Catalytic Activity of cGMP-Dependent Protein Kinase Type I in Intact Cells Is Independent of N-Terminal Autophosphorylation

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Abstract

Although cGMP-dependent protein kinase type I (cGKI) is an important mediator of cGMP signaling and upcoming drug target, its in vivo-biochemistry is not well understood. Many studies showed that purified cGKI autophosphorylates multiple sites at its N-terminus. Autophosphorylation might be involved in kinase activation, but it is unclear whether this happens also in intact cells. To study cGKI autophosphorylation in vitro and in vivo, we have generated phospho-specific antibodies against major in vitro-autophosphorylation sites of the cGKI isoforms, cGKIα and cGKIβ. These antibodies detected specifically and with high sensitivity phospho-cGKIα (Thr58), phospho-cGKIβ (Thr84), or phospho-cGKIβ (Thr56/Ser63/Ser79). Using these antibodies, we show that ATP-induced autophosphorylation of cGKI in purified preparations and cell extracts did not require or induce an enzyme conformation capable of substrate heterophosphorylation; it was even inhibited by pre-incubation with cGMP. Interestingly, phospho-cGKI species were not detectable in intact murine cells and tissues, both under basal conditions and after induction of cGKI catalytic activity. We conclude that N-terminal phosphorylation, although readily induced in vitro, is not required for the catalytic activity of cGKIα and cGKIβ in vivo. These results will also inform screening strategies to identify novel cGKI modulators.

Introduction

Cyclic guanosine monophosphate (cGMP) acts as a second messenger in various cell types and tissues of eukaryotes [1,2]. The intracellular concentration of cGMP depends on the rate of its synthesis and degradation. cGMP is generated by cytosolic soluble guanylyl cyclases in response to NO or by membrane-bound particulate guanylyl cyclases that are activated by natriuretic peptides and some bacterial toxins. cGMP is hydrolyzed to GMP by phosphodiesterases, whose catalytic activity is often regulated by binding of cGMP or cAMP. At least three classes of cGMP effector proteins are known: cyclic nucleotide-gated cation channels, which transduce changes in cGMP concentrations into changes of membrane potential; cGMP-regulated cAMP-hydrolyzing phosphodiesterases, which mediate a cross-talk of cGMP and cAMP signaling; and cGMP-dependent protein kinases, which upon binding of cGMP phosphorylate a variety of target proteins at Ser/Thr residues.

The cGMP-dependent protein kinase type I (cGKI, also known as PKG-I or PRKG1) is considered a major mediator of cGMP signaling in mammals. Many studies suggest that pharmacologic regulation of cGKI might interfere with diverse patho-physiological processes [3,4]. Thus, small-molecule modulators of cGKI for in vivo-use are of great interest to basic and clinical research. However, the development of such drugs has been hampered, in part, because the in vivo-biochemistry of cGKI is not well understood.

cGKI is composed of an N-terminal regulatory domain that contains two non-identical cGMP-binding pockets with different affinities for cGMP and a C-terminal catalytic domain with binding sites for ATP and protein substrates [5–7] (Fig. 1A). The mammalian ptk6 gene encodes two cGKI isoforms, cGKIα and cGKIβ. Each isozyme forms a homodimer of two ≈75 kDa subunits. cGKIα and cGKIβ have identical cGMP-binding and catalytic domains, but differ in their N-terminal regions (=100 amino acids). This region mediates dimerization via a leucine zipper motif, regulates the affinity of the cGMP-binding pockets via allosteric mechanisms, and interacts, presumably in an isoform-specific manner, with anchoring and substrate proteins. It also contains an autoinhibitory/autoactivation region that might be involved in enzyme activation (Fig. 1).

Experiments conducted about 35 years ago revealed that purified cGKI undergoes autophosphorylation of its N-terminal region in the presence of radioactively labeled Mg2+-ATP [8–10]. In cGKIα, major in vitro-autophosphorylation sites were identified as Ser50, Thr58, Ser72, and Thr84, and a lower extent of autophosphorylation was observed on Ser1, Ser63, Ser44, and Ser64 [11–13]. In cGKIβ, Ser63 and Ser79 were identified as in vitro-autophosphorylation sites [14,15]. Note that, in these reports, the numbering system used to identify amino acid residues omitted
the N-terminal Met. This nomenclature was also adopted in the present study.

According to the current working model of cGKI (Fig. 1B), binding of cGMP induces a conformational change that releases the inhibition of the catalytic domain by the autoinhibitory region, perhaps via autophosphorylation of the autoinhibitory region [5,6]. Autophosphorylation of certain sites increases basal phosphotransferase activity and the affinity for cGMP, but it can also promote the degradation of the enzyme [16]. Moreover, the introduction of phosphates in the N-terminal region could influence the interactions of cGKI isoforms with other proteins. Taken together, the previous in vitro studies indicated that N-terminal autophosphorylation regulates various biochemical properties of cGKI, with both positive and negative effects on cGMP signaling. Pharmacologic modulation of cGKI autophosphorylation, possibly in an isoform-specific manner, might therefore be a promising strategy to influence cGMP signaling in vivo. However, very little is known about the relevance of cGKI autophosphorylation in vivo in intact cells and tissues [5].

In the present study, phospho-specific antibodies were generated that detect autophosphorylated cGKIα and cGKIβ with high sensitivity. Our results indicate that N-terminal autophosphorylation of cGKI does readily occur in purified protein preparations or cell extracts but not in intact cells.

Materials and Methods

Ethics statement

All animal procedures were in compliance with the European Community guidelines for the use of experimental animals and had been approved by the committee on animal care and welfare of the Regierungspräsidium Tübingen, Baden-Württemberg, Germany.

Materials

cGMP, 8-Br-cGMP, 8-Br-PET-cGMP, and 8-Br-cAMP were purchased from Biolog Life Sciences Institute. C-type natriuretic peptide, ATP, calyculin A, isoprenaline hydrochloride, and DEA-NONOate were from Abbiotec, AppliChem, Cell Signaling, Sigma-Aldrich, and ENZO Life Sciences, respectively. PhosSTOP phosphatase inhibitor cocktail tablets were from Roche.

Peptide synthesis and antibody generation

Peptides containing known N-terminal cGKI autophosphorylation sites were selected based on the murine proteins (NP_001013855.1 for cGKIα and NP_035290.1 for cGKIβ) and synthesized with and without the corresponding phospho-amino acid. The peptides were synthesized as single peptides and as multiple antigen peptides, (peptide)_{8-(Lys)_{4}-Lys_{2}-Lys-Lys}_{β-Ala-OH}, using standard Fmoc/tBu chemistry [17] on a multiple peptide synthesizer, Syro II (MultiSynTech, Witten, Germany). The single peptides were purified using reversed phase-HPLC and their
identity was confirmed using ESI-MS and MALDI-TOF-MS. Peptide purities were >95% as determined by analytical reversed phase-HPLC. The single peptides were coupled to keyhole limpet hemocyanin using the glutardialdehyde method. The antisera were obtained after repeated immunization of rabbits with a 1:1 mixture of the peptide–keyhole limpet hemocyanin conjugate and the multiple antigen peptide. A total of seven antigenic phospho-peptides were injected (Pineda Antibody-Service GmbH). 10 mg of phospho- or nonphospho-peptide pool. Peptide sequences were derived from murine cGKI and cGKI, and the amino acids were numbered without counting the N-terminal Met.

| Table 1. Phospho-peptides used to generate antibodies against phospho-cGKI species. |
|---|---|---|---|---|
| cGKI | Peptide | Peptide | Peptide | Rabbit |
| isozyme | pool | ID | sequence | antiserum |
| Ix | I | 550 | SVLPVPpSTHG | 1–3 |
| Ix | T88 | GPRTpTRAQGISA | |
| II | 572 | AEQPYTRpSFHDLRQA | 4–6 |
| Ix | T84 | RQAFRKFpTKSERSK | |
| II | 567 | IRPAPQpQAOQK | 7–9 |
| II | 563 | QAQKQpSASTL | |
| II | 579 | RTRKRQpAQSAEP | |

Phosphorylation of cGKI in intact cells and tissues

Cultured cells were serum-starved (MEFs for 3 h and VSMCs for 48 h) in DMEM containing 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C and 6% CO2. Then, test compounds were added in PBS in the absence or presence of 100 nM calyculin A for various times at 37°C and 6% CO2 as specified in the respective figure legends. At the end of the treatment, cells were washed twice with ice-cold PBS, lysed in lysis buffer A (21 mM Tris-Cl, pH 8.3, 0.7% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and one PhosSTOP tablet per 10 mL) and heated for 5 min at 95°C. Samples were stored at −20°C.

Cell culture

Wild-type and cGKI-deficient (genotype: cGKIL−/−) [22] mouse embryonic fibroblasts (MEFs) and primary vascular smooth muscle cells (VSMCs) were obtained as described [23,24]. All cells were from mice on a 129/Sv genetic background. They were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C and 6% CO2. MEFs were used for experiments between passage 20 and 39. VSMCs were isolated from aortae of 5- to 6-week-old mice and analyzed in primary culture without passaging.

Phosphorylation of purified cGKI isozymes

Recombinant bovine cGKIα and cGKIβ were expressed in Sf9 insect cells and purified by affinity chromatography as described [20,21]. Purified proteins were incubated at 30°C for 15 min in a total volume of 80 µL. The reaction mix contained 50 mM Mes, 0.4 mM EGTA, 1 mM magnesium acetate, 10 mM NaCl, 10 mM diisothreitol, and 6 µg cGKIα or cGKIβ. Autophosphorylation was initiated by adding 0.1 mM ATP or 0.1 mM ATP combined with 0.1 mM cGMP. In some experiments, aliquots of the reaction mixtures were pre-incubated with cGMP or subsequently treated with lambda protein phosphatase (200 units/µg protein; NEB) at 30°C for 90 min. The reactions were stopped by adding 1x SDS-PAGE loading buffer and heating for 5 min at 95°C. Samples were stored at −20°C.
Phosphorylation of cGKI in cell extracts

MEFs were serum-starved for 3 h in DMEM containing 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C and 6% CO₂. Using a cell scraper, cells were harvested in ice-cold buffer C (20 mM Tris, pH 8.3, 100 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and 200 µg/mL DNAse I) containing 0.1 mg/mL ATP and 0.1 mg/mL ATP combined with 0.1 mg/mL CMP. In some experiments, the reaction mixtures were pre-incubated with cGMP before ATP was added. The reactions were stopped by adding 1x SDS-PAGE loading buffer and heating for 5 min at 95°C. Samples were stored at −20°C.

Western blot analysis

Proteins were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Millipore). Antibodies used were rabbit anti-cGKI common (DH) (1:5000), a pan-specific (nonphospho-specific) antisera detecting both cGKIα and cGKIβ [26], rabbit anti-VASP (1:1000, Cell Signaling, 9A2, 3132), rabbit anti-GAPDH (1:5000, Cell Signaling, 14C10, 2118), and rabbit anti-Akt (1:1000; Cell Signaling, 9272). The polyclonal rabbit antisera against phosho-cGKI species that were generated and characterized in this study were allPS3 (1:1000, PS6 (1:2000), and PS7 (1:2000). According to the detected phospho-site(s), allPS3, PS6, and PS7 are also designated as anti-cGKIα (phospho-Thr58), anti-cGKIα (phospho-Thr84), and anti-cGKIβ (phospho-Thr56, phospho-Ser63, phospho-Ser79), respectively. As secondary antibody, goat anti-rabbit horseradish peroxidase-conjugated IgG (1:3000; Cell Signaling, 7074) was used.

Statistics

Data are expressed as mean ± SEM. Significance was determined by using Student’s t test.

Results

The phosho-specific antisera detect autophosphorylated cGKI isoforms with high specificity and sensitivity

To detect N-terminally phosphorylated cGKI species, we sought to generate phosho-specific rabbit polyclonal antisera against the major in vitro-autophosphorylation sites reported previously: phospho-Ser50, phospho-Thr58, phospho-Ser72, and phospho-Thr84 in cGKIα [11–13] and phospho-Ser63 and phospho-Ser79 in cGKIβ [14,15]. The potential for phosphorylation of these sites was confirmed in silico by a group-based phosphorylation predicting and scoring method [27]. The in silico method did also identify Thr56 of cGKIβ as a potential autophosphorylation site and, therefore, this site was also selected for antibody generation.

As depicted in Table 1, for each selected autophosphorylation site, a phosho-peptide was synthesized that contained the respective phosho-Ser or phosho-Thr residue, and 2 to 3 different peptides were pooled to immune rabbits. The specificity of the antisera was evaluated by ELISAs with the non-phosphorylated and phosphorylated antigenic peptides as well as by Western blot analysis using purified non-phosphorylated and autophosphorylated cGKI isoforms. Polyclonal serum 3 (PS3), which recognized both phospho-Thr58 and nonphospho-Thr58 of cGKIα (data not shown), was subjected to affinity purification against the antigenic phosho-peptide, yielding affinity purified PS3 (AffPS3). Indeed, ELISA results showed that AffPS3 specifically detects the antigenic peptide containing phospho-Thr58, but not the corresponding non-phosphorylated peptide or any other of the tested peptides (Fig. 2A). In addition, two additional non-purified antisera with good specificities for phosphorylated over non-phosphorylated sites were identified by ELISAs: polyclonal serum 6 (PS6) detects phospho-Thr84 of cGKIα (Fig. 2B), and polyclonal serum 7 (PS7) detects phospho-Thr56, phospho-Ser63, and phospho-Ser79 of cGKIβ (Fig. 2C). We did not obtain antisera that recognized specifically phospho-Ser50 or phospho-Ser72 of cGKIβ (data not shown).

Western blots with purified cGKIα and cGKIβ confirmed the isoform- and phosho-specific detection by the respective antisera (Fig. 2D). Autophosphorylation of the purified cGKI isoforms was induced by incubation with ATP (0.1 mM). The non-specific lambda protein phosphatase was added to dephosphorylate the cGKI proteins, confirming that the antibodies indeed recognized the phosphorylated epitopes. As compared to a pan-cGKI antibody [26] that detects ng-amounts of the non-phosphorylated protein (e.g., 20 ng were loaded in Fig. 2D, and 4 ng were loaded in Fig. 3, right panels), the newly generated antisera appeared to recognize phospho-cGKIα species with equal sensitivity in the lower ng-range. In good correlation with the ELISA data (Fig. 2A-C), AffPS3 selectively detected phosphorylated cGKIα, while PS6 showed weak cross-reactivity with non-phosphorylated cGKIα. PS7 recognized predominantly the phosphorylated cGKIβ isoform, but not the non-phosphorylated protein, and showed weak cross-reactivity to phosphorylated cGKIα (Fig. 2D). The ELISA and Western blot results indicated that we obtained three phosho-specific antisera detecting distinct autophosphorylated cGKI species with high specificity and sensitivity; AffPS3 detects phospho-Thr58 of cGKIα, PS6 detects phospho-Thr84 of
cGKIα, and PS7 detects phospho-Thr56, phospho-Ser63, and phospho-Ser79 of cGKIβ (Table 2). These antisera were used for all further experiments.

The antisera do not detect phosphorylation of cGKI in intact mouse cells and tissues

To study cGKI autophosphorylation in vivo, we analyzed murine cell types and tissues that express a functional endogenous cGMP-cGKI signaling system with our phospho-specific antibodies. In the first set of experiments, cultured mouse embryonic fibroblasts (MEFs) and primary aortic vascular smooth muscle cells (VSMCs) were investigated. In general, these cells express both cGKIα and cGKIβ, but MEFs express predominantly cGKIα and VSMCs express more cGKIβ than cGKIα [24]. The status of N-terminal cGKI phosphorylation was monitored under basal conditions and after stimulation of cGKI catalytic activity in intact cells. It was expected that the latter maneuver would increase autophosphorylation of cGKI [6]. The intracellular endogenous cGKI was activated by incubation of the cells with cell-permeable 8-Br-cGMP, with 8-Br-cGMP in combination with 8-Br-cAMP, or with C-type natriuretic peptide (CNP). CNP stimulates GC-B, a particulate guanylyl cyclase, and thereby increases the intracellular cGMP concentration in both MEFs [24] and VSMCs [28]. As readout for cGKI activation, we monitored the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) a known cGKI substrate. VASP can be phosphorylated on Ser157 by multiple kinases including cGKIα, cGKIβ, and cAMP-dependent protein kinase [29,30]. Phospho-VASP (Ser157) migrates at an apparent higher molecular weight compared to dephospho-VASP, which is conveniently detected on Western blots with VASP antibodies. Note that cells and tissues were lysed in buffer containing SDS and phosphatase inhibitors to block potential dephosphorylation of proteins during sample preparation.

As shown in Fig. 3A (upper panels), VASP phosphorylation was induced in wild-type MEFs by treatment with 8-Br-cGMP, 8-Br-cGMP and 8-Br-cAMP, or CNP for 15 min at 37°C. In cGKI-deficient MEFs, 8-Br-cGMP and CNP did not induce VASP phosphorylation, confirming that the cGMP-induced VASP phosphorylation observed in wild-type cells was indeed mediated by cGKI. Combined treatment with 8-Br-cGMP and 8-Br-cAMP still resulted in an increase of phospho-VASP in cGKI-deficient MEFs, most likely because 8-Br-cAMP had activated the cAMP-dependent protein kinase. Surprisingly, although our stimulation protocol clearly induced cGKI phosphotransferase activity in intact MEFs, our phospho-specific cGKI antisera did not detect epitopes on the Western blot that could potentially represent autophosphorylated cGKI species, both under basal and stimulated conditions, and when huge amounts of protein (50 μg) were...
Figure 3. Analysis of N-terminal cGKI phosphorylation in intact MEFs (A) and VSMCs (B). Cells from wild-type (WT) and cGKI-knockout (KO) mice were incubated for 15 min at 37°C under control conditions (PBS), or in the presence of 1 mM 8-Br-cGMP (8-cG), 1 mM 8-Br-cGMP and 10 mM 8-Br-cAMP (8-cG+8-cA), or 100 nM CNP. Then, cells were lysed in denaturating buffer and cell lysates were subjected to Western blot analysis with a pan-(nonphospho-specific) cGKI antibody, anti-VASP, anti-GAPDH, and the phospho-specific antisera AffPS3, PS6, and PS7. Phosphorylation of VASP at Ser157 (p-VASP) was monitored by immunodetection of the band shift to a higher apparent molecular weight. The protein amounts of MEF
lysates loaded were 15 μg for immunostaining with anti-cGKI and anti-VASP, and 50 μg for immunodetection with the phospho-specific antisera; 12 μg of VSMC lysates were loaded; GAPDH was used as respective loading control. Purified proteins (4 ng) were loaded as controls for non-phosphorylated (cGKIα, cGKIβ) and autophosphorylated (p-cGKIα, p-cGKIβ) cGKI isoforms. Similar results were obtained in three independent experiments. doi:10.1371/journal.pone.0098946.g003

Table 2. Phospho-sites detected by the antisera generated in this study.

| Antiserum | Phospho-site detected          |
|-----------|--------------------------------|
| AffPS3    | Thr58 of cGKIα                 |
| PS6       | Thr84 of cGKIα                 |
| PS7       | Thr56, Ser63 and Ser79 of cGKIβ|

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Figure 4. Effect of inhibition of protein Ser/Thr phosphatases on N-terminal cGKI phosphorylation in intact cells. Wild-type MEFs were incubated at 37°C under control conditions (1% DMSO in PBS for 15 min; Ctr), or for 15 min in the presence of 100 nM of the PP1/PP2A inhibitor, calyculin A (Cal A), or for 15 min in the presence of 100 nM calyculin A followed by 15 min with 1 mM 8-Br-cGMP (Cal A+8-cG) or 1 mM 8-Br-PET-cGMP (Cal A+PET-cG). Then the cells were lysed in denaturing buffer and cell lysates (10 μg) were analyzed by Western blotting with the indicated antibodies. GAPDH was used as loading control. The arrows indicate the positions expected for phospho-cGKI species as determined by co-loading of purified proteins on the same gel. Similar results were obtained in three independent experiments. doi:10.1371/journal.pone.0098946.g004
Under in vitro conditions, autophosphorylation of cGKI is preferred as compared to phosphorylation of exogenous substrates.

To get more insights into the apparent difference in cGKI autophosphorylation in vitro versus in vivo, and to evaluate the utility of the new phospho-cGKI antibodies as experimental tools, we performed further experiments with purified proteins and broken-cell preparations. Purified cGKIα was incubated with ATP alone, cGMP and ATP simultaneously, cGMP alone, or it was first pre-incubated with cGMP before ATP was added. Autophosphorylated cGKIα was then detected with antisera AffPS3 and PS6 (Fig. 6A). As expected (Fig. 2D), addition of ATP alone was sufficient to induce efficient autophosphorylation. Interestingly, compared to ATP alone both phospho-specific antisera detected a decreased level of phospho-cGKIα in the presence of cGMP, particularly after pre-incubation of the enzyme with cGMP (Fig. 6A). In contrast, pre-incubation of purified cGKIβ with cGMP did not significantly affect its autophosphorylation (data not shown) suggesting that inhibition of autophosphorylation by cGMP is a specific property of the cGKIα isofrom.

ATP-induced phosphorylation of endogenous cGKIα and its inhibition by cGMP were also observed when cell extracts prepared from MEFs were incubated in vitro with the respective compounds (Fig. 6B). This experiment showed also that our antibodies can be used for the specific detection of phospho-cGKIα species in complex protein mixtures such as cell extracts. Importantly, a comparison of the levels of phospho-VASP and phospho-cGKIα in the presence of ATP alone (Fig. 6B, lane 2) indicated that, at least in in vitro, (a) cGKIα can undergo N-terminal phosphorylation in a non-cGMP-activated state and (b) autophosphorylation alone does not activate heterophosphorylation of substrate proteins like VASP.

Discussion and Conclusions

In this study, phospho-specific antibodies were generated that detect autophosphorylated cGKIα and cGKIβ with high specificity and sensitivity. Autophosphorylation of cGKI could be induced in purified preparations or cell extracts by addition of ATP. However, phospho-cGKI species could not be detected in intact cells and tissues, both under basal conditions and after induction of cGKI catalytic activity. These findings challenge the physiological relevance of the current cGKI activation model that is based on in vitro experiments with purified cGKI. According to this model (Fig. 1B) [6], autophosphorylation is a preferential process as compared to phosphorylation of exogenous substrates. It can be stimulated by cGMP or cAMP and increases the basal catalytic activity of cGKI even after release of the activator. Thus, one would expect that intact tissues contain cGKI in both phosphorylated and non-phosphorylated forms. In contrast to this model, the results of the present study with intact cells and tissues indicate that neither cGKIα nor cGKIβ is phosphorylated in its N-terminal region in vivo, at least at the residues detected by our antisera. Even under conditions that stimulate the catalytic activity of cGKI, no autophosphorylation could be detected. Our phospho-specific antisera detected two of four major in vitro-autophosphorylation sites of cGKIα (phospho-Thr58 and phospho-Thr84) and three potential phospho-sites of cGKIβ (phospho-Thr56, phospho-Ser63, and phospho-Ser79). We cannot exclude phosphorylation of cGKI at Ser/Thr residues that were not recognized by our antibodies. Alternative in vivo phosphorylation sites of cGKI under baseline and activating conditions could be identified by a hypothesis-free approach based, for instance, on mass spectrometry. However, autophosphorylation of these alternative sites would be expected to coincide with autophosphorylation of at least some of the major sites that were detected by our antisera.

Our failure to demonstrate N-terminal autophosphorylation of cGKI in intact cells with phospho-specific antibodies is in...
agreement with previous studies that used other methods to determine the in vivo-phosphorylation status of cGKI. Hou et al. [33] studied the phosphorylation of cGKIα in transfected HEK-293 cells loaded with [32P]PPi and could not detect an increase of [32P]-cGKIα after treatment of the cells with 8-Br-cGMP. Pinske et al. [12] determined the phosphorylation state of purified bovine lung cGKIα by mass spectrometry. The enzyme was completely phosphorylated on Thr516, but no major phosphorylation site was detected in the N-terminal region under basal conditions. Thr516 is localized in the activation loop within the catalytic core of the protein kinase, and its phosphorylation is essential for enzymatic activity [20]. In line with the mass spectrometric data, purified cGKI contains 1.1–1.4 mol phosphate/mol subunit [11]. Together, the results of the present and previous studies strongly suggest that cGKI occurs in vivo as a phosphoprotein that is mainly phosphorylated at Thr516, but shows no N-terminal autophosphorylation.

Our experiments with purified proteins (Figs. 2D and 6A) and broken-cell preparations (Fig. 6B) confirmed that cGKI autophosphorylates its N-terminal region in vitro. Phosphorylation of cGKI could be induced by addition of ATP (0.1 mM) to the purified enzymes or cell extracts. Interestingly, ATP-induced autophosphorylation of cGKIα was inhibited by pre-incubation with saturating amounts of cGMP (0.1 mM). These data are in line with previous studies that reported cGKI autophosphorylation in the presence of ATP alone and its inhibition by the addition of cGMP [8,9]. Indeed, a recent analysis of the interactions of cGKIα with cGMP and ATP by mass spectrometry showed that ATP can enter the ATP-binding site already in the basal state of cGKI in the absence of cGMP, and that the cGKI-ATP interaction is weakened in the cGMP-activated conformation of the kinase [34]. The apparent discrepancy of these results with other studies reporting that cGKI autophosphorylation can be stimulated by cGMP [5,6] might be explained by different cGMP concentrations that were used in the respective autophosphorylation reactions. High and low cGMP concentrations might induce different protein conformations that hinder or boost autophosphorylation, respectively [35,36]. Another interesting finding of our study was that addition of ATP alone led to efficient cGKI phosphorylation in cell extracts without an apparent increase in phosphorylation of the cGKI substrate, VASP (Fig. 6B, lane 2). Taken together, our data indicate that N-terminal phosphorylation of cGKI (a) does
not require, and can be even inhibited by a cGMP-activated conformation of the kinase and (b) does not increase the basal catalytic activity of the kinase toward exogenous substrates in the absence of cGMP.

Why does cGKI readily autophosphorylate in vitro but not in vivo? Considering that purified cGKI autophosphorylates in the presence of 0.1 mM ATP, and that the intracellular ATP concentration is typically 1–10 mM, one would expect that autophosphorylated cGKI occurs in vivo already under basal conditions. However, we did not detect phospho-cGKI in intact cells. This suggests that the conformation and/or environment of the kinase in intact cells differ fundamentally from purified protein and broken-cell preparations, in which autophosphorylation occurred. The balance between auto- and heterophosphorylation could be influenced by the availability of physiological partner proteins of cGKI, such as anchoring and substrate proteins. Purified cGKI preparations lack these factors and cell extracts contain them in much lower concentrations than intact cells. Interestingly, cell extracts showed cGKI autophosphorylation in the absence of VASP phosphorylation (Fig. 6B, lane 2), whereas intact cells demonstrated VASP phosphorylation in the absence of autophosphorylation (Figs. 3, 4, 5). Thus, it appears that under in vitro conditions autophosphorylation is preferred as compared to phosphorylation of exogenous substrates. However, autophosphorylation is obviously prevented in intact cells by the interaction of cGKI with other proteins, and after cGMP activation only heterophosphorylation of substrate proteins occurs. This also implies that autophosphorylation is not involved in cGKI activation in vivo, and we propose to revise the working model of cGKI accordingly (Fig. 1B). The finding that cGKI is most likely not N-termally autophosphorylated in intact cells does also inform screening strategies aiming to identify novel cGKI-binding drugs based on in vitro assays with purified cGKI protein. Contrary to what would be suggested by the previous model that incorporated autophosphorylated cGKI as a relevant enzyme species, our present results strongly suggest that these assays should not be performed with autophosphorylated cGKI.

In conclusion, this study provides important new insights into the structure-function relationship of cGKI in intact cells. Although readily induced in vitro, autophosphorylation of cGKIα and cGKIβ does most likely not occur in vivo. Thus, the catalytic activity of cGKI in intact cells appears to be independent of N-terminal autophosphorylation. These findings also support the general notion that the in vitro- and in vivo-biochemistry of a given protein can be fundamentally different.

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Author Contributions
Conceived and designed the experiments: RV HK RF. Performed the experiments: RV. Analyzed the data: RV HK RF. Wrote the paper: RV HK RF.

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