

# A Novel PDZ Protein Regulates the Activity of Guanylyl Cyclase C, the Heat-stable Enterotoxin Receptor\*

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**Secretory diarrhea is the leading cause of infectious diarrhea in humans. Secretory diarrhea may be caused by binding of heat-stable enterotoxins to the intestinal receptor guanylyl cyclase C (GCC). Activation of GCC catalyzes the formation of cGMP, initiating a signaling cascade that opens the cystic fibrosis transmembrane conductance regulator chloride channel at the apical cell surface. To identify proteins that regulate the trafficking or function of GCC, we used the unique COOH terminus of GCC as the “bait” to screen a human intestinal yeast two-hybrid library. We identified a novel protein, IKEPP (intestinal and kidney-enriched PDZ protein) that associates with the COOH terminus of GCC in biochemical assays and by co-immunoprecipitation. IKEPP is expressed in the intestinal epithelium, where it is preferentially accumulated at the apical surface. The GCC-IKEPP interaction is not required for the efficient targeting of GCC to the apical cell surface. Rather, the association with IKEPP significantly inhibits heat-stable enterotoxin-mediated activation of GCC. Our findings are the first to identify a regulatory protein that associates with GCC to modulate the catalytic activity of the enzyme and provides new insights in mechanisms that regulate GCC activity in response to bacterial toxin.**

Guanylyl cyclase C (GCC) is the receptor for heat-stable enterotoxins (STa)<sup>1</sup> secreted by *Escherichia coli* and other enteric bacteria. STa binding to GCC increases intracellular cGMP and initiates a signaling cascade, leading to the phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) at the apical surface of gastrointestinal epithelial cells. Phosphorylation of CFTR opens the channel, re-

sulting in the net efflux of ions and water into the intestinal lumen. The endogenous ligands for GCC include guanylin, uroguanylin, and lymphoguanylin, which are thought to regulate ion transport in epithelial tissues (1–3).

GCC is a member of a family of transmembrane proteins that includes receptors for natriuretic peptides and egg-activating peptides as well as several orphan receptors (4). All receptor GCs with a single transmembrane domain share a common topology. There is an NH<sub>2</sub>-terminal extracellular ligand-binding domain and a large cytosolic domain composed of a kinase homology domain and a catalytic domain. Following the catalytic domain, GCC contains an extended COOH terminus of 63 amino acids (COOH-terminal extension peptide (CTEP)) that is not found in the natriuretic peptide receptors (5). The CTEP is well conserved and contains a consensus protein kinase C phosphorylation site that potentiates cGMP-mediated signaling by phorbol esters (6). GCC proteins lacking the 63-amino acid CTEP lose the ability to respond to STa (6, 7), suggesting that this unique sequence plays a role in GCC activation. Since GCC is the only receptor guanylyl cyclase localized predominantly at the apical membrane of epithelial cells, CTEP may also play a role targeting the receptor to the apical cell surface.

To determine whether the COOH terminus of GCC participates in protein-protein interactions that may regulate its targeting or function, we screened a human intestinal epithelial enriched yeast two-hybrid library using CTEP as “bait.” We found that GCC associates via its COOH terminus with a novel protein containing four PDZ domains. Based on its domain organization and restricted mRNA distribution, we named this protein IKEPP (intestinal and kidney enriched PDZ Protein). IKEPP is accumulated at the apical membrane of human intestinal epithelial cells and associates with GCC in a cellular context. Mutagenesis studies indicate that association with PDZ proteins is not required for efficient targeting of GCC to the apical surface. Rather, the interaction of IKEPP and GCC inhibits receptor activation by STa. Thus, GCC activity may be modulated by interaction with accessory proteins, thereby providing additional means to regulate signaling via guanylyl cyclase receptors.

## EXPERIMENTAL PROCEDURES

**cDNA Library Generation, Plasmid Construction, Two-hybrid Screens**—All cDNA inserts were generated by PCR, cloned into complementary restriction endonuclease sites of the appropriate plasmids, and verified by sequencing; specific details are available upon request. A human intestinal epithelial enriched cDNA library was generated by cloning poly(dT)-primed cDNA into the HybriZAP bacteriophage  $\lambda$  vector followed by amplification and *in vivo* mass excision to generate a two-hybrid library in pAD-GAL4 (Stratagene). The yeast binding domain (BD) plasmid pPC86BD was generated by digesting the parental vectors, pPC97 (GAL4BD and LEU2) and pPC86 (GAL4AD and TRP1) (8), with *Apa*I and *Bam*HI. These fragments were then ligated into the opposite backbone vector to give pPC86BD and pPC97AD.

cDNA encoding full-length CTEP was amplified by PCR using

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number AY047359.

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<sup>1</sup> The abbreviations used are: STa, heat-stable enterotoxin; CTEP, COOH-terminal extension peptide; PDZ, postsynaptic density-95, disks large, zonula occludens-1; BD, binding domain; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; TRITC, tetramethylrhodamine isothiocyanate.

pBS.GCC as template; the PCR products were inserted in frame to the corresponding sites in pPC86BD. The yeast strain AH109 was sequentially transformed with pPC86BD.CTEP and 20  $\mu$ g of a human intestinal cDNA library as described (9). A ~2.2-kb cDNA clone encoding a novel protein was isolated twice in the screen. After sequencing and Northern blot analysis, this clone was named IKEPP (intestinal and kidney enriched PDZ protein). To obtain upstream coding sequences we performed 5' rapid amplification of cDNA ends using Marathon-Ready Human Kidney cDNA (CLONTECH); products were cloned into pTAdv (CLONTECH) and sequenced. Human multiple tissue northern blots and a multiple expression array blot (CLONTECH) were probed with  $^{32}$ P-labeled random-primed cDNA probe corresponding to the IKEPP 3'-untranslated region (nucleotides 1565–2120) as described (10).

**Antisera Generation and Immunoblot Analysis**—Rabbit antisera directed against the COOH terminus of human IKEPP were generated in rabbits using residues 484–505 of IKEPP coupled with keyhole limpet cyanin as immunogen. Rabbit polyclonal antisera were also generated using His-IKEPP fusion protein as immunogen. The pET.IKEPP plasmid was transformed into BL21(DE3, pLysS) *Escherichia coli* and grown to the appropriate cell density at 37 °C. IKEPP expression was induced by the addition of 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside for 3 h at 37 °C and purified from the insoluble fraction.

To prepare cell lysates, cultured cells were washed with ice-cold phosphate-buffered saline (50 mM NaPO<sub>4</sub>, 150 mM NaCl, pH 7.4) and isolated by scraping in ice-cold homogenization buffer containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin. The homogenates were centrifuged at 100,000  $\times$  g for 1 h to generate soluble and particulate fractions. Protein concentrations were determined using the BCA protein assay kit (Pierce); samples were fractionated by SDS-PAGE and transferred to Immobilon-P (Millipore Corp.). Western blots were performed using rabbit anti-IKEPP IgG (NC368 or NC369; 1:2000) and visualized using ECL.

**Protein Interaction Assays**—*In vitro* binding assays and co-immunoprecipitations were performed as described (11). For immunoprecipitation of overexpressed HA-GCC and IKEPP, COS7 cells were transfected with cDNAs encoding IKEPP and HA-GCC or HA-GCC $\Delta$ 4 with FuGENE6 (Roche Molecular Biochemicals). After 48 h, the cells were lysed in TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), 1% Triton X-100, and protease inhibitors. Mouse anti-HA or purified normal mouse IgG (2  $\mu$ g) was added to the cell lysate and incubated overnight at 4 °C. Immune complexes were collected on protein G-agarose and washed extensively in TBS buffer plus 0.1% Triton X-100. Bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with HA or IKEPP antisera.

**Confocal Microscopy**—Stable MCDK type II cell lines expressing HA-GCC or HA-GCC $\Delta$ 4 were generated as described (10). MDCK or Caco2 cells were grown on Transwell filters (Costar) until confluent monolayers were observed, and transepithelial resistances, with filter subtraction, were greater than 1000 ohms-cm<sup>2</sup> or 400 ohms-cm<sup>2</sup>, respectively. Immunofluorescent staining was performed as described (10, 11). The localization of IKEPP was also studied in sections of formalin-fixed human colon and small intestine. Sections were prepared as described previously (12), stained with rabbit anti-IKEPP IgG (NC369; diluted 1:1500), and processed using the Vectastain Elite ABC kit (Vector Laboratories, Inc.); sections were counterstained with methyl green to label nuclei.

**GCC Activity Assays**—COS7 cells, plated on six-well culture dishes at a density of 4  $\times$  10<sup>5</sup> 24 h prior to transfection, were incubated in FuGENE 6 as described in the instruction manual. After 48 h, the culture medium was removed, the cells were incubated in serum-free Dulbecco's modified Eagle's medium/F-12 containing 100  $\mu$ M isobutylmethylxanthine for 15 min, and 25 units/ml STa (Sigma) was added to each well for 20 min. The cells were washed twice in ice-cold phosphate-buffered saline, lysed in 0.1 M HCl for 20 min, and collected by centrifugation at 4 °C. For dose-response curves, cells were handled as described except that 2  $\times$  10<sup>5</sup> cells were seeded in 12-well culture dishes 24 h prior to transfection. Following transfection, STa was added to the cells at various concentrations for 30 min in the presence of 100  $\mu$ M isobutylmethylxanthine. Cells were harvested, and cGMP production was measured in both the cell lysate and culture medium using a Correlate-EIA Direct Cyclic GMP Enzyme Immunoassay Kit (Assay Designs, Inc.).

## RESULTS

**Cloning and Characterization of a Novel GCC-interacting Protein**—In an attempt to isolate GCC-interacting proteins, we used the yeast two-hybrid system to identify proteins that

interact with the CTEP of GCC. Screening of a human epithelial enriched intestinal cDNA library yielded several potential interactors that were His<sup>+</sup>, and we further analyzed two clones that exhibited robust  $\beta$ -galactosidase activity. The specificity of the interaction in yeast was verified by transforming the activation domain plasmid along with the original bait, an empty bait vector, or a plasmid encoding an unrelated bait (data not shown).

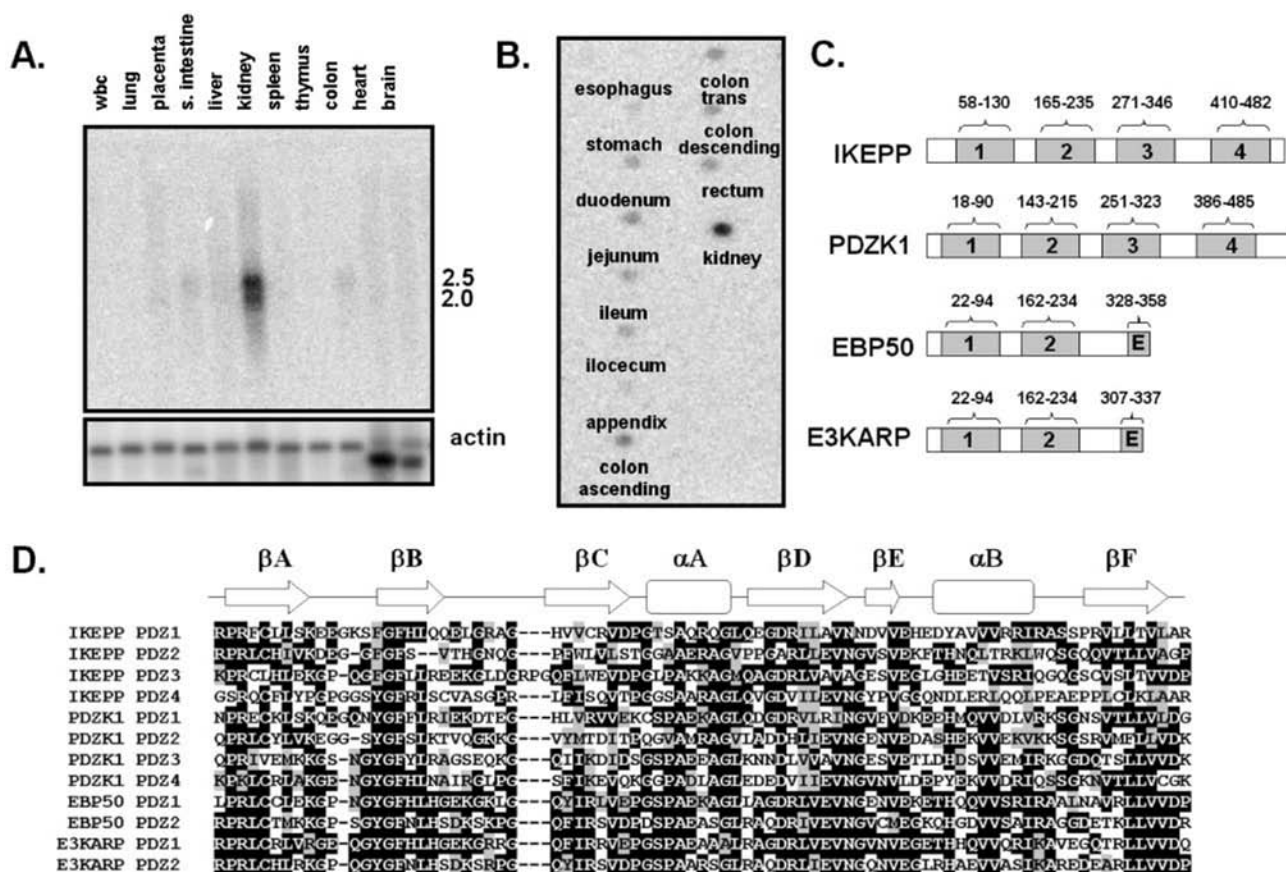
Sequence analysis revealed that the cDNA inserts were ~2.4 kb and contained identical cDNA sequence with an open reading frame of 1503 nucleotides. A protein pattern search using Pfam indicated that the open reading frame encoded a protein containing four PDZ domains. The gene was mapped to a region of chromosome 11q23 when searched against the human genome draft data base. The full-length cDNA with an open reading frame of 1518 nucleotides was predicted from genomic DNA and confirmed by 5' rapid amplification of cDNA ends using human kidney cDNA as template. The open reading frame predicts a protein of 505 amino acids with a theoretical molecular mass of 54.2 kilodaltons and a pI of 5.46.

On Northern blots, we detected ~2.3- and 2.5-kb messages in human kidney (Fig. 1A), although prolonged exposures of the blots revealed that the mRNAs were also expressed in the small intestine and colon. Since GCC mRNA is abundantly expressed in the intestine (5), we also probed a human expression array containing poly(A)<sup>+</sup> RNA prepared from multiple gastrointestinal tissues. We found that mRNA was easily detected in the kidney and along the entire gastrointestinal tract, from the duodenum to the colon (Fig. 1B). The mRNA was not detected in any other human tissue including brain, heart, skeletal muscle, or cells of hematopoietic origin (data not shown). Based on the relatively restricted distribution of the mRNA and the domain structure of the predicted protein, we named this novel protein IKEPP.

**IKEPP Is Related to Other Epithelial Enriched PDZ Proteins**—A BLAST search of the nonredundant GenBank<sup>TM</sup> data base with the IKEPP protein sequence revealed that IKEPP is most closely related to PDZK1 (PDZ domain containing protein-1; also called CAP70), a protein with four PDZ domains (13, 14). IKEPP and PDZK1 are closely related to two other human epithelial PDZ proteins, EBP50 (ezrin-radixin-moesin-binding phosphoprotein-50; also called NHERF1) and E3KARP (NHE3 kinase A regulatory protein; also called NHERF2), which each contain two PDZ domains (Fig. 1C) followed by a COOH-terminal domain that associates with the NH<sub>2</sub> terminus of ezrin, radixin, and moesin to link these proteins to the actin cytoskeleton (15). An analysis of the sequence identity between the individual IKEPP PDZ domains and the PDZ domains of EBP50, E3KARP, and PDZK1 indicates that PDZ1 and PDZ4 of IKEPP are most unique, whereas PDZ2 and PDZ3 of IKEPP share between 30 and 50% identity with the PDZ domains of these related proteins (Fig. 1D). Furthermore, IKEPP is probably the human orthologue of the mouse type IIa sodium/inorganic phosphate cotransporter-associated protein (Na/Pi-Cap2), since both proteins contain four tandem PDZ domains, share 77% sequence identity, and are expressed in the kidney and intestine (16).

PDZ domains are composed of six  $\beta$  sheets ( $\beta$ A– $\beta$ F), capped by two  $\alpha$  helices ( $\alpha$ A and  $\alpha$ B), which form a peptide-binding groove that interacts with, at least, the last four C-terminal amino acids of interacting proteins (17). In general, PDZ domains recognize peptide sequences that contain a hydrophobic residue at the extreme COOH terminus through a conserved carboxylate-binding pocket most often formed by the sequence Arg/Lys-X-X-X- $\Phi$ -Gly-Phe (where  $\Phi$  represents a hydrophobic amino acid) (18). The carboxylate loop of PDZ domains in





**FIG. 1. Identification of a novel PDZ protein preferentially expressed in the intestine and kidney.** A, a multiple tissue Northern blot (CLONTECH) was probed with a random primed  $^{32}$ P-labeled probe generated against the IKEPP 3'-untranslated region. The blot was stripped and incubated with a  $\beta$ -actin probe. Similar results were obtained in two separate blots. B, a multiple tissue array was probed with the same IKEPP probe used in A; all other tissues showed no signal and were deleted from the figure. C, schematic representation of IKEPP and related PDZ proteins. PDZ domains are numbered, and ezrin-radixin-moesin binding motifs are indicated by the letter E. The proteins are drawn to scale, and the amino acid numbers are as shown. D, the amino acid sequences of the individual PDZ domains of IKEPP, PDZK1, EBP50, and E3KARP were aligned using DNASTAR software. At each position, the most commonly conserved residues between sequences are shown in *black boxes*, whereas similarly charged residues are *shaded in gray*. The predicted secondary structures of the PDZ domains, based on the crystal structure of EBP50 PDZ1 are also shown.

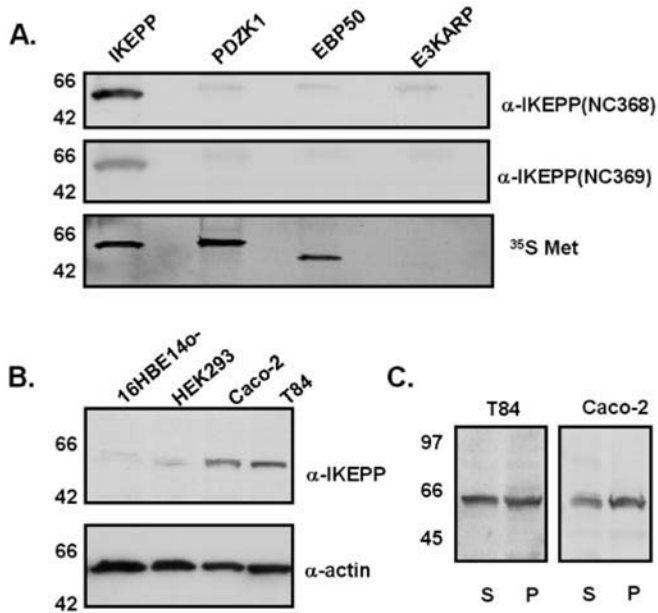
IKEPP, PDZK1, EBP50, and E3KARP contain the general consensus, Arg/Lys-X-X-Tyr/Phe-Gly-Phe, with the exception of IKEPP PDZ4, which possesses a Pro residue rather than the Arg/Lys (Fig. 1D). In the carboxylate-binding pocket, the Arg/Lys residue is responsible for ordering a water molecule that interacts with the terminal carboxylate of the ligand (17). Therefore, the C-terminal residue(s) of proteins that associate with IKEPP PDZ4 will probably differ from the ligands recognized by PDZK1, EBP50, E3KARP, and IKEPP PDZ domains 1–3.

The –2-position of the preferred peptide ligand is used to categorize the PDZ domains as class I (–2 Ser/Thr), class II (–2 hydrophobic), and a lesser defined class III, which deviate from class I and II (18–20). The specificity of the –2 interaction is coordinated by the first residue of the second  $\alpha$  helix of the PDZ domain ( $\alpha$ B1) (18). At the  $\alpha$ B1 position, class I PDZ domains contain a conserved His residue (17, 21), whereas class II domains possess a hydrophobic residue (22). To the best of our knowledge, all of the published binding partners of PDZK1, EBP50, and E3KARP, as well as the binding partners our laboratory has identified for IKEPP, contain a Ser/Thr at the –2-position of the PDZ binding motif. Based on this structural similarity, we predict that IKEPP is a member of the superfamily of class I PDZ proteins. Sequence analysis of the individual PDZ domains of IKEPP, however, reveals that IKEPP PDZ1 and PDZ4 lack the conserved His residue characteristic

of class I PDZ domains. In the  $\alpha$ B1 position, a Tyr residue (IKEPP PDZ1) has been shown to prefer ligands containing a –2 Asp residue, whereas an Asp residue (IKEPP PDZ4) interacts with peptides with a –2 Tyr (20, 23).

**Localization and Distribution of IKEPP in Human Cells and Tissues**—To evaluate the subcellular distribution of IKEPP, we generated rabbit polyclonal antisera directed against the COOH-terminal 15 amino acids of human IKEPP or the recombinant full-length protein. These antisera were first tested by Western blot analysis using full-length human IKEPP generated by coupled *in vitro* transcription/translation. Whereas pre-immune sera did not detect proteins in the reticulocyte lysates, both antibodies reliably detected full-length IKEPP (Fig. 2A). We further tested the specificity of our IKEPP antisera by Western blot analysis of EBP50, E3KARP, and PDZK1 and found that both IKEPP antisera specifically recognize recombinant IKEPP and do not cross-react with these related proteins (Fig. 2A). Recombinant E3KARP contains fewer methionine residues than IKEPP, PDZK1, and EBP50 and was visualized with prolonged exposure to the PhosphorImager screen.

We first examined the expression of IKEPP in cultured human cell lines and found that the protein was expressed in whole cell lysates of two intestinal epithelial cell lines, T84 and Caco2 (Fig. 2B); much less protein was detected in an airway epithelial cell line (16HBE14o–) or in hEK293 cells. A significant

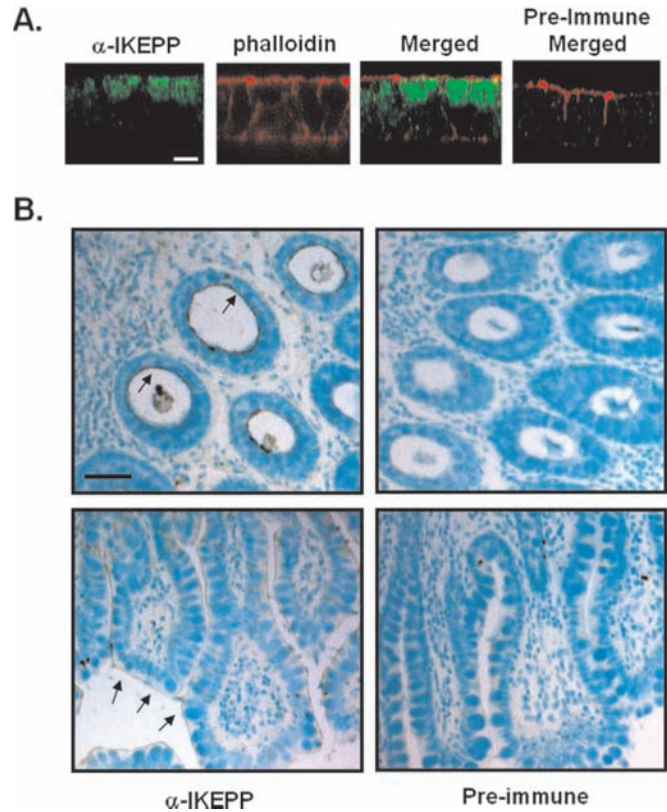


**FIG. 2. Characterization of IKEPP antisera and analysis of IKEPP protein expression.** *A*, radiolabeled IKEPP, PDZK1, EBP50, and E3KARP were generated by *in vitro* transcription/translation in the presence of [<sup>35</sup>S]methionine. Five  $\mu$ l of each reaction was analyzed by Western blot with antisera generated using full-length recombinant IKEPP (NC368) or IKEPP residues 484–505 (NC369); the same membrane was analyzed by PhosphorImager analysis (<sup>35</sup>S Met). Recombinant E3KARP contains fewer methionine residues but is visualized with prolonged exposure to the screen. *B*, whole cell lysates (50  $\mu$ g) were analyzed by Western blot with rabbit anti-IKEPP (NC 368; 1:1000) or mouse anti-actin. *C*, cultured T84 and Caco-2 cells were lysed in homogenization buffer plus protease inhibitors, and soluble and particulate fractions were prepared by differential centrifugation. Equal ratios of each fraction were analyzed by Western blot using NC368 (1:1000). Each blot is representative of three or four experiments.

fraction of the IKEPP protein was found in the particulate fraction of Caco2 and T84 cells (Fig. 2C). We next examined the localization of IKEPP in Caco2 cells grown to confluence on Transwell filters and found IKEPP preferentially accumulated in the subapical compartment and at the apical membrane (Fig. 3A); similar results were obtained with colonic T84 cells (data not shown). In normal human ileum and colon, IKEPP was preferentially accumulated at the apical surface and was visualized in cells of the crypt and villus (Fig. 3B). GCC is also expressed at the apical surface of intestinal epithelial cells (24). Thus, the distribution of IKEPP in human intestine is consistent with the possibility that the GCC and IKEPP associate *in vivo*.

**Characterization of the IKEPP-GCC Interaction**—We further characterized the interaction between GCC and IKEPP. Since GCC terminates with the amino acid sequence STYF, a type I PDZ binding motif, we tested whether the COOH-terminal four amino acids of CTEP mediated the interaction with IKEPP. We immobilized GST, GST-CTEP full-length, or GST-CTEP $\Delta$ 4 fusion proteins on glutathione-agarose beads and incubated the affinity resins with radiolabeled IKEPP. We found that IKEPP bound GST-CTEP but not GST or GST-CTEP $\Delta$ 4 (Fig. 4A). We obtained similar results in overlay assays (data not shown), indicating that the last four amino acid residues (SYTF) of GCC are required for the direct association with IKEPP.

IKEPP has four PDZ domains that probably bind different ligands. Therefore, we determined which IKEPP PDZ domains are capable of associating with CTEP. To do this, we generated histidine-tagged fusion proteins consisting of PDZ1, PDZ2, PDZ3, or PDZ4 of IKEPP and tested which of the radiolabeled fusion proteins associated with full-length GST-CTEP immobilized on glutathione-agarose beads. We found that radiolabeled

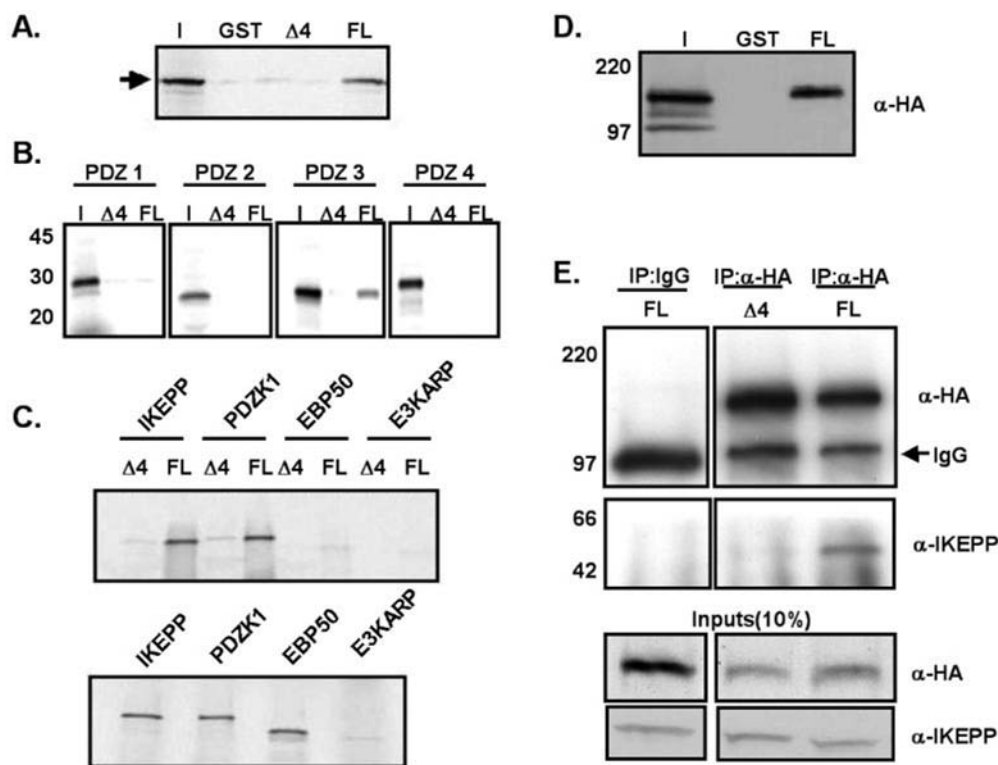


**FIG. 3. Localization of IKEPP.** *A*, Caco-2 cells grown on Transwell filters were fixed, permeabilized, blocked, and stained with preimmune antisera or rabbit anti-IKEPP (NC369; 1:1500) followed by goat anti-rabbit IgG coupled with Oregon Green (1:500). Texas Red-conjugated phalloidin (1:500) was added to stain filamentous actin, and *xz* sections were analyzed by confocal microscopy (scale bar, 10  $\mu$ m). *B*, representative paraffin-embedded 6- $\mu$ m sections of human colon (upper panels) or small intestine (lower panels) were stained with preimmune antisera or NC369 (1:1500). The arrows indicate regions of specific staining. Sections were processed using Vectastain Elite ABC kit (scale bar, 50  $\mu$ m).

PDZ3 bound specifically to full-length GST-CTEP, but not GST or GST-CTEP $\Delta$ 4. This interaction was not detected for PDZ1, PDZ2, and PDZ4 (Fig. 4B). Since IKEPP shares homology with EBP50, E3KARP, and PDZK1, we immobilized GST-CTEP and GST-CTEP $\Delta$ 4 on glutathione-agarose beads and tested whether radiolabeled PDZK1, EBP50, or E3KARP could associate with CTEP. We found that PDZK1, but not EBP50 or E3KARP, associates with GST-CTEP in pull-down assays (Fig. 4C).

To determine whether full-length IKEPP could associate with full-length GCC, we incubated GST or GST-IKEPP with whole cell lysates prepared from cells overexpressing HA-tagged GCC (HA-GCC). HA-GCC associated with GST-IKEPP but not with GST (Fig. 4D). GCC may be tightly associated with the subapical cytoskeleton in the intestinal epithelium (25) and is not easily solubilized from cell membranes in buffers compatible with maintaining protein-protein interactions. Therefore we used an overexpression strategy to study the association of GCC and IKEPP in nonepithelial cells. COS7 cells were transiently transfected with cDNAs encoding IKEPP plus HA-GCC or IKEPP plus HA-GCC $\Delta$ 4. Cell lysates were prepared in buffers containing 1% Triton X-100, which is known to remove GCC from cell membranes in COS7 cells (5), and the cell lysates were incubated with control IgG or HA antibody. We found that IKEPP was not associated with control IgG but was easily detected in HA-GCC immunoprecipitates (Fig. 4E). Moreover, IKEPP was not found in HA immunoprecipitates





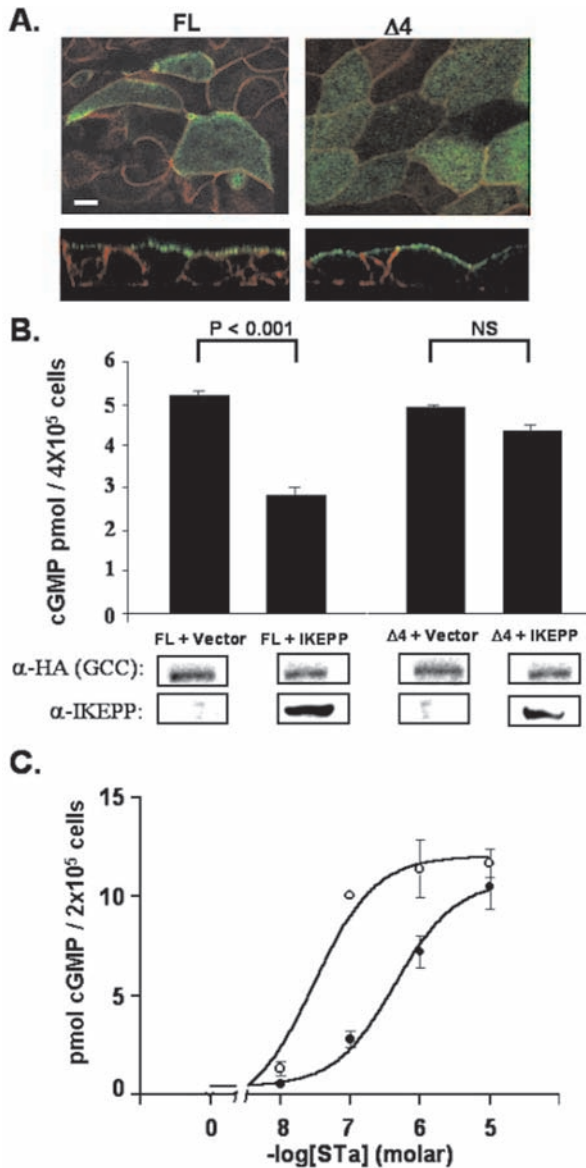
**FIG. 4. Analysis of the GCC-IKEPP interaction.** A, GST, GST-CTEP full-length (FL), or GST-CTEP $\Delta$ 4 ( $\Delta$ 4) were immobilized on glutathione-agarose beads and incubated with 5  $\mu$ l of radiolabeled IKEPP. The bound fractions were resolved by SDS-PAGE and applied to PhosphorImager screen; the *arrow* indicates radiolabeled IKEPP. B, radiolabeled IKEPP PDZ domains were incubated with GST-CTEP full-length or GST-CTEP $\Delta$ 4. The bound fraction was separated by SDS-PAGE and visualized by PhosphorImager analysis. C, GST-CTEP full-length and GST-CTEP $\Delta$ 4 were immobilized on glutathione-agarose beads and incubated with radiolabeled IKEPP, PDZK1, EBP50, or E3KARP. Bound proteins were visualized by PhosphorImager analysis. The *bottom panels* show 20% of the input used in each reaction. E3KARP can be visualized with prolonged exposure only in the input lane. D,  $\sim$ 200  $\mu$ g of total cell lysate prepared from cells expressing HA-GCC was incubated at 4  $^{\circ}$ C overnight with GST or GST-IKEPP. The bound fraction was analyzed by Western blot using HA antibody. I, input, representing 10% of the total cell lysate in each reaction. E, COS7 cells were transfected with FUGENE6 plus 9  $\mu$ g of pCDNA.HA-GCC full-length or HA-GCC $\Delta$ 4 and 13.5  $\mu$ g of pCDNA.IKEPP. Cell lysates were incubated with anti-mouse IgG or monoclonal HA.11. Immunoprecipitates were analyzed by Western blot with HA or IKEPP antisera. The IgG band is indicated by the *arrow*, and molecular weight markers are as shown. IP, the antibody used for immunoprecipitation. All panels are representative of 3–6 similar experiments.

from cells co-expressing HA-GCC $\Delta$ 4 plus IKEPP (Fig. 4E). Thus, we conclude that GCC and IKEPP associate in cells and that the association requires an intact GCC COOH terminus.

**Function of the GCC-IKEPP Interaction**—Interaction with PDZ proteins may be involved in selectively targeting proteins to apical or basolateral cell surfaces in epithelial cells (26–28). Therefore, we tested whether the COOH-terminal STYF sequence in GCC was involved in targeting the receptor to the apical cell surface. We generated stable MDCK cell lines expressing HA-GCC or HA-GCC $\Delta$ 4, lacking the STYF residues that mediate interaction with PDZ proteins. Full-length HA-GCC was targeted to the apical cell surface and was not detected at the basolateral membrane (Fig. 5A). Likewise, HA-GCC $\Delta$ 4 was preferentially accumulated at the apical cell surface of polarized MDCK cells (Fig. 5A). HA-GCC and HA-GCC $\Delta$ 4 were visualized at the apical membrane in nonpermeabilized cells, further suggesting that the HA-GCC and HA-GCC $\Delta$ 4 proteins were on the cell surface (data not shown). Thus, we conclude that interaction with apical membrane PDZ proteins does not play a significant role in the targeting of GCC to the apical cell surface in MDCK cells.

Bakre *et al.* recently compared the STa-induced desensitization of GCC in intestinal epithelial cells and in transfected fibroblasts and suggested that GCC catalytic activity might be regulated by interaction with proteins selectively expressed in epithelial cells (29). Therefore, we tested whether IKEPP modulated STa-mediated activation of GCC in transfected COS7

cells that do not express significant amounts of endogenous IKEPP. Treatment of COS7 cells expressing HA-GCC with 25 units/ml STa for 20 min significantly increased intracellular cGMP, whereas cGMP was undetected in mock-transfected cells (data not shown). In cells co-expressing GCC and IKEPP, 25 units/ml STa also increased intracellular cGMP above background. cGMP levels, however, were reduced by  $\sim$ 1.7-fold in cells co-expressing HA-GCC and IKEPP compared with cells transfected with HA-GCC and empty vector (Fig. 5B). In similar experiments, intracellular cGMP levels were decreased in COS7 cells expressing GCC and IKEPP by 1.5–2.5-fold compared with cells expressing HA-GCC and empty vector following incubation with 25 units/ml STa for 10–30 min (data not shown). This cannot be explained by changes in the expression of HA-GCC in the co-transfected cells, since the receptor was easily detected in membrane fractions prepared from these cells (Fig. 5B). Since the COOH terminus of GCC mediates the interaction with IKEPP, we tested whether IKEPP expression also inhibited STa-mediated activation of HA-GCC $\Delta$ 4. We observed similar levels of STa-mediated cGMP in HA-GCC $\Delta$ 4 cells in the absence or presence of co-expressed IKEPP (Fig. 5B). Therefore, we conclude that IKEPP binding may inhibit the catalytic activity of GCC and that the inhibition requires a physical interaction between the receptor and IKEPP. To begin to understand the mechanism of this inhibition, we transfected COS7 cells with HA-GCC with or without IKEPP and assayed cGMP accumulation over a range of STa concentrations. Appli-



**FIG. 5. Targeting and activation of HA-GCC and HA-GCC $\Delta$ 4.** *A*, localization of HA-GCC in MDCK cells stably expressing HA-GCC (FL) or  $\Delta$ 4 HA-GCC ( $\Delta$ 4); TRITC-conjugated phalloidin was added to visualize actin. *xy* and *xz* sections were analyzed by confocal microscopy (scale bar, 10  $\mu$ m). *B*, COS7 cells were transfected with 6  $\mu$ g of pCDNA.HA-GCC or pCDNA.HA-GCC $\Delta$ 4 plus 9  $\mu$ g of pCDNA.IKEPP or empty vector. cGMP was assayed as described;  $n = 3$  with duplicate measurements for each sample. Each bar represents total cGMP, measured from cell lysates and culture medium. Intracellular cGMP accounted for at least 93% of total cGMP measured for each sample. A corresponding Western blot indicating the relative expression of HA-GCC and IKEPP is also shown. *C*, COS7 cells were transfected with pCDNA.HA-GCC plus pCDNA.IKEPP (closed circles) or pCDNA.HA-GCC plus empty vector (open circles). cGMP was measured from triplicate experiments assayed in duplicate, and each data point represents the mean  $\pm$  S.E.

cation of STa resulted in a concentration-dependent accumulation of cGMP in cells expressing HA-GCC plus vector or HA-GCC plus IKEPP (Fig. 5C). Although we found no change in the  $V_{max}$  of the enzyme in the presence or absence of co-expressed IKEPP, we found that IKEPP significantly increased the amount of STa required for half-maximal activation of the enzyme (Fig. 5C). The half-maximal effect of STa on cGMP accumulation was  $\sim$ 30 nM in the absence of IKEPP and increased  $>10$ -fold with co-expressed IKEPP, indicating that association with IKEPP alters the function of the receptor.

## DISCUSSION

We report the cloning and initial characterization of IKEPP, a novel PDZ protein expressed at the apical membrane of human intestinal epithelial cells. IKEPP directly associates with the COOH terminus of GCC, the heat-stable enterotoxin receptor found at the apical surface of intestinal epithelial cells. Our localization studies (Fig. 3) and co-immunoprecipitation assays (Fig. 4E) support the hypothesis that IKEPP and GCC may associate in cells. Furthermore, the association with IKEPP inhibits the catalytic function of GCC, resulting in a decreased responsiveness to STa (Fig. 5, B and C).

Sequence and structural analysis indicates that IKEPP is most closely related to human PDZK1 (Fig. 1, C and D), a protein identified in a yeast two-hybrid screen as a MAP17-associated protein. PDZK1 also associates with cMOAT, a multidrug resistance transporter (13). The mouse orthologue of PDZK1, named CAP70, was purified from kidney based on its ability to associate with CFTR and was shown to potentiate CFTR Cl<sup>-</sup> channel activity (14). IKEPP and PDZK1 share significant identity with EBP50 and E3KARP (Fig. 1D). These proteins were first cloned as co-factors required for cAMP-mediated inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger 3 (30, 31) and later shown to associate with ezrin, radixin, and moesin (15). EBP50 and E3KARP can interact with receptors, ion channels, transporters, signaling molecules, adaptor proteins, and proteins that regulate membrane trafficking (32–37). Interestingly, EBP50, E3KARP, and PDZK1 associate with CFTR (14, 32, 35), a downstream effector of GCC (33). Therefore, it will be important to test whether IKEPP compartmentalizes GCC and CFTR together in a multiprotein complex at the apical cell surface. It will also be important to compare the expression, subcellular distribution, and binding partners of IKEPP and PDZK1, since we find that CTEP can also bind PDZK1 in biochemical assays (Fig. 4C).

Despite the overall sequence similarity between IKEPP, PDZK1, EBP50, and E3KARP, PDZ1 and PDZ4 of IKEPP differ at critical residues responsible for determining the specificity of the interaction between the PDZ domain and COOH-terminal ligand. Class I PDZ domains coordinate the interaction with a Ser/Thr residue at the  $-2$ -position of their peptide ligands through an  $\alpha$ B1 His residue. IKEPP PDZ4, however, contains an  $\alpha$ B1 Asp residue, which has been shown to preferentially interact with a  $-2$  Tyr, rather than a Ser/Thr (20). In IKEPP PDZ1, the conserved His is replaced by a Tyr residue, which is predicted to result in the preferential binding of Asp at the  $-2$ -position (23). Interestingly, the COOH terminus of IKEPP ends in Ser-Asp-Leu-Leu, which we predict based on sequence analysis, to be a ligand for IKEPP PDZ1. Consequently, this interaction might regulate IKEPP PDZ1 interaction with other proteins via competitive inhibition or serve as the basis for potential IKEPP oligomerization and the formation of a larger signaling complex. Furthermore, the  $\alpha$ B1 Tyr residue of IKEPP PDZ1 is predicted to be phosphorylated (by NetPhos analysis, available on the World Wide Web at [www.cbs.dtu.dk/services/NetPhos](http://www.cbs.dtu.dk/services/NetPhos)), which would alter the binding specificity of the PDZ domain and may serve a regulatory mechanism for PDZ-ligand interaction. Our laboratory is currently examining these potential regulators of IKEPP function.

**Interaction of IKEPP and GCC**—Although there are few antibodies that reliably detect endogenous GCC in sections of intestine, functional studies, *in situ* hybridization, and receptor autoradiography indicate that GCC is expressed in epithelial cells of the gastrointestinal tract (39–41). Although a more complete analysis of IKEPP expression and distribution is needed, our localization studies suggest that GCC and IKEPP may be co-expressed and co-localized at the apical cell surface

in intestinal epithelial cells (Fig. 3). Furthermore, GCC and IKEPP directly interact in biochemical assays, and the interaction requires the COOH-terminal PDZ binding motif of GCC (Fig. 4). When co-expressed in COS7 cells, HA-GCC and IKEPP can be co-immunoprecipitated (Fig. 4E). Taken together, our data support the hypothesis that GCC and IKEPP associate in epithelial cells. However, consistent with previous reports (42), we were unable to extract significant amounts of overexpressed (MDCK cells) or endogenous GCC (T84 cells) from membranes to directly study the GCC-IKEPP interaction in epithelial cells. Therefore, definitive proof that IKEPP and GCC interact in intestinal epithelial cells will require dominant-negative approaches and functional assays.

IKEPP mRNA is also abundantly expressed in the kidney (Fig. 1, A and B). Ligand binding assays and functional studies suggest that GCC may be expressed in the kidney in some species (43, 44). Therefore, IKEPP and GCC may also associate in the kidney; however, renal IKEPP complexes will probably differ from those found in intestinal epithelial cells. Furthermore, proteins that associate with IKEPP may be differentially expressed in distinct regions of the kidney or gastrointestinal tract.

**Functions of the GCC-IKEPP Interaction**—The trafficking, regulation, and function of GCC are poorly understood, and there are many potential roles for the IKEPP-GCC interaction. In well differentiated cultured epithelial cells and in intestinal cell lysates, GCC is found in the detergent-insoluble fraction (45). The insolubility of GCC may be due to a direct or indirect association with cytoskeletal elements enriched at the apical membrane. We find that ~50% of the endogenous IKEPP in cultured intestinal cells is in the cytosolic fraction (Fig. 2C), and membrane-associated IKEPP is easily solubilized in buffers containing 1% Triton X-100 (Fig. 4E). Therefore, IKEPP does not mediate the association of GCC with the detergent-insoluble fraction of epithelial cells. GCC exists as a functional dimer or trimer (46–48), and it is known that interactions with PDZ proteins can facilitate protein oligomerization (14, 36, 49). However, the intracellular domains of GCC are not required for oligomer formation (48), suggesting that association with IKEPP is not likely to play an important role in this process. Harris *et al.* demonstrated that deletion of the PDZ interaction motif at the COOH terminus of the multidrug resistance-associated protein 2 (MRP2/cMOAT) disrupted apical targeting in transiently transfected MDCK cells (50). However, the constructs used in this study contained a COOH terminus green fluorescent protein tag blocking the PDZ interaction motif and interactions with PDZ proteins were not assessed. Moyer *et al.* also reported that the efficient apical trafficking of a green fluorescent protein-CFTR fusion protein required an intact CFTR COOH terminus and association with PDZ proteins (51). In contrast, Benharouga *et al.* find that CFTR proteins lacking the COOH-terminal PDZ binding motif are retained at the apical membrane in polarized MDCK II cells (52). Thus, it is not clear whether apical membrane proteins that bind to PDZ proteins, require the PDZ interaction for apical trafficking or localization. We find that GCCA4 was efficiently targeted to the apical cell surface (Fig. 5A). Thus, GCC must contain apical targeting information in other regions of the protein, and interaction with PDZ proteins is not required for the efficient surface expression or apical targeting of GCC.

Previous mutagenesis studies indicate that the COOH terminus of GCC is required for catalytic function of the enzyme (4, 53). Since association with PDZ proteins has been shown to modulate activation and down-stream signaling of other cellular receptors (36, 54), we tested the hypothesis that association with IKEPP regulated the catalytic activity of GCC. We found

that co-expression of IKEPP with GCC significantly decreases STA-mediated accumulation of cGMP in transfected cells (Fig. 5B). If the GCC-IKEPP interaction occurs within an intracellular compartment, co-expression of IKEPP could decrease the number of receptors present on the cell surface. Our localization studies suggest that IKEPP is associated with the apical cell surface (Fig. 3), but we also observe significant amounts of IKEPP in the subapical compartment of Caco2 cells (Fig. 3A). However, the  $V_{max}$  of the receptor was not significantly different when IKEPP was co-expressed (Fig. 5C), indicating that the GCC-IKEPP interaction does not dramatically change the amount of receptor on the plasma membrane.

IKEPP-mediated inhibition of GCC is only observed in cells expressing full-length GCC and not GCC proteins lacking the PDZ binding motif (Fig. 5B). Thus, a physical interaction between IKEPP and GCC is required for modulation of receptor function. Co-expression of IKEPP and GCC would decrease STA-mediated cGMP accumulation if IKEPP decreases the affinity of the receptor for its ligand. Alternatively, it is possible that intra- or intermolecular interactions between CTEP and the GCC catalytic domain maintain the enzyme in the appropriate conformation for catalytic function. Binding of IKEPP to CTEP may compete for these intra- or intermolecular interactions to decrease the catalytic activity of GCC. Likewise, it is possible that co-expression of IKEPP competes for binding of a cytosolic factor that associates with GCC to stimulate its catalytic function. It is also possible that IKEPP recruits an inhibitory protein to the GCC receptor complex. While the mechanism of the inhibition of GCC catalytic activity by IKEPP is unclear at this time, this interaction may have important implications for understanding the desensitization of GCC. Prolonged application of STA leads to desensitization of the endogenous receptor in T84 cells but not transfected receptors in COS7 or hEK293 cells, suggesting that the desensitization may require the presence of an accessory protein that is not expressed in these cells (29). Thus, it is intriguing to speculate that a regulated interaction with IKEPP or the recruitment of additional proteins to IKEPP-GCC multiprotein complexes is required for GCC receptor desensitization. GCC null mice are resistant to STA but have no obvious phenotype (55, 56), suggesting that we have much to learn regarding the physiological role of GCC and its endogenous ligands. We also have much to learn about mechanisms to control GCC activation and desensitization, since the acute secretory diarrhea caused by STA is a leading cause of pediatric death worldwide. The identification of other proteins in IKEPP-GCC complexes may help elucidate the role of GCC in normal physiology and may provide insights into strategies to control excessive GCC activation by STAs.

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