Entropy-driven cAMP-dependent Allosteric Control of Inhibitory Interactions in Exchange Proteins Directly Activated by cAMP*

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The exchange protein directly activated by cAMP (EPAC) is a guanine nucleotide-exchange factors for the small GTPases Rap1 and Rap2 and represent a key receptor for the ubiquitous cAMP second messenger in eukaryotes. The cAMP-dependent activation of apoEPAC is typically rationalized in terms of a pre-existing equilibrium between inactive and active states. Structural and mutagenesis analyses have shown that one of the critical determinants of the EPAC activation equilibrium is a cluster of salt bridges formed between the catalytic core and helices α1 and α2 at the N terminus of the cAMP binding domain and commonly referred to as ionic latch (IL). The IL stabilizes the inactive states in a closed topology in which access to the catalytic domain is sterically occluded by the regulatory moiety. However, it is currently not fully understood how the IL is allosterically controlled by cAMP. Chemical shift mapping studies consistently indicate that cAMP does not significantly perturb the structure of the IL spanning sites within the regulatory region, pointing to cAMP-dependent dynamic modulations as a key allosteric carrier of the cAMP-signal to the IL sites. Here, we have therefore investigated the dynamic profiles of the EPAC cAMP binding domain in its apo, cAMP-bound, and Rp-cAMPS phosphorothioate antagonist-bound forms using several 1H N relaxation experiments. Based on the comparative analysis of dynamics in these three states, we have proposed a model of EPAC activation that incorporates the dynamic features allosterically modulated by cAMP and shows that cAMP binding weakens the IL by increasing its entropic penalty due to dynamic enhancements.

The exchange protein directly activated by cAMP (EPAC) is one of the key receptors for the ancient and ubiquitous cAMP second messenger in mammals (1–3). The interaction of cAMP with EPAC results in the activation of the guanine-nucleotide exchange in the small GTPases Rap1 and Rap2 (1, 2), leading to the cAMP-dependent control of a wide array of critical signaling pathways underlying diverse cellular functions, ranging from insulin secretion to memory enhancement and cell adhesion (4–10). Two cAMP-dependent EPAC isoforms are currently known (Fig. 1a). Both EPAC1 and -2 are multidomain proteins with an N-terminal regulatory region (RR), including the cAMP binding domains (CBDs) and a C-terminal catalytic region (CR), containing a CDC25-homology module (CDC25HD) that functions as a guanine-nucleotide-exchange factor (GEF) (Fig. 1a). In both EPAC isoforms the cAMP dependence of the GEF function is implemented through the CBD at the C terminus of the RR (Fig. 1, a and b) irrespective of the DEP domain, which serves the primary purpose of controlling the membrane localization of EPAC (4, 9).

The cAMP-dependent structural changes underlying the regulatory function of the EPAC CBD have been previously mapped by the crystal structures of several structurally homologous CBDs solved in the apo and cAMP-bound states (11–18). These CBD structures consistently show that the main conformational change caused by cAMP is a hinge-like rotation of the helix C-terminal to the β-barrel typical of CBDs (17, 18), which is commonly referred to as hinge helix. When cAMP docks into the binding pocket formed by the phosphate binding cassette (PBC) and the base binding β4-β5 region (BBR), the hinge helix approaches the β-barrel core, bringing the region C-terminal to it (i.e. the lid) in proximity of the PBC (18). Based on structural and mutagenesis data, it has been proposed that this hinge-like cAMP-dependent motion contributes to the displacement of the RR away from the CR, thus removing steric impediments for the access of substrates to the catalytic domain and explaining the cAMP-dependent activation of the GEF function of EPAC (18). This mechanism of EPAC activation has been formalized in terms of an equilibrium between active and inactive states in which the RR and CR are in an open and closed relative orientation, respectively. The cAMP-dependent hinge rotation...
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then promotes a shift of this equilibrium toward the active (open) state (18, 19).

The recently solved structure of apo-EPAC2 (16) has also revealed the involvement of the CBD in another fundamental inhibitory mechanism distinct from the hinge rotation. Specifically, the α2 helix in the N-terminal helical bundle (NTHB) of this CBD and the preceding helix (i.e. α1) form a cluster of four salt bridges with the catalytic region. Such a CR/RR salt-bridge cluster is called the ionic latch (IL) (Fig. 1b) and serves two main inhibitory functions. First, it secures the RR and CR in close proximity to each other, sterically blocking the access of effector Rap proteins to the catalytic core (16). Second, the IL sequesters and shields a critical CR arginine (i.e. Arg-886 in EPAC2m) which has been suggested to be required for the recognition of Rap by EPAC, making it unavailable for a crucial interaction with Rap (16).

A recent site-directed mutagenesis study has shown that the equilibrium between active and inactive states of EPAC is not controlled only by the hinge motion of the CBD C-terminal region but also by the IL (16). For instance, a deletion mutant (i.e. EPAC2Δ306) in which the IL is weakened through the removal of one of the CR/RR salt bridges displays a striking 5-fold increase in the maximum exchange activity (kmax) (16), indicating that the integral IL contributes to shifting the EPAC equilibrium toward the inactive state. However, it is currently not clear how the IL sites located in the N-terminal helical bundle are controlled by cAMP, which docks in the distal PBC and BBR, embedded within the β-subdomain. Considering that previous MS investigations (20) support the notion that the region spanning the IL is not subject to major conformational perturbations upon cAMP binding, we propose the hypothesis that the IL is allosterically controlled by cAMP through cAMP-dependent changes in dynamics rather than in structure. To test this hypothesis, we have investigated primarily by classical NMR 15N relaxation experiments as well as by multi-offset NMR dispersion measurements the EPAC1h-(149–318) construct in its apo-, cAMP-bound (holo), and Rp-cAMPS-bound states.

The EPAC1h-(149–318) segment binds cAMP with an affinity similar to that of full-length EPAC (Kp = 4 μM) (21) and includes not only the PBC and BBR, where cAMP binds, but also the NTHB and the C-terminal helical hinge region (Fig. 1b) that is allosterically affected by cAMP. Furthermore, EPAC1h-(149–318) includes in full the α1-helix and the three key sites of the ionic latch (i.e. Gln-168, Asp-172, and Glu-197). A similar N-terminal truncation in EPAC2 (i.e. EPAC2Δ280) did not affect the observed kmax (16), indicating that such an N-terminal deletion mutation does not impair the ability of the IL sites to form inhibitory interactions with the catalytic core. In addition, we have noticed that the α1-helix with its highly polar N-terminal region (Fig. 1b) ensures excellent solubility in both the presence and absence of ligands resulting in high assignment coverage (i.e. >82%) and in an unprecedented picture of allostery and dynamics unavailable for other eukaryotic CBDs due to inherent instabilities that have hampered direct apo versus holo comparisons (19, 22–25).

Overall, our combined comparative NMR analysis of dynamic features in the EPAC1 CBD shows that cAMP binding leads to significant modulations of the CBD dynamic profile in several time scales and at multiple allosteric sites, pointing to a critical link between flexibility and function, in general, and to a pivotal entropic determinant for the allosteric propagation of the cAMP signal from its binding pocket to the distal IL region, in particular.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The human EPAC1 CBD construct EPAC1h-(149–318) was expressed and purified according to previously published protocols (26). Further details are available in the supplemental materials.

**NMR Measurements**—All spectra were acquired at 34 °C using a 700-MHz spectrometer equipped with a TCI cryo-probe. The temperature was calibrated using both a thermocouple and an ethylene glycol sample. The heteronuclear single-quantum coherence (HSQC) spectra were acquired with 128 (15N) and 1024 (1H) complex points and spectral widths of 31.8 and 14.2 ppm for the 15N and 1H dimensions, respectively. 1H chemical shifts were calibrated using 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as a standard. Compounded chemical shifts were computed via the PECAN software (31). Compounded chemical shifts were computed via the following formula: Δδcompound = ((Δδ1H)2 + (Δδ15N/6.5)2)1/2, where Δδ 1H and Δδ 15N are the differences between the proton and nitrogen chemical shifts, respectively.

**15N NMR Relaxation Measurements**—The 15N longitudinal and transverse relaxation rates R1 and R2 as well as the 1H 15N steady state NOEs were measured for 0.1 mM EPAC1h-(149–318) in its apo and Rp-cAMPS- and cAMP-bound forms, with pulse sequences containing water flip back pulses and sensitivity enhancement (32–38). Before the relaxation experiments the NMR samples were filtered through a 100-kDa cut off filter. For the measurements of R1, relaxation rates the following relaxation delays were employed: 100 (×2), 200, 300, 400 (×2), 500, 600, 800, and 1000 ms (where ×2 indicates duplicate spectra). The offset and duty cycle-compensated 15N R2 CPMG experiments (39) were measured using a VCPMG of 472 Hz (40, 41) and CPMG relaxation delays of 8.48, 16.96, 25.44, 33.92, 42.4, 50.88, 59.36, 76.32, and 93.28 ms. For the NOE experiments, a 10-s recycle delay was used that included a 5-s proton saturation period, whereas a recycle delay of 1.5 s was used for
the R₁ and R₂ experiments. The stability of the protein sample was monitored before and after each relaxation experiment via HMQC spectra. To average potential long term instabilities, the entire series of R₁ and R₂ relaxation rates were run in duplicate or triplicate (42), and the \( {\langle {H}\rangle} \) \(^{15}\)N steady state NOEs were collected in 9 or 10 sets of saturated and unsaturated spectra. All spectra were co-added before processing using NMRPipe, resulting in a total of 48 or 72 scans per serial file at each T₂ CPMG length, 32 or 48 scans per serial file at each T₁ inversion recovery delay, and 36 or 40 scans per serial file for each NOE experiment (with and without \(^{1}\)H saturation). The number of dummy scans was always 96 or higher. The \(^{15}\)N and \(^{1}\)H frequencies in the relaxation experiments were digitized with the same number of points as in the HSQC experiments described above. The errors for R₁ and R₂ were evaluated using Gaussian distributed random noise. The steady state NOE values were computed as the ratio of the intensities in saturated to unsaturated spectra. The STDV of the distribution of the differences in fit peaks were not included in the relaxation analyses due to line broadening and/or overlap.

Reduced Spectral Density Mapping—The \(^{15}\)N relaxation data were mapped into reduced spectral densities under the assumption that their high frequency values do not vary: \( J(\omega_{\text{N}} + \omega_{\text{H}}) = J(\omega_{\text{N}}) = J(\omega_{\text{H}}) \), as previously explained (44–47). Assuming these equalities, it is possible to compute the \( H_{\text{NOE}} \) values only from the measured \(^{15}\)N R₁ and \(^{1}\)H\(^{15}\)N NOEs, whereas the J(0) values are derived from the measured \(^{15}\)N R₂ rates as well (47). The value of J(0) calculated in this way also includes contributions from chemical exchange. Error propagation was employed to estimate the errors in the reduced spectral densities starting from the uncertainties in the measured \(^{15}\)N relaxation rates and NOEs.

Hydrodynamic Simulations—The contributions to the relaxation rates and to the reduced spectral densities arising from the overall tumbling and from the effect of diffusion anisotropy were evaluated through hydrodynamic simulations based on the bead method using the HYDRONMR program (48, 49). For this purpose the coordinates of several CBD-spanning fragments of the Protein Data Bank code 2BYV structure of EPAC2\(_{m}\) (16) were utilized (supplemental Table S1). In all simulations hydrogen atoms were added through the program Molmol (50), and the atomic element radius was 3.3 Å, which has been previously shown to best fit multiple hydrodynamic properties (i.e. translational diffusion, sedimentation coefficients, rotational diffusion, and intrinsic viscosity) in a set of model proteins (49). All HYDRONMR computations were carried out at a temperature of 307 K and the water viscosity in centipoises (cP) was calculated as: \( \eta = 1.7753 \times 0.0565 + 1.0751 \times 10^{-7}t^2 - 9.2222 \times 10^{-8}t^3 \), where \( t \) is the temperature in Celsius (49). The HYDRONMR-computed \(^{15}\)N relaxation rates at a static field of 16.44 tesla assume an N-H distance of 1.02 Å and a chemical shift anisotropy of −160 ppm (40, 41). The \( D_{\text{par}}/D_{\text{per}} \) ratios in supplemental Table S1 were calculated as \( 2D_j/(D_x + D_y) \), in which \( D_x \) and \( D_y \) are the two eigenvalues of the rotational diffusion matrix that are closest to each other (48, 49).

Relaxation Dispersion NMR—The \(^{15}\)N relaxation dispersions for backbone amides in the cAMP- and the Rp-CAMPS-bound and apo states of EPAC1\(_{h}\) (149–318) were measured using a constant-time relaxation-compensated CPMG pulse sequence (40, 41, 51). During the total CPMG length (\( T_{\text{CPM}} \)) of 93.3 ms, either 8 or 88 \(^{15}\)N pulses were implemented, resulting in CPMG field strengths (\( n_{\text{CPM}} \)) of 43 and 472 Hz, respectively (40, 41, 51). Six interleaved replicate data sets were recorded and co-added for each CPMG field strength. 128 dummy scans and 16 scans were accumulated per serial file with an interscan delay of 2.2 s. Because of the presence of significant offset effects for the \(^{15}\)N pulses at 700 MHz and at the CPMG RF (radio frequency pulse) strength employed (3.1 kHz), all constant-time relaxation-compensated CPMG experiments were acquired with three different \(^{15}\)N carrier frequencies (110, 119, and 127 ppm) to cover the \(^{15}\)N spectral width through three narrow frequency bands. The NMR relaxation dispersion (\( \Delta R_2^{\text{eff}} \)) was computed using the equation \( \Delta R_2^{\text{eff}} = (1/T_{\text{CPM}}) \ln(I_{472Hz}/I_{110Hz}) \), where \( I_{\text{CPM}} \) is the cross-peak intensity with a CPMG strength \( n_{\text{CPM}} \). NMR dispersion could not be measured for cross-peaks affected by overlap and/or relaxing too quickly to be detected in the relaxation-compensated constant-time CPMG spectra (e.g. several residues in the α3 region).

Sequence and Structure Analyses—Sequence alignments were performed with ClustalW (52), whereas Pymol (53) and/or Molmol (50) were used for the structural analyses.

RESULTS

No Major cAMP-dependent Local Structural Changes Are Observed for the Residues of EPAC1\(_{h}\) (149–318) Spanning the Ionic Latch—The cAMP-dependent structural changes of EPAC1\(_{h}\) (149–318) were probed through the \(^1\)H\(^{15}\)N-compounded chemical shift variations caused by cAMP (Fig. 1, c and d) and the secondary chemical shift-based α-helix and β-strand probabilities measured for both the apo and cAMP-bound states (Fig. 2, a and b). Fig. 1d (black dots) shows that no significant cAMP-dependent \(^1\)H\(^{15}\)N-compounded chemical shift changes are observed for helices α1 and α2, which span the IL region (i.e. 168–197). For most α1 and α2 residues the observed cAMP-induced \(^1\)H\(^{15}\)N-compounded chemical shift variations are below average (Fig. 1d) and are comparable with or smaller than those observed for residues in other structurally invariant regions in all known CBDs, such as β8 (Fig. 1, c and d) (15). In agreement with these observations, the secondary chemical shifts of apo and cAMP-bound EPAC1\(_{h}\) (149–318) (Fig. 2, a and b) further support the absence of significant variations in the 2′ structure for the IL region. Therefore, both the RN and the secondary chemical shifts consistently indicate that the region spanning the IL (i.e. 168–197) is not affected by major structural variations as a result of cAMP binding.

Evaluation of cAMP-dependent Structural Changes beyond the IL Region Based on a Quantitative Analysis of the cAMP-induced Chemical Shift Variations—In the other regions of EPAC1\(_{h}\) (149–318) C-terminal to α2, the observed chemical shift variations (Fig. 1d) are consistent with a model (26, 17)
according to which cAMP docks to the PBC and BBR and relays its signal through the β2-β3 loop and the α6-lid regions. According to this model, cAMP binding causes a hinge-like rotation of α6 (17) perturbing the environment of adjacent residues in the α3 and α4 (Fig. 3) and explaining the cAMP-dependent chemical shift variations observed for these helices. This model is also supported by the NMR-based α/β-probabilities of apo and holo EPAC1h-(149–318) (Fig. 2, a and b) showing that the most significant 2° structure variations occur at the level of the α6 helix C terminus (i.e. residues 305–310), which becomes less helical upon cAMP binding, consistently with a similar helicity profile reported for the cAMP-bound state of CBD-A of PKA (23).

Interestingly, the Rp-cAMPS antagonist, unlike cAMP, does not significantly destabilize the α6 helix C terminus (Fig. 2c), in full agreement with the absence of significant Rp-cAMPS-dependent HN-ppm variations for α6 (Fig. 1d). These observations suggest that Rp-cAMPS does not cause the hinge rotation of α6 and explain also why, upon Rp-cAMPS binding, no appreciable HN-ppm changes are detected for residues in α3 and α4, confirming our interpretation of the cAMP-dependent HN-ppm changes in this region in terms of the α6 hinge motion. Another clear difference between the HN-ppm variations caused by cAMP and by Rp-cAMPS is observed for Gly-238 (Fig. 1d) in the β2-β3 loop, which is very sensitive to cAMP but is only marginally affected by the phosphorothioate antagonist. The HN-ppm changes observed for Gly-238 reflect, therefore, mainly allosteric perturbations.

**General Approach to the Characterization of the Dynamics of the EPAC1 CBD**—The ps-ns and μs-ms dynamic features of EPAC1h-(149–318) were probed in the apo and cAMP- and Rp-cAMPS-bound states through the measurement of $^{15}$N R$_2$ and R$_1$ relaxation rates and of
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**FIGURE 1.** a, EPAC1 and EPAC2 domain organization. The **black circle** indicates the cAMP ligand. The **dotted contour** and the **empty circle** (cAMP) for the CBD-A of EPAC2 mean that this module is not strictly necessary for the regulation of EPAC2 (18). The **question mark** denotes a domain with currently unknown function. The domain investigated here for EPAC1 is **highlighted in orange**, b, sequence alignment of CBDs in human EPAC 1 (EPAC1h), human EPAC2 (EPAC2h), and mouse EPAC2 (EPAC2m). The secondary structure is shown in red, and it was obtained from the crystal structure of apoEPAC2m (Protein Data Bank [PDB] code 1O7F) (17). Strictly conserved residues in all three sequences are **highlighted in green**, whereas residues that are identical only in two of the three sequences are marked in **yellow**. Residues conserved only in terms of side-chain type are highlighted in **cyan**. Residues that play pivotal allosteric roles based on mutational analyses (17) or on the apo-EPAC2m structure (16) are marked by **blue rectangles**. c, representative expansion of the \[^{1}H,^{15}N\]HSQC spectra of EPAC1h-(149–318) (Figs. 4 and 5; supplemental Table S1). Although the best match with the average values of the relaxation rates and of the reduced spectral densities were repeated at three different levels of N-terminal truncation (i.e., starting from residues 149, 161, and 173) (Figs. 4 and 5; supplemental Table S1). Although the best match with the average values of the relaxation rates and of the reduced spectral densities were obtained for the simulations starting at residue 161 (Fig. 4), the data calculated for the constructs truncated at residues 149 and 173 were still employed to provide a conservative estimate of the \[^{1}H,^{15}N\]NOEs, which are reported in Fig. 4a, b, and c, respectively. One of the primary considerations in the \[^{15}N\]R\(_2\), R\(_3\), and NOE data analysis is the pronounced flexibility of most residues in the long N-terminal helix, as suggested by the fast hydrogen exchange rates (26) and the secondary chemical shifts (Fig. 2). It is well known that this type of conformational heterogeneity in the CBD \(\alpha\)-subdomain impinges on the diffusion model describing the overall tumbling of the protein in solution (23). In addition, supplemental Table S1 shows that the \(D_{pp}/D_{pe}\) ratios vary considerably as a function of the degree of N-terminal truncation. The significant conformational plasticity of the N-terminal tail, therefore, hampers the accurate description of the overall motion for the EPAC1 CBD in terms of a single well defined diffusion tensor. Hence, we opted to rely on the reduced spectral density mapping approach, which does not require assumptions on the overall tumbling of the protein or on the exact form of the spectral density functions (44–47). The relaxation data were then translated into reduced spectral density maps, which are provided in Fig. 5. Potential contributions arising from the diffusion anisotropy of the overall tumbling were assessed through the evaluation of the R\(_{pe}/R_{pp}\) product (Figs. 4c), which is essentially insensitive to the anisotropy of the diffusion tensor (54), and/or through hydrodynamic simulations of both the \[^{15}N\]relaxation rates (red lines in Fig. 4) and the corresponding spectral densities (red lines in Fig. 5).

Considering that the \(\alpha\)-helix N-terminal helix is predominantly unstructured, the hydrodynamic modeling of the relaxation rates and of the reduced spectral densities were repeated at three different levels of N-terminal truncation (i.e. starting from residues 149, 161, and 173) (Figs. 4 and 5; supplemental Table S1). Although the best match with the average values of the relaxation rates and of the spectral densities was obtained for the simulations starting at residue 161 (Fig. 4), the data calculated for the constructs truncated at residues 149 and 173 were still employed to provide a conservative estimate of the

**FIGURE 2.** Secondary structure probabilities computed based on the observed chemical shifts for apo (a), cAMP-bound (b), and Rp-cAMPS-bound (c), EPAC1h-(149–318). The experimental conditions are the same as for Fig. 1c. The \(\alpha\)-helix and \(\beta\)-strand probabilities are reported as positive and negative values, respectively. The secondary structure based on the apoEPAC2m crystal structure is reported in panel b, similar to Fig. 1d. The areas **highlighted in gray** mark the C-terminal half of helix \(\alpha\)6 and the region spanning the ionic latch.

**FIGURE 3.** Overlay of the CBDs of cAMP-bound PKA (RIα-A, PDB code 1RGS) (58) and of apoEPAC (PDB code 1O7F) (17) represented as **gray** and **green ribbons**, respectively. Selected secondary structure elements and residues are labeled. The cAMP-dependent hinge rotation of the C-terminal helices affects the environment of the highlighted \(\alpha\)3 and \(\alpha\)4 residues within the N-terminal helical bundle. The C-terminal helix **boxed** by the **dashed contour** is unstable, as indicated by the NMR investigation of PKA CBD-A (22, 23).

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range of rate and spectral density variability associated with the overall tumbling anisotropy (Figs. 4 and 5). In addition, dynamics in the ms time scale were probed in a diffusion anisotropy-independent manner through NMR dispersion (NMRD) measurements reported in Fig. 6. In conclusion, our analysis of the dynamic profile of the EPAC1 CBD is largely structure-independent, as it relies on a combination of HN-NOE, R1R2, reduced spectral densities, and NMR dispersion measurements. HN-NOEs and J{H,N} are used to probe fast local ps-ns motions, whereas J{0}, the R1R2 product, and the NMRD rates report on dynamics in the ms-μs range. This approach was previously successfully applied to the characterization of the PKA CBD-A dynamics (23).

Dynamics of ApoEPAC1h-(149–318)—The dynamic profile of apoEPAC1h-(149–318) was investigated in the ps-ns and ms-μs time scales. For the ps-ns dynamics of apoEPAC1h-(149–318) the most significant feature is the flexibility of the N-terminal moiety of α1, as indicated by the relatively low NOE (Fig. 4d) and high J{ω1+ωN} (Fig. 5c) values observed for the corresponding residues. This marked flexibility at the N terminus explains why the overall tumbling is best modeled by hydrodynamic simulations of the N-terminal-truncated EPAC1 constructs (supplemental Table S1; Figs. 4 and 5). Other sites of apoEPAC1h-(149–318) that appear significantly affected by local ps-ns motions as conservatively indicated by NOE values 0.5 are Asp-236 in the β2-β3 loop and several C-terminal lid residues (Figs. 4d and 5c). The flexibility of Asp-236 is also confirmed by its fast H/H exchange in the apo state (26) despite the fact that its amide hydrogen is involved in a backbone-to-backbone hydrogen bond (17).

ApoEPAC1h-(149–318) displays also multiple sites affected by ms dynamics as revealed by the NMRD data (Fig. 6). Residues with greater than average ΔR{eff} in the apo state cluster at several loci distributed throughout the domain, including the α1 C terminus, the α4-β1 and β2-β3 regions, the PBC (i.e. Ala-277), and the α6 (hinge) helix (Fig. 6). In addition, significant line-broadening is observed for several residues in the PBC and

FIGURE 4. Backbone 15N relaxation data for EPAC1h-(149–318) in its apo (orange), CAMP-bound (black), and Rp-cAMPS-bound (green) states plotted against the residue number. The experimental conditions are the same as for Fig. 1c. a, spin-spin relaxation rate R2. b, spin-lattice relaxation rate R1. c, product of the R1 and R2 relaxation rates. In panels a, c, and d, residues for which the ps-ns or ms-μs dynamics is enhanced or quenched in the CAMP-bound state relative to both the apo and the Rp-cAMPS states are labeled by a star. The red lines in panels a–d indicate relaxation rates calculated based on a hydrodynamic bead model for the overall tumbling motion. The hydrodynamic computations were carried out for three different deletion constructs of the highly homologous EPAC2m, 284–444, 296–444, and 308–444, corresponding to EPAC1h 149–309, 161–309, and 173–309, respectively. Residues for which no relaxation data are available are prolines or are overlapped and/or broadened beyond detection.
in the BBR (supplemental Fig. S1) making them undetectable in the relaxation experiments but also suggesting the presence of ms-μs dynamics at these sites, consistent with their lack of protection from hydrogen/deuterium exchange in the absence of cAMP (19, 26). Other sites affected by ms-μs dynamics in apoEPAC1h-(149–318) are mostly located in the NTHB region spanning helix 3 (Figs. 4 and 5a). For instance, residues Glu-197, His-200, Ile-201, and Ala-203 are highly dynamic in the ms-μs time scale as indicated by their R2 and J0 values, which are significantly higher than the values computed based on hydrodynamic modeling of the overall tumbling (Figs. 4c and 5a). These R2 and J0 enhancements, therefore, cannot be accounted for by diffusion anisotropy effects, as also independently confirmed by the corresponding R2R2 products (Fig. 4). In all three panels, the red lines indicate the reduced spectral densities computed starting from the relaxation rates predicted based on the hydrodynamic bead models, as shown in Fig. 4.

In a previous NMR investigation of a shorter EPAC1 construct (i.e. EPAC1n (169–318)), a minor (i.e. 20%) set of HSQC cross-peaks was detected for 9 residues in the apo state (19). This minor set of apo peaks appears at chemical shifts similar to those observed in the cAMP-bound spectrum for the corresponding residues (19). Even though these minor apo peaks were not reported for other regions with significant cAMP-dependent chemical shift changes such as the α6-helix, they were interpreted as supporting evidence of a dynamic equilibrium between active and inactive states in apoEPAC. For the longer construct EPAC1h-(149–318) and under our experimental conditions, we could not observe this minor set of HSQC peaks. However, minimally populated states that escape direct detection in the HSQC experiment are readily probed through NMRD measurements (Fig. 6) when they exchange with the main set of peaks in the ms time scale (40, 41, 51).

**FIGURE 5.** Reduced spectral densities for EPAC1h-(149–318) in its apo (orange), cAMP-bound (black), and Rp-cAMPS-bound (green) states plotted against the residue number. These spectral densities value were computed based on the relaxation rates reported in Fig. 4. a, plot of J(0) values which include contributions from chemical exchange effects. Secondary structure elements are shown as in Fig. 4. Residues corresponding to the most significant enhancements (reductions) in their J(0) values are highlighted in red (blue). b, plot of J(ωH) with ωH = −γH B0 (B0 = 16.44 tesla at 700 MHz). c, plot of L(ωH + ωN) with ωH = −γH B0. Residues for which J(ωH + ωN) increases (decreases) upon cAMP binding are highlighted in red (blue). Residue labeling follows the same criteria as for Fig. 4. In all three panels, the red lines indicate the reduced spectral densities computed starting from the relaxation rates predicted based on the hydrodynamic bead models, as shown in Fig. 4.

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The ionic latch residue in constant time CPMG measurements and computed as domain. For instance, residues Val-177, Leu-179, Lys-181, and the apo state is highly flexible in the ps-ns time scale, to the fast ps-ns motions. The only overall trend observed for the ms-ndynamics is supported by the reduction in line-broadening observed for (Figs. 4d and 5c), consistent with the destabilization of the C-terminal half of α6 (i.e., residues 305 onward) occurring upon cAMP binding as revealed by the secondary chemical shifts (Fig. 2, a and b).

Rp-cAMPS-dependent Dynamics of EPAC1h-(149–318)—For the purpose of separating cAMP-dependent variations in dynamics merely due to binding from those due to allosteric effects, the 15N R1, R2, and HN NOE together with the corresponding reduced spectral densities (Figs. 4 and 5, green circles) and the NMRD ΔR2,eff. values observed for the antagonist Rp-cAMPS-bound state of EPAC1h-(149–318). Figs. 4–6 show that overall most of the cAMP-dependent changes in dynamics result from allosteric rather than from binding effects. For instance, the ms dynamics at Leu-179, Lys-181, Val-218, Glu-222, and Glu-308 is reduced in both the apo and Rp-cAMPS-bound states relative to the cAMP-bound form, as indicated by the consistently higher ΔR2,eff. values observed for these residues in the cAMP-saturated state compared with both apo and Rp-cAMPS-bound forms (Fig. 6). If these variations in ms dynamics were just the result of binding, the dynamics in the antagonist-bound state should have instead resembled the cAMP-bound rather than the apo state. The NMRD data in Fig. 6 indicate, therefore, that the large majority of the cAMP-dependent enhancements in ms dynamics observed for EPAC1h-(149–318) result from allosteric effects. Similarly, most of the sites affected by a cAMP-dependent quenching in ms dynamics (Figs. 6) are also the result of allosteric perturbations as indicated by the ΔR2,eff. dispersions in the cAMP-bound state being lower than those of both apo and Rp-cAMPS-bound forms (Fig. 6). Furthermore, the R1,R2 analysis shows that allosteric effects account also for the up-regulation of ms-μ dynamics of Arg-169, Glu-197, and Ala-203 (Fig. 4e). Glu-197 mediates critical IL salt bridges, and Arg-169 is adjacent to Gln-168, another IL residue.
The allosteric propagation of the cAMP signal affects the dynamic profile of EPAC1h-(149–318) not only in the ms-μs but also in the ps-ns time scale. This modulation of ps-ns flexibility involves several functionally critical residues such as Asp-172 in the ionic latch, Asp-236 in the 6 helix (Fig. 4d). Specifically, the ps-ns dynamics at Asp-172 and Lys-305 is reduced in both the apo and Rp-cAMPS-bound states relative to the cAMP-bound form (Figs. 4d and 5c). The HN NOE and $J(\omega_{1z} + \omega_{0z})$ values in Figs. 4d and 5c, respectively, indicate therefore that the cAMP-dependent enhancement of ps-ns dynamics at Asp-172 and Lys-305 is an allosteric effect. Similarly, the cAMP-dependent quenching of ps-ns dynamics at Asp-236 (Figs. 4d and 5c) is also the result of allosteric coupling between the β2-β3 loop and the PBC because for this residue the $J(\omega_{1z} + \omega_{0z})$ spectral densities of the apo and Rp-cAMPS-bound states are both significantly higher than that of the cAMP-saturated form.

**DISCUSSION**

**Functional Relevance of the ApoEPAC1h-(149–318) Dynamics**—The ps-ns and ms-μs dynamic “hot spots” of apoEPAC1h-(149–318) are summarized in the three-dimensional map of Fig. 7. To the best of our knowledge this is the first time the full ps-ns and ms-μs dynamic profile of a ligand-free eukaryotic CBD could be investigated at atomic resolution by $^{15}$N relaxation experiments, because previous attempts to analyze the apoCBD of PKA were unsuccessful due to aggregation (24). Fig. 7 shows that these dynamic hot spots cluster into a limited set of patches which match well the previously identified functional sites of the EPAC1 CBD. For instance, not only the N and C termini of the PBC appear dynamic in the ms-μs but also the adjacent β2-β3 loop is affected by both ms-μs and ps-ns motions (Fig. 7a), suggesting that in the absence of cAMP this turn is unstable. Furthermore, two other ms-μs dynamic patches involve the hinge α6 helix and the IL spanning α1–2 helices, respectively (Fig. 7). The patch including α6 also affects the adjacent α4 and that, including α1, extends to the proximal β1 strand as well (Fig. 7). Overall, the high degree of co-localization between the functionally critical regions of the EPAC1 CBD (i.e. the PBC, the β2-β3 loop, the hinge, and the α1–2 helices) and the dynamic patches is fully consistent with the apo state of this domain existing in an equilibrium of active and inactive conformations, as previously hypothesized based on cAMP-dependent bioassays on full-length EPAC (18).

Inspection of Fig. 7 reveals also two major clusters of residues affected by ps-ns dynamics and localized in the vicinity of the N and C termini. Although the flexibility in the C-terminal tail is likely to be to a large extent the result of the C-terminal truncation, the dynamics observed in the N-terminal region is consistent with the elevated B-factors observed for this region in the context of the full-length EPAC structure (16). As shown in Fig. S2, the B-factors for residues 149–165 are significantly higher than the average value observed for the 149–318 segment, suggesting that the flexibility observed for the N terminus of EPAC1h-(149–318) may reflect at least in part an intrinsic property of the EPAC1 CBD rather than just a truncation artifact. In addition, the apo state dynamic profile serves as a key reference to evaluate the changes in flexibility caused by cAMP.

**Functional Relevance of the Allosteric cAMP-dependent Modulations of Dynamics in EPAC1h-(149–318)**—The cAMP-dependent changes in ps-ns and ms-μs dynamics that result from allosteric rather than simple binding effects are summarized in the three-dimensional map shown in Fig. 8, which reveals the presence of multiple clusters of residues for which dynamics is either quenched (referred to as “cold patches”) or enhanced (defined as “hot patches”) by cAMP. Several of these cAMP-dependent dynamic patches match well to the known functional sites of this CBD. For instance, a first striking feature that emerges from Fig. 8 is that the stabilizing effect of cAMP extends well beyond the PBC to the β2-β3 loop. Specifically, the β-turn involving the hydrogen bond between the Asp-236 HN and the Ser-233 CO is stabilized only in the presence of cAMP, which also quenches the ms dynamics of Phe-232 located at the C terminus of the highly distorted β2-strand ($\psi_{1231} = -59\degree$).

Interestingly, the PBC and β2-β3 loop residues for which dynamics is detectably quenched by cAMP (i.e. Ala-277, Asp-236, and Phe-232) cluster around the two highly conserved and...
co-evolved residues, i.e. Gly-238 and Arg-279 (Fig. 8a). These two residues have been proposed to be critical elements of the CBD allosteric network, based on recent extensive evolutionary analyses of genomic CBD sequences revealing that Gly-238 and Arg-279 have co-evolved for the purpose of coupling cAMP binding to distal regulatory regions (55). The chemical shift changes of the Gly-238 site could not be probed through previous spectroscopic analyses (19), but this site is clearly detectable in our spectra (Fig. 4d), which indicate that Gly-238 is highly sensitive to cAMP, although not to the antagonist Rp-cAMPS (Fig. 1d). For both Ala-277 and Phe-232, dynamics are quenched upon cAMP binding. As shown in Fig. 8b, according to the proposed hinge model of cAMP activation, Asn-301 and Arg-302 in the central moiety of the 6 helix approach Val-211 at the N terminus of the 4 helix upon cAMP binding, explaining why for these three residues the ms dynamics is quenched by cAMP. However, the cAMP-dependent 6 rotation also perturbs the 4/6 contacts involving the C termini of these helices, accounting for their enhanced dynamics upon cAMP binding. For instance, the cAMP-dependent hinge rotation of 6 brings the N terminus of the 6 away from Val-218 at the C terminus of the 4 and simultaneously displaces Lys-305 and Glu-308 located in the C-ter-

FIGURE 8. Three-dimensional map of the changes in EPAC CBD dynamics caused by cAMP-dependent allosteric perturbations. Residues for which the ms-μs dynamics is enhanced (quenched) in the cAMP-bound state relative to both the apo and the antagonist Rp-cAMPS-bound states are highlighted in red (blue). Residues for which the ps-ns dynamics is enhanced (quenched) in the cAMP-bound state relative to both the apo and the antagonist Rp-cAMPS-bound states are highlighted in orange (cyan). a, inset showing that two highly conserved CBD residues, Gly-238 and Arg-279, are packed between Ala-277 in the PBC and Phe-232 in the β2-β3 loop. For both Ala-277 and Phe-232, dynamics are quenched upon cAMP binding. b, inset showing the position of the 6 in the cAMP-bound state as modeled according to CBD-A of the regulatory subunit of PKA (PDB code 1RGS). c, inset zooming into the ionic latch region and showing that the sites of cAMP-enhanced dynamics are lined on the α1 helix side where the critical IL Asp-172 resides and which faces another pivotal IL residue, Glu-197.
minal region of α6, away from α4 (Fig. 8b). Consistent with these observations, for all these three residues (Val-218, Lys-305, and Glu-308) cAMP binding results in increased dynamics (Fig. 8).

Notably, Lys-305, which is the only α6 residue for which dynamics is significantly enhanced in the ps-ns time scale (Fig. 8a), marks the beginning of the α6 region that is destabilized by cAMP (Fig. 2, a and b) and includes also Glu-308, which is involved in a backbone-to-backbone hydrogen bond with Lys-305. The reduction of α-helix probability observed for the 305–309 segment upon cAMP binding (Fig. 2, a and b) reflects, therefore, an overall increase in flexibility at this site that connects the EPAC1 CBD to the EPAC1 catalytic region and is critical to control the relative orientation of the regulatory and catalytic regions (i.e. RR and CR in Fig. 1a), as required for the modulation of the GEF activity of EPAC through steric occlusion of its catalytic site.

Another site playing a pivotal role in the activation of EPAC is the IL, which provides additional RR/CR contact points. Interestingly, two of the three IL residues (i.e. Glu-197 and Asp-172) are part of a third major cluster of residues for which dynamics is enhanced by cAMP. This cluster involves part of the α2 helix and mainly the inner side of the α1 helix (Fig. 8c). Considering that the chemical shift maps (Figs. 1 and 2) indicate that cAMP alone does not cause major structural rearrangements in the IL spanning region (i.e. helices α1 and α2), the cAMP-dependent dynamic enhancements observed for Glu-197 and Asp-172 and the adjacent residues suggest that cAMP weakens the inhibitory CR/RR IL interactions primarily by increasing the entropic penalty associated with the formation of the IL salt bridges. It is also possible that this entropic control exerted by cAMP may become even more relevant in full-length EPAC, where the IL dynamics of the apo state is likely to be further quenched by the presence of the catalytic region forming multiple stabilizing salt bridges with the IL (16). Therefore, in full-length EPAC1 the increase in the configuration entropy of the IL region caused by cAMP binding is expected to be even more significant than in the single EPAC1 CBD. In other words, the recognition of cAMP and the formation of the CR/RR IL salt bridges are negatively cooperative events, and dynamics is a key carrier of the allosteric free energy for this negative cAMP/IL cooperativity. Similar allosteric roles of ligand-dependent entropic modulations in the absence of significant structural variations have been previously reported for other systems either on theoretical grounds (57) or based on $^{15}$N NMR relaxation measurements (58, 59).

These conclusions imply that the mechanism of EPAC GEF activation by cAMP should be modified to include multiple allosteric pathways that involve not only conformational and dynamic changes in the region C-terminal to the CBD β-barrel, as previously proposed (18), but also a cAMP-mediated entropy-driven control of the IL interactions. Fig. 9 shows schematically such a modified mechanism of EPAC activation taking into account the allosteric cAMP-dependent changes in dynamics and their impact on the coupled active/inactive and apo/bound equilibria. Upon cAMP binding, the EPAC equilibrium is shifted toward the active state by the combined action of the hinge motion and of the increased entropic cost for the IL between the CR and the NTHB (Fig. 9) as well as by other possible currently uncharacterized cAMP-dependent perturbations in the lid/Ras exchange motif region. Our data show that the Rp-cAMPS ligand does not activate either the α6 hinge motion or the IL entropic weakening, explaining why it functions as an antagonist.

**Dynamics and Allostery in PKA Versus EPAC**—The NMR $^{15}$N relaxation rates of the PKA Rα2 CBD-A have been previously measured in the cAMP- and in the Rp-cAMPS-bound states but not in the apo form due to its poor solubility (23). The lack of this key reference state for the PKA CBD, therefore, limits our EPAC versus PKA comparative analysis of dynamic profiles to the cAMP and to the Rp phosphorothioate antagonist-bound forms. In PKA the main effect of the oxygen-to-sulfur isobolog substitution at the exocyclic equatorial phosphate position is an increase of ms-μs dynamics at the PBC and β2-β3 regions (23). Our data indicate that a similar enhancement of ms-μs flexibility occurs also in EPAC, as indicated by the NMRD dispersions measured for residues Ala-272 and -277 in the EPAC1 PBC and Phe-232 and Ile-243 in the EPAC1 β2-β3 site, which are consistently higher in the Rp-cAMPS-bound state relative to the cAMP-bound form (Fig. 6). Similarly, the $R_{1}$,$R_{2}$ product for Leu-273 is higher in the antagonist-bound state than when the EPAC1 CBD is bound to cAMP (Fig. 6c), pointing to an Rp-cAMPS-specific ms-μs dynamic enhancement at this critical PBC site. Furthermore, in EPAC1 the Rp-cAMPS antagonist causes an increase of the β2-β3
dynamics in the ps-ns time scale as well. This is supported by the low HN NOE value observed for the β2-β3 loop Asp-236 residue in the antagonist-bound state (Fig. 4d). The enhanced PBC and β2-β3 flexibility observed in EPAC and in PKA by the replacement of CAMP with the Rp-antagonist suggests that for both systems the dynamics at these sites are a key allosteric determinant, further confirming the pivotal role of the β2-β3 loop in the CAMP-dependent allosteric control of EPAC.

Another dynamic hot spot common to both the EPAC1 and the PKA CBDs (23) is that observed in the CAMP-bound state for the C terminus of the hinge helix after the β-barrel (Fig. 8b). In PKA the conformational heterogeneity in this region of CBD-A is likely pivotal in the control of the relative orientations of the two CBDs of the regulatory region (CBD-A and -B) (14), whereas in EPAC the dynamic nature of this site may contribute to the re-orientation of the RR relative to the CR as required for the activation of the GEF function (Fig. 9).

As to the dynamics in the helical bundle, which is N-terminal to the β-barrel, at present it is not possible to know whether the CAMP-dependent flexibility enhancements observed in EPAC1 are common to PKA as well due to the limited sequence homology between PKA and EPAC1 in this region. However, in both EPAC and PKA systems the α3 site is likely dynamic in the ms-μs time scale, and also in both EPAC and PKA the α3 flexibility decreases when CAMP is replaced by the Rp antagonist (Figs. 4 and 5) (23). Considering that α3 in the PKA CBD-A is the site of key contacts between the regulatory and catalytic subunits (12), these observations suggest that the CAMP-dependent control of the configuration entropy cost for inhibitory interactions, as proposed here for EPAC1, may represent a more general CBD allosteric mechanism common to both signaling units. This conclusion is also supported by a recent structure of the PKA R:CR complex (14), revealing that CAMP binding to the PKA CBD-B nucleates a network of stabilizing interactions in the C-terminal helices while concurrently causing the disruption of a critical salt bridge that stabilizes the N-terminal helical bundle of CBD-B (14). Overall such a mechanism of CAMP activation proposed for PKA (14) points to the existence of a set of mutually exclusive intra-CBD interactions in full agreement with the enhanced dynamics observed here upon CAMP binding for the N-terminal helices of EPAC1.

Conclusions—We have mapped by classical 15N relaxation and NMRD experiments the dynamic profiles of the EPAC1 CBD in its apo and cAMP- and Rp-cAMPS-bound states. Such a three-state comparative analysis has revealed that CAMP-dependent variations of dynamics in the ms-μs and ps-ns time scales are key carriers of allosteric free energy in this domain. Specifically, we observe positive cooperativity between the PBC and the β2-β3 region, whereby CAMP docking in the PBC results in an extended rigidity of the β2-β3 loop. This turn region emerges as a key allosteric hot spot, in full agreement with the recent finding about the co-evolution of conserved residues at the PBC and β2-β3 sites. In sharp contrast to the quenching of dynamics in the β2-β3 locus upon CAMP-binding, several residue clusters were found in the α-helical subdomain in which dynamics was allosterically enhanced by CAMP. These include not only the C-terminal region of the hinge helix that plays a critical role in defining the RR/CR relative orientation but also the ionic latch spanning region in helices α1–2 that mediates key GEF inhibitory interactions. Considering that CAMP binding alone does not cause any significant structural rearrangement for α1–2, the increase of conformational entropy promoted at this site by CAMP emerges as a key mechanism for a CAMP-dependent weakening of the inhibitory salt bridges mediated by the ionic latch. Based on these results we have proposed a mechanism of EPAC activation that incorporates the dynamic features allosterically modulated by CAMP. According to this model, the CAMP-dependent entropic control of the ionic latch represents an additional allosteric pathway that acts in concert with the previously proposed α6 hinge motion to shift the EPAC equilibrium toward the active (open) state, removing the steric hindrance exerted by the RR on the CR and exposing the catalytic core to the downstream Rap effector. Although a similar three-state comparative analysis of dynamic profiles is currently unavailable for PKA, the existing data suggest that the proposed model for the entropy-driven allosteric cAMP control of the N-terminal helical bundle may be at least in part generalized to the CBDs of PKA.

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REFERENCES
1. de Rooij, J., Zwartkruis, F. J. T., Verheijen, M. H. G., Cool, R. H., Nijman, S. M. B., Wittinghofer, A., and Bos, J. L. (1998) Nature 396, 474–477
2. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiehl, A. M. (1998) Science 282, 2275–2279
3. Kim, C., Vigil, D., Anand, G., and Taylor, S. S. (2006) Eur. J. Cell Biol. 85, 651–654
4. Bos, J. L. (2006) Trends Biochem. Sci. 31, 680–686
5. DiPilato, L. M., Cheng, X. D., and Zhang, J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 16513–16518
6. Li, J., O’Connor, K. L., Cheng, X. D., Mei, F. C., Uchida, T., Townsend, C. M., and Evers, B. M. (2007) Mol. Endocrinol. 21, 159–171
7. Mei, F. C., and Cheng, X. D. (2005) Mol. Biol. Symp. 1, 325–331
8. Mei, F. C., Qiao, J. B., Tsyzgankova, O. M., Meinkoth, J. L., Quilliam, L. A., and Cheng, X. D. (2002) J. Biol. Chem. 277, 11497–11504
9. Qiao, J. B., Mei, F. C., Popov, V. L., Vergara, L. A., and Cheng, X. D. (2002) J. Biol. Chem. 277, 26581–26586
10. Wang, Z., Dillon, T. J., Pokala, V., Mishra, S., Labudda, K., and Stork, P. J. (2006) Mol. Cell. Biol. 26, 2130–2145
11. Clayton, G. M., Silverman, W. R., Heginbotham, L., and Morais-Cabral, J. H. (2004) Cell 119, 615–627
12. Kim, C., Xuong, N. H., and Taylor, S. S. (2005) Science 307, 690–696
13. Su, Y., Dostmann, W. R., Herberg, F. W., Durick, K., Xuong, N. H., Teneyck, L., Taylor, S. S., and Varughese, K. I. (1995) Science 269, 807–813
14. Kim, C., Cheng, C. Y., Saldana, S. A., and Taylor, S. S. (2007) Cell 130, 1032–1043
15. Berman, H. M., Ten Eyck, L. F., Goodsell, D. S., Haste, N. M., Kornav, A., and Taylor, S. S. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 45–50
16. Rehmann, H., Das, J., Knipscheer, P., Wittinghofer, A., and Bos, J. L. (2006) Nature 439, 625–628
17. Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., de Rooij, J., Bos, J. L., and Wittinghofer, A. (2003) Nat. Struct. Biol. 10, 26–32
18. Rehmann, H., Wittinghofer, A., and Bos, J. L. (2007) Nat. Rev. Mol. Cell Biol. 8, 63–73
19. Harper, S. M., Winkel, H., Wechselberger, R. W., Bos, J. L.,Boelens, R., and
