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Review

Class III adenylyl cyclases: molecular mechanisms of catalysis and regulation

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Abstract. Class III adenylyl cyclases are the most abundant type of cyclic AMP-producing enzymes. The adjustment of the cellular levels of this second messenger is achieved by a variety of regulatory mechanisms which couple signals to adenylyl cyclase activity. Because of the divergent nature of stimuli which impinge on these enzymes, highly individualized class III adenylyl cyclases have evolved in metazoans, eukaryotic unicells and bacteria. Regulation usually exploits the dimeric structure of the catalyst, whose active centres form at the dimer interface. The fold of the catalytic domains and the basic catalytic mechanisms are similar in all class III adenylyl cyclases, and substrate binding generally closes the active site by an induced-fit mechanism. Regulatory inputs can result in dramatic rearrangements of the catalytic domains within the dimer, which often are based on rotational movements.

Keywords. Adenylyl cyclase, cAMP, regulation, catalysis, structural transition.

Introduction

Adenylyl cyclases catalyze the formation of the universal second messenger cyclic adenosine-3′,5′-monophosphate (cAMP) from ATP. They are central relay stations which receive and amplify primary signals such as the presence of hormones or alterations in the ionic composition or the pH in the environment of the cell. In turn cAMP activates target proteins such as protein kinases, ion channels and transcription factors, finally resulting in a cellular response to the primary stimulus. Adenylyl cyclases (ACs) are currently grouped in six classes, which share no sequence similarities and are thought to be the product of convergent evolution (for a review see [1]). Class I ACs have been found exclusively in γ-proteobacteria. They are involved in diverse processes such as catabolite repression in *Escherichia coli* or exertion of virulence in *Yersinia enterocolitica*. Class II ACs are toxins secreted by *Bacillus anthracis, Bordetella pertussis* and *Pseudomonas aeruginosa*. They have no known intracellular role in these bacteria. Only few members of class IV, V and VI ACs have been described to date, and these bacterial enzymes have been investigated only to a very small extent.

Class III ACs are universal. They are found in metazoa, protozoa, fungi, eubacteria, some archaebacteria and certain green algae. However, neither class III ACs nor any other type of AC has ever been conclusively identified in higher plants (*Embryophyta*). The widespread occurrence of class III ACs is reflected on the one hand by a large variation in the modular domain compositions of the proteins and thus the signals they receive and on the other hand by a high divergence of the primary structures of their catalytic domains and thus their biochemical properties [2–4]. This review will address the biochemistry of class III ACs with a focus on structure-function relationships and regulation.

Modular structure of class III ACs

Most class III ACs are multi-domain proteins. Figure 1 shows a choice of the many modular structures in which the catalytic domain, often termed cyclase homology domain (CHD), can be found. Two kinds of ACs occur in mammals, membrane-bound ACs represented by nine isoforms (type I to type IX, [4, 5]) and a soluble AC (sAC, [6]). In the membrane-bound ACs two CHDs ($C1_a$ and C2) are tethered to two membrane anchors (M1, M2). The two MC units are linked by a variable cytoplasmatic region ($C1_b$), and the N-terminal part (N) also displays a high heterogeneity in length and sequence. Class III ACs with an identical structure are present in other metazoans such as the worm *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster*, as well as the protozoon *Dictyostelium discoideum* [7]. A half-molecule, i.e. N-M1-C1, exists in the form of the *Mycobacterium tuberculosis* AC Rv1625c and can be regarded as a progenitor of the mammalian ACs [8]. The mammalian sAC gene codes for two N-terminal CHDs in tandem followed by a large unclassified region of ca. 1150 residues, which appears to be an inhibitor of the catalyst [9]. An alternatively spliced messenger RNA (mRNA) codes for the highly active 'small form' of sAC ([10], see below). Similar ACs are found in the genome of the ciliate *Paramecium* and in bacteria such as *Chloroflexus aurantiacus,* indicating that this type of AC is of ancient origin [11].

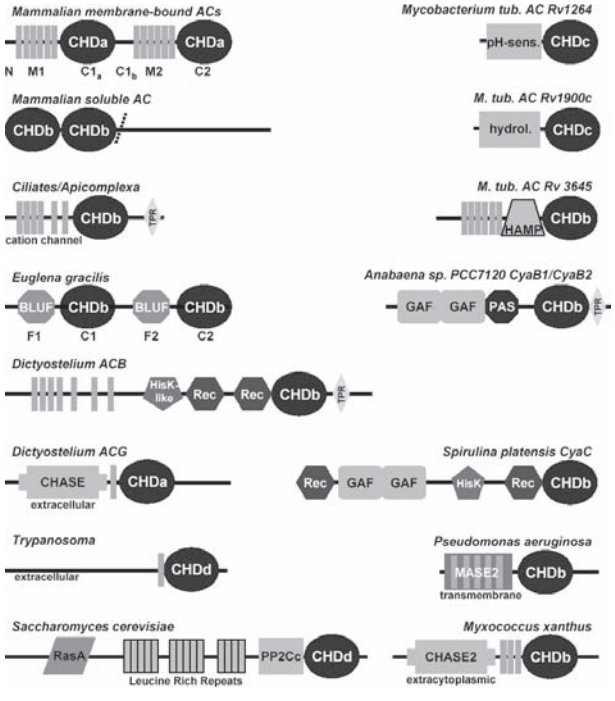

Figure 1. Modular architecture of class III adenylyl cyclases. The black line represents the protein sequence approximately to scale. Domains are coded by shape. CHDs of class IIIa to IIId are labelled accordingly. The dashed line in 'mammalian soluble AC' indicates the C-terminal of the short form. Vertical bars represent predicted transmembrane helices. *BLUF*, sensor of blue light using FAD; *CHASE*, extracellular receptor-like domain [136]; *GAF*, small molecule binding domain found in cGMP phosphodiesterases, adenylyl cyclases, FhlA protein; *HAMP*, signal transmitter module in histidine kinases, adenylyl cyclases, methyl accepting chemotaxis proteins, phosphatases; *HisK*, histidine kinase domain; *hydrol.*, α/β-hydrolase fold; *MASE*, membrane-associated sensor domain; *PAS*, small molecule binding domain originally identified in period clock protein, aryl hydrocarbon receptor, single-minded protein; *pH-sens.*, pH-sensing domain; *PP2Cc*, proteinphosphatase type 2C catalytic domain; *RasA*, RAS-associating domain; *Rec*, receiver domain; *TPR*, tetratricopeptide repeat.

The first class III AC was cloned from *Saccharomyces cerevisiae* [12]. In the yeast AC the CHD is located near the C-terminal of a 228-kDa entity. It is associated with a domain which binds to Ras-GTP and mediates activation of the AC (see below), 14 leucine-rich repeats (LRRs) and a region with similarity to the catalytic domain of protein phosphatase 2c (PP2c). This structure is prototypical for fungal ACs, e.g. the AC of *Ustilago maydis*.

In unicellular organisms complementation cloning and genome projects have provided such a plethora of CHD-associated domains of potential regulatory function that the AC catalytic domain can be termed a multi-purpose signalling module [2]. This indicates the presence of a broad spectrum of signals which are sensed by those ACs. However, only a few regulatory domains have been functionally analyzed, e.g. a mycobacterial pH-sensor domain, blue light-sensor domains in a flagellate, a cyanobacterial chemotactical receiver domain and even cAMP-binding GAF domains also from a cyanobacterium (see below).

The class III AC catalytic mechanism

The catalytic domains of class III ACs must form dimers to be active. Thereby the catalytic centre(s) constitute(s) at the dimer interface [13, 14] (Fig. 2). Accordingly, in mammalian ACs the two CHDs of a single protein form a heterodimeric catalyst with a single ATP-binding pocket [14] (Fig. 2a), whereas ACs with single CHDs homodimerize and form two catalytic pockets [8, 15] (Fig. 2b). CHDs are quite divergent in their primary structures and have been subdivided into four subclasses (class IIIa – class IIId), based on sequence signatures ([2], see Fig. 1). A breakthrough in understanding the catalytic mechanism was the determination of the crystal structures of a chimeric mammalian catalyst (class IIIa) composed of type V AC $C1_a$ and type II AC C2 in complex with various nucleotide inhibitors [14, 16, 17]. Combined with mutagenesis studies, they revealed that eight conserved residues from both monomers participate in substrate binding and catalysis [18–22] (Fig. 2c). Lys938 and Asp1018 bind to the adenine moiety and thus are crucial for selecting the substrate ATP over GTP. Asp396 and Asp440 accomodate the two metal cofactors-ions (Mg^{2+} or Mn^{2+}), which in turn coordinate the triphosphate moiety and position the 3′-OH for

the nucleophilic attack onto the α-phosphate. The reaction appears to proceed via an S_{N2} mechanism in class III ACs, because strict inversion of the configuration has been demonstrated [23]. The presumed trigonal-bipyramidal transition state is stabilized by Arg1029, which neutralizes an excess negative partial charge at the α-phosphate, and by Asn1025, which is believed to stabilize the ribose in a conformation favourable for catalysis to occur. Finally, Arg484 and Lys1065 aid in positioning the pyrophosphate leaving group. Notably, the binding of the 3′ hydroxyl group to the metal cofactor and the transition state were not visible in the crystal structures of V $C1_a$/II C2. Yet, recent structures of protozoan and bacterial ACs corroborate the view that class III ACs adopt a common fold and function by the two-metal-ion mechanism described above: (i) Binding of the 3′ hydroxyl group to a Mg-ion cofactor has been detected in the *Spirulina platensis* AC CyaC (class IIIb) complexed to R_P-ATP-αS [15]. (ii) In the mycobacterial AC Rv1264 (class IIIc), the canonical catalytic residues are arranged in positions almost identical as in the mammalian AC structure [24]. (iii) The model of a CHD dimer of a trypanosomal AC (class IIId) which was derived from the crystal structure of the CHD in a monomeric state also fits this catalytic mechanism [25].

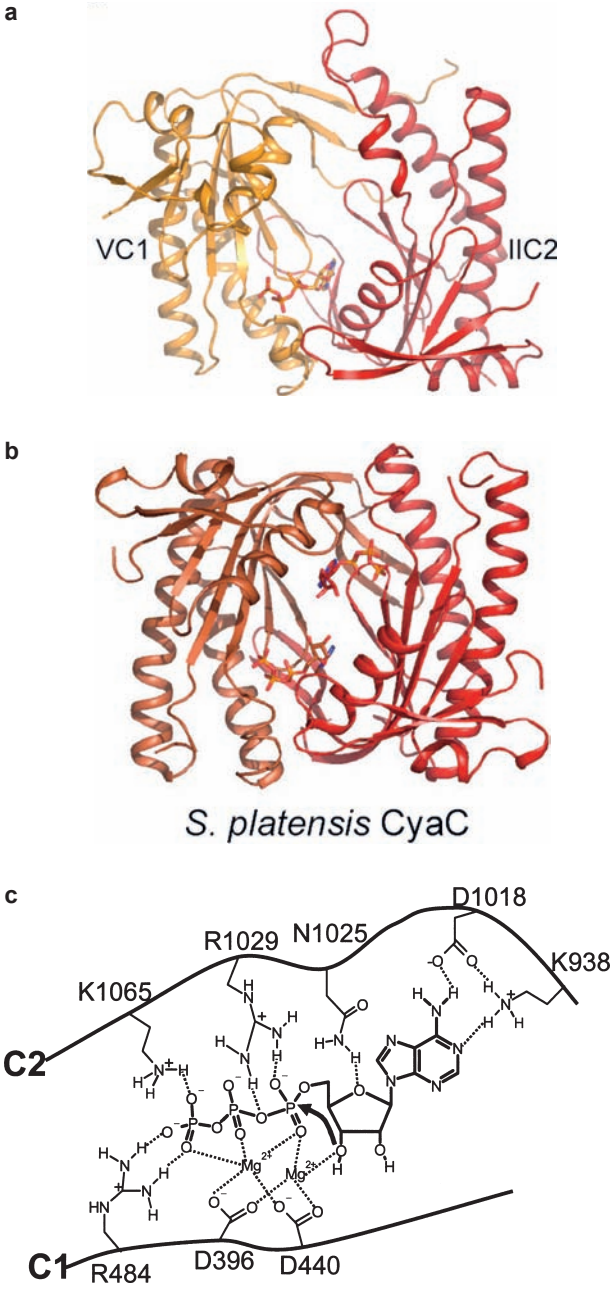

Figure 2. Class III AC catalytic domains function as dimers. (*a*) Heterodimeric mammalian membrane-bound AC: crystal-structure of the type V AC $C1_a$/type II AC C2 heterodimer with the substrate analogue R_P-ATPαS (ATP-α-phosphorothioate) as a stick model (protein databank code: 1CJK). Structure images were generated with *PyMOL* (http://www.pymol.org). (*b*) Homodimeric AC: *Spirulina platensis* CyaC complexed to R_P-ATPαS (PDB: 1WC6). (*c*) Structural model of the catalytic mechanism in mammalian ACs as explained in the text.

The mechanism of substrate selection in class III ACs is variable. Several class III ACs deviate from the purine-binding Lys-Asp consensus, which normally selects ATP over GTP [2]. For example, in class IIIb ACs the Asp is replaced by threonine or serine [2]; in the *M. tuberculosis* AC Rv1900c (class IIIc) an Asn is found in the position of the canonical Lys [26]; and in Rv0386 (class IIIc) the two residues are Gln and Asn [27]. The variant modes of purine-binding by class III cyclases have very recently been reviewed in detail [28] and will not be discussed further here.

In some ACs the cyclization reaction is accomplished without the canonical transition-state stabilizing Asn. The class IIIb AC CyaA from *Myxococcus xanthus* carries a Gly instead of the Asn and appears to be active *in vivo* [29]. In Rv1900c from *M. tuberculosis* the canonical Asn is replaced by His in addition to the alteration in the purine-binding region mentioned above. Nevertheless, Rv1900c is highly active *in vitro* [26]. The His can be mutated to Ala with little reduction of activity, and in the crystal structure of the Rv1900c CHD the residue is not in contact with the ribose moiety of the ATP-analogue α,β-methylene-ATP [26].

Actually, when compiling the sequences of class III ACs with proven activity, the mutagenesis data and the crystal structures, it is evident that only three amino acids constitute the minimal catalytic centre. The two metal-binding Asp residues are essential for catalysis, and the transition-state stabilizing Arg greatly enhances activity.

Conformational flexibility during binding of substrate analogues and nucleotide inhibitors

Substrate analogues and almost all other nucleotide inhibitors of class III ACs are derivatives of adenosine [30]. These nucleotides interfere with the reaction cycle

of ACs at two points as shown in the kinetical scheme in Figure 3a [31]. In mammalian membrane-bound ACs substrate analogues such as α,β-methylene-ATP or R_p-ATPαS bind competitively to the unliganded enzyme, while so-called P-site inhibitors such as 2′d-3′AMP intercept the enzyme-pyrophosphate complex which is the prominent product release intermediate (for a review see [32]). A few very strong inhibitors, e.g. 2′,5′dd-3′ATP, apparently interfere at both points [17]. Comparison of the crystal structures of the mammalian V $C1_a$/II C2 CHD-heterodimer without nucleotide to those in the presence of various inhibitors revealed that the relatively open unliganded dimer closes significantly at the active site upon binding of nucleotide [14, 16, 17] (Fig. 3b, c). The main chain conformations of enzyme-substrate analogue complexes and enzyme-pyrophosphate-nucleotide complexes are quite similar. The main movements accompanying active site closure are a rotation of helix α1 of the C1-CHD towards the helix α4 of C2 accompanied by shifts of the α3-β4 loop of C1 and the β7-β8 unit of C2 (Fig. 3b, c). Larger rearrangements are seen in the class IIIc AC isoform Rv1900c from *M. tuberculosis* [26] (Fig. 3d). The unliganded asymmetric CHD homodimer is much wider open, as in the mammalian AC. Consequently, binding of α,β-methylene-ATP induces major changes in the orientation of the two monomers. They are rotated by 16.6° and translated by 11.4 Å. Moreover, conformational changes are seen in three loops. Taken together the CHDs

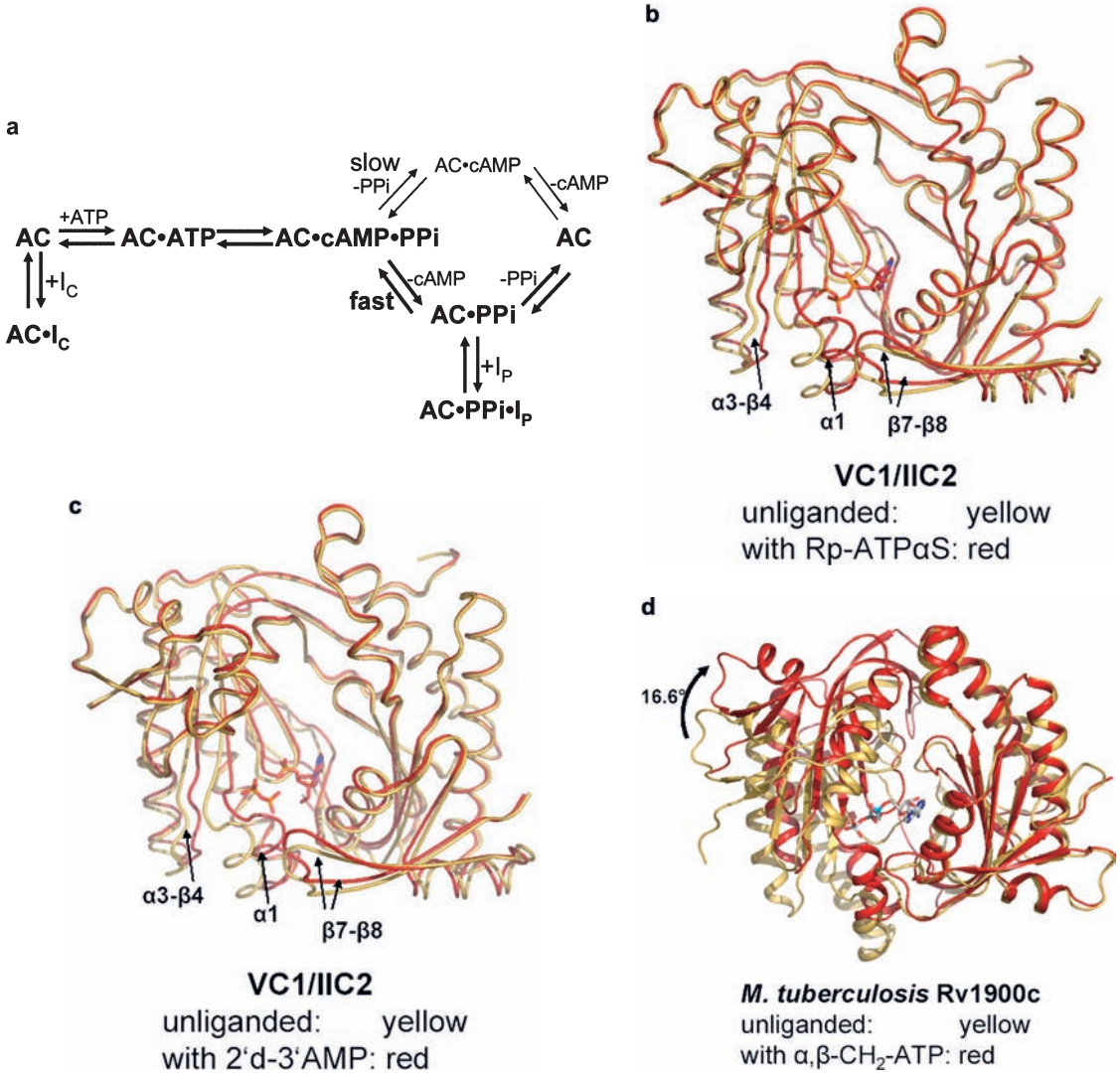

Figure 3. Conformational flexibility upon substrate binding. (*a*) Kinetic model of catalysis in class III ACs. PPi, pyrophosphate; I_C, competitive inhibitor; I_P, P-site inhibitor. Conversion of ATP to cAMP and PPi is usually rate-limiting in class III ACs. (*b*) Overlay of mammalian AC without bound nucleotide (yellow, PDB: 1AZS) and with bound R_P-ATPαS as a competitive inhibitor (red, PDB: 1CJK). (*c*) Overlay of mammalian AC without bound nucleotide (yellow, PDB: 1AZS) and with bound pyrophosphate and 2′d-3′AMP as a P-site inhibitor (red, PDB: 1CS4). (*d*) Overlay of *M. tuberculosis* AC Rv1900c without bound nucleotide (yellow, PDB: 1YBT) and with bound α,β-methylene-ATP (red, PDB: 1YBU).

of class III ACs exhibit a high degree of conformational and orientational flexibility, which reflects induced-fit mechanisms of substrate and inhibitor binding.

Isoform-specific regulation of mammalian membrane-bound ACs

The activity of the nine membrane-bound AC isoforms is differentially modulated by a complex set of primary and secondary regulators. In this section the major concepts are presented as they are established to date (Table 1). For a comprehensive survey see [4].

Heterotrimeric G-proteins are the best-characterized regulators of mammalian ACs. $G_s\alpha \cdot GTP$ stimulates all nine isoforms by a factor of 3 to 20 [33–39]. The crystal structure of V $C1_a$/II C2 in complex with $G_s\alpha \cdot GTP\gamma S$ revealed the binding mode of the activator [14] (Fig. 4b). $G_s\alpha \cdot GTP$ binds to the α-helical backbone of II C2 and to a lesser extent to an N-terminal segment of V $C1_a$. A major interacting epitope on $G_s\alpha$ is the α2-helix, which is part of a structure (switch II) that undergoes a conformational change upon GTP binding. This explains how guanine nucleotide exchange and AC activation are coupled in the G-protein. The structural changes of V $C1_a$/II C2 induced by $G_s\alpha \cdot GTP$ could not be derived directly because no structure of the AC ground state existed for comparison. Consistent with the clasp-like binding of $G_s\alpha \cdot GTP$ to the AC, the dissociation constant of the V C1/II C2 dimer drops from > 5 µM to 0.7 µM upon G-protein stimulation [39]. Although this suggests activation of the AC by enhanced dimerization, this might not be equally important in the membrane-bound holoenzymes, because there the two CHDs are located within a single protein chain anyway. However, based on the structure of an inactive II C2 homodimer as a model of the ground state, it has been calculated that $G_s\alpha \cdot GTP$ induces a 7° rotation of the two CHDs with respect to each other [13, 14]. Thereby the positions of the catalytic residues are assumed to be adjusted towards a state more favourable for catalysis to occur (Fig. 4b).

Table 1. Overview of class III ACs.

Organism	Isoform	Subclass	Soluble?	Dimerization	Regulation
Mammals	I	a	–	hetero	$G_s\alpha\uparrow$, $G_i\alpha\downarrow$, $G_z\alpha\downarrow$, $G_o\alpha\downarrow$, $G\beta\gamma\downarrow$, Ca^{2+}/CaM ↑, PKC ↑, CaMK IV ↓
	II	a	–	hetero	$G_s\alpha\uparrow$, $G\beta\gamma\uparrow$, PKC ↑
	III	a	–	hetero	$G_s\alpha\uparrow$, $G\beta\gamma\downarrow$, Ca^{2+}/CaM ↑, PKC ↑, CaMK II ↓
	IV	a	–	hetero	$G_s\alpha\uparrow$, $G\beta\gamma\uparrow$, PKC ↓
	V	a	–	hetero	$G_s\alpha\uparrow$, $G_i\alpha\downarrow$, $G_z\alpha\downarrow$, ($G\beta\gamma\downarrow$?), $Ca^{2+}\downarrow$, PKA ↓
	VI	a	–	hetero	$G_s\alpha\uparrow$, $G_i\alpha\downarrow$, $G_z\alpha\downarrow$, ($G\beta\gamma\downarrow$?), $Ca^{2+}\downarrow$, PKC ↓, PKA ↓
	VII	a	–	hetero	$G_s\alpha\uparrow$, $G\beta\gamma\uparrow$
	VIII	a	–	hetero	$G_s\alpha\uparrow$, $G\beta\gamma\downarrow$, Ca^{2+}/CaM ↑
	IX	a	–	hetero	$G_s\alpha\uparrow$
	sAC	b	+	hetero	$HCO_3^-\uparrow$, $Ca^{2+}\uparrow$
Yeast		d	+	homo	RAS2-GTP ↑, CAP(↑), Gpa2 ↑
Dictyostelium	ACA	a	–	hetero	indirectly activated via a G-protein coupled receptor in resp. to cAMP
	ACB	b	–	homo	?
	ACG	a	–	homo	high osmolarity ↑
Euglena		b	+	hetero	blue light ↑
Trypanosoma		d	–	homo	stress ↑
Ciliates		b	–	homo	[ion conductance ↑]
Pseudomonas		b	–	homo	?
Myxococcus		b	–	homo	osmotic pressure sensing function
Anabaena	CyaB1	b	+	homo	cAMP ↑, $HCO_3^-\uparrow$
	CyaB2	b	+	homo	cAMP ↑
	CyaC	b	+	homo	involved in light sensing, autophosphorylation ↑
Mycobact. tuberculosis	Rv1264	c	+	homo	acidic pH ↑
	Rv1625c	a	–	homo	?
	Rv1900c	c	+	homo	?
	Rv3645	b	–	homo	regulatable by HAMP domains
Spirulina	CyaC	b	+	homo	$HCO_3^-\uparrow$, $Ca^{2+}\uparrow$

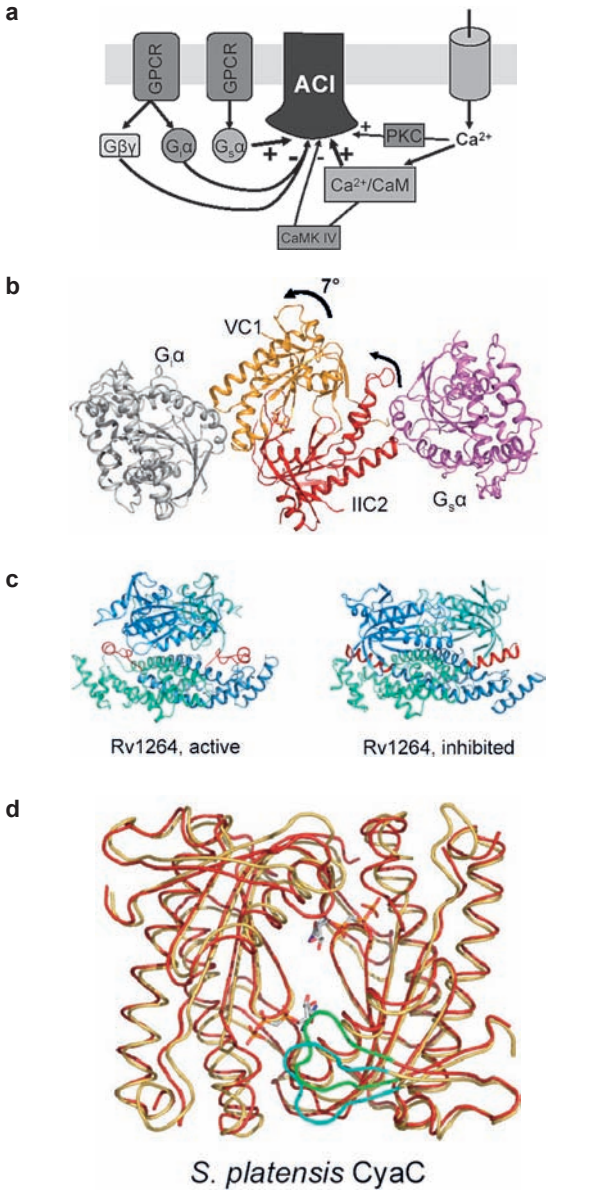

Figure 4. Regulatory mechanisms of class III ACs. (*a*) Regulators of type I AC (ACI) shown in the membrane environment (light grey bar). Type I AC is directly activated either by $G_s\alpha \cdot GTP$ via a G-protein-coupled receptor or by Ca^{2+}/calmodulin upon entry of extracellular Ca^{2+}. Signalling through a GPCR coupled to inhibitory G-proteins counteracts activation by generating $G_i\alpha \cdot GTP$ and $G\beta\gamma$. Protein kinase C and CaMK IV can enhance and weaken activation by Ca^{2+}/calmodulin, respectively. (*b*) Mammalian AC complexed to G-proteins. $G_s\alpha \cdot GTP$ docks to the side distal from the substrate (stick model of R_P-ATPαS, PDB: 1CJK) and supposedly tilts a loop of IIC2 (thin arrow). This generates pressure on VC1 and induces a 7° rotation which narrows the active site [14]. Binding of $G_i\alpha \cdot GTP$ to the opposite side of the dimer is tentatively modelled (gray, PDB: 1CIP) [43]. (*c*) Large conformational changes occur during regulation of *M. tuberculosis* AC Rv1264 as viewed from the side. The active form (PDB: 1Y11) is related to the inhibited state (PDB: 1Y10) by a 55° rotation and 6 Å translation of the catalytic domains. The structural switch in the linker between the two domains is shown in red [24]. (*d*) Bicarbonate induces active-site closure in Spirulina platensis CyaC complexed to α,β-methylene-ATP. Overlay of the open state (yellow, PDB: 1WC0) and the closed state (red, PDB: 1WC5). The most prominent shift is seen with the $\beta7$-$\beta8$ unit (blue: open; green: closed) [15].

In contrast to the direct stimulation of ACs by $G_s\alpha$, inhibitory G-proteins ($G_i\alpha1$, $G_i\alpha2$, $G_i\alpha3$, $G_o\alpha$, $G_z\alpha$) act as secondary regulators [40–43]. They apparently do not inhibit basal AC activity, but only activity stimulated by physiological regulators such as $G_s\alpha$ or pharmacological agents such as forskolin. Thus they can be regarded as non-competitive antagonists of the primary regulators. Only type I, V and VI ACs are affected by inhibitory G-proteins. $G_s\alpha$-activated type V and VI ACs are maximally inhibited 70–80% by $G_i\alpha$ isoforms and $G_z\alpha$ with an IC_{50} (inhibitory concentration that causes 50% of the maximal response) of 50–150 nM *in vitro* [40, 42, 44]. $G_o\alpha$ is ineffective on these two isoforms. In contrast, $G_s\alpha$-activated type I AC is only slightly susceptible, whereas Ca^{2+}/calmodulin-activated type I AC shows a maximal inhibition of ca. 50% by all inhibitory G-proteins, including $G_o\alpha$ [40, 44]. Apparently the potency of these $G\alpha$ subunits as antagonists of activation does not only depend on the AC isoform but also on the mode of activation. This is corroborated by the fact that $G_i\alpha$ is a poor inhibitor of type V AC activated concomitantly by both $G_s\alpha$ and forskolin [43]. A structural model for G-protein inhibition has been suggested based on mutational data of type V AC and considerations based on molecular modelling [43] (Fig. 4b). Accordingly, $G_i\alpha$ docks on to the $C1_a$/C2 dimer opposite of the $G_s\alpha$ binding site in a clasp-like manner as described for $G_s\alpha$ (see above). Thereby it would counteract the rotation induced by the stimulatory G-protein.

The $G\beta\gamma$ unit of heterotrimeric G-proteins shows a complex pattern of AC regulation. AC types II, IV and VII are activated by $G\beta\gamma$, and the action of $G\beta\gamma$ on type II AC has been studied in detail [36, 45]. $G\beta\gamma$ behaves as a secondary or 'conditional' regulator given that basal activity is only stimulated weakly (ca. 1.5-fold, [34]), whereas the $G_s\alpha$-activated type II enzyme is stimulated about 4- to 11-fold with an EC_{50} (effective concentration that causes 50% of the maximal response) in the range of 10–30 nM [34, 42, 45–47]. The site of interaction of $G\beta\gamma$ with the AC has been debated since 1995. Initially peptide competition experiments suggested that $G\beta\gamma$ docks onto a region of the C2 CHD [48, 49], but mutagenesis of this region did not ablate activation [46]. Very recently, a scanning mutagenesis approach unequivocally identified a heptadekapeptide segment in the $C1_b$ region of type II AC as the relevant stimulatory site (aa 493–509; [47]). This segment is highly conserved among type II, IV and VII ACs, but the 3D structural basis of $G\beta\gamma$ activation is yet to be determined.

AC types I, III and VIII are inhibited by $G\beta\gamma$. Again, the G-protein acts secondarily and as an antagonist of activation [42, 45, 47]. Type I AC activated by Ca^{2+}/calmodulin can maximally be inhibited by ca. 80% with an IC_{50} of 5–10 nM. As with inhibition by $G_i\alpha$, maximal inhibition by $G\beta\gamma$ is significantly weaker, with type I AC preactivated by $G_s\alpha$ (50% inh.) or forskolin (30% inh.).

In cotransfection experiments type V and VI ACs also appeared to be inhibited by G$\beta\gamma$, but a direct interaction between the G-protein and the ACs has not been demonstrated so far [50].

Because $G_i\alpha$ is much more abundant than $G_s\alpha$, the G$\beta\gamma$ subunits that regulate AC activity are thought to be released on activation of inhibitory G-proteins [42, 45]. Thus, inhibitory G-proteins inhibit type I AC via $G_i\alpha$ and G$\beta\gamma$, type V and V ACs via $G_i\alpha$ only, type III and VIII ACs via G$\beta\gamma$ only, but they activate type II, IV and VII via G$\beta\gamma$ (see Fig. 4A for a scheme of type I AC regulation).

Ca^{2+}-dependent regulation is the second largely established mechanism of modulation of mammalian AC activity. Ca^{2+}/calmodulin is a strong primary stimulator of type I and VIII ACs. Maximal activation is about 8-fold with an EC_{50} (CaM) of 20 nM for type I AC and an EC_{50} (CaM) of 50 nM for the type VIII isoform [33, 38]. The structural transitions induced by Ca^{2+}/calmodulin are unknown. However, calmodulin binding sites have been mapped to $C1_b$ of type I AC (aa 495–522, [51]), while in type VIII AC calmodulin binds to the cytosolic N-terminal region and to the very C-terminal part of the enzyme [52]. Functional studies indicate that only the C-terminal calmodulin binding site is required for activation of type VIII AC *in vitro*, but both are required for calmodulin stimulation via capacitative Ca^{2+} entry *in vivo* (comp. below, [53]).

Ca^{2+}/calmodulin also appears to act as a weak secondary activator of type III AC, i.e. the enzyme preactivated by $G_s\alpha$ or forskolin is further stimulated 1.5–2-fold [47, 54]. The physiological relevance of this effect is unclear.

The inhibition of mammalian ACs by Ca^{2+} ions has been a matter of debate and is not fully understood. All mammalian ACs are inhibited by unphysiologically high Ca^{2+}; reported IC_{50} values vary from ca. 30 to 250 µM at 1 mM $MgCl_2$ as a cofactor [55, 56]. Kinetic analysis indicated that this low affinity inhibition is due to competition of Ca^{2+} with the metal cofactor *in vitro*. Exclusively type V and VI ACs appear to be partially inhibited by physiological Ca^{2+} concentrations. 1 µM Ca^{2+} reduces the activity of the two isoforms by about 50% if the enzymes are preactivated, e.g. by forskolin [55, 57], whereas basal activity of type V AC is reportedly not affected [58]. Thus Ca^{2+} ions may act as secondary regulators. Mapping of the high-affinity Ca^{2+} site of the type V AC holoenzyme pointed towards the $C1_a$ CHD as the primary site of action [57], yet a soluble form of type V AC lacking the membrane anchors showed no inhibition at physiological Ca^{2+} concentrations [59]. Therefore, the structural and functional basis of mammalian AC inhibition by physiologically relevant Ca^{2+} concentrations remains largely unknown. A physiological role of Ca^{2+} inhibition of type V and VI ACs in cardiac myocytes is discussed, because these two isoforms are dominant in the heart and because during cardiac contraction cytosolic Ca^{2+} concentrations rise from 10 to about 100 nM [60]. For contraction to occur, opening of cAMP-gated HCN-type 'pacemaker' cation channels is essential, which positively links adenylyl cyclase activity to generation of the heartbeat [61, 62]. Thus, inhibition of the two AC isoforms by the rise in cytosolic Ca^{2+} would provide for negative feedback after initiation of contraction. Indeed, cAMP concentrations in the heart do oscillate with the cardiac cycle [63].

Since the early 1990s type-specific regulation of ACs by protein kinases gained significant attention. The effects of the cAMP-dependent protein kinase A (PKA), the Ca2+-stimulated protein kinases C (PKC) and calmodulin kinases (CaMKs) were investigated to some extent, yet are far from being fully understood.

The effect of PKC on AC types I–VI has been investigated *in vitro* and in whole-cell systems. Stimulation of type II AC is the best-characterized effect. A 3–4-fold increase of basal activity can be evoked by phorbol-ester treatment, which activates endogenous PKC, or by addition of purified recombinant PKCα isoform [64, 65]. Thus PKC is a primary stimulator of type II AC. Kinetically, activation is due to an increase in V_{max}. Interestingly $G_s\alpha$-activated type II AC is also stimulated by PKC, whereas secondary G$\beta\gamma$ activation is lost upon kinase treatment [65]. Thus PKC provides for a further level of signal integration by a mammalian AC. Mechanistically, it has been demonstrated that type II AC is directly phosphorylated by PKC, but the site of modification remains to be determined [64]. Type III AC is activated 2-fold by phorbol ester treatment *in vitro*, but 10-fold in whole-cell experiments [66, 67]. *In vitro* basal and prestimulated activities are affected, while in cells PKC acts as a secondary activator only. These differences may be correlated to the apparent lack of direct phosphorylation of the AC by the kinase, which points to a rather indirect mode of action. Treatment of type I AC with phorbol esters yielded ambiguous results. PKC was found to be a secondary activator of forskolin-stimulated type I AC in whole cells in one study, whereas there was no effect in another report [67, 68]. *In vitro* only Ca^{2+}/calmodulin-activated type I enzyme was moderately stimulated, whereas forskolin-activated was not. Type IV, V and VI ACs are not activated by PKC [66]. However, purified PKCα acts as a secondary inhibitor of type IV AC [65]. $G_s\alpha$-activated AC activity is reduced by 70% upon kinase treatment, and responsiveness to G$\beta\gamma$ is ablated. Type VI AC is also secondarily inhibited by PKC [69]. Forskolin-stimulated activity is reduced up to 74% via multiple phosphorylation sites in the N-terminal and catalytic domains. Recent experiments show that this inhibition is blocked by the interaction with Snapin, a component of the cellular vesicle docking and fusion system [70]. Thus PKC increases the repertoire of signal integration as also shown for type II AC (see above).

Type V and VI ACs are secondarily inhibited by *in vitro* phosphorylation using PKA. Type V AC is inhibited by

30% when preactivated by $G_s\alpha$ and by 40% after forskolin stimulation [71]. $G_s\alpha$-activated type VI AC is inhibited by 50% by PKA treatment, whereas forskolin-activated enzyme activity is reduced by 30% [72]. The phosphorylation site in type VI AC has been mapped to a serine residue in the $C1_b$ region. Thus PKA seems to exert some negative feedback regulation on these two AC isoforms.
Negative feedback also appears to be mediated by CaMK isoforms. For example, Ca^{2+}/calmodulin-activated type I AC is inhibited by 50% *in vitro* and in whole cell assays by CaMK IV [73]. Basal activity or activities stimulated by forskolin or $G_s\alpha$ (via isoproterenol) are not affected. Type III AC also appears to be secondarily inhibited by CaMK II *in vivo* [74]. In contrast to type I AC, type III AC is sensitive to the action of the kinase when preactivated with forskolin or $G_s\alpha$. In conclusion, isoform-specific phosphorylation of mammalian membrane-bound ACs provides for further modes of regulation and signal integration but is hardly understood at the molecular and structural level.
Several other secondary regulatory inputs have been described, such as interaction with *protein associated with Myc* (PAM) or S-nitrosylation by nitric oxide. Most of these regulatory mechanisms are not well understood at the molecular level, and some are questionable regarding their physiological relevance (reviewed in [4]).

The role of membrane anchors and subcellular localization in mammalian membrane-bound ACs

The membrane-anchors of mammalian ACs consist of two sets of six putative transmembrane helices (see above). Because they make up for about 40% of the mass of the protein, an additional role beyond a mere anchoring in the plasma membrane was suggested when the first mammalian AC was cloned [5]. Initially it was speculated that the ACs may have a second function as a transporter or channel because the modular structure of the ACs is reminiscent of that of ABC transporters [5]. However, to date such a function has not been detected in any mammalian AC. A first insight into the roles of the transmembrane domains was obtained by construction of chimeric and truncated ACs. In the type V AC an isoform-specific interaction of the two anchors, M1 and M2, has been demonstrated which is needed to yield any basal activity and to obtain full activation by forskolin [37]. M1 and M2 of type V AC could concomitantly be exchanged by the two membrane domains of type VII AC without alteration of activity, but a mixed set of type V and type VII membrane anchors yielded inactive proteins. Thus the M1-M2 membrane complex appears to provide for proper arrangement of the $C1_a$-C2 catalytic dimer [37]. An M1-M2 complex was also detected in type VIII AC using fluorescently tagged constructs [75, 76]. As with type V AC the membrane complex formation was essential to obtain a functional AC. Furthermore, it could be demonstrated that the M1-M2 complex is needed for trafficking of type VIII AC to the plasma membrane, because in the absence of either M1 or M2 the proteins were retained in the endoplasmatic reticulum [75]. Thus the membrane anchors have at least a dual role in orienting the CHDs and in targeting the enzyme to the cell membrane. A third role of the membrane anchors has been suggested in AC oligomerization [76]. Activity of the type VIII AC holoenzyme could be reduced by the coexpression of a type VIII or type VI AC mutant which lacked the $C1_a$ catalytic domain. Thus mammalian ACs apparently dimerize or oligomerize. Deletion mapping, coimmunoprecipitation and FRET (fluorescence resonance energy transfer) assays revealed that the M2 membrane anchors are mainly responsible for these intermolecular interactions. The physiological role of AC oligomerization within the plasma membrane is not clear, but could be related to the formation of multiprotein signalling complexes (see below).
Expectedly, plasma membrane localization is crucial for AC regulation. On the one hand G-protein-coupled receptors (GPCRs) are localized there, so that G-proteins can stimulate ACs in the vicinity, providing for fast propagation of the signal. Indeed, a fusion protein of the β-adrenergic receptor and $G_s\alpha$ efficiently stimulated mammalian AC activity, demonstrating the proximity of the AC and the GPCR [77]. On the other hand, mounting evidence links membrane localization with Ca^{2+} regulation of ACs. In living cells type VI AC is hardly inhibited by Ca^{2+} mobilized from internal stores by thapsigargin treatment, whereas subsequent capacitative calcium entry evoked by addition of Ca^{2+} to the medium is effective. The addition of a Ca^{2+} ionophore did not enhance inhibition [78, 79]. Thus type VI AC was exclusively regulated by Ca^{2+} entering through channels in intimate proximity to the cyclase. Similarly, activation of type VIII AC via Ca^{2+}/calmodulin has also been shown to be dependent on capacitative calcium entry [53]. The localization of Ca^{2+}-sensitive ACs has been further dissected by cell biological methods. Type V, VI and VIII ACs are exclusively targeted to rafts/caveolae, while the Ca^{2+}-insensitive type VII AC is found only in the bulk membrane [80, 81]. The association of type VI and VIII ACs with rafts is essential for Ca^{2+}-dependent regulation. In the case of type VIII AC, binding of the N-terminal to caveolin appears to be a further prerequisite for coupling to capacitative calcium entry [53]. However, surprisingly the localization of Ca^{2+}-sensitive ACs to rafts/caveolae is not dependent on the membrane anchors but is governed by the cytosolic C1 and C2 domains [81]. Thus these ACs seem to be embedded in defined multiprotein signalling complexes, which translate the primary signal to cAMP production. This view is supported by the finding that mammalian AC was detected in a large signalling complex with the β2-adrenergic receptor, an

L-type Ca^{2+} channel, $G_s\alpha$ and $G\beta\gamma$, cAMP-dependent protein kinase and protein phosphatase 2A [82]. To date mammalian ACs have been recognized to perform additional quite divergent protein-protein interactions which may be connected to the formation of more higher-order signalling complexes (for a recent review see [83]). For example, interactions with protein phosphatase 2A, RGS proteins (regulators of G-protein signalling), Snapin and PAM (protein associated with Myc) have been described [70, 84–87], and arterial ATP-sensitive K^+ channels seem to be linked to AC via colocalization in caveolae [88].

Regulation of class III ACs in eukaryotic unicellular organisms

In general, class III ACs in yeast, protozoa and algae have individual multi-domain architectures not found in mammals. This is reflected by different specialized modes of regulation as outlined below (Table 1).

In the yeast *S. cerevisiae* two signals have been identified to activate the single, class IIId AC *in vivo*, a shift from carbohydrate-free to glucose-containing medium and an intracellular acidification upon carbon starvation (for a review see [89]). The small G-protein RAS2 in its GTP-bound form is essential for activation *in vivo* and is a direct stimulator of AC activity *in vitro*. It binds to the RAS-association domain (RA, see above and Fig. 1), but just, how AC is activated by RAS2 remains unclear [90–93]. However, experiments with N-terminally truncated ACs suggested that the N-terminal set of regulatory domains in the yeast AC may have an autoinhibitory function and that autoinhibition is relieved upon the binding of activators such as RAS2 [91]. In addition, full activation by native farnesylated RAS2 *in vitro* requires the presence of a 70-kDa subunit of yeast AC called CAP (cyclase-associated protein) [94]. The glucose-induced signal is mediated via a heterotrimeric G-protein G_α subunit (Gpa2), which in *Schizosaccharomyces pombe* has been shown to bind to an N-terminal region upstream of the RA domain [95]. Thus for full activation of the AC *in vivo* RAS2-GTP, CAP and Gpa2 appear to modulate the enzyme synergistically. Recently the protein Sgt1 was identified as a further interaction partner of the yeast AC, which appears to dock onto the LRR domain. It seems to stabilize or activate the AC and may function in a manner of a cochaperone [96]. Although several regulatory proteins of yeast AC have been identified to date, the molecular mechansisms of signal integration by the AC remain largely unclear. Experiments have mainly been conducted *in vivo* by the use of mutant strains, while *in vitro* data on purified enzyme are scarce.

In the mycetozoan *Dictyostelium discoideum* cAMP signalling is essential for aggregation and development of the amoebae upon starvation. Although ACs of *Dictyostelium* have been investigated for more than 20 years, little is known of the molecular mechanism of their regulation. Three AC genes have been cloned. The first isoform, ACA, has the same architecture as mammalian membrane-bound ACs [7]. It is essential for reacting to and production of cAMP, which in *Dictyostelium* also has an extracellular role as the chemoattractant mediating aggregation [7]. cAMP binds to a G-protein-coupled receptor (reviewed in [97, 98]). However, the activated G-protein (G2) does not directly interact with ACA. Instead the released $\beta\gamma$ dimer activates a phosphatidylinositol-3 kinase, which triggers recruitment of the protein CRAC (cytosolic regulator of AC) to the membrane. CRAC is essential for activation of ACA, but appears not to interact physically with the cyclase [99]. Five other factors have been identified which participate in the process of ACA activation, but none of them seems to bind the enzyme. The second AC isoform, ACG, is essential for germination [7]. Its N-terminus constitutes a large extracellular receptor-like domain (CHASE, Fig. 1), which is linked to the C-terminal class IIIa CHD by a single transmembrane span. The enzyme is activated by high osmolarity, and osmosensing appears to be an intrinsic property of ACG [100]. Yet, the mechanism of activation is unknown. The third isoform, ACB, is expressed during late development and is required for terminal maturation of the spores [101, 102]. It is a multidomain protein containing a membrane anchor, a degenerated histidine kinase-like domain, bacterial-type *receiver* domains and a class IIIb CHD (Fig. 1). The regulation of ACB remains enigmatic to date. Taken together *Dictyostelium* has developed highly specialized pathways of AC activation, and it remains a major challenge to reveal its molecular basis.

In the flagellate *Euglena gracilis* photoavoidance is triggered by cAMP formed by a blue-light activated class IIIb AC [103]. The soluble enzyme is an $\alpha_2\beta_2$ heterotetramer of ca. 400 kDa. Both the α-subunit (112 kDa) and the β-subunit (94 kDa) have the same modular composition [103] (Fig. 1). They contain two BLUF domains (sensors of Blue Light Using FAD, F1 and F2) each fused to a CHD (C1 and C2). Blue-light irradiation activates the AC 80-fold, producing a high substrate turnover rate and a strong substrate affinity ($K_{m,\,ATP} \approx 0.5$ µM) [103]. Sequence analysis indicates that C1 and C2 function as an intramolecular dimer with a single active site similar to mammalian ACs. The BLUF domains, which obviously regulate the activity of the CHD dimer, exhibit maxima at 370 and 450 nm in the excitation spectrum and emission at around 530 nm. The isolated recombinant α-F2 domain, but not F1, binds FAD or FMN and shows phototransformation with a half-life of 34–44 seconds [104, 105]. It will be interesting to learn how light-induced conformational changes in the BLUF-domain(s) are transmitted to the CHD dimer.

Trypanosomatids possess multiple AC isoforms which all have the same architecture [106–108]. A large highly variable extracellular domain (ca. 90 kDa) is linked to a class IIId CHD by a single transmembrane span. It has been speculated that the extracellular domain constitutes a receptor, but ligands are unknown. cAMP seems to be involved in regulation of the life cycle of trypanosomes [109]. It may also play a role in response to stress: AC activity of bloodstream parasites is stimulated 10–100-fold by exposure to pH 5.5 and 5–25-fold by trypsin treatment [110, 111]. This activation is lost upon development to procyclic parasites [111]. The molecular basis of activation is enigmatic, but in vitro experiments support the possibility that the activity of the CHD can be regulated by the N-terminal region. The isolated recombinant CHD of at least one isoform (GRESAG4.4B) appears to dimerize *in vitro* as judged by gel filtration and exhibits only low AC activity [112]. However, fusion to a leucine zipper as an artificial dimerization domain stimulates the maximal activity 20-fold without affecting K_m [112]. Thus the relative orientation of the CHDs in the dimer seems to be rectified by the leucine zipper, and therefore the receptor-like domain may fulfil the same role in the native holoenzyme.

In the ciliate *Paramecium tetraurelia* cAMP production is regulated by the K^+ resting conductance of the cell, i.e. hyperpolarization by K^+ dilution causes a transient up to 5-fold elevation of cAMP levels within seconds [113]. Because a 95-kDa AC purified from ciliary membranes showed an intrinsic K^+ conductance, it was suggested that the cyclase itself acts as a K^+-flux sensor, i.e. as a molecular ammeter [113]. Recently an AC complementary DNA (cDNA) from *Paramecium* was cloned, which codes for a fusion protein of a putative voltage-gated ion channel and a class IIIb CHD [114] (Fig. 1). This modular composition supports the idea of an ion conductance-regulated AC. However, ion permeability remains to be demonstrated for the gene product [114]. Similar ion channel ACs are encoded in the genomes of apicomplexan parasites such as *Plasmodium* and in the green alga *Chlamydomonas reinhardtii*. Thus ion-channel ACs seem to sense ional changes in the environment in a broader spectrum of eukaryotic unicells.

Evidently eukaryotic ACs have evolved into a large variety of signal integrators, while mammalian membrane-bound ACs represent just a small fraction of the modes in which a CHD can be linked to regulators.

Regulation of bacterial class III ACs

Bacterial genomes are a well filled 'treasure box' of class III ACs. About 200 CHDs have been sequenced which are fused to a wealth of various signalling domains (for an overview see [2, 115]). Often multiple class III ACs are found in a bacterial genome. For example, *Sinorhizobium meliloti* contains 26 isoforms. Thus it appears that bacteria can employ individual cAMP signalling pathways to respond to several different stimuli. Often the domain composition of putative class III ACs is highly suggestive for certain types of regulatory inputs and mechanisms [3, 115]. Yet, the physiological roles and the mechanisms of regulation of class III AC isoforms have been elucidated in only a few instances.

In *Pseudomonas aeruginosa*, cAMP is required for expression of the secretion system by which virulence factors are translocated to the host cell. Disruption of a class IIIb AC gene resulted in strong attenuation [116]. The N-terminal half of the cyclase consists of a predicted membrane-associated sensor domain (MASE2), suggesting that it may directly receive the environmental signal(s) which trigger(s) expression of the secretion system (Fig. 1).

Two class III ACs of the soil bacterium *Myxococcus xanthus* have been shown to be involved in sensing high osmotic pressure [29, 117]. Both isoforms have the same architecture. A putative extracellular receptor domain (CHASE2) is linked to a class IIIb CHD by a set of three transmembrane helices (Fig. 1). Whether these CHASE2 domains have osmosensing properties is a question for future research.

The molecular mechanisms of bacterial class III AC regulation have mainly been investigated in *Mycobacterium tuberculosis* and in Cyanobacteria. The cyanobacterium *Anabaena* sp. PCC7120 contains six AC isoforms [118, 119]. Deletion of the class IIIb AC CyaC reduces cellular cAMP levels to 25% of wild-type, and in the mutant cells cAMP levels are not lowered by light as in wild-type cells, but stay constant [118]. CyaC consists of a *receiver* domain, two GAF domains similar to plant ethylene receptor (ETR) domains, a histidine kinase domain, another *receiver* and a CHD (Fig. 1). Analysis of purified recombinant CyaC of *Spirulina platensis*, which has an identical domain composition, has demonstrated a 2–4-fold enhancement of AC activity by the histidine kinase-*receiver* system [120, 121]. The histidine kinase domain autophosphorylates on His572, and subsequently the phosphate is transferred to the second *receiver* domain (Asp895), which is adjacent to the CHD. Mutation of either His572 or Asp895 greatly reduced AC activity [121]. In conclusion CyaC is regulated by an intramolecular two-component signal transduction system, and the question arises how this regulatory scaffold could be linked to sensing of light.

A completely different type of regulation is seen in the class IIIb AC isoforms CyaB1 and CyaB2 of *Anabaena* sp. PCC7120. They are composed of two GAF domains (GAF_A and GAF_B), a PAS domain, a CHD and a single tetratricopeptide repeat (TPR) (Fig. 1). The GAF domains show significant similarity to cGMP binding tandem GAF

domains of mammalian cyclic nucleotide phosphodiesterases (PDEs). Similarly, in CyaB1 the cyanobacterial tandem GAF domains bind cAMP and thereby increase the V_{max} of the AC activity 27-fold, thus functioning as an autoactivating switch and creating a feed-forward stimulatory mechanism [122]. The close relationship between cyanobacterial and mammalian GAF domains was functionally confirmed by the creation of a cGMP-stimulated cyclase upon exchange of the cyanobacterial GAF domains for those of PDE2 [122]. CyaB1 and CyaB2 show some differences in the molecular mechanism of activation [123]. Most prominently, in CyaB1 only GAF_B binds cAMP, while in CyaB2 both GAF_A and GAF_B are cAMP receptors [123, 124]. The crystal structure of the tandem GAF domains of CyaB2 shows an antiparallel dimer, in which GAF_A of one monomer binds to GAF_B of the other and vice versa [124]. In contrast, the GAF domains of PDE2 are organized in parallel, and only the two GAF_A domains dimerize [125]. Whether the GAF tandems are able to switch between such two quartenary structures awaits proof. Nevertheless, the functional equivalence of mammalian and cyanobacterial GAF domains in the AC background suggests that the GAF domains do not bind the CHD, but that the signal is transmitted through the intervening PAS domain. The example of *Anabaena* CyaB ACs demonstrates that not only can the CHD function in multiple regulatory connections, but that also regulatory domains like GAF are connected to a variety of output modules. Such signal transduction units sometimes can be freely assembled like 'lego bricks'.

In the deadly pathogen *Mycobacterium tuberculosis,* 15 putative AC genes are present, 10 of which have yielded active ACs *in vitro* upon expression in *E. coli*. The activities of two isoforms, Rv1264 and Rv3645, have been found to be strongly modulated by adjacent regulatory domains. In Rv3645 a membrane anchor consisting of six putative transmembrane helices is linked to a class IIIb CHD via a HAMP domain (Fig. 1). HAMP domains are small signal transmitter modules which transfer extracellular signals to cytosolic effectors. In this respect the membrane anchor of Rv3645 may serve as a sensor, but a signal or ligand has not been identified. However, the HAMP domain itself has a large stimulatory effect on the activity of the CHD by increasing V_{max} 70-fold [126]. In addition, mutations in the HAMP domain targeted to hydrophobic epitopes further enhanced AC activity by a 7-fold gain in substrate affinity [126]. The strong effect of mutations in the HAMP domain on the CHD suggests that conformational alterations of HAMP elicited by the membrane domain will also modulate the AC activity of the Rv3645 holoenzyme.

Regulation of the mycobacterial AC isoform Rv1264 has been elucidated in detail biochemically and structurally [24, 127]. The enzyme contains a C-terminal class IIIc CHD and a novel N-terminal domain with no similarity to any other known protein except homologous ACs from other Gram-positive bacteria (Fig. 1). Initially the N-terminal domain was identified as an autoinhibitor of the enzyme because the holoenzyme is 200-fold less active than the CHD alone [127]. The signal which relieves autoinhibition was later shown to be a lowering of the pH [24]. Upon a shift from pH 8 to 6, the enzyme is activated 40-fold, mainly by an increase in V_{max}. Thus in biochemical terms Rv1264 is a pH-sensor AC. Curiously, pH sensitivity is not a property of the CHD but is conferred by the N-terminal domain. The molecular basis of regulation has been revealed by crystal structures of the Rv1264 holoenzyme in the active and inhibited states [24] (Fig. 4c). The N-terminal domain is α-helical and forms a tight platform-like dimer. In the active state the CHDs assume a closed dimeric state as required for the catalytic centres to form (see above). There are few contacts between the CHD dimer and the N-terminal platform, and the linker between the two domains adopts a random coil structure. Thus the CHD dimer can freely operate. In contrast in the inhibited state the CHD dimer is disrupted [24] (Fig. 4c). Both CHD monomers are bound to the regulatory platform and thus cannot catalyze. The linker switches to form an extended α-helix, which is essential for inhibition, because disruption by introduction of two proline residues yields a constitutively active mutant. The movement of the catalytic domains between the two states is dramatic: they rotate by 55° and are shifted by 6 Å. This is reminiscent of the proposed mechanism of regulation in mammalian membrane-bound ACs (see above), because in both cases AC activity is tuned by the relative orientation of two CHDs within a dimer. Reorientation of the CHDs occurs mainly by rotation, although to a different extent. Generally, dimerization and orientation of the CHDs appear to be universal features of class III AC catalysis and regulation. Therefore it can be anticipated that rotational mechanisms are also key to regulation of the other eukaryotic and bacterial ACs discussed so far and that more such mechanisms will be discovered in the future.

Mammalian soluble AC and other bicarbonate-regulated class III ACs

In the last 5 years bicarbonate has been recognized as a common modulator of the activity of certain class III ACs among metazoans, eukaryotic unicells and prokaryotes.

Mammalian soluble AC (sAC) was first purified from rat testes in its highly active 48 kDa 'small' form, which consists just of two class IIIb CHDs [6] (Fig. 1, see above). Molecular cloning revealed that the full-length enzyme consists of 187 kDa and contains a large autoinhibitory C-terminal without classified domains and that the small form is a result of alternative mRNA splicing (see above)

[6, 9, 10]. The biological function of soluble AC appears to be widespread, because the protein has been found in the nucleus, in mitochondria, associated with centrioles etc. and because sAC is distributed among a variety of tissues [128]. A *knockout* of sAC in mice causes male sterility by impaired sperm motility, while spermatogenesis is not affected [129]. This phenotype seems to be connected with the molecular mechanism of sAC regulation. Maturation and mobilization of released spermatozoa depends on extracellular bicarbonate and Ca^{2+} ions. Indeed, bicarbonate strongly activates mammalian sAC. The activity of the small form is stimulated 7–30-fold by $NaHCO_3$ (EC_{50} = 10–25 mM) due to an increase in V_{max} [130, 131]. In contrast, the role of activation of sAC by Ca^{2+} ions is less clear. Crude full-length sAC is stimulated about 2-fold by 10 μM Ca^{2+} in the absence of bicarbonate [132]. However, in the presence of bicarbonate a stronger 5-fold activation is observed for full-length and small sAC, which requires millimolar concentrations of Ca^{2+} ions (EC_{50} = 0.3–0.8 mM) [131, 132]. This stimulation is due to an increase in substrate affinity. At first glance it appears questionable whether such Ca^{2+} concentrations are relevant *in vivo*, but one should keep in mind that sAC has been found in mitochondria, which are well-established as calcium stores.

Because the small form of mammalian sAC, which does not contain any domains except two CHDs, is responsive to bicarbonate and Ca^{2+} ions, both regulators obviously act directly on the CHD heterodimer. A 3D structure of mammalian sAC has not yet been obtained, but the molecular mechanism of regulation has been studied with bicarbonate-activated bacterial ACs. The class IIIb AC isoform CyaC of *Spirulina platensis*, which is intramolecularly regulated by its histidine kinase and *receiver* domains (see above), is also stimulated 2.5-fold by bicarbonate (EC_{50} = 20 mM) [130]. Crystal structures of its catalytic domain have been solved with various bound ATP analogues in the absence and presence of bicarbonate [15]. In the absence of bicarbonate the CHD dimer is somewhat open and not apt for catalysis to proceed. Bicarbonate clearly closes the dimer and moves the catalytic residues into an arrangement more favourable for cyclization of ATP. Conformational changes occur in four elements of secondary structure, while the gross relative orientation of the monomers remains unchanged [15] (Fig. 4d). Thus the mechanism of activation is distinct of those for $G_s\alpha$ activation of mammalian membrane-bound ACs and pH activation of mycobacterial Rv1264 and is more similar to the conformational changes seen in CHD dimers upon binding of nucleotides (see above). Surprisingly, bicarbonate ion itself was not detectable in the crystal structures, and thus its binding site and its mode of interaction remain unclear. Bicarbonate activation of bacterial adenylyl cyclases is restricted to class IIIb, where a threonine or serine is in the position of the canonical purine-binding aspartate (Asp1018 in the mammalian AC structure, see above). Mutational analysis of the CHD of *Anabaena* CyaB1, which is activated 2.5-fold by bicarbonate, suggested that the ion indeed acts at the purine binding site to enhance substrate binding [133]. However, in the crystal structures of *Spirulina* CyaC the mode of purine binding is not affected by bicarbonate.

Recently published data show that some fungal class IIId CHDs can also be activated 2- to 20-fold by bicarbonate [134, 135]. Future research will elucidate whether the mechanism of activation of class IIId ACs is similar to that of class IIIb.

Like mammalian sAC *Spirulina platensis* CyaC is also activated by Ca^{2+} ions. The crystal structures show that Ca^{2+} replaces one of the two Mg^{2+} cofactors, which may enhance substrate binding an thus can explain the increase in substrate affinity elicited by Ca^{2+} [15]. Thus not only bicarbonate activation but also stimulation by Ca^{2+} is a conserved feature of some class IIIb ACs retained through evolution, be it in mammals or in bacteria.

Conclusion

Class III ACs show huge variations on a basic theme. Although the primary structures of the CHDs are highly divergent, they all adopt a similar fold, and the catalytic centres form at the interface of CHD dimers. Catalysis is dependent on a conserved two-metal ion mechanism with the aid of a transition-state stabilizing arginine. However, the purine-binding pockets and also ribose binding show a high degree of plasticity among the different types of class III ACs. Substrate binding occurs by an induced-fit mechanism, which reflects a high conformational flexibility of the CHDs that is also exploited in bicarbonate-stimulated ACs. The relative orientation of the two CHDs within the catalytically competent dimer is pivotal for regulation of class III ACs by intramolecular modules such as the pH-sensor domain in the mycobacterial Rv1264 AC isoform and by complex formation with regulatory proteins as in mammalian membrane-bound ACs. Thus regulation of class III ACs appears to exploit a common mechanism. Yet, the variability of the CHDs allowed for the evolution of highly specialized ACs which respond to individual signals and which are tailored to the needs of the respective organism. From a biochemical view there are major question left to be answered. For example, is it possible to trap the transition state in a crystal? What are the regulatory inputs for the many putative class III ACs obtained by genomic sequencing? Which conformational changes are triggered by regulatory domains and how are they transmitted to CHDs? The latter question has only been addressed in a few cases of regulation of mammalian ACs and with the mycobacterial Rv1264 AC. From a medical standpoint class III ACs have not yet been

well appreciated as drug targets. This may be due to the extreme difficulties encountered in the purification of holoenzymes of mammalian membrane-bound ACs, which would be needed for development and testing of isoform-specific activators and inhibitors, because soluble CHD heterodimers from single AC isoforms show very little activity. On the other hand, specific inhibition of ACs from unicellular pathogens may be a strategy for antiinfective therapy once AC isoforms are identified which are essential for the virulence of the respective pathogen, e.g. *M. tuberculosis*. Thus it will probably still take a long time until AC modulators are available which can be applied in medicine.

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1 Barzu, O. and Danchin, A. (1994) Adenylyl cyclases: a heterogeneous class of ATP-utilizing enzymes. Prog. Nucleic Acid Res. Mol. Biol. 49, 241–283.
2 Linder, J. U. and Schultz, J. E. (2003) The class III adenylyl cyclases: multi-purpose signalling modules. Cell. Signal. 15, 1081–1089.
3 Baker, D. A. and Kelly, J. M. (2004) Structure, function and evolution of microbial adenylyl and guanylyl cyclases. Mol. Microbiol. 52, 1229–1242.
4 Sunahara, R. K. and Taussig, R. (2002) Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. Mol. Interv. 2, 168–184.
5 Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W. J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R. and Gilman, A. G. (1989) Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science 244, 1558–1564.
6 Buck, J., Sinclair, M. L., Schapal, L., Cann, M. J. and Levin, L. R. (1999) Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc. Natl. Acad. Sci. USA 96, 79–84.
7 Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R. and Devreotes, P. N. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. Cell 69, 305–315.
8 Guo, Y. L., Seebacher, T., Kurz, U., Linder, J. U. and Schultz, J. E. (2001) Adenylyl cyclase Rv1625c of *Mycobacterium tuberculosis*: a progenitor of mammalian adenylyl cyclases. EMBO J. 20, 3667–3675.
9 Chaloupka, J. A., Bullock, S. A., Iourgenko, V., Levin, L. R. and Buck, J. (2006) Autoinhibitory regulation of soluble adenylyl cyclase. Mol. Reprod. Dev. 73, 361–368.
10 Jaiswal, B. S. and Conti, M. (2001) Identification and functional analysis of splice variants of the germ cell soluble adenylyl cyclase. J. Biol. Chem. 276, 31698–31708.
11 Kobayashi, M., Buck, J. and Levin, L. R. (2004) Conservation of functional domain structure in bicarbonate-regulated 'soluble' adenylyl cyclases in bacteria and eukaryotes. Dev. Genes Evol. 214, 503–509.
12 Kataoka, T., Broek, D. and Wigler, M. (1985) DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. Cell 43, 493–505.
13 Zhang, G., Liu, Y., Ruoho, A. E. and Hurley, J. H. (1997) Structure of the adenylyl cyclase catalytic core. Nature 386, 247–253.
14 Tesmer, J. J., Sunahara, R. K., Gilman, A. G. and Sprang, S. R. (1997) Crystal structure of the catalytic domains of adenylyl cyclase in a complex with $G_s\alpha \cdot GTP\gamma S$. Science 278, 1907–1916.
15 Steegborn, C., Litvin, T. N., Levin, L. R., Buck, J. and Wu, H. (2005) Bicarbonate activation of adenylyl cyclase via promotion of catalytic active site closure and metal recruitment. Nat. Struct. Mol. Biol. 12, 32–37.
16 Tesmer, J. J., Sunahara, R. K., Johnson, R. A., Gosselin, G., Gilman, A. G. and Sprang, S. R. (1999) Two-metal-ion catalysis in adenylyl cyclase. Science 285, 756–760.
17 Tesmer, J. J., Dessauer, C. W., Sunahara, R. K., Murray, L. D., Johnson, R. A., Gilman, A. G. and Sprang, S. R. (2000) Molecular basis for P-site inhibition of adenylyl cyclase. Biochemistry 39, 14464–14471.
18 Yan, S. Z., Huang, Z. H., Shaw, R. S. and Tang, W. J. (1997) The conserved asparagine and arginine are essential for catalysis of mammalian adenylyl cyclase. J. Biol. Chem. 272, 12342–12349.
19 Tucker, C. L., Hurley, J. H., Miller, T. R. and Hurley, J. B. (1998) Two amino acid substitutions convert a guanylyl cyclase, RetGC-1, into an adenylyl cyclase. Proc. Natl. Acad. Sci. USA 95, 5993–5997.
20 Tang, W. J., Stanzel, M. and Gilman, A. G. (1995) Truncation and alanine-scanning mutants of type I adenylyl cyclase. Biochemistry 34, 14563–14572.
21 Sunahara, R. K., Beuve, A., Tesmer, J. J., Sprang, S. R., Garbers, D. L. and Gilman, A. G. (1998) Exchange of substrate and inhibitor specificities between adenylyl and guanylyl cyclases. J. Biol. Chem. 273, 16332–16338.
22 Liu, Y., Ruoho, A. E., Rao, V. D. and Hurley, J. H. (1997) Catalytic mechanism of the adenylyl and guanylyl cyclases: modeling and mutational analysis. Proc. Natl. Acad. Sci. USA 94, 13414–13419.
23 Eckstein, F., Romaniuk, P. J., Heideman, W. and Storm, D. R. (1981) Stereochemistry of the mammalian adenylate cyclase reaction. J. Biol. Chem. 256, 9118–9120.
24 Tews, I., Findeisen, F., Sinning, I., Schultz, A., Schultz, J. E. and Linder, J. U. (2005) The structure of a pH-sensing mycobacterial adenylyl cyclase holoenzyme. Science 308, 1020–1023.
25 Bieger, B. and Essen, L. O. (2001) Structural analysis of adenylate cyclases from *Trypanosoma brucei* in their monomeric state. EMBO J. 20, 433–445.
26 Sinha, S. C., Wetterer, M., Sprang, S. R., Schultz, J. E. and Linder, J. U. (2005) Origin of asymmetry in adenylyl cyclases: structures of *Mycobacterium tuberculosis* Rv1900c. EMBO J. 24, 663–673.
27 Castro, L. I., Hermsen, C., Schultz, J. E. and Linder, J. U. (2005) Adenylyl cyclase Rv0386 from *Mycobacterium tuberculosis* H37Rv uses a novel mode for substrate selection. FEBS J. 272, 3085–3092.
28 Linder, J. U. (2005) Substrate selection by class III adenylyl cyclases and guanylyl cyclases. IUBMB Life 57, 797–803.
29 Kimura, Y., Mishima, Y., Nakano, H. and Takegawa, K. (2002) An adenylyl cyclase, CyaA, of *Myxococcus xanthus* functions in signal transduction during osmotic stress. J. Bacteriol. 184, 3578–3585.
30 Johnson, R. A. and Shoshani, I. (1990) Kinetics of 'P'-site-mediated inhibition of adenylyl cyclase and the requirements for substrate. J. Biol. Chem. 265, 11595–11600.
31 Dessauer, C. W. and Gilman, A. G. (1997) The catalytic mechanism of mammalian adenylyl cyclase. Equilibrium binding and kinetic analysis of P-site inhibition. J. Biol. Chem. 272, 27787–27795.
32 Dessauer, C. W., Tesmer, J. J., Sprang, S. R. and Gilman, A. G. (1999) The interactions of adenylate cyclases with P-site inhibitors. Trends Pharmacol. Sci. 20, 205–210.
33 Tang, W. J., Krupinski, J. and Gilman, A. G. (1991) Expression and characterization of calmodulin-activated (type I) adenylylcyclase. J. Biol. Chem. 266, 8595–8603.
34 Taussig, R., Quarmby, L. M. and Gilman, A. G. (1993) Regulation of purified type I and type II adenylylcyclases by G protein $\beta\gamma$ subunits. J. Biol. Chem. 268, 9–12.

35 Bakalyar, H. A. and Reed, R. R. (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science 250, 1403–1406.

36 Gao, B. N. and Gilman, A. G. (1991) Cloning and expression of a widely distributed (type IV) adenylyl cyclase. Proc. Natl. Acad. Sci. USA 88, 10178–10182.

37 Seebacher, T., Linder, J. U. and Schultz, J. E. (2001) An isoform-specific interaction of the membrane anchors affects mammalian adenylyl cyclase type V activity. Eur. J. Biochem. 268, 105–110.

38 Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M. and Krupinski, J. (1994) Type VIII adenylyl cyclase. A Ca^{2+}/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J. Biol. Chem. 269, 12190–12195.

39 Sunahara, R. K., Dessauer, C. W., Whisnant, R. E., Kleuss, C. and Gilman, A. G. (1997) Interaction of $G_s\alpha$ with the cytosolic domains of mammalian adenylyl cyclase. J. Biol. Chem. 272, 22265–22271.

40 Taussig, R., Iniguez-Lluhi, J. A. and Gilman, A. G. (1993) Inhibition of adenylyl cyclase by $G_{i\alpha}$. Science 261, 218–221.

41 Chen, J. and Iyengar, R. (1993) Inhibition of cloned adenylyl cyclases by mutant-activated G_i-α and specific suppression of type 2 adenylyl cyclase inhibition by phorbol ester treatment. J. Biol. Chem. 268, 12253–12256.

42 Taussig, R., Tang, W. J., Hepler, J. R. and Gilman, A. G. (1994) Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. J. Biol. Chem. 269, 6093–6100.

43 Dessauer, C. W., Tesmer, J. J., Sprang, S. R. and Gilman, A. G. (1998) Identification of a $G_i\alpha$ binding site on type V adenylyl cyclase. J. Biol. Chem. 273, 25831–25839.

44 Kozasa, T. and Gilman, A. G. (1995) Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of α_{12} and inhibition of adenylyl cyclase by α_z. J. Biol. Chem. 270, 1734–1741.

45 Tang, W. J. and Gilman, A. G. (1991) Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Science 254, 1500–1503.

46 Weitmann, S., Schultz, G. and Kleuss, C. (2001) Adenylyl cyclase type II domains involved in $G\beta\gamma$ stimulation. Biochemistry 40, 10853–10858.

47 Diel, S., Klass, K., Wittig, B. and Kleuss, C. (2006) $G\beta\gamma$ activation site in adenylyl cyclase type II. Adenylyl cyclase type III is inhibited by $G\beta\gamma$. J. Biol. Chem. 281, 288–294.

48 Chen, J., DeVivo, M., Dingus, J., Harry, A., Li, J., Sui, J., Carty, D. J., Blank, J. L., Exton, J. H., Stoffel, R. H., Inglese, J., Lefkowitz, R. J., Logothetis, D. E., Hildebrandt, J. D. and Iyengar, R. (1995) A region of adenylyl cyclase 2 critical for regulation by G protein $\beta\gamma$ subunits. Science 268, 1166–1169.

49 Weng, G., Li, J., Dingus, J., Hildebrandt, J. D., Weinstein, H. and Iyengar, R. (1996) $G\beta$ subunit interacts with a peptide encoding region 956–982 of adenylyl cyclase 2. Cross-linking of the peptide to free $G\beta\gamma$ but not the heterotrimer. J. Biol. Chem. 271, 26445–26448.

50 Bayewitch, M. L., Avidor-Reiss, T., Levy, R., Pfeuffer, T., Nevo, I., Simonds, W. F. and Vogel, Z. (1998) Inhibition of adenylyl cyclase isoforms V and VI by various $G\beta\gamma$ subunits. FASEB J. 12, 1019–1025.

51 Vorherr, T., Knopfel, L., Hofmann, F., Mollner, S., Pfeuffer, T. and Carafoli, E. (1993) The calmodulin binding domain of nitric oxide synthase and adenylyl cyclase. Biochemistry 32, 6081–6088.

52 Gu, C. and Cooper, D. M. (1999) Calmodulin-binding sites on adenylyl cyclase type VIII. J. Biol. Chem. 274, 8012–8021.

53 Smith, K. E., Gu, C., Fagan, K. A., Hu, B. and Cooper, D. M. (2002) Residence of adenylyl cyclase type 8 in caveolae is necessary but not sufficient for regulation by capacitative $Ca^{(2+)}$ entry. J. Biol. Chem. 277, 6025–6031.

54 Choi, E. J., Xia, Z. and Storm, D. R. (1992) Stimulation of the type III olfactory adenylyl cyclase by calcium and calmodulin. Biochemistry 31, 6492–6498.

55 Guillou, J. L., Nakata, H. and Cooper, D. M. (1999) Inhibition by calcium of mammalian adenylyl cyclases. J. Biol. Chem. 274, 35539–35545.

56 Gu, C. and Cooper, D. M. (2000) $Ca^{(2+)}$, $Sr^{(2+)}$, and $Ba^{(2+)}$ identify distinct regulatory sites on adenylyl cyclase (AC) types VI and VIII and consolidate the apposition of capacitative cation entry channels and $Ca^{(2+)}$-sensitive ACs. J. Biol. Chem. 275, 6980–6986.

57 Hu, B., Nakata, H., Gu, C., De Beer, T. and Cooper, D. M. (2002) A critical interplay between Ca^{2+} inhibition and activation by Mg^{2+} of AC5 revealed by mutants and chimeric constructs. J. Biol. Chem. 277, 33139–33147.

58 Ishikawa, Y., Katsushika, S., Chen, L., Halnon, N. J., Kawabe, J. and Homcy, C. J. (1992) Isolation and characterization of a novel cardiac adenylylcyclase cDNA. J. Biol. Chem. 267, 13553–13557.

59 Wittpoth, C., Scholich, K., Yigzaw, Y., Stringfield, T. M. and Patel, T. B. (1999) Regions on adenylyl cyclase that are necessary for inhibition of activity by $\beta\gamma$ and $G_{i\alpha}$ subunits of heterotrimeric G proteins. Proc. Natl. Acad. Sci. USA 96, 9551–9556.

60 Petrashevskaya, N. N., Koch, S. E., Bodi, I. and Schwartz, A. (2002) Calcium cycling, historic overview and perspectives. Role for autonomic nervous system regulation. J. Mol. Cell. Cardiol. 34, 885–896.

61 Bucchi, A., Baruscotti, M., Robinson, R. B. and DiFrancesco, D. (2003) I(f)-dependent modulation of pacemaker rate mediated by cAMP in the presence of ryanodine in rabbit sinoatrial node cells. J. Mol. Cell. Cardiol. 35, 905–913.

62 Zagotta, W. N., Olivier, N. B., Black, K. D., Young, E. C., Olson, R. and Gouaux, E. (2003) Structural basis for modulation and agonist specificity of HCN pacemaker channels. Nature 425, 200–205.

63 Krause, E. G., Bartel, S., Beyerdorfer, I., Freier, W., Gerber, K. and Obst, D. (1989) Transient changes in cyclic AMP and in the enzymic activity of protein kinase and phosphorylase during the cardiac cycle in the canine myocardium and the effect of propranolol. Mol. Cell. Biochem. 89, 181–186.

64 Jacobowitz, O. and Iyengar, R. (1994) Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. Proc. Natl. Acad. Sci. USA 91, 10630–10634.

65 Zimmermann, G. and Taussig, R. (1996) Protein kinase C alters the responsiveness of adenylyl cyclases to G protein α and $\beta\gamma$ subunits. J. Biol. Chem. 271, 27161–27166.

66 Jacobowitz, O., Chen, J., Premont, R. T. and Iyengar, R. (1993) Stimulation of specific types of G_s-stimulated adenylyl cyclases by phorbol ester treatment. J. Biol. Chem. 268, 3829–3832.

67 Choi, E. J., Wong, S. T., Dittman, A. H. and Storm, D. R. (1993) Phorbol ester stimulation of the type I and type III adenylyl cyclases in whole cells. Biochemistry 32, 1891–1894.

68 Yoshimura, M. and Cooper, D. M. (1993) Type-specific stimulation of adenylylcyclase by protein kinase C. J. Biol. Chem. 268, 4604–4607.

69 Lin, T. H., Lai, H. L., Kao, Y. Y., Sun, C. N., Hwang, M. J. and Chern, Y. (2002) Protein kinase C inhibits type VI adenylyl cyclase by phosphorylating the regulatory N domain and two catalytic C1 and C2 domains. J. Biol. Chem. 277, 15721–15728.

70 Chou, J. L., Huang, C. L., Lai, H. L., Hung, A. C., Chien, C. L., Kao, Y. Y. and Chern, Y. (2004) Regulation of type VI adenylyl cyclase by Snapin, a SNAP25-binding protein. J. Biol. Chem. 279, 46271–46279.

71 Iwami, G., Kawabe, J., Ebina, T., Cannon, P. J., Homcy, C. J. and Ishikawa, Y. (1995) Regulation of adenylyl cyclase by protein kinase, A. J. Biol. Chem. 270, 12481–12484.

72 Chen, Y., Harry, A., Li, J., Smit, M. J., Bai, X., Magnusson, R., Pieroni, J. P., Weng, G. and Iyengar R. (1997) Adenylyl cyclase 6 is selectively regulated by protein kinase A phosphorylation in a region involved in $G_{\alpha s}$ stimulation. Proc. Natl. Acad. Sci. USA 94, 14100–14104.

73 Wayman, G. A., Wei, J., Wong, S. and Storm, D. R. (1996) Regulation of type I adenylyl cyclase by calmodulin kinase IV in vivo. Mol. Cell. Biol. 16, 6075–6082.

74 Wayman, G. A., Hinds, T. R. and Storm, D. R. (1995) Hormone stimulation of type III adenylyl cyclase induces Ca^{2+} oscillations in HEK-293 cells. J. Biol. Chem. 270, 24108–24115.

75 Gu, C., Sorkin, A. and Cooper, D. M. (2001) Persistent interactions between the two transmembrane clusters dictate the targeting and functional assembly of adenylyl cyclase. Curr. Biol. 11, 185–190.

76 Gu, C., Cali, J. J. and Cooper, D. M. (2002) Dimerization of mammalian adenylate cyclases. Eur. J. Biochem. 269, 413–421.

77 Bertin, B., Freissmuth, M., Jockers, R., Strosberg, A. D. and Marullo, S. (1994) Cellular signaling by an agonist-activated receptor/$G_s\alpha$ fusion protein. Proc. Natl. Acad. Sci. USA 91, 8827–8831.

78 Chiono, M., Mahey, R., Tate, G. and Cooper, D. M. (1995) Capacitative Ca^{2+} entry exclusively inhibits cAMP synthesis in C6–2B glioma cells. Evidence that physiologically evoked Ca^{2+} entry regulates $Ca^{(2+)}$-inhibitable adenylyl cyclase in non-excitable cells. J. Biol. Chem. 270, 1149–1155.

79 Fagan, K. A., Mons, N. and Cooper, D. M. (1998) Dependence of the Ca^{2+}-inhibitable adenylyl cyclase of C6–2B glioma cells on capacitative Ca^{2+} entry. J. Biol. Chem. 273, 9297–9305.

80 Fagan, K. A., Smith, K. E. and Cooper, D. M. (2000) Regulation of the Ca^{2+}-inhibitable adenylyl cyclase type VI by capacitative Ca^{2+} entry requires localization in cholesterol-rich domains. J. Biol. Chem. 275, 26530–26537.

81 Crossthwaite, A. J., Seebacher, T., Masada, N., Ciruela, A., Dufraux, K., Schultz, J. E. and Cooper, D. M. (2005) The cytosolic domains of Ca^{2+}-sensitive adenylyl cyclases dictate their targeting to plasma membrane lipid rafts. J. Biol. Chem. 280, 6380–6391.

82 Davare, M. A., Avdonin, V., Hall, D. D., Peden, E. M., Burette, A., Weinberg, R. J., Horne, M. C., Hoshi, T. and Hell, J. W. (2001) A β2 adrenergic receptor signaling complex assembled with the Ca^{2+} channel $Ca_v1.2$. Science 293, 98–101.

83 Cooper, D. M. (2005) Compartmentalization of adenylate cyclase and cAMP signalling. Biochem. Soc. Trans. 33, 1319–1322.

84 Crossthwaite, A. J., Ciruela, A., Rayner, T. F. and Cooper, D. M. (2006) A direct interaction between the N terminus of adenylyl cyclase AC8 and the catalytic subunit of protein phosphatase 2A. Mol. Pharmacol. 69, 608–617.

85 Roy, A. A., Baragli, A., Bernstein, L. S., Hepler, J. R., Hebert, T. E. and Chidiac, P. (2006) RGS2 interacts with G_s and adenylyl cyclase in living cells. Cell. Signal. 18, 336–348.

86 Abramow-Newerly, M., Roy, A. A., Nunn, C. and Chidiac, P. (2006) RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. Cell. Signal. 18, 579–591.

87 Scholich, K., Pierre, S. and Patel, T. B. (2001) Protein associated with Myc (PAM) is a potent inhibitor of adenylyl cyclases. J. Biol. Chem. 276, 47583–47589.

88 Sampson, L. J., Hayabuchi, Y., Standen, N. B. and Dart, C. (2004) Caveolae localize protein kinase A signaling to arterial ATP-sensitive potassium channels. Circ. Res. 95, 1012–1018.

89 Thevelein, J. M. and de Winde, J. H. (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. 33, 904–918.

90 Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A. and Wigler, M. (1988) Purification of a RAS-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. 8, 2159–2165.

91 Heideman, W., Casperson, G. F. and Bourne, H. R. (1990) Adenylyl cyclase in yeast: antibodies and mutations identify a regulatory domain. J. Cell. Biochem. 42, 229–242.

92 Mintzer, K. A. and Field, J. (1994) Interactions between adenylyl cyclase, CAP and RAS from Saccharomyces cerevisiae. Cell. Signal. 6, 681–694.

93 Colombo, S., Ronchetti, D., Thevelein, J. M., Winderickx, J. and Martegani, E. (2004) Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*. J. Biol. Chem. 279, 46715–46722.

94 Shima, F., Yamawaki-Kataoka, Y., Yanagihara, C., Tamada, M., Okada, T., Kariya, K. and Kataoka, T. (1997) Effect of association with adenylyl cyclase-associated protein on the interaction of yeast adenylyl cyclase with Ras protein. Mol. Cell. Biol. 17, 1057–1064.

95 Ivey, F. D. and Hoffman, C. S. (2005) Direct activation of fission yeast adenylate cyclase by the Gpa2 Galpha of the glucose signaling pathway. Proc. Natl. Acad. Sci. USA 102, 6108–6113.

96 Dubacq, C., Guerois, R., Courbeyrette, R., Kitagawa, K. and Mann, C. (2002) Sgt1p contributes to cyclic AMP pathway activity and physically interacts with the adenylyl cyclase Cyr1p/Cdc35p in budding yeast. Eukaryot. Cell 1, 568–582.

97 Kriebel, P. W. and Parent, C. A. (2004) Adenylyl cyclase expression and regulation during the differentiation of *Dictyostelium discoideum*. IUBMB Life 56, 541–546.

98 Saran, S., Meima, M. E., Alvarez-Curto, E., Weening, K. E., Rozen, D. E. and Schaap, P. (2002) cAMP signaling in *Dictyostelium*. Complexity of cAMP synthesis, degradation and detection. J. Muscle Res. Cell Motil. 23, 793–802.

99 Insall, R., Kuspa, A., Lilly, P. J., Shaulsky, G., Levin, L. R., Loomis, W. F. and Devreotes, P. (1994) CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. J. Cell Biol. 126, 1537–1545.

100 Saran, S. and Schaap, P. (2004) Adenylyl cyclase G is activated by an intramolecular osmosensor. Mol. Biol. Cell 15, 1479–1486.

101 Söderbom, F., Anjard, C., Iranfar, N., Fuller, D. and Loomis, W. F. (1999) An adenylyl cyclase that functions during late development of *Dictyostelium*. Development 126, 5463–5471.

102 Meima, M. E. and Schaap, P. (1999) Fingerprinting of adenylyl cyclase activities during *Dictyostelium* development indicates a dominant role for adenylyl cyclase B in terminal differentiation. Dev. Biol. 212, 182–190.

103 Iseki, M., Matsunaga, S., Murakami, A., Ohno, K., Shiga, K., Yoshida, K., Sugai, M., Takahashi, T., Hori, T. and Watanabe, M. (2002) A blue-light-activated adenylyl cyclase mediates photoavoidance in *Euglena gracilis*. Nature 415, 1047–1051.

104 Ito, S., Murakami, A., Sato, K., Nishina, Y., Shiga, K., Takahashi, T., Higashi, S., Iseki, M. and Watanabe, M. (2005) Photocycle features of heterologously expressed and assembled eukaryotic flavin-binding BLUF domains of photoactivated adenylyl cyclase (PAC), a blue-light receptor in *Euglena gracilis*. Photochem. Photobiol. Sci. 4, 762–769.

105 Yoshikawa, S., Suzuki, T., Watanabe, M. and Iseki, M. (2005) Kinetic analysis of the activation of photoactivated adenylyl cyclase (PAC), a blue-light receptor for photomovements of *Euglena*. Photochem. Photobiol. Sci. 4, 727–731.

106 Alexandre, S., Paindavoine, P., Tebabi, P., Pays, A., Halleux, S., Steinert, M. and Pays, E. (1990) Differential expression of a family of putative adenylate/guanylate cyclase genes in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 43, 279–288.

107 Paindavoine, P., Rolin, S., Van Assel, S., Geuskens, M., Jauniaux, J. C., Dinsart, C., Huet, G. and Pays, E. (1992) A gene from the variant surface glycoprotein expression site encodes one of several transmembrane adenylate cyclases located on the flagellum of *Trypanosoma brucei*. Mol. Cell. Biol. 12, 1218–1225.

108 Sanchez, M. A., Zeoli, D., Klamo, E. M., Kavanaugh, M. P. and Landfear, S. M. (1995) A family of putative receptor-adenylate cyclases from *Leishmania donovani*. J. Biol. Chem. 270, 17551–17558.

109 Seebeck, T., Schaub, R. and Johner, A. (2004) cAMP signalling in the kinetoplastid protozoa. Curr. Mol. Med. 4, 585–599.

110 Nolan, D. P., Rolin, S., Rodriguez, J. R., Van Den Abbeele, J. and Pays, E. (2000) Slender and stumpy bloodstream forms of *Trypanosoma brucei* display a differential response to extracellular acidic and proteolytic stress. Eur. J. Biochem. 267, 18–27.

111 Rolin, S., Hanocq-Quertier, J., Paturiaux-Hanocq, F., Nolan, D., Salmon, D., Webb, H., Carrington, M., Voorheis, P. and Pays, E. (1996) Simultaneous but independent activation of adenylate cyclase and glycosylphosphatidylinositol-phospholipase C under stress conditions in *Trypanosoma brucei*. J. Biol. Chem. 271, 10844–10852.

112 Naula, C., Schaub, R., Leech, V., Melville, S. and Seebeck, T. (2001) Spontaneous dimerization and leucine-zipper induced activation of the recombinant catalytic domain of a new adenylyl cyclase of *Trypanosoma brucei*, GRESAG4.4B. Mol. Biochem. Parasitol. 112, 19–28.

113 Schultz, J. E., Klumpp, S., Benz, R., Schürhoff-Goeters, W. J. and Schmid, A. (1992) Regulation of adenylyl cyclase from *Paramecium* by an intrinsic potassium conductance. Science 255, 600–603.

114 Weber, J. H., Vishnyakov, A., Hambach, K., Schultz, A., Schultz, J. E. and Linder, J. U. (2004) Adenylyl cyclases from *Plasmodium*, *Paramecium* and *Tetrahymena* are novel ion channel/enzyme fusion proteins. Cell. Signal. 16, 115–125.

115 Shenoy, A. R. and Visweswariah, S. S. (2004) Class III nucleotide cyclases in bacteria and archaebacteria: lineage-specific expansion of adenylyl cyclases and a dearth of guanylyl cyclases. FEBS Lett. 561, 11–21.

116 Smith, R. S., Wolfgang, M. C. and Lory, S. (2004) An adenylate cyclase-controlled signaling network regulates *Pseudomonas aeruginosa* virulence in a mouse model of acute pneumonia. Infect. Immun. 72, 1677–1684.

117 Kimura, Y., Ohtani, M. and Takegawa, K. (2005) An adenylyl cyclase, CyaB, acts as an osmosensor in *Myxococcus xanthus*. J. Bacteriol. 187, 3593–3598.

118 Katayama, M. and Ohmori, M. (1997) Isolation and characterization of multiple adenylate cyclase genes from the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. 179, 3588–3593.

119 Kasahara, M., Unno, T., Yashiro, K. and Ohmori, M. (2001) CyaG, a novel cyanobacterial adenylyl cyclase and a possible ancestor of mammalian guanylyl cyclases. J. Biol. Chem. 276, 10564–10569.

120 Kasahara, M., Yashiro, K., Sakamoto, T. and Ohmori, M. (1997) The *Spirulina platensis* adenylate cyclase gene, cyaC, encodes a novel signal transduction protein. Plant Cell Physiol. 38, 828–836.

121 Kasahara, M. and Ohmori, M. (1999) Activation of a cyanobacterial adenylate cyclase, CyaC, by autophosphorylation and a subsequent phosphotransfer reaction. J. Biol. Chem. 274, 15167–15172.

122 Kanacher, T., Schultz, A., Linder, J. U. and Schultz, J. E. (2002) A GAF-domain-regulated adenylyl cyclase from *Anabaena* is a self- activating cAMP switch. EMBO J. 21, 3672–3680.

123 Bruder, S., Linder, J. U., Martinez, S. E., Zheng, N., Beavo, J. A. and Schultz, J. E. (2005) The cyanobacterial tandem GAF domains from the cyaB2 adenylyl cyclase signal via both cAMP-binding sites. Proc. Natl. Acad. Sci. USA 102, 3088–3092.

124 Martinez, S. E., Bruder, S., Schultz, A., Zheng, N., Schultz, J. E., Beavo, J. A. and Linder, J. U. (2005) Crystal structure of the tandem GAF domains from a cyanobacterial adenylyl cyclase: modes of ligand binding and dimerization. Proc. Natl. Acad. Sci. USA 102, 3082–3087.

125 Martinez, S. E., Wu, A. Y., Glavas, N. A., Tang, X. B., Turley, S., Hol, W. G. and Beavo, J. A. (2002) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. Proc. Natl. Acad. Sci. USA 99, 13260–13265.

126 Linder, J. U., Hammer, A. and Schultz, J. E. (2004) The effect of HAMP domains on class IIIb adenylyl cyclases from *Mycobacterium tuberculosis*. Eur. J. Biochem. 271, 2446–2451.

127 Linder, J. U., Schultz, A. and Schultz, J. E. (2002) Adenylyl cyclase Rv1264 from *Mycobacterium tuberculosis* has an autoinhibitory N-terminal domain. J. Biol. Chem. 277, 15271–15276.

128 Zippin, J. H., Chen, Y., Nahirney, P., Kamenetsky, M., Wuttke, M. S., Fischman, D. A., Levin, L. R. and Buck, J. (2003) Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. FASEB J. 17, 82–84.

129 Esposito, G., Jaiswal, B. S., Xie, F., Krajnc-Franken, M. A., Robben, T. J., Strik, A. M., Kuil, C., Philipsen, R. L., van Duin, M., Conti, M. and Gossen, J. A. (2004) Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. Proc. Natl. Acad. Sci. USA 101, 2993–2998.

130 Chen, Y., Cann, M. J., Litvin, T. N., Iourgenko, V., Sinclair, M. L., Levin, L. R. and Buck, J. (2000) Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science 289, 625–628.

131 Litvin, T. N., Kamenetsky, M., Zarifyan, A., Buck, J. and Levin, L. R. (2003) Kinetic properties of 'soluble' adenylyl cyclase. Synergism between calcium and bicarbonate. J. Biol. Chem. 278, 15922–15926.

132 Jaiswal, B. S. and Conti, M. (2003) Calcium regulation of the soluble adenylyl cyclase expressed in mammalian spermatozoa. Proc. Natl. Acad. Sci. USA 100, 10676–10681.

133 Cann, M. J., Hammer, A., Zhou, J. and Kanacher, T. (2003) A defined subset of adenylyl cyclases is regulated by bicarbonate ion. J. Biol. Chem. 278, 35033–35038.

134 Mogensen, E. G., Janbon, G., Chaloupka, J., Steegborn, C., Fu, M.S., Moyrand, F., Klengel, T., Pearson, D. S., Geeves, M. A., Buck, J., Levin, L. R. and Mühlschlegel, F. A. (2006) *Cryptococcus neoformans* senses CO_2 through the carbonic anhydrase Can2 and the adenylyl cyclase Cac1. Eukaryot. Cell 5, 103–111.

135 Klengel, T., Liang, W. J., Chaloupka, J., Ruoff, C., Schroppel, K., Naglik, J. R., Eckert, S. E., Mogensen, E. G., Haynes, K., Tuite, M. F., Levin, L. R., Buck, J. and Mühlschlegel, F. A. (2005) Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signaling and virulence. Curr. Biol. 15, 2021–2026.

136 Zhulin, I. B., Nikolskaya, A. N. and Galperin, M. Y. (2003) Common extracellular sensory domains in transmembrane receptors for diverse signal transduction pathways in bacteria and archaea. J. Bacteriol. 185, 285–294.