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Isotype-specific Activation of Cystic Fibrosis Transmembrane Conductance Regulator-Chloride Channels by cGMP-dependent Protein Kinase II*

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Pim J. French, Jan Bijman, Marcel Edixhoven‡, Arie B. Vaandrager‡, Bob J. Scholte, Suzanne M. Lohmann§, Angus C. Nairn¶, and Hugo R. de Jonge‡‖

From the Departments of Cell Biology and ‡Biochemistry, Faculty of Medicine and Health Sciences, Erasmus University, 3000 DR Rotterdam, The Netherlands, the §Laboratory of Clinical Biochemistry, Medical University Clinic, 97080 Würzburg, Federal Republic of Germany, and the ¶Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York 10021-6399

Type II cGMP-dependent protein kinase (cGKII) isolated from pig intestinal brush borders and type Iα cGK (cGKI) purified from bovine lung were compared for their ability to activate the cystic fibrosis transmembrane conductance regulator (CFTR)-Cl^- channel in excised, inside-out membrane patches from NIH-3T3 fibroblasts and from a rat intestinal cell line (IEC-CF7) stably expressing recombinant CFTR. In both cell models, in the presence of cGMP and ATP, cGKII was found to mimic the effect of the catalytic subunit of cAMP-dependent protein kinase (cAK) on opening CFTR-Cl^- channels, albeit with different kinetics (2–3-min lag time, reduced rate of activation). By contrast, cGKI or a monomeric cGKI catalytic fragment was incapable of opening CFTR-Cl^- channels and also failed to potentiate cGKII activation of the channels. The cAK activation but not the cGKII activation was blocked by a cAK inhibitor peptide. The slow activation by cGKII could not be ascribed to counteracting protein phosphatases, since neither calyculin A, a potent inhibitor of phosphatase 1 and 2A, nor ATPγS (adenosine 5′-*O*-(thiotriphosphate)), producing stable thiophosphorylation, was able to enhance the activation kinetics. Channels preactivated by cGKII closed instantaneously upon removal of ATP and kinase but reopened in the presence of ATP alone. Paradoxically, immunoprecipitated CFTR or CF-2, a cloned R domain fragment of CFTR (amino acids 645–835) could be phosphorylated to a similar extent with only minor kinetic differences by both isotypes of cGK. Phosphopeptide maps of CF-2 and CFTR, however, revealed very subtle differences in site-specificity between the cGK isoforms. These results indicate that cGKII, in contrast to cGKIα, is a potential activator of chloride transport in CFTR-expressing cell types.

Guanosine 3′,5′-cyclic monophosphate (cGMP) has been identified as an important intracellular mediator of salt and water secretion in intestinal epithelium (1–3). Secretagogues acting through the cGMP-signaling pathway include the family of heat-stable enterotoxins (STs),[1] low molecular weight peptides secreted by enteropathogenic bacteria, and guanylin, a recently discovered endogenous ST-like peptide hormone (3, 4). Binding of ST or guanylin to the receptor domain of an intestine-specific isoform of guanylyl cyclase (GC-C) triggers cyclase activation, cGMP accumulation, and stimulation of net fluid secretion through the activation of apical Cl^- channels in parallel with inhibition of coupled NaCl transporters (3, 5, 6). The cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial Cl^- channel mutated in CF patients (7, 8), appears to be involved in the Cl^- secretory response to ST and cGMP analogues, as evidenced by the absence of this response in CF intestine (9, 10).

Several mechanisms have been proposed to link cGMP to the CFTR-Cl^- channels, including (i) cGMP cross-activation of cAMP-dependent protein kinase (11–13) followed by multisite-phosphorylation of CFTR (14), (ii) direct interaction of cGMP with the CFTR protein (15), and (iii) cGMP activation of an intestine-specific isoform of cGMP-dependent protein kinase (type II cGK; Refs. 16–19). cGKII was discovered as a cGMP-sensitive 86-kDa phosphoprotein localized in intestinal brush border membranes (16), which comigrated with a cGMP receptor protein on one- and two-dimensional gels (17, 18). The intestinal isoform is clearly distinct from the homodimeric type Iα and Iβ cGK (153–156 kDa) identified in other mammalian tissues, as illustrated by differences in subcellular localization, subunit composition, isoelectric point, phosphopeptide maps, immunoreactivity, and affinity for cyclic nucleotide analogues (17–19). Recently, molecular cloning of cGKII from mouse brain (20) and rat intestine (21) demonstrated that cGKII is a different gene product than cGKIα and Iβ (22, 23).

In the present study, evidence for a functional difference between cGK isoenzymes was obtained from studies of the activation of CFTR-Cl^- channels in excised membrane patches of an intestinal cell line (IEC-CF7; Ref. 24) or NIH-3T3 fibroblasts stably expressing recombinant CFTR (25). In both models, exposure of patches to a combination of cGMP and ATP failed to elicit Cl^- channel activity. However, the further addition of purified cGKII, but not cGKIα, resulted in almost full activation of CFTR-Cl^- currents.

Differential activation of the CFTR-Cl^- channel by cGKII is the first example of isotype specificity in cGK regulation of

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‖ To whom reprint requests should be addressed: Dept. of Biochemistry, Cardiovascular Research Institute COEUR, Medical Faculty, Erasmus University, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. Tel.: 31-104087324; Fax: 31-104360615.

[1] The abbreviations used are: ST, heat-stable enterotoxin; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; PKI, Walsh inhibitor peptide (PKI(5–24)-amide); ATPγS, adenosine 5′-*O*-(thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.

cellular functions and provides a plausible explanation for the prominent role of cGMP as a regulator of Cl^- transport in intestinal epithelium in comparison to other CFTR-expressing cell types in which cGKII expression is marginal or absent (19–21).

EXPERIMENTAL PROCEDURES

Materials—Calyculin A was obtained from Calbiochem, San Diego, CA. PKI, the Walsh inhibitor peptide (PKI(5–24)-amide) was obtained from Dr. U. Walter, Würzburg, Germany. CF-2, a cloned R domain peptide of CFTR (AA645–835), was produced in bacteria and purified as described (26). Adenosine 5′-triphosphate, sodium salt (ATP), and cGMP were obtained from Boehringer Mannheim. [γ-^{32}P]ATP was obtained from Amersham, UK. All other chemicals were from Sigma.

Cells and CFTR Expression Systems—Two cell types that stably express CFTR were prepared and maintained as described previously (24, 27). IEC-CF7 cells were obtained by stable transfection of the rat fetal intestine-derived IEC-6 cell line with a plasmid encoding CFTR (24); NIH-3T3 cells expressed CFTR after infection with a retroviral vector encoding CFTR (27).

Isolation of Protein Kinases—cGKII was purified from the small intestine of adult pigs (donated by the Department of Experimental Cardiology, Erasmus University). The small intestine was dissected from anesthetized pigs, rinsed with ice-cold 0.9% NaCl, and frozen in liquid nitrogen. Brush border membrane vesicles were prepared from the intestinal pieces by a freeze-thaw procedure and subsequent differential Mg^{2+} precipitation and centrifugation as described previously (28). cGKII extraction from the vesicles and purification by affinity chromatography on 8-(2-aminoethyl)-amino-cAMP-Sepharose was performed essentially as described (17, 18) with a slight modification. To obtain detergent-free enzyme for use in the patch clamp experiments, elution of cGKII from the affinity gel with 1 mM cGMP was performed in the presence of 4 mM octyl glucoside rather than Triton X-100. Subsequently, cGMP and octyl glucoside were removed by dialysis in detergent-free patch clamp medium (see below).

The catalytic subunit of type II cAK and cGKI (characterized by antibody analysis to be primarily the Iα isoform; Ref. 29) were purified from bovine heart and bovine lung, respectively, as described (30, 31). The specific activities (units/mg protein) of the purified protein kinases as determined by the Kemptide phosphorylation assay (32) were 4.2 (cAK), 2.0 (cGKI), and 1.6 (cGKII), respectively. A monomeric constitutively active cGKI fragment was obtained by limited trypsinization as described (33).

Patch Clamp Technique—Patch clamp experiments were performed as described by Hamill *et al.* (34). Glass (borosilicate) pipettes were pulled to a resistance of 308 megohms and heat polished. Pipette potential refers to the voltage applied to the pipette interior with reference to the bath potential. Upward deflections denote negative charge flowing out of the pipette. A List EPC-7 amplifier was used for current amplification and voltage clamping. Membrane voltage was continuously clamped at −40 mV, except when performing current-voltage relationships. Data were monitored on an oscilloscope and stored on a VCR. The recorded data were filtered at 50 or 100 Hz, digitized at 200 Hz, and analyzed on a personal computer. Data analysis was performed as described by Kansen *et al.* (35). In view of the fact that the high density of CFTR-Cl^- channels in membrane patches of the 3T3-CFTR fibroblasts hinders the accurate determination of open state probability or number of channels, channel activity in these patches was expressed in pA, rather than as the number of open channels. The composition of the bath and pipette solutions was (in mM): 140 *N*-methyl-D-glucamine, 1 EGTA, 3 $MgCl_2$, and 10 Hepes-HCl (pH 7.3, final Cl^- concentration 147 mM). In some experiments a low Cl^- pipette buffer was used containing (in mM): 140 *N*-methyl-D-glucamine, 100 L-aspartic acid, 2 $MgCl_2$, 5 $CaCl_2$, and 10 Hepes-HCl (pH 7.3; final Cl^- concentration 49 mM). Excised patches were studied in a solution exchange compartment (volume 1 ml), as described previously by Kansen *et al.* (36). Experiments were performed at room temperature.

In Vitro Phosphorylation of CFTR and CF-2 and Phosphopeptide Mapping—CFTR was immunoprecipitated from T84 cells using specific CFTR antibodies and protein A-Sepharose beads as described (26). CFTR attached to protein A-Sepharose beads (10 μl of suspension) or CFTR antibody plus beads alone was incubated at 30 °C for 40 min in 100 μl of buffer containing 10 mM $MgCl_2$, 1 mM EGTA, 10 mM Hepes, pH 7.3, 50 μM MgATP, 20 μM cGMP, 10 Ci/mmol [γ-^{32}P]ATP, and purified protein kinases (catalytic subunit of cAK, 2 milliunits/ml; cGKI, 7.5 milliunits/ml; cGKII, 9.4 milliunits/ml). The phosphorylated samples were washed, resuspended in SDS-stop solution, and analyzed on 6% SDS-PAGE as described previously (26). Phosphorylation conditions for CF-2 (5 μM) were similar to those for CFTR. For kinetic experiments, linear incorporation of ^{32}P with time was ensured by (i) restricting the incubation time to 5 min, (ii) varying the CF-2 concentrations between 0.1 and 0.5 μM, (iii) increasing the [γ-^{32}P]ATP concentration to 500 μM (1 Ci/mmol), and (iv) using equal concentrations (25 nM) of each protein kinase. Reactions were terminated by the addition of 20 μl of 70% trichloroacetic acid, and protein pellets were washed three times with 0.2 ml of ice-cold H_2O, suspended in 50 μl of SDS-stop buffer, boiled for 2 min, and subjected to 12% SDS-PAGE as described (26). ^{32}P-Labeled CF-2 or CFTR were excised from the dried gels, washed with two changes of 10% acetic acid/30% methanol and three changes of 50% methanol, and lyophilized. In the kinetic experiments, the incorporation of ^{32}P into CF-2 was quantified by liquid scintillation spectrometry. For two-dimensional phosphopeptide mapping of CF-2 and CFTR, 1 ml of 50 mM NH_4CO_3, pH 8.0, containing L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (50 μg/ml) was added to the dried gel pieces and the mixture was incubated at 37 °C for 20 h (26). The gel pieces were washed with 0.5 ml of 50 mM NH_4HCO_3 at 37 °C for 4 h, and the collected supernatants were lyophilized. Phosphopeptides were separated on thin layer cellulose sheets (20 × 20 cm, Eastman Kodak Co.) by electrophoresis in the first dimension, followed by chromatography in the second dimension. Dried sheets were subjected to autoradiography.

RESULTS

Activation of CFTR-Cl^- Channels by Protein Kinases—In agreement with earlier studies of Cl^- channel activation in excised, inside-out membrane patches from 3T3-CFTR fibroblasts (25, 37, 38), the addition of catalytic subunit of cAK (2 milliunits/ml) to the bath, in the presence of 2 mM MgATP, resulted in rapid activation (lag time < 1 min) of multiple anion-selective channels (average current increase per patch 40 ± 38 pA at −40 mV holding potential; n = 15) showing characteristic properties of the CFTR-Cl^- channel (*i.e.* Cl^- selectivity, linear current-voltage relationship in symmetrical Cl^- concentrations, 8 pS single channel conductance; results not shown). A similar low conductance channel, occurring at a much lower density (2–6 channels/patch) was activated by cAK in excised patches from the rat intestinal IEC-CF7 cell line stably expressing CFTR (24). In addition to cAK, the effect of cGK on CFTR-Cl^- channel opening was examined in excised, cell-free patches from both cell lines. Addition of MgATP (2 mM) and cGMP (50 μM) together did not activate current in 3T3-CFTR patches during 5–15 min observation (Fig. 1*A*). Inclusion of the cGKI isoform purified from bovine lung (10 milliunits/ml) likewise failed to open the CFTR-Cl^- channel (Fig. 1*A*), confirming earlier observations by Berger *et al.* (38). However, the subsequent addition of saturating concentrations of the cGKII (10 milliunits/ml) elicited a large Cl^- current, reaching a value after 15 min that was 78 ± 24% (n = 5) of the maximal current attained upon addition of saturating amounts of cAK (2 milliunits/ml) to the same patch (Fig. 1*A*). Half-maximal cGKII-activation (48 ± 9% of the maximal cGKII response at 15 min; n = 5) was observed in the presence of 2 milliunits/ml cGKII, whereas the threshold for current activation (10 ± 6%; n = 5) was found at 0.5 milliunits/ml cGKII (data not shown). In comparison to cAK, CFTR-Cl^- current activation by cGKII was a relatively slow process (time required to reach half-maximal activation following addition of 10 milliunits/ml cGKII: 8 ± 1 min; for 2 milliunits/ml cAK: 0.7 ± 0.2 min, n = 6; Fig. 1, *A* and *B*). This slow activation was unlikely to result from the activity of counteracting protein phosphatases, since neither calyculin A (10^{-7} M), a potent inhibitor of phosphatase 1 and 2A (recently implied in CFTR regulation; Refs. 38 and 39), nor the additional presence of ATPγS (1 mM) to produce stable thiophosphorylation, was able to enhance the CFTR activation kinetics (results not shown).

The cGKII isoform, but not cGKI, was capable of activating CFTR-Cl^- channels also in excised patches from IEC-CF7 cells

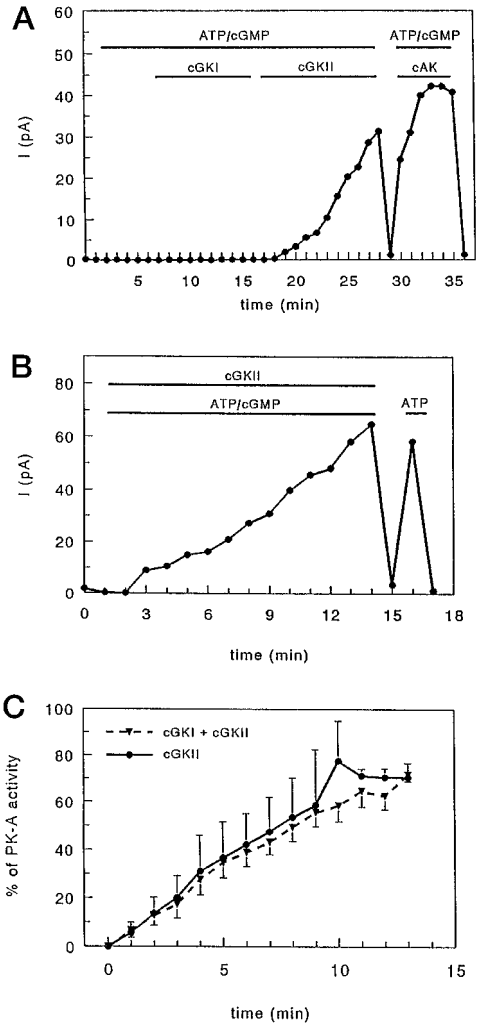

FIG. 1. **cGKII but not cGKI activates CFTR-Cl⁻ channel current in excised, inside-out membrane patches from 3T3-CFTR fibroblasts.** ATP (2 mM), cGMP (50 μM), cGKII (10 milliunits/ml), and catalytic subunit of cAK (2 milliunits/ml) were present in the cytosolic (bath) solution during the times indicated by the *bars*. *Panel A*, comparison of the effects of cGKI, cGKII, and cAK. The amount of current activated by cGKII was 78 ± 24% (n = 5) of the current measured after subsequent addition of cAK. *Panel B*, a representative example of an experiment showing that the cGK-activated currents rapidly returned to near base-line values upon removal of the kinase and ATP from the bath (also shown for the cAK-activated currents in *panel A*), but could be restored almost instantaneously by the readdition of ATP alone. *Panel C*, time course of activation of CFTR-Cl⁻ current by cGKII alone (●; 10 milliunits/ml) or a combination of cGKI (10 milliunits/ml) and cGKII ( ); n = 6. Current levels are expressed as a percentage of the maximal CFTR-Cl⁻ current observed upon subsequent addition of 2 milliunits/ml cAK.

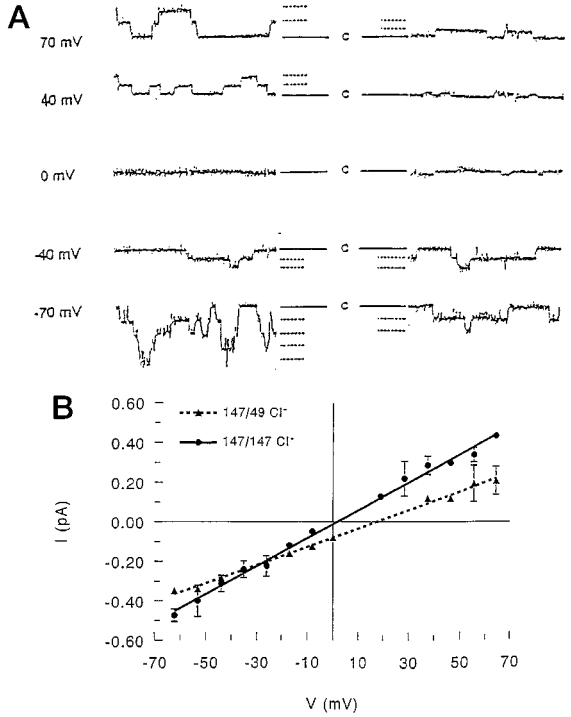

FIG. 2. **Biophysical characteristics of the cGKII-activated channel in excised, inside-out membrane patches from IEC-CF7 cells.** *Panel A*, current tracings of cGKII-activated channels. *Left*, symmetrical 147/147 mM chloride solutions. *Right*, pipette buffer was replaced by a low (49 mM) chloride buffer. Tracings were obtained at the indicated voltages. *C*, all channels closed; *dotted line*, single-channel current levels. *Panel B*, I-V characteristics of the channel. The channel conductance was 8.0 ± 0.6 pS (n = 5). ●, symmetrical Cl⁻ solution (147/147 mM); , reduction of Cl⁻ in the pipette to 49 mM by replacement with aspartic acid.

(Fig. 2). In this low expression model, single-channel events could be monitored (Fig. 2*A*), which had a linear I-V relation in symmetrical Cl⁻ concentrations, a channel conductance of 8 pS, and a rightward shift in current reversal potential upon lowering of the Cl⁻ concentration in the pipette (Fig. 2*B*). The mean open probability (P_o) of CFTR-Cl⁻ channels measured at the plateau phase of activation by saturating concentrations of cGKII (0.22 ± 0.09; n = 9) did not differ significantly from the P_o of cAK-activated channels (0.23 ± 0.12; n = 8).

Additional proof of the identity of the cGKII-activated channel as CFTR came from the observation that cGKII could not further enhance channel activity in excised patches from 3T3-CFTR cells following their pre-phosphorylation by cAK (2 milliunits/ml) and ATP (2 mM) (results not shown). Another similarity between CFTR-Cl⁻ channel regulation by cAK and cGKII was the observation that the currents rapidly returned to near base-line values upon the removal of either kinase and ATP from the bath, but could be restored almost instantaneously by the readdition of 2 mM ATP alone, confirming the crucial role of ATP in CFTR-Cl⁻ channel functioning (Fig. 1*B*; *cf.* Refs. 25, 38, and 40).

To eliminate the possibility that cGKII activation of CFTR resulted from a contamination of the cGKII preparation with cAK, a specific peptide inhibitor of cAK, PKI (0.1 μM) was added to the bath. Under this condition CFTR-Cl⁻ channel activation in 3T3 membrane patches by cAK (2 milliunits/ml) was completely abolished, whereas channel activation by cGKII (2 milliunits/ml) was not significantly affected (42 ± 3% of the maximal channel activity evoked by cAK in the same patch following removal of PKI from the bath, as compared to 37 ± 5% in the absence of PKI; n = 5). Moreover, PKI was unable to inhibit phosphorylation of Kemptide or CF-2 by cGKII (not shown).

Finally, the possibility was considered that cGKI, in spite of its failure to open CFTR-Cl⁻ channels by itself, could interfere with the activation of CFTR-Cl⁻ channels by the other cGK isoform. However, neither preincubation of 3T3 patches with cGKI (10 milliunits/ml, 15 min; results not shown) nor the simultaneous addition of cGKI (10 milliunits/ml) and cGKII (10 milliunits/ml) had any effect on the rate or extent of CFTR-Cl⁻ channel activation as compared to cGKII alone (Fig. 1*C*).

Phosphorylation of CFTR and CF-2 by cGK Isotypes—Phosphorylation studies carried out with CFTR immunoprecipitates (Fig. 3*A*) and CF-2, the recombinant R domain of CFTR (Fig. 3*B*) confirmed previous reports that both proteins are excellent *in vitro* substrates for cAK and cGKI (26, 38). This discrepancy

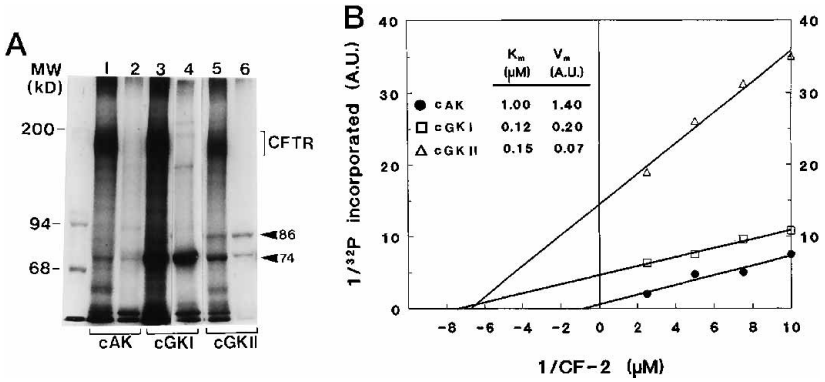

FIG. 3. **Phosphorylation of CFTR (*panel A*) and CF-2 (*panel B*) by purified protein kinases.** *Panel A*, CFTR was immunoprecipitated from T84 cells and phosphorylated as described under "Experimental Procedures." The reactions also contained: catalytic subunit of cAK (2 milliunits/ml; *lanes 1* and *2*), cGKI (7.5 milliunits/ml; *lanes 3* and *4*), and cGKII (9.4 milliunits/ml; *lanes 5* and *6*). *Lanes 2*, *4*, and *6*, control experiments in which CFTR was omitted. The ^{32}P-labeled proteins were separated by 6% SDS-PAGE. The gel was dried and exposed to x-ray film. CFTR migrates as a broad band of 180 kDa ("band C"; see Ref. 14). The 86- and 74-kDa bands represent residual amounts of autophosphorylated cGKII (*lanes 5* and *6*, intact 86-kDa form + 74-kDa proteolytic fragment; *cf.* Ref. 17) and cGKI (*lanes 3* and *4*, intact 74-kDa form), respectively, remaining following the washing steps. *Panel B*, Lineweaver-Burk plots of CF-2 phosphorylation by equal concentrations (25 nM) of cAK, cGKI, and cGKII. The experimental conditions needed to ensure linear rates of ^{32}P incorporation are specified under "Experimental Procedures." The *inset* shows the kinetic constants (K_m, V_{max}) calculated from the Lineweaver-Burk plots. *A.U.*, arbitrary units. Data represent the mean of three experiments.

between phosphorylation and functional studies was even more apparent from the kinetics of *in vitro* phosphorylation of CF-2 by cGKI and cGKII isoenzymes (Fig. 3*B*). CF-2 was a better substrate for cGKI than for cGKII (similar K_m; 3-fold higher V_{max}), and the plateau level of phosphate incorporation into CFTR (Fig. 3*A*) was also higher for cGKI (140 ± 11% of the cGKII level; n = 8).

Two-dimensional Phosphopeptide Mapping of CF-2 and CFTR Phosphorylated by cAK, cGKI, and cGKII—Up to 10 serine residues within the R domain have been suggested to be involved in the activation of CFTR (for review, see Ref. 39). In order to investigate whether the isotype specificity of cGK activation of the CFTR-Cl^- channel might be related to the pattern of specific serine residues that are phosphorylated, two-dimensional phosphopeptide mapping was performed of CF-2 and CFTR phosphorylated by cAK, cGKI, and cGKII (Fig. 4). All three kinases phosphorylated the same peptides in CF-2 with very slight differences being observed in the intensities of phosphorylation of individual peptides (Fig. 4, *A–C*). All three kinases also phosphorylated the CFTR on a subset of the peptides phosphorylated in CF-2 (Fig. 4, *D–F*), arguing against the location of novel cGKII sites outside the R domain. Several sites, in particular those located on peptides 1, 2, and 4 (the latter containing serine 700; see Ref. 26), were phosphorylated to a similar level. In contrast, one prominent peptide, labeled 9 (see Ref. 26 for numbering) was preferentially phosphorylated by cAK and cGKII, but was phosphorylated to a much lower level by cGKI (Fig. 4, *D–F*, and data not shown).

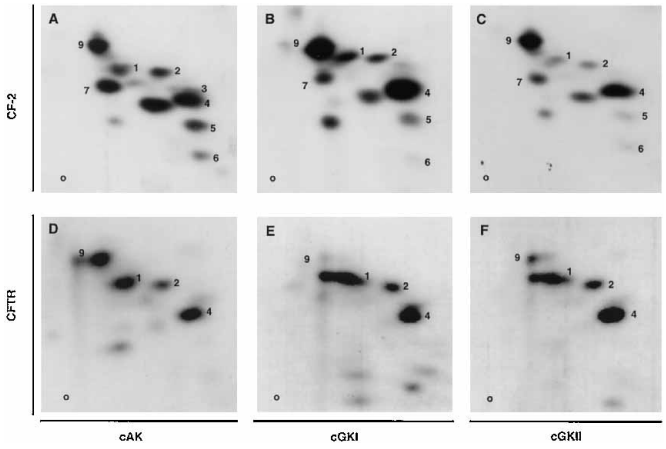

FIG. 4. **Two-dimensional tryptic phosphopeptide maps of CF-2 and CFTR.** *Upper* and *lower panels*, phosphopeptide maps of CF-2 and CFTR, respectively, showing phosphorylation by cAK (*A* and *D*), cGKI (*B* and *E*), and cGKII (*C* and *F*), respectively. Purified CF-2 and immunoprecipitated CFTR were phosphorylated for 40 min at 30 °C in the presence of cAK (2 milliunits/ml), cGKI (7.5 milliunits/ml), or cGKII (9.4 milliunits/ml) as described under "Experimental Procedures." The ^{32}P-labeled proteins were separated by 6% (CFTR) or 12% (CF-2) SDS-PAGE and visualized by autoradiography. Radioactive bands of CF-2 (pooled middle and upper bands running at 30 and 32 kDa, respectively; *cf.* Ref. 26) and CFTR (see Fig. 3*A*) were excised from gels, washed, and digested for 20 h with 50 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. Tryptic digests were separated by electrophoresis in the first dimension at 400 V in 10% acetic acid, 1% pyridine, pH 3.5, and by chromatography in the second dimension using pyridine: 1-butanol:water:acetic acid (10:15:12:3%). *O*, origin. *Left*, +; *right*, −. Phosphopeptides are numbered from 1 to 9, and correspond to those in Ref. 26. The results shown are representative of three independent experiments. In two other sets of CF-2 maps, an additional phosphopeptide migrating closely to peptide 9 could be distinguished, indicating that the peptide 9 spot may contain more than one peptide or more than one phosphoacceptor site in one peptide, occasionally giving rise to alternative tryptic digestion.

DISCUSSION

In this study a recently cloned cGK isoform, cGKII (20, 21), expressed at high levels in the luminal membrane of intestinal epithelial cells (16–19, 21, 41) and to a lower extent in kidney and brain (21), is identified as a novel potential regulator of the CFTR-Cl^- channel. Using a reconstitution assay consisting of a detergent-free preparation of solubilized and purified cGKII, inside-out membrane patches from CFTR-transfected intestinal cells (IEC-CF7), or NIH-3T3 fibroblasts, Mg-ATP and cGMP, the enzyme could almost fully mimic the effect of cAK on CFTR-Cl^- channel opening, albeit with a slower time course. It is possible, however, that the rate-limiting step in the *in vitro* assay is the anchoring of the cGKII to the membrane, and that this delayed opening does not necessarily imply a similar kinetic disadvantage for cGKII *in vivo*, considering its colocalization with CFTR in the luminal membrane (19, 41). The amount of Cl^- channel current reached (78 ± 24% of that measured after the subsequent addition of cAK) was considerably greater than the current level reported after addition of another activating kinase, PKC, using similar assay conditions (15 ± 8% of cAK; Ref. 38). In accordance with these functional data, the phosphopeptide maps made from CF-2 and CFTR phosphorylated *in vitro* by cGKII and cAK were virtually iden-

tical and clearly different from the pattern generated by PKC (26). The plateau level of phosphate incorporation into CFTR reached with cAK and cGKII was also similar (Fig. 3*A*), although the rate of CF-2 phosphorylation by cGKII was slower (Fig. 3*B*). However, such a close correlation between *in vitro* phosphorylation and functional data should be interpreted with caution: cGKIα, another mammalian cGK isoform expressed in many non-intestinal cell types (30, 31) failed to activate CFTR-Cl^- channels in this and an earlier study (38) or to potentiate cGKII activation of the channel, in spite of its ability to phosphorylate *in vitro* three of the four major cAK/cGKII sites in CFTR (Fig. 4).

The molecular basis for the differential activation of the CFTR-Cl^- channel by cGK isotypes is presently unclear. One possibility is that cGKIα may not recognize one or more phosphoacceptor sites in CFTR that are crucial for its activation. However, the results from the two-dimensional peptide mapping studies indicate that the pattern of phosphorylation of CF-2 is similar for cGKI and cGKII and essentially the same as that of cAK. Furthermore, the pattern of phosphorylation of CFTR by the three kinases was also similar with the exception that one peptide (peptide 9) was phosphorylated to only a very low level by cGKI (Fig. 4). Although initial mutagenesis studies suggested that the multisite phosphorylation of the R domain is degenerate and that no single phosphorylation site is critical for CFTR-Cl^- channel function (14), more recent studies provide evidence for a distinct role of subsets of phosphorylation sites in controlling the function of the two nucleotide binding domains in CFTR (39), and for the existence of stimulatory and inhibitory phosphorylation sites in the R domain (42). However, the possible presence of a predominant inhibitory site phosphorylated by cGKIα but not by cGKII or cAK seems unlikely since CFTR-Cl^- channel activation by cGKII or cAK was not inhibited by cGKI (Fig. 1*C*). A second possibility is that the CFTR protein in its natural membrane environment is differentially accessible to the cGK isoenzymes and that this difference is lost following immunoprecipitation or in CF-2 peptide studies. Conceivably, differences in size or quaternary structure between the monomeric cGKII protein (17–19) and the cGKI dimer do not play a major role because limited trypsinization of cGKIα generating a monomeric C-terminal fragment failed to improve CFTR-Cl^- channel activation. On the basis of secondary structure analysis, it has been argued that the structural determinants accounting for the tight association of the type II cGK with membranes may reside in the N-terminal region (21). Earlier topological studies in intestinal brush borders have also pointed to a role of a 15-kDa N-terminal fragment in anchoring cGKII to the microvillar cytoskeleton (17, 18). In particular the presence of a consensus sequence for N-terminal myristoylation in cGKII (21) may have functional significance. This sequence is absent in cGKIα (43), which may explain in part its inability to activate CFTR. As a third possibility, cGK activation of the CFTR-Cl^- channel may depend on the additional phosphorylation of a CFTR-associated regulatory protein ubiquitously expressed in cells of epithelial origin (IEC-CF7) and non-epithelial cells (3T3 fibroblasts). Small phosphorylatable proteins have been recently implicated in the regulation of a different class of Cl^- channels (44, 45). However, the opening of CFTR-Cl^- channels in lipid bilayers by cAK does not require an auxiliary protein (46). Similar studies performed with the cGK isotypes are clearly needed to discriminate between a direct and indirect model of CFTR regulation.

The demonstration of cGKII regulation of CFTR-Cl^- channels in membrane patches together with the high expression level of cGKII and virtual absence of cGKI in intestinal epithelium (17–19, 41) strongly support a model depicting cGKII as the major effector of the action of cGMP and cGMP-linked secretagogues (ST, guanylin) in this tissue. Recent immunological and ion transport experiments (41) also show a tight correlation between cGKII expression and 8-Br-cGMP-provoked, but not 8-Br-cAMP-provoked Cl^- secretion in rat intestinal segments and human colonic cell lines (T84, CaCo-2). With one possible exception (47), the latter cell lines do not express detectable levels of cGKII (41) but are still able to activate CFTR in response to ST, apparently as a consequence of excessive cGMP accumulation followed by cross-activation of cAK (11–13). However, in a previous study no low affinity binding of cGMP to cAMP receptors in the brush border membrane could be detected following luminal exposure of rat small intestine to ST *in vivo* (48). Moreover, in contrast to 8-Br-cAMP, ST did not further enhance Cl^- secretion in rat small intestinal mucosa and proximal colon *in vitro* beyond the level reached in the presence of exogenous cGMP analogues (41). The apparent need for coexpression of cGKII and CFTR may also explain why cGMP activation of Cl^- secretion is not universally observed in other CFTR-expressing cell types, including human airway epithelium (38).

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