

Autoinhibition and Isoform-specific Dominant Negative Inhibition of the Type II cGMP-dependent Protein Kinase*

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In the absence of cyclic nucleotides, the cAMP-dependent protein kinase and cGMP-dependent protein kinases (cGKs) suppress phosphotransfer activity at the catalytic cleft by competitive inhibition of substrate binding with a pseudosubstrate sequence within the holoenzyme. The magnitude of inhibition can be diminished by autophosphorylation near this pseudosubstrate sequence. Activation of type I cGK (cGKI) and type II cGK (cGKII) are differentially regulated by their cyclic nucleotide-binding sites. To address the possibility that the distinct activation mechanisms of cGKII and cGKI result from differences in the autophosphorylation of the inhibitory domain, we investigated the effects of autophosphorylation on the kinetics of activation. Unlike the type I cGKs (cGKI α and β), cGKII autophosphorylation did not alter the basal activity, nor the sensitivity of the enzyme to cyclic nucleotide activation. To determine residues responsible for autoinhibition of cGKII, Ala was substituted for basic residues (Lys¹²², Arg¹¹⁸, and Arg¹¹⁹) or a hydrophobic residue (Val¹²⁵) within the putative pseudosubstrate domain of cGKII. The integrity of these residues was essential for full cGKII autoinhibition. Furthermore, a cGKII truncation mutant containing this autoinhibitory region demonstrated a nanomolar IC₅₀ toward a constitutively active form of cGKII. Finally, we present evidence that the dominant negative properties of this truncation mutant are specific to cGKII when compared with cAMP-dependent protein kinase α and cGKI β . These findings extend the known differences in the activation mechanisms among cGK isoforms and allow the design of an isoform-specific cGKII inhibitor.

The cyclic nucleotide-dependent protein kinases possess a regulatory domain that suppresses activity at the catalytic site through competitive inhibition of a pseudosubstrate sequence within the regulatory domain (1). The inactive cAMP-dependent protein kinase (cAK)¹ holoenzyme is activated by cAMP

binding to two tandem cyclic nucleotide-binding sites (CNBSs) on the regulatory subunit (R). This event reduces the affinity between the R and catalytic (C) subunits, thus releasing the C subunit from the holoenzyme (2). cGMP-dependent protein kinase (cGK) activation is mechanistically similar to the cAKs, although the regulatory and catalytic portions of the kinase are linked domains of the same polypeptide rather than separate subunits (3, 4). For both cAKs and cGKs, the autoinhibitory interactions of the holoenzyme complex play a key role in kinase activation by cyclic nucleotides.

cAK and cGK autoinhibition is dependent upon the electrostatic interaction between positively charged residues of the pseudosubstrate sequence within the regulatory domain and negatively charged residues within the catalytic cleft (4, 5). Autophosphorylation of cAK lowers the K_a for cAMP and decreases the rate of association between the type II form of the R and C subunits (6, 7). This is due in part to electrostatic repulsion between the acidic residues within the catalytic cleft and the negatively charged phosphates incorporated into the autophosphorylated regulatory domain. Both splice variants of the type I isoform of cGK (α and β) share several autoinhibitory characteristics with cAK. First, autophosphorylation within or adjacent to the autoinhibitory (AI) sequence in cGKI β elevates kinase activity in the absence of cyclic nucleotide and lowers the K_a of cAMP and cGMP (8). Second, cGKI α autophosphorylation decreases the K_d of cAMP from one of the cyclic nucleotide-binding sites, lowering the K_a for cAMP (9, 10). Some residues responsible for autoinhibition of cAK are conserved in cGKI and -II, including the presence of at least one of the basic residues that make up the pseudosubstrate sequence.

The specifics of cGKII activation differ from the cGKI isoform (11) in that the low affinity CNBS is most critical for cGKII activation. The distinct mechanism of activation in cGKII may be because of differences in cGMP regulation of autoinhibition. Alternatively, this distinction may result from similar CNBSs that regulate very distinct autoinhibitory subdomains, as exemplified by the difference in the autoinhibition between the splice variants cGKI α and - β (12, 13), which possess identical CNBSs but distinct NH₂-terminal autoregulatory domains.

The goal of this study was to define the autoinhibitory mechanisms of cGKII. The effects of autophosphorylation and mutation of residues within the putative cGKII autoinhibitory subdomain were examined by monitoring the basal activity of the kinase and its sensitivity to cyclic nucleotide activation. Unlike cGKI α and - β isoforms (8, 9, 14) this study found that cGKII autoinhibition was independent of autophosphorylation, but dependent upon residues proximal to the conserved pseudosubstrate sequence. Additionally, this study demonstrates that a truncated regulatory domain of cGKII possesses

cGMP; VASP, vasodilator-A-kinase stimulated phosphoprotein; AI subdomain; autoinhibitory subdomain.

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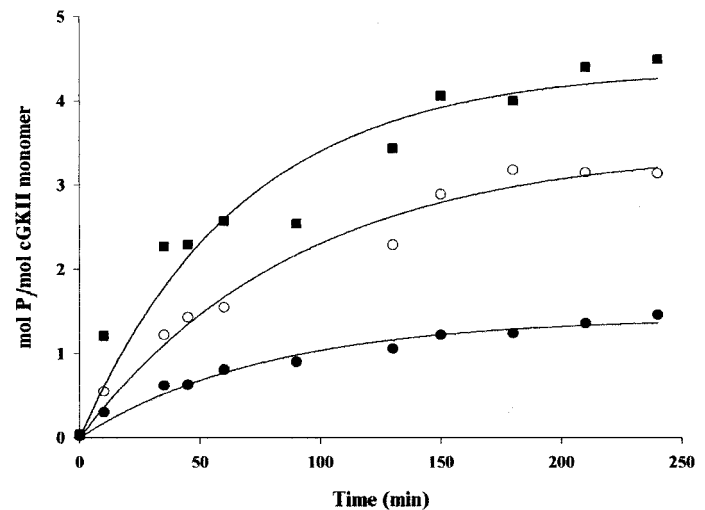
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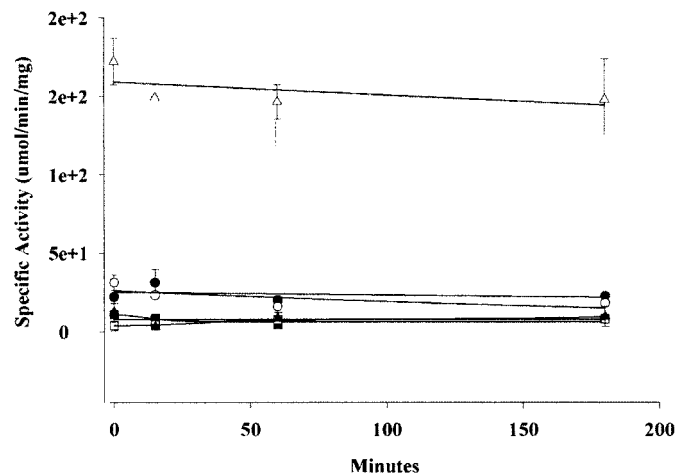
¹ The abbreviations used are: cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; PKI, protein kinase inhibitor; CPT-cGMP, 8-(4-chlorophenylthio)-cGMP; CRE, cAMP response element; CNBS, cyclic nucleotide-binding site; 8-Br-cGMP, 8-bromo-

FIG. 1. Autophosphorylation in cGKII. A, phosphate incorporation in cGKII under autophosphorylation conditions and its effect on basal activity. Freshly purified enzyme from baculoviral Sf9 cells overexpressing histidine-tagged cGKII was incubated in the presence of 100 μ M ATP and 4.5 μ M magnesium acetate, and either 5 μ M cGMP or 10 μ M cAMP, if indicated (see "Experimental Procedures"). At the indicated time points, the stoichiometry of autophosphorylation at 30 °C under different cyclic nucleotide conditions was determined. Compared with autophosphorylation conditions in the absence of cyclic nucleotide (\bullet), the addition of cGMP (\circ) enhances the stoichiometry of autophosphorylation. Further enhancement is seen when the enzyme is incubated under autophosphorylation conditions with cAMP (\blacksquare). B, the effect of autophosphorylation on basal kinase activity. For all conditions, the *filled symbols* show the presence of ATP in autophosphorylation, whereas the *empty symbols* show the response to identical conditions, but in the absence of ATP. The influence of cGMP-induced autophosphorylation in the presence of ATP is shown with *filled circles* (\bullet), and in the absence of ATP is shown with *empty circles* (\circ). The effect of cAMP-induced autophosphorylation in the presence of ATP is shown with *filled squares* (\blacksquare), and the absence of ATP is shown with *empty squares* (\square). The effect of ATP in the absence of cyclic nucleotide is shown with *filled triangles* (\blacktriangle), and the maximum kinase activity is shown with *empty triangles* (\triangle). C, effect of autophosphorylation on K_a . At the indicated cGMP concentration, enzyme autophosphorylated in the presence of cGMP for 1 h was assayed for kinase activity for 8 min at 30 °C (see "Experimental Procedures") with H2Btide (RKRSRAE) as the substrate. Autophosphorylated, \bullet ; nonautophosphorylated, \circ . These data represent one of at least three similar experiments. The *curves* were fitted using the mean values of triplicate samples at each cyclic nucleotide concentration and the *error bars* represent the mean \pm S.D. The data represented in all three figures are from the same preparations of enzyme.

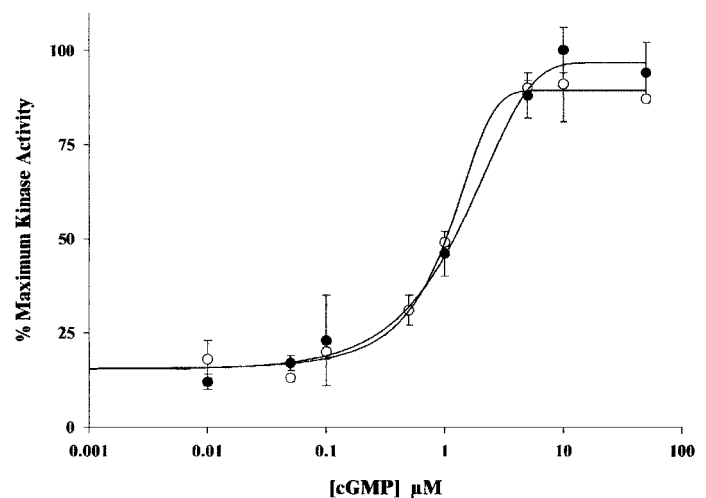
A.



B.



C.



-14	-11	-6-5	-3-2	P+1		
PPNPVVK	GRRRR	GAT	SAEV	RI α		
TPNPVVK	ARRRR	GGV	SAEV	RI β		
DLDVP	IPGRF	DRR	VSVCAET	RII α		
AFNAP	VINRFR	TTRAS	VCAEA	RII β		
YADF	IASGR	TGRR	NAIHDI	PKI α		
ITSF	ASSAR	AGRR	NALPD	PKI β		
YSDF	ISCD	RTGRR	NAVDP	PKI γ		
VPST	HIGPR	TTRAQ	GISAEP	cGKI α		
SAST	LQGE	PRTKR	QAISAEP	cGKI β		
SGLV	SLHS	RRGAK	AGVSAEP	cGKII		

FIG. 2. Alignments for mutagenesis strategies and the effect of mutagenesis at the putative cGKII autophosphorylation site. Alignment of autophosphorylation sites and pseudosubstrate sites in cAK RI subunits, cGKs, and PKIs. The black box outlines the residues that are in the position of known autophosphorylation sites in cAK RI subunits and cGKI β . Highlighted in gray are residues that align with arginine of PKI at the P-6 and arginine of RI α at the P-5 position. Also highlighted are the residues that align with the basic P-2 residue conserved in some, but not all cyclic nucleotide-dependent protein kinases and the highly conserved hydrophobic P + 1 residue, demonstrated to be a positive determinant of cAK and cGKI autoinhibition.

TABLE I

Sites of mutations in the cGKII autoinhibitory domain and their effects on enzyme kinetics

K_a and V_{max} were calculated as the average of at least three experiments and showed a standard error of less than 17%. Basal activity was also calculated from at least three experiments and had a standard error of less than 1.5%. V_{max} is expressed as micromole/min/mg, K_a as micromolar cGMP, and basal activity as % of V_{max} .

Mutant	Autoinhibitory Domain	K_a (μ M)	V_{max}	Basal (%)
Wild type	SRRGAKAGVSAEP	1.11	0.89	3.0
K122A	SRRGAAAGVSAEP	0.26	0.40	10.0
R118A/R119A	SAAGAKAGVSAEP	0.15	0.55	10.0
V125A	SRRGAKAGASAE	0.17	0.59	8.9
R118A/R119A/V125A	SAAGAKAGASAE	—	0.41	72.9

—, Not determined.

dominant negative inhibitory properties *in vitro* and *in vivo*. Inhibition by the truncated cGKII regulatory domain was diminished in the presence of cGMP or by mutagenesis of residues critical for autoinhibition. *In vivo*, the truncated regulatory domain of cGKII showed dominant negative properties toward wild type cGKII in the presence of lower concentrations of 8-Br-cGMP. We also show that the inhibitory properties of the truncated cGKII regulatory domain are specific to cGKII, and did not influence the activity of cGKI or cAK C α . Given the lack of specificity of many inhibitors structurally analogous to ATP or cyclic nucleotides (15), these findings offer an isoform-specific, dominant negative inhibitor of cGKII.

EXPERIMENTAL PROCEDURES

Construction of cGKII and cGKI β Mutants—Plasmid constructs encoding alanine substitutions for candidate residues responsible for regulation of cGKII autoinhibition (R118A/R119A, K122, V125A, S126D, and R118A/R119A/V125A) were constructed using a PCR site-directed mutagenesis protocol as previously described (16). The construction of the truncated cGKII regulatory domain mutant denoted IIR Δ 417–762 was performed using primers and a cGKII template in a one-step PCR site-directed mutagenesis method as described (11). The IIR Δ 417–762 construct contained an amino-terminal hexahistidine tag (N-terminal His-tag) and was truncated at Lys⁴¹⁷ with COOH-terminal addition of either a FLAG epitope sequence (DYKDDDDK) or the last five amino acids of the RI α subunit of cAK (VSLSV). The primers for the amino-terminal truncation of the cGKII regulatory domain were designed with

a BamHI site at the 5' end that was used to ligate the fragment into pcDNA3.0 (Invitrogen). Mutations of the autoinhibitory region were introduced by subcloning a fragment of the R118A/R119A/V125A (called R118A/R119A/V125A IIR Δ 417–762) construct into vectors that contained the 5' end of the IIR Δ 417–762 construct.

Construction of the His-tagged cGKI β truncation mutant, I β R Δ 341–686, was carried out using a sense primer that encoded an NH₂-terminal hexahistidine tag for I β and an antisense primer that contained the BamHI site after the codon for Gly³⁴⁰ to allow the COOH-terminal fusion to either the triple-FLAG epitope encoded by the pFLAG-CMV-14 vector (Sigma) or the c-Myc epitope encoded by pCMVTag5a (Stratagene). The University of Michigan Biomedical Research Core Facilities synthesized the primers used for this approach and sequenced all final plasmid constructs.

Expression and Purification of cGKII Mutants—For *in vitro* studies, hexahistidine-tagged wild type and mutant cGKII proteins were purified from HEK-T cells following transfection (17) unless otherwise indicated. Purification was performed essentially as described (11). Briefly, the cells were harvested and sonicated in Buffer A (10 mM Tris, pH 8.0, 0.1% Triton X-100, 200 mM NaCl) containing CompleteTM EDTA-free protease inhibitors (Roche Molecular Biochemicals) 36 h post-transfection. The solution was centrifuged at 20,000 \times g for 30 min at 4 $^{\circ}$ C, after which 20 mM imidazole in Buffer A was added to the solution, and the supernatant was decanted into fresh tubes to be spun again for another 30 min. The supernatant was then loaded onto a 500- μ l column of nickel affinity resin (Qiagen) and washed with 20 volumes of 20 mM imidazole in Buffer A. The hexahistidine-tagged protein was eluted by stepwise application of 1 ml of 30, 60, and 90 mM imidazole, with the highest concentration of cGKII protein found within the first 500 μ l of the 1 ml of 60 mM imidazole elution step. The protein concentration was determined as described (11).

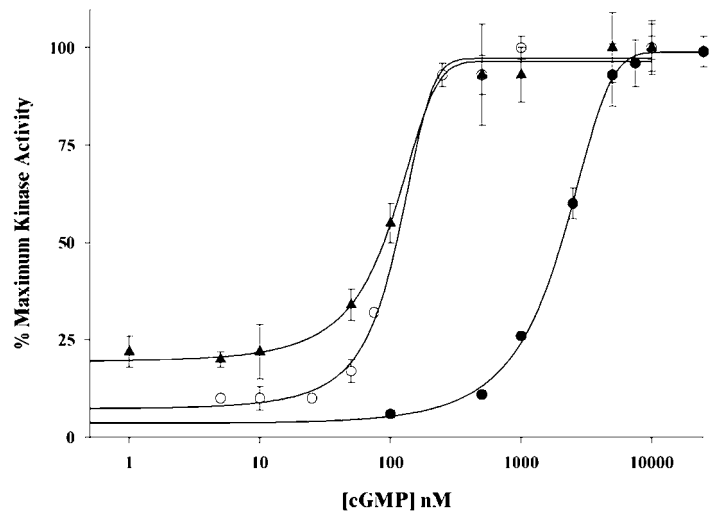
The purification of the previously characterized FLAG-tagged cGKI-C (18) from a 15-cm² plate of transfected HEK-T cells was performed by washing the cells three times with phosphate-buffered saline prior to harvesting in 4 ml of extraction buffer (0.9% phosphate-buffered saline, 10 mM NaPO₄, pH 7.5, 1% Triton X-100, 0.1% Nonidet P-40, 1% Tween 20) and homogenizing with 10 strokes of a Dounce homogenizer. The extract was centrifuged at 13,000 rpm for 10 min and the supernatant was collected into 14-ml Falcon tubes. One-hundred microliters of M2-agarose beads (Sigma) were added to the supernatant and the mixture was allowed to rotate for 1 h at 4 $^{\circ}$ C. After washing the beads three times with extraction buffer, the enzyme was eluted in Buffer A by adding an excess of FLAG-peptide (Sigma). The units of active cGKI-C were determined using the substrate Kemptide and matched with the activity of the R118A/R119A/V125A constitutively active cGKII in the *in vitro* kinase assay.

All purified enzymes were stored at –20 $^{\circ}$ C in a storage solution containing a final concentration of 50% glycerol, 50 mM KPO₄, pH 6.8, 25 mM β -mercaptoethanol, 1 mM EDTA, and 0.1% bovine serum albumin. Prior to the addition of storage solution, all mutant kinases were assayed to be certain that both basal and maximum kinase activity remained linear over all time periods. Additionally, kinase activity assays were performed to ensure that the K_a and V_{max} of the freshly purified kinase was consistent with kinetics assayed after storage, and all freshly purified truncation mutants were assayed for stoichiometry of [³H]cGMP binding and IC₅₀ against the constitutively active R118A/R119A/V125A full-length cGKII (also called constitutively active cGKII).

Determination of Kinase Activity—Kinase activity was determined by varying the concentration of cyclic nucleotide using the conditions and methods described (11). The reaction mixture consisted of 20 mM Tris, pH 7.5, 0.5 mM isobutylmethylxanthine, 10 mM magnesium acetate, 10 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 200 μ M unlabeled ATP, 11 nM [γ -³²P]ATP (specific activity 200–300 cpm/pmol; Amersham Biosciences), and 100 μ M synthetic H2Btide (Arg-Lys-Arg-Ser-Arg-Ala-Glu) as the phosphate acceptor unless otherwise indicated. The K_a value for each kinase was calculated using the Lineweaver-Burk analysis of at least three experiments. For IC₅₀ determination, the stock solution of 5 nM enzyme was incubated on ice with varying concentrations of the regulatory truncation mutant between 3 and 5 min prior to dilution into the kinase reaction mixture. All synthetic peptides were acquired from Research Genetics (Huntsville, AL) or the University of Michigan Biomedical Core Facilities. Single tailed Student's *t* tests were applied to determine significance in observed differences of kinase activity.

Autophosphorylation Assay—The determination of phosphate (P_i) incorporation into wild type cGKII was performed in a similar manner to that described (8). Briefly, purified cGKII enzyme at a concentration

A.



B.

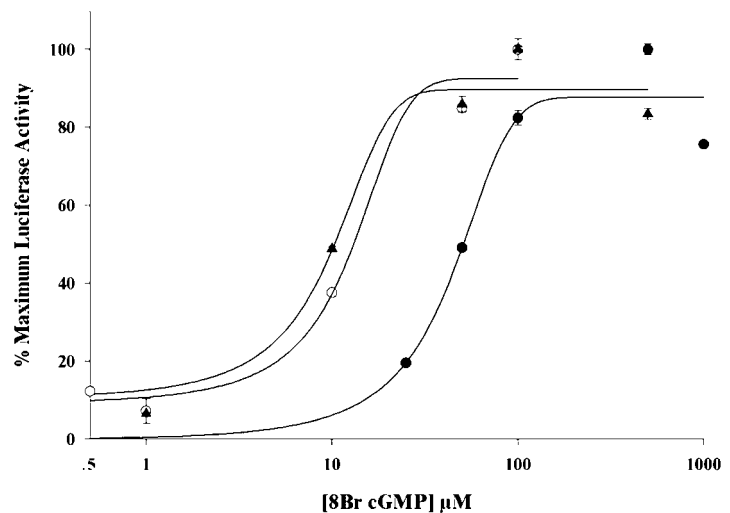


FIG. 3. Effect of mutations of basic residues Arg¹¹⁸, Arg¹¹⁹, or Lys¹²² in the putative AI subdomain on cGKII activity. A, effect of mutagenesis of the basic residues within and proximal to the putative pseudosubstrate sequence of cGKII (¹²¹AKAGV¹²⁵) in the *in vitro* kinase assay. cGMP-induced activation of purified wild type cGKII (●); K122A, which is within the predicted pseudosubstrate domain (○); and R118A/R119A (▲). The *abscissa* shows kinase activity in the presence of the indicated concentrations of cGMP relative to maximum kinase activity (V_{max}). The average values for V_{max} , K_a , and basal activity averaged from all experiments are reported in Table I. B, wild type and mutant cGKII kinase activity *in vivo*. Enzymes were co-transfected with a cAMP response element-dependent luciferase reporter gene in HEK-293T cells under conditions described under "Experimental Procedures." Wild type or AI subdomain mutants were exposed to the indicated concentration of 8-Br-cGMP for 6–8 h and luciferase expression was determined by luciferase assay as described under "Experimental Procedures." The *symbols* are identical to that shown in A, with reporter gene activity expressed as % maximum. The *curves* were fitted using the mean values of triplicate samples at each cyclic nucleotide concentration and the *error bars* represent the mean \pm S.D. These data are representative at least three experiments.

of 1 μ M was incubated in Buffer B (5 mM potassium phosphate, pH 6.8, 1 mM EDTA, 25 mM β -mercaptoethanol) in the presence of 100 μ M ATP and 4.5 mM magnesium acetate (if indicated), under conditions noted in the figure legends. Because bovine serum albumin was found to be a substrate for cGKII, it was not included in the autophosphorylation mixture after it was determined that the linear increase of kinase activity over time was not affected by its omission. The enzyme was then incubated at 30 °C and the autophosphorylation reaction was terminated at the indicated time by adding 18 μ l of the mixture to SDS-PAGE buffer (2% SDS, 12.5% β -mercaptoethanol, 25% glycerol, 0.25 M Tris, pH 6.8, and bromophenol blue). Phosphate incorporation was determined by performing electrophoresis of the reaction mixture on a 10% SDS-polyacrylamide gel and excising the 86-kDa band after Coomassie Blue staining. Analysis of the log of the rate of phosphorylation *versus* log of the enzyme concentration (van't Hoff analysis) indicated that both the basal and cGMP-stimulated autophosphorylations were intramolecular events.

To determine the effect of autophosphorylation on enzyme K_a or V_{max} , the autophosphorylation mixture was diluted 50-fold into an ice-cold solution that contained Buffer B, 0.1% bovine serum albumin, and 25 mM dithiothreitol. After incubating on ice for 2 min, this stock solution of autophosphorylated enzyme was used in phosphotransferase reactions carried out as described above.

[³H]cGMP Dissociation Assay—Dissociation rates of [³H]cGMP from wild type and IIR Δ 417–762 variants were determined using the cyclic

nucleotide dissociation assay as described (11). Briefly, a final concentration of 450 nM of purified enzyme was incubated for 10 min at 30 °C in a mixture of KPEH buffer (5 mM potassium phosphate, pH 6.8, 1 mM EDTA, 25 mM β -mercaptoethanol, and 0.5 mg/ml histone IIA), 50 μ M cGMP, and 3.2 μ M [³H]cGMP (specific activity, 15.6 Ci/mmol; Amersham Biosciences). The mixture was then chilled for 5 min on ice before 30- μ l samples were precipitated in 100% saturated, ice-cold (NH₄)₂SO₄ and filtered over a 0.45- μ m nitrocellulose filter (Millipore). Dissociation rates were determined by sampling the mixture in an identical manner after the addition of 100-fold excess of unlabeled cGMP.

In Vivo Assays of cGKII and cGKI Activity—Two assays were used to determine the activity of the kinase *in vivo*. To study the effect of mutations on the regulation of gene transcription, a luciferase reporter gene construct was used (11). Briefly, expression vectors for the kinase, FLAG-tagged PKI α and a human chorionic gonadotropin luciferase reporter gene containing two cAMP response elements, were co-transfected with vectors encoding the truncated cGKII mutant inhibitor (if indicated) and the Rous sarcoma virus β -galactosidase to control for transfection efficiency. Transfected cells were cultured for 24–36 h in the presence of 10% fetal calf serum, exposed to 8-Br-cGMP at the indicated concentration for 6–8 h (also in the presence of 10% fetal calf serum), and harvested and assayed for luciferase activity as described (11).

To address *in vivo* activation on a shorter time scale and determine the effects of the mutations on phosphorylation, the vasodilator- Δ -kinase stimulated phosphoprotein (VASP) assay was used as described

A.

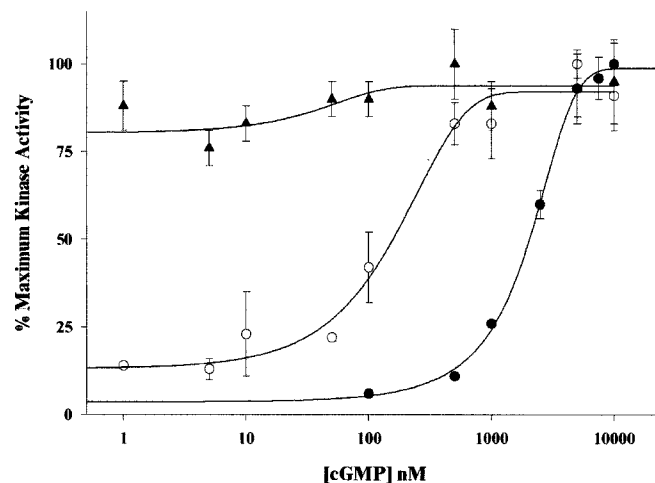
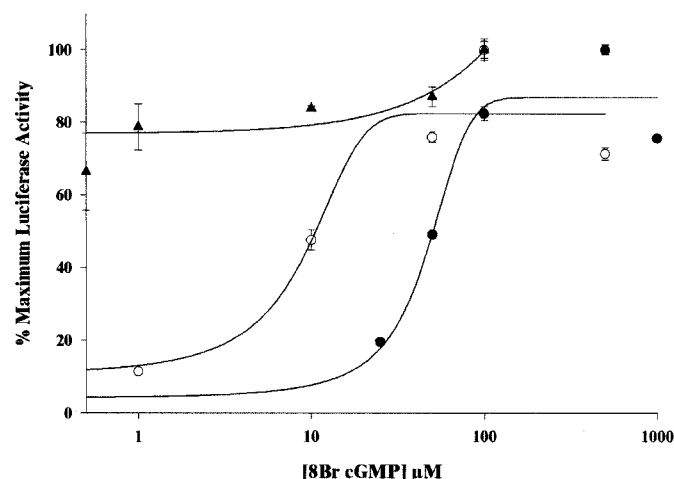


FIG. 4. Effect of mutagenesis of residues at the extreme ends of the putative cGKII AI subdomain. A, *in vitro* kinase activity was determined for the wild type (●) cGKII, in addition to the V125A mutant (○), and the R118A/R119A/V125A mutant (▲). B, *in vivo* kinase activity was determined using the cAMP response element-dependent luciferase reporter gene assay. The symbols are identical to those in A. Results are representative of at least three independent experiments.

B.



(18). The expression vectors for kinase, as well as FLAG-tagged VASP and FLAG-tagged PKI α , were co-transfected with vectors for enhanced green fluorescent protein (p-EGFP C1; CLONTECH) and β -galactosidase (Rous sarcoma virus- β gal) to control for transfection efficiency. After 24 h the cells were serum starved for at least 4 h prior to harvesting with ice-cold homogenization buffer. The samples were then immediately sonicated, a sample was taken for protein and β -galactosidase assay determination, and the remainder diluted into RIPA buffer (20 mM Tris-HCl, pH 8.0, 1% HPO $_4$, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate) followed by storage at -20°C . SDS loading buffer was added to the samples and electrophoresed on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose, and blotted with the indicated antibodies. Chemiluminescent intensity was determined using Bio-Rad Fluro-S MaxiImage (Diabetes Center, University of Michigan). Results are expressed as % maximum phospho-VASP, which was calculated as the percent ratio of VASP phosphorylation in the experimental condition over the maximum amount of VASP phosphorylation when the constitutively active kinase was expressed alone. The specific conditions and mass of DNA added to the transfection conditions are similar to that noted (11, 18) for both assays. Variations of these conditions are noted in the figure legends.

RESULTS AND DISCUSSION

Autophosphorylation Does Not Diminish Autoinhibition—For cGKI α and -I β , autophosphorylation disrupts autoinhibition, allowing the catalytic domain to phosphorylate substrate in the absence of cGMP. To determine the kinetics of autophosphorylation in cGKII, purified wild type cGKII was incubated

with [γ - ^{32}P]ATP and assayed for incorporation of radioactivity. Fig. 1A shows that 5 μM cGMP induced phosphate incorporation at a maximum of 3 P $_i$ /mol of cGKII monomer and that cAMP induced incorporation of an additional mole of P $_i$ /mol of cGKII monomer even though maximum kinase activations in the two conditions were identical (data not shown). However, autophosphorylation of cGKII in the presence of either cAMP or cGMP did not elevate basal kinase activity (Fig. 1B) in contrast to similar experiments with cGKI β (8) and -I α (9). Additionally, autophosphorylation in the presence of cGMP did not shift the sensitivity of the kinase for activation (K_a) by cGMP (Fig. 1C), as has been shown for cGKI β (8). This was also observed when assayed with Kemptide, which has a lower K_m for cGKII (19), or varying concentrations of ATP (data not shown).

Determination of Residues That Are Necessary for cGKII Autoinhibition—Autophosphorylation in all other cyclic nucleotide-dependent protein kinases results in a moderate release of autoinhibition. The inability of cGKII autophosphorylation to alter the activity of the kinase may be because of unique autoinhibitory interactions between its regulatory and catalytic domains. We set out to determine critical residues for cGKII autoinhibition by examining the alignment of pseudosubstrate inhibitors of the cyclic nucleotide-dependent

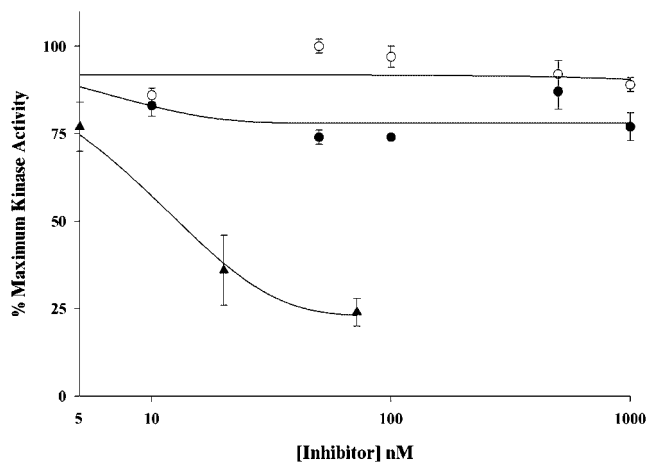


FIG. 5. The inhibitory properties of peptides designed from the AI subdomain of cGKII β and cGKII. The activity of constitutively active cGKII in the presence of varying concentrations of cGKII-AI peptide (SRRGAKAGVSAEPTTR; ○) and IIR4417-762 (▲). The activity of cGKI-C in the presence of varying concentrations of cGKI-AI peptide (EPRTKRQAISAEPTAF; ●). Inhibitors were coincubated with their respective constitutively active mutant kinases for 5 min on ice prior to determination of kinase activity at 30 °C using the *in vivo* kinase assay. The curves were fitted using the mean values of triplicate samples at each cyclic nucleotide concentration and the error bars represent the mean \pm S.E. These results are similar to at least three other experiments.

protein kinases (Fig. 2). Many of the determinants of the inhibitory potency of the cAK protein kinase inhibitor (PKI) (20) and the RI (21, 22) and -II (23) subunits of cAK are known. Alignment of RI α , cGKII β , and cGKII α with cGKII (Fig. 2) revealed that the sequence ¹²¹AKAGV¹²⁵ of cGKII aligns with the RI and PKI pseudosubstrate motif RRXA Ψ , where Ψ represents a hydrophobic residue and the underlined residue is the designated "P-site." Determinants for binding of RI α and the pseudosubstrate inhibitor PKI to the catalytic cleft of cAK consist of several elements, including a P-6 arginine (found in RII α , RII β , and the PKIs), the P-3 and P-2 arginine residues and a P + 1 hydrophobic residue (16, 20, 24). Electrostatic interactions between glutamate residues in the catalytic cleft and the P-3 and P-2 residues of PKI are responsible for their inhibitory potency toward the C subunit. These electrostatic interactions are supplemented by the hydrophobic interactions between the P + 1 residue and a hydrophobic pocket within the substrate-binding site (20, 25). On the basis of these alignments and previous studies, a series of mutants were designed to substitute an alanine for the P-6, P-5, and P-2 basic residues of cGKII, Arg¹¹⁸, Arg¹¹⁹, and Lys¹²², respectively, as well as the P + 1 hydrophobic residue Val¹²⁵ (see Fig. 2 within the gray box).

Our first goal was to determine whether the residue at the P-2 position (Lys¹²², see Fig. 2 in the gray box under the P-2 position) was required for autoinhibition of cGKII. If so, we expected to observe an increase of basal activity in the Lys¹²² mutant, indicative of disrupted autoinhibition. However, we observed only a small change in basal activity (10%; Table I). The other conserved basic residues, the P-6 and P-5 arginine residues (Arg¹¹⁸ and Arg¹¹⁹), were also targeted for alanine substitution. The P-6 residue was targeted because the homologous residue in PKI has been shown to be a major determinant of high affinity binding at a highly conserved glutamate in the catalytic cleft. Also, arginine at the P-5 position was targeted because the homologous residue in RI α and cGKII β contributes to the autoinhibitory potency of their regulatory domains (21, 22). The R118A/R119A cGKII mutant showed a 3-fold increase in the basal activity of the enzyme (Table I), suggesting that

residues distal to the predicted pseudosubstrate sequence contribute to cGKII autoinhibition.

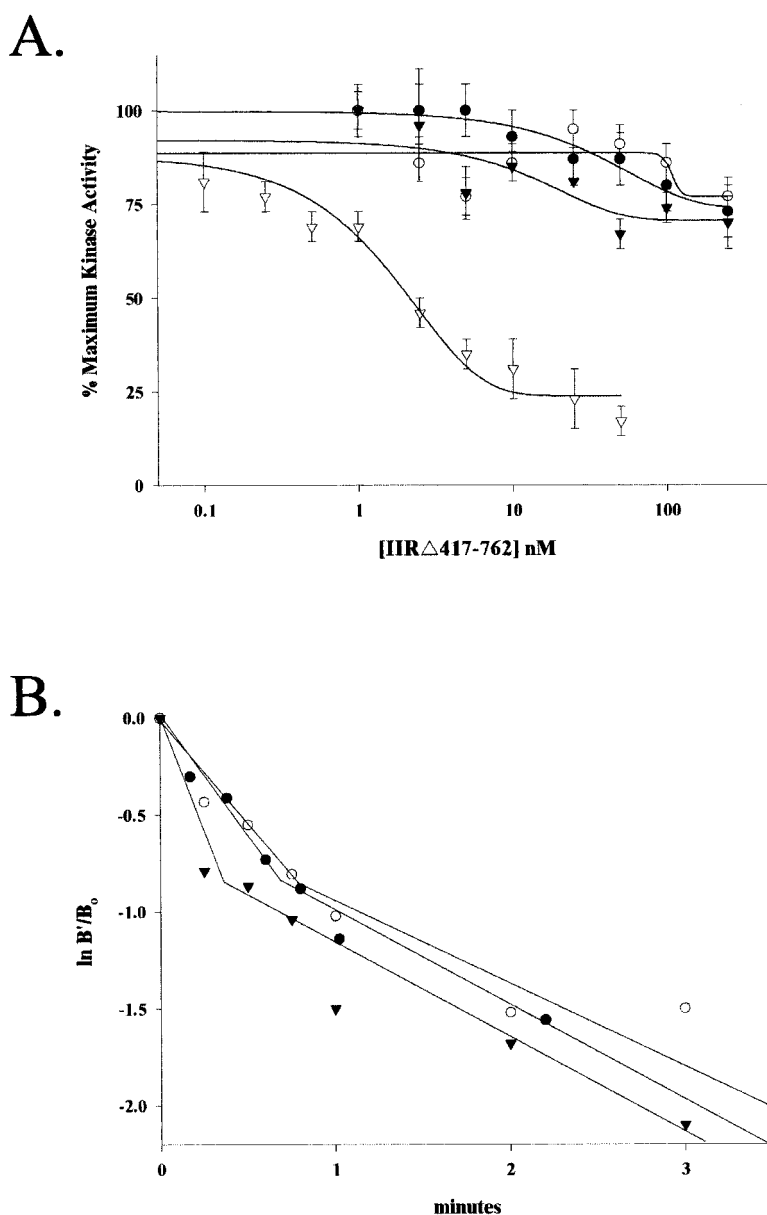
In other cyclic nucleotide-dependent protein kinases, disruption of autoinhibition can enhance the ability of cyclic nucleotide binding to activate the enzyme (26), indicated by a decrease in the K_a for a cyclic nucleotide. To determine whether K122A or R118A/R119A demonstrated increased sensitivity to cGMP, the activities of these mutants were monitored in the presence of varying concentrations of cyclic nucleotide. Fig. 3A illustrates the significant increase in the sensitivity of the enzyme to cGMP (4.2-fold for K122A and 7.4-fold for R118A/R119A; Fig. 3A and Table I). The mutant kinases were also monitored *in vivo* to ensure that the purification procedure did not introduce an experimental artifact. Similar to our *in vitro* observations, K122A and R118A/R119A cGKII mutants showed a 5-fold leftward shift in the 8Br-cGMP induction of luciferase expression (Fig. 3B; EC₅₀ of 10 μ M for each mutant, relative to an EC₅₀ of 50 μ M for wild type).

Thus, compared with cGKII β , cGKII autoinhibition is unique because residues that are relatively distant from the conserved serine residue of the putative pseudosubstrate sequence (P-5 and P-6) contribute to autoinhibition as much as residues within the pseudosubstrate sequence (21, 22). Because substitution of Lys¹²² in cGKII abolishes the only basic residue that aligns with the autoinhibitory pseudosubstrate sequence in cAK and cGK, it was expected that this mutant would have a significant impact on autoinhibition, potentially creating a constitutively active kinase. Instead, K122A showed modest activity in the absence of cyclic nucleotides, demonstrating that other components contribute to autoinhibition.

Because mutation of the charged residues Arg¹¹⁸/Arg¹¹⁹ or Lys¹²² did not completely abolish autoinhibition, we also considered the importance of a hydrophobic residue at the P+1 position in cGKII. This residue is highly conserved in cGKII α and -I β and cAK inhibitors (see Fig. 2). A hydrophobic residue at the P + 1 position has been shown to participate in autoinhibition of cGKI (27) and has also been shown to be important in the inhibitory potency of peptides designed from the pseudosubstrate sequence in RI α and PKI (22, 24). If the residue homologous to the P + 1 position (Val¹²⁵) contributed to autoinhibition like Arg¹¹⁸/Arg¹¹⁹ or Lys¹²², an alanine substitution at that site would decrease the potency of autoinhibition. Indeed, the V125A mutant of cGKII showed a significant elevation of basal activity relative to wild type cGKII (2.9-fold; Table I), in addition to an increase in sensitivity to cGMP (6.6-fold; Fig. 4A and Table I). This shows that the P + 1 residue contributes to autoinhibition, indicating that the interaction of the cGKII catalytic domain with the ¹²¹AKAGV¹²⁵ pseudosubstrate sequence was analogous to that seen in other cyclic nucleotide-dependent protein kinases.

To determine the contribution of adjacent residues, we targeted Arg¹¹⁸/Arg¹¹⁹ and Val¹²⁵ of the cGKII sequence ¹¹⁸RRGAKAGV¹²⁶ to test whether a combination of Ala substitutions could produce a constitutively active kinase. If these residues were primary factors contributing to autoinhibition, then combined disruption of these three residues would yield a constitutively active enzyme. A cGKII enzyme with alanine substitutions for Arg¹¹⁸, Arg¹¹⁹, and Val¹²⁵ demonstrated a dramatic increase in basal activity relative to wild type (25-fold; Fig. 4A and Table I). The increase between the basal and cGMP-stimulated activation for the R118A/R119A/V125A mutant enzyme (from 0.30 to 0.41 μ mol/min/mg) may represent residual autoinhibition mediated by Lys¹²² or to additional, less potent inhibitory elements in the regulatory domain. *In vivo*, these mutants also displayed a pattern of activation that is consistent with that seen *in vitro* (EC₅₀ of 10 μ M for V125A

FIG. 6. IIR Δ 417–762 truncation mutant inhibitory potency toward constitutively active cGKII and its CNBS kinetics. A, comparison of kinase activity of constitutively active cGKII incubated with the ^{R118A/R119A/V125A}IIR Δ 417–762 (●), heat denatured (○), cGMP bound (▼) truncation mutant IIR Δ 417–762, or native IIR Δ 417–762 (▽). Kinase activity was determined by mixing the truncation mutant and constitutively active cGKII together on ice and immediately adding to the kinase assay for a 10-min incubation at 30 °C (see “Experimental Procedures”). B, [³H]cGMP dissociation from wild type cGKII and the IIR Δ 417–762 truncation mutants. Wild type, ●; IIR Δ 417–762, ○; ^{R118A/R119A/V125A}IIR Δ 417–762, ▼. Results for both panels A and B are representative of at least three independent experiments.



and constitutive activity for R118A/R119A/V125A; Fig. 4B). Taken together, these data define Arg¹¹⁸, Arg¹¹⁹, and Val¹²⁵ as major determinants of cGKII autoinhibition. The impact of these residues on autoinhibition would not have been predicted from previous models of regulatory and catalytic domain interactions in cyclic nucleotide-dependent protein kinases (22). Our data show that for cGKII, the integrity of these residues is essential for autoinhibition and defines a region from Arg¹¹⁸–Val¹²⁵ that we call the autoinhibitory subdomain (AI subdomain).

The AI Subdomain Is Not Sufficient for cGKII Autoinhibition—To determine whether the AI subdomain would be sufficient to inhibit cGKII, we created a 16-mer COOH-terminal acetylated peptide that included the AI subdomain (SRRGAK-AGVSAEPTTR; cGKII AI-peptide) that was based on the sequence of cGKII between Ser¹¹⁷ and Arg¹³². For comparison, we also tested a peptide derived from the corresponding cGKI β AI sequence (EPRTKRQAISAEPTAF; cGKI β AI-peptide) to determine whether it was sufficient for inhibition of a constitutively active cGKI mutant that contained only the catalytic domain of the kinase (cGKI-C (18)). Fig 5 shows that the cGKII AI-peptide did not suppress the activity of the constitutively

active cGKII. Also, the cGKI β AI-peptide was not able to inhibit the cGKI-C, as expected from previous studies (22).

Because the AI subdomain was not sufficient for inhibition of cGKs, we wanted to determine whether the larger regulatory domain, which includes the AI subdomain as well as the cyclic nucleotide-binding sites, would be sufficient for inhibition as is seen by the R subunits of cAK. Studies of cAK have shown important interactions between the residues near the CNBS of the R and C subunits of cAK (23, 28–30). We anticipated that these secondary interactions may be important for inhibition and constructed the IIR Δ 417–762 truncation mutant representing the amino-terminal regulatory domain of cGKII. To determine whether the IIR Δ 417–762 truncation mutant inhibited the constitutively active cGKII, the proteins were purified from transfected cells and subjected to *in vitro* kinase assays. Fig. 5 shows that preliminary *in vitro* experiments demonstrated that the IIR Δ 417–762 protein was a significantly more potent inhibitor of cGKII than the AI peptides.

Characterization of the Inhibitory Activity of the cGKII Regulatory Domain—Detailed analysis of the IIR Δ 417–762 inhibition showed suppression of the constitutively active cGKII with an IC₅₀ of 4.5 nM (Fig. 6A). The ^{R118A/R119A/V125A}IIR Δ 417–762

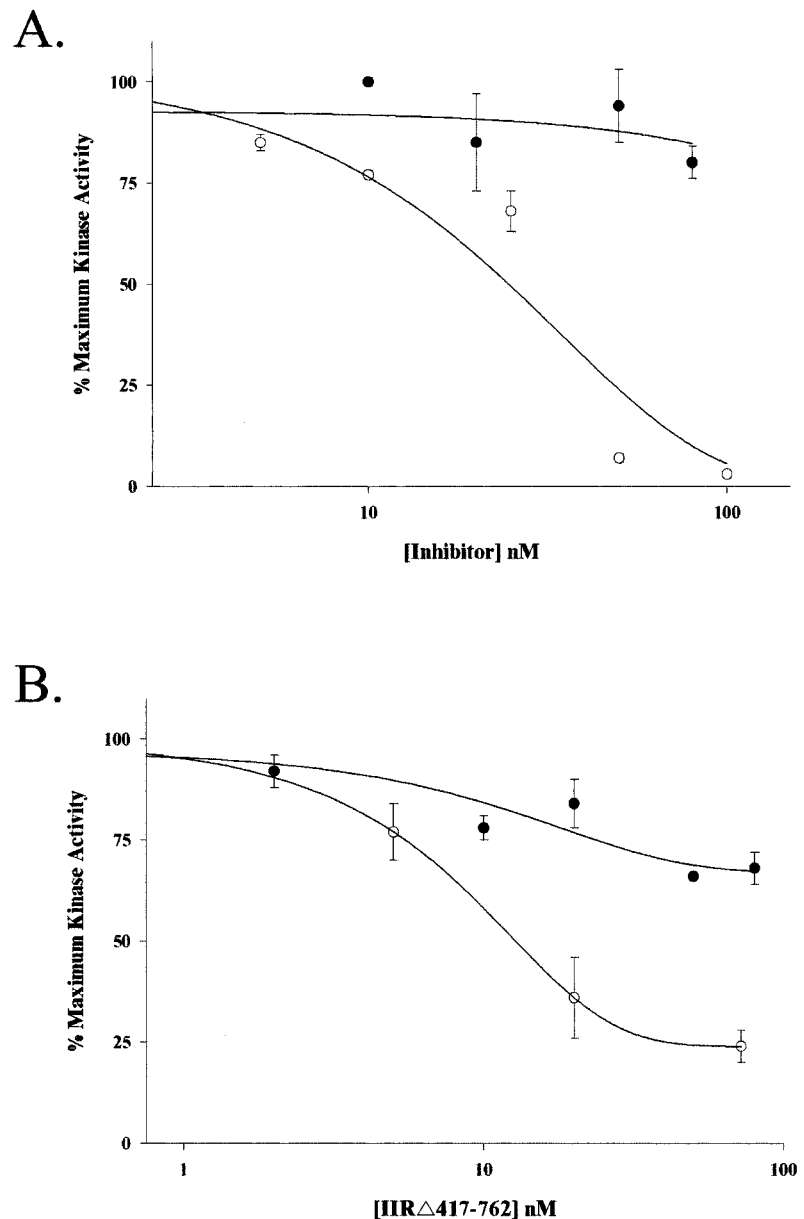


FIG. 7. The isoform specificity of IIRΔ417-762 inhibition. A, the specificity of the inhibitory properties of IIRΔ417-762 (●) and PKIα(5-24) (○) was determined by testing the activity of cAKCα under increasing concentrations of the inhibitor. B, the inhibitory properties of IIRΔ417-762 against the catalytic domain truncation mutant cGKI-C (●) and the constitutively active cGKII mutant (○). All inhibitors were coincubated with the constitutively active kinase for 5 min on ice prior to determination of kinase activity at 30 °C using the *in vitro* kinase assay. The curves were fitted using the mean values of triplicate samples at each cyclic nucleotide concentration and the error bars represent the mean ± S.E. These results are representative of at least three experiments.

protein (Fig. 6A) did not inhibit the constitutively active cGKII, demonstrating that the residues outside of Arg¹¹⁸, Arg¹¹⁹, and Val¹²⁵ were not sufficient for inhibition by the IIR truncation mutant. In addition, heat denatured IIRΔ417-762 (Fig. 6A) did not show significant inhibition of the constitutively active kinase. Also, the ability of cyclic nucleotides to abrogate the inhibitory properties of the IIRΔ417-762 fragment was demonstrated by the addition of 5 μM cGMP to the incubation mixture (Fig. 6A), the amount necessary to fully activate wild type cGKII. The most direct explanation for these results is that cGMP binding to the IIRΔ417-762 truncation mutant altered the conformation of the protein and rendered it incapable of inhibiting full-length cGKII, likely through mechanisms that have been described for cGMP release of inhibition in cGKI (31).

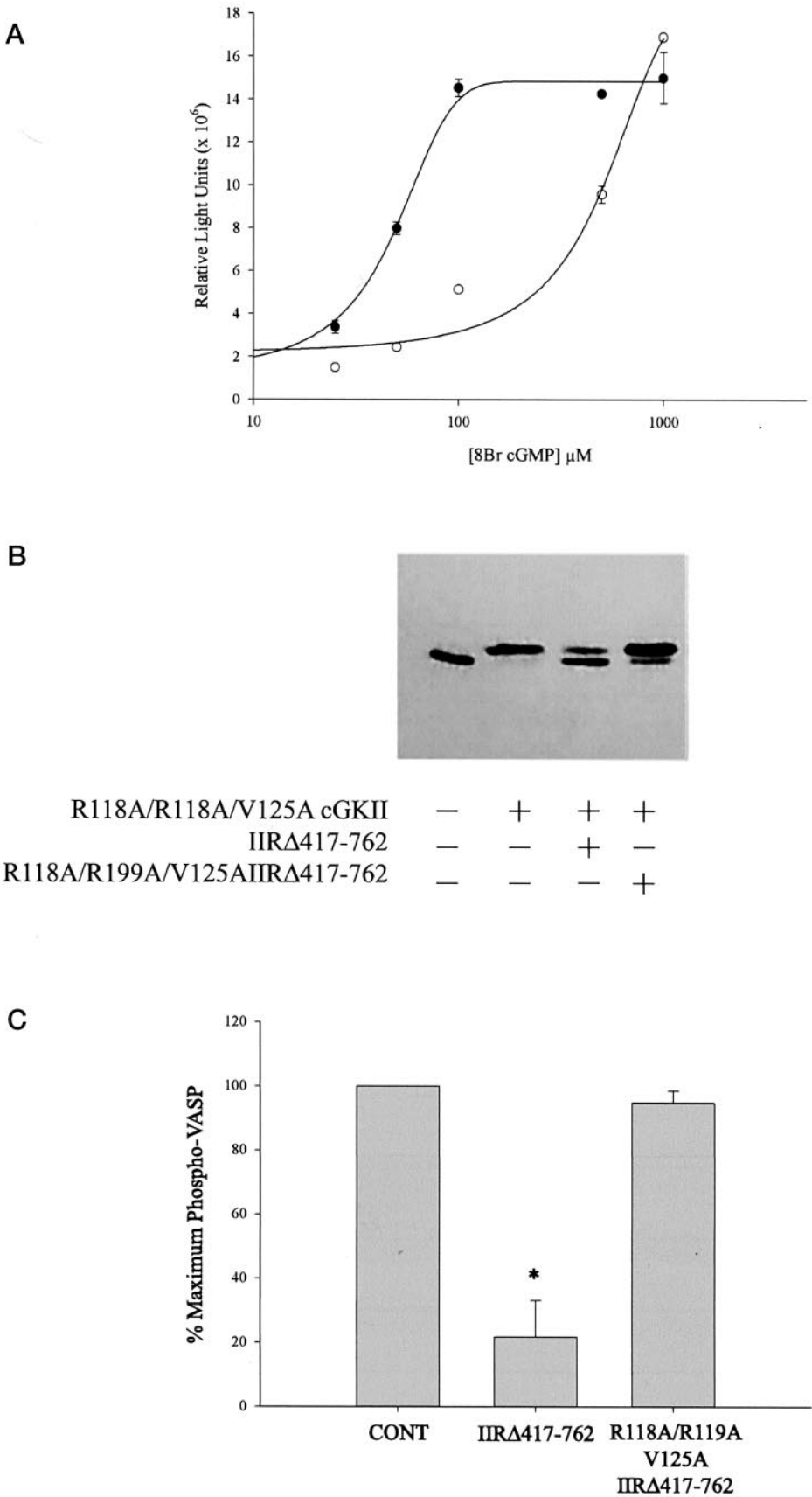
To demonstrate the similarities of cGMP binding kinetics between the wild type kinase and the truncation mutant, and that the loss of inhibition by the R118A/R119A/V125A IIRΔ417-762 was not because of a disruption of the overall conformation of the regulatory domain, we examined the dissociation of [³H]cGMP from the CNBSs. Both forms of IIRΔ417-762 showed identical stoichiometry of [³H]cGMP binding and sim-

ilar kinetics of [³H]cGMP dissociation compared with the wild type cGKII, IIRΔ417-762, and R118A/R119A/V125A IIRΔ417-762 (Fig. 6B). These results demonstrated that the catalytic domain of cGKII was not required for effective cGMP binding and that the R118A/R119A/V125A mutations of the IIRΔ417-762 did not introduce general structural defects that would have compromised its integrity.

Taken together, these data demonstrate a surprising affinity between the regulatory and catalytic domains of cGKII. The similarity of the mechanism of cGKII autoinhibition and inhibition by IIRΔ417-762 was demonstrated by the necessity for the integrity of the AI subdomain in both conditions (compare the effect of the R118A/R119A/V125A mutation on autoinhibition in Fig. 4A and R118A/R119A/V125A IIRΔ417-762 inhibition of constitutively active cGKII in Fig. 6A). These data demonstrate that the regulatory domain, which includes the AI subdomain, is sufficient for autoinhibition, and that the AI subdomain is necessary but not sufficient for autoinhibition.

Determination of Isoform Specificity of IIRΔ417-762 and IβRΔ341-686—Because there is significant homology between the catalytic domains of cGKs and cAKs, we tested whether the IIRΔ417-762 could inhibit the catalytic subunit of cAK. Fig. 7A

FIG. 8. Analysis of the inhibitory properties of the cGKII regulatory domain truncation mutant IIRΔ417-762 *in vivo*. *A*, effect of overexpression of the IIRΔ417-762 truncation mutant on cGKII induction of gene expression. Using the luciferase reporter gene assay, the suppression of cGKII by the IIRΔ417-762 truncation mutant results in a dramatic shift in responsiveness to 8-Br-cGMP. In this assay the amount of IIRΔ417-762 expression vector is in 2-fold excess of the full-length wild type kinase. Wild type, ●; wild type and IIRΔ417-762, ○. The curves were fitted using the mean values of triplicate samples at each cyclic nucleotide concentration and the error bars represent the mean ± S.D. *B*, effect of overexpression of IIRΔ417-762 truncation mutations on VASP phosphorylation. FLAG-tagged VASP phosphorylation was monitored by Western blotting using an M2 antibody (1:5000) and horseradish peroxidase mouse antibody (1:5000). 12 μg of VASP expression vector was co-transfected with the indicated amounts of constitutively active cGKII expression vector and 8.75 μg of the R118A/R119A/V125A IIRΔ417-762, IIRΔ417-762, or empty expression vector. *C*, quantification of VASP conversion to slower migrating phosphorylated species. The ratio of VASP conversion to the slower migrating band under varying co-transfection conditions is expressed as a percentage of VASP conversion seen in the presence of the constitutively active kinase alone. These data are the average of at least three independent experiments with the error bars representing mean ± S.E.; *, indicates significant difference of $p < 0.05$ by the Student's *t* test between the noted condition and the control (constitutively active cGKII alone).

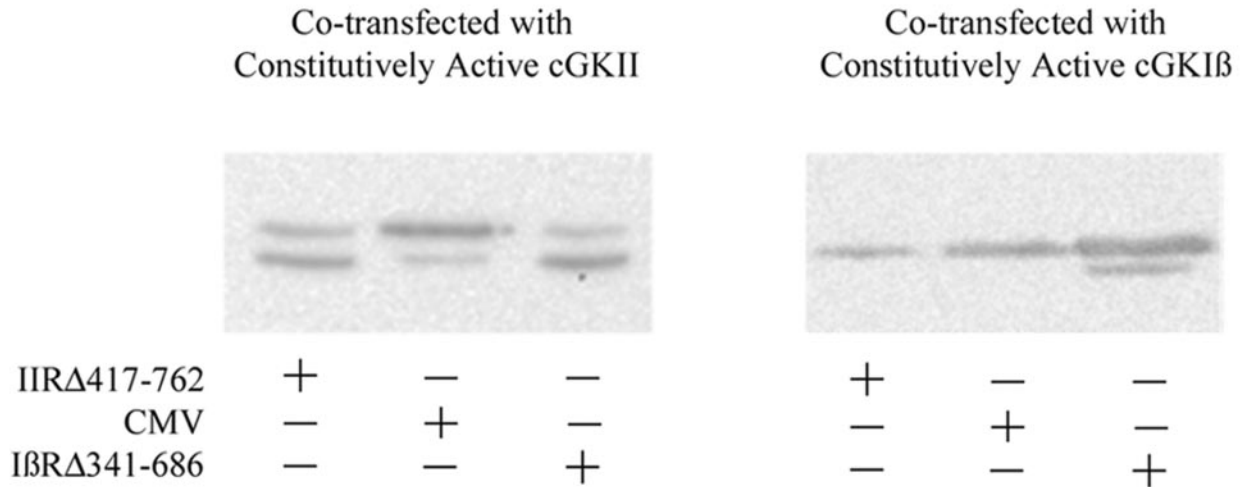


shows that IIRΔ417-762 does not inhibit $C\alpha$, even at concentrations that maximally inhibited the constitutively active cGKII (100 nM IIRΔ417-762). However, the $C\alpha$ is almost completely inhibited by equimolar amounts of PKI α -(5-24) (Fig.

7A), as demonstrated previously (32).

To determine whether IIRΔ417-762 could inhibit cGKI activity, we tested its ability to inhibit a previously characterized truncation mutant of cGKI (cGKI-C) that possessed only the

A.



B.

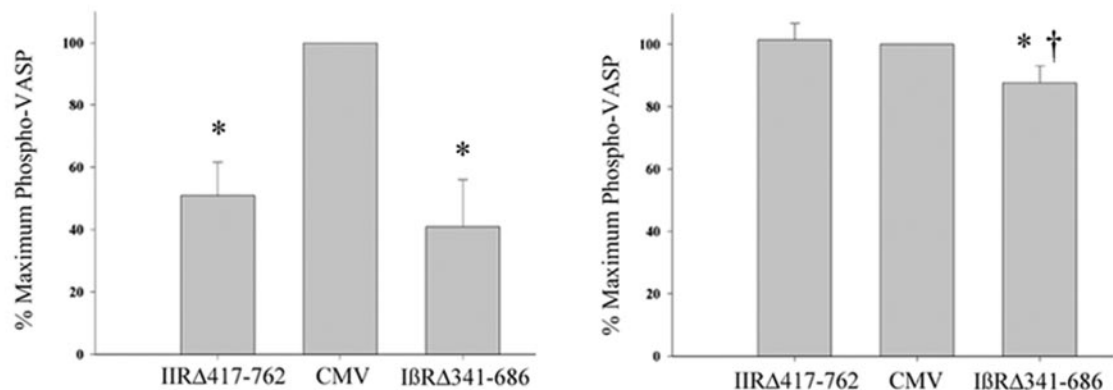


FIG. 9. Isoform-specific effect of the regulatory domain truncation mutants IIRΔ417-762 and IβRΔ341-686 on constitutively active cGKs *in vivo*. A, 2 μg of the constitutively active cGKII expression vector (either R118A/R119A/V125A cGKII or S79D cGKIβ) was co-transfected with 6.6 μg of FLAG-tagged VASP expression vector and 15 μg of either IIRΔ417-762 or IβRΔ341-686 expression vectors. These samples were collected as described under "Experimental Procedures." B, quantification of *in vivo* isoform specificity. The *abscissa* expresses the percent conversion of VASP under the indicated condition relative to maximal conversion seen with the constitutively active kinase (either R118A/R119A/V125A cGKII or S79D cGKIβ), determined from the average for three independent experiments. The *error bars* represent the mean ± S.E.; *, indicates a significant difference of $p < 0.05$ (Student's *t* test) between the indicated conditions and the maximum VASP phosphorylation; † indicates the significant difference ($p < 0.05$; Student's *t* test) between the conditions that have the presence of IIRΔ417-762 and IβRΔ341-686.

catalytic domain (18). The catalytic activity of the cGKI-C showed a small but consistent inhibition at the highest concentrations of IIRΔ417-762 (25% inhibition; Fig. 7B) and this inhibition was not seen in the presence of cGMP (data not shown). The magnitude of inhibition of cGKII by the IIRΔ417-762 was significantly greater (75% inhibition; Fig. 7B). The specificity of IIRΔ417-762 inhibition for the constitutively active cGKII relative to cAK Cα and cGKI-C was also observed using Kemptide as substrate (data not shown).

In Vivo Inhibition of Wild Type and Constitutively Active cGKII by Overexpression of a Regulatory Domain—The *in vivo* inhibition of cGKII activity by IIRΔ417-762 was examined by co-transfection experiments in HEK-T cells. Fig. 8A shows that overexpression of the IIRΔ417-762 caused a 12-fold rightward shift in the 8-Br-cGMP-dependent induction of gene transcription by cGKII, suggesting that IIRΔ417-762 co-expression in-

hibited the wild type kinase. Quantitative Western blot analysis demonstrated that the levels of wild type cGKII expression were identical in all conditions (data not shown), which was consistent with the observation that the maximum relative light units in both conditions were equivalent. However, these results did not distinguish between competitive, indirect, or noncompetitive modes of inhibition by IIRΔ417-762. Direct binding and inhibition of the full-length cGKII would mediate competitive inhibition by the IIRΔ417-762 protein. However, instead of competing directly at the catalytic cleft for substrate, co-expression of the IIRΔ417-762 might have indirectly inhibited the activity by simply binding cGMP or co-expression may have reduced cGKII kinase activity in a noncompetitive manner by forming a nonfunctional heterodimer with a full-length cGKII protein. These indirect and noncompetitive modes of inhibition are not likely, because the large increase (12-fold) of

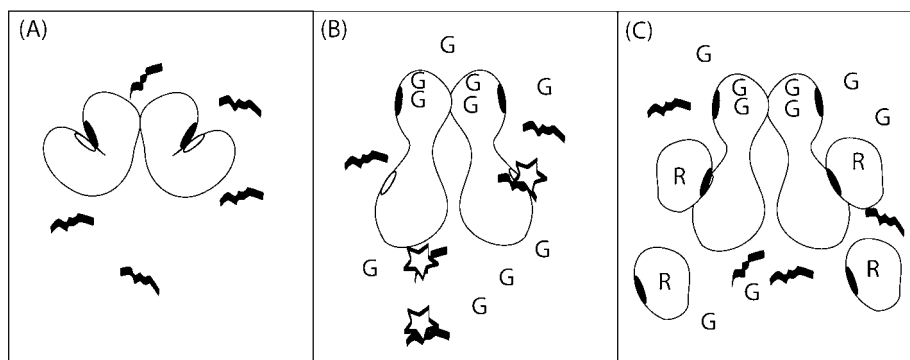


FIG. 10. **Model for IIRΔ417-762 inhibition of cGKII.** A, in the absence of cGMP (G), the kinase activity of the catalytic domain is sterically inhibited by the regulatory domain of the enzyme. B, in the presence of cGMP, the regulatory domain undergoes a conformational change to relieve the steric inhibition of the catalytic domain to increase phosphorylation (stars) of the substrate (irregular lines). C, in the presence of both cGMP and the IIRΔ417-762 protein (R), the IIRΔ417-762 protein is able to bind to the catalytic domain *in vivo* and sterically inhibit enzyme activity. In the absence of cGMP, IIRΔ417-762 has a nanomolar inhibition constant (Fig. 6A) *in vitro* but in the presence of cGMP IIRΔ417-762 has an inhibition constant above 100 nM (Fig. 6B). *In vivo*, cGMP IIRΔ417-762 is proposed to inhibit cGKII enzyme activity (Figs. 8 and 9) because the IIRΔ417-762 protein is estimated to be present at micromolar concentrations.

the cGKII EC_{50} in the presence of the IIRΔ417-762 likely exceeded the amount of suppression expected if the two binding sites of the truncation mutant were absorbing cGMP. Second, active monomers of cGKII resulting from endogenous proteolysis have been observed in some preparations (33), suggesting that each monomer of the cGKII dimer is independently capable of catalysis. Additionally, high affinity interactions observed between the two domains *in vitro* (Fig. 6A) show that heterodimerization is not required for IIRΔ417-762 inhibition.

It should be noted that the inhibitory efficacy of IIRΔ417-762 toward wild type cGKII in the presence of a cyclic GMP analog is likely because of two factors that are unique to the *in vivo* assay. First, because IIRΔ417-762 and wild type cGKII are overexpressed, the intracellular concentration of each would be much higher than the IC_{50} of their interaction, which increases the likelihood of complex formation by the high probability of interaction with a binding partner. Second, there is likely a limited availability of endogenous substrate relative to the IIRΔ417-762 inhibitor, which increases the likelihood of the enzyme remaining bound to inhibitor, rather than forming an enzyme-substrate complex.

To confirm the mode of inhibition by the IIRΔ417-762 mutant *in vivo*, a second *in vivo* approach was used. A phosphorylation dependent shift in the SDS-PAGE mobility of the cGK substrate VASP has been used to quantify the changes of cGKIβ activity *in vivo* (18). We used this *in vivo* phosphorylation assay to determine the inhibitory activity of IIRΔ417-762 by coexpressing it with both a constitutively active cGKII (R118A/R119A/V125A cGKII) and VASP in the absence of cyclic nucleotide.

The Western blot in Fig. 8B shows that in the absence of transfected cGKII, VASP migrates at the more rapidly migrating dephospho form. Co-transfection with the constitutively active cGKII results in the full conversion to the slowly migrating phosphorylated form of VASP. Co-transfection of the constitutively active cGKII with the IIRΔ417-762 truncation mutant significantly inhibits VASP phosphorylation when an 8-fold excess of IIRΔ417-762 DNA was expressed ($79 \pm 11.3\%$ inhibition; the ratio of expression of IIRΔ417-762 to constitutively active cGKII was confirmed by quantitative Western blot analysis). The intensity of the upper and lower bands was quantified and the % maximum conversion of VASP to phospho-VASP (% conversion relative to the condition with VASP expressed with the constitutively active cGKII alone) is expressed on the ordinate axis of Fig. 8C.

To determine whether the Arg¹¹⁸, Arg¹¹⁹, and Val¹²⁵ resi-

dues were required for the inhibitory properties of this regulatory domain truncation mutant *in vivo*, similar experiments were performed with the R118A/R119A/V125A IIRΔ417-762 truncation mutant (Fig. 8B). At levels of expression equivalent to IIRΔ417-762, the R118A/R119A/V125A IIRΔ417-762 mutant demonstrated a lack of inhibition of the constitutively active cGKII ($5 \pm 4\%$ inhibition; $p < 0.05$ when comparing the difference between % inhibition between IIRΔ417-762 and R118A/R119A/V125A IIRΔ417-762).

Taken together, these data show that the IIRΔ417-762 truncation mutant could suppress cGKII kinase activity *in vivo* even at a low stoichiometry of IIRΔ417-762. In addition, the residues that were necessary for inhibition *in vitro* are also required *in vivo*, suggesting that inhibition by the IIRΔ417-762 truncation mutant was not simply because of cGMP binding or heterodimerization with cGKII.

Isoform Specificity of IIRΔ417-762 and IβRΔ341-686 *in Vivo*—The VASP phosphorylation assay was used to assess the *in vivo* isoform specificity of the cGK regulatory domains. To do so, the cGKIβ regulatory domain truncation mutant (IβRΔ341-686) was co-expressed with the constitutively active cGKII or with a constitutively active form of cGKIβ (S79D cGKIβ) that has been characterized previously (18). Interestingly, it was demonstrated that the IβRΔ341-686 inhibits constitutively active cGKII ($59 \pm 15\%$ inhibition) to an extent comparable with IIRΔ417-762 ($49 \pm 10\%$ inhibition; Fig. 9, A and B), in addition to inhibiting the constitutively active form of cGKIβ ($13 \pm 5\%$, $p < 0.05$ when compared with activity in the absence of inhibitor). IIRΔ417-762 was exclusive in its inhibitory influence toward the constitutively active cGKII, and did not influence the constitutively active cGKIβ ($101 \pm 5\%$; Fig. 9B), matching observations seen in the *in vitro* assay (Fig. 8B, closed circles). Thus the cGKII truncation mutant showed isoform-specific inhibition, whereas the corresponding cGKIβ truncation mutant inhibited both cGKII and cGKIβ.

Conclusions—A major goal of this study was to characterize the residues required for autoinhibition in the type II cGMP-dependent protein kinase. Our results showed that cGKII did undergo significant autophosphorylation in the presence of cAMP and cGMP. However, cGKII autoinhibition was distinct from other cyclic nucleotide-dependent protein kinases in that autophosphorylation did not lead to activation of the cGKII kinase. These findings suggested that the AI subdomain of cGKII might involve residues that are not conserved with the cAK and cGKI isoforms.

To identify potentially unique interactions between the reg-

ulatory and catalytic domains, we characterized the contribution of several cGKII residues near the AI subdomain identified by sequence comparison of autoinhibitory sequences. It was found that cGKII was unique because autoinhibition was dependent upon residues that reside outside the conserved pseudosubstrate site. Because the presence of basic residues at both P-5 and P-6 is not conserved with cGKI isoforms, these observations may explain the distinct modes of activation between cGKI and II, as is reflected in their differences in the contribution of each CNBS toward kinase activation (11) and their response to autophosphorylation.

However, the residues identified within the AI subdomain of cGKII were not sufficient for inhibition of the enzyme as determined from *in vitro* studies using synthetic peptides derived from the AI subdomain. However, a truncation mutant of cGKII containing only the regulatory domain was a potent inhibitor of the full-length enzyme *in vitro* and this inhibition required the same residues (Arg¹¹⁸, Arg¹¹⁹, and Val¹²⁵) shown to be required for autoinhibition.

The conclusions concerning cGKII autoinhibition were extended to *in vivo* experiments with the cGKII truncation mutant. *In vivo* inhibition of the constitutively active cGKII by this truncation mutant was demonstrated to be a high affinity, isoform-specific interaction that could be disrupted by cGMP or by mutation of residues surrounding the AI subdomain (Fig. 10). Although 5 nM cGMP reversed the inhibition of the IIRΔ417–762 protein when used to inhibit low concentrations of cGKII (Fig. 6A), significantly higher concentrations of 8-Br-cGMP were required to overcome IIRΔ417–762 inhibition *in vivo* (Fig. 8A), presumably because of the much higher concentrations of cGKII and IIRΔ417–762 found *in vivo*. These findings are clearly applicable to the design of isoform-specific inhibitors of cGKs as well as the more detailed elucidation of the activation mechanisms for the cyclic nucleotide-dependent protein kinases. Pharmacological attempts to discriminate cGK contribution to physiological phenomena have been limited by cross-reactivity of inhibitors between cAKs and cGKs (15), and thus far there is no isoform-specific inhibitor of any cGK available. The present studies offer a molecular probe for cGKII inhibition *in vivo* in an isoform-specific manner, a tool that would be useful in discriminating cGK activity in cells where the two cGK isoforms have been indicated to be co-expressed (34, 35).

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