Characterization of a novel signal transducer element intrinsic to class IIIa/b adenylate cyclases and guanylate cyclases

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Adenylate cyclases (ACs) are signaling proteins that produce the second messenger cAMP. Class III ACs comprise four groups (class IIIa–d) of which class IIIa and IIIb ACs have been identified in bacteria and eukaryotes. Many class IIIa ACs are anchored to membranes via hexahelical domains. In eukaryotic ACs, membrane anchors are well conserved, suggesting that this region possesses important functional characteristics that are as yet unknown. To address this question, we replaced the hexahelical membrane anchor of the mycobacterial AC Rv1625c with the hexahelical quorum-sensing receptor from Legionella, LqsS. Using this chimera, we identified a novel 19-amino-acid cyclase transducer element (CTE) located N-terminally to the catalytic domain that links receptor stimulation to effector activation. Coupling of the receptor to the AC was possible at several positions distal to the membrane exit, resulting in stimulatory or inhibitory responses to the ligand Legionella autoinducer-1. In contrast, on the AC effector side functional coupling was only successful when starting with the CTE. Bioinformatics approaches established that distinct CTEs are widely present in class IIIa and IIIb ACs and in vertebrate guanylate cyclases. The data suggest that membrane-delimited receiver domains transduce regulatory signals to the downstream catalytic domains in an engineered AC model system. This may suggest a previously unknown mechanism for cellular cAMP regulation.

Introduction

Class III adenylate cyclases (ACs) are signaling proteins that produce the second messenger cAMP [1]. They are subcategorized into four groups, denoted class IIIa–d. Classes IIIa and IIIb are found in bacteria and eukaryotes, whereas classes IIIc and IIId are present in bacteria only [2]. Vertebrate genomes encode 10 AC isoforms, nine of which belong to class IIIa and are characterized by two different hexahelical transmembrane domains (6TM) and two complementary catalytic domains [3]. The pseudo-heterodimeric vertebrate ACs have monomeric bacterial homologs that require homodimerization for activity, such as the mycobacterial class IIIa AC Rv1625c, a prototype of the mammalian congeners [4]. The membrane anchors of such ACs comprise approximately 40% of the total protein. One feature of note is that they lack significant extramembranous loops connecting individual transmembrane spans [5]. Recently,
membrane receptors with known ligands have been described that possess 6TM bundles with minimal-length x-helices and short connecting loops [6–8]. They are essentially identical in layout to the membrane anchors of 6TM ACs [3,4,9,10]. This receptor type is represented by bacterial quorum-sensing (QS) receptors present in *Vibrio*, *Legionella*, *Burkholderia* and other eubacteria [6,8,11,12]. The ligands for the QS receptors from *Vibrio*, CqsS, and *Legionella*, LqsS, have been identified as aliphatic hydroxy-keto lipids [8,11,12]. In the absence of sizeable extramembranous loops it is most likely that this type of ligand binds into the membrane space of the receptor protein.

Recently, we replaced the hexahelical membrane anchor of the Rv1625c AC by the QS receptor from *Vibrio* and demonstrated that the receptor conferred direct regulation by the extracellular ligand cholera autoinducer-1 (CAI-1) [5]. Short of the identification of a ligand for a membrane anchor of class IIIa ACs, this is a proof of principle for a receptor function of the 6TM AC anchors [5]. This assertion requires that the mechanism of intramolecular signal transduction from receptor to cytosolic effector in class IIIa ACs shares similarities with that in LqsS and CqsS histidine kinases [5,13].

So far, in membrane-delimited class IIIa ACs, cytosolic sequence elements that are potentially involved in signal transduction are unknown. Such elements should be located within the 60–80 residues that bridge the distance between the membrane exit and the start of the catalytic domain. Generating a chimera between the QS receptor from *Legionella pneumophila* and the Rv1625c AC, we asked: What are the sequence requirements for a functional coupling? Here we identify and characterize a conserved segment of 19 amino acids, the cyclase transducer element (CTE), which is indispensable for signal transduction and which had escaped detection in the past. Bioinformatic analyses demonstrate that CTEs are present in class IIIa and IIIb ACs, and also in ligand-regulated guanylate cyclases (GCs) from vertebrates.

**Results**

**Connecting the QS receptor LqsS to Arg218 of the Rv1625c AC**

Recently, we have demonstrated that CqsS, the QS receptor from *Vibrio*, can replace the 6TM membrane anchor of the Rv1625c AC [5]. AC activity in such a CqsS–Rv1625c chimera is directly stimulated 190% by 1 µM of the ligand CAI-1 ((S)-3-hydroxy-tridecan-4-one; [5]). Here, we replaced the 6TM bundle of Rv1625c AC with the QS receptor from *Legionella*, LqsS, and initially used the equivalent sequence positions in LqsS (Met191) and in Rv1625c (Arg218) for linking (Fig. 1). AC activity was stimulated in the chimera by the *Legionella* autoinducer-1 (LAI-1; (S)-3-hydroxy-pentadecan-4-one). However, its maximal stimulation did not exceed 120%. Half-maximal activation (EC$_{50}$) was graphically estimated to be 48 nM LAI-1 (Fig. 1). The rather weak, albeit statistically significant stimulation might have indicated that Rv1625c partners with LqsS less efficiently than with the CqsS receptor from *Vibrio*, or that the point of connection between LqsS and Rv1625c was suboptimal for intramolecular signal transduction.

Therefore, LqsS was joined with Rv1625c at 11 different positions distal to the predicted cytosolic exit of transmembrane helix 6 at Tyr178 while the point of connection to the cyclase, Arg218, remained unchanged. The results were unexpected (Fig. 2A). Basal activities ranged from 15 to 64 nmol cAMP·mg$^{-1}$·min$^{-1}$, comparable to the activity of the unbridged membrane-

![Image](image-url)

**Fig. 1.** Stimulation of the chimera LqsS–Rv1625c by the QS ligand LAI-1. The scheme for the chimeric construct is shown at the top, sequence information of the critical junction point at the bottom, with respective numbering for LqsS and Rv1625c. The basal activity (100%) was 26.4 nmol cAMP·mg$^{-1}$·min$^{-1}$. The EC$_{50}$ concentration was estimated to be 48 nM. *P < 0.001*. For clarity further significances are omitted. The catalytic domain of Rv1625cR218V443 (14.3 nmol cAMP·mg$^{-1}$·min$^{-1}$) was not affected by 10 µM LAI-1 (open squares). The inset shows the western blot of the protein. Results are presented as means ± SEM (n ≥ 5). At the right is the structure of the ligand.
anchored Rv1625c AC [4]. Levels of protein expression were equivalent for these constructs, as analyzed by western blotting (data not shown). The chimera linked at Tyr178, i.e. directly at the presumed C terminus of TM6, was only modestly active (4.9 nmol cAMP mg⁻¹ min⁻¹). However, with almost 70% inhibition, it was significantly affected by LAI-1 (estimated IC₅₀ = 65 nM; Fig. 2A). Inhibition of chimeras was also observed in three other constructs conjoined four, six and eight amino acids distal to the membrane exit of LqsS, respectively (at Met182, Gln184 and Gln186; Fig. 2A). Notably, in the latter chimeras, basal AC activities were considerably higher. This may indicate that formation of a productive AC dimer was facilitated with greater distance from the predicted membrane exit. Significant stimulation was observed at connection positions more distal to the membrane exit, such as Gln185, Lys187, Met191 and Ala192 (Fig. 2A). Notably, in the latter chimeras, basal AC activities were considerably higher. This may indicate that formation of a productive AC dimer was facilitated with greater distance from the predicted membrane exit. Significant stimulation was observed at connection positions more distal to the membrane exit, such as Gln185, Lys187, Met191 and Ala192 (Fig. 2A). Maximal stimulation of 190% was achieved in the LqsSₜ₉₅₋₉₈₇–Rv1625c₉₂₈₋₉₄₄ chimera (Fig. 2B). The estimated EC₅₀ was 48 nm LAI-1.

Taken together, mostly inhibitory responses were observed when the point of connection was within eight residues of the membrane exit, whereas stimulatory responses prevailed with longer linker sequences (Fig. 2). The estimated ligand concentrations required for half-maximal responses were in the same order of magnitude for all constructs (response curves not shown). This indicated that the modifications did not significantly affect receptor–ligand interactions. Lys187 in LqsS was used as the point of connection in subsequent constructs.

Connecting the AC Rv1625c to Lys187 of the QS receptor LqsS

Next, we analyzed the connecting point on the side of the Rv1625c AC. Inspection of the linker sequences between membrane domains and catalytic domains of type IIIa ACs indicated a conserved segment characterized by an invariant central proline. Its likely C terminus was evident from a distinct drop of sequence conservation. The N terminus could not be identified as clearly (Fig. 3). Earlier experiments had successfully used Arg218 of Rv1625c as the fusion point for chimeras between the AC and CqsS [5]. Similarly, the
corresponding position in the class IIIa AC CyaG from *Arthrospira platensis* (Arg456) was successfully fused to the 2TM chemotaxis receptor Tsr from *Escherichia coli*, resulting in a serine-sensitive AC chimera [14]. We tested LqsS-Lys187–Rv1625c constructs connected at up to four additional amino acids prior to Arg218 (residues 214–217 in Fig. 4). The resulting chimeras had high basal activities, but were unresponsive to LAI-1 (Fig. 4). Connection via Arg218 resulted in a 75% drop of basal activity, but highly significant stimulation by LAI-1 (Figs 2 and 4). The importance of Arg218 in Rv1625c seemed contrary to the high sequence variability at this position in homologs (Fig. 3). To clarify its functional role, we generated three point mutants, replacing the positively charged Arg218 with a hydrophobic (R218L), a polar (R218Q), or an acidic (R218D) residue. In all instances, the stimulation of the chimeric protein and ligand potency were comparable to the Arg218 parent construct (190% stimulation), i.e. 210% for R218L, 241% for R218Q and 206% stimulation for R218D. This indicated that stimulation was dependent on the length of this segment rather than on the physical properties of this particular amino acid. Stepwise deletion of Arg218 and six downstream residues (Ser219 to Ala224) resulted in a progressive loss of AC activity and regulation (Fig. 4). The outright deletion of 19 residues downstream of Arg218 in Rv1625c, up to Lys236, resulted in low basal AC activity (Lys236 in Fig. 4; 170 pmol cAMP·mg⁻¹·min⁻¹) and a loss of regulation. This indicated that residues Arg218 to Lys236 have an essential function in the regulation of Rv1625c AC activity. We termed this region the CTE for its putative role in intramolecular signal transduction.

**Bioinformatic characterization of a universal cyclase transducer element**

Asking whether the CTE is in fact a novel signaling element, rather than a constitutive component of the cyclase fold, we comprehensively analyzed its presence in sequences of nucleotide cyclases by bioinformatics. We found it N-terminal to catalytic domains of class IIIa and IIIb ACs, including the membrane-bound and soluble isoforms of vertebrates (Fig. 3). CTEs were absent in class IIIc and IIId ACs. CTEs were also present in the ligand-regulated eukaryotic GCs, which are closely related to class IIIa ACs, but not in diguanylate cyclases, which form an outgroup to class III ACs. Crystal structures of CTEs exist from a GC from rat, where it was solved together with the upstream signaling helix [S-helix; Protein Data Bank (pdb) : 3hls], and of the class IIIb soluble AC from human (hAC10), where both CTEs of the pseudo-heterodimer were solved together with the catalytic domains (pdb : 4clf; [15,16]). We conclude that the CTE is a distinct element, which likely evolved in the common ancestor of modern class IIIa and IIIb cyclases.

We noted that the CTE sequences are to some extent divergent (Fig. 3). The invariant proline, which had originally caught our attention, is conserved only in CTEs of class IIIa ACs. In the class IIIb AC10 from vertebrates, this proline is conserved in CTEs of the first catalytic (C1) domain, whereas it is absent in CTEs of the C2 domain. It is also absent in CTEs of the homodimeric bacterial class IIIb ACs. Secondary structure predictions, however, are highly similar for all CTEs, irrespective of the presence of this proline residue. This is in agreement with the aforementioned crystal structures, which superimpose to less than 1.5 Å root mean square deviation, even though the C-terminal CTE of the hAC10 is divergent and lacks the central proline. In pseudo-heterodimeric ACs from vertebrates, CTEs of C1 catalytic domains contain an invariant serine three residues upstream of the central proline, whereas this position is occupied by an invariant asparagine in C2 CTEs (Fig. 3). CTE sequences from bacterial ACs and eukaryotic GCs are intermediate, having either serine or asparagine, and probably reflect the ancestral state.

Prompted by this speciation we conducted a cluster analysis of CTEs from class IIIa ACs. The sequences separated, as expected, into three groups along their association with bacterial ACs, vertebrate C1 or vertebrate C2 domain (Fig. 5). The vertebrate groups were further subdivided according to their origin from the nine membrane-delimited isoforms, due to minor, yet conserved differences between their sequences, closely mirroring a cluster map of the catalytic domains (data not shown). This implies that CTEs must have coevolved together with their respective catalytic domains and membrane anchors because the isoform designations were originally based on sequence variations in the transmembrane domains [1,2].

**Exchange of transducer elements between adenylate cyclase isoforms**

The close coevolution of CTEs with their cognate catalytic domains prompted us to ask whether, in the homodimeric LqsS–Rv1625c chimera, the CTE might be functionally replaceable by either of the two CTEs of the mammalian AC5 isoform (Fig. 6). In all cases, introducing an AC5 CTE into the LqsS–Rv1625c chimera resulted in a dramatic drop in basal AC activity...
This emphasized the close relationship between catalytic domains and their cognate CTEs. Nevertheless, we noted that the CTE from AC5-C1 passed the LAI-1 signal to the catalytic domains, both as a homodimer and as an engineered heterodimer in conjunction with the Rv1625c CTE, resulting in significant stimulation. All constructs in which the CTE from AC5-C2 was involved were barely or not at all responsive to LAI-1 (Fig. 6A). We propose that CTEs are critically involved in controlling the formation of an active AC complex between the catalytically inactive monomers. To explore whether this depended

Fig. 3. Sequence diversity of the CTE. The sequence logos are grouped according to classification, phylogeny and origin of the catalytic subunit. Bacterial ACs are labeled with ‘Bact’, vertebrate ACs with ‘Vert’. For vertebrate pseudoheterodimers, C1 and C2 denote the catalytic domains to which the CTE is coupled. Vertebrate class IIIb ACs represent exclusively the soluble AC10 isoform. Note that all subgroups show specific sequence features, most prominently the presence or absence of a central invariant proline. Asterisks denote groups with a solved crystal structure. The following numbers of sequences were used in preparation of the logos: IIIa bacterial: 314; IIIa vertebrate C1: 469; IIIa vertebrate C2: 482; IIIa GCs: 624; IIIb vertebrate C1: 38; IIIb vertebrate C2: 51; IIIb bacterial: 1008. The data set is available at Figshare.
on the presence of the membrane domains, we employed a soluble Rv1625c construct, linked by an established tetradecapeptide used previously for this purpose [4,17]. In these soluble constructs, the catalytic domains are mutated such that hetero-association is required for activity [4]. The affinity-purified Rv1625c AC204–443 heterodimer had high AC activity (380 nmol cAMP mg⁻¹ min⁻¹; Fig. 6B). Replacement of the Rv1625c CTE by the human AC5 CTEs reduced AC activity by 80–95% compared with the parent construct (Fig. 6B). This implies that even without membrane attachment, formation of an active catalyst apparently required a delicate CTE interaction. In the context of the Rv1625c catalytic domains, the conditions for a seamless interaction of the two AC5 CTEs are missing, and the formation of the Rv1625c catalytic homodimer was impaired. This finding may be relevant concerning the enormous difficulties in the past to obtain soluble active AC dimers from a single AC isoform. Mostly, dimers composed of C1 and C2 domains from different vertebrate AC isoforms were used for enzymatic assays and for crystallization [17–22]. As a role for the CTEs was not appreciated at the time they were generally not included in these constructs.

In this context it is further notable that functional coupling of LqsS to the Rv1625c AC via the Rv1625c CTE was partly possible although the His-kinase from which the LqsS receptor domain was derived has no CTEs. To us, this suggested that CTEs operate as cyclase-specific signal transducers, which convert incoming signals into conformational motions that regulate the dimerization of the catalytic domains (see below).

### Interaction of the cyclase transducer element with the S-helix

With the CTE as an independent transducer element it should be possible to connect it functionally to other independent signal transducing domains. The S-helix is a conserved coiled-coil domain of up to 50 residues, found between receiver and output domains in diverse sensory proteins, including histidine kinases and nucleotide cyclases [23,24]. In the retinal GC an S-helix is located directly in front of the CTE [15]. In a construct connecting the receiver domain of the E. coli serine receptor Tsr via a HAMP domain, a CTE and an S-helix to the catalytic domain of the AC CyaG from A. platensis, robust regulation by serine was observed, i.e. all participants interacted productively [14,25]. Removal of the S-helix inverted the sign of the output signal [14].

To investigate whether the CTE of Rv1625c would functionally interact with a heterologous S-helix, we incorporated the 25-amino-acid-long S-helix of CyaG into the LqsS–Rv1625c chimera (Fig. 7). These ‘triple’ chimeras, consisting of the LqsS receptor, the S-helix from CyaG and the CTE and cyclase domain of Rv1625c, were well expressed in E. coli (see western blots in Fig. 7). In the protein with the S-helix in front of the CTE, the sign of the receptor stimulation was inverted compared with the parent construct, i.e. LAI-1 inhibited AC activity by almost 50% in a concentration-dependent manner (Fig. 7). The estimated ligand concentration required for a half-maximal inhibition (48 nM) was similar to the EC50 for half-maximal stimulation of the parent construct (21 nM; Fig. 7). This means that the ligand–receptor interactions remained unimpaired by insertion of an additional transducer element.

As a control, the length of the S-helix was mimicked by a respective extension of LqsS by 25 residues (LAG-MAAAAGMIAHELRSPLLGIKSG) in front of the CTE. This stretch is predicted as helical but shares no sequence similarity with the S-helix. This led to an unregulated chimera, which had a basal AC activity of 0.58 nmol cAMP mg⁻¹ min⁻¹ (Fig. 7). As a second control, the S-helix was inserted posterior to the CTE. AC activity was considerably diminished in the chimeric protein, although expression of the chimera was equivalent to the other constructs (see western blots in Fig. 7). On the basis of the attenuated basal activity LAI-1...
stimulated AC activity with an identical potency as the parent construct (Fig. 7). Taken together, the data demonstrated that the effect of ligands on the output domain was dependent on the order of the transducer elements. Similarly, the data support the notion that the CTE represents a signal transducer element capable of functional interaction with other transducer elements. The interaction appears to be independent of the nature of the membrane domain [14,25].

**A structural model for CTE signal transduction**

Next, we wondered how CTEs might mechanistically transduce the signal. Our considerations were guided by the conspicuous parallels between the CTE and the analogous transducer element of histidine kinases, the dimerization and histidine phosphotransfer domain (DHp). Both are frequently connected at their N-termini to signal transduction domains, such as HAMP, GAF, PAS, or S-helices, from which they receive their input [23,26,27]. Both elements invariably connect directly at their C-termini to the catalytic output domains, whose level of activity they set. In DHp, setting of the output, i.e. phosphatase versus kinase activity, has been shown to involve transitions between symmetric and asymmetric conformations [26,28]. We envisage similarly concerted transitions between nearly isoenergetic states for the CTE. Given the diversity of domains capable of conveying signals to both DHp and CTE, it appears that these efficiently convert different conformational inputs into defined structural changes that determine the activity of the output domains. Polar linkers appear to be a key feature in this process connecting the upstream signaling domains to the transducer elements [26,28]. In DHp, mutations in this linker region are known to severely impair signaling [14]. Accordingly, we note the widespread presence of such linkers N-terminal to the CTEs of type IIIa and IIIb ACs and GCs. The functional analogies in signaling between CTEs and DHp provide a rationale for our ability to generate chimeras between class IIIa ACs and His kinases.

Based on this analogy, we have considered possible conformational changes of CTEs during signal transduction. The available structures of CTEs show two helices, separated by a central bend of approximately 45° [15,16]. In the pseudo-heterodimeric CTE of human AC10 the N-terminal helices form a short
coiled coil, from which the C-terminal helices extend into opposite directions. Thus, the CTE is directly compatible with upstream domains that end in a coiled-coil domain, notably HAMP and the S-helix. The hydrophobic register of the N-terminal helices extends with only minor disturbances into the C-terminal helices, suggesting that the CTE could switch between bent and straight conformations (Fig. 8). In the future this might be amenable to experimental testing by introduction of appropriate mutations.

An analogous structural transition was reported for the pH-sensing Rv1264 AC from Mycobacterium tuberculosis [29], which, being a class IIIc AC, lacks a CTE. In the active state, the segment N-terminal to the catalytic domain constitutes a partly unwound α-helix and the catalytic domains are free to self-

![Fig. 6. Replacement of the Rv1625c CTE with those from human AC5-C1 and/or -C2.](image)

(A) Homodimers: note the drop of activity upon insertion of hAC5 CTEs in LqsS–Rv1625c AC chimeras. Heterodimers: by introduction of inactivating, yet complementing, mutations into Rv1625c (AC-C1 and AC-C2; [4]) exclusively the desired heterodimers can form a catalytic center. White box, LqsS receptor domain M1-K187; black icons, domains from Rv1625c AC; blue icons, CTE from AC5-C1; red icons, CTE from AC5-C2. The respective CTE sequences are shown at the lower right. Results are means ± SEM (n ≥ 3; *P < 0.05; **P < 0.01; †P < 0.001). (B) Soluble heterodimers of AC Rv1625c with CTE replacements from human AC5-C1 and AC5-C2 as controls. Each replacement by a CTE from AC5 resulted in a statistically significant (P < 0.001) drop of activity of 80–95%. Results are means ± SEM (n ≥ 6). Sequence information is available in Table S1.
assemble into an active dimer. In the inactive state the N-terminal segment forms a rigid helix that holds the inactive monomers apart [29]. Similarly, the CTE might affect the conformational flexibility needed by the catalytic domains to assemble the active dimer, suggesting that the straight conformation of CTE correlates with the inactive state. The pairwise adaptation required for such conformational switching provides an attractive explanation for the coevolution of membrane domains, CTEs and catalytic domains (Figs 3 and 5). In this context, the differences between the CTEs of pseudo-heterodimers are particularly striking, because they suggest that, in vertebrates, CTEs may have diverged to enable the decoding of more complex, possibly asymmetric signals.

**Discussion**

The hexahelical membrane anchors of class IIIa ACs, in particular of the nine mammalian isoforms, have remained a sort of biochemical mystery for the past 27 years. At the time of the initial sequencing of a mammalian AC, a transporter or channel function was proposed [9]. At present, we know that the membrane anchors in eukaryotic ACs are conserved in an isoform-specific manner from coelacanth to man, separated by approximately 400 million years of evolution [5]. However, a defined role beyond that of a passive membrane anchor has remained elusive. Most recently, we have provided evidence for a role as a ligand receptor by biochemical characterization of a chimera between the LqsS receptor from *Vibrio harveyi*, CqsS, and the Rv1625c AC. A bioinformatic analysis of shared properties of the membrane domains supported the proposed receptor function [5]. For a receptor to transmit an extracellular signal to a cytosolic effector domain, a suitable molecular signal transducer or converter for a shared AC effector domain is likely to be required. Such a molecular transducer entity present in class IIIa and IIIb ACs has been identified and characterized in this study.

Examination of the connecting points between the LqsS receptor and the AC effector domain identified a conserved segment of about 19 residues present in class IIIa and IIIb ACs. It is termed CTE. Probably, a centrally located proline is important for structure and function. The functional interaction between CTE and the S-helix bolsters the claim that CTEs are signaling devices involved in regulation of class IIIa and b ACs. This function probably is not restricted to membrane-delimited ACs but may well operate in cytosolic ACs composed of receiver and effector domains [2,13]. The sequence differences between CTEs probably are functionally significant (Fig. 6A). This indicates that the receptor, the CTE and the effector output domain communicate in a concerted manner reminiscent of bacterial signaling through HAMP domains [30,31]. Most likely, the receptor–CTE–effector trio is tuned to each other such that the small energetic differences caused by ligand binding to the sensory domain are scaled to trigger the conformational changes required in the respective enzymatic output domain. Such mechanisms provide a high degree of signaling specificity.

On the other hand, one may ask whether the CTEs could not play a rather passive role in the allosteric connection with a sensor domain and that the catalytic...
domains and CTEs are ‘finely tuned’ to each other for the purpose of catalysis only, but not allosteric signal transmission between the ligand-binding receptor and the catalytic dimer. We consider this a rather remote possibility because sequence and structural information indicates essentially identical catalytic mechanisms in all class III ACs \[18,29,32,33\]. In addition, it would be difficult to rationalize the reasons for the high degree of isoform-specific evolutionary conservation, which is apparent in CTEs from vertebrate ACs (Fig. 5).

Remarkably, in soluble Rv1625c proteins without CTE specific activities drop by up to 90% \[4\] whereas in soluble mammalian AC-5C1:AC-2C2 heterodimeric constructs, in which the CTEs are absent, basal AC activity is low, but synergistically stimulated by forskolin/Gs \[~10 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}; \[34\]\]. However, the failure to functionally combine soluble C1 and C2 catalytic domains from a single vertebrate isoform may actually suggest a conformational inability to properly interact in the absence of the respective CTEs. Finally, the functional interaction between the CTE and the established S-helix (Fig. 7) strongly implies an intrinsic signal transducing capacity of CTEs.

In summary, the data provide a stringent argument that membrane-delimited ACs will turn out to be primary signal receivers. For the nine membrane-delimited isoforms of vertebrates, this implies that next to the well-characterized indirect regulation by G-proteins, yet unknown direct regulatory signals impinge on the AC membrane domains. This will establish an entirely new regulatory pathway of cAMP biosynthesis. This argument is further supported by recent reports of two mutations in the human AC type 5 that cause familial dyskinesia and facial myokymia. The point mutations are located between the hexahelical membrane anchors and the respective CTEs \[35–37\]. These single point mutations do not affect AC activity in HEK293 cells transfected with wild-type or respective AC5 mutants, indicating that expression or protein folding is unimpaired. Along the model proposed here, it appears plausible that an as yet uncharacterized mode of AC regulation is dysfunctional in these mutants. A similarly located mutation is also known in the human retinal GC, causing autosomal dominant cone–rod dystrophy \[38,39\].

Fig. 8. Top: CTEs in available crystal structures. (A) Structure of a CTE from a rat guanylate cyclase together with the upstream signaling helix (S-helix) (pdb: 3hls). (B) Structure of the class IIIb soluble AC from human (AC10), where both CTEs of the pseudo-heterodimer were solved together with the catalytic domains (pdb: 4cflf) \[16\]. (C) Superposition of the CTEs in (A) and (B). The CTEs are structurally highly similar and superimpose at a root mean square deviation of less than 1.5 Å. Bottom: a structural model for signal transduction through CTEs. It is proposed that the CTE operates as a hinge that can adopt a bent or a straight conformation. (D) A computational model of the S-helix-CTE-cyclase constructs based on the structures in (A) and (B). The CTEs (highlighted in blue and orange) are in a bent conformation. (E) The transition progresses through an asymmetric state in which one CTE has adopted a hypothetical straight conformation while the other remains bent. (F) Both CTEs adopt the hypothetical straight conformation. The hydrophobic residues in the C-terminal helices interact, thereby extending the coiled-coil formed by the N-terminal helices.
Experimental procedures

Materials

A plasmid containing the LqsS His-kinase receptor DNA (UniProt entry Q5ZRY7) was a gift from H. Hilbi (LMU, Munich, Germany). *Mycobacterium tuberculosis* AC Rv1625c (UniProt entry P9WQ35) and mutant clones with point mutations in catalytic residues were available in the laboratory [4]. Radiochemicals were from Hartmann Analytik (Braunschweig, Germany) and Perkin Elmer (Waltham, MA, USA). All enzymes were purchased from New England Biolabs (Ipswich, MA, USA) or Roche Diagnostics (Basel, Switzerland). Other chemicals were from Sigma-Aldrich (St. Louis, MO, USA), Roche Diagnostics, Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). LAI-1 was synthesized in-house according to [12] and dissolved in DMSO. Because the ligand has surfactant properties, concentration–response curves usually were run up to 1 μM LAI-1. The 25-amino-acid S-helix was from the *A. platensis* AC CyaG (UniProt entry Q427QERLLLSVLPRHVAMEMKA446 and Y1035NRRLL443(D256S, D300S, S301T). This lin- (2017) 1204–1217

Plasmid construction

Standard molecular biology methods were used for DNA manipulations (a list of primers is available as Table S2). DNA fragments and vectors were restricted at their 5'-ends by BamHI or EcoRI and at the 3'-ends by HindIII sites and inserted into pQE80 (ΔXhol; ΔNcoI). When necessary, silent restriction sites were introduced to facilitate cloning. Constructs carried an N-terminal MRGS-His6-tag for detection in western blots. In the pETDuet-3 vector the His6-tag-Rv1625c204 terminal S-tag for western blot detection. The linked heteroimers in catalytic residues were available in mammalian AC-C1 and AC-C2 domains. The linked dimeric construct was His6-tag-Rv1625c204-443(N372T, R376H)-TRAAGGPP AAGGRS-Rv1625c204-443(D256S, D300S, S301T). This linker has been successfully used in a mammalian and a mycobacterial class IIIa AC [4,17]. The fidelity of all constructs was confirmed by double-stranded DNA sequencing by GATC (Konstanz, Germany).

Protein expression

Vectors with DNA constructs were transformed into *E. coli* BL21(DE3). Strains were grown overnight in LB medium (20 g LB broth-L⁻¹) at 30 °C containing 100 μg mL⁻¹ ampicillin. A 200 mL volume of LB medium (with antibiotic) was inoculated with 5 mL of a preculture and grown at 37 °C. At an *A*₆₀₀ of 0.3 the temperature was lowered to 22 °C and expression was started with 500 μM (PET vectors) or 100 μM isopropyl β-D-thiogalactopyranosid (pQE vectors) for 3–4 h. Cells were harvested by centrifugation, washed once with 50 mM Tris/HCl, 1 mM EDTA, pH 8 and stored at −80 °C. For preparation of cell membranes, cells were suspended in lysis buffer (50 mM Tris/HCl, 2 mM thioglycerol, 50 mM NaCl, pH 8) and disintegrated with a French press (1100 p.s.i.). After removal of cell debris (4300 g, 30 min, 4 °C), membranes were collected at 100 000 g (1 h at 4 °C). Membranes were suspended in buffer (40 mM Tris/HCl, pH 8, 1.6 mM thioglycerol, 20% glycerol) and assayed for AC activity. For preparation of cytosolic proteins cell debris and membranes were removed (48 000 g, 30 min, 4 °C) and 200 μL Ni-NTA slurry (Qiagen, Hilden, Germany) added to the supernatant. After gentle rocking for 3 h on ice, the resin was poured into a column and washed (2 mL per wash). Wash buffer A was 50 mM Tris/HCl pH 8, 10 mM β-mercaptoethanol, 2 mM MgCl₂, 400 mM NaCl, 5 mM imidazole; wash buffer B was 50 mM Tris/HCl pH 8, 10 mM β-mercaptoethanol, 2 mM MgCl₂, 400 mM NaCl, 15 mM imidazole; and wash buffer C was 50 mM Tris/HCl pH 8, 10 mM β-mercaptoethanol, 2 mM MgCl₂, 10 mM NaCl, 15 mM imidazole. Proteins were eluted with 0.5–1 mL of buffer C containing 150 mM imidazole. The eluates were dialyzed overnight against 50 mM Tris/HCl pH 7.5, 2 mM β-mercaptoethanol, 10 mM MgCl₂ and 20% glycerol and assayed for AC activity. Purified proteins were stored in dialysis buffer at 4 °C.

Adenylate cyclase assay

Adenylate cyclase activity was determined for 10 min in 100 μL at 37 °C. The reactions contained 1.5 μg protein, 50 mM Tris/HCl pH 8, 22% glycerol, 3 mM MnCl₂, 6 mM creatine phosphate and 230 μg creatine kinase, 75 μM [γ-³²P]ATP, and 2 mM [2,8-³H]cAMP to monitor yield during cAMP purification [4,40]. Substrate conversion was kept below 10%. Ligand was added in DMSO not exceeding 1 μL solvent per tube. Respective controls were carried out throughout.

Western blot analysis

The integrity of all expressed proteins was monitored by western blotting. Sample buffer was added to 1 μg of membrane proteins and applied to SDS/PAGE (12%). Proteins were blotted onto a poly(vinylidene difluoride) membrane, incubated with an RGS-His₅-antibody (Qiagen) or S-tag antibody (Novagen R&D Systems, Darmstadt, Germany) and subsequently with a 1 : 2500 dilution of the fluorophore conjugated secondary antibody Cy3 (ECL Plex goat-α-mouse IgG-Cy3; GE Healthcare, Chicago, IL, USA). Detection was performed by John Wiley & Sons Ltd on behalf of Federation of European Biochemical Societies.
carried out with the Ettan DIGE Imager (GE Healthcare). Proteolysis of expressed products was not observed.

**Bioinformatic methods**

**Sequence analysis**

Sequences were taken from the Uniprot Reference Proteomes database (release 2015_04). Nucleotide cyclases were identified in a HHM3R search (E-value cutoff $1 \times 10^{-5}$; [41]) using EMBL SMART’s CYCc family alignment [42]. We extracted the sequences of catalytic domains and CTEs, as segments N-terminal to the former, and analyzed them using EMBL SMART’s CYCc family alignment [42]. We extracted the sequences of catalytic domains and CTEs, as segments N-terminal to the former, and analyzed them using the WEBLOGO 3 server [44] based on the respective clusters from the cluster analysis. For the analysis of the taxonomic distribution of CTEs, we searched the uniprot20 database (version 2016_2; available at http://toolkit.tuebingen.mpg.de; [45]) with alignments built from CTE sequences of bacteria, vertebrate C1, and vertebrate C2 units using the highly sensitive tool HHBLITS [46].

**Structure models**

Structural models were created using MODELLER (version 9.16; [47]) from crystal structures of Rv1625c (pdb: 4p2f), a soluble type 10 AC from rat (pdb: 4clf), and the Bst2-Thetherin Ectodomain (pdb: 2x7a) for the S-helix. The straight CTE was modelled without a template using structural constraints for this segment.

**Statistical analysis**

Data are presented as means ± SEM where applicable. Student’s t test was used for comparisons. Numbers of experiments are given in the legends of the figures. The estimated EC$_{50}$/IC$_{50}$ concentrations were derived from respective concentration–response curves.

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**Conflict of interest**

The authors declare that no conflict of interest exists.

**Data Accessibility**

Research data pertaining to this article is located at figshare.com: https://dx.doi.org/10.6084/m9.figshare.5001914 [Correction added after online publication on 13 Jun 2017: Data Accessibility section added].

**Author contributions**

JES conceived and coordinated the study and wrote the paper together with all coauthors. MZ and SB designed, performed and analyzed experiments shown in Figs 1 and 2. MZ designed, performed and analyzed experiments shown in Figs 4, 6 and 7. JB and JES designed and analyzed data in Figs 3 and 5. JB and ANL designed and analyzed data in Fig. 8. AS provided cloning assistance and analyzed data. All authors reviewed the results and approved the final version of the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Sequences of constructs described in this work.

Table S2. List of primers used in this work.