A Guanylyl Cyclase from Paramecium with 22 Transmembrane Spans

EXPRESSION OF THE CATALYTIC DOMAINS AND FORMATION OF CHIMERAS WITH THE CATALYTIC DOMAINS OF MAMMALIAN ADENYLYL CYCLASES

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Paramecium has a 280-kDa guanylyl cyclase. The N terminus resembles a P-type ATPase, and the C terminus is a guanylyl cyclase with the membrane topology of canonical mammalian adenylyl cyclases, yet with the cytosolic loops, C1 and C2, inverted compared with the mammalian order. We expressed in Escherichia coli the cytoplasmic domains of the protozoan guanylyl cyclase, independently and linked by a peptide, as soluble proteins. The His6-tagged proteins were enriched by affinity chromatography and analyzed by immunoblotting. Guanylyl cyclase activity was reconstituted upon mixing of the recombinant C1a- and C2-positioned domains and in a linked C1a-C2 construct. Adenylyl cyclase activity was minimal. The nucleotide substrate specificity was switched from GTP to ATP upon mutation of the substrate defining amino acids Glu1681 and Ser1748 in the C1-positioned domain to the adenylyl cyclase specific amino acids Lys and Asp. Using the C2 domains of mammalian adenylyl cyclases type II or IX and the C2-positioned domain from the Paramecium guanylyl cyclase we reconstituted a soluble, all C2 adenylyl cyclase. All enzymes containing protozoan domains were not affected by Go/GTP or forskolin, and P site inhibitors were only slightly effective.

Adenylyl and guanylyl cyclases are key proteins in intracellular signaling of essentially all eukaryotic cells. In the fresh-water protozoans Paramecium and Tetrahymena cAMP and cGMP levels depend on the ion composition of the extracellular milieu. Whereas adenylyl cyclase activity in vivo is stimulated by membrane hyperpolarization (1, 2), cGMP formation is enhanced by a depolarizing Ca\(^2+\) inward current (3). Recently, we reported on a group of guanylyl cyclases of 280 kDa that is present in the ciliates Paramecium and Tetrahymena but also in the genome of the malaria parasite Plasmodium (4). This novel signal transduction protein is bifunctional. It has a 155-kDa N-terminal P-type ATPase-like domain and a 115-kDa C-terminal guanylyl cyclase domain. Both are linked by a cytosolic loop of about 110 amino acids (see Fig. 1A). Surprisingly, the guanylyl cyclase domain is topologically identical to mammalian adenylyl cyclases, i.e. it is composed of two pseudosymmetric modules of six putative transmembrane spans (M1 and M2) that serve as membrane anchors for the cytosolic, catalytic segments C1 and C2 (4). The sequences of both cytosolic subdomains are similar to each other and resemble the catalytic regions of metazoan adenylyl and, to a lesser extent, guanylyl cyclases. Sequence comparisons show that the order of the protozoan C1a- and C2-positioned loops is reversed compared with the corresponding mammalian adenylyl cyclase loops (4). Data based on the crystal structure of a soluble adenylyl cyclase type VC1a/type IIC2 chimera demonstrated that Lys938 and Asp1018 in the catalytic pocket of the C2 domain (adenylyl cyclase type II numbering) are responsible for ATP substrate specificity (5, 6). In the Paramecium guanylyl cyclase, those two amino acids have evolved as Glu1681 and Ser1748 in the C1-positioned domain, i.e. specify GTP as a substrate, whereas the surrounding amino acid residues are more akin to metazoan adenylyl cyclases (see Fig. 1B for an abbreviated sequence alignment). The expressed membrane-bound Paramecium guanylyl cyclase domain uses MgGTP as a substrate; it accepts MnATP as a substrate only slightly and is inactive with MgATP (4).

Following successful strategies by others to produce soluble heterodimeric adenylyl cyclases (7–13), we wished to assemble a protozoan heterodimeric guanylyl cyclase by expression in Escherichia coli of the catalytic domains either separately or covalently linked by a peptide linker. Here, we report on the expression and activity of such constructs. We then changed the purine nucleotide substrate specificity in this ciliate guanylyl cyclase with mammalian adenylyl cyclase topology by site-directed mutagenesis of two amino acids in the C1-positioned cytosolic loop. The inversion of the cytosolic loops was definitively proven by the successful generation of active adenylyl cyclase chimeras, which consisted only of cytosolic C2 cyclase domains, i.e. the mammalian adenylyl cyclase IIC2 or IXC2 and the C2-positioned domain of the Paramecium guanylyl cyclase.

EXPERIMENTAL PROCEDURES

Materials—Radiochemicals were purchased from ICN or Amersham-Pharmacia Biotech; pQE vectors and nickel-NTA1 resin were from Qiagen. All enzymes were from Roche Molecular Biochemicals and New England Biolabs.

Mammalian Adenylyl and Protozoan Guanylyl Cyclase Plasmid Constructs—To obtain the soluble constructs of the Paramecium guanylyl cyclase cytosolic loops, we used a clone as a template in which all TAA/TAG-Gln codons had been mutated to the universal CAA/CAG-Gln triplets (GenBank™ accession number AJ238859; Ref. 4). For cloning purposes the C1a- (ParaGC-C1a) and C2- (ParaGC-C2) positioned cytosolic domains were fitted with SacI, MluI, and AscI restriction sites.

1 The abbreviations used are: NTA, nitrilotriacetic acid; G protein, heterotrimeric guanine nucleotide-binding protein; GTPyS, guanosine 5’-O-(3-thiophosphate); ParaGC, guanylyl cyclase from Paramecium.
The exact segment boundaries including the N-terminal His6-sequence and N- and C-terminal stuffer amino acids added to accommodate respective restriction sites are depicted in Fig. 2A. Conventional experimental protocols in molecular biology were followed to obtain the DNA constructs. A list of oligonucleotide sense and antisense primers and of synthetic mutagenic primers that were used in this study is available on request. The peptide linker AAGGPPAAGG in the construct ParaGC-C1aLParaGC-C2 was generated with two complementary oligonucleotide primers. The correctness of all constructs was verified by double-stranded DNA-sequencing. The constructs were cloned into a pQE30 expression vector that was modified to contain only a 5′-StuI and a 3′-AcHiindII cloning site.

The C2 clone from type II adenylyl cyclase (GenBank™ accession number M80550; IIC2) and a mouse type IX adenylyl cyclone clone (GenBank™ accession number Z50196) were kindly provided by Dr. C. Kleuss (Berlin) and Dr. F. Antoni (Glasgow), respectively. The C2 regions were cloned using the polymerase chain reaction with respective cDNAs as templates and appropriate specific primer pairs with 5′- and 3′-terminal restriction sites suitable for cloning of the products into the pQE30 expression vector (see Fig. 2B).

**Mutagenesis**—Site-directed mutagenesis (ParaGC-El681K and S1748D) was carried out by the method of Deng and Nickoloff (14) following the transformer site-directed mutagenesis kit (CLONTECH) and by polymerase chain reaction techniques (23–25) to (KQQ) to KQQ). cDNAs encoding the C1a- and C2-positioned loops of the Paramecium guanylyl cyclase domain (Fig. 2A) served as templates.

**Expression, Purification, and Stability of Protozoan Guanylyl and Mammalian Adenylyl Cyclase in E. coli**—The constructs in the pQE30 expression plasmid were transformed into the E. coli strain BL21(DE3)[pREP4]. Cultures were grown at 23 °C in 600 ml of Luria broth containing 50 mg/liter ampicillin and 50 mg/liter kanamycin and induced with 30 μM isopropyl-1-thio-β-D-galactopyranoside at an A600 of 0.5. Cells were harvested after 5 h and frozen in liquid N2. For purification (9) frozen cells were suspended in 20 ml of cell lysis buffer (50 mM Tris-HCl, pH 8, 10 mM MgCl2, 100 mM NaCl) containing a mixture of protease inhibitors (22 μg/ml 1-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanol, 22 μg/ml 1-1-chloro-3-[4-tosylamido]-7-amino-2-hepta none-HCl, 22 μg/ml phenylmethylsulfonyl fluoride, 3.2 μg/ml trypsin inhibitor from lima beans, 0.5 μg/ml aprotinin). Cells were sonicated (3 × 15 s), and 0.2 mg/ml lysozyme were added. After 30 min 20 μg/ml DNase I and 5 mM MgCl2 were added, and the suspension was incubated for 30 min. Cell debris was removed by centrifugation (31,000 × g, 30 min). The lysates were immediately made 300 mM in NaCl, and 200 μl of nickel-NTA was added. After 45 min at 0 °C, the resin was poured into a column and washed twice with buffers (1.5 M, 25 kBq). Substrate activity was assayed using [γ-32P]purine triphosphates (75 μM, 25 kBq). Substrate and product were separated using DOWEX and alumina chromatography (15, 16).

**RESULTS AND DISCUSSION**

**Bacterial Expression of Soluble Guanylyl Cyclase**—The membrane-bound guanylyl cyclase from the Paramecium consists of two domains (Fig. 1A). The 155-kDa N-terminal domain resembles a P-type ATPase, and the C-terminal codes for a guanylyl cyclase of 115 kDa with a structure characteristic of canonical mammalian adenylyl cyclases yet with reversed cytosolic C1a- and C2-positioned loops (4). For many investigations soluble chimeric constructs of cytosolic adenylyl cyclase loops have been invaluable tools (7–13, 17–19). We wished to reconstitute a soluble protozoan guanylyl cyclase from its C1- and C2-positioned loops and expressed the recombinant proteins in E. coli either individually or linked by a short peptide in analogy to earlier experiments (17–19). We used a His-tagged Paramecium C1a-positioned construct with an expected molecular mass of 25 kDa starting at Asp1607, i.e. one amino acid in front of the 1608LSILLP1614 sequence, which corresponds to a slightly conserved LLENVLP motif present, for example, in C2 of rat type II adenylyl cyclase. The His-tagged protozoan C2-positioned loop with an expected molecular mass of 29 kDa consisted of the entire region downstream of the last transmembrane span (Fig. 2A). After Ni2+-NTA enrichment of the recombinant proteins, a Western blot analysis identified the expected bands as the most dominant proteins and only a very minor immunoreactive contaminant of 19 kDa, probably a proteolytic product, was present in C1a (Fig. 3). Guanylyl cyclase activity of individual catalytic loops was not detected, whereas a mixture of the C1a- and C2-positioned loops had high activity using either MgGTP or MgATP as substrate (Table I). Obviously, the protozoan guanylyl cyclase loops had a high affinity for each other to form a nonchimeric active dimer. This is in contrast to results with the catalytic domains from mammalian adenylyl cyclases where reconstitution of robust soluble adenylyl cyclase activity usually requires mixing of cytosolic domains from different isoforms and the addition of activators (9).

It was demonstrated that the activity of soluble constructs of mammalian adenylyl cyclases is retained or even increased when the catalytic loops are linked by a short peptide (7). Therefore, we linked the protozoan cytosolic domains via a tetradecapeptide maintaining the sequential order of the domains present in the parent enzyme, i.e. reversed compared with mammalian adenylyl cyclases (construct ParaGC-C1aL2C; Fig. 2A). After affinity purification the expected product was identified by Western blotting as a single band (Fig. 3). Although guanylyl cyclase activity was retained, it did not exceed that obtained in a mixture of the individually expressed loops (Table I).

The K<sub>m</sub> values of the mixture of ParaGC-C1a/ParaGC-C2 for MgGTP and MnGTP were 76 and 9 μM, respectively, and thus not very different from the K<sub>m</sub> values determined with the linked ParaGC-C1aL2C2 construct (60 and 51 μM, respectively) and corresponded closely to the K<sub>m</sub> determined for the membrane-bound guanylyl cyclase domain expressed in HEK293 or for the native enzyme (4, 15). Double reciprocal plots of substrate kinetics were linear from 9 to 230 μM MgGTP and did not indicate kinetic peculiarities apart from a slight substrate inhibition at GTP concentrations exceeding 230 μM. The Hill coefficient was close to 1, indicating no cooperative interactions with the substrate. In assays with 75 μM GTP as a substrate even a 10-fold excess of ATP barely inhibited guanylyl cyclase activity, indicating a high specificity for GTP as a substrate. The temperature optimum of 32 °C was rather low. A molar activation energy of 150 kJ/mol was derived from the linear leg of an Arrhenius plot (1/Τ versus ln(𝑣)).

**Adenylyl Cyclase Activity of Soluble Guanylyl Cyclase**—To examine the substrate specificity of the soluble guanylyl cyclase, we assayed all constructs for adenylyl cyclase activity (Table I). Using MgATP as a substrate, adenylyl cyclase activ-
ity was 5% or less of the respective guanylyl cyclase activity (Table I). With MnATP adenylyl cyclase activity was about 20% of guanylyl cyclase activity, and the  

\[ K_m \]

value was similar to that found with GTP as a substrate (Table I).

**Change of Substrate Specificity—** The crystal structure of a rat adenylyl cyclase type IIC2 homodimer and of a \( G \alpha_s \) VC1a/IIC2 (dog and rat isoforms, respectively) heterodimer bound to \( 2'd,3'\)-AMP, and enzymatic and modeling studies pinpointed two or potentially three amino acids in the cyclases as being responsible for purine nucleotide triphosphate specificity (5, 6, 20−22). These amino acids are individually conserved among all mammalian adenylyl and guanylyl cyclases and are never shared between both. In adenylyl cyclases Lys\(^{938}\) and Asp\(^{1018}\) in the IIC2 catalytic domain interact with the N-1 and N-6 nitrogen atoms of the adenine (6, 23). In guanylyl cyclases the corresponding amino acids are \( \beta_1\)Glu\(^{473}\) and \( \beta_1\)Cys\(^{541}\) (\( \beta_1\) refers to the \( \beta_1\)-subunit of rat soluble guanylyl cyclase), which probably interact with the N-1 nitrogen and the O-6 oxygen atoms of the guanine ring. An exchange of these amino acid residues switches the respective substrate specificities of mammalian guanylyl and adenylyl cyclases (21, 22). With regard to the \( Paramecium \) guanylyl cyclase, the C1-positioned domain most likely is the functional equivalent of the catalytic \( \beta_1\)-subunit of soluble guanylyl cyclases. Glu\(^{1681}\) in the C1-positioned loop is tantamount to \( \beta_1\)Glu\(^{473}\), and Ser\(^{1748}\) is tantamount to \( \beta_1\)Cys\(^{541}\) (Fig. 1B). In the ciliate, serine replaces a cysteine residue that otherwise is invariant in mammalian guanylyl cyclases (Fig. 1B). This Cys/Ser replacement apparently did not affect the specific accommodation of GTP in the substrate-binding fold, possibly because a hydrogen bridge can be formed between the O-6 oxygen of the guanine ring and the serine hydroxyl side chain. This was surprising because a \( \beta_1\)C541S mutation reduced the bovine guanylyl cyclase activity to almost zero despite the conservative nature of the substitution (24). To exam-

**FIG. 1.** Model of the guanylyl cyclase topology from \( Paramecium \) and comparison of catalytic domains with mammalian cyclases. A, the 155-kDa N-terminal of the ciliate guanylyl cyclase conforms to the structure of a P-type ATPase, and the 115 kDa C-terminal cyclase entity has the topology of mammalian adenylyl cyclases (4). The sites for membrane anchoring and catalysis are designated according to the current nomenclature. The cytosolic linker connecting both halves is about 10 kDa. B, abbreviated sequence alignment between cyclase regions (rat type II adenylyl cyclase, rat soluble guanylyl cyclase, and \( Paramecium \) guanylyl cyclase) that are important for substrate specificity. Inverted residues are implicated in defining substrate specificity (21, 22) and have been mutated in the present study. Residues marked by triangles are implicated in metal ion binding and catalysis (23).

**FIG. 2.** Amino acid sequences of the constructs used in this study. A, constructs from the protozoan guanylyl cyclase. The numbers refer to the full-length clone (GenBank\textsuperscript{TM} accession number AF288859). B, amino acid sequences of the C2 constructs from adenylyl cyclases type II from rat (GenBank\textsuperscript{TM} accession number M80550) and type IX from mouse (GenBank\textsuperscript{TM} accession number Z60190).
In the participation of Glu^{1681} and Ser^{1748} in the *Paramecium* C1a-positioned domain in substrate specification, we mutated them to the adenylyl cyclase specific residues Lys and Asp (double mutant E1681K/S1748D). In addition, we generated the single S1748D mutant; the E1681K single mutant was not accessible. The constructs (Fig. 2) were enriched by Ni^{2+}-NTA affinity chromatography and positively identified by Western blotting. All recombinant proteins displayed the expected molecular mass, and degradation products were more or less absent (Fig. 3).

Mutation of Ser^{1748} to Asp (ParaGC-C1aD) virtually abolished all Mg^{2+}-supported cyclase activities, regardless of ATP or GTP as substrates (Table I). Even with 2 mM Mn^{2+} as a divalent cation, guanylyl cyclase activity was diminished by more than 98%, and adenylyl cyclase activity was diminished by more than 83% (Table I). Thus, the ratio of the remaining low adenylyl and guanylyl cyclase activities was only 0.43, which means that efficient substrate discrimination was almost lost. The data indicate then that the interaction with the amino acid corresponding to position 1748 contributes much to the energy needed for ATP or GTP substrate binding and little to substrate discrimination. The ParaGC-C1aD/ParaGC-C2 dimer was in essence a low activity, unspecific purine nucleotide triphosphate cyclase. Similar mutants in one of these two amino acids in mammalian membrane-bound or soluble guanylyl cyclases and in the mammalian VC1a/IC2 heterodimer had lost almost all of their cyclase activity (21, 22). In the guanylyl cyclase double mutant E1681K/S1748D (ParaGC-C1aKD/ParaGC-C2), the nucleotide specificity was completely switched from GTP to ATP. Independently of Mg^{2+} or Mn^{2+} guanylyl cyclase activities were less than 1% of wild type activities, whereas adenylyl cyclase activity increased to 0.97 (Mg^{2+}) and 14.5 (Mn^{2+}) nmol cAMP/mg/min (Table I). This experiment unequivocally demonstrated the functional inversion of the arrangement of the cytosolic loops in the ciliate guanylyl cyclase because the mutated, substrate-defining amino acids were localized in the C1a-positioned domain, whereas in mammalian adenylyl cyclases they are localized in the C2 domain (21). Further, it demonstrated that Ser^{1748}, which replaced a Cys that is invariant in all mammalian guanylyl cyclases, is at its correct and needed location because a change to Asp had the expected profound functional consequences. In addition, it constituted the final proof that the *Paramecium* guanylyl cyclase with a mammalian adenylyl cyclase membrane topology truly is a guanylyl cyclase because we could switch substrate specificity by site-directed mutagenesis of the protozoan C1a-positioned domain as predicted by the available structural knowledge of the mammalian adenylyl cyclase C2 region (5, 6, 23). A novel observation for a cyclase with a canonical mammalian adenylyl cyclase topology was the almost total switch in purine nucleotide specificity. This has so far been accomplished only for mammalian guanylyl cyclases (21, 22). The attempt to change the substrate specificity of a soluble mammalian VC1/IC2 adenylyl cyclase heterodimer resulted in a nonspecific purine nucleotide cyclase (21).

In the C1 domain of adenylyl cyclases Gln^{503} (dog type V adenylyl cyclase) was identified as a potentially important point of contact for substrate recognition by modeling and by mutational studies (5, 21, 22). In adenylyl cyclases I–VIII this Gln residue is part of an invariant tripeptide motif KWQ.

**Table I.**

| Cyclase activities (Km values) | MgGTP | MgATP | MnGTP | MnATP |
|-------------------------------|-------|-------|-------|-------|
| ParaGC-C1a/ParaGC-C2          | 5.1 (76) | 0.25  | 27.5 (9) | 5.9 (26) |
| ParaGC-C1aL/C2                | 9.3 (60) | 0.25  | 19.8 (51) | 3.1 (22) |
| ParaGC-C1aD/ParaGC-C2         | 0.03  | 0.03  | 0.42  | 1.0 |
| ParaGC-C1aKD/ParaGC-C2        | ND    | 0.97  | 0.04  | 14.5 (32) |
| ParaGC-C1a/ParaGC-C2KWQ       | 1.21 (500) | 0.05  | 8.5 (23) | 1.4 (58) |
| ParaGC-C1aD/ParaGC-C2KWQ      | ND    | ND    | 0.2   | 0.2 |
| ParaGC-C1aKD/ParaGC-C2KWQ     | ND    | 0.62  | 0.05  | 11.5 (38) |

**Fig. 3.** Western blot analysis of recombinant and affinity purified cyclase C1, C2, and C1-C2 constructs. A, native and mutant protozoan C1-positioned domains detected with a monoclonal mouse anti-RGS-His antibody. B, protozoan and mammalian C2-positioned domains detected with a monoclonal mouse anti-His6 antibody. The amount of protein layered onto the 15% SDS-PAGE gels was 2–3 μg for ParaGC-C1, ParaGC-C1D, ParaGC-C1K, ParaGC-C2D, ParaGC-C2KWQ, and IC2 and 4 μg for ParaGC-C1IC2 and ICX2.
solute guanylyl cyclases the corresponding residue is an invariant \( \alpha_1\text{Arg}^{2347} \) which corresponds to Arg\(^{2347}\) in the Paramecium C2-positioned loop. Therefore, we mutated \( ^{2345}\text{LVR}^{2347} \) (Table II). This was particularly pronounced in the C2-positioned domain to the adenylyl cyclase equivalent KWQ (only Arg is invariant in guanylyl cyclases) in the ciliate C2 was almost inactive (Table II), which further supports the latter suggestion. The quintuple mutant ParaGC-C1aKD/ParaGC-C2 was almost inactive (Table II), which further supports the KWQ motif is adenylyl cyclase-specific this may have been expected. Yet it was contrary to our expectations that also the KWQ loop is not at all involved in substrate definition. In the protozoan enzyme the molecular arrangement of the cytosolic loops in the Paramecium guanylyl cyclase has now been established on an additional functional level, because the C2-positioned domain from the ciliate guanylyl cyclase fully substituted for a mammalian adenylyl cyclase domain. Irrespective of the sequence differences between the Paramecium C2-positioned and mammalian C1a domains, the structural C1-like epitopes, which are required to form a stable interdomain interface, obviously were fully retained in the ciliate C2 loop. Next, we used the ParaGC-C2KWQ triple mutant for adenylyl cyclase reconstitution with IIC2 or IXC2. Whereas adenylyl cyclase activity was considerably enhanced, the pronounced preference for MnATP as a substrate was unchanged (Table II). This demonstrates that the KWQ motif most likely contributes to the interaction of the C1 and C2 domains in adenylyl cyclases, possibly stabilizing and/or enhancing dimer formation. Although not investigated exhaustively, the ParaGC-C1a domain did not reconstitute an active cyclase catalyst with mammalian C1a domains (data not shown).

**Effect of Cyclase Modulators**—All membrane-bound mammalian adenylyl cyclases share three regulatory features: (i) activation by the GTP-bound form of \( \text{G}_{\alpha} \), and (ii) the diterpene forskolin, and (iii) inhibition by so called P site inhibitors, mainly analogues of adenosine (25). Next, we tested the effect of these canonical adenylyl cyclase modulators. Neither forskolin nor \( \text{G}_{\alpha}/\text{GTP}\gamma\text{S} \) or combined, affected any of the assayed guanylyl cyclase constructs. This was not totally surprising because inspection of the Paramecium guanylyl cyclase sequence showed that amino acid residues considered to be critical for cyclase activation by forskolin and for \( \text{G}_{\alpha} \) binding such as Phe\(^{779}\) (adenyl cyclase V numbering) are altered (6). Further, all protozoan/mammalian chimeras were also unaffected by forskolin and \( \text{G}_{\alpha}/\text{GTP}\gamma\text{S} \). In respective control experiments a 80-fold activation of a VIIC1a/IIC2 heterodimer was achieved under these conditions (data not shown). The data are reminiscent of a membrane-anchored chimera consisting of IIM2C2 and the \( \alpha_1\)-subunit of a soluble rat guanylyl cyclase, which also was insensitive to the adenylyl cyclase activators (26). Taken together, adenylyl cyclase activation by forskolin and \( \text{G}_{\alpha}/\text{GTP}\gamma\text{S} \) so far remains restricted to constructs involving the interactions between mammalian C1 and C2 domains.

P site inhibitors stabilize the complex of the cyclases with its product pyrophosphate (11). Inhibition is strongly activity-dependent (25). The most potent commercially available P site inhibitor for adenylyl cyclases is 2′,3′-AMP with a \( K_i \) of approximately 1 mM for the unstimulated enzyme and in the micromolar range upon activation by forskolin/\( \text{G}_{\alpha}/\text{GTP}\gamma\text{S} \). In a similar fashion, NO-stimulated guanylyl cyclase is inhibited

### Table II

**Mammalian/Paramecium adenylyl cyclase chimeras**

| Enzyme               | Activity remaining with 1 mM inhibitor |
|----------------------|---------------------------------------|
|                      | 2′,3′-AMP     | 2′,3′-GMP     |
| Guanylyl cyclase     | 90           | 38           |
| ParaGC-C1a/ParaGC-C2| 88           | 42           |
| IIC2/ParaGC-C2       | 46           | 71           |
| IXC2/ParaGC-C2       | 41           | 85           |
| IIC2/ParaGC-C2       | 64           | 70           |
| IIXC2/ParaGC-C2      | 42           | 55           |
| IXC2/ParaGC-C2       | 65           | 77           |

Enzymes were in agreement with recent structural data concerning substrate binding of the adenylyl cyclase VC1a/IIC2 chimera in which the KWQ motif appeared uninvolved (23). Of course we cannot exclude the possibility that a direct contribution of the KWQ motif to substrate definition may exist in other cyclase isoforms (see below). In the Paramecium guanylyl cyclase the exclusive amino acid residues for substrate determination appear to be Glu/Ser (and Lys/Asp) for guanylyl (for adenylyl) cyclase activity.

**Chimeras of Paramecium Guanylyl and Mammalian Adenylyl Cyclases**—In the protozoan enzyme the molecular arrangement of the cytosolic C1- and C2-positioned loops is reversed (see above and Ref. 4). To probe the cross-over of function on another functional level, we tested the potential of the C2-positioned domain for reconstitution of cyclase activity with the catalytic C2 loop derived from mammalian adenylyl cyclases of rat type II (IIC2; 29 kDa) and mouse type IX (IXC2; 36 kDa; Fig. 2B). After expression and Ni\(^2+\)-NTA chromatography, IIC2 appeared as a single band on a Western blot, whereas the expected major 36-kDa band of IXC2 remained contaminated by smaller His\(_6\)-immunoreactive proteins, probably products of proteolytic degradation (Fig. 3). No guanylyl cyclase activity was detectable in protozoan C2/mammalian C2 chimeras, whereas adenylyl cyclase activities were measured in C2/C2 chimeras with either IIC2 or IXC2 (Table II). The activities were higher with MnATP as a substrate as compared with MgATP (Table II). This was particularly pronounced in the ParaGC-C2/IXC2 chimera, which was 50-fold more active with MnATP (Table II). Thus, the topological inversion of the cytosolic loops in the Paramecium guanylyl cyclase has now been established on an additional functional level, because the C2-positioned domain from the ciliate guanylyl cyclase fully substituted for a mammalian adenylyl cyclase C1a domain. Irrespective of the sequence differences between the Paramecium C2-positioned and mammalian C1a domains, the structural C1-like epitopes, which are required to form a stable interdomain interface, obviously were fully retained in the ciliate C2 loop.

### Table III

**Inhibition of cyclase activities by 2′-deoxy-nucleotide-3′-monophosphates**

| Enzyme               | Activity remaining with 1 mM inhibitor |
|----------------------|---------------------------------------|
|                      | 2′,3′-AMP     | 2′,3′-GMP     |
| Guanylyl cyclase     | 90           | 38           |
| ParaGC-C1a/ParaGC-C2| 88           | 42           |
| IIC2/ParaGC-C2       | 46           | 71           |
| IXC2/ParaGC-C2       | 41           | 85           |
| IIC2/ParaGC-C2       | 64           | 70           |
| IXC2/ParaGC-C2       | 42           | 55           |
| IXC2/ParaGC-C2       | 65           | 77           |

Enzyme activities were measured in C2/C2 were higher with MnATP as a substrate as compared with MgATP (Table II). This was particularly pronounced in the

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**Mammalian Adenylyl/Protozoan Guanylyl Cyclase Chimeras**

Assays were performed with 75 \( \mu \)M of the respective NTP, 2 mM MnCl\(_2\), or 10 mM MgCl\(_2\). Guanylyl cyclase activity was not detectable with either Mn\(^2+\) or MgGTP. All chimeras involving the ParaGC-C1a domain were inactive. Mutated residues are highlighted in bold (see Table I for further details).
by 2'd3'-GMP at micromolar concentrations (21). We examined the effects of 2'd3'-GMP and 2'd3'-AMP on the constructs generated above. In general, P site inhibition involving soluble Paramecium guanylyl cyclase constructs was modest (Table III). 1 mM 2'd3'-AMP did not significantly affect guanylyl cyclase activity, and 1 mM 2'd3'-GMP inhibited by about 20% when guanylyl cyclase was assayed with 10 mM Mg2+ as a metal cation, i.e. in a low activity state. When guanylyl cyclase activity was tested with 2 mM Mn2+ as an activating cation, i.e. in a higher activity state, 2'd3'-GMP was clearly more inhibitory, i.e. inhibition was activity-dependent and specific as far as the purine moiety was concerned (Table III). The replacement of the Paraga-C2-positioned subunit by the Paraga-C2KWQ triple mutant in the reconstitution of a soluble guanylyl cyclase had no effect on the extent of inhibition (Table III). Next, we examined the Mn2+-stimulated adenylyl cyclase activities reconstituted from the mutated Paramecium domains Paraga-C1aKD/Paraga-C2 and Paraga-C1aKD/Paraga-C2KWQ and from the mammalian/protozoan chimeras, i.e. IIC2/Paraga-C2, IIC2/Paraga-C2KWQ, and IXC2/Paraga-C2KWQ. 1 mM 2'd3'-AMP inhibited adenylyl cyclase activities of all reconstituted enzymes by about 50% (Table III). 2'd3'-GMP was less inhibitory (about 20% at 1 mM), i.e. we see that substrate and P site inhibitor specificity tends to go along in these constructs as reported earlier for mammalian constructs (21). The determination of Ki values appeared meaningless considering the poor potency of the P site inhibitors compared with mammalian cyclases. The question arises then of why this was the case. The protein cleft that is implicated in the collapse into a high activity state which then could be affected by P site inhibitors much like mammalian adenylyl cyclases are. So far, intense efforts have failed to turn-up evidence for the presence of members of the 40-kDa heterotrimeric G protein family in Paramecium and Tetrahymena.2 In fact, in hindsight the existence of a modulator and a modulated protein that use the same substrate, GTP, would have been surprising. Therefore, we have to wait for the emergence of a protozoan G protein orthologue that may conceivably bestow the Ca2+ stimulation observed in the native guanylyl cyclase (15). In this respect the currently enigmatic role of the 155-kDa N-terminal P-type ATPase domain seems the more interesting. The fusion of a P-type ATPase with a guanylyl cyclase disguised as a mammalian adenylyl cyclase may in fact provide a Rosetta Stone sequence to decipher novel binding/regulating partnerships as proposed most recently (27).

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2 J. U. Linder, T. Hoffmann, U. Kurz, and J. E. Schultz, unpublished data.
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