THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 12, Issue of March 22, pp. 7025–7029, 1996

Printed in U.S.A.

N-terminal Myristoylation Is Required for Membrane Localization of cGMP-dependent Protein Kinase Type II*

(Received for publication, November 28, 1995)

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The apical membrane of intestinal epithelial cells harbors a unique isozyme of cGMP-dependent protein kinase (cGK type II) which acts as a key regulator of ion transport systems, including the cystic fibrosis transmembrane conductance regulator (CFTR)-chloride channel. To explore the mechanism of cGK II membrane-anchoring, recombinant cGK II was expressed stably in HEK 293 cells or transiently in COS-1 cells. In both cell lines, cGK II was found predominantly in the particulate fraction. Immunoprecipitation of solubilized cGK II did not reveal any other tightly associated proteins, suggesting a membrane binding motif within cGK II itself. The primary structure of cGK II is devoid of hydrophobic transmembrane domains; cGK II does, however, contain a penultimate glycine, a potential acceptor for a myristoyl moiety. Metabolic labeling showed that cGK II was indeed able to incorporate [^{3}H]myristate. Moreover, incubation of cGK II-expressing 293 cells with the myristoylation inhibitor 2-hydroxymyristic acid (1 mM) significantly increased the proportion of cGK II in the cytosol from 10 ± 5 to 35 ± 4%. Furthermore, a nonmyristoylated cGK II Gly^2 → Ala mutant was localized predominantly in the cytosol after transient expression in COS-1 cells. The absence of the myristoyl group did not affect the specific enzyme activity or the K_a for cGMP and only slightly enhanced the thermal stability of cGK II. These results indicate that N-terminal myristoylation fulfills a crucial role in directing cGK II to the membrane.

Cyclic GMP-dependent protein kinases (cGK)[1] play an important role in cGMP-mediated signaling pathways which are triggered by various hormones and neurotransmitters including nitric oxide (NO), natriuretic peptides, and guanylin (1, 2). In mammalian tissues, two types of cGK have been identified. Type I cGK, consisting of α and β isoforms, possible splice variants of a single gene, is more generally expressed and acts as a key regulator of cardiovascular homeostasis (1, 2). In contrast, type II cGK was described originally as an intestine-specific form (3). Molecular cloning demonstrated that cGK II is indeed a distinct gene product expressed predominantly in epithelial cells of the intestine (4), although its mRNA was also detected in kidney and brain (4–6). Its widespread distribution in various areas of the brain suggests an important role of cGK II in the NO/cGMP signaling in the central nervous system (6).

In the intestine, cGMP is involved in the regulation of ion and water transport. It inhibits the uptake of NaCl and stimulates the secretion of chloride by activating the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel which is mutated in CF patients (7, 8). Heat-stable enterotoxins increase cGMP and elicit a severe secretory diarrhea by activating an intestine-specific isoform of guanylyl cyclase (9). Guanylin, a heat-stable enterotoxin-like peptide, may function as the physiological activator of the cGMP-mediated signaling pathway in intestine (10). Recent electrophysiological and localization studies suggest a key role for cGK II as a mediator of the cGMP-provoked intestinal chloride secretion (8, 11, 12).

The positioning of cGK II in the apical membrane of the enterocyte, facilitating its interaction with transport systems in the same compartment, may contribute importantly to its efficacy as an ion transport regulator. However, the mode of cGK II membrane anchoring has not been elucidated. The primary sequence of cGK II, like the sequence of type I cGK, a known cytosolic and/or peripheral protein, reveals no obvious hydrophobic transmembrane domains (2, 4). Instead, the membrane localization of cGK II might be determined by its association with a distinct anchor protein. A family of anchor proteins exists for the regulatory subunit of cAMP-dependent protein kinase (cAK) (13), and the intermediate filament protein vimentin may function as a receptor for cGK I (14). Alternatively, cGK II itself may acquire hydrophobic properties through the attachment of a lipid moiety. A number of proteins involved in signal transduction, *e.g.* Src, Ras, and trimeric G-proteins require lipid modifications like myristoylation, palmitoylation, or isoprenylation for membrane binding (15). Type II cGK lacks a consensus sequence for isoprenylation, but the penultimate glycine in cGK II might serve as an acceptor for a myristoyl group (15). We report here that recombinant cGK II expressed in HEK-293 and COS-1 cells is indeed myristoylated and that this lipidation is required for its attachment to the membrane.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and G418 sulfate (Geneticin^R) were obtained from Life Technologies, Inc., 3-isobutyl-1-methylxanthine and 2-hydroxymyristic acid from Sigma, L-[^{35}S]methionine (Tran^{35}S-label^{TM}) from ICN, [^{3}H]myristate (54 Ci/mmol), [^{3}H]palmitate (50 Ci/mmol), the enhanced chemiluminescence (ECL) system and Amplify^{TM} from Amersham, protein A-Sepharose from Pierce, and DEAE-dextran

* This study was supported by a grant from the Netherlands Organization for Scientific Research (NWO) and by Grant SFB 355 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: cGK, cGMP-dependent protein kinase, cAK, cAMP-dependent protein kinase; CFTR, cystic fibrosis transmembrane conductance regulator; PAGE, polyacrylamide gel electrophoresis.

from Pharmacia Biotech Inc. A polyclonal cGK II antibody, raised against recombinant rat cGK II expressed in *Escherichia coli*, was prepared as described (11). Preparation of the cGK II expression vector pRc/CMV-cGK II was described earlier (4). The cGK substrate peptide 2A3 (RRVSKQE) and the Walsh inhibitor peptide (PKI-(5–24)-amide) were synthesized by D. Palm (University of Würzburg, Germany). Purified oligonucleotides were obtained from Eurogentec.

Construction of a Nonmyristoylatable cGK II Mutant G2A—A cGKII-G2A mutant in which Gly^2 was changed to Ala was generated directly from the pRc/CMV-cGK II plasmid using the Transformer™ site-directed mutagenesis kit (Clontech), with CTGAGCAACATGGCAAATG-GTTCAGTG as the mutagenic primer and CACTAGTTCTAGTGGGC-CCTATTC as the selection primer targeted to the unique *Xba*I site. The mutation was confirmed by sequencing the G2A plasmid using the Sequenase® DNA Sequencing Kit (United States Biochemical Corp.).

Expression of cGK II in HEK 293 and COS-1 Cells—The pRc/CMV-cGK II expression vector was transfected into HEK 293 cells as described (4). Stable transfectants were selected from individual clones growing in the presence of G418 sulfate. The clone expressing the highest level of cGK II was used throughout this study and cultured in the presence of G418 sulfate (200 μg/ml). COS-1 cells were transfected 1 day after subculturing at 80–90% confluency, by means of a 20-min incubation at 25 °C with 0.5 mg/ml DEAE-dextran in phosphate-buffered saline containing 1 μg of vector DNA per 10^6 cells. Cells were harvested 2 days after transfection as described for the individual analyses that follow.

Membrane Isolation and Subcellular Fractionation—Confluent cells were washed twice with ice-cold phosphate-buffered saline, scraped with a rubber policeman in buffer A (150 mM NaCl, 10 mM $NaPO_4$, pH 7.4, 1 mM EDTA, 100 μg/ml trypsin inhibitor, and 20 μg/ml leupeptin), and processed directly or frozen in liquid N_2 and stored at −80 °C. The cells were homogenized by brief sonication (three bursts of 3 s, peak-to-peak amplitude 15–20 μm). Cytosol and membranes were separated by centrifugation at 150,000 × *g* for 60 min at 4 °C in an airfuge.

Metabolic Labeling and Immunoprecipitation of cGK II—Confluent cells were incubated at 37 °C for 4 h in Dulbecco's modified Eagle's medium or in Dulbecco's modified Eagle's medium without methionine, supplemented with 2% fetal calf serum and either 0.1 mCi/ml L-[^{35}S]methionine, 0.25 mCi/ml [^{3}H]myristate, or 0.5 mCi/ml [^{3}H]palmitate. Labeled cells were washed twice with phosphate-buffered saline, and solubilized for 5 min with 1% Triton X-100 in ice-cold buffer A in the presence of 0.5 M NaCl. After a short spin (5 min at 20,000 × *g*), the supernatant fluid was incubated for 30 min at 0 °C with cGK II antibody (1:200) and centrifuged for 45 min at 20,000 × *g*. The proteins bound to the antibody were precipitated with protein A-Sepharose (5 μl of packed gel/μl of antiserum) and visualized by indirect autoradiography using Amplify™ as described (16).

Immunoblotting and Protein Kinase Assays—Immunoblotting was performed as described earlier (16). Samples (10–20 μg of protein) were separated by SDS-PAGE and blotted onto nitrocellulose. Blots were incubated with the cGK II antibody (1:3000), labeled using the ECL method, and cGK II was quantitated by densitometric scanning (Bio-Rad, model 620).

Protein kinase activity was determined by incubating samples (5–10-μl aliquots containing 5–10 μg of protein) at 30 °C for 10 min in 50 μl of 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM 3-isobutyl-1-methylxanthine, 200 nM protein kinase A inhibitor, 0.1 mM [γ-^{32}P]ATP (200 cpm/pmol), and a 0.1 mg/ml concentration of a cGK-specific substrate peptide 2A3 (RRKVSKQE) as described (17). To reduce high nonspecific background phosphorylation and to facilitate the exposure of membrane-bound cGK II to the exogenous substrate, 1% Triton X-100 and 0.5 M NaCl were added to all fractions prior to assay.

Thermostability Assays—Homogenized cells (15-μl aliquots containing 15–30 μg of protein) were incubated for 3 min at various temperatures prior to determination of protein kinase activity by incubation for 5 min at 30 °C with the phosphorylation mixture (25 μl) described above additionally containing 0.3% Triton X-100.

RESULTS AND DISCUSSION

High speed centrifugation of homogenates of HEK 293 cells stably expressing rat cGK II resulted in recovery of 90–95% of expressed cGK II in the membrane fraction as detected by Western blotting (*cf.* Fig. 3). The enzyme could be released from the membranes by a combination of detergent (1% Triton X-100) and high salt (0.5 M NaCl) but not by detergent (Fig. 1) or high salt alone (not shown). This suggests that recombinant

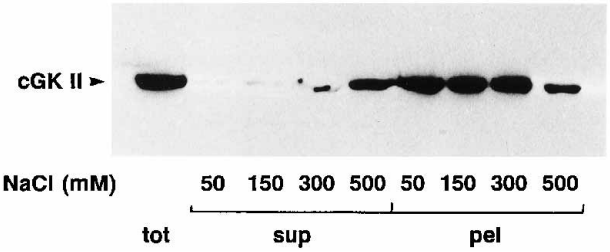

FIG. 1. **Western blot demonstrating that solubilization of cGK II requires high salt in combination with Triton X-100.** HEK 293 cells stably transfected with pRc/CMV-cGK II were homogenized and centrifuged at 20,000 × *g* to obtain a heavy membrane fraction which was then incubated with 1% Triton X-100 for 5 min at 0 °C in the presence of various concentrations of NaCl as indicated. Subsequently, samples were removed before and after centrifugation (15 min, 20,000 × *g*) for detection of cGK II present in the total extract (*tot*) and supernatant (*sup*) and pellet (*pel*) fractions by immunoblotting. The results shown are representative of three experiments.

cGK II is attached both to the membrane and the cytoskeleton in HEK 293 cells, resembling native cGK II in rat or pig intestinal brush-border membranes (3), and argues against the need for an intestine-specific factor for targeting the enzyme to the membrane. Conceivably, membrane attachment could occur through cGK II binding to a more ubiquitous anchor. However, we did not detect any proteins associated with cGK II in immunoprecipitates from HEK 293 cells metabolically labeled with [^{35}S]methionine (Fig. 2*A*). Furthermore, native cGK II still displays hydrophobic properties after its solubilization and purification (3). Therefore, the membrane binding of cGK II is unlikely to depend solely on its association with anchor proteins.

The alternative possibility that cGK II may be lipid-modified was tested by incubating HEK 293 cells stably expressing cGK II with [^{3}H]myristate. As shown in Fig. 2*B*, a protein with the correct M_r (86,000) for cGK II was the major radioactively labeled protein in a total lysate. This myristoylated protein could be precipitated with a cGK II specific antibody, further confirming its identity as cGK II. No evidence was obtained for a second covalent modification by palmitoylation, since no radioactivity was detected in cGK II immunoprecipitated from transfected 293 cells metabolically labeled with [^{3}H]palmitate (not shown).

In order to investigate whether myristoylation plays a role in the membrane attachment of cGK II, the cGK II-expressing 293 cells were incubated for 48 h with a 1 mM concentration of the myristoylation inhibitor 2-hydroxymyristic acid (18). Inhibitor treatment significantly increased the amount of cGK II recovered in the cytosolic fraction (35 ± 4%) compared with that of control cells (10 ± 5%) (n = 5 for each group; Fig. 3). Metabolic labeling of the cells with [^{3}H]myristate in the presence of 2-hydroxymyristic acid showed that the form of cGK II accumulating in the cytosol was nonmyristoylated (Fig. 3), indicating that shift in cGK II topology from the membrane to the cytosol was indeed due to inhibition of myristoylation. High salt (0.5 M NaCl) had no effect on the amount of cGK II recovered in the cytosol fraction of cells preincubated with 2-hydroxymyristic acid, indicating that the fraction of cGK II which remained bound to the membrane was anchored by hydrophobic interactions.The lack of a complete reallocation of cGK II to the cytosol most likely reflects an incomplete blockade of myristoylation by the inhibitor, since [^{3}H]myristate incorporation was still detectable in the membrane-bound cGK II pool after treatment with 2-hydroxymyristic acid (Fig. 3).

To further investigate the membrane binding properties of nonmyristoylated cGK II, we mutated the penultimate glycine to an alanine. As shown in Fig. 4*A*, this G2A mutant, in con-

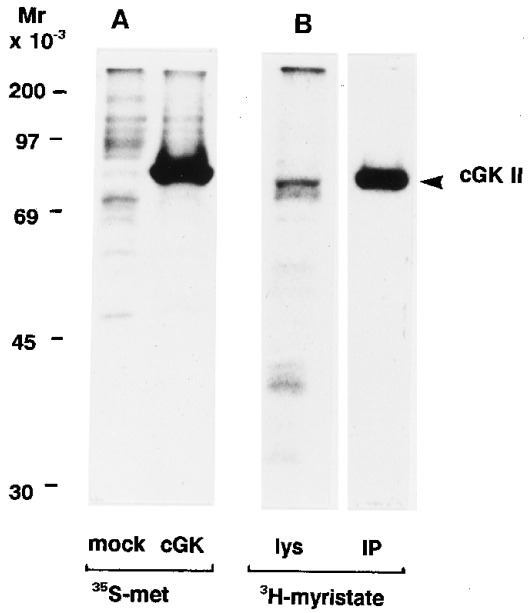

FIG. 2. **Autoradiogram of cGK II immunoprecipitated from HEK 293 cells metabolically labeled with [^{35}S]methionine or [^{3}H]myristate.** *A*, HEK 293 cells mock-transfected or stably transfected with pRc/CMV-cGK II were incubated for 4 h with [^{35}S]methionine and lysed with 1% Triton X-100 and 0.5 M NaCl. cGK II was immunoprecipitated with a specific antibody and analyzed using 10% SDS-PAGE and autoradiography (4 days). *B*, HEK 293 cells stably transfected with pRc/CMV-cGK II were incubated for 4 h with [^{3}H]myristate, lysed with 1% Triton X-100 and 0.5 M NaCl, and analyzed by 10% SDS-PAGE prior to (*lys*) or after immunoprecipitation (*IP*) with cGK II antibody. Label incorporated was detected by autoradiography (30 days).

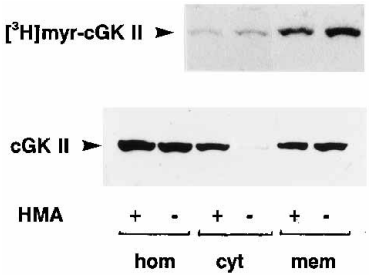

FIG. 3. **2-Hydroxymyristic acid promotes cytosolic localization of cGK II by inhibiting myristoylation.** HEK 293 cells stably transfected with pRc/CMV-cGK II were incubated for 48 h with or without 1 mM myristoylation inhibitor 2-hydroxymyristic acid (*HMA*). Subsequently, cells were either homogenized and cGK II was determined in the homogenate (*hom*), cytosolic (*cyt*), and membrane (*mem*) fractions by immunoblotting (*lower panel*) or cells were incubated for an additional 4 h with [^{3}H]myristate prior to homogenization and separation into a cytosolic or membrane fractions. Incorporation of [^{3}H]myristate into cGK II was detected by autoradiography (20 days) following immunoprecipitation of cGK II and subsequent separation on 7.5% SDS-PAGE (*upper panel*).

trast to wild type cGK II, was unable to incorporate [^{3}H]myristate after transient expression in COS-1 cells, although wild type and mutant cGK II were expressed to similar levels as detected by immunoblotting (Fig. 4*B*). As observed earlier in HEK 293 and intestinal cells, wild type cGK II was predominantly bound to the membrane fraction of COS-1 cells (Fig. 4*C*). The removal of Gly^{2}, however, resulted in the shift of a major proportion of cGK II from a membrane to a cytosolic localization (Fig. 4*C*), providing further evidence that myristoylation is required for membrane binding of cGK II. The small amount of the G2A-cGK II mutant recovered in the membrane fraction most plausibly represents cytosolic enzyme entrapped in incompletely lysed cells or in vesiculated membranes.

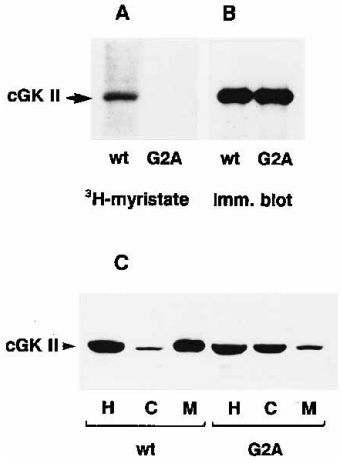

FIG. 4. **Localization of the nonmyristoylated cGK II mutant G2A in COS-1 cells.** COS-1 cells were transiently transfected with pRc/CMV-cGK II (*wt*) or a mutant-cGK II in which Gly^{2} was changed to Ala (G2A). *A* and *B*, 2 days after transfection, cells were labeled with [^{3}H]myristate and cGK II was immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography (20 days) (*A*) or immunoblotting (*B*). *C*, 2 days after transfection, cGK II was determined in the homogenate (*H*), cytosolic (*C*), and membrane (*M*) fractions by immunoblotting.

Paradoxically, mouse brain cGK II, which displays a high level of homology to rat intestine cGK II (4, 5), was reported to reside predominantly (98%) in the cytosol when expressed in COS-1 cells (5). This subcellular localization of mouse cGK II, however, was based on measurements of protein kinase activity rather than on immunological detection. Therefore, we compared the kinase activities of rat cGK II and the G2A mutant in the homogenate, cytosolic, and membrane fraction of COS-1 cells. As shown in Fig. 5, only a small proportion of the cGK II activity was present in the cytosol compared to the membrane fraction of COS-1 transfected with wild type cGK II, whereas most of the activity of the nonmyristoylated G2A mutant was cytosolic. The kinase activities correlated well with the levels of immunodetectable cGK II in the various fractions (compare Figs. 4 and 5), indicating that the specific activity of the myristoylated and nonmyristoylated forms of cGK II are not substantially different. Likewise, the absence of a myristoyl group did not alter the K_a for cGMP (0.7 μM for both wild type cGK II and the G2A mutant; Fig. 6). The K_a for cGMP observed here for rat cGK II resembles the K_a (0.3 μM) reported for mouse brain cGK II (5). These data therefore failed to provide a clear explanation for the difference in localization found for rat and mouse cGK II transiently expressed in COS-1 cells. A species difference in cGK II membrane anchoring properties was also considered, but found unlikely because: (i) the first nine amino acids of mouse brain and rat intestine cGK II, which cover the region normally containing the myristoylation sequence are identical, and (ii) native cGK II from rat and mouse intestine as well as from rat brain were found to be largely membrane-associated (data not shown).

While these results indicate that myristoylation is a prerequisite for membrane binding of cGK II, additional factors might contribute as well. Some myristoylated proteins like the catalytic subunit of cAK are soluble (19), or reversibly attached to the membrane (*e.g.* MARCKS; Ref. 20). Therefore, a second membrane binding motif in combination with the myristoyl group is thought to be required for membrane attachment (21). This second motif was shown to be either a polybasic region, which interacts with the negatively charged inner membrane surface, or a second lipid moiety like palmitic acid (21). No evidence for palmitoylation of cGK II was obtained in this

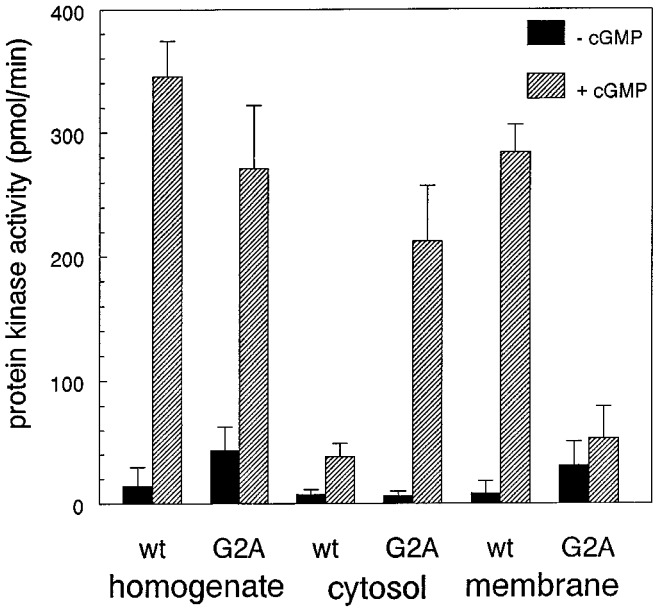

FIG. 5. **Subcellular distribution of the kinase activity of cGK II and the nonmyristoylated cGK II mutant G2A.** COS-1 cells were transiently transfected with either pRc/CMV-cGK II (*wt*), a cGK II mutant in which Gly[2] was changed to Ala (*G2A*), or the pRc/CMV vector. Two days after transfection, cells were harvested, homogenized, and fractionated. Kinase activity in the presence or absence of cGMP (5 μM) was determined after solubilization of cGK II with 1% Triton and 0.5 M NaCl, as described under "Experimental Procedures." cGK II kinase activity was expressed per mg of homogenate protein and corrected for basal activity found in cells transfected with the pRc/CMV vector. The basal kinase activities of samples from mock-transfected COS-1 cells were 92, 94, 13, 17, 61, and 58 pmol/min, respectively, for the homogenate −/+-cGMP, the cytosol −/+-cGMP, and the membrane fraction −/+-cGMP. Data are expressed as means ± S.E. of three experiments.

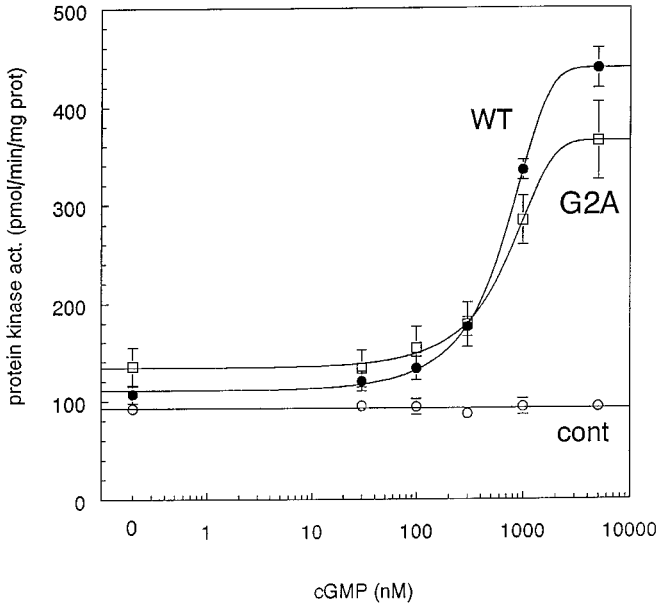

FIG. 6. **Activation of cGK II and the nonmyristoylated cGK II mutant G2A by cGMP.** COS-1 cells were transiently transfected with either pRc/CMV-cGK II (*WT*; ●), a cGK II mutant in which Gly[2] was changed to Ala (*G2A*; □), or the pRc/CMV vector (*cont*; ○). Two days after transfection, cells were harvested and homogenized. Kinase activity in the presence of various concentrations of cGMP was determined in the homogenates after solubilization of cGK II with 1% Triton and 0.5 M NaCl, as described under "Experimental Procedures." Data are expressed as means ± S.E. of three experiments.

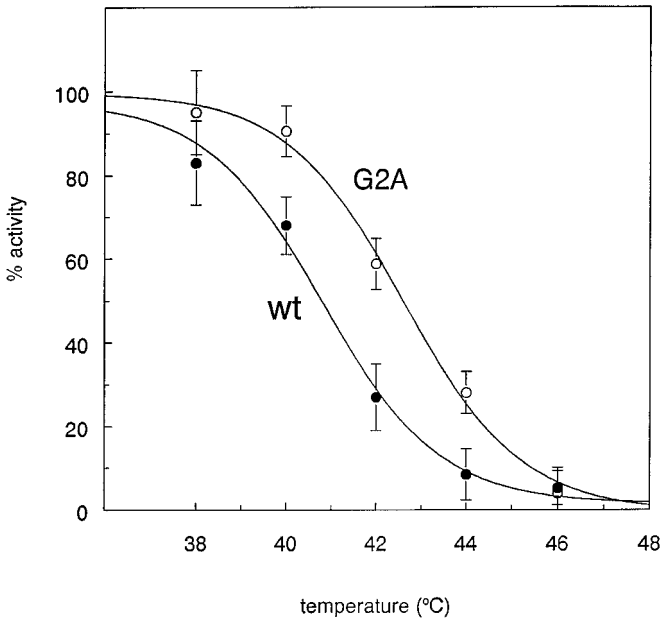

FIG. 7. **Temperature sensitivity of cGK II and the nonmyristoylated cGK II mutant G2A.** COS-1 cells were transiently transfected with either pRc/CMV-cGK II (*wt*; ●) or with a cGK II mutant in which Gly[2] was changed to Ala (*G2A*; ○). Two days after transfection, cells were harvested and homogenized. The homogenates were incubated for 3 min at various temperatures prior to determination of kinase activity in the presence or absence of 10 μM cGMP for 5 min at 30 °C as described under "Experimental Procedures." The cGMP-stimulated kinase activity is expressed as a percentage of the cGMP-stimulated kinase activity in homogenates which were preincubated for 1 min at 30 °C prior to the kinase assay. Data are expressed as means ± S.E. of three experiments.

study. However, cGK II, similar to cGK I (2), appears to function as a dimer under physiological conditions,[2] suggesting that two myristoyl groups per molecule of cGKII are available for membrane binding. The first three lysines in cGKII (Lys[7], Lys[9], Lys[12]), located at similar positions as the lysines involved in membrane binding of Src (21), may also contribute to the membrane binding.

In addition to its role in membrane anchoring, myristoylation was also reported to enhance the stability of certain proteins. Notably, the myristoylated catalytic subunit of cAK is considerably less temperature-sensitive compared to the nonmyristoylated form (22). We therefore compared the thermal stability of cGK II and the nonmyristoylated G2A mutant. As shown in Fig. 7, G2A was slightly more stable than wild type cGK II (1.5–2 °C), indicating that myristoylation does not stabilize cGK II. This suggests that the myristoyl group in cGK II, in contrast to its counterpart in the catalytic subunit of cAK (23), is not involved in intramolecular stabilization, but is fully accessible to membrane interaction.

Conceivably, the intrinsic hydrophobicity of myristoylated cGK II promotes its association with membrane-bound substrates. Two physiologically relevant substrates of cGK II have been described so far, *i.e.* a 25-kDa proteolipid detected in intestinal brush-border membranes whose function is still unknown (24), and the CFTR-Cl channel, which is responsible for the cGMP-mediated electrogenic chloride secretion in the intestine (8, 12). Interestingly, purified cGK II, but not cGK Iα, is able to activate the CFTR-Cl channel in excised membrane patches of cells stably expressing CFTR (12), whereas both isotypes phosphorylate immunoprecipitated CFTR *in vitro* (12, 25). Since the N terminus of cGK Iα is acetylated rather than myristoylated (26), it is tempting to speculate that the different

[2] A. B. Vaandrager, unpublished results.

lipidation and membrane binding properties of cGK II and I, rather than their substrate specificities, account for the preferential activation of CFTR by cGK II. By analogy, modulation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor channel by endogenous cAK was shown to be dependent on the binding of cAK to an anchoring protein, presumably localized in close proximity to the channel (27).

The availability of a soluble, catalytically active, nonmyristoylated mutant version of cGK II may greatly facilitate future studies designed to investigate the putative role of membrane anchoring in cGK II regulation of transport functions by a more direct approach.

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