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Abstract—Cyclophilin A (CypA), a receptor for the immunosuppressive agent cyclosporin A, is a *cis-trans*–peptidyl-prolyl isomerase (PPIase). It accelerates the *cis-trans* isomerization of prolyl-peptide bonds. CypA binds and regulates the activity of a variety of proteins. Atrial natriuretic factor (ANF) and its receptor membrane-bound guanylate cyclase-A (GC-A) are involved in the regulation of blood pressure. We examined whether CypA affects the activation of GC-A by ANF. The results showed that CypA associated with GC-A. Interestingly, binding of ANF to GC-A released CypA. Transfection of CypA inhibited ANF-stimulated GC-A activity, indicating that CypA functions as an endogenous inhibitor for GC-A activation. CypA also inhibits the activity of guanylate cyclase-C (GC-c), the catalytic domain of GC-A, indicating that CypA interacts with the catalytic domain of GC-A. In contrast, transfection of CypA R55A, a CypA mutant expressing low PPIase activity, did not significantly attenuate the activity of GC-c and the activation of GC-A. Inhibition of PPIase activity of CypA with cyclosporin A also blocks the inhibitory effect of CypA on GC-c activity. These results demonstrate that PPIase activity is required for CypA to inhibit GC-c activity and GC-A activation by ANF. Furthermore, mutation of Pro 822, 902, or 958 in GC-c abolished its activity. Therefore, it is likely that CypA binds to GC-A and catalyzes the *cis-trans* isomerization of Pro 822, 902, or 958, which keeps GC-A in the inactive state, and that binding of ANF to GC-A alters the conformation of the catalytic domain that releases CypA from GC-A leading to enzyme activation. **(*Hypertension*. 2004;44:963-968.)**

Key Words: cyclosporin ■ cyclic GMP

Cyclophilin A (CypA), the cytosolic receptor for cyclosporin A (CsA), is a widely used immunosuppressant.[1,2] CypA also possesses peptidyl-prolyl isomerase (PPIase) activity, which is essential for protein folding and conformational change in vivo.[3–6] It is known that CsA binds to CypA and blocks its peptidyl proline *cis-trans* isomerase activity.[7]

Besides acting as a receptor for CsA, CypA acts as a molecular chaperone facilitating the assembly/disassembly of protein complexes, protein trafficking, and regulation of protein activity.[3–6] CypA has been shown to associate with a variety of proteins and regulate their functions.[7–20] For instance, CypA interacts with caveolin-1,[10] peroxiredoxin,[9] prolactin receptor,[8] apoptosis-inducing factor (AIF),[11] protein tyrosine kinase Itk,[19] the HIV capsid protein,[12–14] and the retinoblastoma gene product.[20] Recently, CypA has also been shown to be secreted in response to oxidative stress and functions as a growth factor of smooth muscle cells.[21]

Membrane-bound guanylate cyclase-A (GC-A), a receptor for atrial natriuretic factor (ANF), is known to be involved in the regulation of blood pressure and other cardiovascular functions.[22,23] GC-A contains a kinase-like domain and a catalytic domain in the intracellular region.[22] We and others have shown that the catalytic domain of GC-A is suppressed by the kinase-like domain.[24–26] It is possible that ANF activates GC-A by altering the conformation resulting in the release of the inhibitory constraint imposed on the active site. Because CypA can trigger protein conformational changes by catalyzing a *cis-trans* isomerization of specific Pro residues, we examined its involvement in the activation or inactivation of GC-A by ANF in porcine kidney proximal tubular LLC-PK1 cells. We further examined whether the PPIase activity of CypA is involved in the regulation of GC-A activation by ANF. Our results indicate that CypA interacts with the catalytic domain of GC-A and functions as an inhibitor of ANF-stimulated GC-A activity, and that the PPIase activity is required for the inhibition of ANF-stimulated GC-A activity by CypA.

Materials and Methods

Materials

RPMI medium 1640 was obtained from Gibco. Protein assay reagent was obtained from Bio-Rad. CypA antibody was purchased from

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Calbiochem. Enhanced chemiluminescence kit was obtained from Amersham. Immobilon-polyvinylidene fluoride (PVDF) membrane was purchased from Fisher. GenePORTER transfection reagent was purchased from Gene Therapy Systems. Other common chemicals were purchased from Sigma.

CypA Coimmunoprecipitation With GC-A

The immunoprecipitation was performed as described previously.[27] In brief, LLC-PK1 cells were washed with serum-free RPMI medium 1640 and then challenged with or without 0.1 μmol/L ANF for 10 minutes. Cells were lysed (50 mmol/L Tris, pH 7.6, containing 150 mmol/L NaCl, 20 mmol/L $MgCl_2$, 1% Triton, 1 mmol/L PMSF, 1 μg/mL leupetin, and 1 μg/mL pepstatin), and guinea pig polyclonal GC-A antibodies (1:2000 dilution)[27,28] were added to cell lysates at 4°C for 60 minutes with gentle agitation. Immunocomplexes were collected with protein A Sepharose 4B at 4°C for 30 minutes, washed gently in cold lysis buffer, subjected to SDS-PAGE, and then transferred to PVDF membranes. Membranes were blocked with 5% dry nonfat milk in TBST buffer (50 mmol/L Tris buffer, pH 8.5, containing 150 mmol/L NaCl plus 0.1% Tween-20) and immunoblotted overnight at 4°C with CypA antibodies (1:2000 dilution). Blots were then washed 3× with TBST buffer. PVDF membranes were next incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:4000 dilution) for 1 hour at room temperature. The immunoreactive proteins were visualized using enhanced chemiluminescence.

cGMP Determination

LLC-PK1 and transfected cells were grown to confluence in 6-well plates (35 mm). Cells were washed with 2 mL of serum-free RPMI medium 1640 containing 10 mmol/L Hepes, pH 7.3, and then preincubated at 37°C for 10 minutes with 900 μL of RPMI medium 1640 containing 0.5 mmol/L isobutylmethylxanthine. Various concentrations of ANF were added to the cells and incubated for another 10 minutes at 37°C. After incubation, the medium was aspirated and 0.75 mL cold 10% trichloroacetic acid (TCA) was added to the plates. Cell extracts were scraped, centrifuged for 15 minutes at 2000*g*, and the supernatant fractions were extracted with water-saturated ether to remove TCA. cGMP levels in the supernatants were determined by radioimmunoassay.[26–29]

Expression of Guanylate Cyclase-C, CypA, and CypA R55A

Twelve micrograms of guanylate cyclase-C (GC-c),[26,28] or CypA/CypA R55A[30] plasmids were transfected into 60% to 80% confluent monkey fibroblast COS-7 cells by the GenePORTER transfection reagent according to instructions of the manufacturer. Two days later, transfected cells were used for cGMP determination.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit from Stratagene. This method uses polymerase chain reaction (PCR) with pfuTurbo DNA polymerase, which has 6-fold higher fidelity than *Taq*DNA polymerase in DNA synthesis. PCR was performed using pfuTurbo DNA polymerase, GC-c plasmids, and a pair of primer containing the desired mutations (ie, Pro to Ala). PCR parameters were as follows: denaturation 95°C for 30 sec; annealing 55°C for 1 minute; and extension 68°C for 12 minutes (for GC-c; 18 minutes for GC-A) for 18 cycles. PCR products were then digested with *Dpn*I endonuclease to remove the parental DNA template. DNA templates containing the mutation were used to transform XL1-Blue competent cells. Mutations on GC-c were confirmed by DNA sequencing.

Statistics

All error bars represent the SD from the mean of 4 experimental replicates.

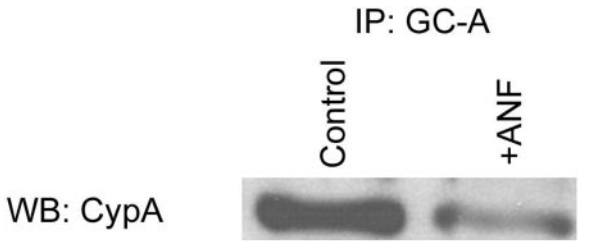

Figure 1. Coimmunoprecipitation of CypA with GC-A in LLC-PK1 cells. LLC-PK1 cells were incubated with and without 0.1 μmol/L ANF for 10 minutes. After incubation, cell lysates were immunoprecipitated (IP) with GC-A antibodies. Immunoprecipitates were subjected to 10% SDS-PAGE and Western blot (WB) analysis by CypA antibodies. CypA associated with GC-A. However, CypA was released from GC-A in the presence of ANF.

Results

CypA Association With GC-A in LLC-PK1 Cells

CypA has been shown to associate with a variety of proteins and affect their functions.[7–20] To examine whether CypA associates with GC-A, we incubated LLC-PK1 cells with and without 0.1 μmol/L ANF for 10 minutes. After incubation, GC-A and its associated proteins were immunoprecipitated with a GC-A antibody.[27] Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis using CypA antibodies. Figure 1 shows that CypA associated with GC-A. Interestingly, addition of ANF caused the dissociation of CypA from GC-A, suggesting that CypA may regulate activation of GC-A by ANF.

ANF-Stimulated GC-A Activity Inhibition by CypA in LLC-PK1 Cells

To examine whether CypA affects the activation of GC-A by ANF, we transfected CypA plasmids into LLC-PK1 cells. Two days after transfection, the effect of CypA on 0.1 μmol/L ANF-stimulated GC-A activity was measured. Figure 2 shows that transfection of CypA had little effect on basal GC-A activity. However, CypA substantially inhibited ANF-stimulated GC-A activity. Interestingly, transfection of CypA into LLC-PK1 cells did not significantly affect the activation of soluble guanylate cyclase by 10 nmol/L bradykinin (Figure 3).

GC-c Activity Inhibition by CypA in COS-7 Cells

GC-A contains a catalytic domain and a kinase-like domain in the intracellular region.[23] We and others have shown that the catalytic domain (GC-c) functions like a constitutively active GC-A.[24–26] To examine whether CypA associates with the catalytic domain of GC-A and inhibits its activity, we measured the effect of CypA on the activity of GC-c in COS-7 cells. GC-c with or without CypA plasmids was transfected into COS-7 cells that express little endogenous guanylate cyclase activity. COS-7 cells transfected with empty pcDNA3.1 were used as the control. Two days after transfection, cGMP levels in transfected cells were measured. Figure 4 shows that expression of GC-c substantially increased cGMP levels in COS-7 cells. Transfection of CypA inhibited the activity of GC-c (Figure 4), indicating that CypA interacts directly with the catalytic domain of GC-A.

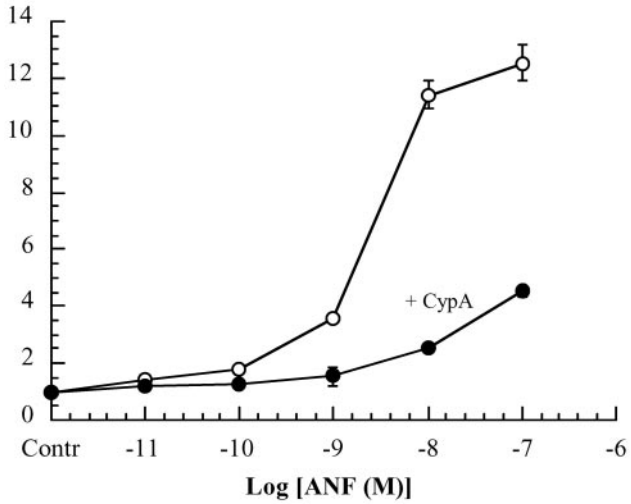

Figure 2. Inhibition of ANF-stimulated GC-A activity by CypA in LLC-PK1 cells. LLC-PK1 cells were transfected with or without CypA plasmids. Two days later, cells were exposed to 0.5 mmol/L isobutylmethylxanthine at 37°C for 10 minutes and various concentrations of ANF for another 10 minutes. The reaction was stopped with 10% TCA. Generated cGMP was measured by radioimmunoassay.

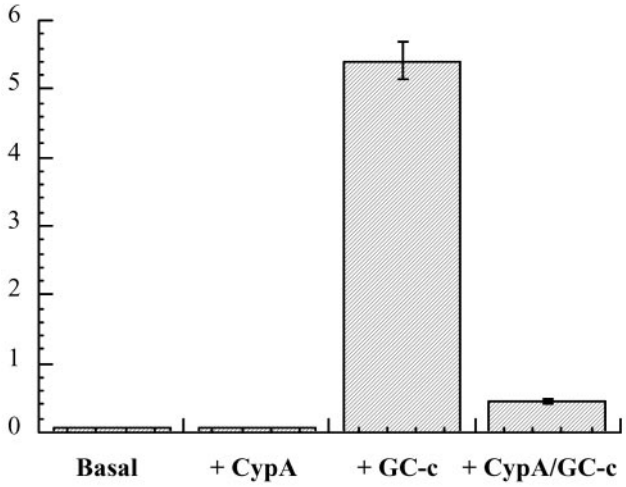

Figure 4. Inhibition of GC-c activity by CypA in COS-7 cells. COS-7 cells were transfected with GC-c in the presence and absence of CypA plasmids. Two days later, cells were exposed to 0.5 mmol/L isobutylmethylxanthine at 37°C for 10 minutes. The reaction was stopped with 10% TCA. Generated cGMP was measured by radioimmunoassay.

PPIase Activity of CypA Requirement for Inhibition of GC-c Activity and GC-A Activation

CypA possesses PPIase activity.[3–6] To explore whether PPIase activity is essential for inhibition of GC-c activity by CypA, we transfected GC-c with and without CypA R55A plasmids, a CypA mutant that expresses very low PPIase activity, into COS-7 cells. Cells transfected with empty pcDNA3.1 plasmids were used as the control. Two days after transfection, cGMP levels in control and transfected cells were measured. Figure 5 shows that unlike wild-type CypA, CypA R55A mutant had little effect on the activity of GC-c. Transfection of CypA R55A into LLC-PK1 cells also failed to inhibit ANF-stimulated GC-A activity (Figure 6). These results suggest that PPIase activity is important for CypA to inhibit GC-c activity.

CsA, an immunosuppressant, is known to bind to CypA and inhibit its PPIase activity.[7] To examine whether CsA treatment can block the inhibitory effect of CypA on GC-c activity, we preincubated CypA/GC-c transfected COS-7 cells with or without 1 μmol/L CsA for 14 hours. Figure 5 shows that CsA treatment reversed the inhibitory effect of CypA on GC-c activity. These results demonstrate that PPIase activity is required for the inhibitory effect of CypA on GC-c activity.

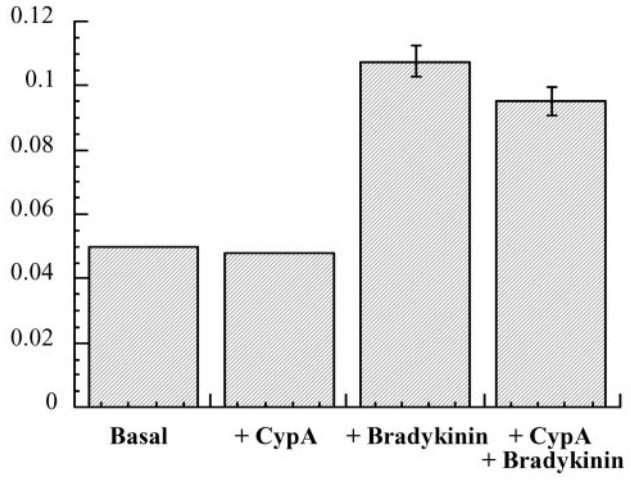

Figure 3. Effect of CypA on bradykinin-stimulated GC-A activity in LLC-PK1 cells. LLC-PK1 cells were transfected with or without CypA plasmids. Two days later, cells were exposed to 0.5 mmol/L isobutylmethylxanthine at 37°C for 10 minutes and 10 nmol/L for another 10 minutes. The reaction was stopped with 10% TCA. Generated cGMP was measured by radioimmunoassay.

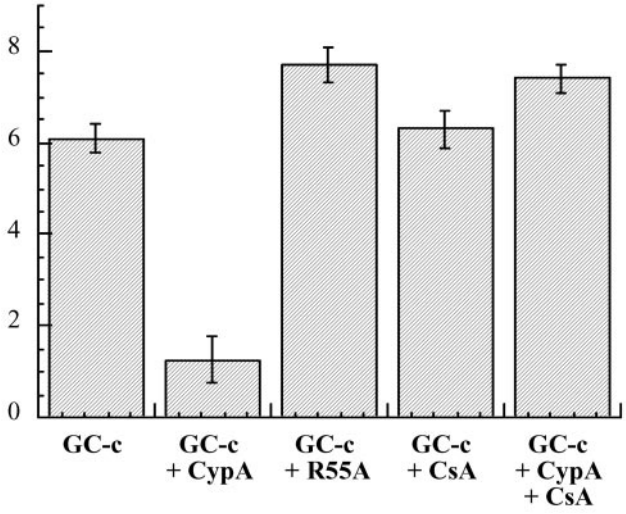

Figure 5. Requirement of PPIase activity for CypA to inhibit GC-c activity in COS-7 cells. COS-7 cells were transfected with GC-c with or without CypA or CypA R55A plasmids. Two days later, cells were exposed to 0.5 mmol/L isobutylmethylxanthine at 37°C for 10 minutes. The reaction was stopped with 10% TCA. Generated cGMP was measured by radioimmunoassay.

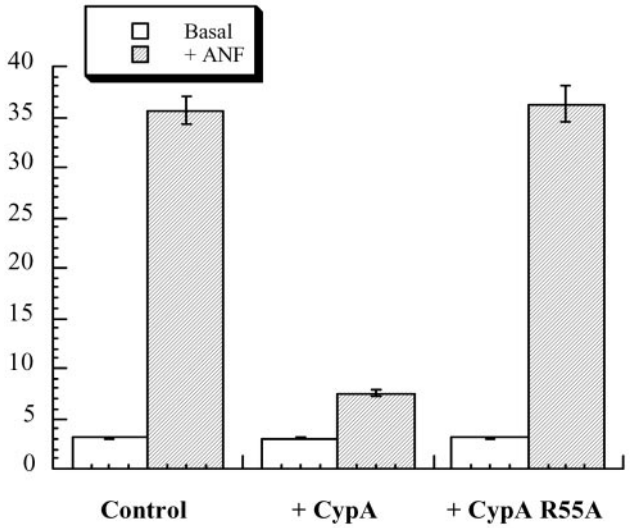

Figure 6. Blockage of the effect of CypA on ANF-stimulated GC-A activity by CsA in LLC-PK1 cells. LLC-PK1 cells were transfected with or without CypA or CypA R55A plasmids. Two days later, cells were then exposed to 0.5 mmol/L isobutylmethylxanthine at 37°C for 10 minutes and then 0.1 μmol/L ANF for another 10 minutes. The reaction was stopped with 10% TCA. Generated cGMP was measured by radioimmunoassay.

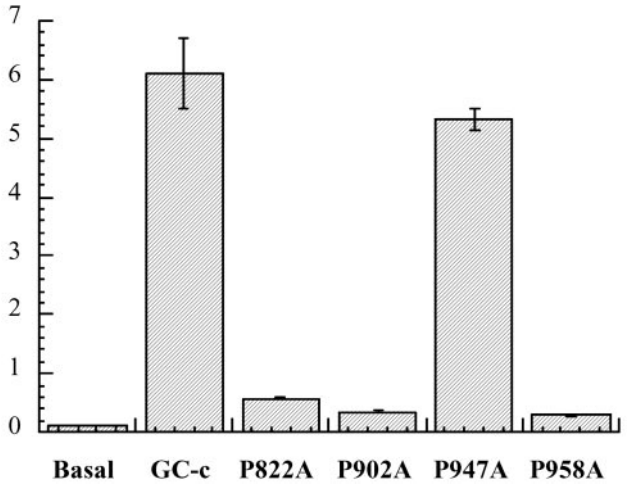

Figure 7. Loss of GC-c activity by substitution of Pro 822, 902, or 958 with an Ala residue. Pro 822, 902, 947, or 958 on GC-c was replaced with an Ala residue by site-directed mutagenesis. Wild-type GC-c or GC-c mutants were transfected into COS-7 cells. Two days after transfection, cultured cells were exposed to 0.5 mmol/L isobutylmethylxanthine at 37°C for 10 minutes. The reaction was stopped with 10% TCA. Generated cGMP was measured by radioimmunoassay.

Proline Residues Within GC-c Determined as Potential Targets of CypA

If CypA inhibits GC-c and ANF-stimulated GC-A activity by catalyzing the *cis-trans* isomerization of prolyl peptide bonds, some Pro residues in the catalytic domain should be targets of CypA. The catalytic domain of GC-A contains 6 Pro residues at positions 822, 862, 902, 933, 947, and 958. To determine the potential target of CypA on GC-A, we performed site-directed mutagenesis to substitute Pro residue 822, 902, 947, or 958, with an Ala residue on GC-c, and then transfected wild-type GC-c or the resulting mutants into COS-7 cells. Figure 7 shows that mutation of Pro 822, 902, or 958 abolished the GC-c activity. However, GC-c P947A expressed enzyme activity similar to wild-type GC-c. These results suggest that Pro 822, 902, and 958 may be the substrates of CypA.

Discussion

Membrane-bound GC-A is a receptor for ANF and possesses guanylate cyclase activity. Although GC-A is a bifunctional protein, the mechanisms by which ANF activates GC-A are not fully understood. CypA, a protein involved in protein folding, is expressed abundantly in the kidney and in epithelial cells of renal proximal tubules.[31] In this study, we investigated the effects of CypA on the activation of GC-A by ANF in porcine proximal tubular LLC-PK1 cells. We found that CypA inhibits ANF-stimulated GC-A activity by interacting with the catalytic domain of GC-A, and that the PPIase activity of CypA is required for this inhibitory effect.

GC-A contains 3 domains: an ANF-binding domain in the extracellular region, a kinase-like domain, and a catalytic domain in the intracellular region.[22,23] Coimmunoprecipitation and guanylate cyclase assay indicate that CypA associates with GC-A and inhibits ANF-stimulated GC-A activity in LLC-PK1 cells. Similar results were also observed in rat kidney proximal tubular epithelial cells (our unpublished data, 2004). To determine whether CypA interacts with the catalytic domain of GC-A, we cotransfected CypA and GC-c plasmids into COS-7 cells and found that CypA transfection inhibits GC-c activity. These results indicate that CypA associates with the catalytic domain of GC-A and inhibits its enzyme activity. A precedent that CypA can interact with a hormone receptor has been reported for the prolactin receptor.[8]

Previous deletion mutagenesis and limited proteolysis studies indicated that the catalytic domain of GC-A is suppressed by the kinase-like domain.[24–26] Therefore, it is believed that the binding of ANF to GC-A triggers a conformational change on GC-A that relieves the inhibitory constraint imposed on the active site of GC-A. Thus, GC-c that does not contain the kinase-like domain would function as a constitutively active GC-A. Our results indicate that CypA inhibits GC-c activity, suggesting that CypA interacts with the activated GC-A rather than inactive GC-A. Consistently, our results showed that CypA inhibits ANF-stimulated GC-A activity without significantly affecting basal GC-A activity in LLC-PK1 cells.

A variety of proteins, including receptors, channels, and protein kinases, has been shown to associate with and be regulated by CypA.[7–20] CypA is a *cis-trans*-PPIase.[3–6] However, whether the PPIase activity of CypA is required for these regulations varies with its associated proteins or ligands. For instance, the PPIase activity of CypA is not involved in the immunosuppressive effect of CsA[3–6] and its cooperation with AIF for triggering apoptosis.[11] However, the PPIase activity of CypA is involved in the production of reactive oxygen species and muscle differentiation induced by CsA, opening of the cystic fibrosis transmembrane conductance regulator channel, inhibition of the protein tyrosine

kinase Itk, promotion of Zpr1 nuclear export, and catalysis of *cis*/*trans* isomerization of HIV-1 capsid.[30,15–18] We used 2 different approaches to examine whether PPIase activity of CypA is required for its inhibitory effect on GC-c activity. Transfection experiments showed that unlike wild-type CypA, CypA R55A, a CypA mutant expressing very low PPIase activity,[30] did not inhibit GC-c activity. Furthermore, CsA treatment that inhibits PPIase activity of CypA blocks the inhibitory effect of CypA on GC-c activity in COS-7 cells. Thus, PPIase activity of CypA is required for inhibition of GC-c activity. It is likely that CypA catalyzes the *cis-trans* isomerization of the prolyl-peptide bond on the catalytic domain of GC-A and thus affects conformation and catalytic function of the active site of GC-A.

The substrate specificities of CypA have been determined using a synthetic substrate *N*-carboxypropionyl-Ala-Xaa-Pro-Phe-p-nitroanilide.[32] CypA is effective when Xaa is Gly, Ala, Val, Leu, Phe, His, Lys, or Glu. Sequence analysis reveals that there are 6 proline residues in the catalytic domain of GC-A. Among them, Pro 962 and 933 are preceded by Thr and Arg, respectively, and are probably not the CypA substrates. Therefore, we mutated Pro 822, 902, 947, and 958 into an Ala residue that tends to favor the *trans*-peptidyl conformation.[18] Results showed that mutation of Pro 822, 902, or 958 into an Ala residue on GC-c abolishes its activity. However, GC-c P947A still expresses significantly high enzyme activity. Therefore, it is likely that CypA associates with the catalytic domain of GC-A and catalyzes the *cis-trans* isomerization of peptide bond of Pro 822, 902, or 958 residue. These isomerization events alter conformation of the active site, leading to inhibition of enzyme activity. The 3D structure of the active site of GC-A remains unknown. Our studies suggest that the active state favors the *cis* configuration of peptide bond of Pro 822, 902, or 958, and that CypA converts the *cis* configuration into *trans* and keeps the enzyme in the inactive state.

The mechanisms of GC-A activation by ANF remain unclear. Accumulated studies indicate that the catalytic domain of GC-A is subjected to an inhibitory constraint exerted by the kinase-like domain.[24–26] Besides the inhibitory constraint exerted from the kinase-like domain, an endogenous inhibitor for GC-A has been suggested.[33] However, this postulated inhibitor has not been yet identified. We showed that CypA associates with GC-A and that addition of ANF causes the release of CypA from GC-A. Furthermore, transfection of CypA inhibits GC-c activity in COS-7 cells and ANF-stimulated GC-A activity in LLC-PK1 cells. Because CypA can inhibit GC-c activity (which is free of the kinase-like domain), the kinase-like domain plays no role in the inhibitory effect of CypA on GC-A activation. Therefore, CypA is likely the proposed endogenous inhibitor by Dr Garber's laboratory. Thus, GC-A is subjected to 2 different inhibitory constraints: 1 from its kinase-like domain and the other from CypA. It is likely that binding of ANF to GC-A alters its conformation and thus releases the inhibitory constrains from CypA and the kinase-like domain on the active site.

In summary, we have shown that CypA functions as an inhibitor for GC-A activation stimulated by ANF. CypA associates with catalytic domain of GC-A. Addition of ANF to GC-A alters the conformation of the catalytic domain that releases CypA from GC-A. Because the PPIase activity is required for inhibition of GC-c activity, and mutation of Pro 822, 902, or 958 abolishes GC-c activity, it is likely that CypA catalyzes the *cis-trans* isomerization of peptide bond of Pro 822, 902, or 958 that keeps GC-A in the inactive state. Binding of ANF to GC-A alters its conformation and triggers the release of CypA leading to enzyme activation.

Perspectives

The mechanisms by which ANF activates GC-A are not fully understood. ANF is known to activate GC-A by releasing the inhibitory constraint exerted from its kinase-like domain. Additionally, an endogenous inhibitor has also been suggested to regulate activation of GC-A or catalytic activity of GC-A. Our studies indicate that CypA functions as the proposed inhibitor for GC-A. Therefore, understanding how CypA inhibits GC-A activation would help uncover the mechanisms of GC-A activation by ANF. Because the ANF/GC-A pathway is involved in the regulation of blood pressure, inhibition of the CypA–GC-A interaction may have therapeutic applications.

Acknowledgments

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