Functional Chimeras of the Phosphodiesterase 5 and 10 Tandem GAF Domains*

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The tandem GAF domain of hPDE10A uses cAMP as an allosteric ligand (Gross-Langenhoff, M., Hofbauer, K., Weber, J., Schultz, A., and Schultz, J. E. (2006) J. Biol. Chem. 281, 2841–2846). We used a two-pronged approach to study how discrimination of ligand is achieved in human (h)PDE10A and how domain selection in the phosphodiesterase GAF tandems is determined. First, we examined which functional groups of cAMP are responsible for purine ring discrimination. Changes at the C-6 ring position (removal of the amino group; chloride substitution) and at the N-1 ring position reduced stimulation efficacy by 80%, i.e. marking those positions as decisive for nucleotide discrimination. Second, we generated a GAF tandem chimera that consisted of the cGMP-binding GAF-A unit from hPDE5A1, which signals through cGMP in PDE5, and the GAF-B from hPDE10A1, which signals through cAMP in PDE10. Stimulation of the reporter enzyme exclusively was through the GAF-B domain of hPDE10A1 (EC_{50} = 7 \mu M cAMP) as shown by respective point mutations. The PDE5 GAF-A domain in the chimera did not signal, and its function was reduced to a strictly structural role. Signaling was independent of the origin of the N terminus. Generating 10 additional PDE5/10 tandem GAF chimeras surprisingly demonstrated that the length-conserved linker in GAF tandems between GAF-A and GAF-B played an unforeseen decisive role in intramolecular signaling. Swapping the linker sections between PDE5 and PDE10 tandem GAF domains abrogated signaling completely pointing to specific domain interactions within GAF tandems, which are not visible in the available crystal structures with bound ligands.

The second messengers cAMP and cGMP transduce extracellular stimuli into intracellular responses (1). Obviously, efficient and precise regulation of biosynthesis and degradation of these messengers is an important issue. For this purpose a multitude of adenylyl cyclases (ACs) and phosphodiesterases (PDEs) is available to rapidly adjust cyclic nucleotide levels in the cell (2–4). In mammals 11 PDE families exist that are encoded by no less than 21 genes. Using different transcription start sites, alternative splicing, and secondary modifications, more than 50 PDE isozymes have been counted so far (5, 6). Although the catalytic domains of mammalian PDEs are rather similar, the N-terminal regulatory domains differ (3). For example, PDE1 has a tandem calmodulin-binding ensemble; PDE4 has a tandem arrangement of uniformly conserved regions, and PDE2, -5, -6, -10, and -11 have N-terminal tandem GAF domains (6), i.e. two GAF domains, A and B, spaced by a length, yet not the sequence conserved linker of 36 amino acids (Fig. 3B). Generally, the >4500 GAF domains that are present in all kingdoms of life are considered small molecule-binding domains. In mammals, GAF domains are exclusively found in PDE2, -5, -6, -10, and -11.

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Albeit individual GAF domains within a particular GAF tandem and between different mammalian PDEs share considerable sequence similarities, it is impossible to predict ligand specificity based on sequence features. In addition, in a GAF tandem the individual GAF domains in PDEs differ functionally in that only one appears to bind cyclic nucleotide and signal, whereas the other appears to be necessary to stabilize a certain structural state because halving GAF tandems abolishes signaling and regulation (13, 14). In PDE2, the GAF tandem signals via the B domain (9), in PDE-5 and -11, cGMP binds and signals via GAF-A, and in PDE10 cAMP signals via GAF-B (7, 8). This raises questions such as whether binding of cNMPs in individual GAF domains is accomplished in an identical manner or whether chimeras consisting of signaling GAF-A and -B units from different PDEs retain both functionalities.

First, we characterized the specificity for cAMP signaling in the PDE10 GAF tandem using structural analogues. Second, we generated chimeric GAF tandems from GAF-A PDE5, which signals via cGMP, and GAF-B of PDE10, which signals via cAMP. In using the cyanobacterial AC CyaB1 as an established reporter enzyme, we avoided the problem that substrate and allosteric activator are identical (15). We obtained partially functional chimeras and, surprisingly, found that the linker between the GAF-A and -B domains appears directly involved in signaling.
GAF Chimeras from PDE5 and PDE10—Experiments with the right.

**EXPERIMENTAL PROCEDURES**

Recombinant DNAs—The *cybA1* gene (gi: 15553050) was from Dr. Ohmori (University of Tokyo), hPDE5A1_2 (gi: 61744434) from Dr. Friebe (University of Bochum), and a cDNA clone of hPDE10A1 (gi: 4894715) from Dr. Quintini (Nycoderm, Konstanz, Germany). All numbering refers to these genes. For generation of chimeric and PDE5/PDE10 tandem GAF domains, each tandem was subdivided into four segments. These were as follows: PDE5A1 N terminus, 5N, residues 1–143; GAF-A, 5A, 144–310; linker, 5L, 311–346; GAF-B, 5B, 347–513. For hPDE10A1: N terminus, 10N, residues 1–72; GAF-A, 10A, 73–230; linker, 10L, 231–266; GAF-B, 10B, 267–422. For *CyaB1* adenylyl cyclase, AC, 386–859. The mean of basal activities (*n* = 4–6) and the EC50 concentrations for cAMP-stimulated constructs (except for the PDE5 tandem GAF construct, here cGMP) are on the right. n.d., not determinable; n.a., not activated. At the far right the fold activation for the activated constructs is given.

FIGURE 1. Schematic overview of all chimeric PDE5/PDE10 tandem GAF domains. The domain boundaries were as follows: for hPDE5A1: N terminus, 5N, amino acids 1–143; GAF-A, 5A, 144–310; linker, 5L, 311–346; GAF-B, 5B, 347–513. For hPDE10A1: N terminus, 10N, 14–72; GAF-A, 10A, 73–230; linker, 10L, 231–266; GAF-B, 10B, 267–422. For *CyaB1* adenylyl cyclase, AC, 386–859. The mean of basal activities (*n* = 4–6) and the EC50 concentrations for cAMP-stimulated constructs (except for the PDE5 tandem GAF construct, here cGMP) are on the right. n.d., not determinable; n.a., not activated. At the far right the fold activation for the activated constructs is given.

| 10N | 10A | 10L | 10B | Basal activity (nmol cAMP/min) | EC50 concentrations for cAMP activation | Fold-activation by cAMP over basal |
|-----|-----|-----|-----|-------------------------------|---------------------------------------|---------------------------------|
| 5N  | 5A  | 5L  | 5B  | 10N  | 10A  | 10L  | 10B  | 5N  | 5A  | 5L  | 5B  | 10N  | 10A  | 10L  | 10B  |
| 51.7 ± 5.3 | 18.1 ± 1.0 | 16.5 ± 1.4 |
| 12.6 ± 1.0 | 2.4 ± 0.1 | 24.4 ± 3.0 (cGMP) |
| 3.6 ± 0.4 | 7.2 ± 0.3 | 7.2 ± 0.5 |
| 24.7 ± 2.9 | 7.0 ± 1.7 | 4.9 ± 0.5 |
| 3.7 ± 0.1 | 144 ± 66 | 1.7 ± 0.1 |
| 10.7 ± 2.3 | 17 ± 7.5 | 2.4 ± 0.2 |
| 69.3 ± 7.4 | n.d. | 1.2 ± 0.1 |
| 62.8 ± 11 | n.a. | --- |
| 4.0 ± 1.3 | n.a. | --- |
| 65.0 ± 5.4 | n.d. | 1.2 ± 0.04 |
| 45.1 ± 0.3 | n.d. | --- |
| 42.0 ± 0.4 | 1.4 ± 0.2 |
| 33.7 ± 6.0 | n.a. | --- |

Expression and Purification of Recombinant Proteins—Expression was in *E. coli* BL21 (DE3) [pRep4] grown in Luria-Bertani medium with 50 μg/ml kanamycin and 100 μg/ml ampicillin. Induction was with 200 μM isopropyl β-thiogalactoside at an *A600* of about 0.6. Cells were grown in the presence of 10 mM MgCl2 at 24 °C for 5–7 h. Bacteria were harvested by centrifugation (10 min, 4100 × g, 4 °C), washed once with 50 mM Tris/HCl, pH 8.0, 1 mM EDTA (4 °C), collected by centrifugation, and stored at −80 °C.

Cells were broken at 4 °C with a French press at 1000 p.s.i. in cell lysis buffer (50 mM Tris/HCl, 20% glycerol, 50 mM NaCl, 25 mM imidazole, pH 8.0, and Complete EDTA-free protease inhibitor tablets (Roche Diagnostics)). The homogenate was centrifuged (41,000 × g, 60 min, 4 °C), and the supernatant was incubated with 0.25 ml of Profinity™ IMAC nickel-charged resin (Bio-Rad) for 2–4 h on ice. The affinity material was poured into a mini-column and successively washed twice with 6 ml of buffer A (50 mM Tris/HCl, 20% glycerol, 400 mM NaCl,
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2 mM MgCl₂, 35 mM imidazole, pH 8.0), twice with 4 ml of buffer B (buffer A + 55 mM imidazole), and twice with 4 ml of buffer C (buffer A with 10 mM NaCl and 75 mM imidazole). Protein elution was with 0.3 ml of buffer A containing 10 mM NaCl and 300 mM imidazole. The eluate was immediately dialyzed for at least 2 h (4 °C) against 50 mM Tris/HCl, pH 7.5, 35% glycerol, 10 mM NaCl, and 2 mM MgCl₂. Proteins were stored at −20 °C.

Adenylyl cyclase activity was measured for 10 min in a volume of 100 μl at 37 °C (17). The reactions contained 22% glycerol, 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 50 μg of bovine serum albumin, 50−150 ng of recombinant protein, and 75 μM [α-32P]ATP (25 kBq; Hartmann Analytic, Braunschweig, Germany). [2.8-3H]cAMP (150 Bq; GE Healthcare) was added after the reaction stop to monitor yield during product isolation. The reaction was started by addition of ATP. ATP conversion was limited to less than 10% to guarantee linearity. We consistently examined reaction kinetics of the reporter AC under all conditions. cNMP additions did not affect Kₘ values for ATP but altered Vₘₐₓ. Values are means ± S.E. of 4–6 experiments. A t test was used for statistical evaluation. Cyclic nucleotide analogues were purchased from BioLog (Bremen, Germany).

Western Blot Analysis—Proteins were mixed with sample buffer and subjected to SDS-PAGE (12.5%). Proteins were blotted onto polyvinylidene difluoride membranes and probed with an anti-RGS-H4- (Qiagen) and with a 1:5000 dilution of a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Dianova, Hamburg, Germany). Peroxidase detection was with the ECL Plus kit (GE Healthcare). Western blots of affinity-purified proteins are depicted to visualize the presence of intact constructs. N-terminally degraded proteins would not have bound to the affinity material. C-terminally truncated proteins, which occasionally were present, are catalytically inactive, i.e., Western blots faithfully showed the amount of active proteins.

RESULTS

Activation of hPDE10-GAF Tandem CyaB1 Adenylyl Cyclase Chimera by Cyclic Purine Nucleotides—So far, six N- and C-terminal PDE10 splice variants have been detected in humans with PDE10A1 and -A2 being the major isoforms. Both are identical starting from Leu-14. Therefore, we used Leu-14 as the start of the PDE10 N terminus. hPDE10A1-(14–422)/CyaB1-(386–555) AC (Fig. 2A) had been used to identify cAMP as a ligand (7). We used this chimera to examine seven cyclic purine monophosphates for their potency to activate the GAF tandem domain to identify functional groups at the cAMP moiety, which affect cyclic nucleotide binding affinity or discrimination. All cAMP analogues were less potent (10−83%) to stimulate the CyaB1 AC than cAMP itself (Fig. 2B). Dose-response curves up to 300 μM indicated three groups of compounds (Fig. 2B). cAMP and 7-deaza-cAMP (7-CH-cAMP) were nearly equipotent; the calculated EC₅₀ concentrations were 30 μM. This showed the absence of major interactions between N-7 of the imidazole ring and indicated that preferentially the pyrimidine ring is involved in ligand selection. Equiportent were 2-chloro-cAMP and 2’-deoxy-cAMP which, however, stimulated significantly less over the entire concentration range. The EC₅₀ concentrations increased significantly to 100 μM. The 2-position is a critical spot for ligand discrimination because cGMP has an amino group at this position. The loss of efficacy of 2-chloro-cAMP probably was because of steric hindrance by the substituent rather than to the slight electron withdrawing effect. The removal of the 2’-hydroxyl from the ribose considerably reduced potency indicating that the ribose entity contributed to the overall binding energy, although it is not involved in ligand discrimination as this position is identical in all cyclic nucleotides. This is in agreement with the observed H-bonding to Thr-364 observed in the recent crystal structure (18). The third group of compounds, 1-NO-cAMP, 6-Cl-cPuMP, cPuMP, and 2-amino-cPuMP, stimulated the attached reporter enzyme even less, and EC₅₀ concentrations could not be calculated (Fig. 2B). Removal of the 6-amino group (cPuMP) or its replacement by chloride (6-Cl-cPuMP) highlighted the crucial importance of the 6-NH₂ group. This amino group thus appeared to be the expected crucial discriminator between cAMP and cGMP, which has a carbonyl function at this position (cGMP itself was inactive). Modification of the N-1 position (1-NO-cAMP) had a similarly strong effect (Fig. 2B) probably because this dramatically altered the charge distribution around this critical region and abolished a position for a hydrogen bond. 2-Amino-cPuMP was even less effective than the 2-chloro-cAMP. Because both substituents have approximately the same size, the steric impediment should be identical. The 2-amino group most likely released electrons into the amido ring heavily involved in the amido structure resulting in reduced affinity. This demonstrated that the 6-amino function cannot be shifted to the 2-ring position. Cyclic inosine and cyclic xanthosine monophosphates had
no activity (not shown). In these compounds the charge distribution around the pyrimidine ring is altered because of the lack of the amino substituent (inosine) or the additional oxygen (xanthosine).

Characterization of PDE5/PDE10/CyaB1 Chimeras—12 of 14 individual GAF domains in mammalian PDEs share an NKFDE motif that has been implicated in formation of the cyclic nucleotide binding pocket (3, 16). This motif is present in either GAF domain of the PDE5 tandem. In the PDE10 GAF tandem, only the B-domain has an NKFDE motif, whereas in GAF-A these positions are RHFFHQ (Fig. 3A) (13). Mutational studies of the NKFDE motif established that PDE5 signals via GAF-A and PDE10 via GAF-B (7, 19). Therefore, the question was whether a chimera, which consists of the cGMP-signaling GAF-A from PDE5 and the cAMP-signaling GAF-B domain from PDE10, could be stimulated by cGMP and cAMP. In view of the important role of the N terminus in signaling of GAF tandem domains, we designed 11 PDE5/PDE10 GAF tandem chimeras to individually examine the contribution to intramolecular signal transduction (Fig. 1).

In 10N-5A-10L-10B-AC, GAF-A from hPDE10A1 was replaced by the one from hPDE5A1 (Fig. 4A). The affinity-purified protein had a basal activity of 3.6 ± 0.4 nmol of cAMP mg⁻¹ min⁻¹. AC activity was stimulated mainly by cAMP (7.3 ± 0.6-fold), whereas cGMP was much less potent (3.8 ± 0.3-fold; Fig. 4A). The EC₅₀ for cAMP was 7.2 ± 0.3 μM, compared with 18.1 ± 1.0 for the PDE10-tandem GAF (7). For cGMP, no EC₅₀ concentration could be determined. On one hand, the data were surprising in that such a chimera could be stimulated at all. On the other hand, they were disappointing because the PDE5 GAF-A domain, which is responsible for cGMP binding and signaling, apparently was not functional. In the next chimeras, the N terminus of hPDE5A1 was introduced into the former one (5N-5A-10L-10B-AC; see Fig. 4B). This did not affect the stimulation by cAMP and cGMP. cAMP stimulated 4.9 ± 0.5-fold and cGMP 2.5 ± 0.2-fold. The EC₅₀ concentration for cAMP remained at 7.0 ± 1.7 μM. A major difference was an increase in basal activity indicating that the N terminus of the GAF domain probably interacted with the C-terminal catalytic domain as reported previously. To rule out that the differences were because of changes in enzymatic properties, Kₘ values derived from a Lineweaver-Burk plot (19.9 ± 1.4 and 21.9 ± 2.6 μM ATP, respectively) and Hill coefficients (both <1) were determined. These values are almost identical to the Kₘ values of the parent constructs PDE5-GAF/CyaB1 AC (21.2 ± 1.4 μM ATP) and PDE10-GAF/CyaB1 AC (22.2 ± 1.2 μM ATP).

To probe whether cAMP and the minor cGMP stimulation were mediated exclusively by the PDE10 GAF-B subdomain, we introduced a D397A mutation into PDE10A1 GAF-B of the 5N-5A-10L-10B-AC,
which is known to abrogate cAMP signaling in a PDE10-GAF/CyaB1 AC construct (see Fig. 4B for scheme and inset for data) (7). The D397A mutation in 5N-5A-10L-10B-AC led to a drastic reduction of cyclase activation by cAMP (EC$_{50}$ = 144 ± 66.4 μM) and abolished stimulation by cGMP (Fig. 4B, inset). This point was further corroborated by using a D299A mutation in 5A of 5N-5A-10L-10B-AC. The activation by cAMP (EC$_{50}$ = 17.5 ± 7.5 μM) was unaffected as was the minor effect of cGMP strongly indicating signaling via 10B in 5N-5A-10L-10B-AC.

It is known that phosphorylation of Ser-102 in the PDE5 N terminus leads to an enhancement of cGMP signaling (16, 20). Therefore, a 5N-S102D-5A-10L-10B-AC construct was generated. AC activation by cAMP and cGMP as examined with the recombinant protein was not different from the parent in 5N-5A-10L-10B-AC protein. This result indicated that 5N could not productively interact with 10B in a manner comparable with the PDE5 GAF tandem and suggested again that 5N-5A in this construct was devoid of an intrinsic biochemical function. Because the $K_m$ value of the cyclase in these constructs (22.8 ± 5.2 μM ATP) was not affected, this showed that the major stimulation by cAMP and the minor one by cGMP in the parent chimera were both mediated via the GAF-B domain of PDE10, whereas the PDE5 GAF-A domain did not signal and probably was reduced to a strictly structural role because cyclase chimeras with only a single GAF domain are means structs (22.8 ± 5.2 μM ATP) was not affected, this showed that the major stimulation by cAMP and the minor one by cGMP in the parent chimera were both mediated via the GAF-B domain of PDE10, whereas the PDE5 GAF-A domain did not signal and probably was reduced to a strictly structural role because cyclase chimeras with only a single GAF domain are not regu-

DISCUSSION

To date, the structures of a PDE2 tandem GAF domain with cGMP, the CyaB2 tandem GAF domain with cAMP, and a homodimer of the GAF-B from PDE10 with bound cAMP are publicly available (18, 21, 22). In all instances, ligands are deeply buried in a pocket in these proteins with less than 10% of the ligand surface solvent-accessible. This implies that to bind or release the ligands, these domains must open. Because no empty structure of a PDE tandem GAF was available, we do not know how much the contacts visible in the resolved structures contribute to purine discrimination. Irrespective of these uncertainties, we used cNMP analogues to identify functional groups of the ligand that are used for purine discrimination by the PDE10 GAF tandem. The region around N-1 and N$^6$ is most important as follows: (i) N-1, because first, N-1 extends a hydrogen bond to Arg-286, which cannot form with the 1-N-oxide cAMP, and, second, probably because of the electron withdrawing effect of the N-oxide, which affected the neighboring N$^6$ group; and (ii) N$^6$, because its removal as in cPUmp or a shift to the 2-position as in 2-amino-cPUmp or its replacement with a chlorine as in 6-Cl-cPUmp disrupted formation of an H-bond to the main chain carbonyl of Cys-287 (18). Thus, the data confirmed and extended the novel structural data (18). This agreement extends to the minor cross-reactivity of cGMP, which apparently binds with much less affinity because it lacks these specific H-bond interactions in the binding pocket and therefore the entropy loss is rather small. The 2-position appeared to discriminate against cGMP and favor cAMP possibly via steric hindrance because 2-substitution with an amino group (same as in cGMP) or chlorine significantly reduced potency (Fig. 2). These effects are not readily explained by recent structural data as the available space appears to be sufficient to accommodate these side chains (18). The 7-nitrogen (7-CH-cAMP) or the ribose ring (2'-cAMP) appeared to be less important for purine ring discrimination. The above inter-
pretation appears to agree with available structural data of the binding pocket that identified C-6 and N-1 as main interaction sites (18, 21, 22). However, this may reflect more the differences in loss of entropy once the ligand is buried in the binding pocket than the actual process of ligand discrimination.

PDE5 specifically hydrolyzes cGMP. It is allosterically activated by its substrate cGMP that binds to GAF-A (8). PDE5, which is prevalent in skeletal and smooth muscle, brain, kidney, lung, heart, and platelets, is the target of sildenafil and similar drugs (5, 24–26). PDE10, which is prominent in testes, thyroid, putamen, and nucleus caudatus (27–29), is a dual specificity enzyme that hydrolyzes cAMP and cGMP about equally well. It is activated by cAMP that binds to the GAF-B domain (5, 7). Remarkably, the cAMP-binding GAF-B domain of hPDE10 shares 44.7% sequence identity with the cGMP-binding GAF-A of hPDE5 (13). Thus, one might have envisaged that combining a cGMP-binding GAF-A from hPDE5 with a rather similar cAMP-binding GAF-B from hPDE10 might result in a chimera that is stimulated equally well by both second messengers. This was not the case. Only GAF-B from PDE10 in the tandem was able to activate the reporter AC, mediating the cAMP and the minor cGMP responses with an efficiency comparable with the PDE10 tandem (7) (Fig. 4B). Thus, the PDE5/PDE10 GAF tandem chimera follows the known pattern in PDEs in that only one domain of the GAF tandem signals, whereas the other apparently has an indispensable structural role. Only the GAF tandem of the CyaB2 AC appears to signal via both subunits as demonstrated by structural and biochemical data (14, 22).

As reported previously (7, 16, 30, 31), we note that the basal activities of different constructs vary considerably. Thus, one may argue that the expression of the chimeras may result in loss of entropy once the ligand is buried in the binding pocket, yet its presence alone is not sufficient for cyclic nucleotide binding and signaling.

The above arguments do not fully exclude the possibility that elevated basal activities might, at least in part, reflect the relief of an inhibitory function associated with one or more of the structural elements in the regulatory domain of the GAF-PDEs (e.g. see Ref. 23). Another interesting point to note is that most of the high basal activity mutants have the PDE5 linker (5L) incorporated with the PDE10 GAF domains (particularly 10B).

Our data imply that interactions between the two GAF domains for the mammalian PDE2, -5, -6, -10, and -11 play an important yet mechanistically not fully understood role in signaling. In this study, the PDE5-GAF-A domain obviously was able to substitute the structural role that is usually played by the PDE10-GAF-A unit, yet it lost its own signaling capability. Similarly, the PDE5 N terminus did not affect signaling anymore as demonstrated by the lack of an effect in the phosphomimetic S102D mutant. Such a mutation in the PDE5 GAF tandem increases cGMP affinity as does phosphorylation of this serine (16, 20). The finding that the source of the linker between GAF-A and -B is very important was unexpected (compare data in Figs. 4 and 5) because the linkers are length-conserved in the seven known tandem GAF domains (Fig. 3B). Obviously the sequence differences in the linkers determine essential interactions between upstream and downstream GAF domains.

Therefore, in the PDE5 GAF tandem, the 36-amino acid-long linker of PDE10 was not a functional equivalent. Similarly, although PDE5 signals via GAF-A, replacement of its GAF-B by that from PDE10 blocked signal transmission, i.e. PDE10-GAF-B did not operate as a structural substitute for PDE5-GAF-B. These complicated interdomain interactions are not apparent from the two GAF tandem structures available so far. One reason may be that only structures with cNMPs bound into the binding pockets are available, i.e. which are in the signaling or on-state. The transition to the off-state without bound ligand may provide a clue about the importance of the linker between GAF-A and -B. It was also notable that we did not observe a gain in function in chimeras between the inactive GAF domains of PDE5 and -10, i.e. PDE10-GAF-A and PDE5-GAF-B, although the latter carried an unbridged NKFDE motif considered essential for ligand binding and signaling (see Fig. 1 for scheme; data not shown). This demonstrated that the NKFDE motif might be necessary for formation of a correct cyclic nucleotide binding pocket, yet its presence alone is not sufficient for cyclic nucleotide binding and signaling.

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