Arginine 75 in the Pseudosubstrate Sequence of Type Iβ cGMP-dependent Protein Kinase Is Critical for Autoinhibition, Although Autophosphorylated Serine 63 Is Outside This Sequence*

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Autoinhibitory domains in many protein kinases include either a phosphorylatable substrate-like sequence or a pseudosubstrate sequence. This study shows that Iβ cGMP-dependent protein kinase (cGK) autophosphorylates Ser-63, which is in an atypical cGK substrate sequence (-79AQKQSAS-) that is amino-terminal to the pseudosubstrate motif (-74RRVSV-). cGMP increases the rate of autophosphorylation (∼0.8 phosphate/cGK monomer) 3-fold. Autophosphorylation is an intramolecular process since it is independent of cGK concentration. cGMP activation of cGK enhances proteolysis within and near the pseudosubstrate site; treatment of dimeric cGK with three proteases produces three cGK monomers (∼67-70 kDa each). Their amino-terminal sequences are RQAISAEP-, QAISAEPFA-, and DIQDSLXV- respectively. cGMP stimulates these kinases by 10-, 25-, and 1.4-fold, respectively, compared with a 10-fold effect on intact cGK. Increased basal activity accounts for the diminished stimulation. Thus, the primary autophosphorylation site of Iβ cGK is well outside the pseudosubstrate site, but Arg-75 in the pseudosubstrate site is critical for autoinhibition. Autoinhibition also involves elements that are carboxy-terminal to Arg-75.

Protein kinases commonly undergo autophosphorylation, and in many instances the modified residues are located in their autoinhibitory domains (1–4). Substrate-like motifs in the autoinhibitory domains of protein kinases are suggested to compete with protein substrates for access to the catalytic site (5, 6). This autoinhibitory mechanism was first shown for type IIα regulatory subunit (RII)1 of cAMP-dependent protein kinase (cAK), which is autophosphorylated in a substrate-like sequence (-RRVSV-) (7, 8) that is critically important in autoinhibition of cAK (1, 7, 9). This substrate-like sequence is partially conserved in autoinhibitory domains of all known cyclic nucleotide kinases (Table I) (10–28). For some of these kinases, the homologous sequence within the autoinhibitory domain lacks a phosphorylatable residue and is referred to as a pseudosubstrate sequence. Autophosphorylation of RII in its autoinhibitory domain decreases its affinity for the catalytic subunit, and the affinity of the holoenzyme for cAMP is increased (7, 9, 29, 30).

Autoinhibition in cGMP-dependent protein kinases (cGK) is less well understood. Autoinhibitory domains of cGKs lack well-conserved substrate consensus motifs that might serve as autophosphorylation and/or autoinhibitory sequences. The autoinhibitory domains of types Iα, Iβ, and II cGKs are highly dissimilar (11–13) despite the similar roles of these regions to inhibit kinase activity in their respective catalytic domains. In contrast, the primary structures of the catalytic domains of Iα and Iβ cGKs are identical, and the type II cGK catalytic domain is very similar.

Both types Iα and Iβ cGKs undergo autophosphorylation (31–35). In the presence of cGMP, Iα cGK autophosphorylates primarily at Thr-58, which is just amino-terminal to a sequence that is homologous to the autophosphorylation site/autoinhibitory domain in RII (Table I) (12). Other autophosphorylation sites (Ser-50, Ser-72, and Thr-84) are significantly removed from the pseudosubstrate motif. In the presence of CAMP, all four sites are phosphorylated, and both basal kinase activity and affinity for CAMP are increased (36, 37). Extensive autophosphorylation of Iβ cGK also increases basal kinase activity and increases affinity for cGMP and cAMP (38, 39, see accompanying paper by Smith et al. (70)). Two autophosphorylation site(s) in Iβ cGK have now been identified, and the contributions of these sites to the activation that occurs with autophosphorylation have been determined (as reported here and in the accompanying paper by Smith et al. (70)). Identification of these sites, as well as the sequences that provide for autoinhibition in Iβ cGK, provides insight into the autoregulation of this enzyme. The current study was designed to identify the site that is rapidly autophosphorylated in Iβ cGK in the presence of cGMP and to determine the sequence(s) that provide for autoinhibition.

EXPERIMENTAL PROCEDURES

Purification of cGKs

cGKs were purified to apparent homogeneity from bovine tissues as described previously (40) and had kinase specific activities of ∼3 μmol/min/mg. Purity was analyzed using SDS-PAGE followed by either Coomassie Brilliant Blue stain or silver stain (41).

Protein Kinase Assay

Catalytic activity was assayed as described previously (∼10 μM cGMP) using heptapeptide substrate (RKRSRAE) (34). The Kₘ for...
TABLE I

Comparison of substrate and pseudosubstrate sequences from the autoinhibitory domains of cyclic nucleotide-dependent protein kinases and the protein kinase inhibitor

| Protein Kinase | Substrate | Pseudosubstrate |
|---------------|-----------|-----------------|
| cGK Type 3 | 1 | cGMP-dependent protein kinases |
| cGK Type 2 | 2-5 | cAMP-dependent protein kinases |
| cGK Type 1 | 6-10 |  |

**Preparation of Phosphorylated 1β cGK and Trypsin Digestion of the Radiolabeled 1β cGK for 32P-Phosphopeptide Studies**

Purified 1β cGK (2.5 nmol) was incubated with 10 mM KH2PO4, pH 6.8, 2 mM EDTA, 25 mM 2-mercaptoethanol (KPEM), and 0.2 M NaCl was incubated with 12 μM cGMP at 4°C for 30 min prior to addition of a reaction mixture to achieve a final concentration of 75 μM [γ-32P]ATP (specific radioactivity ~ 200 cpm/pmol) and 10 mM MgCl2. In some instances unlabeled ATP was used. For preparation of labeled trypptic phosphopeptides, a specific radioactivity of ~1000 cpm/pmol was used. Autophosphorylation reaction mixtures were incubated at 30°C for 2 h. Aliquots from the reactions were spotted onto phosphocellulose papers (Whatman P-81, 1.5 x 2 cm) to determine 32P incorporation into cGK. Papers were then washed with six changes of 75 mM phosphoric acid, dried, and counted. Aliquots for protein determination were taken throughout the incubation.

For isolation of 32P-phosphopeptides, the autophosphorylation proceeded as above. The reaction mixture was then applied to a Sephacryl S200 column (0.46 x 56 cm) in KPEM to separate [32P]phospho-1β cGK from [32P]ATP and its products. Fractions containing 32P-phospho-cGK were pooled and suspended in a boiling water bath for 35 min. Sample was cooled, the pH was adjusted to pH 7.8 with 1 M Tris base, and TPK-trypsin was added at 0.1, 0.5, and 1 h final trypsin:1β cGK, 1:10, w/w. The digestion proceeded for 19 h at 30°C after which a sample was analyzed by SDS-PAGE.

**Determination of 32P-Phospholabeled Amino Acids**

Samples of 32P-phospholabeled peptides from the HPLC C8 column chromatography were taken to dryness in a Speed-Vac rotary evaporator and then partially hydrolyzed by 6 N HCl at 110°C for 4 h. Hydrolysates were taken to dryness repeatedly and resuspended in water, and unlabeled phosphoserine and phosphothreonine standards (10 μM of 10 mg/ml each) were added. Aliquots (~16,000 Cerenkov cpm) from each sample were spotted onto thin layer chromatography plastic sheets (silica gel 60, 0.2-mm thickness), and subjected to high voltage flat bed electrophoresis at pH 1.9 in a system pre-cooled to 4°C (15 min at 100 V and then 2 h at 500 V). The sheet was then dried and stained with ninhydrin to locate the standards. Subsequent autoradiography identified the radiolabeled amino acid(s).

**Proteolytic Cleavage of cGKs by Trypsin, Chymotrypsin, and Endoproteinase Lys-C**

Analytical digestions with each protease were used to establish optimal conditions for conversion of intact cGK (~78 kDa) to a high molecular mass fragment (~65–70 kDa) as assessed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining.

Trypsin Digestion—Purified 1β cGK (300 μg) was treated with TPCK-trypsin for ~30 min at 30°C at a 1:40 ratio of trypsin to enzyme (w/w) in 10 mM KH2PO4, pH 6.8, 1 mM EDTA, 25 mM 2-mercaptoethanol, 0.27 M NaCl in the presence and absence of 10 μM cGMP. The sample was then quickly chilled; an aliquot was removed, [3H]H2O was added, and this portion of the digest was applied to a Sephacryl S200 column (0.9 x 55 cm) in KPEM. Fractions (0.5 ml) were collected and assayed for kinase activity in the presence and absence of 10 μM cGMP. In some instances, [3H]GMP was included in the digestion in order to quantitate GMP that was retained by cGK fragments following Sephacryl chromatography. The remainder of the digest was then prepared using 10% trichloroacetic acid at pH 4°C for 60 min followed by centrifugation at 4°C for 15 min. The protein pellet was sequentially washed with two acetone extractions and a single extraction with diethyl ether and then air-dried prior to amino acid sequence analysis by Edman degradation. Aliquots of the original digest and Sephacryl S200 fractions were routinely analyzed on 10% SDS-PAGE in order to verify complete conversion of native 1β cGK to a single high molecular weight fragment, and native cGK was used as control. Gels were stained with either silver stain or Coomassie Brilliant Blue. Trypsin treatment of purified bovine lung 1α cGK produced a similar proteolytic fragment (~65 kDa) that was purified on Sephacryl S200 and assayed for kinase activity.

Chymotrypsin Digestion—Chymotrypsin was added to purified 1β cGK (1:40 w/w) for 30 min at 30°C in the presence of 10 μM cGMP, and

1 β cGK, autophosphorylates Ser-63, which is amino-terminal to the conserved autoinhibitory domain sequences of cyclic nucleotide-dependent protein kinases and a related sequence in the protein kinase inhibitor. The underlined residues in the sequences are either residues in the autoinhibitory domains that are phosphorylated upon interaction with the catalytic component of the kinase or this residue occupies the position in the sequence that is equivalent to the phosphorylated residue in the other sequences and, as such, these constructs are referred to as "pseudosubstrate" sites (6).
the digest was treated as described above for the trypsin digest.

Endoprotease Lys-C Digestion—Iβ cGK (130 µg) was treated with endoprotease Lys-C peptide (sequencing grade) for 30 min at 30 °C at a 1:30 ratio (w/w) of protease to enzyme in the presence of 10 µM cGMP. The extent of the digest was assessed using 10% SDS-PAGE. A portion of the digest (18 µg) was combined with [3H]H2O and then subjected to Sephacryl S200 chromatography. Fractions (0.5 ml) were collected and analyzed for kinase activity (±10 µM cGMP) and by SDS-PAGE. The remainder of the digest was precipitated using 10% trichloroacetic acid at 4 °C as described above, and the protein pellet was then subjected to amino acid sequence analysis by Edman degradation. The endoprotease Lys-C was dissolved in buffer containing 50 mM Tricine, pH 8, 10 mM EDTA. The properties of the 65–70-kDa fragments of Iβ cGK that were produced by these three respective proteolytic digestions were determined utilizing numerous preparations of the digested cGK, and the amino-terminal amino acid sequence of each fragment was determined on protein from at least two preparative digestions. Sequence analyses of the cGK fragments were performed by the Harvard Microchemistry Laboratory, Cambridge, MA and by the core sequencing facility at the University of Washington, Seattle, WA.

Protein Determination

Protein was measured by the method of Bradford using bovine serum albumin as standard. Protein values for cGK that were determined by the Bradford method were multiplied by the 0.63 correction factor previously determined for cGK by Picotag analysis (34). Synthetic peptide concentration (QAQK QSASTLQ) was determined by Pi-tag amino acid analysis.

Materials

[γ-32P]ATP was purchased from DuPont NEN. [3H]cGMP and [3H]H2O were purchased from Amersham Corp. Resins and protein standards were from Pharmacia Biotech Inc. Peptide substrate (RRKRSRAE) was purchased from Peninsula Labs. The peptide (QAQK QSASTLQ) was synthesized by Peptides International, Inc. Phosphocellulose paper was from Whatman. L-1-Tosylamido-2-phenyl-ethyl chloromethyl ketone-treated (TPCK) trypsin and endoprotease Lys-C were from Boehringer Mannheim. 8-(6-Aminohexyl)cAMP-agarose, cGMP, cAMP, ATP, bovine serum albumin, and 2-mercaptoethanol were from Sigma. Plastic silica gel TLC sheets were from Alltech. Trifluoroacetic acid was from Pierce, and acetonitrile was from Burdick and Jackson.

RESULTS

Time Course of Autophosphorylation of Iβ cGK and Effect of Substrates on Autophosphorylation—In the presence of 10 µM cGMP, the rate and extent of autophosphorylation of Iβ cGK is increased 3-fold (Fig. 1A). After 90 min approximately 0.8 mol ± 0.07 (mean ± S.E.) phosphate is incorporated per mol of cGK subunit in the presence of cGMP compared to −0.25 mol of phosphate incorporated in its absence. Similar values were obtained with five different preparations of enzyme. Under these conditions, kinase activity of autophosphorylated cGK is the same as that of control cGK when assayed in the presence of 10 µM cGMP. Protein content was determined throughout the autophosphorylation reaction. The autophosphorylation rate is not enhanced by addition of a 5-fold excess of Iβ cGK that had been pre-autophosphorylated for 2 h in the presence of unlabeled ATP (75 µM) and 10 µM cGMP (Fig. 1B). This implies that autophosphorylation of Iβ cGK is an intramolecular process that is stimulated by the binding of cGMP to the cyclic nucleotide binding domain of the enzyme. This suggests that the site(s) of autophosphorylation are in close proximity to the catalytic site of cGK even when the enzyme is activated.

Autophosphorylation of Iβ cGK is unaffected by 28 µM heptapeptide substrate (RRKRSRAE) (Fig. 2), which is a 150-fold molar excess; at a 440-fold excess, autophosphorylation is decreased by only 10%. However, a 630-fold excess of heptapeptide significantly lowers autophosphorylation —70%. Under these conditions, phosphorylation of heptapeptide is linear with time, and there is no time lag in the initial phase of the assay (data not shown). This suggests that autophosphorylation is not a prerequisite for full activation of cGK. Since a large excess (~400-fold) of high affinity heptapeptide substrate is required to compete with autophosphorylation, it is predicted that under physiological conditions, autophosphorylation would proceed rapidly despite the availability of competing substrates containing more optimal phosphorylation site sequences.

Purification and Sequence Determination of the 32P-Phosphopeptide—HPLC C8 chromatography of the tryptic digest of [32P]phospho-Iβ cGK that has been autophosphorylated in the presence of 10 µM cGMP, as described under “Experimental Procedures,” reveals one major 32P-labeled peptide that elutes at −16% acetonitrile, and several minor 32P-peptides (Fig. 3). The labeled peptide eluting at 16% acetonitrile represents at least 80% of the total label recovered under these conditions. The major peaks contain only [32P]phosphoseryl, consistent with the 32P-phosphoamino acid content of [32P]phospho-Iβ cGK.
The major $^{32}$P-labeled peptide has been sub-digested through Arg-72 in I $\beta$ cGK (SVIRPATQQAQKQSASTLQGEPR) extending from Ser-50. Tandem mass spectrometric analysis determines the amino acid sequence of the Ser-63 site (-QKQSA-) has a single basic residue at P-2, immediately amino-terminal to Ser-63. A synthetic undecapeptide (-RRSRAE) does not conform to that of a cGK consensus substrate motif (-RRXSR-) (44, 45) since there is only one basic residue immediately amino-terminal to Ser-63. A synthetic undecapeptide (QQKQSASTLQ) that includes the sequence surrounding Ser-63 has been tested as a substrate for I $\beta$ cGK. The undecapeptide is included in a kinase assay lacking other substrates (see "Experimental Procedures") and assayed for 40 min at 30 $^\circ$C. A parallel assay utilizing the same cGK dilution and the RKR SRAE heptapeptide that is typically used as substrate is incubated for 3 min. I $\beta$ cGK phosphorylates the undecapeptide with a $K_m$ = 4 $\mu$M (compared with 12 $\mu$M for the RKRSRAE heptapeptide) (34) and a $V_{\text{max}}$ that is 150-fold lower than that for heptapeptide substrate (RKR SRAE) (46, 47).

Although the amino acid sequence around the autophosphorylation site of I $\beta$ cGK is not a typical cGK substrate sequence, it is noteworthy that the three autophosphorylation site sequences in I $\alpha$ cGK are also atypical (35); Thr-58 (in the sequence -54GPRTTRA-) has a single basic residue at P-2,
whereas sequences surrounding two minor autophosphorylation sites in Ia cGK (\(-^{36}\text{LPVPSTH}-\) and \(-^{68}\text{QTYRSTF}-\)) are even more anomalous. Ia cGK has a low affinity for a synthetic peptide whose sequence corresponds to that surrounding Thr-58 \(K_m = 0.58 \text{ mM}\) and a low \(V_{\text{max}}\) \(= 0.07 \text{ mol/min/mg}\) (48). Substitution of the P = 1 Arg (R-59) by Ala in this peptide weakened the affinity of its interaction with Ia cGK by nearly 40-fold. The phosphorylation site peptide from Ia cGK (QAQK QASAITLQ) lacks a basic residue in this position. Since functional domains of cGKs are colocalized in a single polypeptide chain, the interaction of the catalytic site with phosphorylatable residues in its autoinhibitory region may be less constrained by specific sequences compared with that determined for its interactions with exogenous substrates. Some other protein kinases that exhibit intrasteric autoinhibition (49) also autophosphorylate sites whose sequences do not conform to a consensus substrate motif (50–53).

Determination of Sequences That Contribute to Potency of Autoinhibition—Ser-63 in Ia cGK is well outside the conserved pseudosubstrate site \((^{72}\text{KRAI}-)^{(49, 54, 55)}\). The most rapidly autophosphorylated site in Ia cGK (Thr-58) is also amino-terminal to the putative pseudosubstrate site \((-^{59}\text{RAQAIS}-\) which lacks a dibasic motif. By analogy with aCaK, basic residues in the pseudosubstrate sequences of cGKs are presumed to interact intrastERICly with acidic residues in the catalytic site to block catalysis, thus forming the autoinhibitory domain (5, 6, 36, 56, 57). However, structural components that contribute to autoinhibition in cGKs have not been experimentally determined. Therefore, Ia cGK has been partially proteolyzed with three proteases to produce three cGK monomers that vary in the extent of autoinhibition.

Partial proteolysis of Ia cGK by trypsin, endoproteinase Lys-C, or chymotrypsin has been performed as described under “Experimental Procedures” monitored using SDS-PAGE followed by Coomassie Brilliant Blue or silver staining. Each protease converts dimeric Ia cGK to a monomeric cGK of 67–70 kDa (Table III). The rate of proteolysis by all three proteases is significantly increased in the presence of \(10 \mu\text{M cGMP}\) (results with trypsin are shown in Fig. 4). This effect suggests that occupation of the cGMP-binding sites produces a conformational change in the autoinhibitory domain to increase its solvent exposure and sensitivity to proteolytic cleavage. The particular preparation of Ia cGK used in Fig. 4 has a small amount of proteolytic breakdown of the cGK that sometimes occurs with storage. Using a Sephacryl S200 column that has been standardized with proteins of known Stokes radii, the fragment generated by endoproteinase Lys-C is determined to have Stokes radius of 40 Å compared with 53 Å for Ia cGK (Fig. 5). Each of the fragments has been generated at least six times using different preparations of purified Ia cGK. Amino acid sequences for each fragment have been determined in at least two separate analyses. The findings are consistent with reports showing that a leucine zipper located near the amino terminus of cGK is important for dimerization (58), and a proteolytic breakdown product of Ia cGK that is cleaved amino-terminal to Gln-62 is monomeric (59).

Conditions for partial proteolysis of cGK are chosen so that no detectable intact Ia cGK remains. cGK fragments have been purified from the digest by Sephacryl S200 chromatography (see “Experimental Procedures”); eluted fractions are assayed for kinase activity \(= 10 \mu\text{M cGMP}\) (Fig. 5). Purity of the cGK fragments is assessed initially using SDS-PAGE. Sequential Edman degradation verifies homogeneity of the products and identifies the positions in the known primary structure of Ia cGK that have been cleaved (Table III). The specific catalytic activities of the proteolyzed fragments of Ia cGK in the presence of \(10 \mu\text{M cGMP}\) are \(2.4 \mu\text{mol/min/mg}\) (endoproteinase Lys-C fragment), \(5.9 \mu\text{mol/min/mg}\) (trypsin-derived fragment), and \(3.3 \mu\text{mol/min/mg}\) (chymotrypsin-derived fragment), which are essentially the same as that for native Ia cGK \((3 \mu\text{mol/min/mg})\).

The cGK fragment produced by digestion with endoproteinase Lys-C has an activity ratio that is indistinguishable from that of native Ia cGK \((-0.1)\). The amino-terminal sequence of this fragment has been determined to be \((^{59}\text{RAQAIS}-\) The fragments of Ia cGK that are produced by limited digestion with trypsin or chymotrypsin have activity ratios of \(-0.5\) and \(-0.7\), and their amino-terminal sequences have been deter-
fragments produced by endoproteinase Lys-C digestion retain negligible amounts of \(^{3}H\)cGMP. The trypsin- and chymotrypsin-derived fragments of I\(\beta\) cGK retain cGMP that is sufficient to occupy -15\% of the cGMP-binding sites. Dialysis removes all measurable \(^{3}H\)cGMP from these fragments, and the activity ratios for the dialyzed enzymes are -0.5 and -0.7, respectively (Table III).

Partial proteolytic digestion of the bovine lung I\(\alpha\) cGK has also been carried out. Trypsin treatment in the presence of 10 \(\mu\)M \(^{3}H\)cGMP produces a 65-kDa fragment (data not shown). Following gel filtration and dialysis to remove \(^{3}H\)cGMP bound to the enzyme, this I\(\alpha\) cGK monomer is still partially dependent on cGMP (activity ratio = 0.74 \pm 0.01 (mean \pm S.E.). The trypsin-sensitive site in I\(\alpha\) cGK has previously been shown to occur at Arg-77 and has been suggested to render the enzyme fully independent of cGMP (61). However, the current results demonstrate that the autoinhibitory domains of both I\(\alpha\) and I\(\beta\) cGK include interactions that are carboxyl-terminal to the pseudosubstrate sequences.

Increased basal activity of the trypsin-generated I\(\beta\) cGK fragment is consistent with decreased potency of the autoinhibitory domain which could decrease the cGMP concentration required for activation. The concentration of cGMP required to activate the trypsin-derived fragment of cGK is somewhat lower (\(K_a = 0.20 \pm 0.035 \mu\)M) than that required by native cGK (\(K_a = 0.35 \pm 0.02 \mu\)M) (mean \pm S.E.) (Table III). The slightly lower \(K_a\) for cGMP of the cGK fragment could result from the absence of inhibitory interactions involving Arg-75.

Variations in Assay Conditions to Test for cGMP Dependence of the Proteolytically Derived cGK Fragments—The cGMP dependences of the kinase activities of native I\(\beta\) cGK and the fragment of I\(\beta\) cGK produced by trypsin or chymotrypsin cleavage are not altered under a variety of conditions (data not shown). Varied assay conditions including time (15 to 40 min), temperature (4, 30, and 40 \(^\circ\)C), kinase dilution, pH (6.0–8.7), and ATP concentration (2–200 \(\mu\)M) or heptapeptide substrate concentration (16–130 \(\mu\)M) do not significantly alter the activity ratio of chymotrypsin-derived fragment of cGK. Similar results involving enzyme dilution, temperature, and assay time are obtained with the trypsin-derived cGK fragment. Thus, the increased basal kinase activity of these cGK monomers is a stable change.

**DISCUSSION**

Cyclic GMP binding to I\(\beta\) cGK produces at least three effects on the structure/function of the enzyme: (a) inhibition of catalytic activity by the autoinhibitory domain is relieved, (b) the sensitivity of the amino-terminal autoinhibitory domain to limited proteolysis is greatly enhanced presumably due to increased solvent exposure, and (c) the rate of autophosphorylation of Ser-63 located near autoinhibitory sequences is markedly increased.

Autoinhibitory domains of type I cGKs are shown here to involve at least two discrete sequences, a pattern that has been demonstrated in some other protein kinases (62, 63). In I\(\beta\) cGK, a single arginine, Arg-75, that is part of a putative pseudosubstrate sequence (-74KRQAI-) in the autoinhibitory domain provides a critical determinant for potent autoinhibition of catalysis, but additional sequences located carboxyl-terminal to this sequence also contribute to autoinhibition. Autoinhibition of type I cGKs does not require two tandem basic residues in the pseudosubstrate sequence, since removal of Lys-74 in I\(\beta\) cGK has no measurable effect on the activity ratio. A contribution of Lys-74 to the potency with which the autoinhibitory domain binds to the catalytic domain cannot be excluded, but the simplest interpretation is that the autoinhibitory domain of I\(\beta\) cGK begins at Arg-75. Whether Lys-74 in the absence of Arg-75 could provide for potent autoinhibition remains to be deter-
minded. This is a particularly interesting possibility since Iα cGK has a single basic amino acid in its putative pseudosubstrate sequence (RQVAI) (Table I) (12), and the location of Arg-59 is homologous to that of Lys-74 in Iβ cGK (11). Autoinhibitory interactions are also provided by sites located carboxy-terminally to the pseudosubstrate site in Iα cGK, since removal of the pseudosubstrate sequence in our hands does not render this enzyme fully cGMP-independent.

Two tandem basic amino acids are important components in the consensus phosphorylation sequence for protein and peptide substrates for cyclic nucleotide-dependent protein kinases (1, 7, 9, 36, 47, 64, 65), and such an arrangement of basic amino acids is required for potent inhibition of catalytic subunit of cAK by R subunit (1, 7, 9). In cAK, these basic residues are believed to interact with specific acidic residues in the catalytic site (66). However, autoinhibition and autophosphorylation in cGKs may be less dependent than are these processes in cAKs on the veracity with which substrate is mimicked; the cGK regulatory and catalytic domains remain in relatively close contact since they are contained in a single polypeptide chain (12). Critical interactions involving a single basic residue positioned at P-2 is suggested by the effects of partial proteolysis on autophosphorylation of Iβ cGK described herein and by the fact that the primary autophosphorylation sites in type I cGKs involve a single basic residue at P-2, i.e. Thr-58 in Iα cGK (PRRT-) (35) and Ser-63 in Iβ cGK (QKQS) (12). Thus, a single basic amino acid at P-2 will suffice for these autophosphorylations and for a significant portion of autoinhibition. If a doublet of basic amino acids is not required for autoinhibition, then other sequences that involve single basic amino acid have the potential to serve as an autoinhibitory "pseudosubstrate" motif. This might explain the partial autoinhibition observed in the Iβ cGK monomers derived from trypsin or chymotrypsin treatment. The partial autoinhibition that remains following removal of the putative pseudosubstrate site in Iβ cGK could also be conferred by some element(s) in the CAMP-binding sites. Physical linkage of regulatory and catalytic domains in cGKs is likely to enhance the contribution and the detection of weak autoinhibitory interactions compared with such effects in cAKs.

Cyclic GMP binding to Iβ cGK promotes a rapid intramolecular autophosphorylation at Ser-63 which is located 11 amino acids away from the pseudosubstrate site that is critical for autoinhibition. In Iα cGK, Ser-50 is autophosphorylated (35) and is located 8 amino acids outside the pseudosubstrate site (12). Similar positioning of these autophosphorylation sites in the primary sequences of type I cGKs implies that upon activation these serines are shifted closer to the catalytic site coincident with removal of the inhibitory pseudosubstrate site from this location.

The sequence surrounding Ser-63 (QAOQKQASTLQ-) is atypical for a cGK substrate (47), but a synthetic peptide with this sequence is phosphorylated by Iβ cGK, albeit weakly. Autophosphorylation in nonprototypical sequences also occurs in other protein kinases (35, 50, 51, 53), but in cAK autophosphorylation of RII occurs only within the substrate consensus sequence (Table I) that is critical for autoinhibition (1, 7, 9). Thus, although the autophosphorylation domain and the autoinhibitory domain of cGKs overlap somewhat, the sequences that delineate these functional regions are distinct. The increased rate of autophosphorylation of cGK autoinhibitory domains upon activation suggests that these regions remain in close proximity to the catalytic sites. This is in agreement with observations concerning catalytic and autoinhibitory domains of myosin light chain kinase (67). Although Ser-63 is the initial site of autophosphorylation, Iβ cGK is also autophosphorylated at Ser-79 in a slower reaction as described in the accompanying paper (Smith et al. (70)).

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