We analyzed cGMP signaling by the human phosphodiesterase 5 (hPDE5) tandem GAF domain based on a functional activation assay. The C-terminal catalytic domain of the cyanobacterial adenylyl cyclase (AC) cyaB1 was used as a reporter enzyme. We demonstrate functional coupling between the hPDE5 GAF ensemble and the AC resulting in a chimera stimulatory effect on AC activity, which is released upon cGMP activation. Removal of 109 amino acids from the N terminus resulted in partial disengagement of the GAF domain and AC, i.e. in a 10-fold increase in basal activity, and affected cGMP affinity. The Ser-102 phosphorylation site of hPDE5 increased cGMP affinity, as shown by a 5-fold lower \( K_D \) for cGMP in a S102D mutant, which mimicked complete modification. The function of the NKFDE motif, which is a signature of all GAF domains with known cyclic nucleotide binding capacity, was elucidated by targeted mutations. Data with either single and double mutants in either GAF A or GAF B or a quadruple mutant affecting both subdomains simultaneously indicated that it is impossible to functionally assign cGMP binding and intramolecular signaling to either GAF A or B of hPDE5. Both subdomains are structurally and functionally interdependent and act in concert in regulating cyaB1 AC and, most likely, also hPDE5.

In essentially all eukaryotic cells, cAMP and cGMP act as second messengers. Therefore the concentration of these nucleotides is meticulously regulated by the rates of biosynthesis and breakdown (1, 2). Although for years most studies have focused on mammalian ACs (3), and peculiar regulatory features are mediated by differing N-terminal domains. PDEs 2, 5, 6, 10, and 11 contain N-terminal domains. PDEs 2, 5, 6, 10, and 11 contain N-terminal tandem GAF domains (the acronym derives from proteins of initial identification: mammalian cGMP-binding PDEs, Anabaena adenylyl cyclases, and Escherichia coli transcription factor FhlA (4, 5)). To date, GAF domains have been identified in more than 3000 proteins. They bind a variety of small ligands and promote protein dimerization (2, 6–8).

The tandem GAF domains in mammalian PDEs 2, 5, and 6, which bind cGMP, have been analyzed intensively (2, 7–12). The studies have been hampered by the fact that cGMP concurrently serves as a substrate and as an allosteric regulator creating an unsolvable kinetic conundrum. Because the tandem GAF domains of mammalian PDEs are closely related to those of cyanobacterial ACs (6, 13, 14), we have replaced the cyanobacterial tandem GAF domain in the cyaB1 AC, which imparts cAMP regulation, with that of rPDE2a. The chimera is regulated by cGMP acting via the GAF B domain and uses ATP as a substrate (6).

Here, we successfully replaced the tandem GAF domain of the cyaB1 AC with that of the hPDE5, again and surprisingly generating a cGMP-regulated AC. This separation of substrate ATP and allosteric regulator cGMP allowed us to characterize the hPDE5 tandem GAF domain biochemically using the AC as a reporter enzyme.

The allosteric regulation of the PDE5 catalytic domain is reported to occur predominantly via its GAF A domain (11, 15). Yet, considerable discrepancies have been reported in cGMP binding studies, and the role of Ser-102 phosphorylation (hPDE5 numbering) in cGMP binding to the GAF domain is contentious (15–24). We characterized the contributions of the GAF A and B domains as well as the potential phosphorylation site at Ser-102 to the allosteric cGMP regulation of the cyanobacterial cyaB1 AC by the hPDE5 tandem GAF domain.

**MATERIALS AND METHODS**

*Recombinant DNAs—*The gene of hPDE5 1A_2 (GenBank accession number NM001083.3) was a gift of Dr. Friebe (University of Bochum) and the cyaB1 AC gene (gi 15553050) of Dr. Ohmori (University of Tokyo). Throughout this report, the numbering of amino acid residues refers to these genes. The hPDE5 tandem GAF domain (amino acids 1–513) was amplified by PCR and cloned into the BglII and SalI sites of pBluecript II SK(−) upstream of the cyaB1 AC (amino acid 386–859). By transfer to pQE30 the construct was fitted with an N-terminal MRGSH6GS affinity tag.

The hPDE5 GAF-cyaB1 chimera in pQE30 served as a template to generate the mutants hPDE5 D299A, hPDE5 D488A,
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hPDE5 D299A/D488A, hPDE5 K287A, hPDE5 K473A, hPDE5 K287A/K473A, hPDE5 K287A/D299A, hPDE5 D299A/K473A, hPDE5 K287A/D299A/K473A/D488A, hPDE5 S102D, and hPDE5 S102A and the N-terminally shortened constructs starting at Val-30, Ile-72, Ala-89, Asn-91, Gly-95, Ile-101, Pro-109, Thr-121, and Gln-148 of hPDE5. Single amino acid mutations were introduced by fusion PCR with Pfu polymerase and the respective sense and antisense primers using nearby restriction sites (for mutations in GAF A, BglII, XbaI, SalI, or MfEI sites were used; for mutations in GAF B, XbaI and MfEI sites were employed). For generation of S102D and S102A mutants a wobble primer was used, and clones were identified by digestion with Clal (the S102D mutation created a Clal site within the chimera). Double and quadruple mutants were constructed either by digesting the single mutants with BsrGI and MfEII and religation of the respective fragments (D299A/D488A, K287A/K473A, and K287A/D299A/K473A/D488A) or by using the single mutants as PCR templates for fusion PCRs (K287A/D299A and K473A/D488A).

N-terminally shortened constructs were obtained with the corresponding BglII sense primers and XbaI or MfEII and religation of the respective fragments (D299A/D488A, K287A/K473A, and K287A/D299A/K473A/D488A) or by using the single mutants as PCR templates for fusion PCRs (K287A/D299A and K473A/D488A).

Adenyl Cyclase Assay—Activity was tested for 10 min at 37 °C in 100 μl (25) containing 22% glycerol, 50 mM Tris/ HCl, pH 7.5, 10 mM MgCl2, 10 μg of bovine serum albumin, and 75 μM [α-32P]ATP (25 kBq). 2 mM [2,8-3H]cAMP (150 Bq) was added when stopping the reaction to monitor yield during product isolation. Substrate conversion was limited to <10%. For koff measurements, 0.3 μg of hPDE5 cyaB1 AC was preincubated with 100 μM cGMP for 1–15 min (8 data points) at 0 °C in assay buffer. After samples were warmed for 30 s to 37 °C, reactions were started by adding substrate. For koff determinations, samples were incubated with 100 μM cGMP for 10 min at 0 °C, diluted 840-fold to 119 nM cGMP, and assayed after 5–250 min at 0 °C. Activities were compared with controls with 119 nM cGMP added directly. All values are given as means ± S.E. Two-tailed Student’s t tests were used for statistical evaluation.

Western Blot Analysis—Protein was mixed with sample buffer and subjected to SDS-PAGE (10%). Proteins were blotted onto polyvinylidene difluoride membranes and probed sequentially with an anti-RGS-HC antibody (Qiagen) and with a 1:5000 dilution of a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Dianova). Peroxidase detection was carried out with the ECL Plus kit (GE Healthcare). Mostly Western blots are shown because of the need to exclude the presence of degradation products that might adversely affect assay results.

Preparation of Phospho-hPDE5-Proteins—hPDE5 GAF-cyaB1 AC and the hPDE5 S102D and hPDE5 S102A mutants were phosphorylated using the catalytic subunit of cAMP-dependent protein kinase (PKA from New England Biolabs) as described (24). Each protein (0.5 μM) was incubated with 10 mM MgCl2, 2 mM [γ-32P]ATP (7.4 MBq/μmol ATP), 25 μg of bovine serum albumin, and 5000 units of the PKA subunit in 100 μl at 30 °C for 2 h. To separate unused [γ-32P]ATP, the solutions were applied to Quick-Spin columns (Sephadex G-50 from Roche Diagnostics) and centrifuged for 5 min at 1100 × g. [γ-32P]ATP remained on the column, and phosphorylated protein was eluted. Immediately after elution, AC activity and activation by cGMP and cAMP were assayed. A sample without PKA catalytic subunit served as a reference for protein stability.

To check for protein phosphorylation by autoradiography, aliquots were loaded onto a 12.5% SDS-PAGE. After electrophoresis the gel was exposed to an x-ray film (Kodak Biomax MR) for 1 and 28 days, respectively.
(n = 5; Fig. 1). AC activity of the chimera was linear with protein concentration up to 15 min, and the pH optimum was between 7 and 9 (±100 μM cGMP; data not shown). The temperature optima were 54 °C (−cGMP) and 50 °C (±100 μM cGMP). The activation energies derived from Arrhenius plots were 70 and 100 kJ/(mol·K) (±100 μM cGMP); tested range 0–66 °C. The Km for ATP with 10 mM Mg2+ as a cofactor was 13 μM (±100 μM cGMP), i.e. similar to that of the cyaB1 holoenzyme and other PDE-cyaB1 chimeras (6, 27). Vmax values obtained from linearized Michaelis-Menten plots were 51 (−cGMP) and 435 nmol cAMP·mg⁻¹·min⁻¹ (+100 μM cGMP), i.e. cGMP increased Vmax. The hPDE5 GAF-cyaB1 AC chimera was stable at −20 °C for up to 8 months. Longer storage increased basal activity and decreased cGMP activation and maximal activity. This was reminiscent of the poor stability of mPDE5, which could not be activated after 2 weeks of storage (11).

Stimulation of the hPDE5 GAF-cyaB1 AC chimera by cGMP was direct and immediate. Preincubation with cGMP was not required because the reactions were linear up to 15 min, independently of preincubations up to 15 min with 100 μM cGMP prior to substrate addition (data not shown). Similarly, termination of activation was as quick as could be determined experimentally, i.e. within 5 min after removal of cGMP by an 840-fold dilution, because activities were identical when assayed at 5 or 250 min after dilution (8 data points assayed). Those fast kₚₒ and kₒₒ values for hPDE5 GAF-cyaB1 AC are at variance with results for cGMP binding to PDE5, where on and off kinetics were about 15 min for the on-reaction (11) and up to 7 h for the off-reaction (10, 24). Similarly, binding assays with the isolated PDE5 GAF domains have demonstrated that cGMP binding and release are slow (15, 17, 18, 24). We examined cGMP binding to hPDE5 GAF-cyaB1 AC chimera following standard protocols (10, 27). In our hands cAMP or cGMP binding never exceeded 3%. In view of the rapid on and off rates of hPDE5 GAF-cyaB1 AC activation, we assumed that bound cyclic nucleotides were washed off during the washing step. The differences may be rationalized by different interactions between the GAF domain and the PDE5 or cyaB1 AC catalytic domains and/or the use of native protein isolated from lung (28) or recombinant proteins expressed in COS-7 cells (10), in SF9 and High Five insect cells (22), and in E. coli (17).

Effect of the N Terminus on PDE 5 GAF-cyaB1 AC Activation—Using various PDE5 GAF domain constructs, cGMP binding constants between 0.027 and 1.9 μM have been reported in the past (15, 17, 18, 22, 24, 27). Possibly different N- and C-terminal truncations have contributed. Recently, we have demonstrated that the N-terminal domain of the tandem GAF of PDE11A is critical for cGMP stimulation, because activation was observed only with the complete N terminus (26). Therefore, we examined the role of the N terminus of hPDE5 of 147 amino acids that contains a potentially important phosphorylation site (Ser-102). Initially, we tested a construct starting at Gln-148, i.e. devoid of the N terminus (hPDE5 Gln-148-cyaB1 AC). Basal AC activity was 9.8-fold higher (243.1 ± 15.8 versus 24.8 ± 7.5 nmol cAMP·mg⁻¹·min⁻¹; n = 5), and the EC₅₀ value for cGMP was reduced to 0.2 ± 0.07 μM (n = 6; Fig. 2). However, because of high basal activity, cGMP activation (391 ± 25.3 nmol cAMP·mg⁻¹·min⁻¹; n = 9) was only 1.6-fold. Maximal stimulated AC activity was not significantly affected. To exclude the possibility that the results reflected structural changes in the catalytic domain, Kₘ values were determined (11.4 and 14.3 μM ATP ± 1 mm cGMP, respectively), i.e. they were unchanged from the full-length chimera (13 μM ATP). Thus, we can conclude that removal of the N terminus did not affect substrate kinetics. The altered activation of the chimera may then indicate a different interaction between the truncated tandem GAF and the catalytic domain. In fact, AC activity appeared relieved from inhibition by removal of the GAF N terminus.

To narrow the region responsible for this effect, we generated a series of shortened hPDE5 GAF-cyaB1 AC constructs. Structural predictions for the N terminus were used for construct design (DNA Star Protein). Flexible regions are predicted for amino acids 1–30, 70–100, and 135–147. They are interrupted by predicted α-helices and β-strands. Accordingly, chimeras started with Val-30, Ile-72, Pro-87, Ala-89, Asn-91, Gly-95, Ile-101, Pro-109, and Thr-121 (Table 1). With ongoing shortening of the N terminus we initially observed a minor increase in basal AC activity and a decrease in the EC₅₀ values for cGMP (Table 1). A major leap in basal activity occurred between constructs starting at Gly-95, Ile-101, and Pro-109 (19.2, 217, and 815 nmol cAMP·mg⁻¹·min⁻¹, respectively; Fig. 2). Removal of amino acids 101–108 had a dual effect; it released the AC from an apparently inhibited state and increased the cGMP affinity of the GAF domain 4–5-fold (Table 1 and Fig. 2). All constructs with deletions beyond Pro-109 exhibited no further increases in basal AC activity, no additional reductions of the EC₅₀ for cGMP or of maximal cGMP activation (Table 1, Fig. 2). For the shortened constructs the Kₘ values for ATP were found to be unchanged, and the degree of purification for all constructs was similar, making the data comparable (Fig. 2B). Further, AC activation of all constructs
was instantaneous and required no preincubation with cGMP (data not shown).

Influence of Phosphorylation at Ser-102—The hPDE5 N terminus has a phosphorylation site at Ser-102 which is subject to modification by cAMP- or cGMP-dependent protein kinase (PKA, PKG). Results whether phosphorylation is catalyzed by PKA and/or exclusively by cGMP-dependent protein kinase are ambiguous (11, 17, 19, 24, 28). Further, it was reported that occupation of both PDE5 GAF domains by cGMP is required for Ser-102 phosphorylation (22, 24, 28). On the other hand, phosphorylation of Ser-102 was found to enhance cGMP binding or inhibit dissociation and PDE activity (16, 17, 19, 29). Here we examined the effect of Ser-102 phosphorylation on cGMP activation of the cyaB1 AC. For Ser-102 phosphorylation we followed the protocol of Zoraghi et al. (24). A sample without PKA served as a control. Ser-102 phosphorylation modestly shifted the cGMP dose-response curve to the left (Fig. 3). Basal AC activity was not significantly affected ($p = 0.131; n = 4$).
Similarly, the extent of activation and the EC_{50} concentrations for cGMP were not significantly altered (27.5 ± 11.4 versus 16.4 ± 2.8 μM; n = 4; 2 p > 0.05; Fig. 3). At the lower end of cGMP concentrations, the enhancement of cAMP formation in the phosphorylated sample by cGMP was more pronounced (e.g. at 10 μM cGMP p < 0.001, n = 4) in agreement with former results (16). Activation factors were 10.8 ± 1.6 (n = 5) for the unphosphorylated control and 11.3 ± 2.2 (n = 5) for phosphorylated hPDE5 GAF-cyaB1 AC. The quantitative interpretation of the above data is hampered by the fact that we did not have a reliable measure of the extent of phosphorylation. Also we could not exclude additional phosphorylation sites, e.g. in the cyaB1 AC. These issues were addressed by using a S102A mutation that could not be phosphorylated and a S102D mutation that mimicked phosphorylation.

In the S102D mutant, the parameters of substrate kinetics were unchanged, indicating that the reporter enzyme was unaffected (see also below). The EC_{50} of 1.9 ± 0.33 μM for cGMP stimulation was significantly lower compared with the unmutated protein (9.6 ± 1.8 μM; 2 p < 0.05; n = 5) consistent with earlier results where the K_{s} for cGMP binding to the phosphorylated PDE5 holoenzyme was 4-fold lower (14, 16). The mutant S102A cyaB1 chimera displayed diminished stability and therefore was assayed at once after purification. The EC_{50} was 8.0 ± 1.5 μM cGMP (n = 5; not significantly different from the unmutated chimera); the 5.1 ± 0.4-fold activation was, however, significantly lower (n = 6, 2 p < 0.002, n = 5). Attempts to phosphorylate the S102D and S102A mutants failed; autoradiograms were blank after 4 weeks of exposure. This demonstrated unequivocally that Ser-102 was the only site for phosphorylation in the chimera.

Role of the Conserved NKFDE Motif in hPDE5—The tandem GAF domains in the mammalian PDE families 2, 5, 6, 10, and 11 contain a conserved NKFDE motif that is proposed as prototypical for cNMP-binding GAF domains (6, 10, 27). Point mutations in this motif, such as Lys → Ala and Asp → Ala, usually have abrogated cGMP binding, and this has been used to determine which of the two GAF domains predominantly contributes to signal recognition and transmission (10, 23). Stimulation of PDE5 activity via cGMP binding to the GAF domains has been shown unequivocally (11, 24, 30); however, data concerning cGMP binding to GAF A, GAF B, or both subdomains are contradictory (23). Using an antibody directed against GAF A prevents mPDE5 activation and cGMP binding in vitro, implicating GAF A in intramolecular signaling (11). Here, we evaluated the contributions of GAF A and B to cGMP-mediated AC activation using the established mutations in the NKFDE motif. We generated alanine mutants in Lys-287, Asp-299, Lys-473, and Asp-488, i.e. those amino acids that interfered in cGMP binding assays and that, according to the available x-ray structures of the tandem GAF domains of mPDE2A and cyaB2, are involved in formation of a stabilizing salt bridge (8, 14).

Initially, mutants D299A and D488A and the double mutant D299A/D488A were tested (Fig. 4A, Table 2). Basal as well as stimulated AC activities were reduced to about 75% of the unmutated chimera, and none of them was stimulated by cAMP. All mutants were stimulated by cGMP, although with diminished potency (Fig. 4A, Table 2). In the D299A mutant (GAF A), the EC_{50} for cGMP increased 10-fold to 103.1 ± 28.8 μM, in agreement with data from cGMP binding experiments (10). A D488A mutant (GAF B) was stimulated by cGMP with an EC_{50} of 18.2 ± 8.5, which was not significantly different from the unmutated chimera (2 p > 0.1; n = 3), whereas in the D299A/D488A double mutant the affinity for cGMP increased significantly compared with the unmutated protein (EC_{50} = 36.5 ± 7.8 μM; 2 p < 0.01; n = 5), i.e. a complete abrogation of cGMP stimulation was not accomplished in the double mutant, whereas cGMP binding is lost in a corresponding PDE5 double mutant (22).

Next, we investigated K287A, K473A, and K287A/K473A mutants (Fig. 4B, Table 2). The results were similar to the corresponding Asp → Ala mutants; however, the effects were more pronounced (Table 2). In the GAF A mutant K287A, the EC_{50} for cGMP rose 25-fold to 235.6 ± 78.6 μM (n = 5), whereas in the K473A mutant it was reduced to 4.0 ± 1.7 μM cGMP (n = 3; 2 p < 0.14; not significant). The EC_{50} for the double mutant (K287A/K473A) was about the mean of the single mutants, 135.0 ± 20.8 μM cGMP. Obviously, in the K287A mutant cGMP activation was more impaired than in the D299A mutant. A surprising observation was, however, that in the K473A mutant the extent of stimulation of AC activity (29-fold) was tripled (Table 2).

Finally, we mutated both Lys and Asp in GAF A and/or GAF B (Fig. 4, C and D; Table 2). K287A/D299A (in GAF A) had a 13-fold higher EC_{50} than the unmutated chimera (127.2 ± 40.2 μM cGMP), K473A/D488A (in GAF B) showed an EC_{50} of 7.1 ± 2.9 μM cGMP, i.e. in the range of the parent protein, and the extent of stimulation appeared to be increased even further (Table 2). Even in the quadruple mutant (hPDE5 K287A/D299A/K473A/D488A-cyaB1 AC) cGMP activation was maintained, albeit with a reduced affinity for cGMP (Fig. 4D, Table 2). Substrate kinetics of the above mutants was not affected. Taken together the results highlight a more complex structural relationship and cooperation between GAF A and B in regulation and activation of the attached AC than appreciated thus far.

DISCUSSION

We characterized the intramolecular signaling of the tandem GAF domain of hPDE5. This was made possible by the dissociation of the allosteric regulator, cGMP, and the substrate, ATP, allowing us to use the C-terminal cyaB1 AC as a reporter enzyme. The linker between both entities consisted of a cyanobacterial PAS domain (residues 394–464) and the 130-amino acid N terminus of the AC catalytic domain (residues 465–594), which starts with Gly-595. No regulated cyclase activity was obtained when the ~30-amino acid-long linker that connects the PDE5 GAF tandem domain and the PDE5 catalytic domain was used in a hPDE5 GAF/cyaB1 AC chimera (data not shown). This indicates that signaling from the tandem GAF ensemble to the cyaB1 AC requires structural features that are embedded in the 200-amino acid linker region of the cyanobacterial AC.

Previously we reported that the tandem GAF domain of rPDE2 linked to cyaB1 AC generates a cGMP-regulated cyclase. The rPDE2a GAF tandem domain supposedly signals by bind-
FIGURE 4. cGMP-stimulated cyaB1 AC activity using hPDE5 tandem GAF domains with mutations in the conserved NKFDE motif. A, single mutations D299A and D488A in GAF A and B, respectively. B, single mutations K287A and K473A in GAF A and B, respectively. C, double mutants K287A/D299A and K473A/D488A. D, quadruple mutant K287A/D299A/K473A/D488A. Curves in A–C were normalized to cGMP stimulations at 1 μM cGMP, which were 63 (D299A), 50 (D488A), 78 (K287A), 250 (K473A), 65 (K287A/D299A), and 115 (K473A/D488A) nmol cAMP/mg·min. The inserts show Western blots of the affinity purified proteins (70–200 ng/lane) with molecular mass markers on the left. cAMP did not stimulate any of these constructs. Representative experiments are shown; for statistics see Table 2.

TABLE 2

EC<sub>50</sub> values for cGMP and maximal cGMP stimulation for all constructs with mutations in the conserved NKFDE motif of the hPDE5 tandem GAF domain

| Construct                        | EC<sub>50</sub> for cGMP | -Fold activation by cGMP |
|----------------------------------|--------------------------|--------------------------|
| Wild-type GAF tandem domain      |                          |                          |
| hPDE5 GAF-cyaB1 AC               | 9.6 ± 1.8 (n = 5)        | 10.2 ± 1.5 (n = 5)       |
| Mutations in GAF A               |                          |                          |
| hPDE5 D299A-cyaB1 AC             | 103.1 ± 29.8 (n = 3)     | 5.5 ± 0.5 (n = 4)        |
| hPDE5 K287A-cyaB1 AC             | 235.6 ± 78.6 (n = 5)     | 7.4 ± 1.0 (n = 5)        |
| hPDE5 K287A/D299A-cyaB1 AC       | 127.2 ± 40.2 (n = 2)     | 15.2 ± 1.6 (n = 2)       |
| Mutations in GAF B               |                          |                          |
| hPDE5 D488A-cyaB1 AC             | 18.2 ± 8.5 (n = 3)       | 11.2 ± 1.7 (n = 4)       |
| hPDE5 K473A-cyaB1 AC             | 4.0 ± 1.7 (n = 3)        | 28.9 ± 1.5 (n = 3)       |
| hPDE5 K473A/D488A-cyaB1 AC       | 7.1 ± 2.9 (n = 2)        | 42.2 ± 23.4 (n = 2)      |
| Mutations in GAF A and B         |                          |                          |
| hPDE5 D299A/D488A-cyaB1 AC       | 36.5 ± 7.8 (n = 5)       | 10.7 ± 0.9 (n = 7)       |
| hPDE5 K287A/K473A-cyaB1 AC       | 135.0 ± 20.8 (n = 3)     | 13.8 ± 2.7 (n = 3)       |
| hPDE5 K287A/D299A/K473A/D488A-cyaB1 AC | 96.2 ± 31.3 (n = 3) | 16.5 ± 0.9 (n = 12)     |

Hill coefficients did not indicate cooperativity.
ing cGMP to GAF B (6, 8). Because the PDE5 GAF equivalent reportedly signals via GAF A (11), it was uncertain whether it would couple productively with the cyanobacterial AC. In addition, the available structures of tandem GAF domain dimers, which show either a parallel (8) or antiparallel arrangement (14), are too ambiguous for a confident prediction of functional coupling. The observed 10-fold stimulation by cGMP of AC activity via the hPDE5 GAF tandem domain was, therefore, surprising. The exclusivity of cGMP binding was in agreement with former cGMP binding studies and hPDE5 activation. It demonstrated unequivocally that this essential property of the PDE5 GAF tandem domain was fully retained in the chimeric protein and lent credence to the functionality of the ensuing data.

**Role of the GAF Tandem Domain N Terminus**—The EC$_{50}$ of 10.8 μM for cGMP stimulation of the hPDE5 GAF-cyaB1 AC chimera was higher than $K_p$ values based on cGMP binding ($0.027–1.9$ μM) (18, 24). We hypothesized that the 70-fold range observed in those studies may be due to the variety of constructs used, such as individual GAF A (bovine GAF A-(147–321) (27); hGAF A-(1–403), -(1–321), -(46–403), and -(156–394) (18, 24)) and GAF B subdomains (bovine GAF B, human GAF B-(332–512) (27)) and the tandem ensemble with varying parts of the N terminus (starting at amino acids 36, 46, and 156 (16, 18)) or the bovine and human PDE5 holoenzymes (16, 19, 28). Our data demonstrate that the N terminus has a significant role in intramolecular signaling. Removal of up to 100 amino acids resulted in small differences in the cGMP EC$_{50}$ values (3–15 μM). Basal and stimulated AC activities were within the same range (Fig. 2A). A shortening beyond Ser-102, i.e. removal of the phosphorylation site, caused up to a 25-fold increase in basal activity without affecting the maximal stimulation attainable by cGMP addition. Thus, it appeared that removal of 108 N-terminal amino acids released the cyaB1 AC from inhibition. This may have occurred either through a direct interaction between both parts of the protein or by release of a structural constraint on the adjacent GAF domain. Further, cGMP affinity was significantly lowered to 0.2–0.3 μM upon removal of 108 N-terminal amino acids. Thus, our data may explain the reported 70-fold range of $K_p$ values for cGMP binding observed earlier and as outlined above. Perhaps the N-terminal part of the hPDE5 GAF complex affects the overall architecture and/or accessibility of the cGMP binding pocket. Because the ineffectiveness of cAMP was untouched in all shortened constructs, we suggest that the major specificity filter for cGMP discrimination is not in this area. It appears conceivable that in vivo limited proteolysis at the N terminus might constitute an additional level of PDE5 regulation.

**Effect of Phosphorylation at Ser-102**—Phosphorylation of Ser-102 in the hPDE5 tandem GAF domain has been discussed in terms of enhancement of cGMP affinity and maintenance of a high catalytic state (11). Here, Ser-102 phosphorylation only minimally affected AC stimulation by cGMP. We assume that phosphorylation was not stoichiometric. However, in the S102D mutant, in which the negative charge of the carboxyl group mimics complete phosphorylation, cGMP affinity was enhanced 5-fold. This corroborated earlier binding data that showed an 86% increase in the phosphorylated PDE5 holoenzyme (20). This may be taken as another sign that the basic principles of GAF domain signaling in hPDE5 and cyaB1 AC are highly conserved.

**Role of the NKFDE Motif**—A sequence feature common to all cyclic nucleotide binding GAF domains is a conserved NKFDE motif. Because mutations therein, such as Asp → Ala or Lys → Ala, diminished or abrogated cGMP binding, the NKFDE motif was thought to be involved in cGMP binding. Mutational studies of the NKFDE motif were also used to decipher whether GAF A or B was binding cGMP and thus responsible for signaling. We observed that intramolecular signaling required the intact tandem GAF ensemble, because constructs that contained either GAF A or B alone in front of the cyaB1 AC unit remained unregulated (not shown). Probably an interaction of GAF A and B is necessary for formation of the signaling unit, and the NKFDE motif in either GAF subdomain may be needed for structural integrity of the tandem. Our and earlier data support such a proposal. cGMP binding assays with the PDE5 tandem showed that Asp → Ala or Lys → Ala mutations in GAF A reduced but did not abolish binding (10, 15, 23), whereas a Asp → Ala mutation in GAF B increased cGMP binding (10). Functionally, our experimental data corroborate this finding; cGMP signaling was never completely lost, even in a quadruple mutant with Asp → Ala and Lys → Ala mutations in both subdomains. Therefore, in contrast to data gathered using an antibody directed against the PDE5 GAF A subdomain, we could not confidently assign an exclusive signaling role to either subdomain. In fact, the specific antibody against PDE5 GAF A may have subtly impaired the correct assembly and stability of the tandem unit and inhibited signaling. Such a view would be compatible with the functional results reported here and with findings concerning cAMP signaling by the cyaB2 tandem GAF unit (13). Further, cAMP was bound to all four subdomains of the tandem in the antiparallel crystal structure (14). The preferential binding of the allosteric regulator molecule to either GAF A or B, which is observed using mammalian PDE GAF domains with mutations in the conserved NKFDE motif, has been taken as a sign that signaling may be mediated by either the GAF A or B subdomain. Our data indicate that in the mammalian PDEs and cyanobacterial ACs, both subdomains are structurally and functionally interdependent and act in concert. The NKFDE motif probably plays a role in this interaction. Consequently, we should be able to generate chimeric tandem GAF domains from individual subdomains. This has in fact been accomplished. 3

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