Modeling and mutational analysis of the GAF domain of the cGMP-binding, cGMP-specific phosphodiesterase, PDE5

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Abstract **The GAFa domain of the cGMP-binding, cGMP-specific phosphodiesterase (PDE5A) was modeled on the crystal structure of PDE2A GAF domain and residues involved in cGMP binding identified. Tandem GAFa and GAFb domains of PDE5A, expressed in *Escherichia coli*, bound cGMP (K_d 27 nM). Mutation of aspartate-299 in GAFa, suggested earlier to be critical for cGMP binding, did not abrogate cGMP binding, but mutation of F205, which formed a stacking interaction with the guanine ring of cGMP, led to complete loss of cGMP binding. Therefore, the GAFa domain of PDE5A adopts a structure similar to the GAFb domain of PDE2A, and provides the sole site for cGMP binding in PDE5A.**
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Key words: cGMP; GAF domain; PDE5; Homology modeling; PDE2

1. Introduction

The cGMP-binding, cGMP-specific phosphodiesterase PDE5A[1], identified as the target of the drug sildenafil [1], is a multi-domain protein that appears to be regulated intricately by phosphorylation as well as binding of cGMP to allosteric cGMP-binding sites at the N-terminus of the protein [2,3]. Based on sequence homology in the full-length PDE5A protein, it appears that there are two cGMP-binding domains arranged in tandem that follow an N-terminal domain harboring a site for phosphorylation, and preceding the C-terminal catalytic domain of the enzyme [4]. PDE5A is a dimer, and regions mediating the dimerization have been suggested to be present in the allosteric cGMP-binding domain [5–7].

The cGMP-binding sites are representative of 'GAF' domains, so named because of the presence of similar motifs in the genomes of a number of organisms, and in a variety of proteins including other members of the phosphodiesterase family [8–10]. Biochemical studies performed with the full-length recombinant bovine PDE5A enzyme suggested that there were two cGMP binding sites present in the enzyme, based on kinetic analysis of binding [11]. Site-directed mutagenesis analysis in the full-length enzyme indicated the importance of an aspartate residue at position 289 in the bovine PDE5A GAFa sequence that was critical for binding cGMP, and perhaps providing discrimination between cAMP and cGMP at this site [12,13]. Abolishment of cGMP binding to the GAF domain did not alter the catalytic activity of the enzyme, but regulated the ability of PDE5A to be phosphorylated by protein kinase G, suggesting a regulatory role for this domain in the feed-back control of cGMP levels in the cell [2].

A putative GAF domain protein from yeast, YKG9, was expressed and crystallized to serve as a structural model for understanding GAF domains in other proteins, including the phosphodiesterases [8,14]. The expressed protein did not bind cGMP, but the crystal structure was used to model the GAFa domain of bovine PDE5A, which, when expressed as a fusion protein with glutathione *S*-transferase, bound cGMP with a dissociation constant of 650 nM [9]. More recent studies were directed to expressing the GAFa domain of human PDE5A as a GST-fusion protein, as well as a protein that comprised the entire N-terminus of PDE5A and the two tandem GAFa and GAFb domains. The proteins bound cGMP, but the affinity of the longer protein comprising the N-terminus and the GAFa and GAFb domains was markedly lower, with a dissociation constant of 1.9 μM, suggesting the presence of high and low affinity binding sites in the protein, as was seen in the full-length enzyme [15]. Based on the homology model of the bovine GAFa domain and YKG9, residues comprising $N[KR]X_{(5-14)}FX_{(3)}DE$, the 'NKFDE' motif suggested to be involved in interacting with cGMP were identified [5,9].

The cGMP-stimulated phosphodiesterase, PDE2A, contains two tandem GAF domains, and binding of cGMP is a prerequisite for hydrolysis of cAMP by this enzyme. Recently, a crystal structure of the GAF domains of PDE2A was reported and revealed a dimeric protein with cGMP bound to the GAFb domain, while the GAFa domain provided critical interactions for the dimerization of the protein. Binding analysis revealed a very high-affinity site for cGMP with a dissociation constant of 26 nM [5]. PDE5A GAFa has the highest similarity to the GAFb domain of PDE2A, suggesting that this domain in PDE5A could also bind cGMP with a high affinity. We have therefore used the PDE2A structure as a model for re-evaluating the GAF domains of PDE5A. By expression and mutational analysis of the GAFa and GAFb domains of PDE5A we show here that a single cGMP binding site is present in the GAFa site of PDE5A, and also identify a phenylalanine residue that is critical for providing an interaction with cGMP.

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2. Materials and methods

2.1. Modeling of the GAFa domain of PDE5A

A three-dimensional model of the GAFa of human PDE5A was generated on the basis of the crystal structure of the GAFb domain of mouse PDE2A. The suite of programs encoded in COMPOSER [16] and incorporated in SYBYL (Tripos, St. Louis) was used to generate the 3-D model of the GAFa of human PDE5A. The structures of the conserved regions of the GAFb domain of mouse PDE2A were extrapolated to the equivalent regions of GAFa of human PDE5A. The variable regions were modeled by identifying a suitable segment from a known structure in the data bank. A template-matching approach [17] to rank the candidate loops was also used. The best-ranking loop with no short-contacts with the rest of the protein was fitted using the ring-closure procedure of F. Eisenmenger (unpublished). Sidechains were modeled either by extrapolating from the equivalent positions in the basis structure where appropriate, or by using rules derived from the analysis of known protein structures [18]. The model thus generated was subjected to energy minimization to get a refined model for the GAFa domain in SYBYL using the AMBER force-field [19].

The known crystal structure of the GAFb of mouse PDE2A bound to cGMP (PDB code 1MCO) [5] was used to generate a model of the cGMP-bound complex by first extracting the residues from GAFb of the 1MCO implicated to interact with atoms in cGMP either directly or through water molecules, and then superposing them with equivalent residues in the human PDE5A GAFa domain.

2.2. Generation of the GST–GAF: wild type clone

The cloned full-length human PDE5A cDNA was used as a template for polymerase chain reaction (PCR) using specific primers (sequences available on request) to amplify the GAF domain comprising residues M134–N583. The fragment was cloned initially into pBluescript II KS(+) vector (Stratagene), sequenced, and then subcloned into pGEX-5X-1 (Pharmacia) to obtain plasmid pGEX-PDE5A (M_{134}–N_{583}). This would allow the expression of a protein with an N-terminal fusion to glutathione *S*-transferase (Fig. 2A).

2.3. Generation of D299A and F205 mutations

An overlap PCR-based approach was used to introduce a single amino acid mutation in the GAFa domain (D299 to Ala). Details of primer sequences and PCR conditions are available on request. The PDE5A (M_{134}–N_{583})$_{F205A}$ and PDE5A (M_{134}–N_{583})$_{F205Q}$ mutations were generated using the *Dpn*I method of mutagenesis [20]. The pGEX-PDE5A (M_{134}–N_{583}) plasmid was used as a template to carry out the mutagenic PCR reaction using overlapping forward and reverse primers harboring the mutation. The PCR product obtained was digested with *Dpn*I taken for transformation, and colonies obtained screened for the presence of the mutation by sequencing.

2.4. Expression and purification of the GST fusion proteins

The plasmid expressing the wild type or mutant GAF domains was used to transform BL21 (DE3) C43 strain of *Escherichia coli*. Cells harboring the plasmid were induced at an A_{600} of 0.6 with 800 μM isopropyl β-thiogalactopyranoside (IPTG) for 8–12 h at 30°C. The cells were collected by centrifugation, resuspended in sonication buffer (50 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol (DTT), 2 mM EDTA, 100 mM NaCl, 1 μg/ml benzamidine, 1 μg/ml soyabean trypsin inhibitor and 2 mM phenymethylsulfonyl fluoride) and lysed by sonication, after which Triton X-100 was added to a final concentration of 0.1%. Soluble protein was purified by application of the lysate obtained after centrifugation to a column of glutathione Sepharose 6B beads (Pharmacia). The protein bound to beads was resolved on a 10% SDS–PAGE and the purified protein visualized by Coomassie blue staining (Fig. 2B). The GST–GAF domain protein is predicted to have a molecular weight of 76 kDa.

2.5. cGMP saturation binding assay

Saturation cGMP binding studies were carried out in a 100 μl reaction containing 10 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA and 2.5 mM DTT, in the presence of increasing concentrations of [^{3}H]cGMP (NEN, USA; 5×10^{-7} to 8×10^{-9} M) and 100 nM of the purified protein bound to beads, at 37°C for 1 h. The binding reaction was then filtered through GF/C filters (Whatman) and washed with 10 ml of ice-cold buffer (10 mM potassium phosphate, pH 6.8; 1 mM EDTA). The filters were then dried and radioactivity measured by liquid scintillation counting in scintillation cocktail (1:1, 2-methoxy ethanol: toluene, 5 g/l PPO). The binding data were then analyzed using GraphPad Prism (San Diego, CA, USA).

2.6. cGMP dissociation

For cGMP dissociation analysis, cGMP binding was carried out in binding buffer containing 1 μM [^{3}H]cGMP along with the fusion protein. Binding was carried out for 2 h at 37°C to establish equilibrium, transferred to 4°C and an aliquot was withdrawn for filtration to determine the initial bound radioactivity (B_0). To initiate dissociation of [^{3}H]cGMP, unlabeled cGMP to a final concentration of 100 μM was added and aliquots withdrawn for filtration at different time points (B_t). cGMP dissociation rate was calculated by plotting $\ln(B_t/B_0)$ versus time. The slope of the line determined the k_{off}.

3. Results and discussion

Recently, the crystal structure of the tandem GAFa and GAFb domains of mouse PDE2A indicated that the GAFb domain of PDE2A alone bound cGMP, while the GAFa domain was involved in dimerization [5]. The GAFa domain of PDE5A shows the highest degree of similarity to the GAFb domain of PDE2A, and therefore, by analogy, could be the site for cGMP binding. We therefore modeled the GAFa domain of PDE5A using the crystal structure of the GAFb domain of PDE2A as a template. An alignment of the two sequences, based on the structural properties of the residues of the GAFb domain, is shown in Fig. 1A, and demonstrated 48% identity between the two domains. A model of the complex of the GAFa domain of PDE5A and cGMP was also generated based on the crystal structure (Fig. 1B).

This structure-based alignment showed the conserved nature of substitutions of residues in secondary structure regions of PDE5A GAFa, and except for the β1–β2 loop, all the other loops have comparable sequence length to their equivalent loops in the GAFb domain of PDE2A. Moreover, all residues implicated in cGMP binding to the GAFb domain of PDE2A are conserved or conservatively substituted in the GAFa domain of human PDE5A (Fig. 1B). These include D206 (D439 in PDE2A), which is engaged in both back-bone and side-chain interactions with the guanine base. Interestingly, the adjacent residue F205 (F438 in PDE2A) is positioned in such a way that a stacking interaction could occur with the ring of the phenylalanine and the guanine base, since any group in the sidechain of F205 (other than the CB atom) is within 4.5 Å distance from the base ring of cGMP. These phenylalanine and aspartate residues are replaced by histidine and methionine in the GAFb domain of PDE5A and isoleucine and glycine in the GAFa domain of PDE2A, and these could be substitutions which compromise cGMP binding to these domains.

To confirm the predictions of the model, we cloned the GAF domain of PDE5A based on sequence alignment of this domain with other proteins [5] containing GAF domains and expressed the protein as a fusion with GST to aid in its purification (Fig. 2A). This is a similar approach as taken earlier, but the protein used in our studies extends further to the N-terminus than the earlier construct of the GAFa domain [15], and does not contain the N-terminus of the full-length PDE5A that harbors a site for phosphorylation (Fig. 2A). As was reported earlier [15], the presence of the N-terminus in the GAF–GST fusion protein did not alter the binding affinities to the GAF domain, and we therefore chose

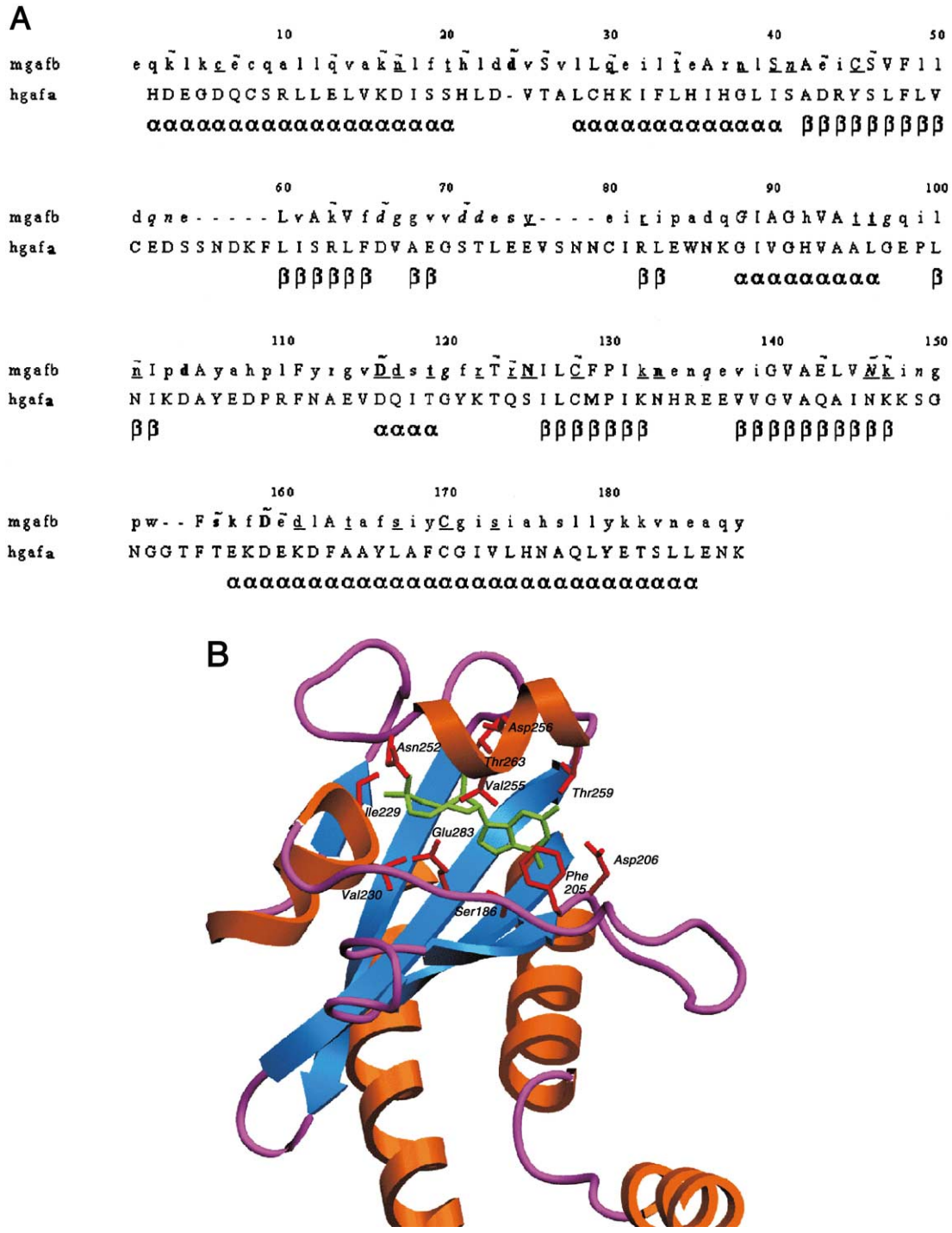

Fig. 1. Model of the GAFa domain of PDE5A. A: Alignment of the GAFa domain of human PDE5A with the GAFb domain of mouse PDE2A. Key to the JOY notation: solvent inaccessible; UPPER CASE (O); solvent accessible, lower case (o); positive ϕ, *italic* (*o*); *cis* peptide, breve (õ); hydrogen bond to other sidechain, tilde (ö); hydrogen bond to main chain amide, **bold** (**o**); hydrogen bond to main chain carbonyl, underline (o); disulphide bond, cedilla (ç). B: A model of the PDE5A GAFa–cGMP complex is shown with the cGMP-binding site, and residues implicated in cGMP binding are highlighted. The figure was generated using SETOR [23].

to omit this region from our construct. Equilibrium binding analysis was performed on the purified fusion protein and the data obtained indicated a single site for cGMP binding with a dissociation constant of 27 nM (Fig. 3A). The affinity that we report here is very similar to that observed for cGMP binding to the GAF domain of PDE2A (26 nM) [5], and is higher than that reported for the earlier GST–GAF domain construct. Dissociation of cGMP from the GST–GAF domain protein revealed a single off-rate for dissociation of cGMP with a k_{off} of 0.34 ± 0.008 h^{-1} (Fig. 3B). These results contrast with those reported earlier that suggested the presence of two binding sites in the GAF domain of PDE5A, with the apparently high-affinity site showing a K_d of 330 nM [15].

The specificity of binding of cGMP and cAMP was studied

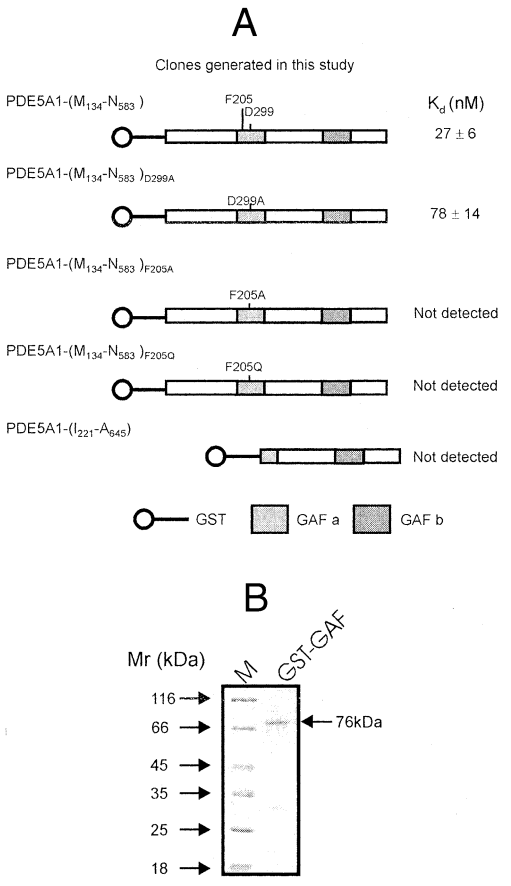

Fig. 2. PDE5A GST–GAF domain proteins. A: A diagrammatic representation of various constructs generated in this study for analysis of the GAF domain of PDE5A. All proteins were expressed as fusion proteins with GST. Also shown is a summary of the binding data obtained using the various proteins. B. Purification of the GST–GAF domain wild type protein. Coommassie stained gel of protein (1 μg) purified from lysates by glutathione affinity chromatography.

and data obtained from inhibition analysis indicated a nearly 1000-fold reduction in affinity for cAMP (Fig. 3C). It is interesting to note that there was only a 10-fold lowering of affinity of cAMP binding to the PDE2A GAFb domain [5], indicating that certain structural features in the PDE5A GAF domains must contribute to the higher specificity that is seen for cGMP over cAMP.

A highly conserved 'N[KR]$X_{(5-14)}$F$X_{(3)}$DE' motif is present in nearly all PDE GAF domains, and was suggested to be critical for cGMP binding, based on mutational analysis of some of these residues in the GAF domain of bovine PDE5A [12], as well as recent homology modeling of the bovine PDE5A GAFa domain on the crystal structure of the yeast GAF domain containing protein, YKG9 [9]. We mutated residue D299 in GAFa to an alanine and monitored the binding to the GST–GAF domain protein. In our current model, this residue lies away from the cGMP-binding site and is therefore predicted not to alter binding of cGMP appreciably. It was observed that while the D299A mutation led to a marked reduction in the B_{max} (Fig. 4A), the affinity of binding of cGMP to the mutant protein was only marginally decreased. This suggested that there may have been some misfolding of the fusion protein as a consequence of this mutation, but that the fraction of protein that retained the ability to bind cGMP did so with an affinity comparable to the wild type protein. This is in contrast to results reported earlier with the full-length bovine PDE5A, where mutation of the corresponding D residue led to a dramatic reduction in the dissociation constant for cGMP [11].

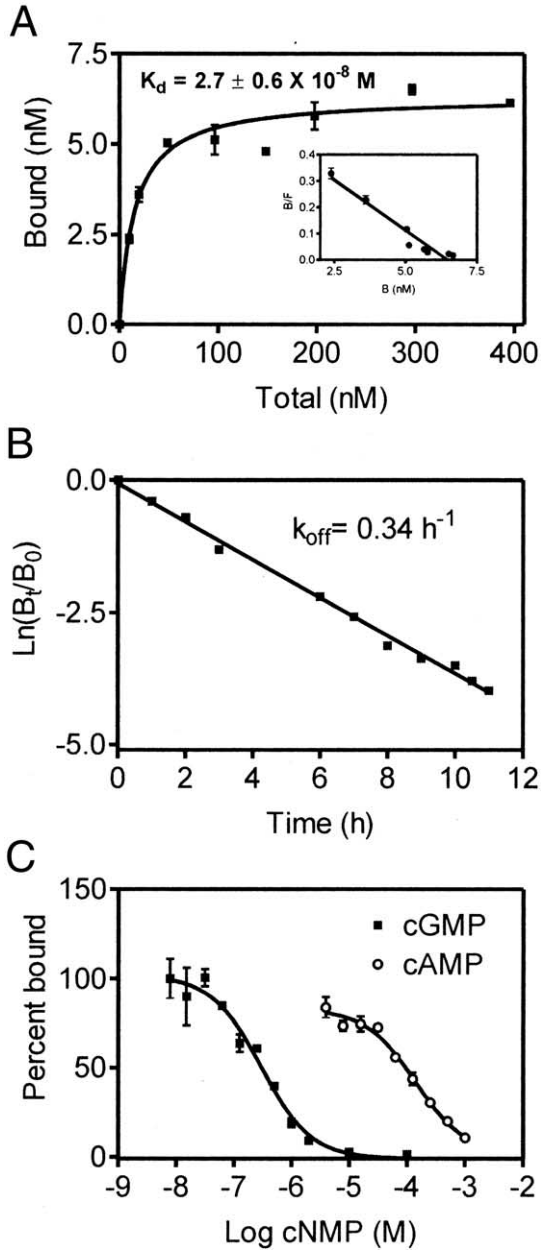

Fig. 3. Binding analysis for wild type GST–GAF. A: Protein bound to beads (100 nM) was incubated in binding buffer in the presence of increasing concentrations of [^{3}H]cGMP at 37°C for 1 h, and binding data were analyzed using GraphPad Prism and fitted best to a one-site binding hyperbola equation. Data shown are representative of five separate experiments and values are the mean ± S.E.M. Inset shows the Scatchard plot of the binding data obtained. B: Dissociation of cGMP bound to the GST–GAF protein was measured as described in the text. Data shown are representative of experiments performed at least twice. C: Various concentrations of either unlabeled cGMP or cAMP were used to inhibit the binding of radiolabeled cGMP to the purified GST–GAF protein. Data shown are representative of experiments performed twice and values are mean ± S.E.M. of duplicate determinations.

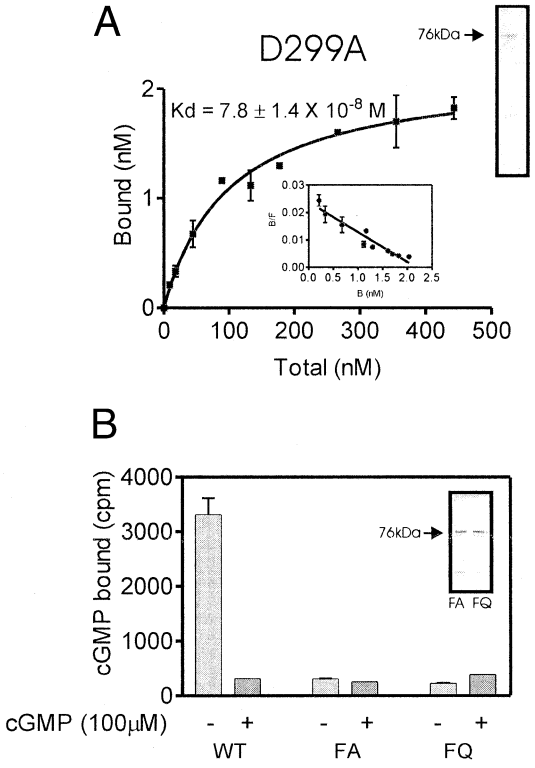

Fig. 4. Binding analysis for mutant GST–GAF_{D299A}, GST–GAF_{F205A} and GST–GAF_{F205Q} proteins. A: GST–GAF_{D299A} protein bound to beads (100 nM) was incubated in binding buffer in the presence of increasing concentrations of [^{3}H]cGMP. The binding data were analyzed using GraphPad Prism and fitted best to a one-site binding hyperbola. Data shown are representative of three separate experiments, and values are the mean ± S.E.M. of duplicate determinations. Insets show a Scatchard plot of the binding data obtained, and a Coomassie stained gel of the purified mutant protein. B: GST–GAF_{F205A} and GST–GAF_{F205Q} proteins were purified and 1 μM used for binding analysis in the presence and absence of unlabeled cGMP, and in the presence of 100 nM [^{3}H]cGMP. Data shown are representative of assays performed twice and values are mean ± S.E.M. of duplicate determinations. Inset shows the Coomassie stained picture of the purified mutant proteins. Around 3 nM of cGMP was bound to the wild type protein, used at 100 nM concentration under the assay conditions, representing less than 5% saturation.

In order to validate the structural model in terms of the implicated similarity between the GAFa domain of PDE5A and the GAFb domain of PDE2A, we generated two mutants where F205 in GAFa was changed to either an alanine or a glutamine residue, in order to dramatically alter the environment of the cGMP binding pocket. Mutations were generated in the GST–GAF domain protein and mutant proteins were purified and monitored for cGMP binding. As seen in Fig. 4B, no binding was detected in either of the mutant proteins, indicating the critical role for the F residue in directly interacting with cGMP, or maintaining the overall conformation of the GAFa domain to allow cGMP binding. Preliminary evidence suggests that the F205A or F205Q mutations do not alter the phosphodiesterase activity when introduced in the full-length PDE5 (data not shown), but the low levels of expression of the GST fusion proteins we obtain in this study preclude a detailed structural analysis of the consequences of this mutation on the overall structure of the GST–GAF fusion.

The results presented here have indicated that an earlier model of the bovine PDE5A GAFa domain that was generated using a yeast GAF domain protein as template may have incorrectly predicted the positioning of the cGMP in the protein. This is justified based on the observation that the earlier model predicted the importance of the 'NK(X)$_n$DE' motif in binding cGMP, but our data on the mutation of the D residue, as well as observation of the position of this motif in relation to cGMP in the current model, preclude a critical requirement for this motif in direct interaction with cGMP, as was also observed in PDE2A GAFb [5].

Also interesting to note is that the GAF domain construct that we have generated has a high affinity for cGMP and is in very close agreement to that observed for the GAF domain of PDE2A (27 nM for PDE5A GAF vs. 26 nM for PDE2A GAF [5]). The protein used in the current study begins at methionine 134 and ends at asparagine 583, with the numbering based on the residues in the human PDE5A1 splice variant [21,22]. In some of our earlier work, a construct was generated that began at isoleucine 221 and extended to alanine 645 (Fig. 2A). This protein showed no binding to cGMP, even though it would have contained the NK(X)$_n$DE motifs (residues 286–300 in GAFa and 515–527 in GAFb), as well as the entire GAFb domain. However, F205, which we have shown here to be critical for cGMP binding, would be absent in this protein and therefore this could have accounted for the loss of cGMP binding.

In earlier studies using GST–GAF domain proteins, constructs began at valine 165 and extended to aspartate 403 for the GST–GAFa domain construct, and this protein was reported to have an affinity for cGMP of 330 nM [15]. A similar construct of the GAFa domain of bovine PDE5A was shown to have an affinity for cGMP of 650 nM [9]. These results suggest that the presence of the GAFb domain may increase the affinity of the GAFa domain for cGMP, as is seen in our constructs, perhaps aiding in the formation of a correctly juxtaposed dimer, as was observed in the crystal structure of PDE2A.

In comparison to the GAF domain of PDE2A GAFb, the GAF domain of PDE5A shows a much higher specificity for cGMP as compared to cAMP [5]. This could perhaps be explained from the model because of the presence of arginine 174 that is substituted by an isoleucine in PDE2A. The model predicts that the sidechain of arginine would be positioned in PDE5A such that there would be a charge repulsion from the N6 atom of cAMP, thereby reducing the affinity of binding. It is likely that the isoleucine residue present in PDE2A may accommodate cAMP more readily, allowing it to bind with an affinity only 10-fold lower than that of cGMP.

Our results therefore suggest that the GAF domain of PDE5A is likely to adopt a structure similar to that of PDE2A, with critical determinants for cGMP binding being retained in both proteins. Given the high-affinity binding that we observe with the PDE5A-GAFa domain construct designed here, additional mutational analyses on full-length PDE5A are warranted. Since the reported affinity of cGMP binding for the full-length PDE is much lower than we see here (K_d 1 μM) it appears that the addition of the catalytic domain, and perhaps the N-terminal domain of PDE5A, alters the conformation of the GAF domain such that it binds cGMP with a lower affinity. This could be an advantage in the cell, where an increase in PDE5A activity, as a consequence of

cGMP binding to the GAF domain and phosphorylation of the enzyme, should occur only on large increases in cGMP levels, and not at the low nM concentrations that are present under basal conditions in most cells.

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