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

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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

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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,

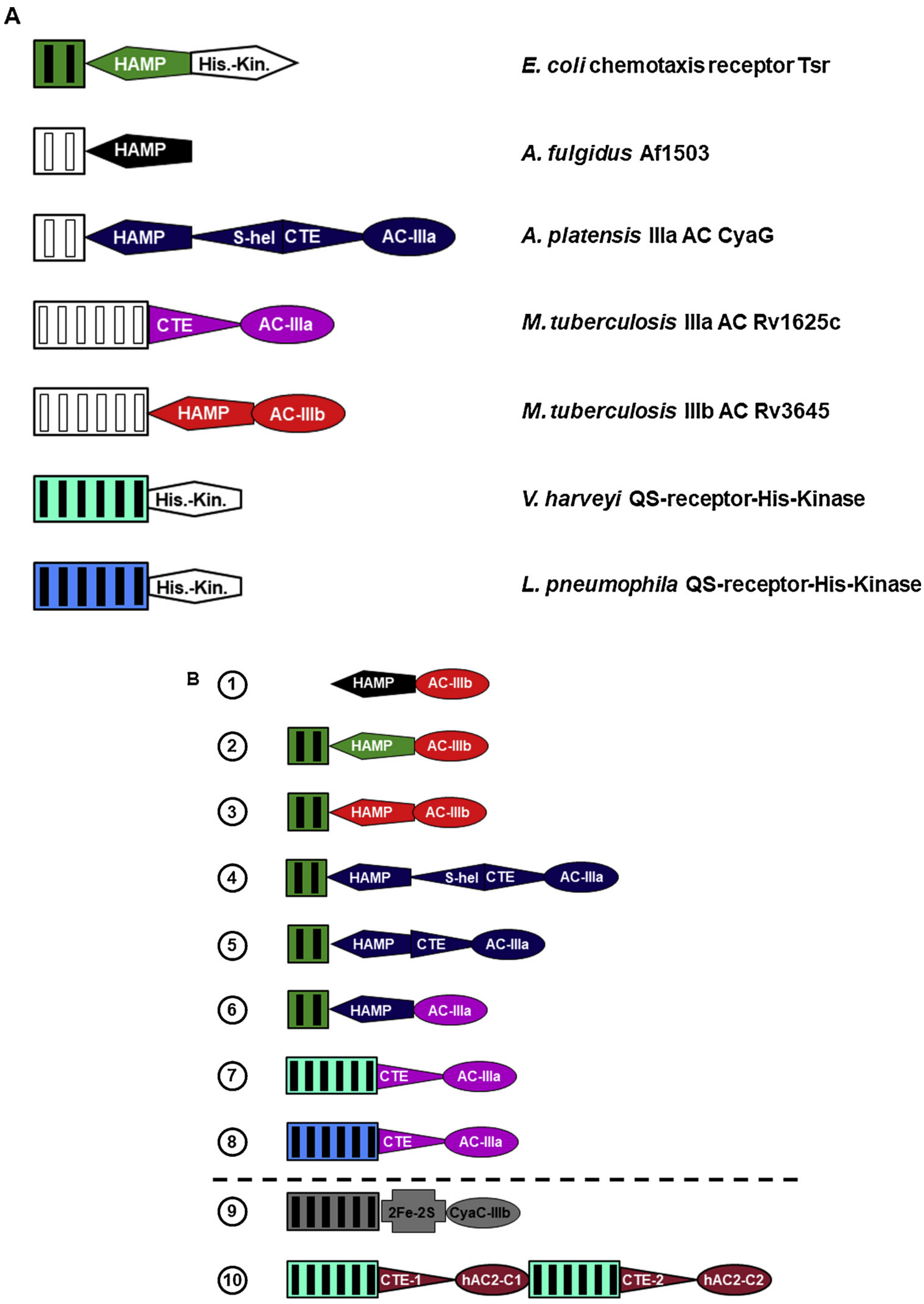

Fig. 1. A) Schematic domain organization of the reference proteins. The colored domains were used as building blocks in subsequent chimeric adenylate cyclases. The empty frames (membrane domains and histidine-kinases) denote domains not used in subsequent constructs. Transmembrane helices are symbolized 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

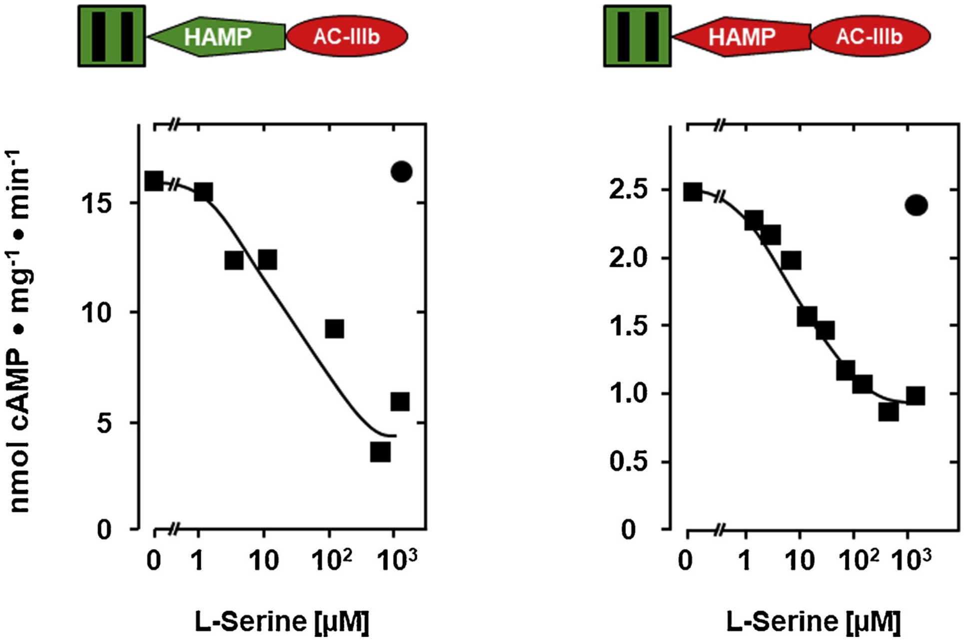

Fig. 2. L-Serine inhibits the class IIIb AC Rv3645 via the *E. coli* Tsr chemotaxis receptor for serine. Above each graph the diagram of the chimera is depicted. Left: Construct with the HAMP domain from Tsr. Maximal serine inhibition was 71 ± 3% (half maximal inhibition was at 18.5 µM). Right: The construct 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

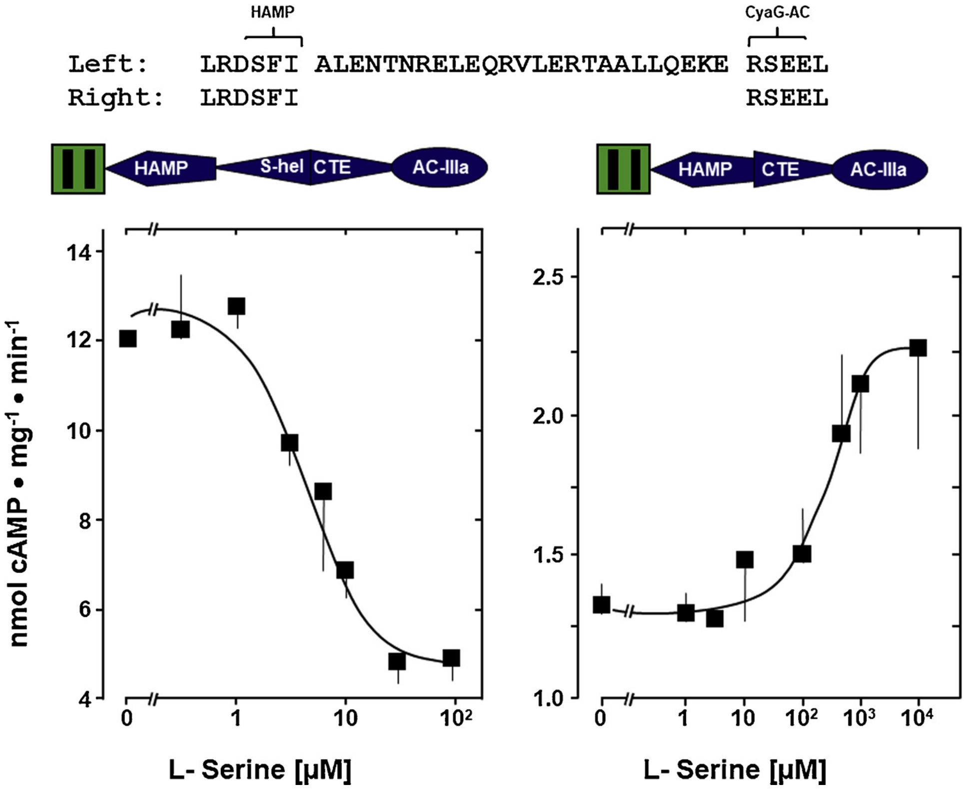

Fig. 3. Class IIIa AC CyaG from *A. platensis* is regulated by L-serine via the *E. coli* Tsr chemotaxis receptor. Left: Tsr linked to CyaG AC via HAMP and S-helix of CyaG AC is regulated by L-serine. Maximal inhibition was 58% (IC_{50} = 6 µM). Right: Removal of the 25 aa long S-helix in the chimera 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. ***Cholera*** **A**uto**I**nducer-1, CAI-1 [(*S*)-3-hydroxytridecan-4-one] and ***Legionella*** **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).

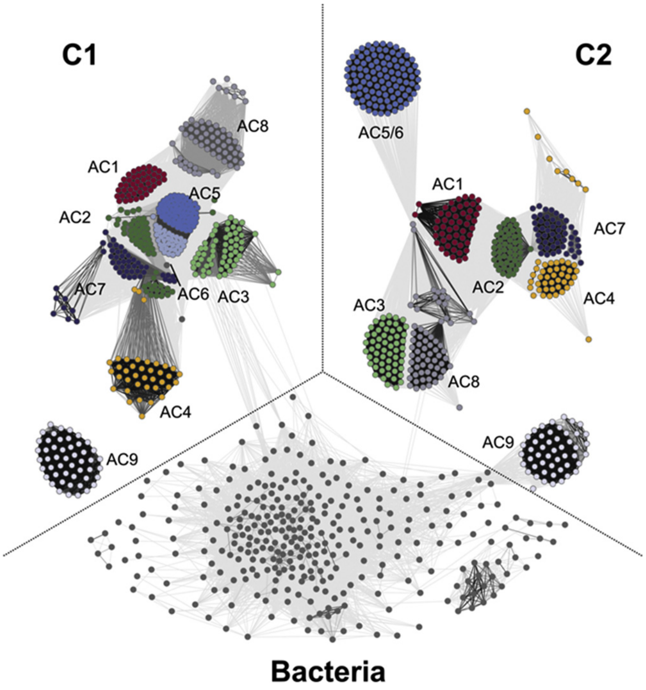

Fig. 5. Cluster map of cyclase-transducer-elements. The sequences of bacterial and vertebrate class IIIa and IIIb CTEs were analyzed using CLANS. Each dot represents a single sequence. Above threshold hits are shown as connecting lines. Darker line color indicates better pairwise blast hits. The three 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).

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

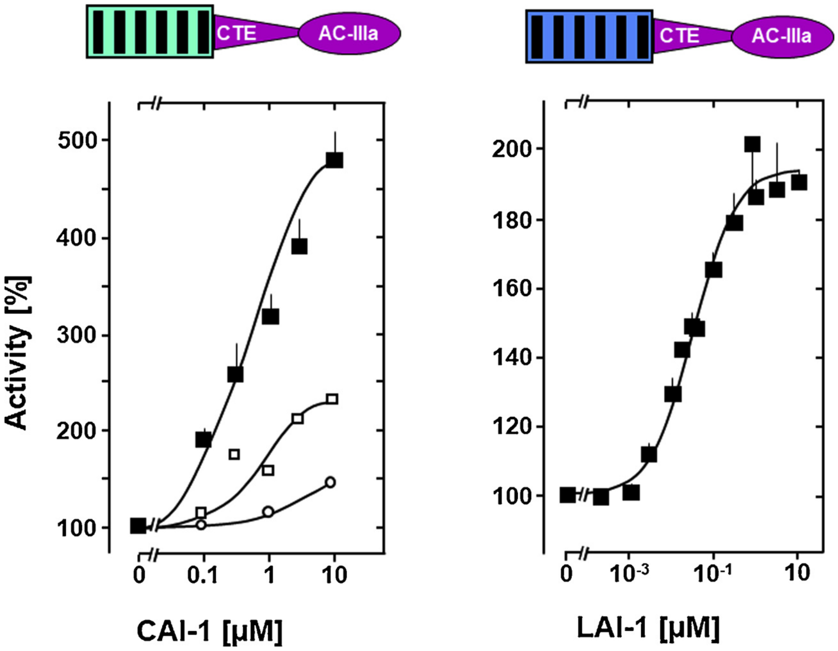

Fig. 4. Left: CqsS-Rv1625c stimulation by the QS-ligand CAI-1. Basal activity was 4 nmol cAMP·mg^{-1} min^{-1}. Filled squares, CAI-1 (n = 5–11; ± S.E.M.); open squares, LAI-1; open circles, 3,4-tridecanediol. The EC_{50} concentration for CAI-1 is 400 nM. CAI-1 stimulations were significant starting 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.

Acknowledgments

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References

Anantharaman, V., Balaji, S., Aravind, L., 2006. The signalling helix: a common functional theme in diverse signalling proteins. Bio Direct 25 (1795-), 1804.

Aravind, L., Ponting, C.P., 1999. The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. FEMS Microbiol. Lett. 176, 111–116.

Barzu, O., Danchin, A., 1994. Adenylyl cyclases: a heterogeneous class of ATP-utilizing enzymes. Prog. Nucleic Acid Res. Mol. Biol. 49, 241–283.

Bassler, J., Schultz, J.E., Lupas, A.N., 2018. Adenylate cyclases: Receivers, transducers, and generators of signals. Cell Signal. 46, 135–144.

Beltz, S., Bassler, J., Schultz, J.E., 2016. Regulation by the quorum sensor from Vibrio indicates a receptor function for the membrane anchors of adenylate cyclases. eLife 5.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry 3rd, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., Barrell, B.G., 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393, 537–544.

Dessauer, C.W., Watts, V.J., Ostrom, R.S., Conti, M., Dove, S., Seifert, R., 2017. International union of basic and clinical pharmacology. ci. structures and small molecule modulators of mammalian adenylyl cyclases. Pharmacol. Rev. 69, 93–139.

Ferris, H.U., Dunin-Horkawicz, S., Mondejar, L.G., Hulko, M., Hantke, K., Martin, J., Schultz, J.E., Zeth, K., Lupas, A.N., Coles, M., 2011. The mechanisms of HAMP-mediated signaling in transmembrane receptors. Structure 19, 378–385.

Ferris, H.U., Zeth, K., Hulko, M., Dunin-Horkawicz, S., Lupas, A.N., 2014. Axial helix rotation as a mechanism for signal regulation inferred from the crystallographic analysis of the E. Coli serine chemoreceptor. J. Struct. Biol.

Guo, Y.L., Seebacher, T., Kurz, U., Linder, J.U., Schultz, J.E., 2001. Adenylyl cyclase Rv1625c of Mycobacterium tuberculosis: a progenitor of mammalian adenylyl cyclases. EMBO J. 20, 3667–3675.

Hazelbauer, G.L., Falke, J.J., Parkinson, J.S., 2008. Bacterial chemoreceptors: high-performance signaling in networked arrays. Trends Biochem. Sci. 33, 9–19.

Hulko, M., Berndt, F., Gruber, M., Linder, J.U., Truffault, V., Schultz, A., Martin, J., Schultz, J.E., Lupas, A.N., Coles, M., 2006. The HAMP domain structure implies helix rotation in transmembrane signaling. Cell 126, 929–940.

Kanchan, K., Linder, J.U., Winkler, K., Hantke, K., Schultz, A., Schultz, J.E., 2010. Transmembrane signaling in chimeras of the Escherichia coli aspartate and serine chemotaxis receptors and bacterial class III adenylyl cyclases. J. Biol. Chem. 285, 2090–2099.

Krupinski, J., Coussen, F., Bakalyar, H.A., Tang, W.J., Feinstein, P.G., Orth, K., Slaughter, C., Reed, R.R., Gilman, A.G., 1989. Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science 244, 1558–1564.

Lancaster, C.R., 2002. Succinate:quinone oxidoreductases: an overview. Biochim. Biophys. Acta 1553, 1–6.

Linder, J.U., Schultz, J.E., 2003. The class III adenylyl cyclases: multi-purpose signalling modules. Cell. Signal. 15, 1081–1089.

Linder, J.U., Schultz, J.E., 2008. Versatility of signal transduction encoded in dimeric adenylyl cyclases. Curr. Opin. Struct. Biol. 18, 667–672.

Ng, W.L., Wei, Y., Perez, L.J., Cong, J., Long, T., Koch, M., Semmelhack, M.F., Wingreen, N.S., Bassler, B.L., 2010. Probing bacterial transmembrane histidine kinase receptor-ligand interactions with natural and synthetic molecules. Proc. Natl. Acad. Sci. U.S.A. 107, 5575–5580.

Ng, W.L., Perez, L.J., Wei, Y., Kraml, C., Semmelhack, M.F., Bassler, B.L., 2011. Signal production and detection specificity in Vibrio CqsA/CqsS quorum-sensing systems. Mol. Microbiol. 79, 1407–1417.

Ng, W.L., Perez, L., Cong, J., Semmelhack, M.F., Bassler, B.L., 2012. Broad spectrum pro-quorum-sensing molecules as inhibitors of virulence in vibrios. PLoS Pathog. 8, e1002767.

Parkinson, J.S., 2010. Signaling mechanisms of HAMP domains in chemoreceptors and sensor kinases. Annu. Rev. Microbiol. 64, 101–122.

Schultz, J.E., Natarajan, J., 2013. Regulated unfolding: a basic principle of intraprotein signaling in modular proteins. Trends Biochem. Sci. 38, 538–545.

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

Schultz, J.E., Kanchan, K., Ziegler, M., 2015. Intraprotein signal transduction by HAMP domains: a balancing act. Int. J. Med. Microbiol. 305, 243–251.

Sinha, S.C., Wetterer, M., Sprang, S.R., Schultz, J.E., Linder, J.U., 2005. Origin of asymmetry in adenylyl cyclases: structures of Mycobacterium tuberculosis Rv1900c. EMBO J. 24, 663–673.

Spirig, T., Tiaden, A., Kiefer, P., Buchrieser, C., Vorholt, J.A., Hilbi, H., 2008. The Legionella autoinducer synthase LqsA produces an alpha-hydroxyketone signaling molecule. J. Biol. Chem. 283, 18113–18123.

Steegborn, C., Litvin, T.N., Levin, L.R., Buck, J., Wu, H., 2005. Bicarbonate activation of adenylyl cyclase via promotion of catalytic active site closure and metal recruitment. Nat. Struct. Mol. Biol. 12, 32–37.

Sutherland, E.W., Rall, T.W., 1958. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J. Biol. Chem. 232, 1077–1091.

Tesmer, J.J., Sunahara, R.K., Gilman, A.G., Sprang, S.R., 1997. Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsalpha.GTPgammaS. Science 278, 1907–1916.

Tews, Findeisen, F., Sinning, I., Schultz, A., Schultz, J.E., Linder, J.U., 2005. The structure of a pH-sensing mycobacterial adenylyl cyclase holoenzyme. Science 308, 1020–1023.

Ulrich, L.E., Koonin, E.V., Zhulin, I.B., 2005. One-component systems dominate signal transduction in prokaryotes. Trends Microbiol. 13, 52–56.

Ulrich, L.E., Zhulin, I.B., 2010. The MiST2 database: a comprehensive genomics resource on microbial signal transduction. Nucleic Acids Res. 38, D401–407.

Vercellino, I., Rezabkova, L., Olieric, V., Polyhach, Y., Weinert, T., Kammerer, R.A., Jeschke, G., Korkhov, V.M., 2017. Role of the nucleotidyl cyclase helical domain in catalytically active dimer formation. Proc. Natl. Acad. Sci. U. S. A. 114, E9821–E9828.

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

Wei, Y., Ng, W.L., Cong, J., Bassler, B.L., 2012. Ligand and antagonist driven regulation of the Vibrio cholerae quorum-sensing receptor CqsS. Mol. Microbiol. 83, 1095–1108.

Winkler, K., Schultz, A., Schultz, J.E., 2012. The S-helix determines the signal in a Tsr receptor/adenylyl cyclase reporter. J. Biol. Chem. 287, 15479–15488.

Wissig, J., Grischin, J., Bassler, J., Schubert, C., Schultz, J.E., Unden, G., 2019. CyaC, a redox-regulated adenylate cyclase of Sinorhizobium meliloti with a fumarate reductase-like diheme-B membrane anchor. Mol. Microbiol in revision.

Ziegler, M., Bassler, J., Beltz, S., Schultz, A., Lupas, A.N., Schultz, J.E., 2017. A novel signal transducer element intrinsic to class IIIa and IIIb adenylate cyclases. eLife 6, 1–5.