Epac1 is a guanine nucleotide exchange factor (GEF) for the small GTPase Rap1 that is directly activated by cAMP. This protein consists of a regulatory region with a cAMP-binding domain and a catalytic region that mediates the GEF activity. Epac is inhibited by an intramolecular interaction between the cAMP-binding domain and the catalytic region in the absence of cAMP. cAMP binding is proposed to induce a conformational change, which allows a LID, an α-helix at the C-terminal end of the cAMP-binding site, to cover the cAMP-binding site (Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., de Rooij, J., Bos, J. L., and Wittinghofer, A. (2003) Nat. Struct. Biol. 10, 26–32). Here we show that mutations of conserved residues in the LID region affect cAMP binding only marginally but have a drastic effect on cAMP-induced GEF activity. Surprisingly, some of the mutants have an increased maximal GEF activity compared with wild type. Furthermore, mutation of the conserved VLVLE sequence at the C-terminal end of the LID into five alanine residues makes Epac constitutively active. From these results we conclude that the LID region plays a pivotal role in the communication between the regulatory and catalytic part of Epac.

A multitude of cellular stimuli, including hormones, growth factors, and neurotransmitters, induce activation of the Ga subunit of heterotrimeric G-proteins, which, in turn, activates adenylyl cyclase to synthesize cyclic AMP (cAMP) from ATP. cAMP acts as a second messenger that binds to and regulates the cAMP-responsive Guanine Nucleotide Exchange Factor Epac*.

Communication between the Regulatory and the Catalytic Region of the cAMP-responsive Guanine Nucleotide Exchange Factor Epac*

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The abbreviations used are: PKA, protein kinase A; GEF, guanine nucleotide exchange factor; Epac, exchange protein directly activated by cAMP; REM, Ras exchange motif; DEP, Dishevelled/ Egl-10/pleckstrin (domain); PKG, cGMP-regulated protein kinase; mamTDP, 2‘(3’)-O-(N-methylanthraniloyl) guanosine diphosphate; C subunit, catalytic subunit; R subunit, regulatory subunit; CAT, catalytic domain; RD, regulatory domain; AC₅₀, half-maximal activity.
times higher. The cAMP solution was in the same buffer as the purified protein. The data were analyzed using the manufacturer’s software. In Vitro Activation of Rap1—In vitro activation of Rap1 was performed as described previously (10). Briefly, 200 nM of RasBpo loaded with the fluorescent GDP analogue 2’3’-O-(N-methylanthraniloyl) guanosine diphosphate (mantGDP) were incubated in the presence of 20 μM GDP (Sigma) and either 100 nM (mutant) Epac or isolated domains as indicated. cAMP (Sigma) was added as indicated for the individual experiments. The nucleotide exchange was measured in real time as decay in fluorescence using a Spek spectrofluorometer (Spek Industries). The decay is caused by the release of protein-bound mantGDP, which shows higher fluorescence intensity in the hydrophobic environment of the protein than in the buffer solution. The obtained data were fitted to a single exponential decay, and the rate constants (kobs) calculated were plotted against the cAMP concentration. The concentration dependence of the rate constants was treated as a normal titration experiment. All data analysis, fitting, and plotting were done with the Graft 3.0 program (Erthiacus Software).

RESULTS

Regulation of GEF Activity via the C-terminal Region of the cAMP Domain—The isolated recombinant catalytic domain of Epac1 (Epac-CAT) is active as a GEF and accelerates release of GDP bound to Rap1. The activity is independent of cAMP but can be inhibited by the addition of recombinant regulatory domain (Epac-RD1–328) (14). The heterodimeric Epac-RD1–328 Epac-CAT complex can be reactivated by cAMP (Fig. 2A). To investigate whether an isolated cAMP-binding domain was sufficient for trans-inhibition, we tested Epac-RD149–328. As expected, this cAMP-binding domain was sufficient to inhibit Epac-CAT. Interestingly however, a cAMP-binding domain that is shorter by only eleven amino acids at the C terminus (Epac-RD149–317) did not inhibit Epac-CAT, not even at much higher concentrations (Figs. 1 and 2B).

To exclude the possibility that Epac-RD149–317 is not properly folded, we determined the affinity for cAMP, because an unfolded protein should not be able to bind cAMP. As determined by isothermal titration calorimetry, the cAMP affinities for Epac-RD149–328 (4 μM) and for Epac-RD149–317 (3 μM) are similar (Fig. 3). From these results we conclude that the additional eleven amino acids are required for the trans-inhibition of Epac-CAT and, thus, for the communication of the cAMP-binding domain to Epac-CAT.

The VLVLE Sequence Is Required for Auto-inhibition—Sequence comparison identifies a VLVLE motif within the additional eleven residues, which is conserved between different Epac proteins from man to nematode and fly (Fig. 1). To inves-
tigate the function of these residues in the regulation of Epac1, we mutated the VLVLE motif in Epac to AAAAA creating Epac(Ala)_5. Epac is inactive in the absence of cAMP but becomes active after the addition of cAMP (Fig. 4A). In contrast, Epac(Ala)_5 is active in the absence of cAMP in a concentration-dependent manner (Fig. 4, B and C). Importantly, cAMP does not stimulate this activity any further (Fig. 4B). From these results we conclude that the VLVLE sequence is required for the inhibition of Epac by the regulatory domain.

To understand in more detail the contribution of the individual residues of the VLVLE motif, mutations were generated as indicated (Table II). First, residues were mutated in a pair-wise alanine scan generating Epac(VAALE), Epac(VLAAE), and Epac(VLVAA). The mutant proteins were characterized by determination of the cAMP concentration necessary to obtain half-maximal activity (AC_{50}) as well as the maximal GEF activity measured as the maximal rate constant k_{max} for dissociation of mantGDP from Rap1-mantGDP, which was achieved under standard experimental conditions and saturating concentrations of cAMP. The results are shown in Fig. 5 and summarized in Table I. All of the mutations were inactive in the absence of cAMP and show cAMP-dependent GEF activity. Importantly, AC_{50} and k_{max} values of the mutants did not differ more than 2-fold compared with the wild type protein. Whereas the k_{max} of Epac(VLVAA) is comparable with wild type, it shows an AC_{50} of 25 μM as compared with 45 μM for wild type. Epac(VAALE) and Epac(VLAAE) are characterized by a reduced k_{max} but an almost unchanged AC_{50}. Apparently, the VLVLE motif tolerates a number of mutational assaults.

In a second series of experiments, more drastic mutations to charged or bulky residues were introduced into the VLVLE
Regulation of Epac by cAMP

The obtained individual reaction rates (as a decrease in fluorescence and fitted to a single exponential decay). The exchange activity was monitored at different concentrations of cAMP. The exchange activity was monitored as a decrease in fluorescence and fitted to a single exponential decay. The obtained individual reaction rates (k_{obs}) were plotted against the cAMP concentration. *wt*, wild type.

### Biochemical properties of mutations in the VLVLE region

The AC_{50} values and the relative k_{max} as determined from Fig. 5 are summarized.

| Mutant | AC_{50} (μM) | Relative k_{max} |
|--------|--------------|-----------------|
| Wild type | 45 | 1 |
| VAALE | 45 | 0.6 |
| VLVAE | 50 | 0.4 |
| VWVLE | 24 | 1 |
| VLVLE | 65 | 0.6 |
| VLRLE | 50 | 0.4 |
| VLVRLE | 17 | 2.3 |

Additional Conserved Amino Acids in the LID Region—From the comparison between the crystal structure of the cAMP-free regulatory domain of Epac2 and the cAMP-bound regulatory domain of PKA (19, 20), a model of the molecular mechanism of cAMP-induced activation was developed (21). It predicts that the cAMP binding induces a conformational change in the phospho-phosphate binding cassette (PBC) and the HINGE of the cAMP-binding domain. This, in turn, induces the C-terminal helix of the cAMP-binding domain, the LID, to move toward the core structure and shield cAMP from the solvent. The VLVLE motif is located at the end of the LID (Figs. 1 and 8). A number of additional conserved residues in the LID (Fig. 1) were analyzed for their possible involvement in binding the adenine base of cAMP and/or mediating the interaction between the regulatory region and the catalytic region. Four mutants were made in Epac-RD148–328 and analyzed by measuring the affinity of cAMP to the isolated cAMP-binding domain by isothermal titration calorimetry. Whereas H317A showed wild type affinity, E308A, T311A, and R313A showed a 2-fold reduction in affinity for cAMP, arguing that these residues make a small contribution to binding of cAMP. In addition, the same mutations were made in Epac to determine cAMP-dependent catalytic activity using the fluorescent GEF assay (Fig. 6 and Table II). The AC_{50} values for the isolated cAMP binding domains (Epac-RD148–328) were determined by isothermal titration calorimetry. The corresponding AC_{50} values and relative k_{max} for cAMP-mediated Epac activation are determined from Fig 6.

| Mutant | AC_{50} (μM) | Relative k_{max} |
|--------|--------------|-----------------|
| Wild type | 3 | 45 | 1 |
| E308A | 6.3 | 15 | 3 |
| T311A | 5.5 | 15 | 2.3 |
| R313A | 6.2 | 40 | 1 |
| H317A | 3.6 | 50 | 0.2 |

**DISCUSSION**

A previous comparison of the structures of the cAMP-free (open) cAMP-binding domain of Epac and the cAMP-bound (closed) cAMP-binding domain of PKA revealed that the main difference between the open and the closed structure is the orientation of the LID. In the closed conformation of PKA, this LID covers the cAMP-binding site and interacts with the adenine group of cAMP. This suggests that this LID region may be the main determinant of the interaction between the cAMP-binding domain and the catalytic region of Epac (21). This would imply that, in the open conformation, the LID is able to interact with the catalytic region, resulting in an inhibition of the activity (see Fig. 8). Here we show that the LID region of Epac1 indeed plays a pivotal role in the communication between the regulatory and catalytic domain of Epac. Most nota-
bly, deletion of the C-terminal region of the LID, containing a conserved VLVLE sequence, completely abolishes the ability of the regulatory domain to inhibit the exchange activity of Epac. This was shown in a trans-inhibition experiment in which an isolated cAMP-binding domain is capable of inhibiting an isolated catalytic region only when the region containing the VLVLE sequence is present. In addition, the Epac(Ala)5 mutant, wherein the VLVLE sequence is replaced by alanine residues, is constitutively active, and cAMP cannot activate this mutant protein further. Thus, the VLVLE sequence is essential for inhibition of the catalytic region by the open cAMP-binding domain even when the two domains are separated. This strongly suggests that this region directly interacts with the catalytic region. Further analysis of the VLVLE sequence revealed that mutating individual residues into alanine had modifying effects on cAMP-induced activation. This is reflected by either a change in the AC50 for cAMP and/or a change in the max activity. Apparently, the sequence requirement for the VLVLE sequence to inhibit the catalytic domain is not very strict. Nevertheless the sequence is highly conserved. This may indicate that the small changes we observe can nevertheless not be tolerated in vivo or imply that the VLVLE sequence, in addition to inhibiting the regulatory domain, serves an additional function. Indeed, single amino acid changes did affect cAMP-induced regulation. This is reflected by either a change in the AC50 for cAMP and/or a change in k max. Most surprisingly, mutating glutamate into the positively charged arginine resulted in a 2–3-fold higher k max of Epac. From these results, we conclude that the VLVLE sequence also serves as an element that is responsible for translating cAMP binding in a correct activation response. Most likely, the VLVLE sequence is conserved to serve both in the inhibition of the catalytic region and the establishment of the correct conformational response to cAMP.

We have also identified conserved residues in the N-terminal part of the LID region that are involved in the regulation mechanism. From their positioning compared with PKA, we assumed that these residues might be involved in the interaction with the base of cAMP. Indeed, the affinity of cAMP for isolated CAMP domains of the E308A, T311A, and R313A mutants is reduced ~2-fold. This difference is relatively minor and suggests that the core structure and the phosphate-binding cassette mainly provide the affinity of cAMP for Epac. As for the single mutations in the VLVLE sequence, mutants with mutations in the N-terminal LID region also affect the AC50 of cAMP to activate Epac and the maximal activation at saturating cAMP levels. The H317A mutant is still responsive to cAMP with a wild type AC50 value but a k max of only one-fifth of wild type. This indicates that H317 plays a key role in releasing Epac from auto-inhibition, perhaps by sensing the correct base
structure of cAMP. A similar interaction is found between cAMP and tryptophane or a tyrosine residue in the LID of PKA (19, 20). In contrast, E308A and T311A show a reduced AC50 value and a higher $k_{\text{max}}$. Apparently, these mutants make Epac more active.

The observation that certain LID mutants have a higher or lower $k_{\text{max}}$ than wild type is very intriguing and reminiscent of our findings that certain cAMP analogues also show either reduced (22) or increased maximal activity. This indicates that the LID region in the presence of cAMP still influences the catalytic region and precludes a simple model as for PKA, wherein the activation of the catalytic region is caused by the dissociation of the regulatory region. For Epac, the interaction between the regulatory region and the catalytic region apparently remains after the binding of cAMP and imposes a restraint on the activity of Epac. One possible reason for this continuing restraint in the presence of cAMP is that Epac has an additional level of regulation that can modulate the effect of cAMP. This additional level of regulation may impinge on Epac through the binding of regulators to, for instance, the DEP domain, affecting the orientation of the LID region.

This explanation for the various levels of maximal activity, which is consistent with the mutational data and thermodynamic considerations, can be put forward by considering the four-state model of Epac activation (Fig. 9) proposed earlier (22). Regulation of Epac is thus described by a system of coupled equilibria between a bound and an unbound state and between an inactive and an active state. Whereas ligand-free Epac exists mostly (but not exclusively) in the inactive conformation, the binding of cAMP to the cAMP-binding domain shifts the equilibrium more (but not totally) to the active conformation. One should note that the AC50 values measured here reflect the overall equilibrium of cAMP binding and activation, whereas $k_{\text{max}}$ reflects the equilibrium between the cAMP-bound inactive and active conformation. The VLVLE-(Ala)5 mutation would thus favor the active conformation even in the absence of ligand, described by $K_{d3}$. Other mutations would influence the conformational equilibrium $K_{d2}$ between cAMP-bound inactive and active conformation. The fact that Epac(VLVLR), Epac(E308A), and Epac(T311A) have a higher maximal activity than wild type protein would indicate, keeping with the same model, that cAMP does not induce maximal activity even in the wild type protein and that Epac can therefore exist in a cAMP-bound but inactive conformation. The fact that maximal activity can be increased 2–3-fold indicates that the equilibrium described by $K_{d2}$ is close to or even a bit lower than unity and that a low energy barrier for the conformational change (described by $K_{d2}$) might be advantageous for cAMP signaling or will be influenced by other cellular components. Preliminary NMR shows that, in the absence and presence of cAMP, there is indeed a fast dynamic inter-conversion between two conformations, which we assume corresponds to the active and inactive conformations.

At this moment, the residues in the catalytic region in contact with the LID are elusive. It could be that the LID, in particular the VLVLE sequence, interacts with the CDC25 homology domain, thereby preventing binding of Rap (Fig. 8).

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2 H. Rehmann, unpublished observations.

3 A. Shimada, unpublished observations.
Alternatively, the LID region may induce a conformational change in the catalytic region, for instance through an interaction with the REM domain. Indeed, recently structural and biochemical evidences were presented that the REM domain could accelerate the intrinsic activity of the CDC25 homology domain of the Ras GEF Sos (24).

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