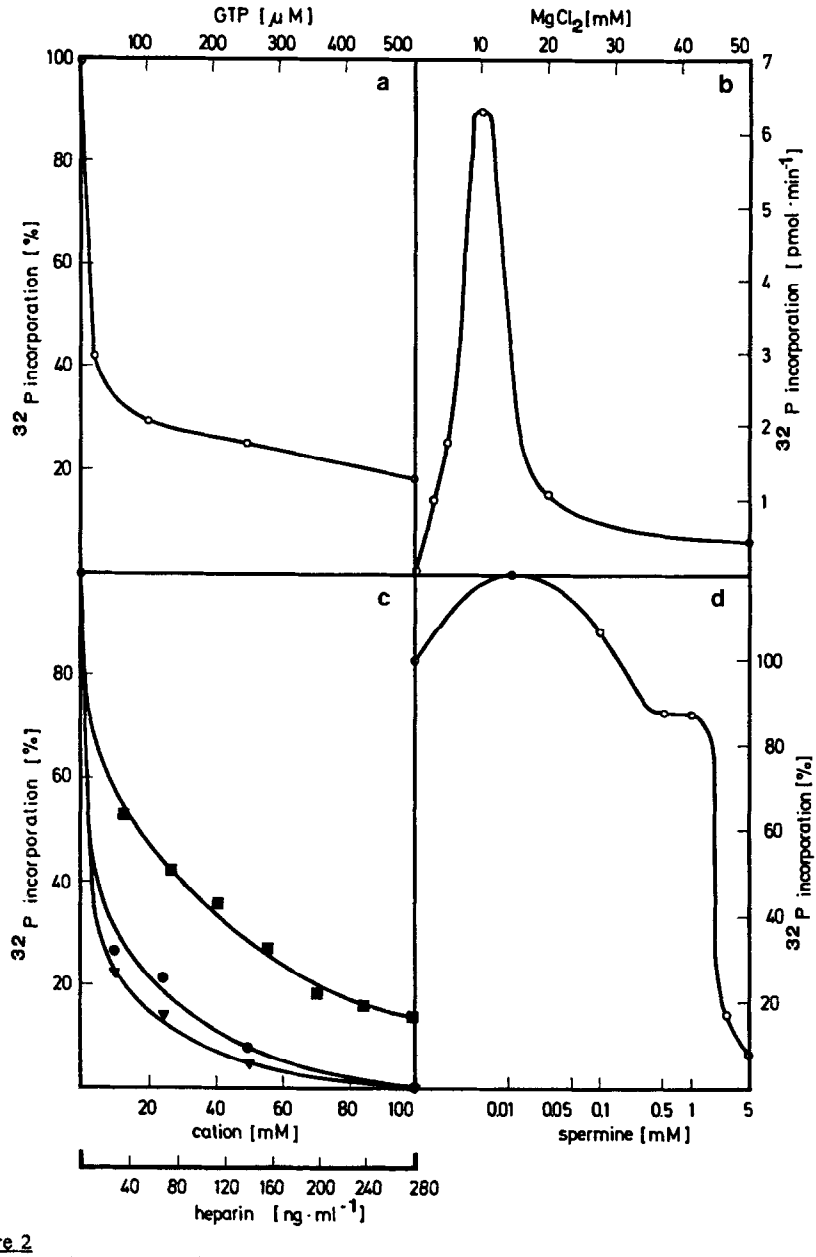

Figure 2
Properties of the casein kinase activity.
The data were obtained by the incubation of standard assays with casein as protein substrate, followed by SDS-PAGE and scintillation counting of the radioactive casein bands identified by autoradiography. a) Reduction of ^{32}P incorporation by nonradioactive GTP. b) Mg^{2+} dependence of the phosphorylation reaction. c) Inhibition of ^{32}P incorporation by KCL (▼), NaCl (●) and heparin (■). d) Influence of spermine on the kinase reaction.

Spermine displays a weak stimulation at 0.01 mM, but becomes strongly inhibitory at higher concentrations (Fig. 2d). A comparable behavior was also obtained with spermidine or polylysine as further polycationic substances. To ensure, that the observed reduction in phosphorylation was not due to the presence of a polyamine stimulated phosphatase (13), the kinase preparation

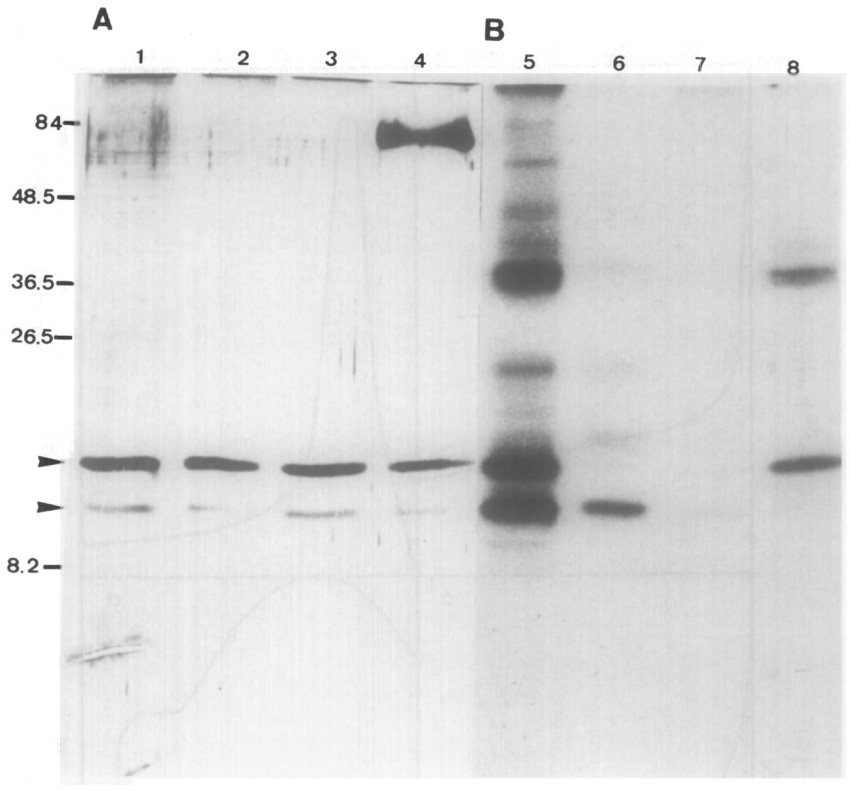

Figure 3
Electrophoretic analysis of phosphorylated HMG proteins.
Standard phosphorylation assays were incubated with HMG proteins (pre-heated for 5 min at 50°C) as protein substrate (0.8 mg/ml) and subjected to SDS-PAGE. The gel was first silver stained (part A) and then autoradiographed (part B). Lanes 1,3,4,5,7, and 8 are from assays containing 3 μCi [^{32}P]ATP as phosphate donor, while lanes 2 and 6 contained instead 3 μCi [^{32}P]GTP respectively. Lane 3 and 7 represent a control assay without addition of kinase preparation. Lanes 4 and 8 represent an assay treated with alkaline phosphatase (1 mg/ml) for 30 min at 30°C after the kinase reaction was performed with [^{32}P]ATP. The arrows indicate the dominant HMG proteins. The numbers on the side indicate molecular weight markers in KDa.

was assayed for the absence of any endogenous phosphatase activity by a test with p-nitrophenyl-phosphate.

The highly negative charged glycosaminoglycan heparin was also found to be a potent inhibitor of the kinase activity, leading to a 50% inhibition when present at 3.2 nM (Fig. 2c), while the addition of 0.01 mM Ca^{2+} (alone or in combination with 0.1 mg/ml calmodulin) did not show an influence on the phosphorylation reaction.

Maize HMG proteins as substrate for the casein kinase activity

In search for nuclear proteins serving as in vivo substrate of the kinase activity, HMG proteins were isolated from endosperm nuclei, purified and tested as protein substrates in phosphorylation assays. As HMG proteins from maize endosperm had not been isolated before, a purification method was established following basically the procedure of Spiker (12). An important modification of this method was the inclusion of a heparin-agarose chromatography

to enrich the crude nuclear protein extract before the final purification for the HMG proteins. This increased the yield and quality of the isolated HMG proteins. The identity of the isolated HMG proteins was confirmed by their conformity to the operational HMG protein definition. This includes their extraction from chromatin at 0.35 M NaCl, their solubility in 2% trichloroacetic acid and their migration in gel electrophoresis (7,8). The described purification procedure yielded a HMG protein preparation containing two major HMG proteins of 11500 Da and 13000 Da as shown by silver staining (14) of the SDS-PAGE separated proteins (Fig. 3A). This is in agreement with the results obtained with HMG proteins from maize leaves (15). Phosphorylation of the isolated HMG proteins by the kinase activity could be achieved with either ATP or GTP as phosphate donor. When analyzed by gel electrophoresis, the two major radioactive bands of [γ-^{32}P]ATP phosphorylated proteins comigrated with the silver stained HMG protein bands (Fig. 3, lane 1,5), confirming a phosphorylation of the HMG proteins. More bands can be seen on the autoradiogram than detectable on the corresponding silver stained gel, indicating that the HMG preparation contains trace amounts of additional proteins which are easily phosphorylated. In the case of [γ-^{32}P]GTP as phosphat donor, only the low molecular weight HMG protein was heavily phosphorylated (lane 6), suggesting a differential action of the kinase preparation on the two HMG proteins, or alternatively, the presence of two kinases. The phosphorylation of the HMG proteins depended strictly on the addition of the kinase preparation as shown in lane 7 of figure 3, eliminating the possibility of a kinase activity in the HMG preparation itself. Treatment of the phosphorylated HMG proteins with alkaline phosphatase led to the dephosphorylation of only the lower molecular weight HMG protein (lane 8), suggesting that the phosphates of the larger size HMG protein may require a specific phosphatase for cleavage. Histones were not phosphorylated by the kinase activity if added to the phosphorylation assay instead of casein.

Discussion

Nuclear extracts of maize endosperm tissue were found to display a strong protein kinase activity, which, after an approximately 100 fold enrichment by heparin-agarose and ATP-agarose chromatographies, could be separated from other kinases and contaminating phosphatase activity. Besides its use of casein as protein substrate, the kinase activity accepts either ATP or GTP as phosphate donors and is inhibited by low concentrations of heparin, suggesting that the kinase belongs to the casein type II kinases (5). However, in contrast to animal casein type II kinases (5) the maize nuclear enzyme is also inhibited by monovalent cations and polyamines such as spermine, and displays a low apparent molecular weight of about 20 kd as determined by gel filtration.

Only few nuclear kinases have been determined so far in plants and all appear to be different from the kinase activity described here (7). In the case of cultured plant cells, a growth associated activity of casein kinase activity was observed (16). A comparison of the maize casein kinase with other nuclear plant casein kinases and representative animal kinases of this type is given in table 1.

An interesting feature of the maize kinase activity relates to its preferential phosphorylation of HMG proteins, since HMG proteins have been implicated in transcriptional active chromatin

Table I
Comparison of the maize casein kinase activity with other casein type kinases

kinase \ characteristics	150 mM KCl [activity]	10 nM heparin [activity]	2 mM spermine [activity]	GTP as phosphate-donor	presence in nuclei	M_r [kd]
maize CK	↓	↓	↓	+	+	about 20
animal CK I (5)	↑	↓↑	↑	-	+	37
animal CK II (5)	↑	↓	↑	+	+	130
tobacco CK I (6)	↓	↓↑	↓↑	-	+	23
tobacco CK II (6)	↓↑	↓	↓↑	+	+	85
wheat CK (17)	↓	↓	↓↑	+	n.d.	37

A stimulation of the kinase activities is indicated by (↑), an inhibition by (↓) and no influence is indicated by (↓↑).

(4,8). Phosphorylation of HMG proteins by casein kinase II has also been seen in animal systems (17), where HMG protein phosphorylation was observed as a developmental stage specific process (4). In addition, animal casein kinase II phosphorylates other nuclear proteins including RNA polymerase, topoisomerase II, nucleolin and myc-protein (5,18,19,20). It is likely that the maize kinase activity described in this communication may also be involved in the phosphorylation of regulatory nuclear proteins. Work is in progress to test this hypothesis.

Acknowledgments: The expert technical assistance of Mrs. A. Schäfer is greatfully acknowledged. We thank Dr. D. Marmé for helpful discussions and Drs. T. Quayle and T. Sarre for critical reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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