A Particulate Guanylate Cyclase (EC 4.6.1.2) from Growing Yeast Cells (*Saccharomyces cerevisiae*)

Hans Eckstein and Heike Schlobohm

Institut für Physiologische Chemie der Universität Hamburg Martinistr. 52 – UKE –, D-20251 Hamburg, Germany

Z. Naturforsch. **52c,** 373–379 (1997); received December 23, 1996/February 11, 1997

Guanylate Cyclase, Signal Transduction, cGMP, (*Saccharomyces cerevisiae*)

The detection of cGMP in yeast (Eckstein 1988), but lacking hints at guanylate cyclase from sequencing of the yeast genome, raised questions about existence, isoform, and regulation of guanylate cyclase from this organism. We found a particulate guanylate cyclase activity in yeast extracts, exhibiting properties of an integral membrane protein. Characteristics are: pH-optimum at pH 6.8, temperature-optimum around 60 °C, only slight stimulation by Mn^{2+}. Sigmoidal enzyme kinetics indicate allosteric regulation, ATP and Ca^{2+} act as negative allosteric effectors. The enzyme activity is increased by yeast alpha-1 mating factor, and by sodium nitrite, thus showing properties of particulate as well as of soluble isoforms from other eukaryotes. The activation by alpha-1 mating factor suggests receptor functions, and a role in ascospore conjugation.

Introduction

The enzyme guanylate cyclase (EC 4.6.1.2) catalyses the synthesis of 3',5'-cyclic guanosine monophosphate (cGMP) from GTP. cGMP interacts as a second messenger with three groups of target proteins, namely phosphodiesterases, cGMP-dependent protein kinases, and cation channel proteins (Corbin *et al.*, 1990; Garbers 1993; Schultz and Klumpp 1993; Biel *et al.*, 1994; Hsu and Molday 1994; McKee *et al.*, 1994). Guanylate cyclases are described from bacteria, protozoa, evertebrata and vertebrata, the genes of some enzymes were cloned (Tremblay *et al.*, 1988; Chinkers and Garbers 1991; Garbers 1993; Matsuoka *et al.*, 1995; Liu *et al.*, 1995). Two groups of guanylate cyclases are known: The predominating, phylogenetically very old isoforms are homodimeric transmembrane glycoproteins with receptor functions (Tremblay *et al.*, 1988; Chinkers and Garbers 1991; Vaandrager *et al.*, 1993; Abe *et al.*, 1993; Koller *et al.*, 1993; Koch *et al.*, 1994). Beyond these, at least three subgroups can be distinguished by their peptide ligands (Chinkers and Garbers, 1991). Phylogenetically younger soluble isoforms represent heterodimeric hemoproteins, which are activated by nitric oxide or other oxidizing agents (Murad *et al.*, 1979; Ignarro *et al.*, 1984; Chinkers and Garbers, 1991; Murad *et al.*, 1993; McKee *et al.*, 1994). The previous findings that growing yeast cells (*Saccharomyces cerevisiae*) contain cGMP (Eckstein, 1988), but that analysis of the yeast genome yielded no sequences homologous to those of guanylate cyclases from other sources, raised questions about existence and isoforms of the presumed guanylate cyclase, its regulation, and its possible biological function. We present data on a particulate enzyme activity from yeast extracts, and on some of its characteristics.

Reprint requests to Dr. Eckstein.
Telefax: 49-040-4717-5430.

Materials and Methods

Chemicals

Rabbit anti cGMP antiserum, [8-^{3}H]guanosine 5'-triphosphate, ammonium salt (259 GBq/mmol), (2,8-^{3}H]adenosine 5'-triphosphate, ammonium salt (1.44 TBq/mmol), [8^{3}H]guanosine 3',5'-cyclic phosphate, ammonium salt (673 GBq/mmol), and [2,8-^{3}H]adenosine 3',5'-cyclic phosphate (1.18 TBq/ mmol), were from Amersham-Buchler, Braunschweig. Unlabeled nucleotides, phenylmethylsulfonyl fluoride (PMSF), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4-morpholine-ethanesulfonic acid (MES), 1,4-piperazinediethanesulfonic acid (PIPES), and 2-amino-

0939–5075/97/0500–0373 $ 06.00

2-(hydroxymethyl)-1,3-propanediol (Tris-HCl) were from Boehringer-Mannheim. Dithiothreitol (DTT), ZWITTERGENT 3–14, and mannitol, were from CALBIOCHEM-NOVABIOCHEM. Triethylamine, and Protoporphyrine IX (PP) were from FLUKA. Theophylline was from SERVA. Atrial natriuretic peptide (rat ANP, synthetic), *E. coli* heat stable enterotoxine STa, Hemin (bovine), 3-isobutyl-1-methylxanthine (IBMX), sodium nitroprusside, and yeast α_1-mating factor (YMF, synthetic) were from SIGMA, Munich. All other chemicals were from MERCK, Darmstadt, Germany.

Yeast culture and cell fractionation

Yeast cells were cultured, and homogenized, according to (Eckstein 1988). A cell clone originally derived from baker's yeast (*Saccharomyces cerevisiae*, "Germania Hefe", Deutsche Hefewerke Hamburg) was used throughout all experiments. The cells were grown overnight in a modified WICKERHAM medium at 31.3 °C with stirring and aerating until 2–3 x 10^7 cells /ml (log phase growth). They were harvested by centrifugation, washed three times with MET-buffer (250 mM mannitol + 1 mM EDTA-Na_2 in 15 mM Tris-HCl, pH 7.5), and resuspended at 1 x 10^9 cells/ml with MET-buffer, which contained 1 mM PMSF and 1.4 mM DTT. The yeast cells were disintegrated immediately by shaking with glass beads in a Merkenschlager homogenizer. The homogenate was filtered through a sintered glass filter (G-1), the filtrate was centrifuged 10 min with 190 x *g* at 2 °C. The resulting supernatant was centrifuged 30 min at 30.900 x *g* at 2 °C. The resulting residue was resuspended at 1/3 volume of the 190 x *g* supernatant with MET-buffer containing PMSF and DTT. The supernatant was centrifuged 120 min at 4 °C at 100.000 x *g* in a Beckman L-7 ultracentrifuge. The resulting supernatant remained unchanged, the residue was resuspended at 1/3 volume of the 190 x *g* supernatant (28–30 ml) with MET-buffer containing PMSF and DTT.

Solubilisation of particle-bound enzymatic activity

a): NaCl: 450 µl of a 100,000 x *g* residue were mixed with 112 µl 5 M NaCl (final concentration 1 M), incubated 60 min in an ice bath, and subsequently centrifuged 30 min at 4 °C at 100,000 x *g*. The resulting supernatant was ultrafiltrated twice with Centricon 30, the residue was washed three times with 1 µM PMSF in MET buffer. Both fractions were refilled to 450 µl with PMSF-MET buffer and tested for guanylate cyclase activity.

b): Zwittergent 3–14: 450 µl of a 100,000 x *g* residue were mixed with 1.55 ml PMSF-MET-buffer, which contained 25% (v/v) glycerol and 10 µmol Zwittergent 3–14 (final concentration 5 mM), and incubated 10 min. at room temperature. For the further procedure see a), except that the buffer always contained glycerol.

c): Triton X-100: 450 µl 100,000 x *g* residue were mixed with 1.55 ml glycerol-PMSF-MET-buffer, which contained 15 µl Triton X-100 (final concentration 12.2 mM), and incubated 10 min at room temperature. For the further procedure see b).

Guanylate cyclase assay

The standard assay contained, in a total volume of 200 µl: 100 mM PIPES as buffer, 0.5 mM IBMX, 0.5 mM theophylline, 1.0 mM $MnCl_2$ x 2 H_2O, 1.0 mM GTP labeled with 74 TBq ^3H, and 10 µl yeast extract (100,000 x *g* residue, 0.2–0.4 mg protein/10 µl). The final pH was pH 6.7. The reaction was started by addition of the yeast extract and incubation at 37 °C in a shaker. After 15 minutes, 10 µl 0.2 M EDTA were added, and the assay was heated for 10 min at 95 °C. After further 10 min in an ice bath, the insoluble material was centrifuged off, and the synthesized ^{3}HcGMP was estimated from the clear supernatant by a radioimmunoassay (RIA), as described below.

All assays were performed in duplicate. Control assays routinely were run without the incubation at 37°.

Radioimmunoassay

^{3}H-cGMP was acetylated, and estimated by a RIA, as described earlier (Eckstein, 1988). 175 µl supernatant from the cyclase assay were mixed with 350 µl sodium acetate, 0.4 M, pH 6.2, and 50 µl of a mixture of 2 vol. triethylamine + 1 vol. acetic anhydride. After 15 min at room temperature, 400 µl potassium phosphate, 1 M, pH 7.4, and 50 µl anti cGMP antiserum, 28 mg protein/ml, were added. The final pH was pH 7.0. The probes were allowed to stand 18 hours at 4 °C. The proteins were then precipitated at 0 °C by 3.0 ml 4 M

$(NH_4)_2SO_4$, and sedimented by centrifugation. The pellet was washed three times with 3 ml $(NH_4)_2SO_4$ and finally redissolved with 800 µl 1 mM NaOH. 750 µl of this solution were mixed with 4 ml scintillation cocktail ("Biofluor", DuPont NEN, Bad Homburg, Germany). The radioactivity was measured in a WALLAC liquid scintillation counter (EG & G BERTHOLD GmbH). The values were adjusted for unspecific ^{3}H-binding, and expressed in terms of pmol cGMP synthesized/min.

There was no interaction between the anti cGMP antiserum and cAMP, nor with AMP, ATP, or GMP, even with a hundredfold excess over cGMP. Unspecific binding of ^{3}H from ^{3}H-GTP was less than 0.1%.

Protein was estimated by the biuret reaction.

Results and Discussion

Incubation of 190 x *g* supernatants from yeast homogenates in a guanylate cyclase assay with ^{3}H-GTP as a substrate results in the synthesis of ^{3}H-cGMP, detectable by a RIA with rabbit anti cGMP antiserum.

With ^{3}H-ATP as substrate instead of ^{3}H-GTP, and with rabbit anti cAMP antiserum in the RIA, a synthesis of ^{3}H-cAMP was not detected. Unspecific cGMP synthesis by yeast adenylate cyclase therefore seems unlikely.

The synthesis of ^{3}H-cGMP shows all characteristics of an enzymic reaction: The amount of ^{3}H-cGMP increases with the incubation time (Fig. 1A), and with the protein concentration (Fig. 1B) in the cyclase assay. The detection fails, when the 190 x *g* -supernatant is omitted, or when it was heated 10 min at 95 °C before use, or when ^{3}H-GTP is replaced by ^{3}H-ATP.

Fractionated centrifugation of the 190 x *g* supernatant results in sedimentation of more than 90% of the enzyme activity together with the insoluble cell constituents. About 30% are sedimented by 30,000 x *g*, and 60% by 100,000 x *g* (Table I). This separation is accompanied by a remarkable increase in enzyme activity, presumably due to the elimination of inhibitors and degrading enzymes. The activity can be solubilized from 100,000 x *g* residues by Triton X-100, but not by 1 M NaCl (Table I), though with a remarkable loss. As can be concluded from its solubility by detergents, the particulate enzyme activity from yeast seems to belong to an integral membrane protein, like the particulate isoforms from bacteria and animal cells (Bradham and Cheung, 1982; Tremblay *et al.*, 1988; Garbers, 1993; Schultz and Klumpp, 1993; Koller *et al.*, 1993).

With 100,000 x *g* residues, maximum enzyme activity is obtained between pH 6.7 and 6.9 (Fig. 2A), and between 55 and 65 °C (Fig. 2B, a). The particulate yeast guanylate cyclase in this regard differs clearly not only from the membrane-bound yeast adenylate cyclase (Varimo and Londesborough, 1982; Casperson *et al.*, 1983), but also

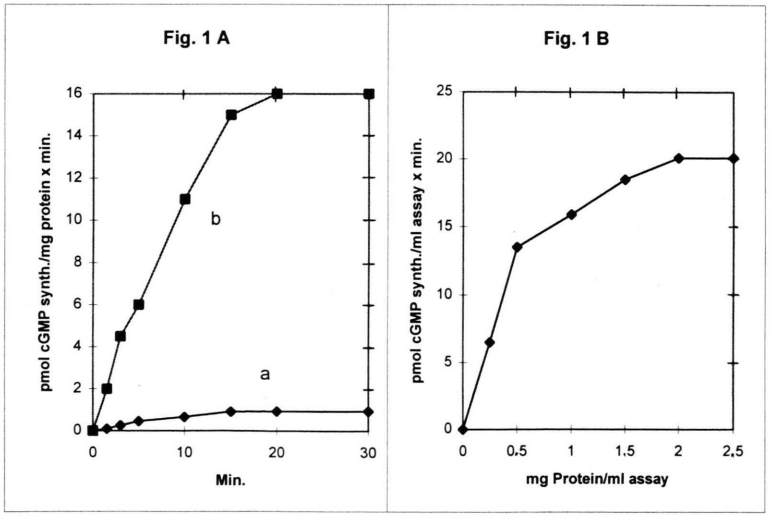

Fig. 1. Synthesis of cGMP by yeast extracts.
A) Dependence on the incubation time. (a): 190 x *g* supernatant, 4 mg protein/ml assay. (b): 100.000 x *g* residue, 0.3 mg protein/ml assay.
B): Dependence on the protein concentration/assay, 100.000 x *g* residue.

Table I. Distribution of the guanylate cyclase activity between fractions from growing yeast cells. Cell fractions were prepared, and guanylate cyclase was estimated from the fractions, as described in "Materials and Methods".

Fraction	pmol cGMP synthesized /ml fraction x min	pmol cGMP synthesized /mg protein x min	pmol cGMP synthesized /fraction x min
190 x *g*			
supernatant	11	0.5	320
residue	6	0.3	90
30,900 x *g*			
supernatant	7	0.6	207
residue	130	13.5	1340
100,000 x *g*			
supernatant	3	0.3	90
residue	370	14.5	3800
100,000 x *g*-residue:			
+ Triton:			
– supernat.		9	1830
– residue		7.5	460
+ NaCl:			
– supernat.		0.2	35
– residue		18	1680

from many guanylate cyclases from other eucaryotic cells, which in general show maximum activities between pH 7.2 and 8.0 and around 37 °C (Bradham and Cheung, 1982; Schultz and Klumpp, 1993). The unexpectedly high temperature optimum cannot be explained by an interference between enzyme activity and any heat-sensitive constituents of the extracts, since the same temperature dependence was obtained with extracts, which were preincubated 10 min at 56 °C before use (Fig. 2B, b).

Another remarkable difference from many other guanylate cyclases results from the behaviour against bivalent cations. Whereas guanylate cyclases from other sources need unphysiologically high Mn^{2+} -concentrations for full activity, we found only a slight stimulation of the yeast enzyme by Mn^{2+}, even at 1 mM (Fig. 3). On the other hand, the enzyme distinctly is inhibited by Ca^{2+} above 0.05 mM, or by Mg^{2+}. The rapid inhibition particularly by Ca^{2+} at concentrations in a physiological range suggests a role of Ca^{2+} as regulator, as is described from other cell systems (Schultz and Klumpp, 1993; Bahnson *et al.*, 1993; Gorczyca *et al.*, 1994; Komalavilas and Lincoln, 1994).

As shown in Fig. 4, increasing GTP-concentrations result in a sigmoidal substrate saturation curve. 50% activity are obtained with 0.3 mM GTP. Presence of 1 mM ATP shifts the curve to the right,

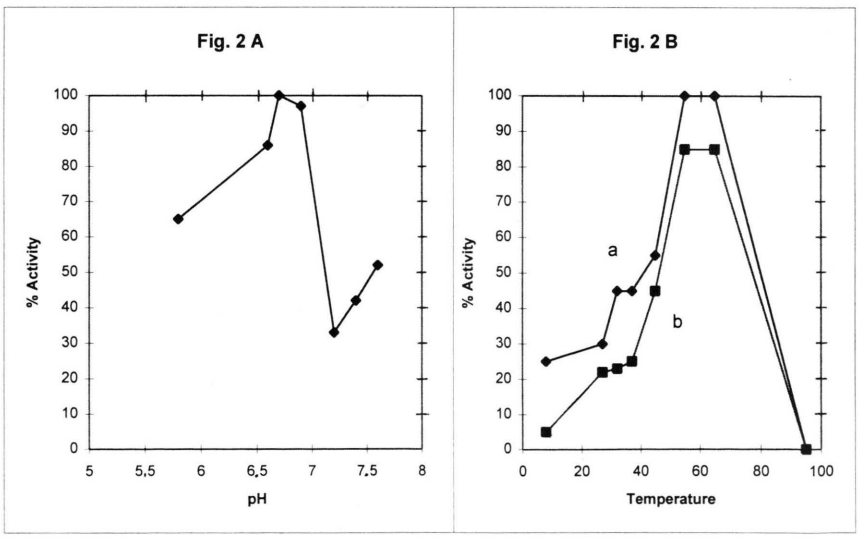

Fig. 2. Synthesis of cGMP by 100.000 x *g* residues from yeast extracts.
A): pH dependence. The buffers used were MES (pH < 6.6), PIPES (pH = 6.7), HEPES (pH = 6.9–7.2), and Tris-HCl (pH > 7.2).
B): Temperature dependence. (a): Standard assay. (b): The residue was preincubated before use for 10 min. at 56 °C. 100% = 16 pmol cGMP synthesized/mg protein x min.

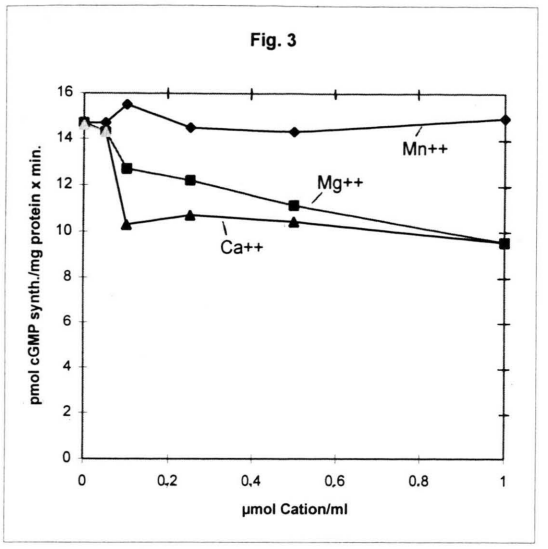

Fig. 3. Guanylate cyclase activity from 100.000 x *g* residues with bivalent cations.
The enzyme assays were supplemented with increasing concentrations of Mn^{2+}, or Mg^{2+}, or Ca^{2+}, as indicated.

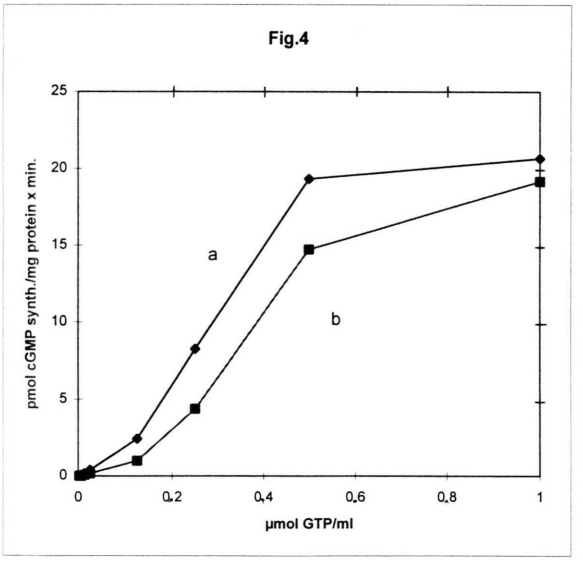

Fig. 4. cGMP formation by 100.000 x *g* residues at increasing GTP concentrations.
a) control, b) + 1 mM ATP.

indicating an allosteric inhibition of the enzyme by ATP. The nonlinear decrease of the enzyme activity with increasing ATP-concentrations (Fig. 5) confirms this interpretation. 50% inhibition are obtained with an ATP concentration, which is

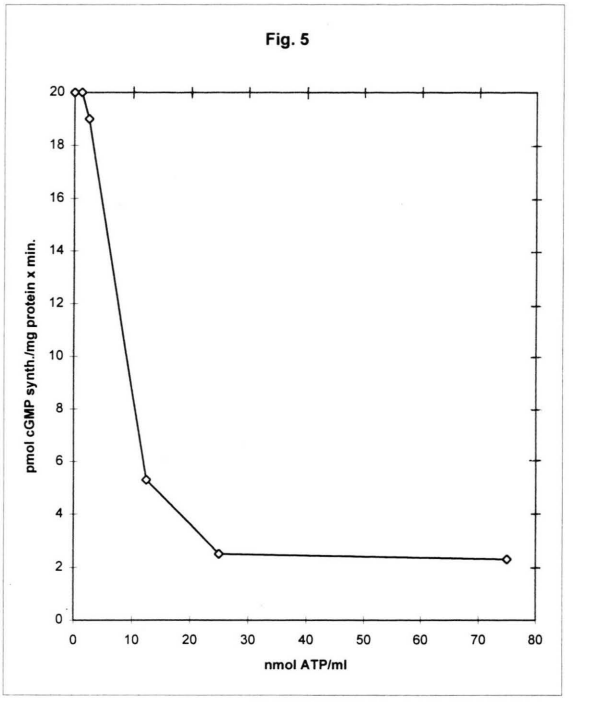

Fig. 5. Inhibition of the cGMP-synthesis by ATP.
Standard guanylate cyclase assays were supplemented with increasing ATP concentrations, as indicated.

Table II. Guanylate cyclase activity with various effectors.

Assay	pmol cGMP synthesized/mg protein x min	p
Control	17	
+ sodium nitrite 20 mM	26	< 0.001
+ YMF *)		
0.025 mM	20	< 0.05
0.1 mM	31	< 0.001

*) YMF yeast alpha-1 mating factor, synthetic.
Significance was calculated using Student's t-test.

three orders of magnitude below the intracellular level of aerobically growing yeast (Hilz and Eckstein, 1964). The enzyme activity in aerobically growing cells therefore seems to be determined essentially by the GTP level. The sigmoidal substrate kinetics shows, that GTP exerts its effects as a positive allosteric effector.

As shown in Table II, the enzyme activity is increased significantly in presence of the alpha-1

mating factor, a peptide produced by yeast ascospores. This increase indicates a peptide binding domain comparable to the receptor domain of other particulate isoforms. A slight, but significant increase also is obtained by 20 mM sodium nitrite, an activator of soluble isoforms from mammalian cells. The consequence of this is not clear. Other activators of particulate or soluble isoforms from mammalians, e. g. atrial natriuretic peptide, *E. coli* enterotoxine, hydroxylamine, hemin, or sodium nitroprusside, show no effects.

Acknowledgements

The excellent technical assistance of Birgit Fluegge is gratefully acknowledged.

This paper includes some results from the PhD thesis of H. S.

Abe T., Nishiyama K., Snajdar R., He X. and Misono K. S. (1993), Aortic smooth muscle contains guanylate-cyclase-coupled 130-kDa atrial natriuretic factor receptor form. Spontaneous switching to 60-kDa C-receptor upon cell culturing. Eur. J. Biochem. **217,** 295–304.

Bahnson T. D., Pandol St. J. and Dionne V. E. (1993), Cyclic GMP modulates depletion-activated Ca^{2+} entry in pancreatic acinar cells. J. Biol. Chem. **268,** 10808–10812.

Biel M., Zong X., Distler M., Bosse E., Klugbauer N., Murakami M., Flockerzi V. and Hofmann F. (1994), Another member of the cyclic nucleotide-gated channel family, expressed in testis, kidney, and heart. Proc. Natl. Acad. Sci. USA **91,** 3505–3509.

Bradham L. S. and Cheung W. Y. (1982), Nucleotide cyclases. Progr. Nucl. Acid Res. **27,** 189–231.

Casperson G. F., Walker N., Brasier A. R. and Bourne H. R. (1983), A guanine nucleotide-sensitive adenylate cyclase in the yeast *Saccharomyces cerevisiae.* J. Biol. Chem. **258,** 7911–7914.

Chinkers M. and Garbers D. L. (1991), Signal transduction by guanylyl cyclases. Annu. Rev. Biochem. **60,** 553 -575.

Corbin J. D., Thomas M. K., Wolfe L., Shabb J. B., Woodford T. A. and Francis S. H. (1990), New insights into cGMP action. In: Biol. Med. Signal Transduction (ed. Y. Nishizuka *et al.*). Raven Press, New York p. 411–418.

Eckstein H. (1988), 3':5'-cyclic GMP in the yeast *Saccharomyces cerevisiae* at different metabolic conditions. FEBS Lett. **232,** 121–124.

Garbers D. L. (1993), Guanylyl cyclase receptors and their ligands. Adv. Second Messenger Phosphoprotein Res. **28,** 91–95.

Gorczyca W. A., Gray-Keller M. P., Detwiler P. B. and Palczewski K. (1994), Purification and physiological evaluation of a guanylate cyclase activating protein from retinal rods. Proc. Natl. Acad. Sci. USA **91,** 4014–4018.

Hilz H. and Eckstein H. (1964), Teilungssynchronisierte Hefezellen, I. Unterschiedliche Wirkungen von Röntgenstrahlen und cytostatischen Verbindungen auf Stoffwechsel und Zellteilung. Biochem. Z. **340,** 351–382.

Hsu Y. and Molday R. S. (1994), Interaction of calmodulin with the cyclic GMP-gated channel of rod photoreceptor cells. J. Biol. Chem. **269,** 29765–29770.

Ignarro L. J., Wood K. S., Ballot B. and Wolin M. S. (1984), Activation of purified soluble guanylate cyclase by protoporphyrin IX. J. Biol. Chem. **259,** 5923–5931.

Koch K.-W., Stecher P. and Kellner R. (1994), Bovine retinal rod guanyl cyclase represents a new N-glycosylated subtype of membrane-bound guanyl cyclases. Eur. J. Biochem. **222,** 589–595.

Koller K. J., Lipari M. T. and Goeddel D. V. (1993), Proper glycosylation and phosphorylation of the type A natriuretic peptide receptor are required for hormone-stimulated guanylyl cyclase activity. J. Biol. Chem. **268,** 5997–6003.

Komalavilas P. and Lincoln Th. M. (1994), Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic GMP dependent protein kinase. J. Biol. Chem. **269,** 8701–8707.

Liu W., Yoon J., Burg M., Chen L. and Pak W. L. (1995), Molecular characterization of two *Drosophila* guanylate cyclases expressed in the nervous system. J. Biol. Chem. **270,** 12418–12427.

Matsuoka I., Mori T., Sato T., Sakai M. and Kurihara K. (1995), Identification of novel guanylyl cyclases from chemosensory tissues of rat and cattle. Biochem. Biophys. Res. Commun. **216,** 242–248.

McKee M., Scavone C. and Nathanson J. A. (1994), Nitric oxide, cGMP, and hormone regulation of active sodium transport. Proc. Natl. Acad. Sci. USA **91,** 12056–12060.

Murad F., Arnold W. P., Mittal Ch. K. and Braughler J. M. (1979), Properties and regulation of guanylate cyclase, and some proposed functions for cyclic GMP. Adv. Cyclic Nucl. Res. **11,** 175–204.

Murad F., Forstermann U., Nakane M., Pollock J., Tracey R.,Matsumoto T. and Buechler W. (1993), The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. Adv. Second Messenger Phosphoprotein Res. **28,** 101–109.

Schultz J. E. and Klumpp S. (1993), Cyclic nucleotides and calcium signaling in *Paramecium.* Adv. Second Messenger Phosphoprotein Res. **27,** 25–46.

Tremblay J., Gerzer R. and Hamet P. (1988), Cyclic GMP in cell function. Adv. Second Messenger Phosphoprotein Res. **22,** 319–383.

Vaandrager A. B., Van der Wiel E. and DeJonge H. R. (1993), Heat-stable enterotoxin activation of immunopurified guanylyl cyclase C. Modulation by adenine nucleotides J. Biol. Chem. **268,** 19598–19603.

Varimo K. and Londesborough J. (1982), Adenylate cyclase activity in permeabilised yeast. FEBS Lett. **142,** 285 -288.