Functional chimeras between the catalytic domains of the mycobacterial adenylyl cyclase Rv1625c and a *Paramecium* guanylyl cyclase

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Abstract The class IIIa adenylyl cyclase (AC) Rv1625c from *Mycobacterium tuberculosis* forms homodimers with two catalytic centres, whereas the *Paramecium* guanylyl and mammalian ACs operate as pseudoheterodimers with one catalytic centre. The functional and structural relationship of the catalytic domains of these related class III cyclases was investigated. Point mutations introduced into Rv1625c to engineer a forskolin-binding pocket created a single heterodimeric catalytic centre, yet did not result in forskolin activation. Chimerization of these Rv1625c point mutants with corresponding mammalian AC domains was impossible. However, it was successful using a complemental *Paramecium* guanylyl cyclase domain and resulted in an AC. The data signify a divergence of structural and functional evolution in class III Acs.

Keywords: Adenylyl cyclase; Guanylyl cyclase; Heterodimer; *Mycobacterium tuberculosis*; *Paramecium tetraurelia*

1. Introduction

In mammals the second messengers cAMP and cGMP are synthesized by adenylyl cyclases (ACs) and guanylyl cyclases (GCs), respectively. The catalytic domains are similar and are often referred to as cyclase homology domains (CHDs). A recent analysis has subclassified mammalian GCs and membrane-bound ACs as class IIIa cyclases, together with protozoan ACs and GCs as well as many eubacterial ACs [1]. The catalytic centre is formed at the interface from either different CHDs (C1 and C2, one catalytic centre) or identical CHDs (two catalytic centres, Fig. 1(a)) [2,3]. The domain compatibility of different isoforms of class IIIa CHDs appears to be high, because active heterodimers can be reconstituted in vitro, e.g., mammalian ACs type V C1 + type II C2, type I C1 + type II C2 and even mammalian type V AC C2 + soluble GC-α_1 [4–6]. This functional compatibility has been further extended by the successful heterodimerization of mammalian CHDs (type II C2, type IX C2) and a corresponding *Paramecium* GC C2 domain, demonstrating a structural as well as functional conservation of catalytic centres from metazoan and protozoan isoforms [7]. Recently, the class IIIa CHD of the membrane-bound AC Rv1625c from *Mycobacterium tuberculosis*, which is topologically identical to one half of a mammalian AC, was characterized [3,8,9]. Here, we ask whether the mycobacterial monomer can functionally interact with eukaryotic CHDs. We demonstrate that an inactive point mutant (D300A) of Rv1625c, which is equivalent to a mammalian C2-CHD, can be reconstituted with an inactive C2 domain (C1 equivalent) of the *Paramecium* GC (Fig. 1(a)). The chimera exclusively uses ATP as a substrate. The results characterize the structural and functional relationship of mammalian, protozoan and bacterial class IIIa CHDs.

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Abbreviations: AC, adenylyl cyclase; GC, guanylyl cyclase; CHD, cyclase homology domain

2. Materials and methods

2.1. Plasmid construction

Cloning of Rv1625cD300A, the C2 domains of rat type II and mouse type IX ACs, and the C2 domain of a GC from *Paramecium tetraurelia 51s* was as reported earlier [3,7]. A pQE60-based expression plasmid for the C1-CHD of type I AC was provided by Kleuss and coworkers [10]. The type V AC C1 domain from rabbit (amino acids 405–645, GenBank Accession No. P40144) was fitted with a 5′-*Sal*I and a 3′-*Mlu*I site and cloned into a modified pQE30-vector [7]. Thereby, an N-terminal $MRGSH_6GVD$ metal-affinity tag and a C-terminal TRLSLIS heptapeptide extension were added. The bovine type VII AC C1 domain (amino acids 222–485, GenBank Accession No. Q29450) was cloned identically.

Mutants Rv1625cD300S, Rv1625cN372A and Rv1625cN372T were generated by PCR using the DNA of the catalytic domain of Rv1625c as a template and cloned into pQE30 [3]. Expression and purification of recombinant proteins was as reported [3,7].

2.2. Cyclase assays

The reactions contained in 100 μl of 50 mM Tris–HCl, pH 7.5, 22% glycerol, 2 mM $MnCl_2$, 75 μM [α-^{32}P]ATP (unless stated otherwise) and 2 mM [2,8-^{3}H]cAMP. Ingredients were mixed on ice 10 min prior to the start of the reaction. AC activity was determined at 30 °C for 10 min. Standard errors of the mean (S.E.M.) of multiple assays were usually <10%. GC activity was determined identically using GTP as a substrate [11,12].

3. Results and discussion

3.1. The catalytic and forskolin-binding sites in class IIIa adenylyl cyclases

In the catalytic domain of the mycobacterial AC, Rv1625c functions are incorporated which in mammalian ACs are distributed between two similar, individually inactive CHDs, C1 and C2 [3]. Consequently, Rv1625c is active as a homodimer with two functional catalytic centres, whereas mammalian ACs

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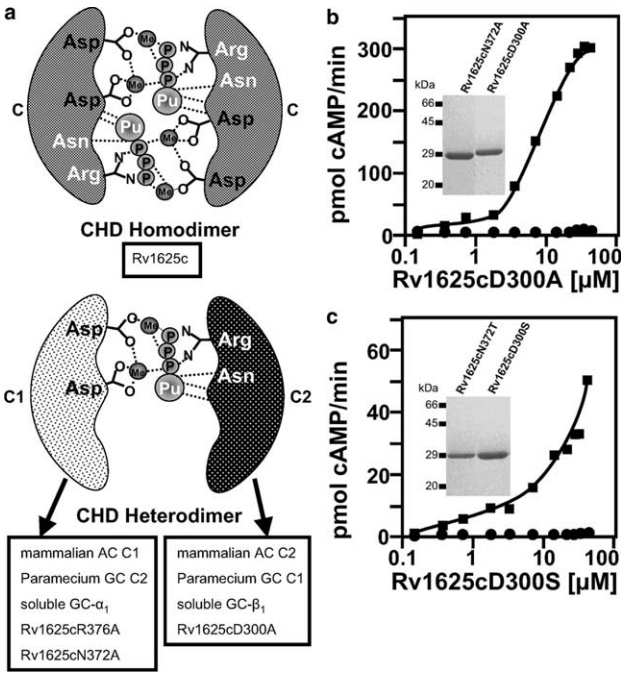

Fig. 1. Heterodimeric class IIIa AC catalysts. (a) Structural comparison of the homodimeric Rv1625c with two catalytic centres versus heterodimeric catalysts harbouring a single catalytic centre. Purine triphosphates are sketched by moieties. Pu: purine; Me: metal ion-cofactor; P: phosphate moiety. C: homodimeric CHD; C1, C2: heterodimeric CHDs. Examples are given in the respective boxes. (b) Titration of 45 nM Rv1625cN372A with an excess of Rv1625cD300A (squares). Circles: Rv1625cD300A alone. Inset: 15% PAGE of purified recombinant proteins (2 μg per lane). (c) Titration of 45 nM Rv1625cN372T with Rv1625cD300S (squares). Circles: Rv1625cD300S alone. Inset: 15% PAGE of purified recombinant proteins (1 and 2 μg per lane, respectively). Both titrations were conducted at 85 μM ATP.

types I–IX have only one catalytic site. With the exception of AC type IX, the second location evolved as a site which makes the heterodimer susceptible to activation by the plant diterpene forskolin [2]. Therefore, in a first step to resolve the structural relationship between mycobacterial and mammalian AC catalysts, we created a mycobacterial heterodimeric AC by introduction of point mutations which effectively generated mycobacterial C1 or C2 equivalents (Fig. 1(a), [3]). For example, Rv1625cN372A and Rv1625cR376A or Rv1625cD256A and Rv1625cD300A correspond to mammalian C1 and C2 domains, respectively, are catalytically incompetent alone and complement each other. Rv1625cN372A and Rv1625cR376A lack transition-state stabilizing amino acids, whereas Rv1625cD256A and Rv1625cD300A each miss a metal-cofactor binding aspartate. For example, Rv1625cN372A, a C1 equivalent, complements Rv1625cD300A resulting in wild-type AC activity of 2.5 μmol cAMP mg^{-1} min^{-1} (Fig. 1(b)). This proved that D300 in a C2-equivalent and N372 in a C1 equivalent were dispensable for dimer formation.

Next, we attempted to create an environment for forskolin binding. Eleven conserved amino acids were identified as forskolin contacts in the structures of mammalian ACs, i.e., Phe394, Tyr443, Val506, Tyr507, Ser508, Val511 and Thr512 in C1 (ACV numbering) and Leu912, Ile940, Gly941 and Ser942 in C2 (ACII numbering) [2,13]. Six of these residues are similar or identical in Rv1625c (see [3] for alignment). Asp300 and Asn372 in Rv1625c are at the positions of Ser942 and Thr512, respectively, and are predicted to impede forskolin binding [2]. Accordingly, we generated the heterodimer Rv1625cD300S/Rv1625cN372T. With now 8 out of 11 amino acids known to contact forskolin either similar or identical, we dismissed further mutations at this point to avoid unpredictable structural changes. In fact, we expected that with this reconstructed binding pocket the diterpene might at least cause a small and consistent activation. Rv1625cD300S and Rv1625cN372T were purified to homogeneity and were inactive as expected. Titration of 45 nM Rv1625cN372T with Rv1625cD300S reconstituted 16% (0.4 μmol cAMP mg^{-1} min^{-1}) of wild-type activity (Fig. 1(c)) and demonstrated that the proteins were properly folded, interacting and active. However, addition of 100 μM forskolin did not activate at all at 75 μM substrate ATP, whereas it strongly activated an appropriate mammalian C1/C2 couple run as a positive control under identical conditions. 75 μM MnATP was used because mammalian ACs are more sensitive to forskolin at low MnATP [14]. Mn^{2+} was also used as a metal-cofactor because Mg^{2+} is ineffective with Rv1625c [3,8,9].The results suggest that the evolution of forskolin-sensitivity of mammalian ACs probably has been accompanied by structural rearrangements going beyond differences in those amino acids which line the non-catalytic pocket and which are in direct contact with forskolin.

3.2. Chimeras of Rv1625c and mammalian ACs

Next, we tested whether the C2 equivalent Rv1625cD300A can complement C1 domains from mammalian ACs types I, V and VII. The recombinant purified proteins were preincubated and AC activity was assayed. In no instance did we observe a functional reconstitution as compared to standard control incubations (see above). We also tested whether mixtures of Rv1625cD300S and the C1 domains of mammalian type I and V ACs would yield an active heterodimer. Again, no reconstitution occurred irrespective of the presence or absence of forskolin. Next, we used Rv1625cN372A and *forskolin-adapted* Rv1625cN372T as C1 equivalent AC domains and the C2 domains of the mammalian ACs types II and IX. As above, robust reconstitution of AC activity with these formally complementary bacterial/mammalian AC domains was not detectable. The absence of AC activity in the mycobacterial-mammalian chimeras prohibits a meaningful discussion beyond the conclusion that the considerable extent of conservation in primary sequence, canonical catalytic amino acids and computed protein structure of Rv1625c and its mammalian AC congeners [1,3,15] stops short of a structural compatibility. The interacting domain surfaces and/or the ATP-binding pockets obviously diverged beyond the minimal requirements for a functionally successful interaction.

3.3. An active chimera of Rv1625c and a Paramecium GC

Earlier we reported that mammalian C2-CHDs from AC types II and IX can functionally interact with the C2-CHD of a GC from the ciliate *Paramecium* which is the equivalent of a mammalian C1-CHD, whereas reverse chimeras (mammalian C1/*Paramecium* C1) are inactive [7,16]. The C2 GC/C2 AC heterodimer operates as a specific AC because the substrate-specifying residues reside in the mammalian C2 domains [7,16]. Does the *Paramecium* GC C2-CHD complement a function-

ally corresponding mycobacterial Rv1625cD300A? At 3 μM, each individual CHD was inactive. However, in the mixture of both domains a robust AC activity of 6.8 nmol cAMP mg^{-1} min^{-1} was detected using Mn^{2+} as a metal-cofactor. Mg^{2+} ions did not support activity. As a negative control, we mixed the inactive Rv1625cN372A mutant (a C1 equivalent) with the *Paramecium* GC C2 domain, also a C1 equivalent. As expected no AC activity was detectable although Rv1625cN372A was otherwise capable to restore activity with the Rv1625cD300A mutant proving its structural and functional integrity.

As expected, the chimeric heterodimer did not use GTP as a substrate, because Rv1625cD300A carries the substrate specifying residues (K296 and D365, [3]) and thus enforces ATP substrate specificity. A kinetic analysis (45 nM *Paramecium* GC C2 + 9 μM Rv1625cD300A) yielded a K_M of 75 μM ATP and a V_{max} of 135 nmol cAMP mg^{-1} min^{-1} based on the amount of the *Paramecium* GC C2 (Fig. 2). The Hill coefficient was 1.0, in line with the presence of a single catalytic centre.

The Rv1625cD300A/*Paramecium* GC C2 chimera was insensitive to 100 μM forskolin and was only slightly affected by 1 mM of the P-site inhibitors 2′d–3′AMP and 2′d–3′GMP (20% inhibition for each compound, data not shown).

To determine the affinity of the mycobacterial and ciliate dimerization epitopes, we titrated 155 nM *Paramecium* GC C2 (C1 equivalent) with up to 63 μM Rv1625cD300A as a C2 equivalent (Fig. 3). AC activity increased with the concentration of Rv1625cD300A over three orders of magnitude giving an almost linear graph on a semi-logarithmic plot (Fig. 3). A reciprocal plot characterized this behaviour as being biphasic (Fig. 3, inset). The first phase (up to 5 μM Rv1625cD300A), which obviously corresponded to the formation of heterodimers, had a dissociation constant (K_D) of 1.1 μM. The second phase ($K_D = 5$ μM) may represent the formation of higher order oligomers, e.g., *Paramecium* GC C2/$(Rv1625cD300A)_3$, because oligomerization of the Rv1625c catalytic domains was previously shown at higher protein concentrations [3]. The successful titration experiments demonstrated again that the lack of reconstitution of Rv1625c mutants and mammalian AC domains could not be ascribed to a reduced ability of the proteins used here to dimerize, although such defects have been reported for other Rv1625c mutant proteins [9]. Actually, the successful heterodimerization showed that a sufficient fraction of monomers was present in the dynamic monomer–

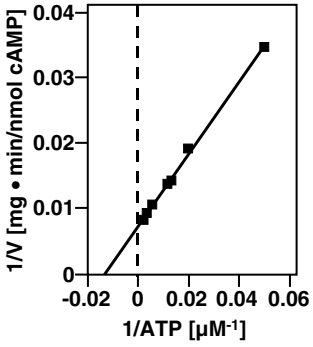

Fig. 2. Kinetic analysis by a Lineweaver–Burk plot of the *Paramecium* GC/Rv1625c chimera. 45 nM *Paramecium* GC C2 was mixed with 9 μM Rv1625cD300A and assayed with 2 mM Mn^{2+} and 20–425 μM ATP.

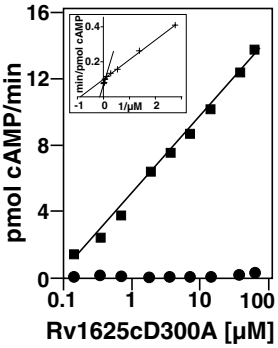

Fig. 3. Titration of 155 nM *Paramecium* GC C2 domain with Rv1625cD300A (squares). Circles: Rv1625cD300A alone. Assays were done with 2 mM Mn^{2+} and 75 μM ATP. Inset: Reciprocal plot demonstrating the biphasic nature of the titration curve.

dimer equilibrium because otherwise active heterodimers would not have formed on a reasonable timescale. The successful reconstitution with the *Paramecium* GC C2 domain substantiated that heterodimers can successfully form between Rv1625c mutants and distantly related cyclases from other organisms, although homodimerization of Rv1625cD300A probably was a concurrent competing process. Because the *Paramecium* GC C1 domain cannot be purified and is unstable [7], the theoretically inverse complementary combination of GC C1 and Rv1625cN372N was not investigated.

The ability of the CHDs from the mycobacterial AC and the ciliate GC to form an active chimeric catalytic centre highlights the structural and functional complementarity between a bacterial and protozoan catalytic domain. From an evolutionary perspective, the protozoan GC is thought to stand between the mycobacterial and the mammalian AC species. It can successfully interact with corresponding domains on both sides of an envisaged class III AC development. This effectively indicates that primary structure, i.e., sequence conservation and functional conservation, was retained over more than 3 billion years. Nevertheless, accompanying changes in amino acid composition altered the fine structure of the dimer surfaces sufficiently to hamper domains interactions. The 3D structure of the mammalian heterodimer has been resolved at 2.3 Å [2] and a structure of the Rv1625c is forthcoming [15]. It would be most interesting to obtain an X-ray structure of the protozoan GC catalytic heterodimer to see how structural and functional parameters evolved independently.

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