Expression, purification, and characterization of the cGMP-dependent protein kinases Iβ and II using the baculovirus system

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Abstract Detailed studies of differences in distinct cGMP kinase isoforms are highly dependent on expression of large amounts of these enzyme isoforms that are not easily purified by conventional methods. Here cGMP-dependent protein kinases, the type Iβ soluble form from human placenta, and the type II membrane-associated form from rat intestine, were each expressed in a baculovirus/Sf9 cell system and purified in milligram amounts by affinity chromatography. The expressed recombinant proteins displayed characteristics like those of their native counterparts. cGK Iβ was expressed as a 76 kDa protein predominantly found in the cytosol fraction, whereas cGK II was expressed as an 86 kDa protein predominantly associated with the membrane fraction. The apparent K_a and V_{max} of cGMP for activation of cGK Iβ were 0.5 μM and 3.4 μmol/min/mg, and for cGK II were 0.04 μM and 1.8 μmol/min/mg.

Key words: cGMP-dependent protein kinase; Baculovirus expression; Affinity purification; Kinase acitivity

1. Introduction

Two major signal transduction pathways, one involving production of nitric oxide (NO), i.e. endothelium derived relaxing factor (EDRF), which stimulates soluble guanylyl cyclases, and the other composed of small peptide hormones such as atrial natriuretic peptide (ANP) which stimulate transmembrane receptor guanylyl cyclases, elevate cGMP and activate cGMP regulated channels, phosphodiesterases, and kinases [1–4]. The vertebrate cGMP dependent protein kinases (cGK) consist of the soluble type Iα and Iβ isoforms, possible splice products derived from the same gene which differ only in about the first 100 N-terminal amino acids, and a membrane-associated type II [5–8]. Whereas cGK I is found in high concentrations in cerebellar Purkinje cells, smooth muscle cells and human platelets [1], cGK II was initially described biochemically as a special membrane-associated form found in intestinal epithelium [9,10]. Recent phylogenetic analysis of the cloned forms of these enzymes and others from *Drosophila* [11] suggest in fact that cGK types I and II derive from different ancestral kinases [7].

In order to facilitate structure–function analyses of the different cGMP kinase forms, the expression of large amounts of specific recombinant forms was undertaken. Since cGK Iα can be readily obtained by purification from bovine lung and has also recently been expressed using the baculovirus/Sf9 insect cell system [12], we chose to express cGK Iβ and cGK II, two less well-studied kinases, using this system. Our own experience and that of others indicated that bacterial expression of cGKs invariably led to insoluble and enzymatically inactive cGK Iα [13] and cGK Iβ and cGK II [14]. The reason for this remains unclear although it could be due to lack of posttranslational modification required for correct kinase folding. One critical modification may be the phosphorylation of a conserved threonine residue found in the catalytic (C) subunit (Thr-197) of cAMP-dependent kinase and cGMP kinases which appeared to be important for correct assembly of C subunit structure and for substrate orientation in cocrystallization studies of C subunit and a peptide of the protein kinase inhibitor PKI [15]. In contrast to expression in *E. coli*, expression of cGK I isoforms in various mammalian cell types (COS-7, CHO, HEK 293) [16,17,18] and cGK II [7] in HEK 293 cells resulted in active enzyme; however, in insufficient amounts for preparative work.

It was particularly important to develop an expression system for cGK Iβ and cGK II to replace the conventional purification methods for these enyzmes. cGK Iβ has been purified from bovine aorta, but is, however, susceptible to proteolysis which converts about half of the native dimer form to an N-terminal truncated monomer [19]. cGK II is extremely susceptible to proteolysis and therefore has only been successfully purified from intestine removed from surgically prepared pigs [9,10], a very elaborate source not widely available. Furthermore the activation constants of conventionally purified cGK II could not be evaluated due to the presence of bound cGMP as a result of the purification protocol [9,10]. Here we report the expression and purification of both cGK Iβ and cGK II in active forms without bound cGMP and the analysis of their activation and kinetic properties in comparison to cGK Iα.

2. Experimental

2.1. Materials

TC-100 medium, antibiotics, amphotericin B and L-glutamine were obtained from Gibco/BRL, fetal calf serum from C.C.PRO, BaculoGold DNA from Dianova, DOTAP from Boehringer-Mannheim, restriction enzymes from New England Biolabs, aprotinin (Trasylol) from Bayer, [γ-^{32}P]ATP from Amersham, [^{125}I]protein A from ICN, and P81 paper from Whatman. All cyclic nucleotides were purchased from

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Abbreviations: cGK, cGMP-dependent protein kinase; 8-Br-cGMP, 8bromo-cGMP; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; PET-cGMP, β-phenyl-1,N^2-etheno-cGMP; 8-Br-cAMP, 8-bromo-cAMP; Sp-5,6-DCl-cBiMPS, Sp isomer of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′, 5′-monophosphorothioate; Rp-8-pCPT-cGMPS, Rp isomer of 8-(4-chlorophenylthio)guanosine 3′,5′-monophosphorothioate; Rp-cAMPS, Rp isomer of adenosine-3′,5′-monophosphorothioate; IBMX, 3-isobutyl-1-methyl-xanthine; NP40, ethylphenolpoly(ethyleneglycolether)$_n$; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate; PMSF, phenylmethylsulfonylfluoride; MOI, multiplicity of infection; p.i., post-infection.

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Biolog. Other chemicals were obtained from Sigma in analytical grade. Preparation of purified cGK Iα from bovine lung [20] and generation of polyclonal antibodies against cGKIβ and cGK II [14] have been described previously. The peptides Kemptide and VASPtide were synthezised in the laboratory of D. Palm, University of Würzburg, Germany. Sf9 cells were a kind gift of S. Bräutigam, University of Würzburg, Germany, and 8-(2-aminoethyl)-amino-cAMP-Sepharose was a kind gift from H. De Jonge, University of Rotterdam; 8-(6-aminohexyl)-amino-cAMP-Sepharose was synthesised as described [21].

2.2. Construction of recombinant transfer vectors

A recombinant transfer vector pVL-GK II, containing the 2286-bp open reading frame of rat intestinal cGK II flanked by 52 bp of the 5′-untranslated and 652 bp of the 3′-untranslated sequence, was constructed by cloning a 3.0 kb *Eco*RI/*Eco*RI fragment of cGK II cDNA [7] into the *Eco*RI site of the baculovirus transfer vector pVL1392. Similarly, pVL-GK Iβ containing nucleotides −62 to 3682 of human placenta cGK Iβ-cDNA [6] including the entire coding region (nt 1 to 2058) was cloned as a 3.7 kb *Eco*RI/*Eco*RI fragment into pVL 1392. The coding sequences were properly orientated for transcription from the viral polyhedrin promotor as confirmed by restriction site analysis and dideoxy sequencing of the constructs.

2.3. Sf9 cell culture and purification of recombinant baculoviruses

Standard methods for heterologous gene expression in the baculovirus/Sf9 insect cell system were used [22]. *Spodoptera frugiperda* (Sf9) cells were maintained as monolayers and in suspension cultures at 27°C in TC-100 insect medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B. To obtain recombinant viruses, cotransfection of Sf9 cells with 250 ng BaculoGold linearized baculovirus genomic DNA carrying a deletion in an essential gene for replication and 2.5 μg purified recombinant transfer vector (pVL-GK II or pVL-GK Iβ) was performed according to instructions of the PharMingen BaculoGold transfection kit or using a liposome-mediated method with DOTAP. Recombinant baculoviruses were identified by Western blot analysis of whole cell extracts of transfected Sf9 cells harvested 48 h post-transfection using polyclonal antibodies raised against bacterially expressed cGK fusion proteins [14]. Purification of virus-encoding recombinant rat intestinal cGK II was carried out by plaque assay, whereas clones of recombinant virus encoding human placental cGK Iβ were obtained by three series of end-point dilution. Purified viruses were amplified and titered by end-point dilution before use for protein production.

2.4. Infection and subcellular fractionation of Sf9 cells

Suspension cultures (130 ml) of Sf9 cells in 250 ml spinner flasks (Techne) were seeded at a cell density of 3×10^5 cells/ml and grown with good aeration to a density of 2×10^6 cells/ml. Cells were collected, subsequently either left uninfected (control), or were infected with recombinant baculovirus clones at an MOI > 10, and then added to fresh medium to again obtain 130 ml of suspension culture. Aliquots (10 ml) of these cultures were harvested at different times p.i. for determination of cell densities. Cells were centrifuged at $800 \times g$, washed 2 times with PBS and resuspended in 1 ml buffer A (10 mM PIPES, pH 7.4, 2 mM EDTA, 50 U/ml Trasylol, 0.7 μg/ml leupeptin, and 0.5 μg/ml pepstatin A). Cells were lysed by 30 passages through a 0.33 × 12 mm needle, and complete lysis was controlled by phase-contrast light microscopy. The lysates were adjusted to 150 mM NaCl and centrifuged at $100{,}000 \times g$ for 1 h. Supernatants were saved and pellets were resuspended in the same volume of buffer A containing 150 mM NaCl. Identical protein amounts of both fractions were analyzed for recombinant cGMP kinase by Western blots.

2.5. Expression and purification of recombinant human placental cGK Iβ

Sf9 cells (2×10^7) were seeded in a 650 ml cell culture flask, grown in monolayers and infected with the recombinant baculovirus cGK Iβ clone at an MOI > 10. At 48 h p.i. cells were harvested, washed twice with PBS, and resuspended in 1 ml of cold buffer B (30 mM Tris, pH 7.4, 2 mM EDTA, 150 mM NaCl, 15 mM β-mercaptoethanol, 1 mM IBMX, and 50 mM benzamidine). All further steps were carried out at 4°C. Cells were homogenized by shear stress as described above and the lysates were centrifuged at $100{,}000 \times g$ for 1 h. The supernatant was loaded on a chromatography column (inner diameter 9 mm) containing 8-(2-aminoethyl)amino-cAMP-Sepharose (0.5 ml bed volume) and equilibrated with buffer B. After washing the column three times with 1 ml of buffer B, bound protein was eluted in a batch procedure by suspension of the Sepharose matrix in 1 ml of buffer B containing 30 mM cAMP. The eluate was dialysed three times against buffer C (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 5 mM β-mercaptoethanol, and 20 U/ml Trasylol) followed by buffer C containing 25% and subsequently 50% glycerol for storage of kinases at −20°C. Protein concentration was determined by comparison of purified kinases to standard amounts of BSA using SDS-PAGE.

2.6. Expression and purification of recombinant rat intestinal cGK II

The procedures for expression and purification of cGK II were the same as for cGK Iβ except for the following modifications. Infected Sf9 cells expressing recombinant cGK II were harvested at 60 h p.i. and

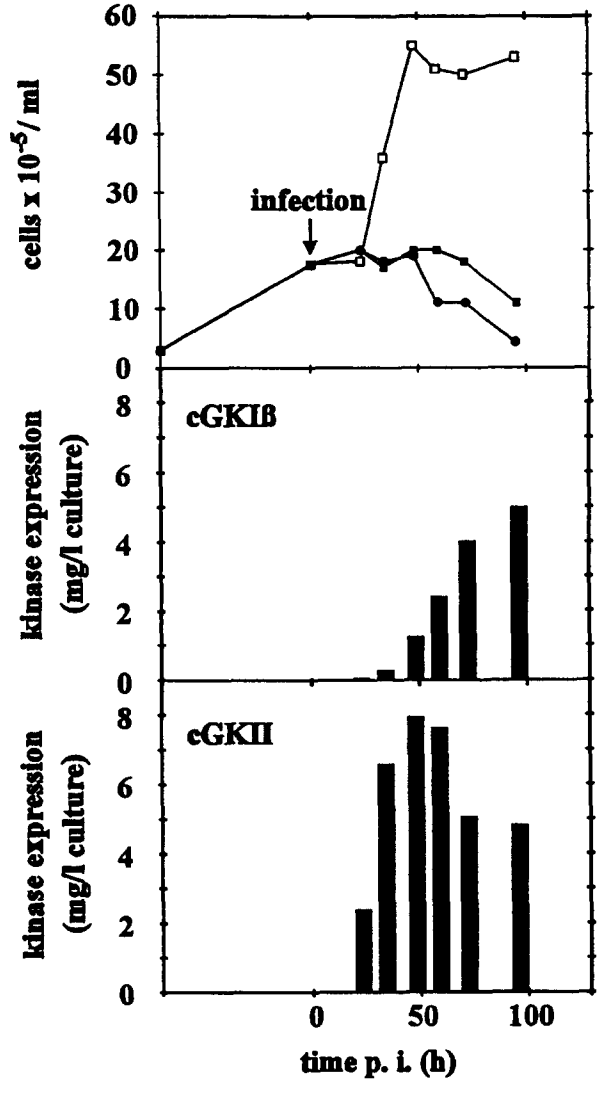

Fig. 1. Time course of cGK Iβ and cGK II expression in Sf9 suspension cultures. The relationship between the growth of uninfected and infected Sf9 suspension cultures (top) and the amount of expression of cGK Iβ (middle) and cGK II (bottom) is shown. Suspension cultures of Sf9 cells were seeded with a cell density of 3×10^5 cells/ml, grown to 2×10^6 cells/ml, and at $t = 0$ either left untreated (□ control) or infected with an MOI > 10 with baculovirus coding either for cGK Iβ (■) or for cGK II (●). At the p.i. times shown, aliquots of cultures were taken for determination of cell densities and the amount of expressed cGK Iβ or cGK II detected in whole cell lysates by Western blots using ^{125}I-labelled protein A. The amounts of kinases per liter suspension culture were calculated from radioactivity bound to full-length (undegraded) cGKs compared to defined kinase standards.

homogenized in buffer D (10 mM PIPES, pH 7.4, 2 mM EDTA, 250 mM $(NH_4)_2SO_4$, 300 mM sucrose, 0.5% Triton X-100, 1 mM IBMX, 50 mM benzamidine, 50 U/ml Trasylol, 0.7 μg/ml leupeptin, and 0.5 μg/ml pepstatin A). The supernatant obtained after centrifugation of the lysate at 100,000 × *g* for 1 h was applied to an 8-(6-aminohexyl)amino-cAMP-Sepharose column as described above. After washing the column with buffer B containing 600 mM NaCl, bound protein was batch eluted with buffer B containing 30 mM cAMP. Dialysis was performed as described for cGK Iβ except that the dialysate and dialysis buffer contained 0.015% NP40.

2.7. Immunoblots

Samples of homogenates and cytosolic and particulate subcellular fractions made from Sf9 cells expressing cGK II or cGK Iβ, as well as purified cGK II or cGK Iβ, were subjected to SDS-PAGE using a 4% stacking gel and 8% separating gel and subsequently transfered to nitrocellulose filters. The filters were incubated with polyclonal rabbit antisera raised against the cGMP kinases [14] and radiolabelled with [^{125}I]protein A. Radioactivity was localized after autoradiography and quantitated by γ-counting of excised protein bands.

2.8. In vitro kinase assay

In vitro measurement of cGMP dependent protein kinase activity was measured as described [23] with minor modifications. Aliquots of cGK (10 or 50 ng) were incubated in a total volume of 100 μl containing 20 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.01% (w/v) bovine serum albumin, 10 μg VASPtide (RRKVSQE) [18,24,25] and various concentrations of cyclic nucleotides. The reaction was started by adding 50 μM ATP containing 100 cpm/pmol [γ-^{32}P]ATP, incubated for 5 min at 30°C, and stopped with 100 mM EDTA. Kinase activity (μmol/min/mg protein) was measured in triplicates in all experiments.

K_a' values, defined as the concentration of cyclic nucleotides producing half-maximal cGK activation, were determined from plots of kinase phosphotransferase activity versus log concentration of cyclic nucleotide. For inhibitors, an apparent inhibition constant K_i' was determined from Dixon plots of reciprocal enyzme activity (velocity) versus inhibitor concentration. With the moderate concentrations of cGMP used, we assumed that only one site of cGK is saturated as has been reported for the bovine lung enzyme [26,27].

3. Results

3.1. Expression of recombinant cGMP-dependent protein kinase type II and type Iβ in Sf9 cells

Compared to control Sf9 cells, infected cells expressed proteins of either 86 kDa (cGK II) or 76 kDa (cGK Iβ) which were labeled by specific antibodies to the respective kinases on immunoblots (cf. Fig. 2). Whereas uninfected control Sf9 cell suspension cultures showed optimal cell growth with a doubling rate of about 24 h, infection of Sf9 cells with recombinant baculovirus at an MOI>10 resulted in immediate arrest of cell growth followed by a rapid decline in cell viability which culminated in lyses of the majority of cells by about 5 days p.i. (Fig. 1, top). cGK Iβ protein was first detectable by immunoblots of infected cells between 23 and 34 h p.i. and thereafter steadily increased in amount (Fig. 1, middle panel). The maximal amount of cGK Iβ (76 kDa protein) expression in Sf9 cells at 96 h p.i. was quantitated by comparing Sf9 total cell lysates on Western blots to standard amounts of recombinant cGK Iβ expressed in and purified from *E. coli* (not shown), and was calculated to be about 5 mg enzyme per liter (10^9 cells) of Sf9 culture, equivalent to 10% of Sf9 cell protein. Note, however, that already at 72 h p.i., smaller bands representing possible cGK Iβ proteolytic products began to appear (Fig. 2, top).

In contrast to the peak of cGK Iβ expression, that of cGK II detected by immunoblotting appeared much earlier at 48 h p.i. (Fig. 1, lower panel). The amount of Sf9-expressed cGK II, quantitated by comparison of Sf9 lysates with a purified standard of cGK II obtained from expression in *E. coli* (not shown), was calculated as 8 mg cGK II per liter (10^9 cells) Sf9 culture (≈15% of total Sf9 cell protein). A small amount of possible cGK II proteolysis was detected beginning at about 48 h (Fig. 2, bottom).

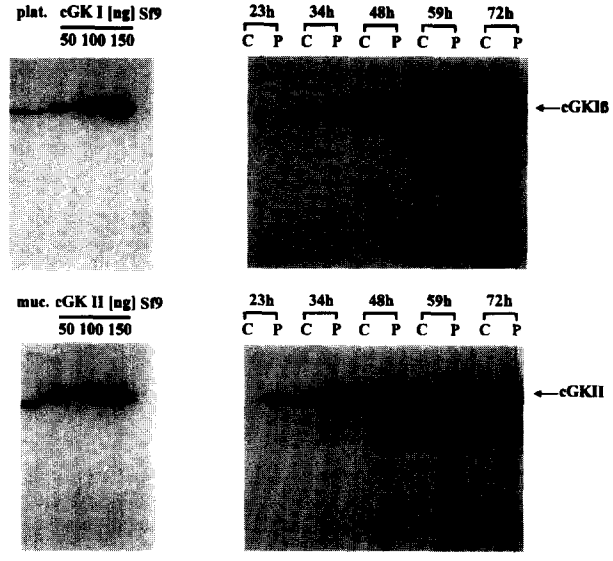

Fig. 2. Immunoblots demonstrating distinct subcellular localizations of cGK Iβ (upper right) and cGK II (lower right) expressed in Sf9 cells. Aliquots were removed from suspension cultures at the p.i. times shown and cells were lysed in low ionic strength buffer. Shown are identical protein amounts of cytosol (C) and particulate (P) fractions obtained by 100,000 × *g* centrifugation of infected Sf9 cells as described in section 2.4. Arrows indicate the positions of cGK Iβ and cGK II, respectively. The migration of recombinant cGK Iβ purified from Sf9 cells in comparison to the endogenous isoform expressed in human platelets (plat.) is shown at the upper left. Similarly the migration of recombinant cGK II purified from Sf9 cells is compared to that of rat intestinal mucosa (muc.) at the lower left. No expression of either cGK Iβ or cGK II was observed in uninfected cells (Sf9 lane).

3.2. Subcellular localization and purification of recombinant cGK Iβ and cGK II

The molecular masses of recombinant cGK Iβ and cGK II proteins expressed in and purified from Sf9 cells were similar to that of these kinases in homogenates of human platelets and rat intestinal mucosa, respectively (Fig. 2, left panels). The slight depression of the migration of cGK II in intestinal mucosa homogenate was not routinely observed. Also in Sf9 cells the two cGK forms showed a subcellular localization (Fig. 2, right panels) similar to that observed in native tissue in which cGK I has been described mainly as a soluble enzyme and cGKII as a particulate one [3,7]. Both enzymes from Sf9 cell lysates were functionally active in in vitro kinase activity assays (data not shown). For subcellular fractionation studies, Sf9 cells were lysed by shearing in low ionic strength buffer A, then the lysate was adjusted to contain 150 mM NaCl to prevent unspecific sticking of proteins to membranes before centrifugation at 100,000 × *g* as described in section 2.4. Analysis of soluble and particulate subcellular fractions by Western blotting (Fig. 2) revealed that the major amount of cGK Iβ protein was recovered in the supernatant fraction and only a small amount in the particulate cell fraction late in infection. In contrast to

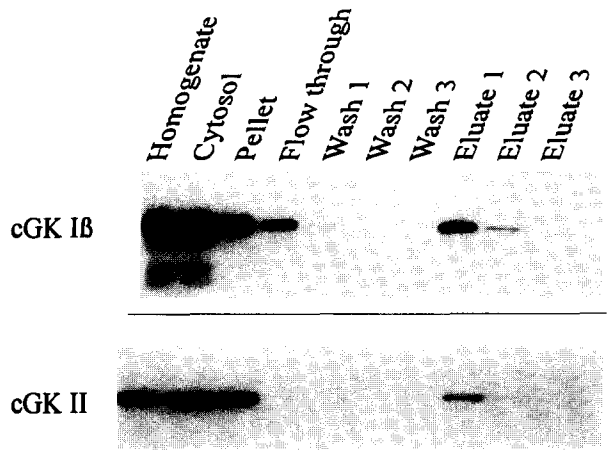

Fig. 3. Purification of recombinant cGK Iβ and cGK II from infected Sf9 cells. The kinases were purified by affinity chromatography with elution by cAMP as the final step, as described in detail in section 2. Equivalent fractions of each purification step were analyzed by SDS-PAGE and Western blots as shown here.

cGK Iβ localization, the majority of recombinant cGK II was detected in the particulate fraction of Sf9 cells and the small amount of soluble cGK II protein observed appeared to decrease over time.

Extraction experiments were carried out to solubilize cGK II protein for purification of the enzyme. However neither incubation of Sf9 cell homogenates with high salt concentrations (0.6–2 M NaCl), nor use of various detergents (0.1–5% Triton X-100, 0.4–2% laurylsucrose, 0.1–5 mM deoxycholate), nor a combination of both were effective in solubilization of cGK II (data not shown). cGK II was partially solubilized in an enzymatically active form and recovered in the cytosolic fraction by extraction of the homogenate with buffer D containing 250 mM ammonium sulfate, 300 mM sucrose and 0.5% Triton X-100. However, a varying amount of cGK II still sedimented in the high-speed pellet (Fig. 3, bottom, lane 3) and could not be solubilized even after repeated extraction.

For purification of the kinases from infected Sf9 cell cultures, cells were harvested at 48 h p.i. (for cGK Iβ) or 60 h p.i. (for cGK II). After cell lysis, cGK Iβ and solubilized cGK II were further purified from supernatants of 100,000 × *g* centrifugation by affinity chromatography on 8-(2-aminoethyl)-amino-cAMP-Sepharose or 8-(6-aminohexyl)-amino-cAMP-Sepharose, respectively. The solubilized enzymes bound with high affinity to the columns and could be specifically and quantitatively eluted in an enzymatically active form using 30 mM cAMP. After dialysis, the overall recovery for both enzymes varied between 15% and 33% of the initial amount measureable in the homogenates. A minor fraction of cGK Iβ did not bind to the affinity matrix (Fig. 3, top, lane 4), perhaps due to denaturation of the enzyme, since this material was again recovered in the flow-through fraction after its reapplication to the column. A representative Coomassie blue-stained SDS gel shown in Fig. 4 demonstrates that purified recombinant human placental cGK Iβ and rat intestinal cGK II were proteins of molecular masses 76 and 86 kDa, respectively. The doublet of cGK Iβ shown in Fig. 2 (upper right panel) may have been a gel artifact since it was not consistently observed (e.g. Fig. 2, upper left panel). The minor bands of 69 kDa and 74 kDa observed in Fig. 4 in the purified enzymes appear to represent stable degradation products since they show immunoreactivity on Western blots with the respective cGMP kinase antisera (data not shown).

Table 1
Activation properties of recombinant cGMP-dependent protein kinase Iβ and II

	K_a' GMP (μM)	V_{max} (μmol/min/mg)	Hill coefficient
cGK Iβ	0.50 ± 0.03 (4)	3.4 ± 0.40 (3)	1.9 ± 0.07 (3)
cGK II	0.04 ± 0.01 (6)	1.8 ± 0.38 (6)	1.2 ± 0.07 (6)

Results represent the means ± S.E.M. for three to six separate kinase assays (number in parentheses), each performed with triplicate determinations. Basal enzyme activity (μmol/min/mg) was 0.24 ± 0.08 for cGK Iβ and 0.23 ± 0.03 for cGK II.

3.3. Enzymatic characterization of recombinant cGK Iβ and cGK II

Purified recombinant cGK Iβ was highly stimulatable by cGMP (14-fold over basal activity) and had a K_a' and V_{max} for cGMP using VASPtide as a substrate of 0.5 μM and 3.4 μmol/min/mg protein, respectively (Fig. 5A, Table 1). The K_a of cGMP for activation of recombinant cGK Iβ (0.5 μM) agrees very well with that reported for the purified native enzyme (0.4 μM; [19,32]). In comparison, preparations of purified recombinant cGK II were stimulated about 8-fold by cGMP and had a K_a' and V_{max} for cGMP using VASPtide as a substrate of 0.04 μM and 1.8 μmol/min/mg, respectively. The apparent Hill coefficient for cGMP activation of cGK Iβ was 1.9 and for cGK

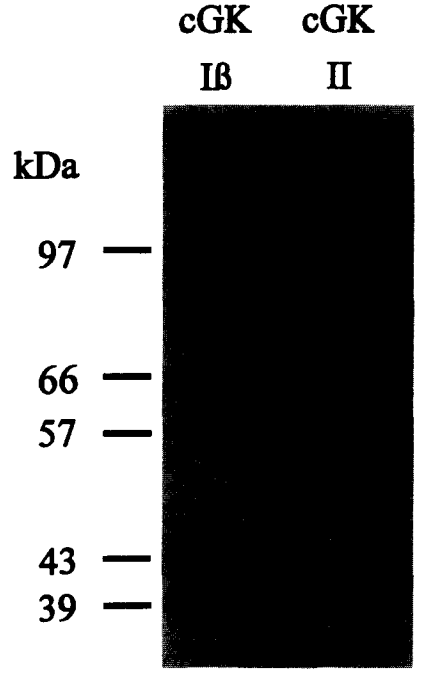

Fig. 4. Comparison of purified recombinant cGK Iβ and cGK II. The kinase preparations purified as shown in Fig. 3 were tested for homogeneity by SDS-PAGE. The Coomassie staining of 2 μg of each preparation is shown in comparison to the location of protein size markers. The smaller bands in the kinase preparations most likely represent degradation products since they are recognized by the antisera of the respective kinases.

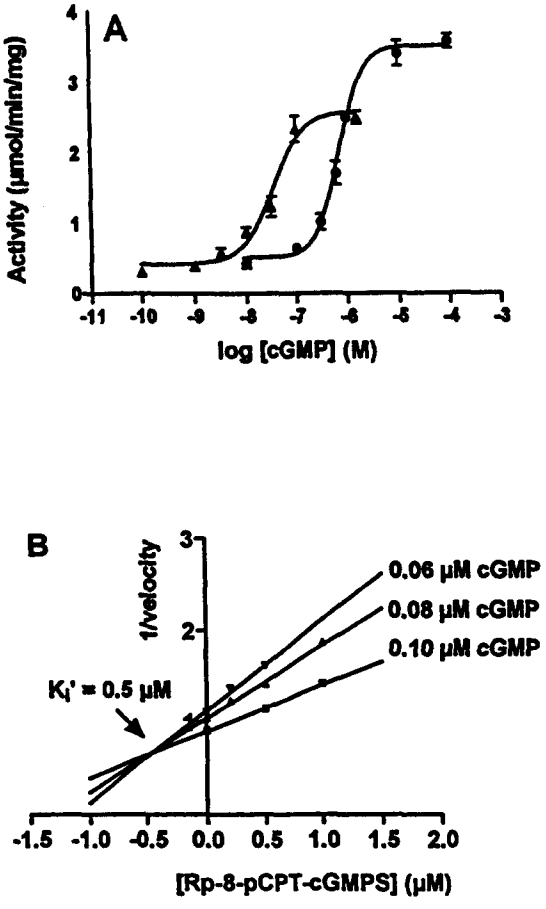

Fig. 5. Kinetic characterization of recombinant cGK Iβ and cGK II enzyme activity. (A) Phosphotransferase activity of purified recombinant cGK Iβ (●; 50 ng) and cGK II (▲; 25 ng) in the absence and presence of the indicated cGMP concentrations. The data (± S.D.) shown are representative of 4 separate kinase assays, each containing triplicate determinations. (B) Dixon plot demonstrating competetive inhibition of recombinant cGK II by Rp-8-pCPT-cGMPS. Plotting inhibitor concentration versus the reciprocal phosphotransferase activity (velocity, μmol/min/mg) obtained with VASPtide as substrate gave a similar K_i' in two separate experiments.

II was 1.2 (Table 1), suggesting strong co-operativity between the two cGMP-binding sites, within the enzyme monomer or on different subunits, for cGK Iβ only, whereas very little for cGK II. Determination of maximal enzyme activities using two different synthetic substrate peptides, VASPtide and Kemptide, showed that cGK Iβ catalysed the phosphorylation of VASPtide at a three times greater rate than Kemptide, whereas cGK II showed no preference for one substrate over the other (data not shown).

The K_a' values of activation of purified recombinant cGK types Iβ and II by certain cGMP analogs were clearly different (Table 2). cGK II was considerably more sensitive to 8-substituted cGMP derivatives than was cGK Iβ (about 40-fold for 8-Br-cGMP and 260-fold for 8-pCPT-cGMP). The decreasing order of potency for stimulation of cGK II was 8-pCPT-cGMP>>8-Br-cGMP>cGMP>PET-cGMP. PET-cGMP activated cGK II and cGK Iβ with similar potency. Although none of the cGMP derivatives tested were found to be specific activators of cGK II, 8-pCPT-cGMP stimulated cGK II (K_a' = 0.004 μM) much better than either cGK Iα or Iβ.

In contrast to the K_a' values for cGMP and its analogs, those for cAMP and two of its derivatives, 8-Br-cAMP and Sp-5,6-DCl-cBiMPS, were very high for all cGMP-kinases. With Sp-5,6-DCl-BiMPS, a specific activator of cAMP-dependent protein kinase, complete activation of cGK II could not be achieved probably because high concentrations of the analog interfere with the kinase ATP binding site and thus maximal catalytic activity [28].

Furthermore, a recently developed antagonist for cGK Iα, Rp-8-pCPT-cGMPS [29], was tested for its ability to inhibit cGK Iβ and II. Analysis of the plots of reciprocal enzyme activity versus analog concentration at different unsaturating concentrations of cGMP (Fig. 5B) revealed that Rp-8-pCPT-cGMPS is also a competitive inhibitor of purified recombinant cGK II (K_i' = 0.5 μM), as well as cGK Iβ and Iα (Table 2).

4. Discussion

In this study we have shown that the baculovirus/Sf9 insect cell expression system could be used to produce milligram quantities of rat intestinal cGK II and human placental cGK Iβ for purification to apparent homogeneity by affinity chromatography on cAMP-Sepharose. The expression levels achieved ranged from 5 mg/l for cGK Iβ to 8 mg/l for cGK II, corresponding roughly to 10–15% of total Sf9 cellular protein. This is a substantial improvement in comparison to the much smaller amounts which could be purified by conventional techniques from tissues which posed problems with proteolysis and availability.

The properties of the expressed recombinant enzymes reflected those of the native ones. The kinases had the appropriate molecular masses, were recognized by specific antibodies, and had the same subcellular distribution as their respective endogenous counterparts in mammalian tissues. Like cGK II expressed in Sf9 cells, cGK II endogenous to rat intestine epithelium or expressed from cDNA in HEK 293 cells was also associated with membranes [7,9]. It is not clear whether this characteristic subcellular localization of cGK II results from a

Table 2
Apparent activation constant (K_a') or inhibition constant (K_i') of cyclic nucleotide analogs for recombinant cGK Iβ and cGK II

Derivative	cGK Iα*	cGK Iβ	cGK II
	K_a' (μM)		
cGMP	0.11	0.5	0.04
8-Br-cGMP	0.01	1.0	0.025
8-pCPT-cGMP	0.04	0.9	0.0035
Pet-cGMP	0.03	0.05	0.06
cAMP	39	18	12
8-Br-cAMP	5.8	10	6
Sp-5,6-DCl-cBiMPS	10	27	–
	K_i' (μM)		
Rp-8-pCPT-cGMPS	0.5	0.6	0.5
Rp-cAMPS	53	14	50

K_a' and K_i' values were calculated from individual curves like those shown in Fig. 5 using VASPtide as substrate for cGK I and cGK II. The data represent means of triplicate determinations from one to three separate experiments.
*The data for cGK Iα were obtained using purified bovine lung enzyme and Kemptide as substrate [18,29].

common posttranslational modification of the protein or from binding to a ubiquitous or homologous putative anchor protein in different host cell types. The availability of purified recombinant cGK II will allow us to address this question in more detail. Nevertheless, solubilization of cGK II from Sf9 cell membranes proved more difficult than had been reported for intestinal epithelium [9,10]. Whereas solubilization of the latter required a combination of 1% Triton and 0.5 M NaCl, this was insufficient for Sf9 cell membranes which required $(NH_4)_2SO_4$ and 0.5% Triton (in buffer D) reported to extract cytoskeleton-attached proteins [30]. Cytoskeletal attachment of native intestinal cGK II had also been suggested [10].

For the measurement of kinase activity we used two synthetic peptides, VASPtide (RRKVSKQE), previously shown to be a relatively specific substrate for cGK Iα, and Kemptide (LRRASLG) (latter data not shown), a peptide derived from pyruvate kinase having a higher affinity for cAMP-dependent protein kinase [31]. Our data indicated that cGK II accepted VASPtide and Kemptide equally well, whereas cGK Iα and cGK Iβ showed a moderate preference for VASPtide. All of the cGMP kinases seem to possess a similar requirement for multiple basic amino acid residues N-terminal to the phosphorylated residue as was deduced for cGK Iα [31]. The strong similarity of the primary amino acid sequence in the substrate binding region within the kinases' catalytic domains may partly explain their highly similar substrate binding properties.

The activation of purified recombinant cGK Iβ from Sf9 cells by cyclic nucleotide analogs was similar to that of native cGK Iβ from bovine aorta smooth muscle [19,32], suggesting that Sf9 cells are capable of expressing a correctly folded and modified enzyme.

The activation constants of purified cGK II with respect to cGMP and its analogs were reported here for the first time. Although cGK II had been purified previously from intestinal epithelium, it could only be eluted from the affinity column with cGMP which is difficult to remove from the enzyme. Therefore enzyme activity was usually measured in membranes as autophosphorylation or phosphorylation of an endogenous intestinal epithelium substrate, p25 [9,10,33]. In our purification protocol, cGK II could be eluted with cAMP which has a lower affinity than cGMP for the enzyme and can be removed by dialysis. In the in vitro kinase activity assay, cGK II was extemely sensitive to 8-pCPT-cGMP (K_a' = 3.5 nM); however, 10–260-fold higher concentrations of this analog could activate cGK Iα and cGK Iβ, respectively. Rp-8-pCPT-cGMPS behaved as a competitive inhibitor of cGKII, however demonstrated no selectivity towards this isoform since it inhibited the two other cGMP kinases equally well.

The relative order of cGMP activation of specific cGMP kinase forms we observed (recombinant cGK II, K_a = 0.04 μM > purified cGK Iα, K_a = 0.1 μM > recombinant cGK Iβ K_a = 0.5 μM) agrees with the cGMP binding observed previously (cGK II, K_d = 5 nM > cGK Iα, K_d = 4 nM, 70 nM; distinguished for the 2 sites > cGK Iβ, K_d = 153 nM) [9,26,16]. For cGK II, there appears to be no cooperativity of cGMP binding [9] or recombinant kinase activation, whereas there is positive cooperativity for both parameters for cGK Iα [34,35] and for cGMP activation of native [32] and our recombinant cGK Iβ. It remains to be shown whether activation of specific cGMP kinase forms by different intracellular cGMP concentrations might be of physiological relevance in intact cells as has been postulated [19,36]. A perspective given by our present results is that further studies can now be undertaken with the purified recombinant kinases characterized here, as well as mutants thereof, in order to test their distinct function in biological systems accessible, for example, to microinjection or patch clamp methods.

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