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Biochemical evidence for a cGMP-regulated protein kinase in *Pharbitis nil*

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Abstract

It is known that the level of cGMP is modulated in response to a number of stimuli in plant cells but intracellular events distal to cGMP metabolism are not clear. Cyclic GMP-dependent protein kinase (Pk-G) is a major effector of cGMP action in animals and yeasts. We wanted to determine whether such kinase is present in plant cells. A soluble protein kinase was isolated from seedlings of *Pharbitis nil* and purified following purification methods including anion-exchange and affinity-chromatography. The enzyme consists of a single polypeptide of M_r 70 kDa as determined by SDS–PAGE. From conventional modulators only cyclic GMP, when applied in low concentration, was able to accelerate the enzyme activity in the presence of histones. The enzyme autophosphorylated on serine and threonine residues and phosphorylated some substrates only on serine residues. Mixture of histones and histones H2B, H3 were the best phosphate acceptors. The process of autophosphorylation was accelerated by a low concentration of cGMP and reduced by high concentration of this second messenger. Antibodies raised against catalytic domain of animals Pk-G I α and β cross-reacted with protein kinase from *Pharbitis nil* tissue. These data, taken together, demonstrate the presence of functional enzyme, which activity is regulated by cGMP and allow to classify this protein kinase as a member of the second messenger regulated group of enzymes.

Keywords: Pharbitis nil; Convolvulaceae; Japanese morning glory; Protein purification; cGMP; Protein kinase; Protein phosphorylation

1. Introduction

Cyclic nucleotides (cAMP and cGMP) are important signalling molecules in both eukaryotic and prokaryotic cells (Reggiani, 1997). The existence and role of cyclic nucleotides in plants at first was very controversial, but for a couple of years the knowledge about these second messengers in plants has been growing.

Several reports have implicated cGMP in various aspects of cellular regulation in plants. For example, indirect evidence indicates that cGMP may be important in light detection (Brown et al., 1989), signal transduction (Bowler et al., 1994), ion channels regulation (Dzeja et al., 1999) and pathogenesis (Durner et al., 1998).

Despite the recent progress in understanding the diverse biological function of cGMP, it has been difficult to identify the cGMP-effectors that mediate the cGMP signal in a given tissue.

In animal cells three major targets for cGMP have been identified: cGMP-dependent protein kinase (types Iα, Iβ and II) (Lincoln et al., 1988; Wolfe et al., 1989); cGMP-gated cation channels (Kaupp et al., 1989); and various cyclic nucleotide phosphodiesterases (Søderling and Beavo, 2000).

Cyclic GMP-dependent protein kinase is a key enzyme in the intracellular signal transduction in a variety of eukaryotic cells ranging from the unicellular organism, for example, *Paramecium* to humans (Francis and Corbin, 1994; Lohmann et al., 1997; Pfeifer et al., 1999). With mammalian tissues, a soluble type I

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contributes to the control of intracellular calcium levels by phosphorylation of inositol 1,4,5-trisphosphate receptors (Komalavilas and Lincoln, 1994; Archer et al., 1994), whereas a membrane-bound type II is involved in the control of chloride channels (French et al., 1995).

Protein phosphorylation/dephosphorylation in plant cells is also established as one of the major regulatory mechanisms by which intracellular signalling and the resultant cell response are controlled. In many cases, activation of protein kinase and subsequent promotion of protein phosphorylation are mediated by cyclic nucleotides.

Although the cyclic nucleotide protein kinase group, the AGC kinases, is known to exist in plant cells (Hanks and Hunter, 1995), the investigation of cyclic nucleotide-dependent protein kinase in plants is currently limited and needs detailed examination.

During our research we are studying the transduction of light signal in photoperiodic flower induction in the model short-day plant, *Pharbitis nil*, particularly the role of protein kinases and cyclic nucleotides.

As a part of this research we isolated a protein kinase and the present paper reports the characterization of this enzyme and the regulatory role of cGMP on its activation.

2. Results and discussion

In this study, we have examined the importance of cGMP for enzyme activation. To do so, we isolated soluble protein fractions from *Pharbitis nil* seedlings and analyzed if their activity could be modulated by cyclic GMP. Also the biochemical characterization of such enzyme was done.

2.1. Purification of protein kinase

Enzyme has been partly purified from 5-day-old *Pharbitis nil* seedlings. Two column chromatography steps on DEAE-cellulose and 8-(6-aminohexyloamino)-cAMP-agarose were used to purify proteins. Such chromatographies were previously described by Francis and co-workers (1991) to separate cGMP-dependent protein kinase (Pk-G) from cAMP-dependent protein kinase (Pk-A) in animal tissue. In our experiment we made the same steps to purify cyclic nucleotide regulated protein kinase. Starting from supernatant some proteins were partially separated from others by chromatography on DEAE-cellulose, followed by affinity chromatography on cAMP-substituted agarose. The specific binding of proteins to the resin was ensured by washing the column with buffer containing 2 M NaCl. This washing step would elute all non-specifically and weakly bound proteins. Proteins specifically bound to matrix were eluted with increasing concentration of cAMP, and fractions were analysed for cGMP-dependent kinase activity. The results of purification are summarized in Table 1. The standard purification procedure allowed to receive approximately 93 μg of purified enzyme from 1 kg of plant material. In the presence of cGMP and mixture of histones as substrates, specific activity values were greater than 0.543 nmol min^{-1} mg^{-1}. So, enzyme activity was stimulated two-fold by cGMP. Similar results have been reported for cGMP-dependent particulate protein kinase from yeast (Eckstein and Flügge, 1999); its activity was stimulated 2.4-fold by addition of 0.77 mM cGMP. Also Pk-G isolated from bovine lung was activated 1.9 to 3.5-fold by 0.1 mM cGMP (De Jonge and Rosen, 1977). However, the activity of the enzyme isolated by Nakazawa and Sano (1975) from pig lung was five times higher in the presence of cGMP.

2.2. Characterization of protein kinase

2.2.1. Determination of the molecular mass

SDS–PAGE was used to determine the homogeneity and molecular mass of the purified enzyme. Protein fraction purify by affinity chromatography exhibited few bands on SDS gel. The main ones had molecular masses of 55 and 70 kDa on 10% SDS gel (Fig. 1A). These values correspond to those reported for Pk-G from animals (74–86 kDa) (Francis and Corbin, 1994; Vaandrager and de Jonge, 1994; Gamm et al., 1995) and regulatory subunit of the cAMP-dependent protein kinase $M_r = 55$ kDa (Erlichman et al., 1974).

Table 1
Summary of purification of protein kinase from *Pharbitis nil*

Purification steps	Volume (ml)	Protein (mg)	Specific activity (nmol min^{-1} mg^{-1})		Total activity (nmol mg^{-1})
			−cGMP	+cGMP	
1. Crude homogenate	3500	2780	—	—	—
2. Crude supernatant	3400	2713	—	—	—
3. DEAE-cellulose	265	447.4	0.0055	0.0065	2.90
4. cAMP-agarose	3	0.0935	0.275	0.543	0.05

2.2.2. Characterization of protein enzyme by immunoblotting and in-gel kinase assay

In order to look for any immunological relationship between protein kinase from *Pharbitis nil* and Pk-G from animals, Western-blot analysis has been carried out using rabbit or guinea pig polyclonal antibodies made against the Pk-G I α and β (Keilbach et al., 1992; Firestein and Bredt, 1998). Fig. 1B shows that the rabbit antibody recognized 55 and 70 kDa polypeptides (lane 4) or proteins of 70 and 80 kDa (lane 5) in case of guinea pig antibodies. So, two different antisera against the regulatory subunit of Pk-G I from animals cross-reacted with enzyme from *Pharbitis nil*.

However, this immunoblotting experiment did not allow us to unequivocally identify the band corresponding to the protein kinase. To solve this problem we did an immunoprecipitation using anti-Pk-G antibodies from guinea pig and the immunoprecipitated proteins were analysed by in-gel kinase assay. The results show that only 70 kDa polypeptide had kinase activity and this activity disappeared when enzyme was removed (Fig. 2). The absence of kinase activity for 80 kDa peptide recognised by the anti-Pk-G guinea pig antibodies suggests that the band corresponded either to non-Pk-G protein with homology to these antibodies or to an unsuccessfully renatured kinase.

2.2.3. Autophosphorylation, substrate and effector specificity

Most protein kinases can phosphorylate themselves in the presence of ATP (Smith et al., 1993). In animal cells binding of cyclic nucleotide to or autophosphorylation of cGMP-dependent protein kinase activates this enzyme, but the molecular mechanism of activation for either process is unknown (Chu et al., 1998). The phosphorylation shown in Fig. 3 occurred when no exogenous substrate was added, indicating that the enzyme is capable of autophosphorylation. Proteins were incubated in the absence or presence of different concentration of cGMP and then separated on the gel. At least two proteins with kinase activity were revealed. Their apparent molecular masses were 35 and 70 kDa. While a phosphorylation process of 35 kDa protein was on the same level in all analyzed samples, phosphorylation of 70 kDa has changed depending on cGMP concentration. In the absence of cGMP the autophosphorylation was very

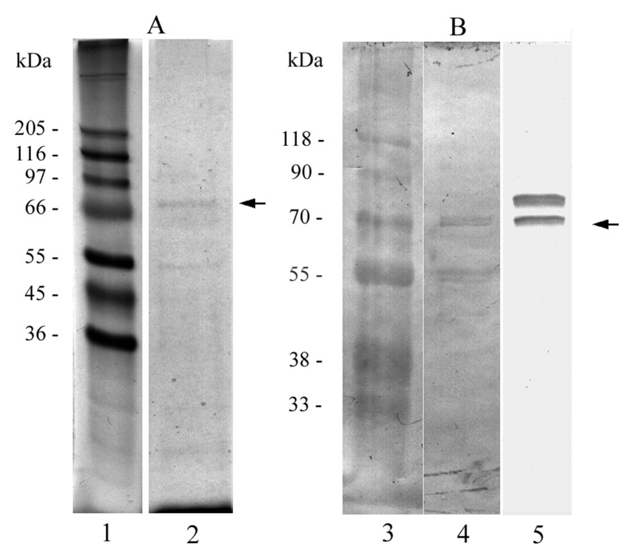

Fig. 1. SDS–PAGE and Western-blotting analysis of proteins from the last stage of purification (see Table 1). Proteins (1.2 μg) were resolved by electrophoresis in a 10% poliacrylamide gel in the presence of SDS and visualized by silver staining (panel A). Resolved proteins were transferred to nitrocellulose and immunoassayed with rabbit polyclonal antibodies (panel B, lane 4) or guinea pig polyclonal antibodies (panel B, lane 5) raised against the regulatory domain of animal Pk-G I α and β.

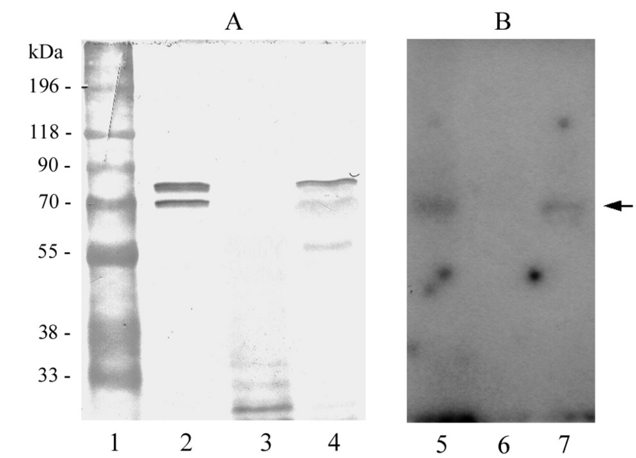

Fig. 2. Immunoprecipitation of proteins from the last stage of purification by anti-guinea pig Pk-G antibody. Proteins were analysed by Western-blotting (panel A) and in-gel protein kinase assay (panel B). Lane 1, molecular mass markers; lanes 2 and 5, the proteins fraction before immunoprecipitation; lanes 3 and 6, soluble fraction after enzyme removing; lanes 4 and 7, immunoprecipitated enzyme. The arrow shows the kinase activity.

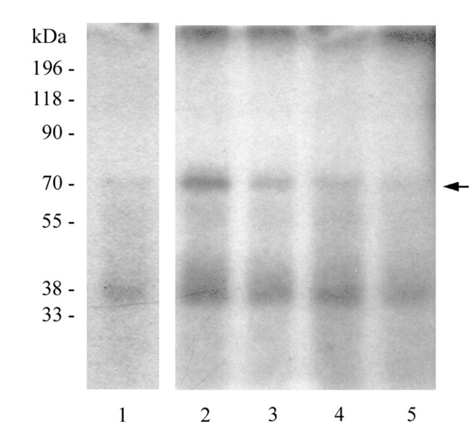

Fig. 3. Effects of various cGMP concentrations on autophosphorylation process. The enzyme autophosphorylation process was assayed in-gel in the absence (lane 1) or presence of various concentration of cGMP (lane 2, 1 μM; lane 3, 10 μM; lane 4, 50 μM; lane 5, 100 μM) as described in Section 4.

weak (Fig. 3, lane 1). When cGMP level increased the autophosphorylation process was stronger, reaching the maximal activation when 1 μM cGMP was added (Fig. 3, lane 2), further elevation (10–100 μM) of this second messenger led to decreasing of autophosphorylation (Fig. 3, lanes 3–5). From these results we can conclude that 70 kDa enzyme is a substrate for its own phosphorylation and cyclic GMP modulates the process of self-phosphorylation. A similar observation was made by Hofmann et al. (1985) and Smith et al. (2000) for the Pk-G from mammalian cells. They analysed the role of the two different cGMP-binding sites in Pk-G activity. Their data has shown that as cGMP level progressively increased the autophosphorylation was stimulated maximally because of cGMP-binding to the high affinity "slow" site. However, further elevation in cGMP led to cGMP-binding to the low affinity "fast" site as well and thereby decreased this process, even though the rate was still faster than that with no cGMP. Thus, occupation of both cGMP-binding sites of the enzyme was required for maximal stimulation of heterophosphorylation, whereas occupation of the slow site alone was sufficient for stimulation of autophosphorylation, and additional occupation of the fast site reduced the autophosphorylation process (Smith et al., 2000). These data also supports the proposal that our enzyme has a cGMP-binding site.

During the next step we examined the catalytic activity of the 70 kDa protein using different exogenous acceptors of phosphorus in the presence of cGMP. As a control, the enzyme activity was measured without substrate addition. Under the chosen assay conditions some of widely known exogenous proteins prove to be suitable substrates (Table 2). The enzyme favoured the histone H2B, but it also phosphorylated, at a lower rate, the mixture of histone, histones H2A and H3. Some differences between enzyme from *Pharbitis nil* and mammalians Pk-G appeared. Whereas H2B and H2A were the best substrates for the kinase from mammals (see Francis and Corbin, 1994), only H2A serves as a good substrate for Pk-G from *Paramecium* (Francis et al., 1991) and *Saccharomyces* (Eckstein and Flügge, 1999).

Table 2
Substrate specificity of protein kinase from *Pharbitis nil*

Substrate	Activity (%)
Histones mix	100
H3	88
H1	71
H2B	124
H2A	85
Syntyd-2	61
Without substrate	50

The protein kinase activity was assayed for each substrate in the presence of *iso*-butyl-methylxantine and cGMP as described in Section 4. Activity was expressed as a percentage of that obtained with mixture of histones.

As was shown earlier the mixture of histones could be used as the exogenous substrate for protein kinase from *Pharbitis nil*. The kinetic data were obtained by assaying kinase in standard assay conditions at the fixed cGMP (10 μM) and ATP (25 μM) concentrations and by varying the concentration of protein substrates. As demonstrated in Fig. 4 increasing concentration of histones leads to substrate saturation at the optimum pH = 7.4 at 30 °C. It should be noted that kinase activity without histone addition was quite high. Probably this value represents the autophosphorylation process. However, phosphorylation was enhanced when mixture of histones was added to the reaction. The same observation led De Jonge and Rosen (1977) to suggest that cyclic nucleotide dependent protein kinase from bovine lung is a substrate for its own phosphorylation and that other protein substrates (e.g. histones) and cyclic nucleotides modulate the process of autophosphorylation. Much later Smith et al. (1996) revealed that autophosphorylation of type Iα and Iβ Pk-G is affected minimally by the presence of high concentrations of substrate, suggesting that autophosphorylation occurs in preference to heterophosphorylation.

The effect of cyclic nucleotide concentration on the protein kinase activity was measured and the results are shown in Table 3. The exogenously applied cAMP had no effect on enzyme activity in the presence of histones (data not shown), however the activity was stimulated by the increasing concentration of cGMP. These data demonstrate that cGMP alone is sufficient to stimulate the activity of this protein kinase.

The earlier mentioned results are in accordance with data reported for animals and *Paramecium* Pk-G (see Francis and Corbin, 1994) and Pk-G from fungi

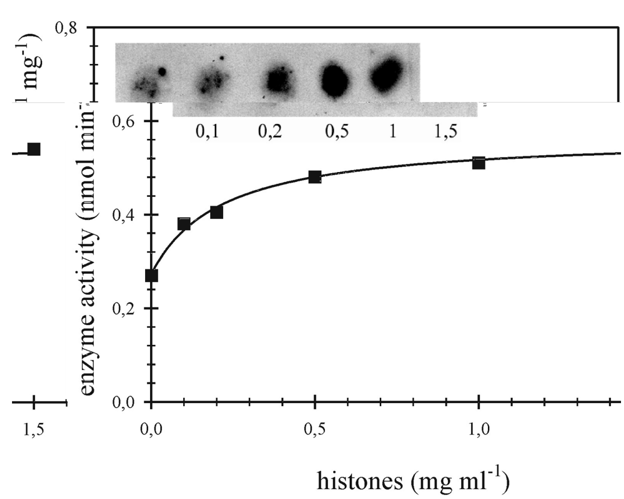

Fig. 4. Determination of kinase activity with different concentrations of substrate. The protein kinase activity was measured against mixture of histones as substrate (range 0.1–1.5 mg ml^{-1}). The inset shows autoradiograms of the dot blots.

sequence of cDNA clones was found and described. However, each year new data are published which suggest the existence of such enzyme in plant tissues (Cousson and Vavasseur, 1998; Penson et al., 1996; Bowler et al., 1994).

We succeeded in partially purifying a protein kinase from *Pharbitis nil* by DE-52 and cAMP-agarose chromatographies. At the moment it is possible to conclude that (1) it is an enzyme with an apparent molecular mass of the 70 kDa according to SDS–PAGE, (2) it cross-reacts with polyclonal antibodies raised against animals' Pk-G, (3) its activity is regulated by cGMP, (4) it is better autophosphorylated in low concentration of cGMP, (5) it preferentially phosphorylated histone H2B.

This enzyme had several biochemical features in common with other eucaryotic Pk-G and it may possibly be included in the "AGC" group of protein kinase, which are regulated by second messengers. Further work is required now to establish the sequence of amino-acids and search for the gene that codes this enzyme.

4. Experimental

4.1. Plant material and reagents

Seeds of Morning glory (*Pharbitis nil* Chois. cv. Violet) (Marutane Co., Kyoto, Japan) were soaked in concentrated sulphuric acid for 50 min and then washed with running tap water for 3 h. They were left in water at 25 °C overnight. The swollen seeds were planted on a mixture of vermiculite and sand (2:1) in plastic pots, covered with Saran Wrap to maintain high humidity. For soluble protein kinase isolation seedlings were grown for 5 days in the light/dark conditions, then whole seedlings or various organs were harvested and frozen in liquid nitrogen.

^{32}P-Labelled adenosine triphosphate ([γ-^{32}P] ATP) (4500 Ci mmol^{-1}) was obtained from ICN. DEAE-cellulose and P-81 filter paper were from Whatman. TLC-cellulose (20 cm$\times$20 cm$\times$0.1 cm) was from Merck. Calibration proteins for sodium dodecyl sulphate-poliacrylamide gel electrophoresis (SDS–PAGE) were from Sigma and BioRad. Other reagents were purchased from Sigma.

4.2. Enzyme purification

Protein kinase was purified by methods of Francis and co-workers (1991) modified for our purpose. All steps were carried out at 4 °C. *Pharbitis nil* seedlings were put into 3 l homogenization buffer A (10 mM phosphate buffer, 1 mM EDTA, 25 mM β-mercaptoethanol, pH 6.8) with 1 mM PMSF, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin, homogenized for 3 min and then filtered through a double layer of muslin. To the resulting crude homogenate 2% PVP, 10% PEI were added and then centrifuged at 8000$\times g$ for 45 min. The crude supernatant fraction was applied to a column of DEAE-cellulose equilibrated in buffer A. The column was washed with 2.5 l of buffer A containing 0.05 M NaCl, and all proteins absorbed were eluted with 2.0 l of buffer A containing 0.4 M NaCl. The fractions containing proteins were pooled and solid ammonium sulphate was added to 60% saturation. The mixture was stirred for 1 h and precipitated proteins were pelleted by centrifugation at 8000$\times g$ for 40 min. The pellet was resuspended in 100 ml of buffer A and dialysed for 3 h against the same buffer. The solution was clarified by centrifugation at 15,000$\times g$ for 15 min. The supernatant was loaded on 3.5 ml column of 8-(6-aminohexyl) amino-cAMP-agarose that was equilibrated in buffer A. The resin was washed sequentially with 200 ml buffer A containing 2 M NaCl and 10 ml buffer A. Protein kinase was eluted using buffer A containing 10 mM cAMP. The fractions containing kinase activity were pooled.

For storage, 10% glycerol was added to the purified protein kinase, which was divided into aliquots and frozen at −80 °C.

4.3. Protein determination

Protein concentrations were measured by dye binding protein (Bradford, 1976), with bovine serum as a standard at 595 nm.

4.4. SDS gel electrophoresis and immunoblotting

Proteins were electrophoresed in 10% acrylamide gels (Laemmli, 1970) to determinate the following: the homogeneity of protein kinase, the existence of the subunits and their M_r, autophosphorylation and phosphorylation of exogenous substrates, distribution in organs. After electrophoresis some gels were silver stained using Sigma system.

For Western-blotting analysis proteins were transferred to nitrocellulose by semi-dry system (BioRad) (15 min at 15 mA) using 25 mM Tris, 192 mM glycine and 20% (v/v) methanol (pH 8.3). Nitrocellulose was blocked in TBS (20 mM Tris, 100 mM NaCl) containing 5% non-fat dry milk and rinsed for 1 h with primary polyclonal antibodies to the I α and I β isoform of Pk-G raised in rabbit (1:600, in TBS containing 1% non-fat dry milk) (Keilbach et al., 1992) or anti-Pk-G I antibodies raised in guinea pig (1:1000) (Firestein and Bredt, 1998). After three times washing in TBS, membrane was incubated for 1–2 h with alkaline phosphatase-conjugated secondary goat antibodies (1:15 000) and visualized using NBT/BCIP as a substrate in 10 ml buffer containing 100 Tris–HCl (pH 9.5) and 5 mM $MgCl_2$. Rinsing the blots in water terminated the reaction.

4.5. Enzyme assay

Activity was determined in vitro by measuring the incorporation of ^{32}P from [γ-^{32}P] ATP into mixture of histones. Protein kinase assays were performed using 50 μl of an assay mixture, which contained 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.5 mg ml^{-1} mixture of histones, 0.1 mM 3-isobutyl-methylxanthine, 1 μM protein kinase A inhibitor fragment 6-22 amide (Sigma) and 25 μM [γ-^{32}P] ATP (260–420 cpm pmol^{-1}). The reaction was conducted in the absence or presence of 10 μM cGMP. A 0.5 μg of protein was added to the assay mixture, and the reaction was allowed to proceed at 30 °C for 15 min. All assays were made in duplicate. Spotting 40 μl on P-81 filter terminated the reaction. The filters were washed with 75 mM phosphoric acid and 95% ethanol, dried and added to scintillation vials containing 4 ml scintillation cocktail and counted in a liquid scintillation counter (Wallac 1407).

For the other assays, reactions were carried out as described earlier but reaction mixtures contained additional effectors: (1) exogenous substrates: histone H3 (0.5 mg ml^{-1}), histone H2A (0.5 mg ml^{-1}), histone H2B (0.5 mg ml^{-1}), histone H1 (0.5 mg ml^{-1}), syntide-2 (50 μM), (2) various cGMP concentration (1, 10, 50, 100 μM) and (3) different concentration of mixture of histones (0.1–1.5 mg ml^{-1}).

For some experiments the reaction was terminated by addition of SDS sample buffer. The samples were boiled for 5 min and subjected to 10% acrylamide gels. The dried gels were autoradiographed with FOTON X-ray film, which was exposed at −80 °C for 1–3 days.

4.6. ^{32}P-amino acid analysis

Phosphorylation of protein kinase itself and some exogenous substrate was performed as described earlier. Gel fragments containing phosphoproteins were cut out of the gel and rehydrated in 10% methanol. The cellophane was removed and the fragments were redried. The proteins on the gel were subjected to hydrolysis in 6 M HCl for 2 h at 110 °C in sealed glass tubes. After hydrolysis, samples were lyophilized and the residue was washed two times with 100 μl distilled water and relyophilized. Samples were then dissolved in 10 μl distilled water. Thin-layer chromatography was performed on cellulose plates (20×20 cm; 0.1 mm thick on glass) in the following solvent system: 1 M propionic acid/ NH_4ON/isopropanol (45:17.5:17.5) according to (Neufeld et al., 1989). Standards of phosphoserine (Ser-P) and phosphothreonine (Thr-P) (5 μg) were run in parallel and detected by reaction with 0.1% ninhydrin, whereas the ^{32}P-labelled amino acids were detected by autoradiography.

4.7. In-gel protein kinase assay

For in-gel autophosphorylation assay the enzyme was subjected to 10% SDS–PAGE. For heterophosphorylation assay histone H3 (0.5 mg ml^{-1}) was added to the separation gel just prior the polymerization. After electrophoresis, SDS was removed by washing the gel for 30 min at room temperature with 20% isopropanol in 50 mM Tris (pH 8.0) and then 2×30 min in 50 mM Tris (pH 8.0), 5 mM β-ME (buffer A). Proteins were denatured by treating the gel with 6 M guanidine–HCl in buffer A for 1 h at room temperature and then renaturated with 0.04% Tween 40 in buffer A at 4 °C overnight. Next, the gel was preincubated with 25 ml of 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 0.1 mM 3-isobutyl-methylxanthine, 1 μM protein kinase A inhibitor fragment 6-22 amide (Sigma) and then in 4 ml of the same buffer with 25 μM ATP and 50 μCi [γ-^{32}P] ATP (4500 Ci mmol^{-1}) for 1.5 h at 30 °C. The reaction was conducted in the presence of 10 μM cGMP. Reaction was stopped by washing the gel with 1% sodium pyrophosphate in 5% trichloroacetic acid. The washed gels were dried and subjected to autoradiography.

4.8. Immunoprecipitation

For immunoprecipitation analyses 1% Triton X-100, 0.5% NP-40 were added to 5 μg of protein fraction. It was incubated with 10 μl of antibodies against Pk-G from guinea pig for 4 h at 4 °C in rotating condition. After adding 60 μl of 10% Protein A-sepharose incubation was carried out for 2 h in the same conditions. Then, complexes were collected by centrifugation at 13000×*g* for 10 min and washed four times in buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100 and 0.5% NP-40. Immune complexes bound to the beads were eluted with four times sample buffer. The solubilized proteins were subjected to 10% SDS–PAGE and Western-blotting and in-gel heterophosphorylation assay were performed.

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