Guanosine 3':5'-Cyclic Monophosphate -Dependent Particulate Protein Kinase Activity from Yeast (*Saccharomyces cerevisiae*)

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Continuing our studies on cGMP in growing yeast we detected a particulate cGMP dependent protein kinase (Pk-G), which was solubilized by detergents and NaCl. It achieves maximum activity at 25 °C and pH = 6.8, high concentrations of substrate proteins or cGMP produce saturation. Casein and histones are appropriate substrates, phosphatase-pretreated histone H-2a provokes outstandingly high activity. Pk-G differs from cAMP-dependent protein kinase (Pk-A) with respect to pH optimum, temperature tolerance above 50 °C, and stability. Partial purification is achieved by chromatography with DEAE-cellulose, Sepharose, and cGMP-substituted Sepharose. The latter step also markedly removes Pk-A. At least three proteins with Pk-G-activity and high cGMP-affinity are separated by polyacrylamide-gel-electrophoresis. Their apparent molecular masses, as deduced from comigrating marker proteins, differ considerably from those of other Pk-G's, but also of Pk-A's.

Introduction

Protein kinases are essential components in the regulation of metabolism and cell proliferation. Their activity often is controlled by certain effectors, especially cAMP, cGMP, Ca^{2+}/Calmodulin and inositol-trisphosphate. cGMP-dependent protein kinases have been identified from mammalian as well as from some nonmammalian species including *Paramecium* (Francis, 1994). With mammalian tissues, a soluble type I contributes to the control of intracellular calcium levels by phosphorylation of inositol 1,4,5-trisphosphate receptors (Komalavilas *et al.*, 1994; Archer *et al.*, 1994), whereas a membrane-bound type II is involved in the control of chloride channels (French *et al.*, 1995).

With yeast, protein kinases dependent on cAMP, Ca^{2+}/calmodulin, 1,4,5-inositol-trisphosphate, cyclin and the CDC7- gene product are described, besides from protein kinases, which obviously are independent of these effectors (Londesborough *et al.*, 1987; Levin *et al.*, 1987; Kolarov *et al.*, 1988; Zoller *et al.*, 1988; Jazwinski, 1988; Stack *et al.*, 1995; Glover III, 1997; Morgan, 1997). Nothing is known hitherto about cGMP dependent protein kinases from yeast. Our recent reports on particulate guanylate cyclase and cGMP in growing yeast (Eckstein, 1988; Eckstein *et al.*, 1997) raise questions about the target proteins of this effector. Several different intracellular receptor proteins are to consider, including protein kinases (Pk-G), phosphodiesterases (PDE) and ion channel proteins (Lincoln *et al.*, 1993). We report on a particulate cGMP-dependent protein kinase from yeast, which is distinguishable from cAMP-dependent protein kinase, but which also differs somewhat from mammalian and other Pk-Gs.

Materials and Methods

Chemicals

Acrylamide-Bis was from BioRad. cGMP, cAMP, ATP, γ-S-ATP, Histones, Histone Mix and phenylmethylsulfonylfluoride (PMSF) were from Boehringer-Mannheim. Dithiothreitol (DTT) and

Abbreviations: BSA, bovine serum albumin; cAMP, 3':5'-cyclic adenosine monophosphate; cGMP, 3':5'-cyclic guanosine monophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PK-A, cAMP-dependent protein kinase activity; PK-G, cGMP-dependent protein kinase activity; PK-I, protein kinase activity without cyclic nucleotides; IBMX, isobutylmethylxanthine; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecylsulfate; Tris, tris(hydroxymethyl)aminomethane.

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Zwittergent 3–14 were from Calbiochem. Triethylamine was from FLUKA. Sephadex G-200, epoxyactivated Sepharose 6B, Sepharose CL4B and CL6B, protein calibration kits and silver stain kit were from PHARMACIA. Sodium dodecylsulfate (SDS), theophylline and Triton X-100 were from SERVA. Bromphenol blue, Coomassie brillant blue R and 8-bromo-cGMP were from SIGMA. DEAE cellulose DE-32 and cellulosephosphate P-81 were from WHATMAN. [8-^{3}H]-cGMP, ammonium salt, 470 GBq/mmol, was from Amersham Buchler. Adenosine 5'(γ-thio)triphosphate, 35-S (γ-35-S-ATP), 47 TBq/mmol, was from NEN. All other chemicals were from MERCK.

Buffers

PEM: 100 mM sodium EDTA + 25 mM mercaptoethanol in 5 mM sodium phosphate, pH = 6.8. PEM-PMSF: PEM + 1 mM PMSF. PGP: PEM + 25% glycerol + 1 mM PMSF. Buffer A: 0.5 mM IBMX + 1 mM DTT + 5 mM EDTA + 2 g/l BSA in 25 mM potassium phosphate, pH = 7.2.

Yeast culture and cell fractionation

A cell clone formerly derived from baker's yeast (*Saccharomyces cerevisiae,* "Germania Hefe", DHW Hamburg, (Eckstein 1988)), was used for all experiments. The cells were grown up to 20–30×10^6 cells/ml (log phase growth) in a modified WICKERHAM medium (Hilz *et al.,* 1964) at 31.3 °C with stirring and aerating. They were then harvested by centrifugation, washed two times with PEM buffer, resuspended at 1–2×10^9 cells/ml with PEM-PMSF, and broken by shaking with glass beads (0.45 mm in diameter) in a MERKENSCHLAGER cell homogenizer. The glass beads were filtered off, the filtrated homogenate was centrifuged 10 min at 190 x *g*. The resulting supernatant ("crude extract", fraction 1) was centrifuged 30 min at 30,000 x *g*, yielding fractions 2a (supernatant) and 2b (residue). The residue was resuspended with PEM-PMSF at 1/3 of the original volume. The supernatant was centrifuged 120 min. at 100,000 x *g* yielding a soluble fraction 3a (supernatant) and a particulate fraction 3b (residue). The residue was resuspended at 1/5 of the original volume with PEM-PMSF. If not used immediately, all fractions were stored at −80 °C.

Extraction of particle bound materials

a) Extraction by Zwittergent 3–14

270 µl of fraction 3b were diluted with 1540 µl Zwittergent 3–14 (3 mM) in PGP-buffer, and incubated 10 min at room temperature and 5 min. in an ice bath. The insoluble material was sedimented by centrifugation 30 min at 100,000 x *g*, washed two times with 1.0 ml PGP buffer, and resuspended with PGP ad 1080 µl, yielding fraction 4b. The supernatant was ultrafiltrated through a Diaflo YM-30 membrane, washed two times with 1.0 ml PGP, and diluted ad 1080 µl, yielding fraction 4a. Fraction 4a, or in some cases fraction 5a, was taken for further purification steps.

b) Extraction by NaCl

270 µl fraction 3a were diluted ad 3.6 ml with PGP-buffer, the assay was made 0.5 molar with NaCl, and incubated 10 min at room temperature and 5 min in an ice bath. The assay was then centrifuged 30 min at 100,000 x *g* and further treated, as described in Section **a)**, yielding fractions 5a (supernatant) and 5b (insoluble residue).

Ion exchange chromatography

A column of DEAE cellulose (DE 32 WHATMAN), 350 x 16 mm, was equilibrated with PGP-buffer, loaded with ca. 250 mg protein (8 ml fraction 4a), washed with 150 ml PGP, and eluted stepwise with 0.2 and 0.5 M NaCl in PGP-buffer. Fractions of 200 drops were collected. The fractions containing the bulk of PK-G activity were pooled and reconcentrated ad 8 ml by ultrafiltration with a YM-30 membrane, yielding fraction 6a.

Separation with Sepharose Cl-6B

A column of Sepharose Cl-6B (PHARMACIA), 350 x 16 mm, was equilibrated with the 5–6 fold volume of PGP-buffer, and loaded with 200 mg protein (ca. 6.5 ml) of fraction 6a. The proteins were eluted with PGP, fractions of 90 drops were collected. The fractions containing the bulk of Pk-G activity were pooled and concentrated at 4.0 ml by ultrafiltration, yielding fraction 7.

Affinity chromatography

a) Synthesis of the matrix

A cGMP affinity matrix was prepared from epoxy-activated Sepharose 6B (PHARMACIA) and 8-(2'-hydroxyethylthio)-cGMP analogous to (Weber *et al.*, 1979).

8-(2'hydroxyethylthio)-cGMP was synthesized analogous to (Muneyama *et al.*, 1971): 500 mg 8-Br-cGMP were heated in a reflux apparatus 5 hrs with 9.62 ml methanol + 1.92 ml mercaptoethanol + 312 mg sodium methoxide. The preparation was evaporated in a rotary vacuum evaporator, resolved with 1 M ammonia, brought to pH = 3 with 25% HCl, and crystallised. The crystals were separated by centrifugation, recrystallised, washed with HCl-water, and finally dried in a vacuum exsiccator. The product was controlled by thin layer chromatography on silica gel 60 F-254 with propanol-1/ammonia/water (7:1:2, v:v:v), and with butanol/acetic acid/water (5:2:1, v:v:v), respectively, as eluents.

The matrix was synthesized from 380 mg freshly prepared 8-(2'-hydroxyethylthio)-cGMP and 5.47 g epoxy-activated Sepharose 6B by stirring 20 hrs. at 45 °C in 20.9 ml sodium carbonate buffer, 0.2 M, pH = 11). The product was sedimented by centrifugation 10 min at 2,000 x *g*, resuspended with 1 M ethanolamine-HCl buffer pH = 9.0, and stirred 3 hrs at 45 °C. Then it was collected by centrifugation, washed three times with 0.1 M sodium borate in 0.5 M NaCl, pH = 8.5, and three times with 0.1 M sodium acetate in 0.5 M NaCl, pH = 4. It was stored as a suspension with 50 mM sodium borate in 0.5 M NaCl, pH = 8.5, containing 0,02% sodium azide. The product was controlled by hydrolysis of an aliquot with 0.1 M HCl 45 and 120 min at 100 °C, and determination of the UV absorbance of the supernatant after centrifugation.

b) Separation of Pk-G

Yeast PK-G was separated by affinity chromatography as follows: The cGMP-Sepharose was equilibrated with PGP-buffer containing 1.38 mM DTT, mixed with 5 ml fraction 7 (60–65 mg protein), and incubated overnight at 4 °C. The product was filled into a small column and washed with 20 ml buffer, yielding fraction 8a. The proteins were eluted stepwise with 20 ml and 10 ml 5 mM cGMP in PGP + DTT, 20 ml buffer, and 20 ml and 10 ml 10 mM cAMP in buffer. The cGMP- and cAMP-eluates each were pooled, the resulting fractions 8b and 8c were ultrafiltrated with YM-30 membranes and Centricon C-30 tubes, washed three times with PGP-buffer, and refilled to 5.0 ml. They were stored at −80 °C.

Polyacrylamide-gelelectrophoresis (PAGE)

SDS-PAGE was performed according to (Laemmli, 1970), with 4% stacking gel and 7.5% running gel in 192 mM glycine + 25 mM Tris + 1% SDS, pH = 8.7. The fractions 8a–8c were supplemented with 1.7% SDS, 3.6 mM DTT, 0.9 mM PMSF, and 0.0014% bromophenol blue. Alternatively, the proteins were stained with silver according to BIO-RAD. 10–20 µg protein/lane were applied to the gel. The proteins were separated in two steps, 30 min at 100 V and 120 min at 200 V. "Native" PAGE was performed without SDS.

Elution of proteins from polyacrylamide gels

Pk-G activity and cGMP-affinity of the separated proteins were tested after elution of the proteins from the gel. For this purpose an additional lane was prepared containing 150 µg protein (60 µl) without dye. The lane was cut after electrophoresis into 5 mm pieces, these were incubated 5 min at 6 °C with 5 µl 2% bromophenol-blue in 300 µl sodium phosphate buffer (20 mM, pH = 7.3) and transferred into a HSB-Elutor (BIOMETRA, Göttingen) containing the same buffer. The buffer was underlayered with 3 M sodium acetate, pH = 7.3. The proteins were eluted 2 hrs at 50 mA into the sodium acetate, washed three times with PGP-buffer in Centricon C-30 tubes, and resuspended at 100 µl with PGP. They were stored at −80 °C.

Chromatography with SEPHADEX G-200

A Sephadex G-200 column, 320 x 16 mm, was equilibrated with PGP-buffer, and loaded with 3 ml of fraction 7 (19 mg protein, 2000 units Pk-G). The proteins were eluted with PGP at a flow rate of 2 drops/min, fractions of 100 drops (about 2 ml) were collected. The fractions containing PK-G activity (fractions no. 9–16) were pooled, concentrated by ultrafiltration with Centricon-30,

washed with PGP, and refilled to 3.0 ml with PGP (fraction 7a).

Affinity of yeast proteins to cyclic nucleotides

The assay contained, in a total volume of 200 µl: 77 pmol (51.8 TBq) [8-^{3}H]cGMP, or [8-^{3}H]cAMP, respectively, glycerol-PEM-buffer diluted 1 : 6.67, buffer A diluted 1 : 2, and 40 µl (4–400 µg protein) probe. The assay was incubated 21 hrs at 4 °C, the proteins were precipitated with 50% ammonium sulfate, sedimented by centrifugation, washed two times with 1 ml 40% ammonium sulfate, and redissolved with 1.2 ml 1 mM NaOH. The radioactivity of this solution was counted in a WALLAC LSC counter (EG&G BERTHOLD) and expressed in terms of µmol cGMP, or cAMP, respectively, bound per 1 mg protein.

cGMP-dependent proteinkinase activity

Proteinkinase activity was measured by incorporation of radioactivity from γ-phosphate-labeled ATP into substrate proteins. The standard assay contained, in a volume of 67 µl: 100 mM sodium acetate buffer pH = 6.4, 77 mM magnesium acetate, 1.5 mM theophylline, 6.6 mM dithiothreitol, 17.7 mM sodium fluoride, 17.7 mM sodium vanadate, 0.4 mM γ-S-ATP labeled with 46 Bq 35-S/nmol, and 50 µg Histone-Mix (Boehringer) as substrates. In parallel, additional assays were supplemented ad 0.77 mM with cGMP, or with cAMP. All assays were made in triplicate. The reaction was started by addition of 50 µl sample (2–200 µg protein) and incubation at 30 °C. After 0, 1, 2.5 and 5 min of incubation were 30 µl-aliquots transferred to filter papers (cellulose phosphate P-81, 20 mm in diameter, Whatman) and transferred into cold 10% TCA. The filters were washed, twice with 5% TCA and once with ethanol, and counted for radioactivity. The counts were converted into units of enzyme activity. 1 unit incorporates 1 µmol phosphate/min. from ATP into the acid-insoluble material (protein). The units obtained from the standard assay were called cyclic nucleotide independent (Pk-I). The difference between Pk-I and the units from assays with cGMP, or with cAMP, respectively, was considered as Pk-G, and Pk-A, respectively.

Results and Discussion

Extracts from growing yeast cells contain a protein kinase activity, which is resistant to dialysis, and does not require cGMP, or cAMP in the assay, and apparently is independent of cyclic nucleotides (Pk-I, Table I). Additional protein kinase activities are triggered, when the assay is supplemented with cGMP, or with cAMP, respectively, indicating the existence also of Pk-G and Pk-A activities. The surprising finding, that the cGMP-induced activity exceeds those of Pk-A and Pk-I, might be explained by the test conditions. But the question arises, whether the Pk-G activity only represents an unspecific reaction of known protein kinases from yeast, particularly of Pk-A. The following investigations may answer this problem.

The PK-G activity is sedimented quantitatively by centrifugation with 100,000 x *g* (40.000 r.p.m., Table I). In contrast, PK-A-activity, like Pk-I, partially remains in the supernatant, according to the results from (Kudlicki *et al.*, 1978; Müller *et al.*, 1987; Zoller *et al.*, 1988; Behrens *et al.*, 1988). The association of Pk-G to the particulate cell fraction on principle seems adequate to Pk-G type II, though type I also can be present in the particulate fraction (Francis *et al.*, 1994).

The particle bound PK-G-activity is solubilized in part, but with a remarkable loss by 3 mM Zwittergent 3–14 (Table II). The activity also becomes solubilized by incubation of the residue with 0.5 M NaCl, again with a remarkable loss. Similar results are obtained with the particulate fraction of Pk-A. The solubility of the enzyme protein by detergents

Table I. Protein kinase activity in different yeast cell fractions.
Fraction 1 = 190 x *g* supernatant, 2a = 30,000 x *g* supernatant, 2b = 30,000 x *g* residue, 3a = 100,000 x *g* supernatant, 3b = 100,000 x *g* residue. PK = protein kinase activity: PK-A = cAMP-dependent, PK-G = cGMP-dependent, PK-I = basal activity without cyclic nucleotides in the assay. For further details see "Materials and Methods".

Fraction	Protein	Units/Fraction			Units/mg protein		
	mg/fraction	PK-G	PK-A	PK-I	PK-G	PK-A	PK-I
1	2755	4090	2590	650	1.5	0.94	0.24
2a	2300	9410	4180	160	4.1	1.82	0.07
2b	450	4870	1370	1340	10.8	3.04	2.98
3a	1710	0	3030	2050	0	1.77	1.20
3b	1030	10960	4300	4540	10.6	4.17	4.40

Table II. Solubilisation of the Pk-G- and Pk-A-activities from the 100,000 x *g* residue.
270 µl fraction 3b (Table I) were incubated with 3 mM Zwittergent 3–14, and with 0.5 M NaCl, respectively, centrifuged 30 min. at 100,000 x *g*, dialyzed, and concentrated. Control assays were incubated with PEM buffer. Fractions 4a and 5a are the resulting supernatants, 4b and 5b represent the remaining insoluble residues. For further details see "Materials and Methods".

Solvent	Fraction		Protein	Units		% activity		Units/mg protein	
	no.	µl	mg	Pk-G	Pk-A	Pk-G	Pk-A	Pk-G	Pk-A
Control	3b	270	12.3	131	51	100	100	10.6	4.1
Zwitter-	4a	1080	8.8	58	15	44	29	6.6	1.7
gent	4b	1080	3.5	35	16	27	31	10.0	4.6
NaCl	5a	1080	8.4	50	34	38	67	6.0	4.0
	5b	1080	3.9	3	0	2	0	0.8	0

suggests an integration of Pk-G into membranous structures rather than an association, although the solubility by high salt concentrations appears somewhat contradictory. But similar results have been reported from type II isoforms from mammalian cells (Francis *et al.*, 1994).

Increasing concentrations of yeast extract in the assay result in corresponding increases of cGMP- and cAMP-dependent protein phosphorylation. But whilst the cAMP-dependent reaction *in* vitro proceeds linearly at least for 10 min, the cGMP-dependent protein phosphorylation begins to decrease just after 2 min (Fig. 1). The reasons for this increasing inhibition of the enzyme are unknown. With *Paramecium* as well as with mammalian tissues the Pk-G's undergo slow autophosphorylation, which could affect their enzymatic function (Francis *et al.*, 1994). Whatever it might be, we as a consequence generally determined the Pk-G-activity only from the first minute of incubation.

Fig. 1

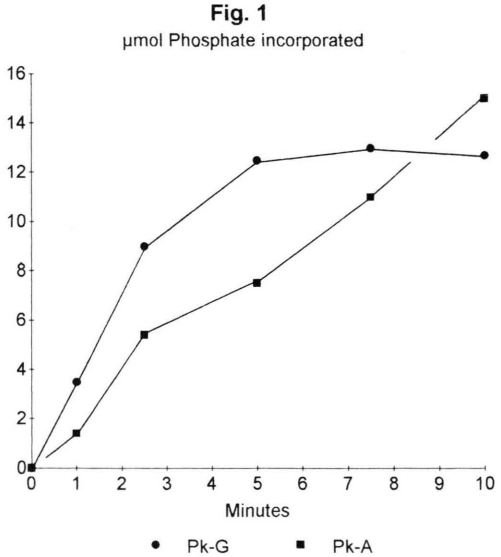

Fig. 1. Time kinetics of the histone mix phosphorylation by Pk-G and Pk-A.
Ordinate: µmol phosphate incorporated by 1 mg yeast protein. 150 µl yeast extract were incubated with the threefold assay volume. For further details see "Materials and Methods". Mean from 4 experiments.

As demonstrated in Table III and Figs. 2 and 3, the cGMP-triggered phosphorylation of substrate proteins shows all characteristics of an enzymic reaction. Increasing concentrations of histones for substrates lead to substrate saturation (Fig. 2A). Saturation also can be obtained with the effector cGMP (Fig. 2B). Secondly, the reaction proves to be temperature sensitive. It is restricted to temperatures between 0° and 65 °C (Pk-A: 0°–45 °C), with a maximum between 20° and 25 °C (Pk-A: 20 °C, Fig. 3A). And it is destroyed, like the cAMP-dependent histone phosphorylation, by preincubation of the yeast extract for 10 min at 95 °C. Thirdly, the cGMP-dependent reaction is restricted to a certain pH range, which refers to pH = 6 to pH = 7.6 (Pk-A: 6–8.5, Fig. 3B), with an optimum pH at 6.8 (Pk-A: 8.1). The substrate specificity, on the other hand, is pronounced only moderately. Casein, histone mix and most of single histones prove to be suitable substrates (Table III). But remarkable differences between single histones emerge, if these were pre-treated with alkaline phosphatase. In this case an outstandingly high phosphorylation is obtained with histone H-2A (Table III). H-2A also serves as a good substrate for Pk-G

Table III. cGMP- and cAMP-dependent protein kinase activity in 100,000 x *g* residues with different substrate proteins.
Where indicated, histones were pretreated as follows: 500 mg histone / 2 ml diethanolamine-HCl (1 M, pH = 9,8, supplemented with 0.5 mM $MgCl_2$) were incubated successively 30 min at 37 °C and 10 min at 95 °C with 5 units alkaline phosphatase. Controls were incubated a) without histones but with phosphatase, and b) without phosphatase but with histones, respectively. The proteins were centrifuged off and resuspended at 10 mg/ml with 0.1 M sodium acetate pH = 6.4. 5 µl each were taken for the protein kinase assays. For further details see "Materials and Methods".

Substrate	Pretreated with phosphatase	Units/mg protein Pk-G	Pk-A
Casein	–	16	
Protamine	–	07	
Histone-Mix	–	11	4.5
	+	14	19
Histone H-1	–	13	0.1
	+	15	21
Histone H-2a	–	9	8
	+	50	41
Histone H-2b	–	11	5
	+	9	12
Histone H-3	–	27	7
	+	21	17
Histone H-4	–	4	5
	+	21	17

from *Paramecium* (Francis *et al.*, 1994). It seems doubtful, whether this reflects a special role of Pk-G *in vivo*, since a similar result comes from the cAMP-triggered reaction.

The difficulties with detection and exact estimation of Pk-G activity from yeast extracts prompted us to a series of experiments aiming at separation of the enzyme from Pk-A and other cell constituents. The results are summarized in Table IV.

Starting from a Zwittergent extract, both Pk-G and Pk-A partially are separated, but with remarkable loss, from other proteins by chromatography with DEAE-Cellulose (DE 32, Whatman) followed by Sepharose CL-6B. Comparable results are obtained with a NaCl-extract (not shown here). Surprisingly both kinase activities are eluted from the ion exchanger just by buffer. The reason for this is not clear. Subsequent affinity chromatography with cGMP-substituted Sepharose results in separation of Pk-G- from Pk-A-activity. About 75% of the Pk-G-activity are eluted by cGMP (Fraction 8b, Table IV). Most of Pk-A, however, subsequently is eluted by cAMP (Fraction 8c, Table IV). This finding substantiates the assumption that Pk-G- and Pk-A-activities from yeast extracts belong to different enzyme proteins. As a whole, extraction and chromatographic steps result in a remarkable purification of both en-

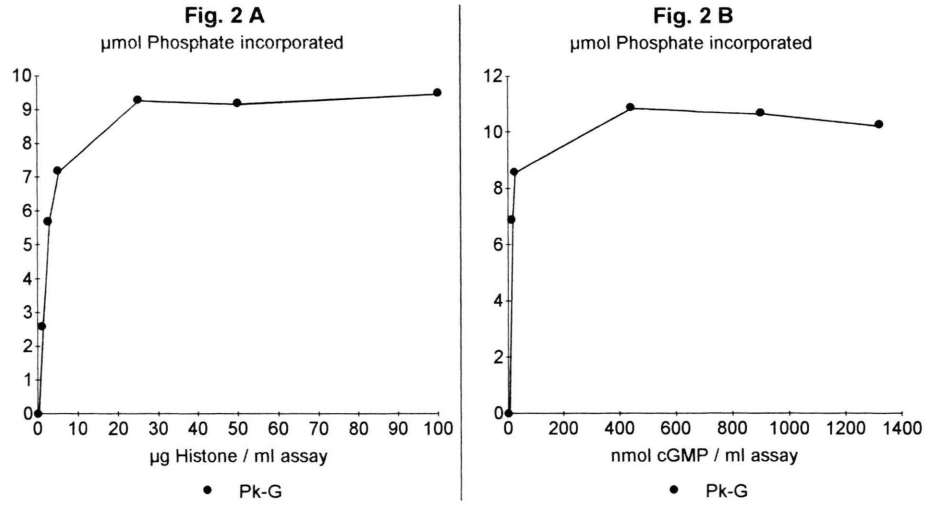

Fig. 2. Activity of cGMP dependent protein kinase with increasing concentrations of:
A : Histones,
B : cGMP.
Ordinates: µmol phosphate incorporated by 1 mg yeast protein (fraction 3b). For further details see "Materials and Methods". Mean from 4 experiments.

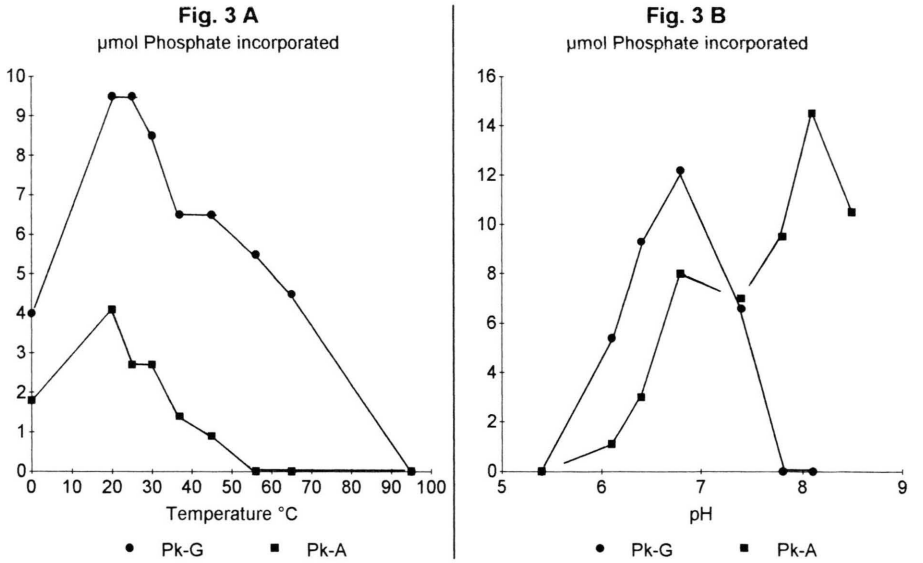

Fig. 3. cGMP- and cAMP-dependent protein kinase activity at various test conditions.
A : Temperature. Assays were incubated simultaneously at the indicated temperatures and the Pk-G activity determined, as described in "Material and Methods". Ordinate: µmol phosphate incorporated by 1 mg yeast protein. Mean from three experiments.
B : pH. The pH values were adjusted by the following buffers: MES (pH = 5–6.4), PIPES (pH = 6.3–7.5), HEPES (pH 7.5–8.5). For further details see "Materials and Methods". Ordinate: µmol phosphate incorporated by 1 mg yeast protein. Mean from three experiments.
MES : 2-[N-morpholino]ethanesulfonic acid.
PIPES : 1,4-piperazinediethanesulfonic acid.
HEPES : N-[2-hydroxyethyl]piperazine-N′-[4-butanesulfonic]acid.

Table IV. Partial purification of the cGMP- and cAMP-dependent protein phosphorylating activities by chromatography.
Fraction 3b (100,000 x *g* residue) was extracted by Zwittergent 3–14, as described in "Materials and Methods", the resulting fraction 4a was purified further by the following chromatographic steps: DE-32 (–> fractions 6 a–c), Sepharose CL6B (–> fraction 7), cGMP-substituted Sepharose 6B (–> fractions 8 a–c). For details see "Materials and Methods".

Fraction	Eluent	V	Protein	Units		Units/mg protein		Enrichment	
		ml	mg	Pk-G	Pk-A	Pk-G	Pk-A	Pk-G	Pk-A
3b		22.6	1030	11000	4600	10.7	4.5	1.0	1.0
4a	Zwittergent	40	737	4870	1350	6.6	1.8	0.6	0.4
6a	Buffer	40	552	7500	1244	13.6	2.3	1.3	0.5
b	0.2 M NaCl	15	104	67	139	0.6	1.3	0.0	0.3
c	0.5 M NaCl	10	68	42	49	0.6	0.7	0.0	0.2
7	Buffer	4	113	7498	1387	66.4	12.3	6.2	2.7
8a	Buffer	4	103	1912	152	18.6	1.5	1.7	0.3
b	5 mM cGMP	8	2.5	5156	323	2062	129.2	193	28.7
c	10 mM cAMP	8	1.4	0	1332	0	951.0	0	211.3

zymes, 193-fold with Pk-G and 211-fold with Pk-A relative to the particulate cell fraction (Fraction 3b). 47% of the Pk-G-activity and 29% of the Pk-A-activity have been recovered.

The Pk-G-activity from fraction 8b can be further separated by polyacrylamide gel-electrophoresis. With a "native" gel two pronounced peaks comigrating with bovine serum albumin, and with

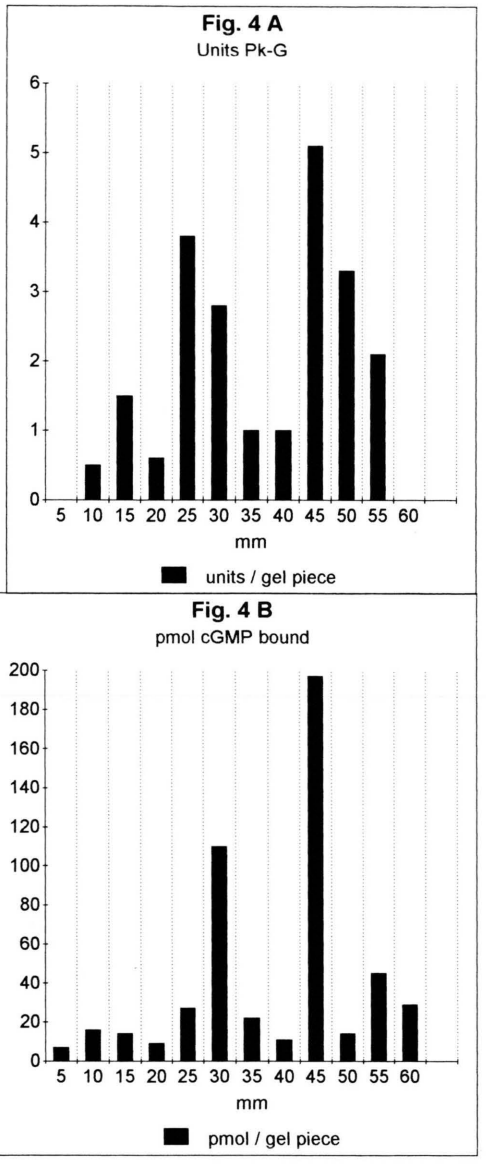

Fig. 4. cGMP-dependent protein phosphorylating activity and cGMP binding proteins after polyacrylamide gel electrophoresis. PK-G was separated from a yeast extract according to Table IV. 60 µl of fraction 8b (27 units Pk-G) were applied to a 7.5% polyacrylamide gel and the proteins separated by electrophoresis, as described in "Materials and Methods". Control lanes were run with 2.5, 5, and 10 µl eluate, and with 10 µl (5 µg protein) calibration protein solution, respectively, and stained with a silver-staining kit. Beginning at the top, the lane was cut into 5 mm pieces, and the proteins were eluted and tested. For further details see "Materials and Methods".
A : cGMP-dependent protein kinase activity. Ordinate: units/gel-piece.
B : Affinity for cGMP. Ordinate: pmol cGMP bound/ gel-piece.

a mixture of β-galactosidase + bovine carboanhydrase + catalase, respectively, are found (Fig. 4A). The proteins from these positions also bind outstanding amounts of cGMP (Fig. 4B). At least three fractions are separated in presence of 7.5% SDS, which after "renaturation" exhibit Pk-G-activity (positions 25, 35, and 45 mm, Fig. 5). Their apparent molecular masses, as deduced from comigrating marker proteins around 232 kDa (catalase), 116 kDa (β-galactosidase) and 66 kDa (bovine serum albumin), pretend mono-, di- and tetramers. The smallest one is positioned between the subunits of yeast Pk-A (52 kDa (Zoller *et al.*, 1988)) and mammalian and other Pk-G monomers (74–86 kDa (Vaandrager *et al.*, 1994; Gamm *et al.*, 1995)).

Taken together, the data indicate a cGMP-dependent protein kinase in extracts from growing yeast cells, which can be separated from cAMP-dependent kinase by affinity chromatography.

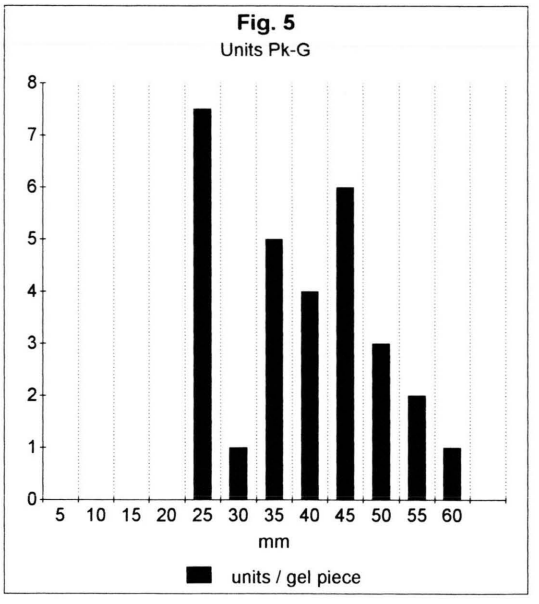

Fig. 5. Separation by electrophoresis of the cGMP de= pendent protein phosphorylating activity (Pk-G) with a SDS-polyacrylamide gel.
Pk-G was eluted by cGMP from a cGMP-Sepharose-matrix, applied to a 7.5% polyacrylamide gel containing 1% SDS, and the proteins separated by electrophoresis, as described in "Materials and Methods". Beginning at the top, the lane was cut into 5 mm pieces and the proteins eluted and tested for cGMP-dependent protein kinase activity. Molecular weights were derived from marker proteins on control lanes. For further details see "Materials and Methods". Ordinate: units/gel-piece.

Like Pk-G type II from other cell systems (French *et al.*, 1995; Jarchau *et al.*, 1994), it obviously is attached to membranous structures. But it differs from other Pk-G's with respect to optimum pH, optimum temperature, substrate specificity and apparaent molecular masses. The Pk-G-activity must be ascribed to different proteins, as revealed by polyacrylamide-gel electrophoresis. One of these isozymes exhibits a deduced molecular mass between those of Pk-A-subunits and Pk-G-monomers. The meaning of this is not clear. Additional investigations, especially immunochemical and genetic studies are necessary to enlighten this problem. An analysis of the role of the system guanylate cyclase/cGMP/Pk-G and its possible interdependence with other regulatory systems, e. g. the inositol trisphosphate system or ion channels (Komalavilas *et al.*, 1994; Archer *et al.*, 1994; French *et al.*, 1995), in the regulation of growing yeast cells, should be an objective of further work.

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