Characterization of transmembrane domains of class IIIa adenylyl cyclase: A three state model

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Anubha Seth

aus

Delhi, India

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Dedicated to my Dad

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TABLE OF CONTENTS

Abbreviation

AC	Adenylyl cyclase
BSA	Bovine serum albumin
CAI-1	*Cholera*-Autoinducer-1
CqsS	*Cholera* quorum sensing sensor
CTE	cyclase-transducing-element
FSK	forskolin
G protein	heterotrimeric guanine nucleotide regulatory protein
GPCRs	G-protein coupled receptors
Gsα	constitutively active $Q_{227}L$ mutant of G protein α subunit (used in assays in this thesis work)
hAC	human adenylyl cyclase
HS	human serum
LAI-1	*Legionella* Autoinducer-1
LqsS	*Legionella* quorum sensing sensor
mAC	mammalian adenylyl cyclase
Sf9	*Spodoptera frugiperda*, insect cells
TCS	two-component system
TH	thyroid hormone
TM	transmembrane domain

SUMMARY

cAMP uses a plethora of ways to be integrated into regulatory systems hence becoming a universal second messenger. Generation of such a crucial moiety is via a widespread signaling protein, adenylyl cyclase (AC). Classically, a membrane-bound AC has a N-terminal transmembrane domain (TM) and a transducer domain which are linked to the C-terminal catalytic domain. And, these features are dominantly displayed in pharmacologically most relevant species i.e. mammalian ACs.

Mammalian ACs (mACs) have nine membrane-bound isoforms, with two dissimilar hexahelical membrane anchors that are isoform-specifically conserved for ›500 million years and which hold ≈40% protein. For which a physiological function is not known. So, a typical mode of regulation for this enzyme known is indirect i.e. via GPCRs (where G-protein α subunit, Gsα, is released and binds to the catalytic domain of an AC).

Since, understanding the myriad aspects of cAMP signaling concerning the regulation of its enzyme, the role of the hexahelical membrane domains has remained enigmatic. Despite many speculations that were made regarding the role of these huge transmembrane helices no role was substantiated. A direct investigation for the regulatory function of these TMs was seemingly unapproachable as no ligand was known.

Therefore, I exchanged both membrane anchors of the human adenylyl cyclase isoform 2 (hAC2) with the membrane domain of the quorum-sensing receptor from *Vibrio harveyi*, CqsS, for which the ligand is known, CAI-1. A functional chimera was attained, where cyclase activity was stimulated by Gsα but CAI-1 by itself had no effect.

Here I showcase a new layer of regulation of adenylyl cyclase: where CAI-1 inhibited Gsα-stimulated cyclase activity thus shifting the concentration-response curve to the right and attenuating the maximal response.

The results identify a potential regulation of mACs by the membrane anchors, as receptors, and Gsα; thus, making each mAC isoform approachable for the search of the ligands.

Further bolstering the hypothesis, I report serum inhibiting AC activity in membranes isolated from rat brain cortex. Concomitantly, another cyclase stimulating factor, forskolin, was tested. Using hAC2 expressed in Sf9 cells a similar pattern of inhibition was observed in the presence of serum i.e. lowering stimulation by both, forskolin and forskolin + Gsα. Thus, marking the serum as a potential fluid to search for the ligands.

Taken together, the data determine a putative pathway of signaling via AC membrane anchors as receptors to the catalytic domain. This resulted in a three-state model, defining a new orchestrated form of AC regulation and an approach to search for ligands, 31 years after its sequencing.

Zusammenfassung

cAMP nutzt eine Vielzahl von Möglichkeiten, um in Regulierungssysteme integriert zu werden und wird so zu einem universellen sekundären Botenstoff. Die Biosynthese einer solchen zentralen Komponente erfolgt, Adenylylcyclasen (ACn). Die membrangebundene AC hat einen cytosolische N-Terminus gefolgt von einer hexahelikalen Transmembraneinheit (TM). Daran schließt sich die Sequenz eines„ cyclase-transducing element“ (CTE) an, die mit einer katalytischen Domäne verknüpft ist. Zwei dieser miteinander verbundenen Gesamteinheiten ergibt eine pseudoheterodimere Säugetier-AC (Mammalia-AC = mACs).

mACs gibt es neun Isoformen membrangebundener ACn mit jeweils zwei unterschiedlichen Membrananker, die seit über 500 Millionen Jahren isoform-spezifisch konserviert sind und etwa 40% des Proteins ausmachen. Eine regulatorische Funktion dieser Domänen ist unbekannt. Die Regulation für mACn erfolgt indirekt über GPCRs. Hierbei wird die G-Protein-Untereinheit Gsα freigesetzt wird und das katalytische Dimer aktiviert.

Trotz Spekulationen über eine regulatorische Funktion der Membrandomänen konnte diese bisher nicht bestätigt werden. Eine direkte Untersuchung der Bestimmung dieser TMs war unerreichbar, da kein Ligand bekannt war.

Ich tauschte beide Membrananker der humanen Adenylylcyclase Isoform 2 (hAC2) gegen die isostere Membrandomäne des *Quorum-Sensing*-Rezeptors von *Vibrio harveyi*, CqsS, für den CAI-1 als Ligand bekannt ist, aus. Es wurde eine funktionelle Chimäre erzeugt, bei der die Cyclase durch Gsα stimuliert wurde, CAI-1 selbst jedoch keinen Effekt hatte.

Ich zeige eine neue Regulationsebene von mACn auf: CAI-1 hemmte die Gsα-stimulierte Cyclase-Aktivität. Die Konzentrations-Wirkungs-Kurve wurde nach rechts verschoben und die maximale Aktivierung verringert.

Die Ergebnisse identifizieren beide Membrananker als eine Rezeptoreinheit; somit wird jede membrangebundene mAC-Isoform für die Suche nach Liganden zugänglich.

Um die Hypothese weiter zu untermauern, konnte ich zeigen, dass Serum die AC-Aktivität in Membranen hemmt, die aus der Hirnrinde von Ratten isoliert wurden. Gleichzeitig wurde ein weiterer Cyclase-stimulierender Faktor, Forskolin, getestet. Unter Verwendung von in Sf9-Zellen exprimierter hAC2 wurde ein ähnliches Inhibitionsmuster in Gegenwart von Serum beobachtet, d. h. eine Verringerung der Stimulation sowohl durch Forskolin als auch durch Forskolin + Gsα. Daher kann Serum für die Suche nach Liganden verwendet werden.

Zusammengefasst zeigen die Daten einen mutmaßlichen Signalweg über AC-Membrananker als Rezeptoren auf. Die Ergebnisse führen zu einem Modell mit drei definierten Aktivitätsniveaus, welches 31 Jahre nach der ersten AC-Sequenzierung eine neue Form der AC-Regulation aufzeigt und einen Ansatz zur Ligandensuche etabliert.

1 INTRODUCTION

1.1 Signal Transduction

Communication is the most essential facet of biological life. All cells, whether prokaryotic or eukaryotic, are enveloped by a membrane. To communicate across, relay of signals are transduced which allow the cell to receive and respond to the constantly changing environment. This transduction is maintained via continuous biochemical modification within the cell or change in the membrane potential due to the mobility of ions in and out of the cell. One of the prominent modes of transmission is by protein phosphorylation in which a protein (receptor/sensor) detects the stimuli, initiated upon receptor binding of a ligand (primary messenger), which in turn activates the biochemical cascade carried out by intracellular second messengers or signaling intermediates (Dennis, 2003). These biochemical changes that are brought about by either direct changes in intrinsic enzymatic activities or by stimulating formation of intracellular messenger molecules (Heldin et al., 2016).

One-component systems are a mode of transduction in which signal input and response output are managed by the same protein where the activity is defined by binding or removal of the ligand (Ulrich et al., 2005). Other systems, mostly in prokaryotes and in some eukaryotes, possess a two-component system, in which sensory domain and response regulator are physically distinct. The transduction of signal is achieved by phosphotransfer between the two proteins, a sensor histidine kinase and a response regulator (Stock et al., 2000).

Now, depending on the signal received, transmitted and produced the domains are termed as input or receiver, transducer, and output domains. Depending on these cues the signaling proteins are categorized into different families such as adenylyl cyclase, histidine kinase, etc. Similarly, there are transducer domains such as HAMP, CTEs, etc. that are present in their respective families. In a similar fashion, each signaling pathway has second messengers, exclusive in prokaryotes (cyclic diguanylate (c-di-GMP), etc.) and some present in both pro- and eukaryotic

cells. One such universal messenger is known as 3',5'-cyclic adenosine monophosphate (cAMP) (Tamayo et al., 2007).

1.2 Adenylyl Cyclase

In late 1950s Sutherland and Rall discovered cAMP while studying glycogen metabolism in the liver that led to our existing notion of hormone signaling through second messengers (Sutherland and Rall, 1958). Irrespective of the discovery of other second messengers, cAMP has never left the limelight (Hurley, 1999). Not only because of being extensively involved in the regulation of diverse physiological responses but also because of the limitations associated with the understanding of direct regulation of the enzyme, adenylyl cyclases (ACs).
Adenylyl cyclases are the most polyphyletic known enzyme (Barzu and Danchin, 1994), all catalyzing the same reaction, using ATP as a substrate and Mg^{2+}/ Mn^{2+} as a metal cofactor, which leads to the generation of cAMP and pyrophosphate (Tesmer et al., 1999).

1.2.1 Class III AC: multipurpose signaling unit

Despite all the AC proteins catalyzing the same reaction the assumption of the homologous enzyme was not true. ACs were divided into six distinct classes mostly on a sequence basis, out of which the most extensively studied class is class III. The other five classes had a rather small share of the attention mostly because they're present in a limited range of prokaryotic species (Finkbeiner et al., 2019; Linder and Schultz, 2003; Sinha and Sprang, 2006). Class III ACs are the most diverse, structurally and functionally, numerically the largest and pharmacologically the most substantial one. Not only it is the only class present in mammals, but also in most eukaryotes, eubacteria and in many prokaryotes (Barzu and Danchin, 1994; Finkbeiner et al., 2019; Sinha and Sprang, 2006). Therefore, class III is best characterized, structurally and biochemically. Furthermore, they're divided into

soluble and membrane-bound ACs, with a major emphasis on mammalian ACs. The most broadly recognized subclassification of class III ACs is built on sequence similarities. This resulted in four subclasses, IIIa-IIId (Bassler et al., 2018). In general, the catalytic domains are rather conserved and are also related to bacterial diguanylate cyclase, which give us a hint on a common ancestry (Figure 1).

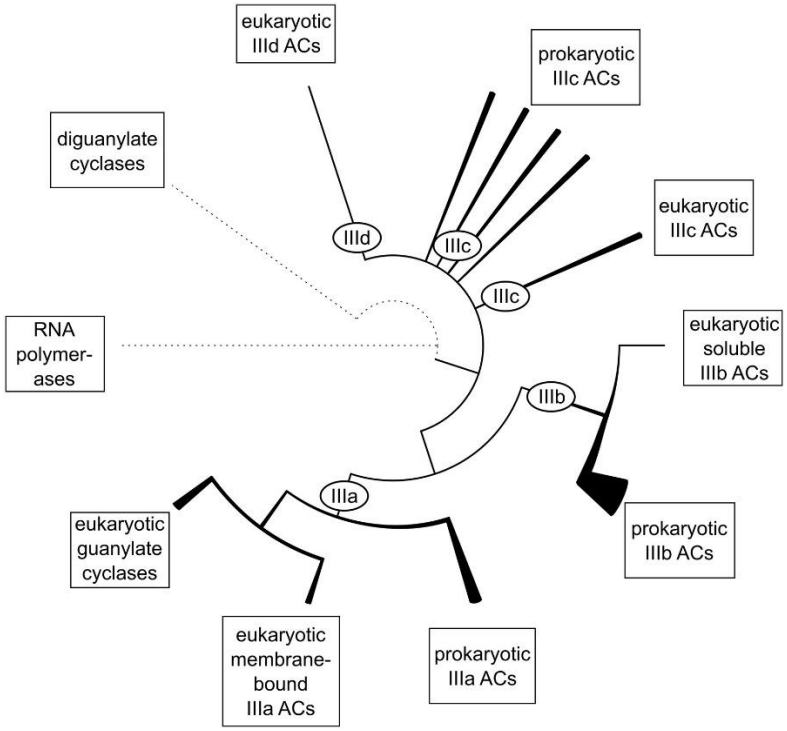

Figure 1. *The evolutionary linkage of class III ACs between the catalytic domains. (solid line) indicate relations between major subdivisions, (line thickness) indicate the diversity within a branch among domain architectures and (dotted line) represent remote homology to other protein groups (*source Bassler et al., 2018).

Major domain organization in class III ACs

All ACs in this class must dimerize for activity (Finkbeiner et al., 2019; Steegborn et al., 2005; Tesmer et al., 1999). At the interface of the dimer the catalytic reaction take place for which both cyclase homology domain (CHD) provide catalytic residues (Linder and Schultz, 2003; Tesmer et al., 1999).

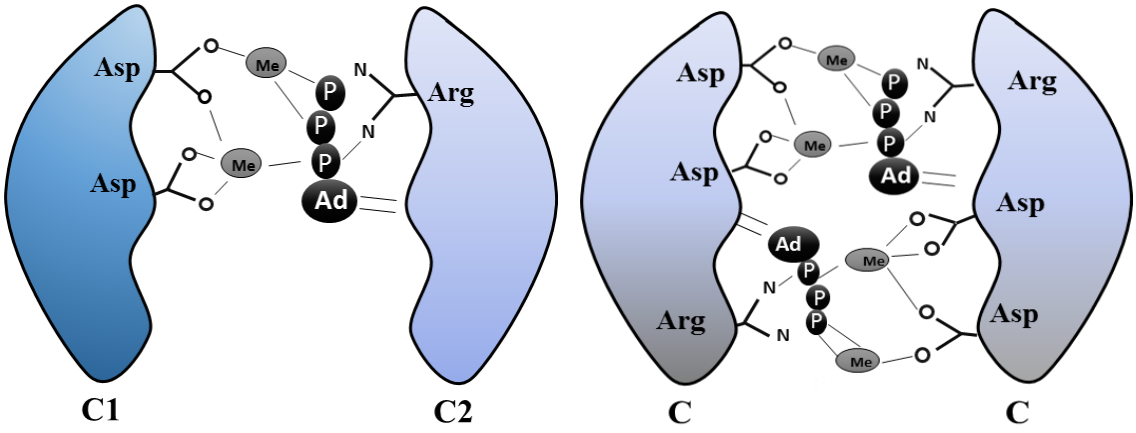

Figure 2. *Schematic representation of class III adenylyl cyclase catalytic domains.* ***Left:*** *Is a heterodimer, present in eukaryotic ACs, with one catalytic center forming at the interface of the catalytic domain (C1 and C2); ATP binds to the catalytic pocket whereas forskolin binds to second, non-catalytic, pocket (not shown).* ***Right****: Homodimeric catalytic domain representing two catalytic centers. Ad: adenosine, Me: divalent metal cofactor, P: phosphate group. Six amino acid residues are known in ACs to play a major role in catalysis. Two aspartates (Asp) bind with to cofactors and stabilize the transition state by pairing with an arginine (Arg) residue provided by the contralateral cyclase domain (adapted* from Linder and Schultz, 2003).

The crucial differences between ACs from eukaryotes and prokaryotes are that the former membrane-bound ACs are pseudoheterodimers with two transmembrane domains connected, respectively, C-terminally to catalytically inactive domains which form a single catalytic center, and the latter is homodimeric where two

monomers dimerize for activity and form two catalytic centers (Guo et al., 2001; Krupinski et al., 1989; Whisnant et al., 1996; Zhang et al., 1997).

As we know, bacterial ACs show large architectural and domain diversity in comparison to mACs (Bassler et al., 2018). The diversity in membrane bound ACs is predominantly restricted to the two hexahelical membrane domains (Figure 3).

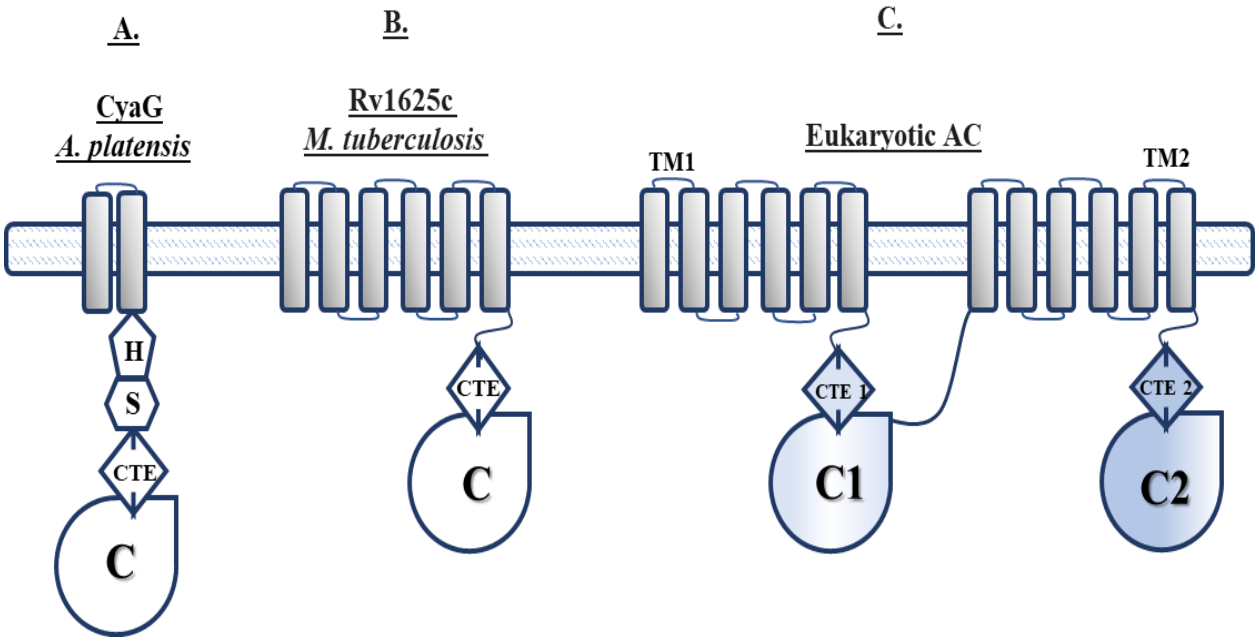

Figure 3: *Schematic representation of class IIIa adenylyl cyclases membrane domains. Bacterial ACs (A; B) are monomeric. (C) represent a typical eukaryotic pseudoheterodimer with 2 hexahelical transmembrane domains. Rv1625c represents half of a membrane-bound, eukaryotic AC. TM helices are denoted by bars. H = HAMP domain, C = catalytic domain, S = S helix, CTE= cyclase transducing element. C1 = Catalytic domain 1, C2 = catalytic domain 2 (adapted* from figure 1 Finkbeiner M, Grischin J, Seth A, Schultz JE, 2019*)*

1.2.2 Modularity in bacterial signaling

Bacterial class III ACs are monomers that must dimerize to become active. These monomers have a single cyclase homology domain hence all six catalytic residues critical for an active dimer are present on a single protein subunit in many bacterial species (e.g.: mycobacterial Rv1264, Rv1625c) (Guo et al., 2001; Linder and Schultz, 2003). Typically, they are modular multi-domain proteins comprising additional domains (Tesmer et al., 1997; Zhang et al., 1997) with domain organizations from 2TMs, 4TMs to 6TMs, they display features related to mammalian membrane-delimited ACs (Bassler et al., 2018; Schultz and Natarajan, 2013). Mammalian ACs are known to generally be regulated indirectly by GPCRs. Such a general principle of regulation is missing in bacterial ACs, certainly due to lack of G-proteins. In fact, the regulation of bacterial ACs is poorly understood in comparison to mammalian ACs. So far, only a few factors, all cytosolic, are identified that regulate the activity of full-length bacterial ACs (Kanacher et al., 2002; Linder et al., 2002; Ohki et al., 2016; Tews et al., 2005).

Generally, bacterial two-component sensory systems retort to environmental cues, such as changes in osmolarity, pH, or redox potential e.g. mycobacterial Rv1264 is activated by low pH (Linder et al., 2002;Tews et al., 2005).

As the field advanced, transmembrane domains were more and more considered as likely input domains. For example, bacterial ACs possessing 2TMs and 4TMs have sizable extracellular loops that often are recognized as domains that have been identified as sensors in other protein signaling families (Anantharaman and Aravind, 2001, 2000; Mougel and Zhulin, 2001; Zhulin et al., 2003). Analogous to 2TMs and 4TMs bacterial ACs, 6TMs are also considered to be putative input domains (Kanchan et al., 2010), yet no direct ligand for these enormous 6 bundles in adenylyl cyclase is known.

Irrespective of these speculations the role of TM domains in ACs remained enigmatic.

1.2.3 Mammalian ACs

The first structures of the mammalian AC catalytic core (Tesmer et al., 1997; Zhang et al., 1997) established a new biochemical understanding of the actions of regulators (G-protein, forskolin, etc.). The insights on the catalytic pathway of an enzyme is a prerequisite to understanding its regulation (Hurley, 1999).

Eukaryotic ACs are much less diverse in comparison to bacterial ACs and their domain organization is rather conserved. In mammals, only two types are present throughout; membrane-bound and soluble ACs. There are ten distinct isoforms of mACs in which isoforms 1-9 are membrane bound proteins while isoform 10 supposedly is a soluble protein. The nine isoforms share the same domain organization, an intracellular N-terminus than six transmembrane α-helices (TM1) which are followed by a domain specific transducer, CTE-1, connecting to the cytosolic catalytic domain (C1), which in turn is connected to another hexahelical domain (TM2) that is linked via a specific CTE-2 to the second cytosolic catalytic domain (C2) (figure 3C and 4) (Krupinski et al., 1989; Ziegler et al., 2017).

Catalytic domains

The catalytic domains represented as C1 and C2 are subdivided into C1a and C1b; and C2a and C2b. The C1b is a large region (~15 kDa), acts as a linker and contains several regulatory sites (Hurley, 1999). C1a and C2a are conserved, similar, and have all the catalytic gear (Tang and Gilman, 1995). This means changes in the relative orientation of C1 and C2 domains to each other by any factor will affect the structure of the active site which in turn will affect the enzymatic activity. The hybrid crystal complex between mAC5(C1) and mAC2(C2) in presence of G protein and forskolin shows that the catalytic site is present at the interface between both domains (Tesmer et al., 1997). Since the catalytic domains are not identical, mACs display only one catalytically active pocket (Dessauer and Gilman, 1996; Tesmer et al., 1999, 1997; Whisnant et al., 1996).

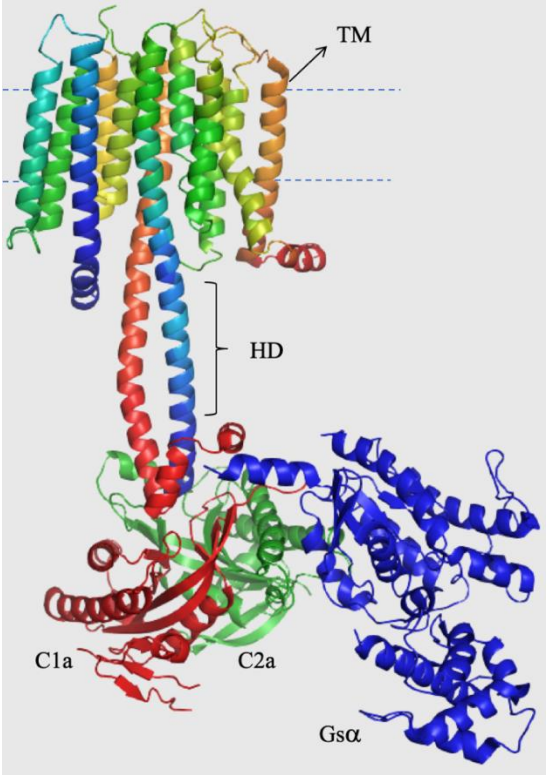

Figure 4. Cryo-EM based 3D model of the bovine AC9 bound to Gsα (3.4 Å resolution, figure adapted from Qi et al., 2019). The structural elements represented are TM: transmembrane domain. C1a/C2a: parts of two catalytic domain C1/C2. HD: helical domain (coiled-coiled) including the CTEs designated by Ziegler et al., 2017.

On the membrane side, TM1 and TM2 have 6 transmembrane helices each, which makes around forty percent of the total protein. This large structure with a strong evolutionary lineage is present only for anchoring was something which remained perplexing and justified the search for a physiological function beyond membrane-anchoring (Bassler et al., 2018; Beltz et al., 2016; Ziegler et al., 2017).

1.2.3.1 Understanding the mechanism in mACs

In 1971 E. Sutherland won the Nobel prize for, "the mechanisms of the action of hormones" involving the second messenger cAMP. Later, it was demonstrated that binding of agonists to an appropriate receptor (GPCR) causes activation of a G-protein α-subunit, Gsα, which in turn stimulates the ACs (Hepler and Gilman, 1992; Sunahara et al., 1996).

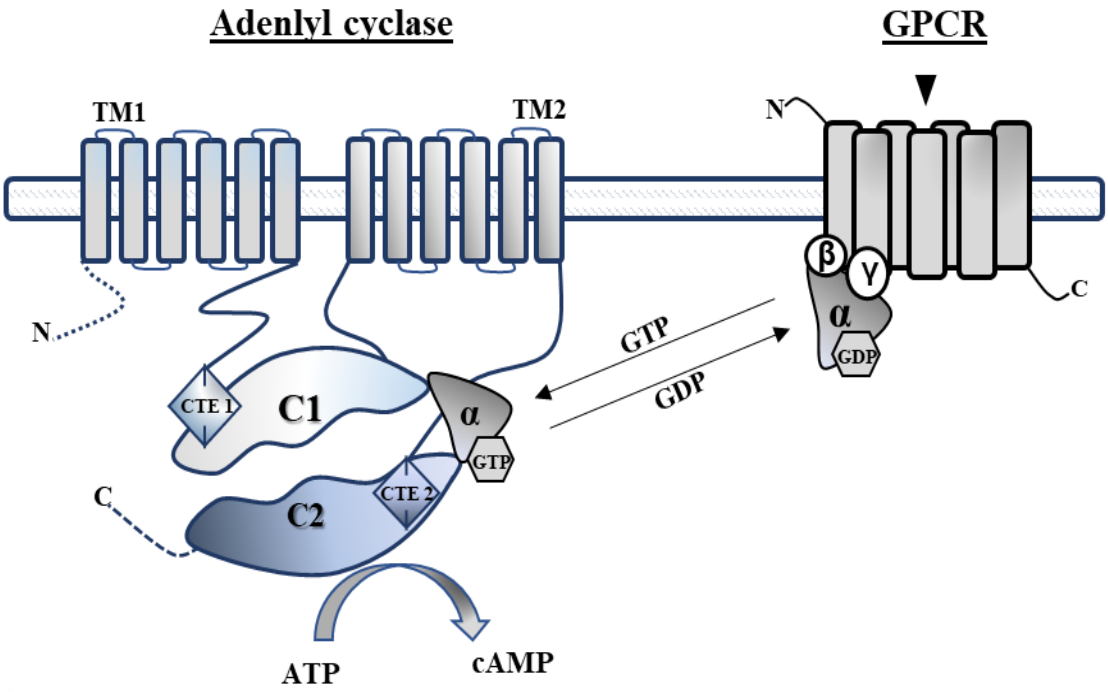

Figure 5. *Adenylyl cyclase activated indirectly by GPCRs (G protein-coupled receptors, right) as an example of a two-component system (TCS) in eukaryotes. GPCR/ G-Protein trimer: α, β, γ (adapted from* Boularan and Gales, 2015; Pulliainen et al., 2012).

Activation by Gsα

The pathway of activation of ACs by GPCRs, the heptahelical receptors, has been investigated extensively. Binding of an agonist to GPCR catalyzes the exchange of

GDP for GTP on the G protein α-subunit. GTP-bound Gsα then undergoes a conformational change and dissociates from the G protein trimer. This allows the α-subunit to bind with and activate AC. α-subunits have intrinsic GTPase activity, and hydrolysis of Gsα-GTP to GDP eventually terminates AC activation (Hepler and Gilman, 1992; Sunahara et al., 1996).

The universal form of activation of all nine ACs isoforms is by Gsα, in addition these isoforms are also, except for isoform 9, activated by the plant diterpene forskolin, individually and synergistically in the presence of Gsα (Hurley, 1999). A recent study of AC isoform 9 also indicated sensitivity towards forskolin (Qi et al., 2019).

All the isoforms differ in their expression pattern and are affected differently by several cytosolic factors (Ca^{2+}, calmodulin, etc.) (Dessauer et al., 2017; Tang and Gilman, 1995; Vorherr et al., 1993). The emphasis on Gsα regulation may only be a partial aspect to understand the mechanism of these ACs because the role of membrane anchors is considered inert, a speculation which remained unsettled. Hence, the focus in my thesis and for many years in our lab is based on the hypothesis: ACs-membrane domains possess a function beyond anchoring.

1.3. TMs of ACs: Orphan receptor?

As noted, this indirectness in the mode of AC regulation is a bit disconcerting as ACs have features of membrane proteins potentially indicating other functions. In 1989 the field advanced after the first AC amino acid sequence was published in conjunction with a prediction of a secondary structural model (Krupinski et al., 1989). Since then several theories have been proposed for the function of 6xTM domains in ACs such as an ion channel, a transporter (Krupinski et al., 1989), a voltage sensor (Reddy et al., 1995), a dimerization factor (Cooper and Crossthwaite, 2006). Up to now, none of these suggestions has panned out.
To think for a receptor function was considered farfetched mainly because the predicted 6TM bundles of the membrane anchor have rather short extra-membranous loops which may be insufficient for a complex ligand.
The notion of this belief was challenged when lipid-based ligands were identified in an analogous system with a similar 6TM domain, in bacterial quorum sensing histidine kinase complexes (Higgins et al., 2007; Spirig et al., 2008).

1.3.1 Quorum sensing Receptor

Quorum sensing is a process enabling the bacterial population to communicate using extracellular signal molecules termed as autoinducers (Henke and Bassler, 2004). *Vibrio harveyi*, *Vibrio cholerae* and *Legionella pneumophila* are bioluminescent bacteria that have displayed a quorum sensing relay. These species include membrane-bound quorum receptors (QS) which display a transmitter domain which is divided into dimerization histidine phosphotransfer (DHp) and a catalytic and ATP-binding (CA) domain with the conserved histidine residue (Lorenz et al., 2017).

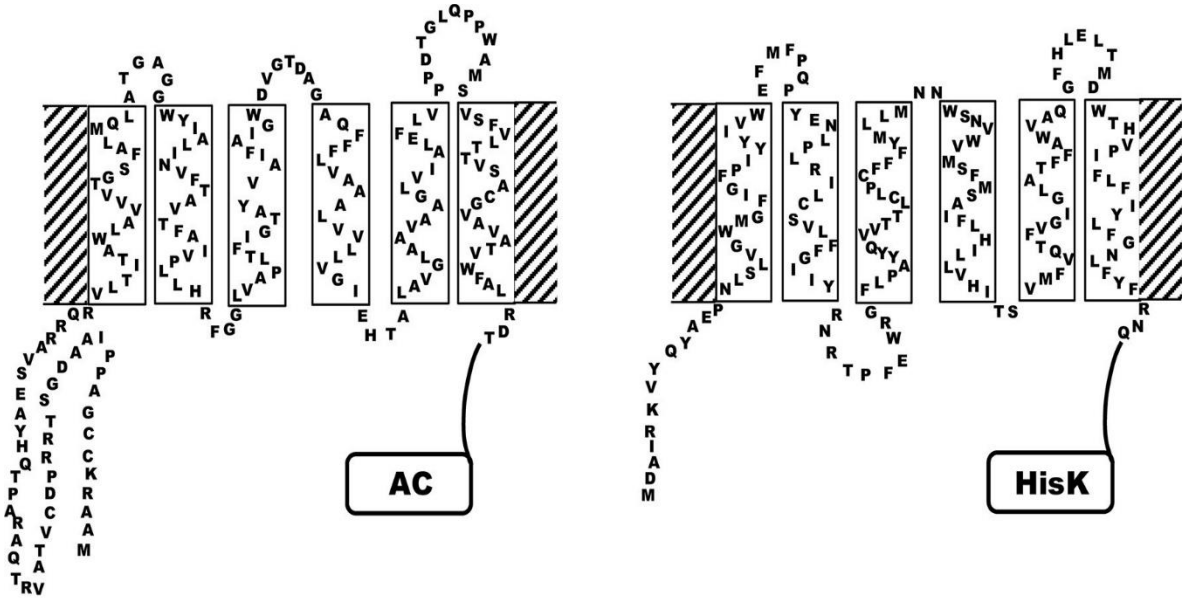

Figure 6. *Two-dimensional models representing (**Left**) Rv1625c AC from M. tuberculosis and (**right**) the QS receptor from V. harveyi, CqsS. Ligand for ACs is unknown in contrast to the QS receptor where ligands are known. For the CqsS the ligand is CAI-1,* ((S)-3-hydroxy-tridecan-4-one) *a lipophilic compound (Image source* Beltz et al., 2016).

These QS receptors possess adenylyl cyclase look-alike, N-terminal 6TM domain with minimal short transmembrane helices and almost no extended loops which connects individual transmembrane spans (figure 6). The striking similarity was also displayed by computational analysis (Beltz et al., 2016). The ligands for ACs are unknown whereas for the QS receptors, *Vibrio* sp. and in *Legionella pneumophila*, *Cholera* and *Legionella* quorum sensing sensor, CqsS and LqsS, lipophilic ligands were discovered, CAI-1, the *Cholerae* autoinducer-1 and LAI-1, the *Legionella* autoinducer-1 (Ng et al., 2010; Spirig et al., 2008).

Like bacterial class III AC, QS receptors are also homodimers, they are coupled to histidine kinases as cytosolic domains. The autoinducer molecule binds to the receptor domain and initiates signal transduction (Linder and Schultz, 2003; Ng and Bassler, 2009).

1.3.2 Progenitor to a chimeric mAC

Mycobacterium tuberculosis encodes an uncommonly large number of biochemically diverse ACs (McCue et al., 2000). One such specie is mycobacterial AC Rv1625c which belongs to class IIIa. It is an AC monomer that dimerizes to become active. The predicted domain organization of this monomer is exactly one-half of a eukaryotic ACs, thus Rv1625c is considered as a prototype for vertebrate ACs (Guo et al., 2001). Similarly, CqsS is only functional as a dimer.

Beltz et. al. (2016) replaced the 6TM domain from Rv1625c by the QS-receptor from *Vibrio*, generating a CqsS-AC Rv1625c chimera (here CqsS is referred for only the transmembrane ligand binding domain not the whole CqsS). The chimera was expressed in *E. coli* and was directly regulated by its ligand CAI-1 via the receptor CqsS. In addition to the biochemical approach, bioinformatic analysis demonstrated that these CqsS domains possibly are distantly related to hexahelical domains of ACs. Altogether the data proposed a receptor function for the 6TM domains in mycobacterial Rv1625c AC (Beltz et al., 2016).

If considered to be a receptor the presence of signal converter or a transducer unit is most likely anticipated. One such entity was identified, when Zeigler et. al. (2017) made a chimera, LqsS-Rv1625c, in which she identified a 19 amino acid long cyclase transducing element, CTE. These elements are not only conserved at N-termini of catalytic domains in all class IIIa, IIIb ACs, and mammalian GCs, but also displayed high specificity, wherein mACs they are isoform and C1/C2 domain-specific, and highly conserved, indicating the AC membranes as potential ligand receptors. The crystal structure shows that CTEs have two helices parted by a bend of ≈ 45°. A model has indicated that CTEs can adopt a coiled-coil structure. It was also shown that CTEs were involved in conformational flexibility and thus participate in dimerization (Bassler et al., 2018; Vercellino et al., 2017; Ziegler et al., 2017). Such pattern of conservation and recent findings of mutations in this segment, hampering signaling, associates them with several hereditary diseases thus suggesting a direct role of CTEs in regulating the enzymatic activity of these cyclases (Finkbeiner et al., 2019; Ziegler et al., 2017).

Taken together, the data clearly bolstered the hypothesis that the transmembrane domain of Rv1625c are putative receptor.

2. OBJECTIVE

Purification and peptide sequencing of mammalian adenylyl cyclase (mACs) lead to cloning studies which marked the existence of nine AC isoforms (reviewed by Sunahara et al., 1996). So far, the membrane anchors appeared to be dispensable for catalysis and regulation, as the catalytic domains of mACs, when expressed separately are regulated by Gsα (Tang and Gilman, 1995).

So when, 1 or 2 TMs are sufficient for anchoring the catalytic domains, why is there a need of 2x6 transmembrane helices which are conserved isoform specifically for > 500 millions of years (Finkbeiner et al., 2019; Kanchan et al., 2010). Due to the lack of any known ligand, direct investigation of the function of 6TMs in mACs was rather unapproachable.

This brings us to the center question of this thesis, an investigation of the role of membrane anchors in hAC by replacing both of their transmembrane domain with the QS-receptor CqsS.

In this context, there were several pressing questions which are going to be addressed in the thesis:

1) Can the CqsS receptors functionally and conformationally replace the two hexahelical membrane anchors of mammalian AC pseudoheterodimer?
    a) As bacterial expression of mammalian ACs has so far proven difficult, so can we achieve a successful expression of such a chimera in *E. coli*.
    b) Can we achieve an active chimera, i.e., is the most conserved form of regulation by Gsα maintained?
2) Will the regulation by CqsS receptor-ligand be maintained in chimera?
3) Which are the interactions between ligand and G-protein (Gsα) regulation exist?
4) Are the results restricted to the bacterial-mammalian chimera CqsS-hAC2?
5) Can we demonstrate a similar regulation in full-length mammalian ACs and where to expect potential ligands?

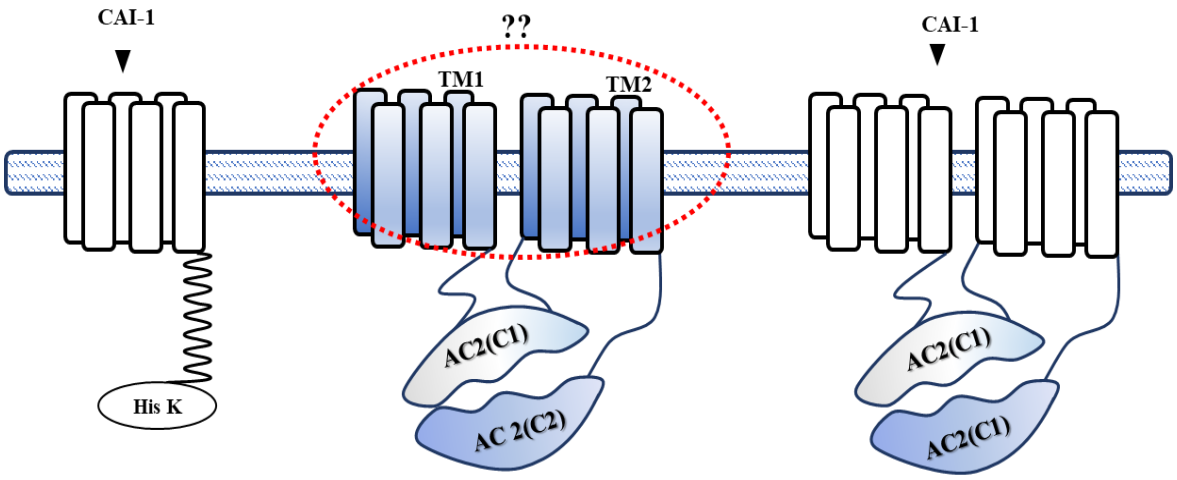

Figure A: *General model representing the center question. (Left) Quorum sensing receptor from V. harveyi with the known ligand, CAI-1. (Center) Human adenylyl cyclase 2 with unknown ligand and receptor function. (Right) Chimeric cyclase, in which TM1 and TM2 of hAC2 were replaced by the quorum-sensing receptor CqsS generating a concatenated pseudoheterodimer.*

In the process to answer the above questions, I established a completely different outlook. I unveiled a new approach towards a regulatory network of mACs which bolstered our hypothesis of a function beyond anchoring: A three state model and putative approach to search for ligands for mammalian adenylyl cyclase was developed.

3. RESULT AND DISCUSSION

3.1 Chapter 1: Manuscript Seth et al., 2020

Title:

Gsα stimulation of mammalian adenylate cyclases regulated by their hexahelical membrane anchors

Author contribution:

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Personal contribution:
Designed, carried out and analyzed experiments for all the data and figures except 4a and 5a in the main manuscript.
Designed, carried out and analyzed experiments for supplementary figure 4 and 5 right. Drafting and revising the manuscript with all authors.

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Gsα stimulation of mammalian adenylate cyclases regulated by their hexahelical membrane anchors

Anubha Seth[a,b], Manuel Finkbeiner[b], Julia Grischin[b], Joachim E. Schultz[a,*]

[a] Pharmazeutisches Institut der Universität Tübingen, Tübingen, Germany
[b] Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany

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ABSTRACT

Mammalian adenylate cyclases (ACs) are pseudoheterodimers with dissimilar hexahelical membrane-anchors, isoform-specifically conserved for more than half a billion years. We exchanged both membrane anchors of the AC isoform 2 by the quorum-sensing receptor from *Vibrio harveyi*, CqsS, which has a ligand, *Cholera*-Autoinducer-1 (CAI-1). In the chimera, AC activity was stimulated by Gsα, CAI-1 had no effect. Surprisingly, CAI-1 inhibited Gsα stimulation. We report that Gsα stimulation of human AC isoforms 2, 3, 5, and 9 expressed in Sf9 cells is inhibited by serum as is AC activity in membranes isolated from rat brain cortex. AC2 activation by forskolin or forskolin/Gsα was similarly inhibited. Obviously, serum contains as yet unidentified factors affecting AC activity. The data establish a linkage in ACs, in which the membrane anchors, as receptors, transduce extracellular signals to the cytosolic catalytic dimer. A mechanistic three state model of AC regulation is presented compatible with all known regulatory inputs into mammalian ACs. The data allow designating the membrane anchors of mammalian ACs as orphan receptors, and establish a new level of AC regulation.

1. Introduction

The first amino acid sequence of a mammalian adenylate cyclase identified two similar catalytic domains (C1 and C2) and two dissimilar hexahelical membrane anchors (TM1 and TM2) which were proposed to possess a channel or transporter-like function, properties, which were never confirmed [1]. Subsequently, nine genes for mAC isoforms were identified, indicating substantial subfunctionalization during evolution (mAC isoforms 1–9; [2,3]). Membrane-delimited ACs (mACs) are the cellular effector proteins for many hormones that signal via G-protein-coupled receptors and their regulation has received broad attention [2,3]. The catalytic center of mACs is formed at the C1/C2 dimer interface. Most biochemical studies have used the startling observation that the separately expressed C1/C2 catalytic domains are regulated by Gsα, i.e. the membrane anchors appear dispensable for catalysis and regulation [4]. Why then 2 × 6 transmembrane spans, when 1 or 2 would have been sufficient for membrane-anchoring? The evolutionary conservation of the membrane domains for more than half a billion years justifies searching for a physiological function beyond membrane-anchoring [5–7].

Recently, we replaced the 6TM domain of the mycobacterial Rv1625c AC, a monomeric progenitor of mACs, by the hexahelical quorum-sensing receptor CqsS from *Vibrio*, generating a CqsS-AC Rv1625c chimera (here and in the following, CqsS is used to denote the ligand-binding membrane domain of CqsS, not the full receptor protein [6,7]). The ligand for CqsS, *Cholera*-Auto-Inducer-1 [CAI-1; (*S*)-3-hydroxy-tridecan-4-one], stimulated AC activity in the chimera [6]. Subsequently, we characterized a family of conserved cyclase-transducing-elements (CTEs) which are indispensable for signal transduction [7]. They are isoform-specifically conserved in mACs, supporting the notion that the AC membrane domains may be ligand receptors [5–7]. Here, we asked whether AC regulation by CqsS is maintained in a chimera, in which the TM1 and TM2 domains in human AC2 (hAC2) are replaced by CqsS, generating a CqsS-hAC2 chimera. We report that stimulation by Gsα is preserved whereas the ligand CAI-1 does not, by itself, affect basal AC activity. Surprisingly, CAI-1 inhibits Gsα stimulation in CqsS-hAC2. We further show that the Gsα stimulation of the hAC2 holoenzyme is similarly inhibited by as yet unidentified factors present in human serum. Serum also inhibited Gsα activation of hAC isoforms 3, 5, and 9, and AC activity in rat brain cortical membranes indicating that the AC membrane domains are orphan ligand receptors.

Abbreviations: AC, adenylate cyclase; hAC, human adenylate cyclase; mAC, membrane-delimited adenylate cyclase; TM, hexahelical membrane domain; CTE, cyclase-transducing-element; CAI-1, *Cholera*-Autoinducer-1; CqsS, quorum-sensing receptor CqsS from *Vibrio*

* Corresponding author at: Pharmazeutisches Institut der Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany.
E-mail address: joachim.schultz@uni-tuebingen.de (J.E. Schultz).

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2. Materials and methods

2.1. Reagents and materials

CqsS of *V. harveyi* (acc. # AAT86007) and hAC2 and hAC9 (acc. # Q08462 and NM_001116.3) sequences were used. Position Phe166 in CqsS was mutated to Leu (F166 L) [6]. Radiochemicals were from Hartmann Analytic and Perkin Elmer. Enzymes were from either New England Biolabs or Roche Molecular. Other chemicals were from Sigma, Merck and Roth. CAI-1 was synthesized in-house [8]. The constitutively active GsαQ227L point mutant was from Dr. C. Kleuss, Berlin [9]. It was expressed and purified as described earlier [9–11]. Human serum (catalog # 4522 from human male AB plasma), heat-inactivated human serum (i.e. inactivated complement; catalog # H3667 from human male AB plasma), and serum albumin (catalog # A3782) were from Sigma-Aldrich, fetal bovine serum was from Gibco, Life Technologies, Darmstadt, Germany (catalog #: 10270; lot number: 42Q8269K).

2.2. Plasmid construct

CqsS-hAC2 was generated using standard methods. 5'-BamHI or *Eco*RI and 3´-*Hin*dIII sites restriction sides were used and inserted into $pQE80_L$ (Δ *Xho*I; Δ *Nco*I). Gly-Ser and Arg-Ser were introduced for internal restriction sites. An N-terminal MRGS-hexa-His-tag was used to for Western blotting. The construct boundaries were: $MRGSHis_6$-GS–CqsS $(F166L)_{1–181}$-$hAC2_{221–603}$-RS-CqsS $(F166L)_{1–181}$-$hAC2_{836–1091}$. Genes for hACs 2, 3, 5, and 9 were obtained from GenScript. For virus production, hACs were inserted into pLIB. The plasmid was amplified in *E. coli* XL1blue and transformed into *E. coli* EMBacY cells generating the bacmid for Sf9 transfection.

2.3. Protein expression

CqsS-hAC2 was expressed in *E. coli* BL21 (DE3) in Luria-Bertani broth at 30 °C with 100 μg/ml ampicillin. 200 ml medium were inoculated with a preculture (to A_{600} of 0.2) and grown at 30 °C with antibiotics. At A_{600} 0.8 expression was induced with 500 μM isopropyl β-D-1-thiogalactopyranoside (22 °C, 4 h). Cells were harvested, washed with buffer and stored at −80 °C. Cell membranes were prepared in lysis buffer (50 mM Tris/HCl, 0.021% thioglycerol, 50 mM NaCl, pH 8) after disintegrating cells in a French press (1100 psi). After removal of cell debris (4300 × g, 30 min, 4 °C) membranes were collected (100,000 × g; 1 h, 4 °C). Membranes were suspended in buffer (40 mM Tris/HCl, 0.016% thioglycerol, 20% glycerol, pH 8). Virus-infected Sf9 cells expressing hACs were grown in Sf900 III medium, harvested after three days and membranes were isolated and stored at −80 °C. Each hAC isoform was expressed once in Sf9 cells and membranes were used in multiple assays (in triplicates). Membrane preparation from rat brain cortex was according to [12].

2.4. Adenylate cyclase assay

Activity of CqsS-hAC2 was assayed for 15 min at 30 °C–37 °C in 100 μl with 40 μg membrane protein, 50 mM Tris/HCl pH 8.3, 5 mM $MnCl_2$, 6 mM creatine phosphate, 230 μg/ml creatine kinase, 750 μM [α-^{32}P]-ATP, and 2 mM [2,8-^{3}H]-cAMP to monitor yield during cAMP purification [13]. Substrate conversion was kept below 10%. CAI-1 was dissolved in DMSO. Incubations with DMSO were carried out as controls. Activity of hACs was determined in a volume of 10 μl using 1 mM ATP, 2 mM $MgCl_2$, 3 mM creatine phosphate, 60 μg/ml creatine kinase, 50 mM MOPS, pH 7.5 using an Assist-Plus pipetting robot (Integra Biosciences, Germany) and a cAMP assay kit from Cisbio (Codolet, France) according to the supplier's instructions (see controls of standard curves in Supplemental fig. 5).

2.5. Western blot analysis

For Western blotting an RGS-His_4-antibody (Qiagen) and a 1:2500 dilution of the fluorophore-conjugated secondary antibody Cy3 (ECL Plex goat-α-mouse IgG-Cy3, GE Healthcare) was used. Proteolysis was not observed.

2.6. Data analysis and statistical analysis

All incubations were in duplicates (CqsS-hAC2) or triplicates (hACs). S.E.M values are given for experiments with CqsS and refer to separately expressed and analyzed proteins. S.D. values apply hACs individually expressed once in Sf9 insect cells. Data analysis was with GraphPad prism 8.1.2.

3. Results

3.1. CqsS serves as a ligand receptor for hAC2

We generated a pseudoheterodimeric chimera of hAC2 in which both 6TMs were isosterically replaced by the 6TM quorum-sensing receptor CqsS from *Vibrio* (CqsS-hAC2). The point of transition between CqsS and hAC2 was at the respective hAC2 CTEs in front of the catalytic domains C1a and C2, thus maintaining all structural features potentially required for signaling (Fig. 1 left; [6,7,14]). Two questions were obvious: a) Is such a chimera expressed in *Escherichia coli* although bacterial expression of mammalian ACs has so far proven impossible, and b) Is regulation by Gsα and the quorum-sensing ligand CAI-1 maintained? The chimera was expressed in *E. coli* and had robust basal activity indicating that native mAC-like features were maintained (Fig. 1). AC activity was stimulated by constitutively active Gsα (Q227L, below termed Gsα [10,11]) demonstrating formation of a productive C1/C2 catalytic dimer. A concentration-response curve showed a 2.3-fold increase in activity with an EC_{50} of around 200 nM Gsα (Fig. 1). By comparison, AC2 expressed in Sf9 cells is stimulated by Gsα about 5 to 12-fold [15,16]. The quantitatively differing responses compared to AC2 may be due to the replacement of the dissimilar TM1 and TM2 domains by two identical CqsS receptors. The quorum-sensor ligand CAI-1, up to 100 μM, failed to affect AC activity. This posed two questions: a) Are the catalytic domains of mACs at all capable of operating as output domains for transmembrane signals? b) Which biochemical differences between bacterial and mammalian ACs exist, which might explain the divergent results obtained with the CqsS-Rv1625c AC chimera [6].

A crucial difference between bacterial and mammalian ACs are the dissociation constants of the catalytic domains. Bacterial catalytic domains usually have a high 'self'-affinity (Kd $\leq 10^{-7}$ M) and are active when conformationally unconstrained [5–7,17]. Similarly, the individual C1 and C2 domains of mACs have a high propensity for self-association, i.e. C1 preferentially associates with C1 and C2 with C2, as documented in the first mAC crystal structure, a C2 homodimer [18]. However, homodimers of C1 or C2 are inactive [19]. The actual affinity between the C1 and C2 catalytic domains in mACs is rather low (Kd $\geq 10^{-5}$ M [20–22]. AC stimulation by Gsα is tantamount to an increase in the apparent affinity of C1 and C2 for each other by approximately two orders of magnitude [20–23]. Therefore, a provocative interpretation for the lack of a CAI-1 effect would be that CqsS receptor activation causes conformational changes which interfere with Gsα stimulation. This was tested next.

3.2. The CqsS receptor regulates stimulation of hAC2 by Gsα

In presence of 5 or 10 μM CAI-1, Gsα activation of CqsS-hAC2 was significantly attenuated (Fig. 2A). Inhibition was instantaneous. The effect was ligand-specific and reversible as determined by re-assaying membranes which were stimulated, washed and re-isolated (Fig. 2B). In

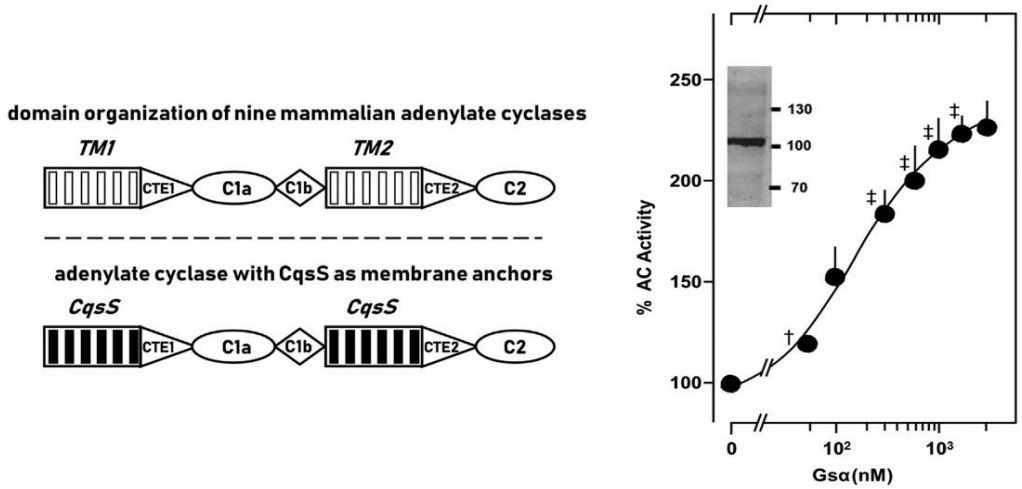

Fig. 1. Stimulation of the chimera CqsS-hAC2 by Gsα. Left: general domain organization of the membrane-delimited adenylate cyclases (top) and scheme of the chimera between CqsS as membrane anchors and hAC2 (bottom). The 6TM regions are idealized, CTE1 and CTE2 denote the cyclase-transducing-elements, indispensable for signal transduction [6,7], C1a and C2 are the respective catalytic domains, C1b connects C1a to TM2 (construct boundaries: MRGSHis$_6$-GS–CqsS (F166 L)$_{1–181}$-hAC2$_{221–603}$-RS-CqsS (F166 L)$_{1–181}$-hAC2$_{836–1091}$). Right: Gsα concentration-response curve. Basal activity (100%) was 27.2 ± 2.6 pmol cAMP·mg^{-1}·min^{1}. Half-maximal stimulation was around 200 nM Gsα. Error bars denote S.E.M. of 2–7 separate experiments (expressions). Significances: ‡: $p < .001$ compared to basal. For clarity, not all significances are marked. Insert: Western blot of the chimera with an anti-His$_6$-antibody indicating absence of proteolysis. MW standards are at right.

presence of 5 or 10 μM CAI-1, the EC_{50} concentrations for Gsα were increased 1.5- and 4-fold, respectively (Fig. 2A). Concomitantly, CAI-1 at 10 μM significantly diminished the maximal Gsα response by 20% (Fig. 2A). The data supported the hypothesis that CqsS receptor activation interfered with activation by Gsα.

Next, a concentration-response curve for CAI-1 in the presence of 1 μM Gsα was carried out. At 35 μM CAI-1, activation of the CqsS-hAC2 chimera by Gsα was almost abrogated (Fig. 3A). The IC_{50} for CAI-1 inhibition was 6.3 μM, i.e. about 15-fold higher than for the stimulatory effect of CAI-1 in the CqsS-Rv1625c AC [6]. This might be due to the fact that we went from a homodimeric CqsS-Rv1625c AC to a linked pseudoheterodimeric CqsS-hAC2 chimera. To exclude that CAI-1 might have obstructed the formation of the catalytic dimer or its interaction with Gsα, the effect of CAI-1 on Gsα stimulation of native hAC2 expressed in Sf9 cells was tested. CAI-1 neither affected basal nor Gsα-stimulated AC activity (Fig. 3B). This unequivocally demonstrated that a) CAI-1 did not interfere in formation of the catalytic dimer in the CqsS-hAC2 chimera, b) CAI-1 did not interact with Gsα and impair its function, and c) CAI-1 did not impair the interactions between the catalytic dimer and Gsα. From these data we can conclude that the effect of CAI-1 in the CqsS-hAC2 chimera was mediated via the CqsS-receptor.

3.3. Gsα stimulation of hAC2 is inhibited by human serum

The above data present a proof-of-concept experiment to demonstrate that a 2x6TM anchor domain can regulate formation of the catalytic dimer of hAC2. The data pose the questions: are mammalian

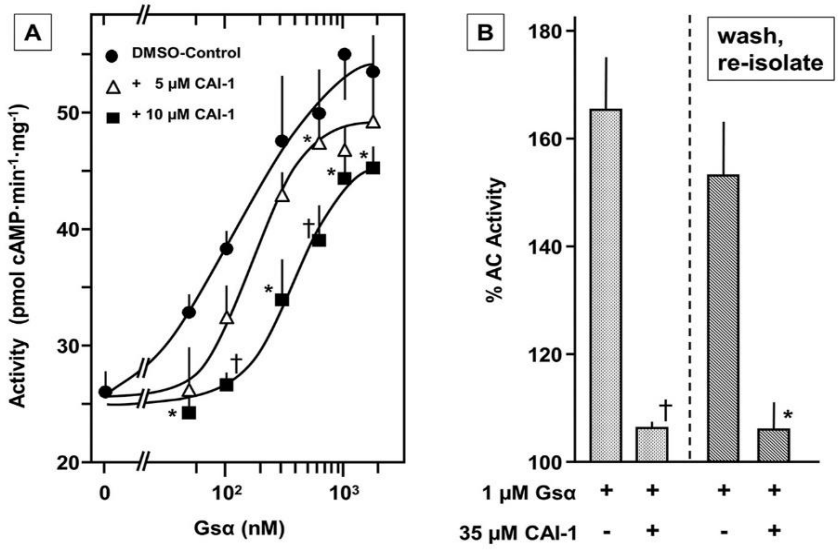

Fig. 2. A) Effect of CAI-1 on the stimulation of the chimera CqsS-hAC2 by Gsα. B) Inhibition of the Gsα response by CAI-1 is reversible. After stimulation of CqsS-hAC2 by 1 μM Gsα ± 10 μM CAI-1 for 15 min (left) membranes were washed and re-isolated by ultracentrifugation (total time required about 150 min). *Re*-stimulation was for 15 min. Basal activity (100%) corresponded to 48.2 ± 9.1 (primary stimulation) and 32.3 ± 5.1 pmol cAMP·mg^{-1}·min^{-1} (washed membranes). Error bars denote S.E.M. of 5 separate experiments. Significances: *, $p < .05$; †, $p < .01$ compared to the respective Gsα stimulation.

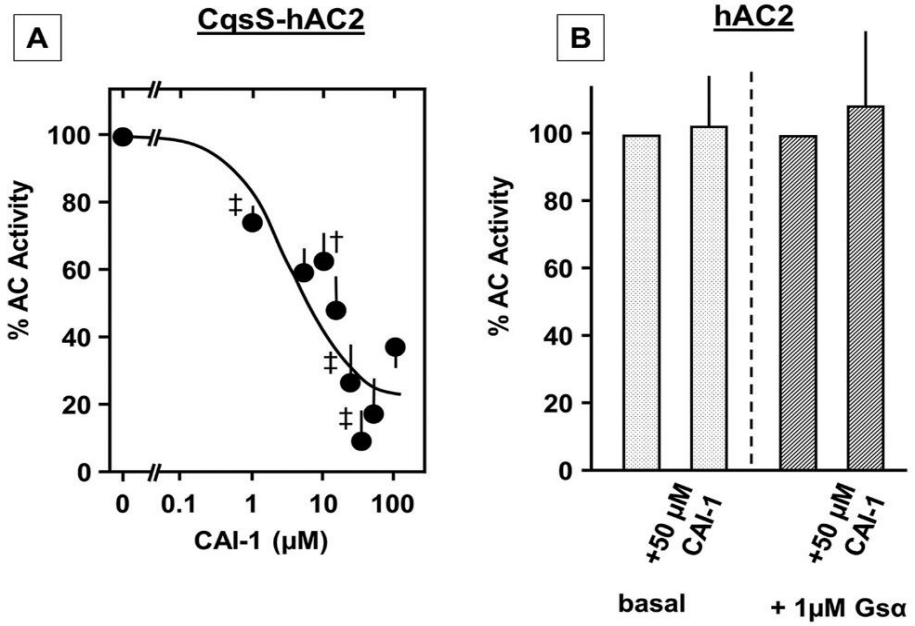

Fig. 3. A) CAI-1 concentration-response curve for the inhibition of Gsα-stimulated CqsS-hAC2. 1 μM Gsα-stimulated activity above basal (100%) was 33 ± 2.6 pmol cAMP·mg^{-1}·min^{-1}. The IC_{50} for CAI-1 was 6.3 μM. Error bars denote S.E.M. of 3–7 separate experiments. Significances: †: p < .01; ‡: p < .001 compared to Gsα-stimulated activity. For clarity, not all significances are indicated. B) CAI-1 has no effect on Gsα stimulation of hAC2. Basal (60 ± 20 pmol cAMP·mg^{-1}·min^{-1}) and 1 μM Gsα stimulated activities (2.7 ± 1.4 nmol cAMP·mg^{-1}·min^{-1}) were set at 100%, respectively; CAI-1 was dissolved in DMSO, 2% final DMSO in all assays. 5 independent assays were carried out. Error bars denote SD.

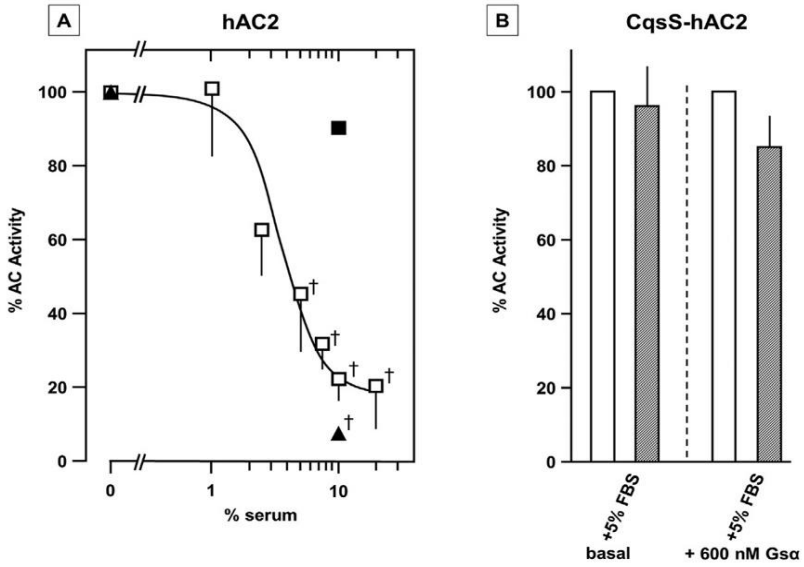

Fig. 4. A) Inhibition of Gsα stimulation (600 nM) of hAC2 by human serum (□) and FBS (▲); human serum albumin (■) at 45 μg/assay. Basal hAC2 activity was 30 pmol cAMP·mg^{-1}·min^{-1}, 100% Gsα-stimulated activity was 1.6 nmol cAMP·mg^{-1}·min^{-1}. Error bars represent SD of 3–5 experiments. †: p < .01 compared to control. B) Basal (open bars; 13.6 ± 2.2) and Gsα-stimulated activity (hatched bars; 27.2 ± 6.2 pmol cAMP·mg^{-1}·min^{-1}) of the CqsS-hAC2 chimera were unaffected by 5% FBS.

mACs regulated in a similar manner and what are potential ligands in mammals and where to expect them? Considering that the mAC membrane anchors are isoform-specifically conserved, prospective ligands are predicted to be similarly primordial [5,7]. Because ligands supposedly access the mACs from the extracellular solvent space, they are expected to be systemically present in the extracellular fluid system of the body. Indeed, human serum significantly inhibited stimulation of hAC2 by 600 nM Gsα (and similarly did heat-inactivated human serum in which complement is inactivated) in a concentration-dependent manner (Fig. 4A). Serum albumin had no effect (Fig. 4A). Fetal bovine serum (FBS) was even more potent indicating a higher concentration of inhibitory factors and almost excluding immunoglobulins as potential ligands because the concentration of immunoglobulins in FBS is substantially lower compared to human serum (Fig. 4 A). Although hAC2 activity with Mn^{2+}-ATP was much higher than with Mg^{2+}-ATP we preferred using Mg^{2+}-ATP as the likely physiological substrate because serum contains 1.25 mM Mg^{2+} and 2.4 mM Ca^{2+}, virtually excluding a chelating effect of divalent cations and, probably, regulatory effects of Ca^{2+} [24]. The data suggested that potential receptor ligands for hAC2 are present in serum. We also carried out concentration-response curves for Gsα stimulation of hAC2 in the absence and presence of 5 and 10% human serum (Supplemental fig. 1). In the presence of serum the efficacy of Gsα was substantially diminished, increasing the required Gsα concentrations for activation and diminishing the maximal responses (Supplemental fig. 1). This was similar to the results with the CqsS-hAC2 chimera (Fig. 2A) suggesting similar mechanisms of action for CAI-1 and specific serum factors, respectively.

Stringent controls are required to unequivocally assign the effect of

serum to the membrane anchors of hAC2: a) exclusion of a salt effect as serum contains around 120 mM NaCl, b) exclusion of an interference of serum components with the dimerization of the C1 and C2 catalytic domains, and c) exclusion of an interference in the interaction between the catalytic dimer and Gsα. 10 mM NaCl in the incubations, equivalent to 10% serum in the assays, did not impair hAC2 basal activity or Gsα stimulation. Similarly, using dialyzed serum did not abrogate inhibition of Gsα stimulation excluding interference by NaCl.

Basal activities of all mACs generally are rather low and difficult to determine reliably [25]. Therefore, we increased the amount of hAC2 membrane protein 27-fold and examined the effect of serum. It inhibited basal hAC2 activity in a concentration-dependent manner with a half-maximal inhibition at 4.9% serum whereas albumin did not (Supplemental fig. 2).

This created a critical issue because components of human or fetal bovine serum might either interfere with dimerization of C1 and C2 domains or with activation of the dimer by Gsα. This was explored using CqsS-hAC2 in which the membrane anchors are from a *Vibrio* quorum-sensor whereas the cytosolic domains are from hAC2 (scheme in Fig. 1). In the CqsS-hAC2 chimera FBS did neither interfere with basal hAC2 activity nor with Gsα stimulation (Fig. 4B). Thus we excluded an effect of serum on the catalytic hAC2 dimer or on Gsα activation of the dimer. The results demonstrated that the action of serum on basal hAC2 activity was contingent on the presence of the membrane anchor of hAC2.

3.4. Human serum inhibits Gsα stimulation of hAC 3, 5, 9 and in brain membranes

Are the above results restricted to the hAC2 isoform or are they indicative of a more general regulatory mechanism applicable to all mACs? Based on pronounced sequence features the nine mACs are subclassified into four subclasses, AC 1, 3 and 8, AC 2, 4, and 7, AC 5 and 6, and the standalone isoform AC9 [3,26,27]. We investigated hAC3, hAC5 and hAC9, i.e. one member of each subclass (including hAC2). Using appropriate enzyme concentrations, serum inhibited basal activities of hACs 3, 5, and 9 with hAC5 being less sensitive to inhibition compared to hAC3 and 9 (IC_{50} = 6.3% serum) indicating either different concentrations of specific inhibitory factors in serum or differences in affinity of a potentially common factor (Supplemental fig. 3). Similarly, Gsα stimulation of hAC isoforms 3, 5, and 9 was inhibited by human serum. The calculated IC_{50} concentrations ranged between 3 and 7% (Fig. 5A). These insignificant differences were not surprising as the commercial human serum is mixed from adult human donors, certainly presenting various physiological states while donating blood.

Next we investigated whether we might have dealt with a serum effect related to the heterologous expression of hACs in Sf9 cells. We prepared membranes from rat brain cortex which contain essentially all mAC isoforms [28]. Serum potently inhibited basal AC as well as Gsα stimulated activity, suggesting that the mACs in brain membranes are similarly regulated as individual AC isoforms expressed in Sf9 cells (Fig. 5B and Supplemental fig. 4).

3.5. Serum inhibits forskolin-stimulated AC activity

mACs are known to be regulated by a number of cytosolic effectors such as Gβγ, calcium / calmodulin, or forskolin and by several secondary modifications such as phosphorylation [3]. These factors generally have divergent, isoform-specific effects, e.g. Gβγ is reported to enhance Gsα or forskolin-stimulated activities of mACs 2, 4, 5, 6, and 7, but to have no effect alone (reviewed in [26,29]). On the other hand, Gβγ inhibits mACs 1, 3, and 8, and is even reported to inhibit AC5 and 6 [26,30,31]. Similarly, calcium and calmodulin have isoform-specific inhibitory or activating effects [26]. In contrast, the plant diterpene forskolin uniformly activates mACs 1 to 8 and it potentiates Gsα activation [3]. In crystal structures of the catalytic dimer, forskolin is bound within the catalytic cleft [21,32]. Therefore, we examined whether serum affects the action of forskolin on hAC2, either alone or in conjunction with Gsα. 25 μM Forskolin stimulated hAC2 about 2.4-fold and human serum significantly inhibited activation (Fig. 6A). 25 μM Forskolin +300 nM Gsα resulted in a 6.6-fold potentiation of activation of hAC2 and serum was significantly inhibitory as well (Fig. 6B). Interestingly, also basal AC activity in rat cortical membranes was inhibited by serum in line with respective results of hACs 2, 3, 5, and 9 activities (Supplemental fig.'s 2, 3 and 4). This demonstrates that regulatory processes of mACs, which are mediated via direct effects on the cytosolic catalytic dimer, are affected by action of specific inhibitory factors present in serum acting via mAC membrane domains. The data support our suggestion that we were dealing with a novel general mechanism of mAC regulation.

4. Discussion

Thus far, studies of regulation of mAC activity mostly dealt with

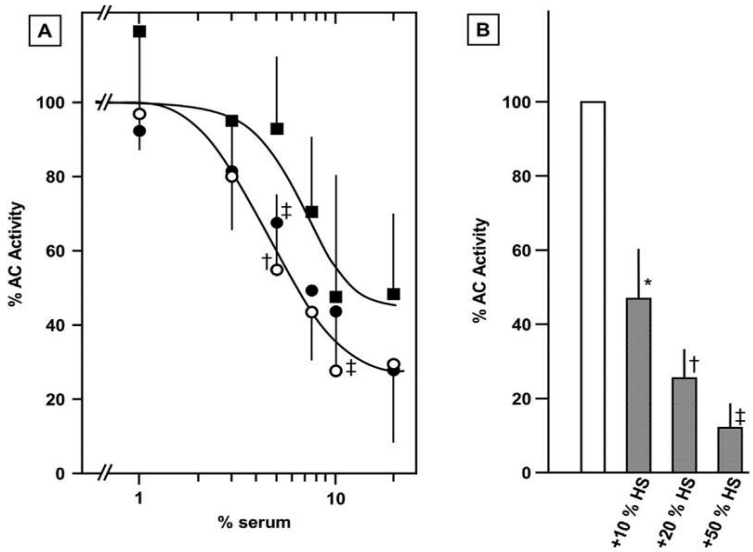

Fig. 5. A) Serum inhibition of hACs 3, 5, and 9 stimulated by 600 nM Gsα (○: hAC 3; ●: hAC5; ■: hAC9). To depict the data in a single graph, activities of hACs 3, 5, and 9 stimulated by 600 nM Gsα were taken as 100% (hAC3 = 0.7, hAC5 = 4.5, AC9 = 1.5 nmol cAMP·mg^{-1}·min^{-1}; for inhibition of basal AC activities see Supplemental fig. 3). B) Serum inhibition of AC activity in rat brain cortical membranes stimulated by 600 nM Gsα. 100% activity corresponds to 1.24 nmol cAMP·mg^{-1}·min^{-1} (3.5-fold stimulation above basal; n = 3). *: $p < .05$: †: $p < .01$: ‡: $p < .001$. Error bars denote SD of 3–4 experiments. (for inhibition of basal activity see Supplemental fig. 4). For clarity, not all significances are indicated.

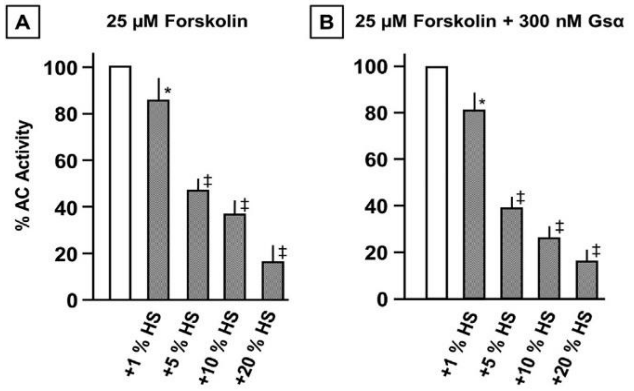

Fig. 6. Serum inhibition of hAC2 activity stimulated by forskolin and forskolin + Gsα. A) Inhibition of 25 µM forskolin stimulated hAC2 activity (100% corresponds to 0.19 nmol cAMP·mg^{-1}·min^{-1}, a 2.5-fold stimulation). B) Inhibition of hAC activity stimulated by 25 µM forskolin and 300 nM Gsα (100% corresponds to 0.5 nmol cAMP·mg^{-1}·min^{-1}, a 6.6-fold stimulation). Error bars denote SD of 4 experiments. *: p < .05; ‡: p < .001.

regulation of the cytosolic catalytic dimer, primarily by uniformly activating Gsα and, secondarily, by variable other inputs (reviewed in [3,27]). The biochemical data emanating from these studies are usually discussed by a two-state model, an active and an inactive state. In this respect, the 2x6TM anchors were considered inert. Potential roles for the membrane anchors were assigned to localization, e.g. in membrane rafts, or as potential interaction sites for scaffolding proteins [33–36]. Our data demonstrate a new level of mAC regulation which is spatially distinct from the catalytic dimer, and, for the first time, confer a regulatory function to all mAC domains. In addition, the regulatory input via the AC membrane domains immediately suggests a possible explanation for the striking evolutionary conservation of the membrane anchors in an isoform-specific manner [5] and requires expanding the previous two-state model of mAC regulation to a three-state model.

CqsS is a hexahelical quorum-sensing receptor from *Vibrio* sensing the extracellular ligand CAI-1 [8,37]. It is isosteric to a 6TM domain of the pseudoheterodimeric mACs [6]. In the CqsS-hAC2 chimera, we observed that CAI-1 attenuated Gsα stimulation in an unequivocally receptor-mediated process (Fig. 2). CAI-1 had no such effect on Gsα-stimulated activity of the hAC2 holoenzyme, because the hAC2 membrane anchor lacks the functionality to sense CAI-1 (Figs. 2, 3). However, stimulation of hAC2 by Gsα was inhibited by serum (Fig. 4A). The effect was dependent on the membrane domain from hAC2 because serum did not affect Gsα stimulation of CqsS-hAC2 as the CqsS receptor cannot sense signaling components present in mammalian serum (Fig. 4B). In addition, this demonstrated that serum did not affect dimerization of C1 and C2 (Fig. 4B). Serum albumin, the major protein in serum, had no effect suggesting the presence of specific, as yet unidentified inhibitory components in serum (Fig. 4A). Inhibition of Gsα stimulation by serum was further demonstrated for hAC isoforms 3, 5, and 9, thus covering one isoform from each mAC subclass (Fig. 5A). Likewise, serum inhibited basal and Gsα stimulated mAC activity present in rat brain cortical membranes (Fig. 5B and Supplemental fig. 4), virtually excluding the possibility of an artifact due to heterologous expression of hACs in Sf9 insect cells. Conceptually, a regulatory input from the extracellular space should affect all cytosolic regulatory inputs impinging upon the catalytic dimer. This was verified with forskolin which stimulates cytosolic catalytic dimer (exception mAC9). Forskolin activation of hAC2 ± Gsα was inhibited by serum (Fig. 6B).

4.1. A three-state model of adenylate cyclase regulation

Based on these data and equilibrium thermodynamic considerations we propose a novel formal concept of regulation of mACs which encompasses all available biochemical, pharmacological and structural data (Fig. 7). Three distinct basal states of mACs exist in equilibrium, state A (inactive), state B (inactive), and state C (active). States A and B differ in the conformational flexibility of their catalytic C1/C2 domains. In state A, the catalytic domains are conformationally constrained and cannot form an active dimer. In state B, the catalytic domains are conformationally unconstrained, yet because of their low affinity for each other, they only occasionally collapse into an active dimer (state C). The highly transient state 'C' is responsible for the very low basal activity observed in all mACs.

Constraining structural flexibility by binding of a ligand at the extracellular side shifts the equilibrium to the inactive 'A'-state (Fig. 7, far left) attenuating basal AC as well as Gsα stimulated activities in hAC2, 3, 5, 9, and in rat brain cortical membranes (Figs. 4, 5 and Supplemental Fig.'s 2–4). Forskolin reversibly increases the apparent affinity of C1 and C2 about tenfold, i.e. it stabilizes the 'C'-state. The 'C'-state is further stabilized by binding of Gsα at the cytosolic dimer thus

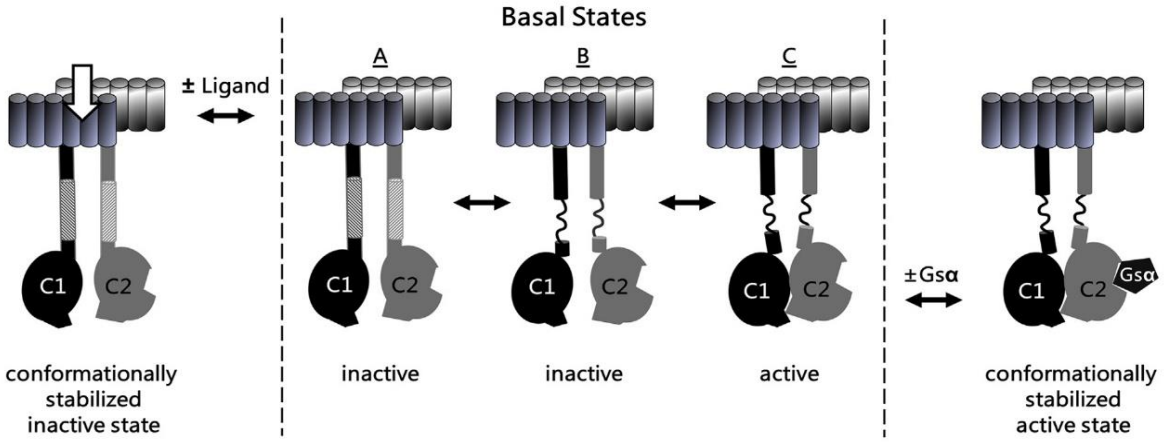

Fig. 7. Scheme of regulation of mammalian adenylate cyclases. Three basal states are in a thermodynamic equilibrium, two inactive, A and B, and one active state, C. The constant low basal enzyme activity is due to fractional formation of an active dimer as symbolized in C. Contact with Gsα in the cytosol stabilizes the active 'C'-state (conformationally stabilized active state, at far right). Conversely, binding of as yet unknown ligands at the extracellular side of the membrane anchor stabilizes the inactive state A (conformationally stabilized inactive state; at far left).

activating mACs and potentially acting in concert with forskolin (Fig. 7, far right).

The proposed three-state model suggests the existence of an allosteric linkage in mACs, in which the membrane anchors, as receptors, transduce extracellular signals across the cell membrane to the cytosolic catalytic dimer. This way, each mAC isoform can be addressed individually by an extracellular ligand and primed for a physiologically measured GPCR/Gsα response. Such a regulatory network would explain the conundrum of why often multiple Gsα-stimulated mAC isoforms are expressed in a single cell. The model is in agreement with the recently published structure of a bovine AC9 isoform which contains features compatible with signal transduction between membrane anchor and catalytic dimer [14]. We are aware of the fact that the equilibrium of states will be subject to ambient conditions such as ion and substrate concentrations, membrane charge and membrane potential. Thus, the model is open to modifications without losing its conceptual validity. Similarly, the lack of chemically identified ligands neither impaired establishing nor does it affect the validity of the three-state model. Considering the isoform-specific conservation of AC membrane anchors nine ligands are expected. These ligands in conjunction with the respective receptors will define a regulatory system which affects GPCR/Gsα actions executed via mACs. In summary then, the AC membrane anchors can now be designated as orphan receptors.

Credit author statement

Anubha Seth, Manuel Finkbeiner, Julia Grischin: Designed, carried out and analyzed experiments; Joachim E. Schultz: Conceptualization, analyzed data and wrote manuscript with all others.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2020.109538.

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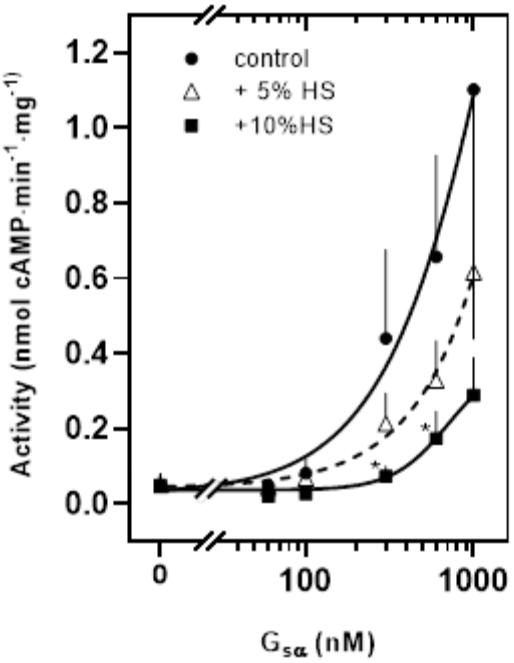
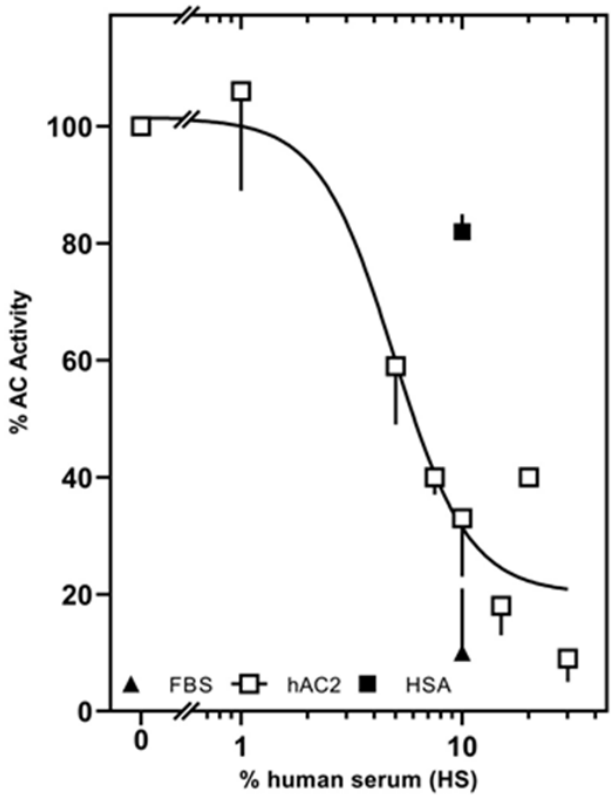
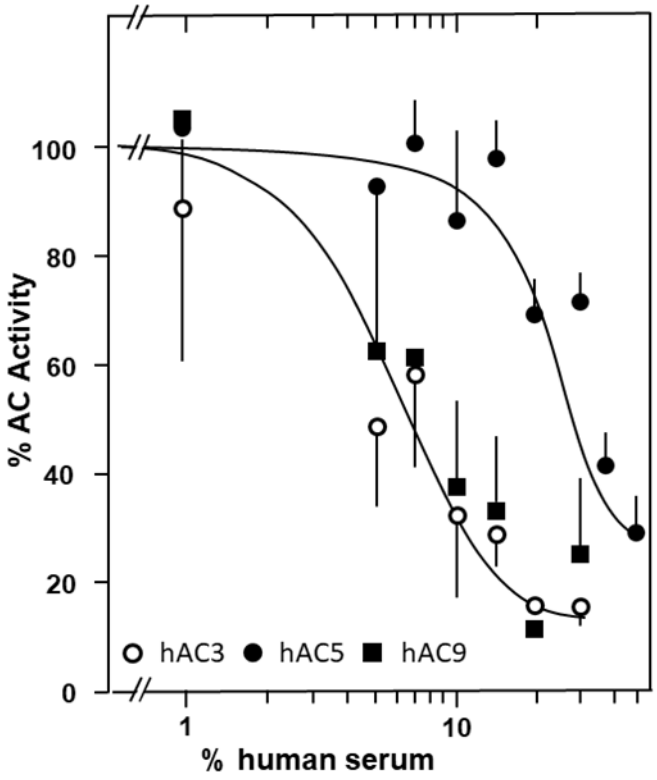
Supplementary data:



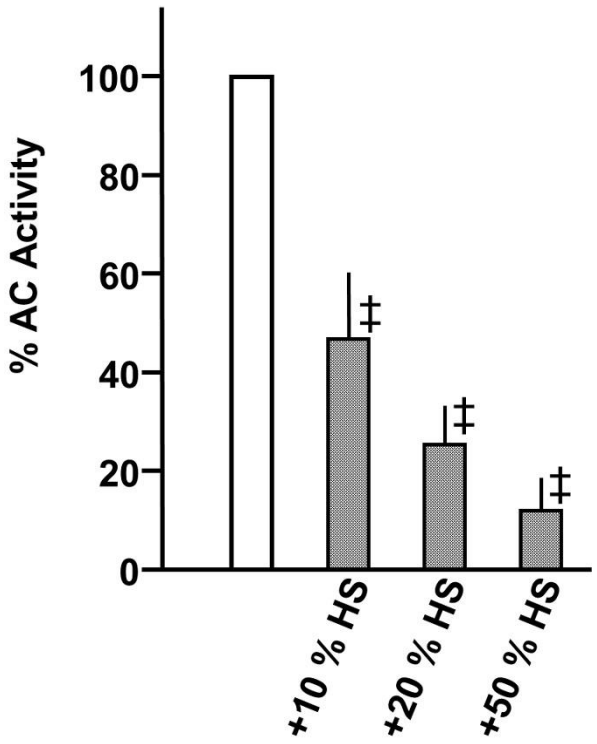
Supplementary Figure 1. Gsα concentration-response curve of hAC2 in presence of 5 and 10 % human serum, respectively. Significances: *: $p \leq 0.05$ compared to control. Error bars denote SD of 3 experiments.



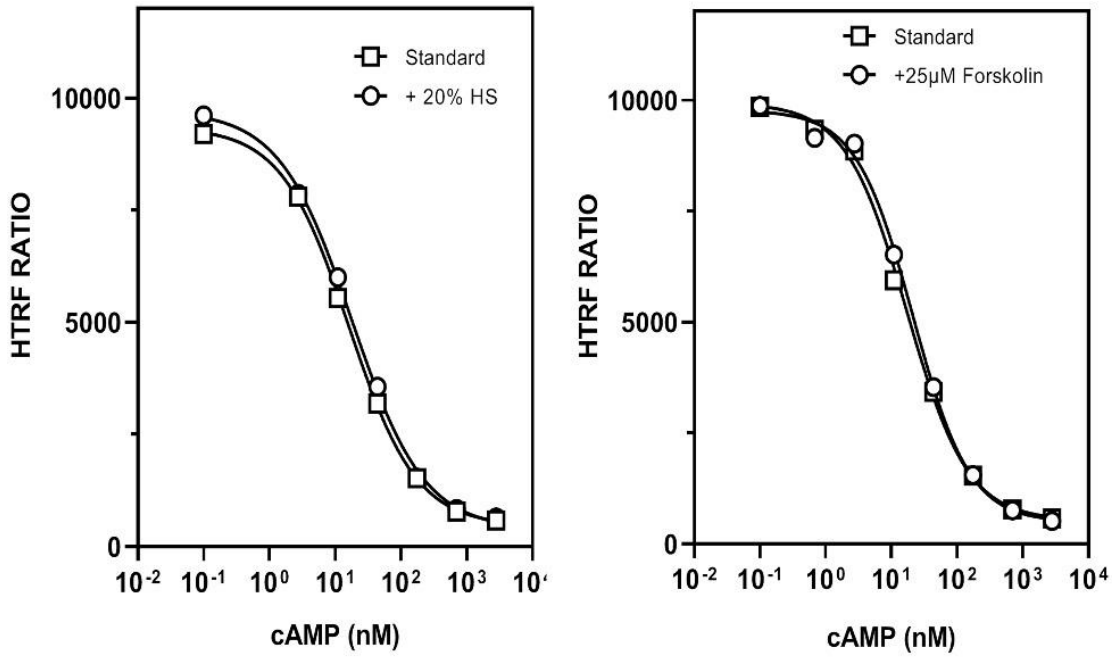
Supplementary Figure 2. Inhibition of basal activity of hAC2 by human serum. Basal activity was 0.038 ± 0.006 nmol cAMP·mg^{-1}·min^{-1}. IC_{50} concentration determined by graph-pad was: 4.9% HS. 2-5 Experiments were carried out, Error bars denote SD



Supplementary Figure 3. Inhibition of basal activity of hACs 3, 5, and 9 by human serum. 2-5 Experiments were carried out. Error bars denote SD. Basal activities were: hAC3 0.025 ± 0.007; hAC5 0.049 ± 0.013; hAC9 0.150 ± 0.047 nmol cAMP·mg^{-1}·min^{-1} IC_{50} concentrations determined by graph-pad were: 6.0% estimated ~40% and 5.9%, respectively



Supplementary Figure 4. Inhibition of basal AC activity in a rat brain cortical membrane preparation. Basal activity (100 %) was 325 pmol cAMP·mg1·min-1. n = 3, Error bars denote SD. Significance: ‡**:** p< 0.001



Supplementary Figure 5. Standard curves for cAMP determination generated with the homogenous-time-resolved fluorescence assay from Cisbio in presence of 20 % human serum or 25 µM forskolin. No interference of serum and forskolin (and other agents used in respective AC assays such as Gsα at 1 µM or DMSO at 2%) was observed.

3. RESULT AND DISCUSSION

3.2 Chapter II: Manuscript Finkbeiner et al., 2019

Title:

In search of a function for the membrane anchors of class IIIa adenylate cyclases.

Author contribution:

Shared first author publication

Personal contribution:
Collected data and putting the outcomes into perspective, (predominantly for figure 1b and sections 3). Extending interpretation with the new data with chimera CqsS_AC2 which suggested current advancement in membrane bound-AC research field. (section: conclusion & outlook).
Drafting and revising the manuscript (with all authors).

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In search of a function for the membrane anchors of class IIIa adenylate cyclases

Manuel Finkbeiner[a,1], Julia Grischin[a,1], Anubha Seth[b,1], Joachim E. Schultz[b,*]

[a] Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany
[b] Pharmazeutisches Institut der Universität Tübingen, Tübingen, Germany

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ABSTRACT

Nine pseudoheterodimeric mammalian adenylate cyclases possess two dissimilar hexahelical membrane domains (TM1 and TM2), two dissimilar cyclase-transducing-elements (CTEs) and two complementary catalytic domains forming a catalytic dimer (often termed cyclase-homology-domain, CHD). Canonically, these cyclases are regulated by G-proteins which are released upon ligand activation of G-protein-coupled receptors. So far, a biochemical function of the membrane domains beyond anchoring has not been established. For almost 30 years, work in our laboratory was based on the hypothesis that these voluminous membrane domains possess an additional physiological, possibly regulatory function. Over the years, we have generated numerous artificial fusion proteins between the catalytic domains of various bacterial adenylate cyclases which are active as homodimers and the membrane receptor domains of known bacterial signaling proteins such as chemotaxis receptors and quorum-sensors which have known ligands. Here we summarize the current status of our experimental efforts. Taken together, the data allow the conclusion that the hexahelical mammalian membrane anchors as well as similar membrane anchors from bacterial adenylate cyclase congeners are orphan receptors. A search for as yet unknown ligands of membrane-delimited adenylate cyclases is now warranted.

1. Introduction

Viability of cells, prokaryotic and eukaryotic alike depends on the ability to sense and respond to changes in the environment in a timely manner. In the late 1950s, Sutherland and colleagues identified adenosine 3′, 5′- monophosphate (cyclic AMP, cAMP) as a perspicuous intracellular second messenger in glycogen metabolism in the liver (Sutherland and Rall, 1958). In the next decades, cAMP was shown to be one of the most universal second messengers in essentially all forms of life. Generally, cAMP is formed in response to 'first messengers', i.e. primary signals such as ligands for respective membrane receptors. The proteins responsible for cAMP biosynthesis from ATP, adenylate cyclases (ACs), were identified as mostly membrane-bound proteins and the diversity of regulatory processes indicated the presence of several isoforms.

With the cloning and sequencing of the first mammalian AC in 1989, the field rapidly advanced (Krupinski et al., 1989). Quickly, it was apparent that the most populated class of AC isoforms (now termed class III ACs) was present in mammals, in most eukaryotes and throughout eubacteria (Barzu and Danchin, 1994). The divergent classes I, II, IV, V and VI ACs are restricted to bacteria. Based on specific sequence variations in class III ACs, the proteins were subclassified into class IIIa, b, c, and d (Linder and Schultz, 2003). Classes IIIc and d ACs are restricted to bacteria whereas classes IIIa and b ACs are shared among eukaryotes and prokaryotes. All class III ACs have a dimeric catalytic center which is formed at the interface of two complementary domains, often termed cyclase-homology-domain (CHD) (Linder and Schultz, 2008). X-ray structures of class III AC catalytic dimers revealed that both monomers contribute to the formation of the catalytic fold (Sinha et al., 2005; Steegborn et al., 2005; Tesmer et al., 1997; Tews et al., 2005). A crucial difference between eukaryotic and prokaryotic ACs is that bacterial isoforms generally are monomeric proteins which must dimerize for activity and form two catalytically competent centers. In contrast, nine mammalian ACs are pseudoheterodimers with two dissimilar hexahelical membrane domains (TM1 and TM2) connected via domain-specific linkers to two catalytically inactive monomers, C1 and C2. The catalytic core is formed at the interface of the two complementary domains with a single active site (Tesmer et al., 1997). Similarly, a number of bacterial congeners possess hexahelical membrane domains (6 T M), obviously much in excess of what would be

* Corresponding author at: Pharmazeutisches Institut der Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany.
E-mail address: Joachim.schultz@uni-tuebingen.de (J.E. Schultz).
[1] Names are arranged alphabetically.

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required for membrane anchoring. Indeed, it was initially speculated that these membrane anchoring devices might possess an ion channel- or transporter-like function (Krupinski et al., 1989). However, a possible regulatory function of these voluminous membrane domains has so far proved elusive.

Since publication of the first amino acid sequence of a mammalian AC our working hypothesis was that such large membrane anchors likely have a physiological function beyond anchoring. Over time we developed several experimental approaches to address and possibly solve this puzzling question. Initially, we were intrigued by the cAMP system in *Paramecium*. In this protozoan, a K^+-current evoked by hyperpolarization of the cell stimulates cAMP biosynthesis (Schultz et al., 1992). The purified AC protein has ion-channel properties when inserted into a black lipid membrane (Schultz et al., 1992). Cloning of the AC identified a canonical voltage-sensor in its hexahelical membrane domain and the motif of a potassium channel (Weber et al., 2004). Such an AC is also present in other protozoans such as *Tetrahymena* or the malaria pathogen *Plasmodium* (Weber et al., 2004). Sequence comparison of these protozoan ACs with mammalian isoforms established that we were most likely dealing with a molecular outlier which did not permit to generalize about such a function in other, similarly membrane-anchored ACs. Over time, the increasing number of sequenced ACs from animals and bacteria allowed well-founded bioinformatic analyses and considerably expanded the number of potential model organisms suitable for addressing the central question of this lab: is there any function of the hexahelical AC membrane anchors beyond anchoring? Below we summarize the data following the timeline since 1999.

The genome of *Mycobacterium tuberculosis HRv37* was sequenced in 1998 (Cole et al., 1998). About 15 genes were predicted to encode class III ACs. These comprise AC isoforms of many stripes, such as the soluble ACs Rv1264, Rv0386 and Rv1900 (AC class IIIc), the class IIIa AC Rv1625c with a hexahelical membrane anchor, a likely monomeric progenitor of the pseudoheterodimeric mammalian ACs, and the class IIIb ACs Rv3645, Rv1318c, Rv1319c and Rv1320c which possess 6 T M anchors and a cytosolic HAMP domain connecting to the catalytic domain (Linder and Schultz, 2003). The acronym HAMP is derived from its occurrence in **H**istidine kinases, **A**denylate cyclases, **M**ethyl-accepting chemotaxis receptors and **P**hosphatases (Aravind and Ponting, 1999). It is a ubiquitously occurring domain in many dimeric signal-transducing proteins in bacteria. HAMP domains are established as signal transducers and their concurrent presence in bacterial ACs is indicative of such a function in these AC isoforms. The hexahelical membrane anchor, the HAMP domain and the catalytic domain give these bacterial ACs an unequivocal tripartite domain organization. We have generated several chimeras between these ACs and bacterial signaling proteins such as chemotaxis receptors and quorum-sensing (QS)-receptors (Fig. 1). In conjunction with comprehensive bioinformatic studies, this has allowed us to tentatively conclude that all membrane delimited ACs are in fact primary signal-transducing proteins which translate extracellular signals into an intracellular cAMP - second messenger response (Bassler et al., 2018).

2. Chemotaxis receptors and HAMP domains as signaling devices for adenylate cyclases

Cytoplasmic HAMP domains are components of bacterial one- and two-component systems such as chemotaxis receptors and histidine kinases (Ferris et al., 2011; Hazelbauer et al., 2008; Parkinson, 2010; Ulrich et al., 2005; Ulrich and Zhulin, 2010). Signaling via chemotaxis receptors (MCPs, methyl-accepting chemotaxis proteins) has been studied extensively (Parkinson, 2010). The HAMP domain connects a two-helical membrane receptor e.g. Tsr (for serine) or Tar (for aspartate) to a methyl-accepting protein involved in the cascade to regulate swimming behavior via control of flagellar beating and in adaptation to a given level of stimulation (Hazelbauer et al., 2008; Parkinson, 2010). In 2006, the predicted coiled-coil structure of the archaebacterial protein Af1503 from *Archaeoglobus fulgidus* was determined by NMR (Hulko et al., 2006). N-terminally, Af1503 has a two-helical membrane anchor of unknown function followed by a cytoplasmic HAMP domain. Somewhat surprisingly, it lacks an output domain (Fig. 1A). We have replaced the HAMP domain in the Rv3645 AC from *M. tuberculosis* by the Af1503 HAMP and introduced several point mutations at a critical alanine position in the HAMP core (Hulko et al., 2006; Fig. 1B, construct 1). The data suggest rotation of the α-helices by 26° in signal transduction (Ferris et al., 2011, 2014; Hulko et al., 2006). Next, we replaced the hexahelical membrane anchor and the HAMP domain of the Rv3645 AC by the Tsr receptor from *E. coli* (Fig. 1B; construct 2). The point of connection in the chimera was based on the alignment of the two HAMP domains (Kanchan et al., 2010). The protein is expressed in *E. coli* and inserted into the membrane. AC activity in isolated membranes is robust (up to 20 nmol cAMP·mg^{-1} min^{-1}). Serine as Tsr receptor ligand inhibits AC activity with a half-maximal inhibitory concentration (IC_{50}) of 18.5 μM, well within the physiological range (Fig. 2). The effect is ligand-specific and depends on an unabridged receptor as demonstrated by inactivating point mutations within the extracellular loop region of the serine-binding side in Tsr (Kanchan et al., 2010). These findings strongly suggest that a bacterial catalytic AC homodimer (class IIIb AC) may be a direct target for regulation by a membrane receptor signal. Signal transmission is not fully specific for the origin of the HAMP domain. It can be either from Tsr or from Rv3645 AC (Fig. 1B, constructs 2 and 3, Fig. 2; Kanchan et al., 2010). In similar constructs with the *E. coli* chemotaxis receptor for aspartate, Tar, AC activity is specifically inhibited by aspartate (Kanchan et al., 2010). The data highlight several points: a) a molecular modularity between bacterial signal transducing proteins such as chemotaxis receptors and ACs exists which enables uncomplicated domain exchangeability between differing signaling pathways; b) the HAMP domains in bacterial ACs operate as signal transducers, i.e. they receive and transduce conformational signals from N-terminal input to C-terminal output domains; c) the catalytic activity of the class IIIb AC Rv3645 in *M. tuberculosis* is probably regulated in response to membrane receptor stimulation (Kanchan et al., 2010).

The experiments summarized above deal with a class IIIb AC. A functional extrapolation to mammalian class IIIa isoforms may be deemed adventurous. Therefore, we have used the cyanobacterial class IIIa AC CyaG from *Arthrospira platensis* as our next model. CyaG has a two-helical membrane anchor of unknown function, a canonical HAMP domain and a 25 aa long spacer between the HAMP and the catalytic domains, termed signaling-helix or S-helix (Anantharaman et al., 2006; Fig. 1A). The S-helix is modeled as a two-helical parallel coiled-coil. In the next construct (Fig. 1B, construct 4), the membrane anchor of CyaG was replaced by the Tsr receptor as above and the HAMP domain and S-helix were retained together with the catalytic domain from CyaG (Kanchan et al., 2010; Winkler et al., 2012). This chimera is inhibited by serine similarly to the Tsr-HAMP-Rv3645 chimera described above (compare Figs. 2 and 3, left). Notably, this regulation requires the presence of the cyanobacterial HAMP domain as a linker (Kanchan et al., 2010). The data establish that a canonical class IIIa AC catalytic dimer can operate as a receiver/output domain for an upstream receptor signal. Surprisingly, in constructs in which the 25 amino acid long S-helix is deleted (Fig. 1B, construct 5) the sign of the signal is inverted, i.e. serine activates (Fig. 3, right). We note that the basal AC activities in these constructs ± S-helix differ substantially. Deletion of the S-helix causes a 10-fold drop in activity from 12 to 1.25 nmol cAMP mg^{-1} min^{-1}. The data are then most plausibly rationalized by assuming two different basal states in the presence or absence of the S-helix (Schultz and Natarajan, 2013). In the presence of the S-helix, basal AC activity is high, and serine inhibits, probably constraining the conformational freedom of the catalytic domains to dimerize. In the absence of the S-helix, basal activity is low, i.e. the catalytic domains cannot properly associate with each other, and serine activates,

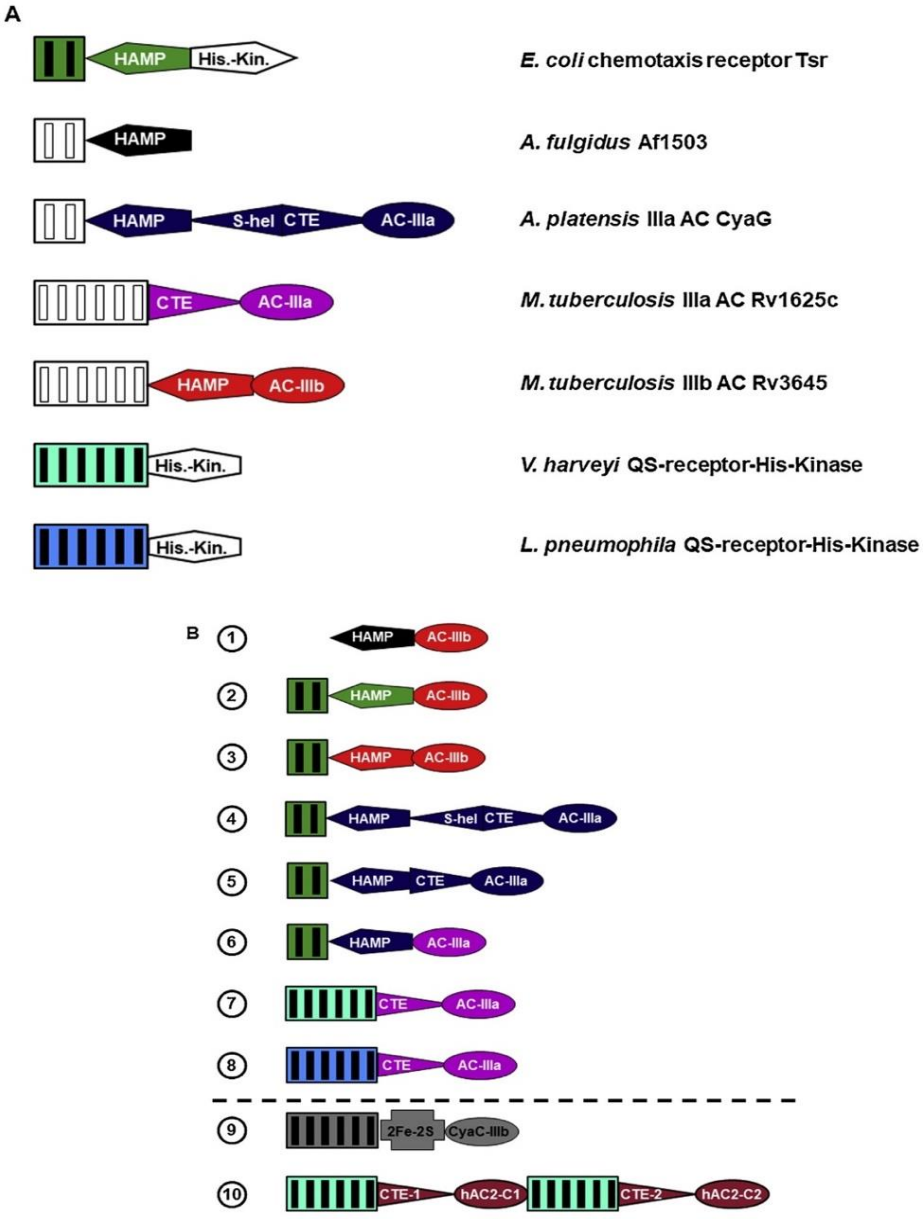

Fig. 1. A) Schematic domain organization of the reference proteins. The colored domains were used as building blocks in subsequent chimeric adenylate cyclases. The empty frames (membrane domains and histidine-kinases) denote domains not used in subsequent constructs. Transmembrane helices are symbolized by narrow vertical bars. S-hel: S-helix of AC CyaG from *A. platensis*. CTE: cyclase-transducing-element. **B) Domain organization of signaling proteins with mycobacterial class IIIb AC Rv3645, cyanobacterial class IIIa AC CyaG, mycobacterial class IIIa AC Rv1625c, and class IIIb AC CyaC from *Sinorhizobium meliloti*.** For exact domain boundaries see references (Beltz et al., 2016; Kanchan et al., 2010; Winkler et al., 2012; Ziegler et al., 2017). The numbering, color and shape coding used in this figure is maintained throughout the review. The numbering of the chimeras approximates the timeline in which they were generated.

probably by releasing the catalytic domains from a conformational constraint and allowing the formation of a productive dimer (Schultz and Natarajan, 2013; Winkler et al., 2012). Generating these slightly more complex chimeras we have noticed the necessity to use clearly defined points of transition between individual domains. The precise transition points between the S-helix and the catalytic domain of CyaG have been of particular importance (Winkler et al., 2012). The experiments summarized above demonstrate that also a class IIIa AC catalytic dimer serves as a signal output domain in response to activation of the chemotaxis receptor much like the closely related IIIb AC variant Rv3645 (Fig. 1B constructs 2–3).

In the mammalian pseudoheterodimeric ACs, HAMP domains are absent (Dessauer et al., 2017). Similarly, the mycobacterial class IIIa AC Rv1625c, a close bacterial progenitor of mammalian ACs, has no HAMP domain (Guo et al., 2001). Therefore, the next question is whether the catalytic domain of Rv1625c can in principle serve as a signal receiver/output domain for a membrane receptor signal. Accordingly, we have generated chimeras which consist of single protein modules from three bacterial species, the chemotaxis receptor Tsr from *E. coli,* the HAMP transducer domain from the AC CyaG from *A. platensis* and class IIIa catalytic domain from *M. tuberculosis* (Fig. 1B, construct 6). As reported earlier, the constructs are expressed in *E. coli* and have AC activity that is activated by serine at physiologically meaningful concentrations (Schultz et al., 2015). Including the S-helix of CyaG in such a chimera inverts the sign of the output signal (Schultz et al., 2015). Taken together, we conclude that a class IIIa AC catalytic domain without a HAMP domain in its original domain organization can also operate as an output domain for a membrane signal. In addition, our data highlight the considerable extent of modularity in these bacterial signaling systems. We have used the data to propose a receptor function for all

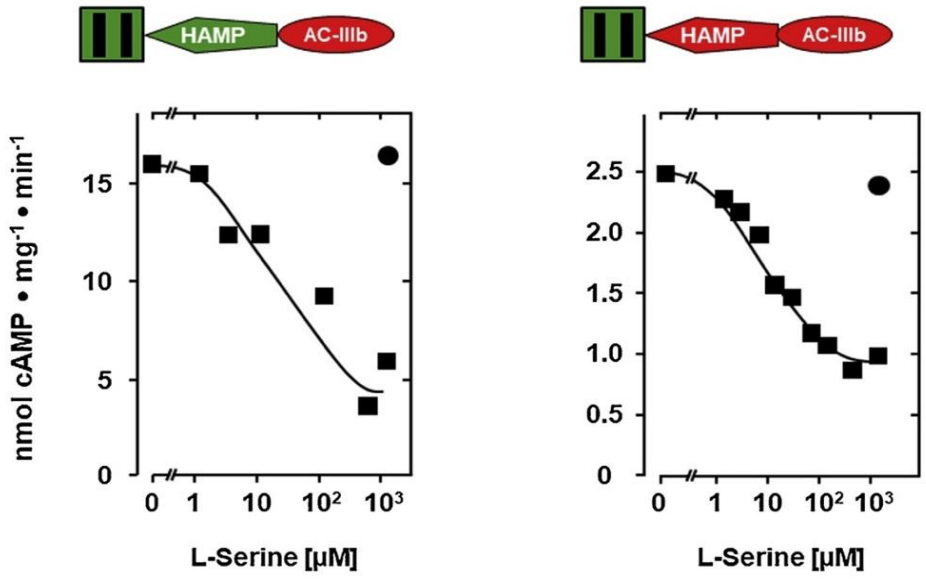

Fig. 2. l-Serine inhibits the class IIIb AC Rv3645 via the *E. coli* Tsr chemotaxis receptor for serine. Above each graph the diagram of the chimera is depicted. Left: Construct with the HAMP domain from Tsr. Maximal serine inhibition was 71 ± 3% (half maximal inhibition was at 18.5 µM). Right: The construct with the HAMP from the Rv3645 AC was inhibited by 45% (half maximal inhibition at 15 µM serine). The filled circles in both graphs are controls with 1 mM L-aspartate (figure was adapted from ref. Kanchan et al., 2010).

6 T M ACs such as AC Rv1625c or the pseudoheterodimeric mammalian ACs. However, at this point we had to acknowledge a major conceptual drawback, i.e. so far we have been dealing with a membrane signal originating from an established receptor with two transmembrane α-helices whereas Rv1625c and all mammalian ACs have hexahelical membrane anchors of unknown function. Playing Lego with protein domains in vitro is not necessarily a compelling argument for the existence of such proteins in evolution, i.e. if something can be made to function it is no proof that this functional entity must exist in nature. Therefore, following up on this point we have used the hexahelical quorum-sensing (QS) receptors from *Vibrio harveyi* and *Legionella pneumophila* which are similar to the 6 T M domains of class IIIa ACs such as Rv1625c or the mammalian congeners. Importantly and fortunately, the ligands for the QS-receptors are known (Ng et al., 2011, 2010; Spirig et al., 2008).

3. Adenylate cyclase regulation by quorum sensing receptors

The hexahelical membrane anchors of essentially all respective bacterial ACs and the nine mammalian isoforms are similar. The α-helices are connected by rather short linkers usually considered problematic for ligand-binding (Beltz et al., 2016). An almost isosteric

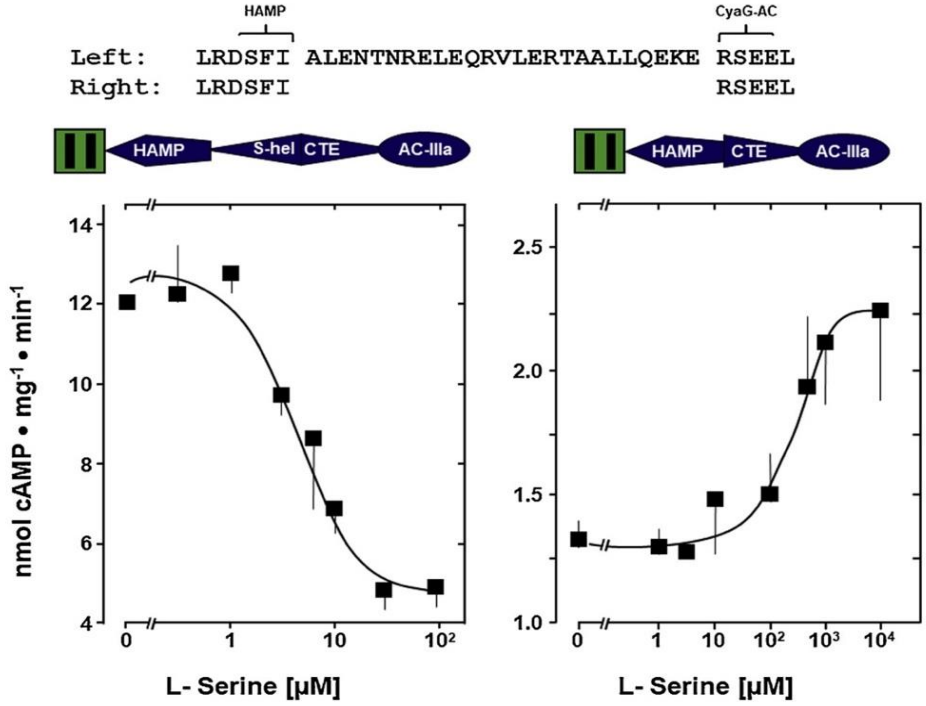

Fig. 3. Class IIIa AC CyaG from *A. platensis* is regulated by l-serine via the *E. coli* Tsr chemotaxis receptor. Left: Tsr linked to CyaG AC via HAMP and S-helix of CyaG AC is regulated by L-serine. Maximal inhibition was 58% (IC_{50} = 6 µM). Right: Removal of the 25 aa long S-helix in the chimera results in activation, i.e. the sign of the signal upon Tsr stimulation is inverted. Half-maximal activation is at 68 µM L-serine (EC_{50} = 305 µM). Top: Partial sequence of CyaG HAMP-S-helix. Error bars denote S.E.M., n = 4; figure was adapted from Winkler et al., 2012).

design, i.e. minimal-length α-helices and short connecting linkers, is known from the hexahelical QS-receptors of *Vibrio harveyi*, CqsS, and *Legionella pneumophila*, LqsS (Beltz et al., 2016). These QS-receptors are the extracellular sensing modules of canonical histidine-kinases which are active as dimers much like the class III ACs (here and in the following, CqsS is used to denote the membrane domain of the CqsS protein; Ng et al., 2012; Wei et al., 2012). For the QS-receptors, the natural ligands have been unequivocally identified. They are highly lipophilic aliphatic acyloins, i.e. ***C**holera* **A**uto**I**nducer-1, CAI-1 [(*S*)-3-hydroxytridecan-4-one] and ***L**egionella* **A**uto**I**nducer-1, LAI-1 [(*S*)-3-hydroxypentadecan-4-one] (Ng et al., 2010; Spirig et al., 2008). In a somewhat daring experimental approach, we have tested whether these QS-receptors can replace the 6 T M anchor function in Rv1625c AC and possibly impose regulation by the ligands CAI-1 or LAI-1 (Beltz et al., 2016; Ziegler et al., 2017). We have generated a large number of chimeras from CqsS and the AC Rv1625c. At the QS-receptor side the optimal point of linkage is short of the canonical H-box which comprises the site of auto-phosphorylation of a critical histidine residue. For the Rv1625c AC a comparison with the above mentioned constructs between Tsr and the CyaG AC from *A. platensis* was useful (Fig. 1B, construct 4 and 5). It revealed a similar site in the Rv1625c AC that starts with the amino acids RSEALL (Winkler et al., 2012). This way, we have at last generated a chimeric class IIIa AC with a hexahelical membrane anchor/QS-receptor (Fig. 1B, construct 7) which is expressed in *E. coli* and specifically regulated, i.e. activity is stimulated by CAI-1 via of a hexahelical membrane receptor, CqsS, at nanomolar concentrations. With a point mutation at the QS-receptor membrane exit (F166 L) stimulation is enhanced up to 5-fold (Fig. 4, left) (Beltz et al., 2016). Stimulation is highly ligand specific as the chemically related LqsS ligand LAI-1 has diminished potency and the 3,4-dihydroxyderivative of CAI-1 is almost inactive (Fig. 4, left). CAI-1 also stimulates cAMP formation in vivo confirming a fully functional chimera (Beltz et al., 2016). Stimulation is irreversible probably because the lipophilic ligand binds into the membrane space of the receptor. An extended bioinformatic analysis shows that these QS-receptors may, in fact, be evolutionarily related to 6 T M anchors of ACs (Beltz et al., 2016).

In a related study using the QS-receptor LqsS from L. *pneumophila*, we have investigated in detail the points of connection between the QS-receptor and the Rv1625c AC (Fig. 1B, construct 9; Ziegler et al., 2017). The QS-receptor ligand LAI-1 significantly stimulates AC activity (Fig. 4 right). The analysis of the fusion points between the QS-receptor and the Rv1625c AC has led to the identification of a highly conserved, 19 aa long element between membrane anchor and catalytic domain which is absolutely required for functional coupling. This element is termed cyclase-transducing-element, abbreviated CTE. It is highly conserved in all class IIIa and IIIb ACs and in mammalian guanylate cyclases which

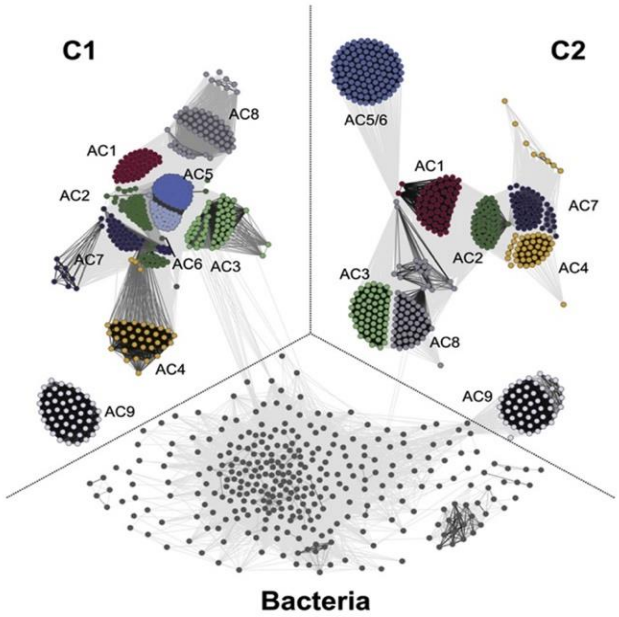

Fig. 5. Cluster map of cyclase-transducer-elements. The sequences of bacterial and vertebrate class IIIa and IIIb CTEs were analyzed using CLANS. Each dot represents a single sequence. Above threshold hits are shown as connecting lines. Darker line color indicates better pairwise blast hits. The three sectors are partitioned by broken lines. Cluster labeling indicates the AC isoform. The segregation shows that CTEs from vertebrate class IIIa ACs are highly specific for the C1- and C2-domain origins as well as for the peculiar AC isoforms. Bacterial sequences display no distinct clustering pattern. The data set comprises a total of 1265 AC sequences (figure from Ziegler et al., 2017).

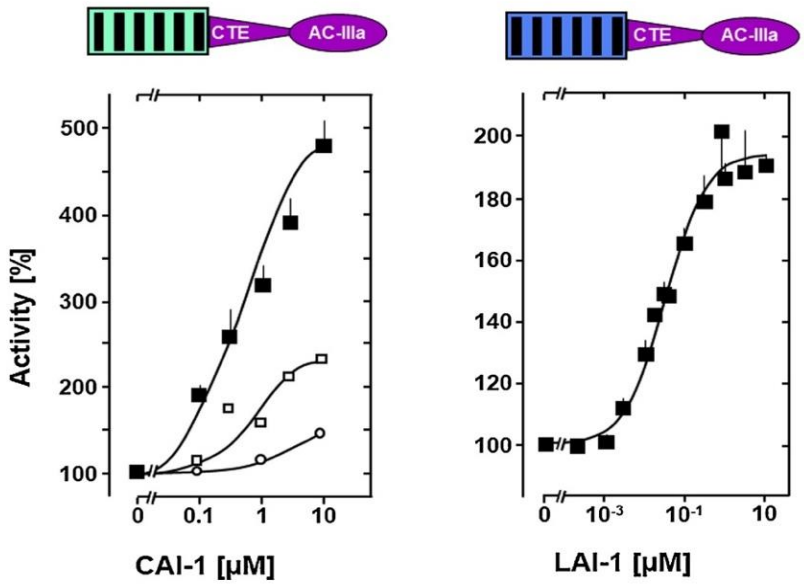

Fig. 4. Left: CqsS-Rv1625c stimulation by the QS-ligand CAI-1. Basal activity was 4 nmol cAMP·mg^{-1} min^{-1}. Filled squares, CAI-1 (n = 5–11; ± S.E.M.); open squares, LAI-1; open circles, 3,4-tridecanediol. The EC_{50} concentration for CAI-1 is 400 nM. CAI-1 stimulations were significant starting at 100 nM.
Right: LqsS-Rv1625c stimulation by the QS-ligand LAI-1. LAI-1 concentration-response curve for the LqsS construct connected to Rv1625c via Lys187. Basal activity is 20.7 nmol cAMP·mg^{-1} min^{-1}. Error bars denote S.E.M.'s. The figure was adapted from references (Beltz et al., 2016; Ziegler et al., 2017).

evolutionarily are related to the mammalian ACs (Bassler et al., 2018; Vercellino et al., 2017; Ziegler et al., 2017). These CTEs are located N-terminal with respect to the catalytic domains. They are present in all nine mammalian ACs. Recently, the structure of the cytosolic domain of the AC Cya from *Mycobacterium intracellulare* including its CTE was elucidated. Cya is 80% identical to that of Rv1625c (Vercellino et al., 2017). In the nine mammalian ACs CTEs are highly isoform specific and, in addition, specific for C1 and C2 catalytic domains as apparent from a cluster analysis (Fig. 5; Ziegler et al., 2017). In contrast, the CTEs of bacterial class IIIa and b ACs do not cluster in a respective bioinformatic analysis possibly indicating that each bacterial AC has its 'personalized' ligand and a thermodynamically optimized CTE (Fig. 5). Further experiments in which the S-helix has been placed either N-terminal or C-terminal of the CTE in an LqsS–Rv1625c chimera revealed how deeply CTEs are entrenched in signal transduction from a membrane receptor to an AC catalytic dimer. When ´N-terminal, S-helix and CTE combine into a single functional unit which inverses the sign of the signal, i.e. the QS-ligand LAI-1 inhibits cAMP formation. Positioned C-terminally of the CTE, the S-helix is without discernible function, i.e. lost its transducer function. AC activity is substantially reduced, albeit stimulation is retained (Ziegler et al., 2017). Taken together, the data can be discussed in a simple mechanistic model (Schultz and Natarajan, 2013). The two catalytic domains required for AC activity, either in homomeric bacterial or heteromeric mammalian ACs, must be conformationally free to associate into a productive dimer. Most likely, regulation by N-terminal receptors such as the chemotaxis receptors Tsr or Tar or the QS-receptors CqsS or LqsS affect the balance between constrained and unconstrained states of the catalytic domains and thus regulate activation or inhibition of ACs. In a conformationally unconstrained state, formation of a productive dimer is enabled whereas in a conformationally constrained state it is inhibited (Schultz and Natarajan, 2013). Summarizing all biochemical and bioinformatic data, one is tempted to suggest that this kind of receptor regulation is an intrinsic property of 6 T M class IIIa ACs, including the mammalian congeners. After tirelessly following many experimental trails we can reasonably conclude that the hexahelical membrane anchors of mammalian ACs in reality are orphan receptors.

4. Conclusion and outlook

In an analysis of various 6 T M domains, a small group of class IIIb ACs has been identified which obviously is an outlier and barely related to other AC membrane anchors (Beltz et al., 2016). The 6 T M domains of these ACs have a cytosolic ferredoxin in front of the catalytic domain (Fig. 1B, construct 9). Bioinformatic analysis of their membrane anchor has turned up a surprise: it is closely related to that present in fumarate reductases and succinate dehydrogenases, two proteins in the respiratory chain (Lancaster, 2002). A sequence comparison identified four fully conserved intramembranous histidine residues which axially coordinate two heme-B molecules (Wissig et al., 2019). Investigating the regulation of the CyaC AC from *Sinorhizobium meliloti*, we found that a) heme B is required for activity; b) all four histidine residues are required for heme B binding; c) AC activity is regulated via redox processes similar to those known in fumarate reductases. A somewhat unconventional interpretation of these findings would denote the heme-B molecules as membrane-integral ligands, faintly reminiscent on the membrane-integral cis-retinal in the G-protein-coupled receptor opsin that is rhodopsin. Although the conjecture might appear somewhat premature and far-fetched, CyaC AC from *S. meliloti* is the first hexahelical membrane domain of a class IIIb AC in which a clear regulatory function has been identified and characterized (Wissig et al., 2019).

Extending the above findings to mammalian ACs we have recently replaced the two dissimilar membrane anchors in the human AC2 by two necessarily identical CqsS receptors from *V. harveyi* (Fig. 1B, construct 10). In such a construct, human AC2 activity is regulated by the ligand CAI-1 and Gsα proteins which in mammalian cells are intracellularly released upon extracellular stimulation of G-protein-coupled receptors (GPCRs). The data strongly suggest that the catalytic dimers of mammalian ACs have retained the capacity to receive and translate extracellular signals from their own membrane receptor (unpublished data). The identification of ligands specific for any pro- or eukaryotic AC membrane domains/receptor remains a pressing problem and major challenge for our future work.

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4. Chapter 3: In search of ligands for the orphan receptors of mammalian adenylyl cyclases.

Outlook

The previous results suggested that in mACs, a signal transduction pathway between the membrane anchors, as receptors, and their catalytic dimer exists, and the TM domains may regulate AC activity in response to unidentified ligands (Seth et al., 2020). In such a way all mACs isoforms could be individually addressed by respective ligands and primed for a physiologically measured GPCR/Gsα response. For the first time a targeted search for mACs-ligand appears to be conceivable (Seth et al., 2020).

In view of the conservation of mACs, CTE1 and CTE2 sequences, and especially the two 6TM domains conserved in an isoform specific manner for > 500 million years, one may reasonably hypothesize that similarly conserved ligands should be present.

Based on the published data the future experimental work is apparent i.e. assay for screening the ligands that affect /regulate Gsα stimulation in an isoform-specific manner. Probably the ligands are systemically present in the blood, lymph, and interstitial fluids which are intimately interconnected. Indeed, a clear indication of the regulation of Gsα stimulation in hACs was observed in the presence of serum (Seth et al., 2020).

Serum is a complex mixture of lipids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, amino acids , hormones, minerals, growth factors, and trace elements (Arora, 2013; von Seefried and MacMorine, 1976).

Taken together, further steps were clear, fractionation of serum and screening of the components present in serum. Following is the list of components present in serum that were investigated.

I) Hormones: as possible ligands?

Hormones are chemical messengers that serve biological harmonization. They circulate systemically in the blood, thereby influencing nearly all aspects of the phenotype, including developmental and physiological process (Cox et al., 2016; Tata, 2005). Major branches of the endocrine system are evolutionarily conserved and these hormones play a role which often varies across taxa, even small changes in the binding pattern to downstream targets offer a subtle regulatory system (Cox et al., 2016; Cox and John-Alder, 2005).

Following are the preliminary data with some of the hormones that were screened in their physiological concentration ranges in the presence of hACs expressed in Sf9 cells. Keeping the serum effect in mind from the published data (Seth et al., 2020) and considering the possibility of a helper protein required by a ligand to bind, 1% dialyzed human serum was also checked in the presence of some of the listed hormones.

Hormones	**Physiological range**
Triiodothyronine (T3) (free)	3.53 – 6.45 pM
Triiodothyronine (T3) (total)	1.08 – 3.08 nM
Triiodothyronine (T3), reverse	0.15 – 0.37 nM
Thyroxine (T4) (total)	94.02 – 213.68 nM
Estradiol	0.036 – 1.28 nM
Progesterone	1.55 – 8.64 nM
Aldosterone	27.7 – 582.5 pM
Insulin	9.7 – 97.2 pM
Glucose	~5.5 mM fasting plasma blood glucose. 7.8 – 11.1 mM is prediabetes. > 11.1 mM is a sign of diabetes mellitus.

Table 1: *List of hormones/compounds and their physiological concentrations.* (Walker, 2010)

Thyroid hormones

Thyroid hormones (THs) are produced by the thyroid gland and are vital for regulation with significant involvement in development and physiology of vertebrates. THs are present in two main forms, T3 and T4. T4 differs from T3 because of the presence of an iodine at the 5' position (Table 2). Healthy humans produce a large amount of T4 and little of T3, the more active form of THs. T3 is produced via deiodination of T_4. (Schroeder and Privalsky, 2014; Visser, 1988)

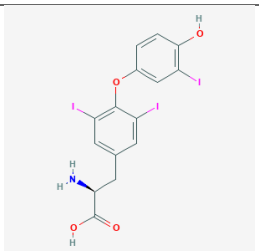
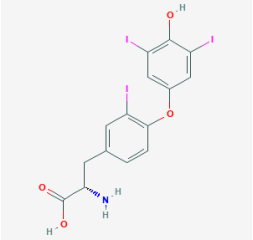
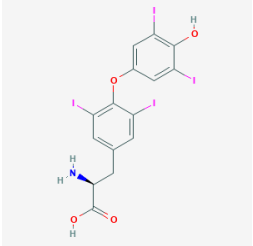
A) Triiodothyronine (T3)/ 3, 3',5-triiodo-L-thyronine	$C_{15}H_{12}I_3NO_4$	
B) Reverse T3 (rT3)	$C_{15}H_{12}I_3NO_4$	
C) Thyroxine (T4)/ 3,5,3',5'-tetraiodo-L-thyronine	$C_{15}H_{11}I_4NO_4$	

Table 2. *Structure and chemical formula of T3, rT3 and T4.*
(source: PubChem, NIH)

A) Thyroid hormones: Triiodothyronine (T3)

	hAC3	hAC5
	AC activity (nmol/mg/min)	
Gsα 600nM	0.93	2.02
Gsα 600nM + T3 1nM	0.84	1.78
Gsα 600nM + T3 3nM	0.85	1.61
Gsα 600nM + T3 10nM	0.67	1.42
Gsα 600nM + HS 1%	0.80	1.49
Gsα 600nM + HS 1% + T3 1nM	0.73	1.58
Gsα 600nM + HS 1% + T3 3nM	0.73	1.40
Gsα 600nM + HS 1% + T3 10nM	0.62	1.49

Table 3: *Different concentrations of T3 were checked in the presence 600 nM Gsα ± 1% Human Serum (HS-dialyzed) on hAC 3 and 5 (basal activities: hAC3* = 0.038 and hAC5 = *0.053 nmol cAMP· mg^{-1}·min^{-1}).*

B) Thyroid hormones: Reverse T3 (rT3)

	hAC3	hAC5
	AC activity (nmol/mg/min)	
Gsα 600nM	0.81	1.31
Gsα 600nM + rT3 0.1nM	0.73	1.43
Gsα 600nM + rT3 0.3nM	0.77	1.21
Gsα 600nM + rT3 1nM	0.71	1.18
Gsα 600nM + HS 1%	0.69	1.25
Gsα 600nM + HS 1% + rT3 0.1nM	0.59	1.14
Gsα 600nM + HS 1% + rT3 0.3nM	0.65	1.16
Gsα 600nM + HS 1% + rT3 1nM	0.63	1.12

Table 4: *Different concentrations of rT3 were checked in the presence of 600 nM Gsα ± 1% Human Serum (HS-dialyzed) on hAC 3 and 5 (basal activities: hAC3* = 0.040 and hAC5 = *0.051 nmol cAMP· mg^{-1}·min^{-1}).*

C) Thyroid hormones: thyroxine (T4)

	hAC3	hAC5
	AC activity (nmol/mg/min)	
Gsα 600nM	0.73	1.29
Gsα 600nM + T4 90nM	0.71	1.27
Gsα 600nM + T4 200nM	0.70	1.11
Gsα 600nM + T4 500nM	0.61	1.18
Gsα 600nM + HS 1%	0.54	1.26
Gsα 600nM + HS 1% + T4 90nM	0.54	1.04
Gsα 600nM + HS 1% + T4 200nM	0.51	1.03
Gsα 600nM + HS 1% + T4 500nM	0.55	1.05

Table 5: *Different concentrations of T4 were checked in the presence of 600 nM Gsα ± 1% Human Serum (HS-dialyzed) on hAC 3 and 5 (basal activities: hAC3 =* 0.041 and hAC5 = *0.052 nmol cAMP· mg^{-1}·min^{-1}).*

Steroid hormones

They are ancestral molecules developed about 2 billion years ago and well recognized as biological regulators. The biosynthetic pathway of these hormones is the same irrespective of the organ producing them (brain, ovary, adrenal cortex, placenta, and testis), but the amount and the type of steroids synthesized/secreted depends on the expression of enzymes specific to each of these organs (Taraborrelli, 2015).

They are generally lipophilic in nature and mostly present in the blood. These hormones are found usually bound to carrier proteins which are specific for them hence increasing their water solubility. Example: corticosteroid binding globulin (CBG) or Sex hormone binding globulin (SHBG). Steroid hormones are categorized to corticosteroids and sex hormones and subcategorized into glucocorticoids (corticosteroids), androgens, estrogens and progestogens (sex steroids) (Holst et al., 2004).

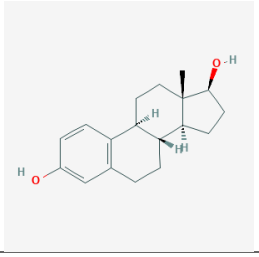
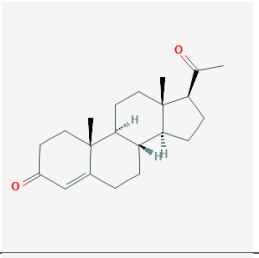
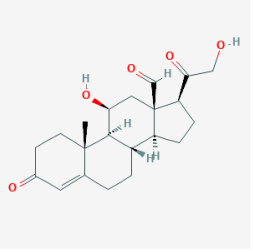
D) Estradiol	$C_{18}H_{24}O_2$	
E) Progesterone	$C_{21}H_{30}O_2$	
F) Aldosterone	$C_{21}H_{28}O_5$	

Table 6. *Structure and chemical formula estradiol, progesterone and aldosterone. (source: PubChem, NIH)*

D) Estradiol

Estrogens are synthesized in all vertebrates and some insects suggesting a long evolutionary history of these hormones (Mechoulam et al., 1984; Ryan, 1982). Females have three main estrogens that have estrogenic activity: estradiol, estriol and estrone. Estradiol is the most potent among all and act as a major female sex hormone. The main synthesis is by the ovary though locally some other organs and tissues are also contributing in synthesis such as adipose tissue, brain, cells of the immune system, and bone (Wise et al., 2009). It is locally synthesis from cholesterol where key intermediate is androstenedione that also converts to testosterone which further converts to estradiol by aromatase (Taraborrelli, 2015)

	hAC3	hAC5
	AC activity (nmol/mg/min)	
Gsα 600nM	0.61	1.79
Gsα 600nM + Estradiol 0.05 nM	0.51	1.58
Gsα 600nM + Estradiol 1 nM	0.50	1.74
Gsα 600nM + Estradiol 3 nM	0.45	1.74
Gsα 600nM + HS 1%	0.48	1.80
Gsα 600nM + HS 1% + Estradiol 0.05 nM	0.45	1.64
Gsα 600nM + HS 1% + Estradiol 1 nM	0.48	1.66
Gsα 600nM + HS 1% + Estradiol 3 nM	0.41	1.83

Table 7: *Different concentrations of estradiol were checked in the presence of 600 nM Gsα ± 1% Human Serum (HS-dialyzed) on hAC 3 and 5 (basal activities: hAC3*= 0.018 and hAC5 = *0.082 nmol cAMP· mg^{-1}·min^{-1}).*

E) Progesterone

It belongs to a group of steroid hormones called progestogens. It is a twenty-one-carbon steroid hormone and its common precursor is cholesterol. Progesterone is commonly known for its role in the reproduction system (for e.g. its role in different stages of ovarian cycle, pregnancy). Around 95–98% of progesterone circulating in the blood is protein-bound, CBG (about 10%) and most of the remaining is bound to albumin. It has a relatively short half-life in the body of only five minutes (Taraborrelli, 2015). It has a range of physiological effects in the presence of estrogen. It plays a significant role throughout body, such as the mammary gland (e.g. preparation for breastfeeding), metabolic effects (e.g. it elevates basal insulin levels and stimulates insulin secretion after intake of carbohydrates), central nervous system, and bones (e.g. in osteoporosis). It increases diuresis through activation of the renin-angiotensin system, relaxes smooth muscle cells, increases excretion of Ca^{2+} and phosphorus, has sedative and analgesic effects (Regidor, 2014). Hence, also known as a neuro-steroid suggesting

its numerous effects and involvement throughout the body (Kuhl, 2005; Ottoson et al., 1984; Taraborrelli, 2015).

	hAC3	hAC5
	AC activity (nmol/mg/min)	
Gsα 600nM	0.47	1.23
Gsα 600nM + Progesterone 10 nM	0.39	1.46
Gsα 600nM + Progesterone 70 nM	0.41	1.25
Gsα 600nM + Progesterone 100 nM	0.37	1.41
Gsα 600nM + HS 1%	0.34	1.46
Gsα 600nM + HS 1% + Progesterone 10 nM	0.33	1.41
Gsα 600nM + HS 1% + Progesterone 70 nM	0.30	1.36
Gsα 600nM + HS 1% + Progesterone 100 nM	0.34	1.38

Table 8: *Different concentrations of progesterone were checked in the presence of 600 nM Gsα ± 1% Human Serum (HS-dialyzed) on hAC 3 and 5 (basal activities: hAC3*= 0.025 and hAC5 = *0.064 nmol cAMP· mg^{-1}·min^{-1}).*

F) Aldosterone

It is the main mineralocorticoid. A hormone created from cholesterol in the adrenal gland. In normal conditions, aldosterone secretion is regulated by the renin-angiotensin system (RAS) and K^+ through negative feedback loops (Williams and Dluhy, 1972). It plays a crucial role in electrolyte and fluid homeostasis and thus in control of blood pressure. The regulation of aldosterone via renin-angiotensin II-aldosterone system is initially activated via a decrease in the mean arterial blood pressure. This decrease is sensed in the kidney which primes for the release of prorenin which in turn is converted to renin. Renin converts angiotensinogen to angiotensin I. Then angiotensin converting enzymes (ACE) converts angiotensin I to angiotensin II in kidney and lungs. Angiotensin II activates type 1 receptors, angiotensin II specific GPCRs. These receptors depending on the location, functions to increase aldosterone, Na^+-H^+ exchange,

anti-diuretic hormone (ADH). Aldosterone together with ADH can cause an increase in water absorption, therefore increasing blood pressure (Hall et al., 2019; Maron and Leopold, 2010; Nehme et al., 2019; Scott and Dunn, 2019).

	hAC3	hAC5
	AC activity (nmol/mg/min)	
Gsα 600nM	0.53	1.58
Gsα 600nM + Aldosterone 0.03 nM	0.40	1.46
Gsα 600nM + Aldosterone 0.6 nM	0.51	1.28
Gsα 600nM + Aldosterone 1 nM	0.40	1.34
Gsα 600nM + HS 1%	0.41	1.61
Gsα 600nM + HS 1% + Aldosterone 0.03 nM	0.34	1.73
Gsα 600nM + HS 1% + Aldosterone 0.6 nM	0.34	1.70
Gsα 600nM + HS 1% + Aldosterone 1 nM	0.31	1.57

Table 9: *Different concentrations of aldosterone were checked in the presence of 600 nM Gsα ± 1% Human Serum (HS-dialyzed) on hAC 3 and 5 (basal activities: hAC3* = 0.018 and hAC5 = *0.070 nmol cAMP· mg^{-1}·min^{-1}).*

The above list of hormones didn't show a significant effect on AC activity of hAC3 and 5. The data are preliminary, different conditions and optimization is required to explore it further for e.g. increase in serum concentration, ± FBS or ± binding globulins.

G) Insulin

Diabetes mellitus is one of the oldest diseases known to mankind. It is a metabolic disorder characterized by chronic hyperglycemia due to lack in insulin production / secretion, insulin action, or both. The classical classification of diabetes is type 1 and type 2. Type 1 is the consequence of insulin deficiency due to progressive destruction of β cells by T-cell mediated autoimmunity. Type 2 diabetes is mainly associated with insulin resistance (Kharroubi, 2015; Olokoba et al., 2012; Tan et al., 2019).

Insulin is a 51 amino acid peptide hormone consisting of A chain (21 amino acid residue) and B chain (30 amino acid residue) bound by disulfide linkages. It is produced by β cells in the pancreas. At the surface of insulin there are several residues that are evolutionarily conserved and involved in conformational flexibility and are likely crucial for determining the insulin receptor binding affinity. Biosynthesis of insulin involves multiple factors and nutrients (e.g. fatty acids, amino acids); glucose metabolism is the central event that stimulates insulin gene transcription and mRNA translation (Fu et al., 2012).

In addition, several hormones (e.eg. estrogen, leptin, and growth hormones) also regulate insulin secretion, making β cells as a central link connecting the endocrine system and nutrient metabolism. Although the primary intracellular signal for insulin secretion is an increase in Ca^{2+} concentrations, cAMP signaling-dependent mechanisms are also critical in the regulation of insulin secretion (Blundell et al., 1972; Fu et al., 2012; Zhao et al., 2010).

Apart from many known and well-studied factors that are involved in oscillations of cAMP in the α and β cells of the islets of Langerhans the role of insulin, glucose and glucagon in respect to cAMP regulation still remains enigmatic (Tian et al., 2011).

It is known that cells express more than one AC isoform (Granneman, 1995). A little is investigated about hAC 9, for which the expression has been described in the pancreas (Leech et al., 1999). Given the potential role of cAMP in regulating insulin secretion, it was worthwhile to study the effect of insulin on AC activity. Hence, following are the data of insulin effects on hAC9 in the presence of Gsα.

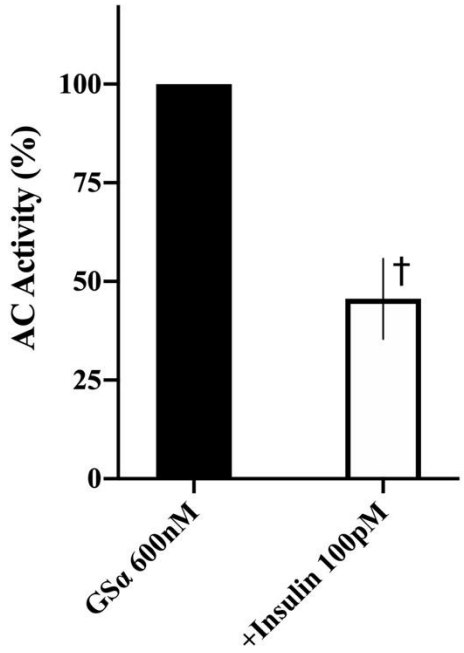


Figure 7. Effect of insulin on hAC9. 100% activity corresponds to 600nM Gsα stimulated hAC9 = 1.31 nmol cAMP· mg^{-1}·min^{-1} (basal activity = 0.27 nmol cAMP· mg^{-1}·min^{-1}). Significance: †: p <.01. Error bar denotes SD of 3 experiments (assay conditions page 69).

At 100pM insulin concentration, Gsα-stimulated hAC9 showed inhibitory effect (figure 7). The above data are preliminary which give us a hint on insulin effect on hAC9 and are in line with the proposed model in Seth et al.(2020), but in the future, the effect has to be explored exhaustively.

Glucose

People with diabetes often show elevated glucagon secretion, which contributes to hyperglycemia (D'alessio, 2011). A recent study shows that glucose controls cAMP and glucagon secretion by a direct effect on the α cells (Yu et al., 2019).
The islet-based study reported the effect on cAMP in the presence of glucose. The data stated, increase in glucose concentration (1 to 20mM) reduces cAMP in α cells and the effect is independent of paracrine influences. 'It remains to be elucidated how glucose lowers cAMP' (Yu et al., 2019).

Considering the above, differences in conditions of membrane-based assay and variability in glucose range depending on each individual, especially with disorders and pre- or postprandial conditions (for glucose range see table 1), it was worthy to check the glucose effect on ACs. So, different concentrations of glucose were checked in the presence of Gsα-stimulated hACs 3,5 and 9 (figure 8).

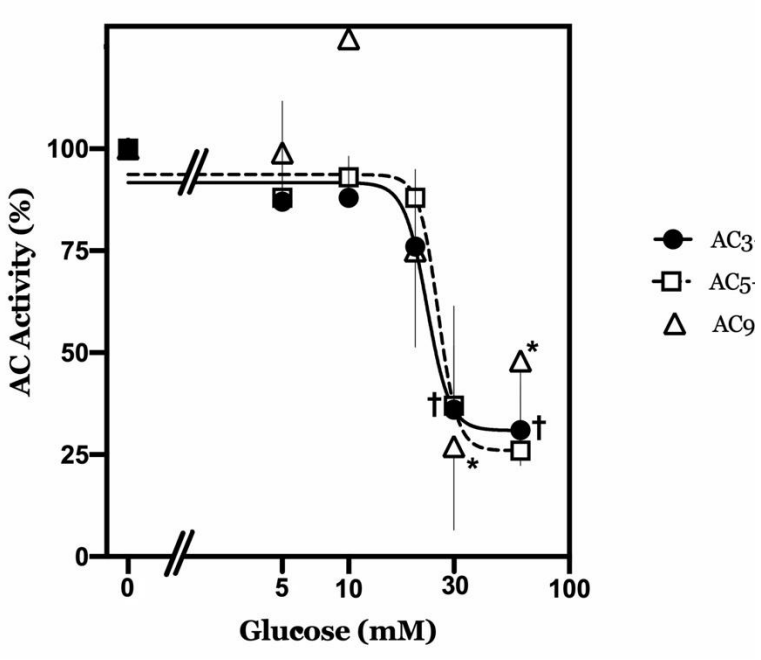


Figure 8. *Effect of glucose on hAC 3, 5, 9. To depict the data in a single graph, activities of hACs 3, 5, and 9 stimulated by 300 nM Gsα were taken as 100% (hAC3 = 0.57, hAC5 = 2.3, AC9 = 0.6 nmol· cAMP ·mg^{-1}·min^{-1}. Significances: *: p < .05, †: p <.01. Error bars denote SD (n= 3). The assay was carried as described in* (Seth et al., 2020).

As we know α cells also have somatostatin receptors which lower AC activity by activating $G_{i/o}$ (G_i protein α subunit is a family of heterotrimeric G protein alpha subunits. This family is often known as $G_{i/o}$. Where $G_i\alpha$ is a well-known inhibitor for AC isoforms 1,5,6) (Kumar et al., 1999; Strowski et al., 2000).

For now, only speculations can be made, in consideration of the data reported in α cells by Yu et al. (2019). Here I report, inhibitory effect of glucose on Gsα-stimulated ACs, hAC 3, 5 and 9. Where IC50 range is 20-25mM and 30mM glucose nearly abrogated the AC activity (Figure 8). The inhibitory effect of $G_{i/o}$ signaling on hAC5, may limit the interpretation for the glucose effect on hAC5 (figure 8).
But due to a lack of understanding of the functional importance and expression profile for different AC isoform in α cells (Tengholm and Gylfe, 2017), the data reported by Yu et al. (2019) and considering that cells express more than one AC isoform (Granneman, 1995) it is worthwhile to report the inhibitory effect of glucose on hAC3 and 9.
The above stated data are preliminary, specific aspects need to be explored such as checking of other sugars, to check if the effect is via TMs or via catalytic domains or due to hampering of Gsα, this can be explored using forskolin and the CqsS_AC2 construct.

II) Serum fractionation

Serum is a complex mix of growth factors, albumins and growth inhibitors (Arora, 2013). Based on Seth et al.(2020) it was clear that serum fractionation is maybe a fruitful strategy. There are various methods to fractionate serum considering the presence of many components which makes it complex (see page 43). One of the many methods considered initially was size fractionation with Amicon® Ultra-4 10K-100K device.
Using this method human serum was fractionated and 10% of each fraction was checked on 600nM Gsα stimulated hAC2. Similarly, dialyzed human serum was fractionated and checked on 600nM Gsα-stimulated hAC2 (data not shown). For both, dialyzed and un-dialyzed serum fractions, the inhibitory pattern was identical (figure 9). The data indicated that the smaller size fractions (*<30kD - <100kD)* were more prone towards reducing the AC activity compared with larger size fractions. This gives us an initial indication of the probable size range of the inhibitory factors present in the serum.

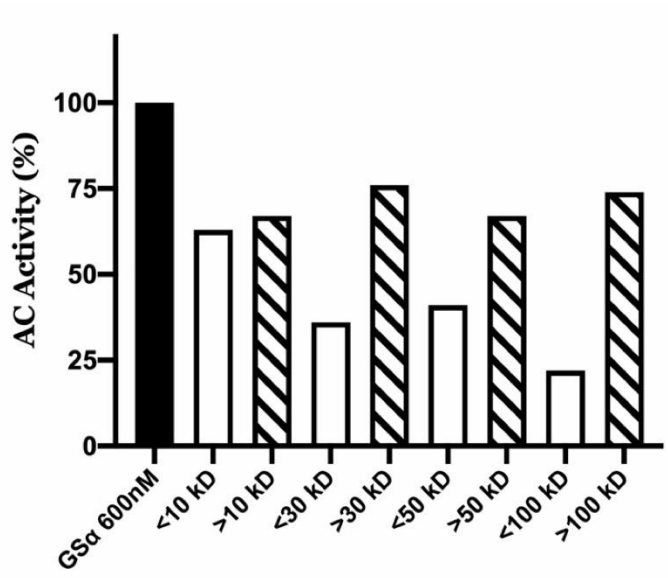


Figure 9. *Effect of human serum fractions on hAC2. Black bar (100%) = hAC2 + 600nM Gsα corresponds to 0.463 nmol cAMP· mg^{-1}·min^{-1} (basal = 0.09 nmol cAMP· mg^{-1}·min^{-1}). Human serum fraction ranges from <10kD to >100kD. 10% of each fraction was checked in presence of hAC2 + 600nM Gsα. The assay was carried as described in (Seth et al., 2020).*

The data reported in this section are preliminary because to search a ligand in serum is like finding a needle in a haystack and many experiments were conducted in different directions. This led us to a more orchestrated search, an ongoing work in the lab.

Altogether, the data help to take a step forward in ligand search i.e. to narrow down the most inhibitory fraction in the serum and to investigate it and layout the components which are most likely to have an evolutionary lineage.

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6. List of figures

(except from result and discussion section, paper 1 and paper 2)

7. List of tables

(except from result and discussion section, paper 1 and paper 2)

8. Material and methods

(for chapter 3)

Reagents and materials

- *Human serum (catalog # 4522 from human male AB plasma).*
- *Rat insulin (Merck-Millipore, catalog 8013-k).*
- *Human Serum (HS-dialyzed): dialysis was done for 48 hours in NaCl (10mM) at 4-7°C. (for Table 2-7) (by Julia Grischin)*
- *Serum fraction by* Amicon® Ultra-4 10K-100K device *(Merck-Millipore).*
- *Human serum dialysis (by Anita Schultz): dialysis was done for 24 hours in NH4HCO3 (10mM) at 4-7°C. (for figure 8)*

Adenylyl cyclase assay

- *(For figure 7)* Activity of hAC 9 was determined in a volume of 10 µl using 1 mM ATP, 4 mM MgCl2, 3 mM creatine phosphate, 60 µg/ml creatine kinase, 50 mM HEPES, pH 7.4, BSA 37.5µM, using an Assist-Plus pipetting robot (Integra Biosciences, Germany) and a cAMP assay kit from Cisbio (Codolet, France).
- Each experiment was run in duplicate/triplicate.