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A Functional Chimera of Mammalian Guanylyl and Adenylyl Cyclases[†]

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ABSTRACT: Adenylyl and guanylyl cyclases synthesize second messenger molecules by intramolecular esterification of purine nucleotides, i.e., cAMP from ATP and cGMP from GTP, respectively. Despite their sequence homology, both families of mammalian cyclases show remarkably different regulatory patterns. In an attempt to define the functional domains in adenylyl cyclase responsible for their isotypic-common activation by Gα_s or forskolin, dimeric chimeras were constructed from soluble guanylyl cyclase α_1 subunit and the C-terminal halves of adenylyl cyclases type I, II, or V. The cyclase−hybrid generated cAMP and was inhibited by P-site ligands. The data establish structural equivalence and the ability of functional complement at the catalytic sites in both cyclases. Detailed enzymatic characterization of the chimeric cyclase revealed a crucial role of the N-terminal adenylyl cyclase half for stimulatory actions, and a major importance of the C-terminal part for nucleotide specificity.

Two cyclic nucleotide-generating enzymes are involved in the cellular responses to extracellular stimuli. In mammals, adenylyl cyclases are regulated by hormones that bind to plasma membrane heptahelical receptors resulting in intracellular changes in metabolism and gene expression (*1*). Mammalian guanylyl cyclases are either membrane-bound homomers or cytosolic heterodimers involved, e.g., in blood pressure regulation and phototransduction (*2*).

Families of either nucleotide cyclase are known by their nucleotide sequence and can be expressed as recombinant proteins (*3*). There are several isoforms of each cyclase family: types I−IX for adenylyl cyclase; α^1-β^1 and α^2-β^1 for soluble guanylyl cyclase; GC-A, -B, -C, -D, -E, and -F for particulate guanylyl cyclase. Adenylyl cyclase is a single protein chain that spans the membrane 12 times; the N- and C-termini as well as two large cytosolic domains reside intracellularly (*4*). The soluble guanylyl cyclase consists of an α- and a β-subunit each, embracing a prosthetic heme group that is indispensable for activation by NO, but not for basal activity (*5*). Sequence alignments between adenylyl and guanylyl cyclases show extensive similarities in their putative catalytic regions, indicating similar reaction mechanisms. However, the residual parts of either cyclase molecule are not homologous. The regulatory features of adenylyl and guanylyl cyclases are diverse. Gα_s, the natural activator, or the diterpene forskolin activate almost all known forms of membrane-bound mammalian adenylyl cyclase, but do not influence any guanylyl cyclase. Insight into the three-dimensional structure of adenylyl cyclase was gained by the recent crystallization of the cytosolic domains of adenylyl cyclase (*6*, *7*).

Work on recombinant adenylyl cyclases showed that the membrane-bound type I enzyme can be dissected into halves [IM1C1 plus IM2C2; see ref (*8*)].[1] Even after removal of the membrane spans, enzymatic activities and regulatory features were retained [IC1−IIC2, see ref (*9*)]. Both cytosolic, catalytic domains were required for enzyme activity as expression of only one membrane-bound half (e.g., IM1C1 or IIM2C2) or its cytosolic counterparts (e.g., IC1 or IIC2) did not result in any detectable adenylyl cyclase activity. In parallel, the catalytic center of the soluble guanylyl cyclase is formed by two catalytic domains contributed by a subunit each. Based on this phenotypic analogy, we coexpressed guanylyl cyclase and adenylyl cyclase halves to investigate the regulatory transposition in the resulting chimera (GC/AC chimera).

MATERIALS AND METHODS

Construction of Adenylyl Cyclase Halves. Adenylyl cyclase halves with the respective tags (see below) were generated by PCR. Sequences of primers are as indicated.[2]

Reactions were performed with standard PCR buffers from Perkin-Elmer or TaKaRa; for the GC-rich VM1C1 construct,

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[1] Abbreviations: IM1C1, N-terminal half of type I adenylyl cyclase; IM2C2, C-terminal half of type I adenylyl cyclase; IC1, cytosolic domain of N-terminal half of type I adenylyl cyclase; IC2, cytosolic domain of C-terminal half of type I adenylyl cyclase; sGC-α, α_1-subunit of soluble guanylyl cyclase; sGC-β, β_1-subunit of soluble guanylyl cyclase; HA, hemagglutinin epitope YPYDVPDYA; myc, amino acids QEKLISQQDL from the c-myc protein. Amino acids are denoted in the single letter code; X, any amino acid except proline.

[2] myc-IM1C1: 5′-GGAGGAACTAGTACCATGGAACAAAAACTG-ATATCGGAAGAAGACCTCGCGGGGGCGCCGCGCGGCCGAGGC-3′ and 5′-GGACGAAAGCTTAGTACCTGTTGACACGTGTGC-3′; IM2C2-HA: 5′-GGAGGAACTAGTACCATGATCGGCCGACTC-CTGGAAGCC-3′ and 5′-GGACGAAAGCTTATGCGTAGTCCG-GCACGTCGTACGGATAAGCCTCCTTCCCAGAGGCTCCAGG-3′; myc-IIM1C1: 5′-GGAGGAACTAGTACCATGGAACAAAAACTG-ATATCGGAAGAAGACCTCCGGCGGCGCCGCTACCTGCGGG-ACC-3′ and 5′-GGACGAAAGCTTACCTTTCATTCAGTTCTTCTT-CAAATCTC-3′; IIM2C2-HA: 5′-GGAGGAACTAGTACCATGATC-CAAGCCATTGATGGGATCAATGCAC-3′ and 5′-GGACGAAAGCT-TATGCGTAGTCCGGCACGTCGTACGGATAGGATGCCAAGT-TGCTCTGAGAAAGG-3′; myc-VM1C1: 5′-GGAGGAACTAGTAC-CATGGAACAAAAACTGATATCGGAAGAAGACCTCTGCAGCAG-CAGCAGCGCCTGG-3′ and 5′-GGACGAAAGCTTAAAATTCATC-CACTTCATCCTCAG-3′; VM2C2-HA: 5′-GGAGGAACTAGTACC-ATGCTGGGCCGCGCCATTGACGCC-3′ and 5′-GGACGAAAGCT-TATGCGTAGTCCGGCACGTCGTACGGATAACTGAGCGGGGGC-CCACCGTTG-3′.

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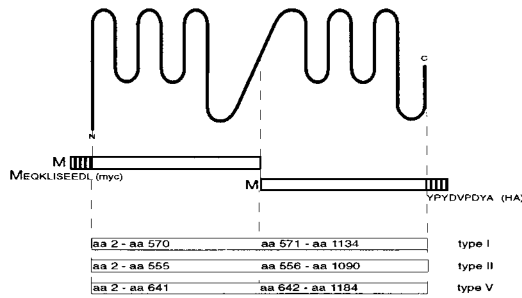

FIGURE 1: Membrane topology of adenylyl cyclases and constructions of halves thereof. The diagram (top) shows the assumed topology of mammalian adenylyl cyclases in the membrane. Bars (middle part) indicate the extensions of N- and C-terminal constructions of adenylyl cyclases including the engineered myc- and hemagglutinin- (HA) tags.[1] The C-terminal half is preceded by an additional methionine (M). The exact numbers of the start and stop amino acids for each subtype (I, II, and V) of adenylyl cyclase are denoted in the boxed lower part of the diagram.

the GC-advantage PCR Kit (Clontech) was used. Amplified DNA was purified on agarose gels, extracted by Jetsorb (Genomed), and ligated with a T-extended cloning vector (TA-cloning, Invitrogen; pGEM TEasy, Promega). Adenylyl cyclase-encoding DNA was cut with *Spe*I and *Hin*dIII and ligated into pFastBac1. The correct nucleotide sequence of the insert was verified by DNA sequencing.

Due to the inability to express enzymatically active soluble guanylyl cyclase in bacteria, insect cells were chosen as an expression system. In Sf9 insect cells, adenylyl cyclase halves could be expressed in their membrane-bound forms, i.e., as M1C1 or M2C2, as depicted in Figure 1. The N-termini were modified by inserting a myc-tag behind the starting methionine. The C-termini were extended by an hemagglutinin epitope. The site of bisection was chosen according to (*8*) with no overlapping amino acids between M1C1 and M2C2. A stretch of 30−50 amino acids before the first putative membrane-spanning domain in M2C2 seemed to be sufficient to ensure the proper insertion of the C-terminal half into the plasma membrane.

Baculovirus Generation. pFastBac1 vectors containing the tagged adenylyl cyclase halves were transformed into bacterial DH10Bac cells for bacmid generation (Gibco BRL). Bacmid DNA for construction of recombinant baculovirus was further processed as advised by the manufacturer. Positive baculoviruses were subcloned and verified by immunoreactivity, migration velocity in polyacrylamide gels, and enzyme activity of the encoded protein.

Expression of Adenylyl Cyclase. Baculoviruses encoding the indicated parts of adenylyl cyclase were used to infect Sf9 insect cells growing at 10^6 cells/mL with a multiplicity of infection (moi) of 5−10. After 48−55 h, cells were harvested, resuspended in buffer A (0.5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 2 mM DTT, 20 mM Na-Hepes, pH 8.0), and lysed by nitrogen cavitation as described (*10*). After removal of nuclear and microsomal material, membranes were washed in buffer A before resuspension in buffer B (1 mM EDTA, 5.7% sucrose, 2 mM DTT, 16 mM Na-Hepes, pH 8.0) at 1−3 mg/mL. Membranes were aliquoted, frozen in liquid nitrogen, and stored at −80 °C. Baculoviruses encoding wild-type adenylyl cyclase types I, II, and V were generous gifts of Dr. A. G. Gilman; plasmids and baculoviruses encoding sGC-α and sGC-β were kindly provided by Dr. D. Koesling, as well as sGC-α-specific antibodies.

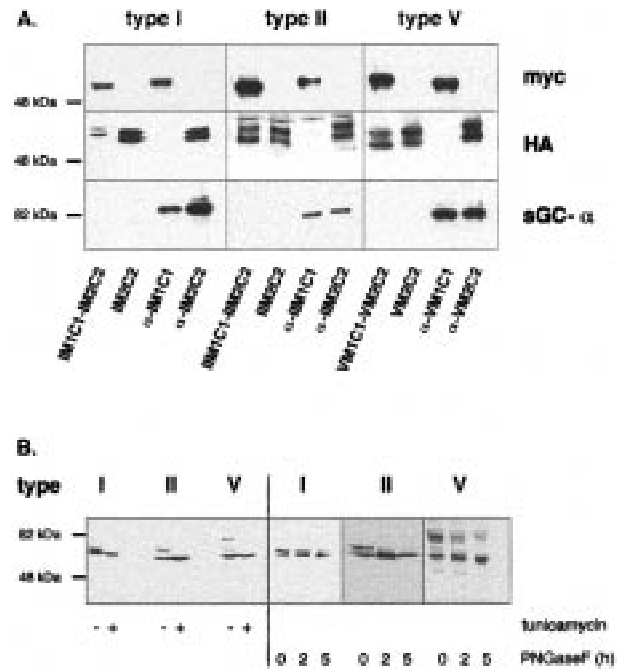

FIGURE 2: Expression of adenylyl cyclase halves. (A) Insect cells were infected with baculoviruses encoding the indicated cyclase moieties: IM1C1, N-terminal half of type I adenylyl cyclase; IM2C2, C-terminal half of type I adenylyl cyclase; IIM1C1, N-terminal half of type II adenylyl cyclase; IIM2C2, C-terminal half of type II adenylyl cyclase; VM1C1, N-terminal half of type V adenylyl cyclase; VM2C2, C-terminal half of type V adenylyl cyclase; α, α_1-subunit of soluble guanylyl cyclase. Shown is the fluorogram of a representative Western blot: Ten milligrams of total membrane protein was alkylated with *N*-ethylmaleimide, separated on an 11% acrylamide gel, blotted onto nitrocellulose, and immunostained with either myc-, HA-biotin-, or guanylyl cyclase-α_1-selective antibodies, as indicated at the right side. (B) Western blot of Sf9 membranes expressing adenylyl cyclase halves of type I (IM1C1−IM2C2), type II (IIM1C1−IIM2C2), or type V (VM1C1−VM2C2). Cells were grown in the absence (−) or presence (+) of 10 mg/L tunicamycin as denoted in the lower left part. For each lane, 5 μg of membrane protein was processed as described in part A and probed with a HA-specific antibody. The right half shows a time course of deglycosylation performed on 5 μg of membrane proteins from Sf9 cells expressing bisected adenylyl cyclases with 0.3 unit of glycosidase F (PNGaseF) at 37 °C.

Miscellaneous. Adenylyl cyclase and guanylyl cyclase assays were performed as described (*11, 12*). Determination of the protein concentration in membrane preparations was performed according to Bradford with bovine serum albumin as standard (*13*). For immunodetection of tagged adenylyl cyclase, commercially available antibodies were used such as biotinylated anti-HA from Boehringer and anti-AC II, anti-AC V, and anti-myc from Santa Cruz.

RESULTS AND DISCUSSION

Expression of Adenylyl Cyclase Halves. Mammalian adenylyl cyclases can roughly be divided into three subfamilies: the Ca^{2+}/calmodulin-activated (types I, III, and VIII), the $G\alpha_i$-inhibited (types V, VI, IX), and the $\beta\gamma$-stimulated enzymes (types II, IV, VII). All members are activated by $G\alpha_s$ and, with the exception of type IX, by forskolin. One type from each subfamily was chosen, i.e., types I, II, and V, to construct the bisected adenylyl cyclase halves for the GC/AC chimera. Halves of all three bisected adenylyl cyclase subtypes were successfully expressed in Sf9 cells and detected in the particulate fraction (Figure 2A).

The C-terminal halves appeared as double bands (ca. 60 and 70−80 kDa) indicating a heterogeneity of the M2C2 halves.

Glycosylation of M2C2. Expression of the three M2C2 halves in the presence of tunicamycin, an inhibitor of dolichol pyrophosphate−oligosaccharide synthesis, resulted in a homogeneous population of M2C2 with an apparent molecular mass of ca. 60 kDa (Figure 2B, left part). Moreover, treatment of the heterogeneous population of adenylyl cyclase halves with glycosidase F reduced the doublet of IIM2C2 or VM2C2 to the lower molecular mass species (Figure 2B, right part). Both features, the sensitivity to tunicamycin and glycosidase F, indicated that the high molecular mass species of IIM2C2 and VM2C2 corresponded to the N-glycosylated form of the 60 kDa species. Indeed, consensus sequences for N-glycosylation are present in the extracellular loops between transmembrane helices 9 and 10 [IIM2C2, see ref (*14*)], and helices 9 and 10 and helices 11 and 12 (VM2C2). The amino acid sequence of IM2C2 also contains the motif N-X-S/T[2] for N-glycosylation. A proline preceding the motif may have prevented the deglycosylation by *N*-glycosidase F, but not the action of tunicamycin. Glycosylation of the C-terminal half of adenylyl cyclase type I (*8*) and glycosylation of the wild type enzyme type II (*15*) have recently been shown. The myc-tagged N-terminal halves of all three cyclase subtypes showed up as single bands in a SDS−PAGE/Western blot and were not altered by glycosidase F or tunicamycin treatment, showing that posttranslational glycosylation occurred exclusively at the C-terminal halves. Glycosylation did not modulate the basal, $G\alpha_s$-, or forskolin-stimulated enzyme activity of bisected adenylyl cyclase (not shown). In summary, N- and C-terminal halves of bisected adenylyl cyclase types I, II, and V were successfully expressed in insect cells with glycosylation patterns and subcellular localization (in the 200000*g* pellet) similar to the respective wild-type enzymes.

Enzyme Activity of Bisected Adenylyl Cyclase. Figure 3B shows the systematic evaluation of bisected adenylyl cyclase types I, II, and V for activity and stimulation. All bisected enzymes were catalytically active and stimulated by $G\alpha_s$ or forskolin, irrespective of additional tag sequences such as myc and HA. The corresponding activities and regulatory features of the wild-type adenylyl cyclases are shown in Figure 3A. It is noteworthy that half-maximal concentrations for stimulation by forskolin were the same for full-length and bisected adenylyl cyclase, but bisected adenylyl cyclases were less sensitive to stimulation by $G\alpha_s$.

From bacteria, it was known that enzyme activity could be successfully reconstituted by mixing the lysates of *E. coli* cultures expressing individual soluble adenylyl cyclase halves [IC1 or IIC2; see ref (*16*)]. In contrast, mixing membranes of Sf9 cells individually expressing M1C1 or M2C2 was not sufficient to restore enzyme activity; but co-infection with viruses encoding both halves was a prerequisite to obtain catalytically active enzymes (not shown). The simultaneous translation probably ensured a heterodimeric organization in the “sticky” membrane environment to a reasonable degree.

The level of expression of adenylyl cyclase halves was equal or higher than that of the wild-type enzymes (tested for type II and type V with C-terminal adenylyl cyclase specific antibodies, not shown). Nevertheless, coexpressed bisected adenylyl cyclases exhibited roughly 10−20% of the activity seen with the wild-type enzyme (Figure 3B). This discrepancy can be explained by the occurrence of homomeric (inactive), homodimeric (inactive), and heterodimeric (enzymatically active) forms of expressed adenylyl cyclase halves. In summary, the data demonstrated that enzyme activity and regulatory features were not inhibited by the engineered tag sequences and did not depend on the covalent linkage between adenylyl cyclase halves found in the wild-type enzyme.

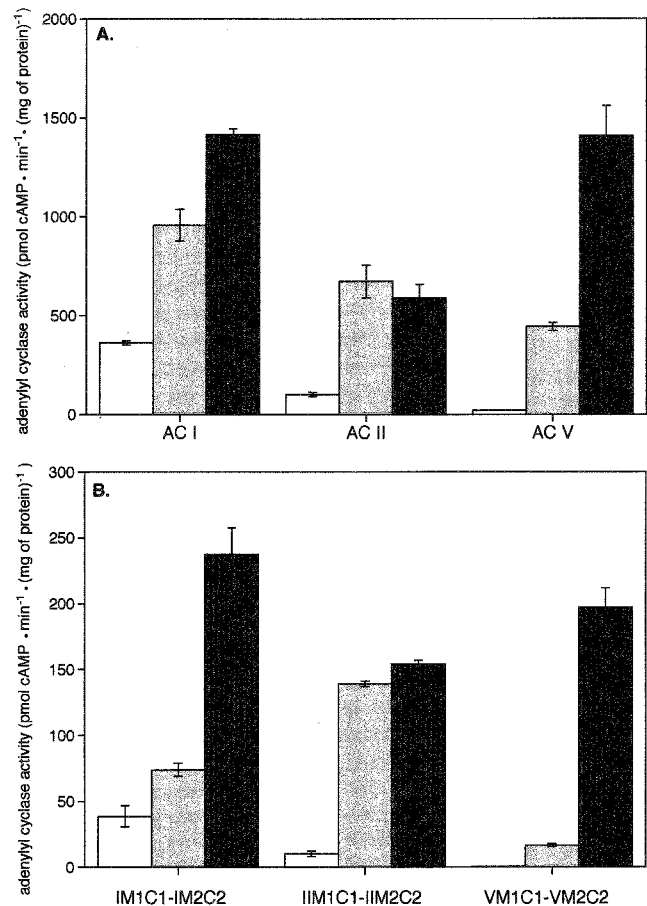

FIGURE 3: Activity of covalently connected (wild-type) and bisected adenylyl cyclases. Insect cells were infected with viruses encoding the denoted cyclases: AC I, AC II, and AC V, wild-type adenylyl cyclase types I, II, or V, respectively; IM1C1−IM2C2, IIM1C1−IIM2C2, and VM1C1−VM2C2, N- plus C-terminal halves of adenylyl cyclase type I, II, or V, respectively. Ten milligrams of membranes was assayed for cAMP generation in the presence of no stimulator (open bars), 50 nM GTPγS-activated $G\alpha_s$ (gray bars), or 100 μM forskolin (black bars). Data were corrected for the endogenous, particulate adenylyl cyclase activity of insect cells by subtracting the specific activities (basal, or $G\alpha_s$- or forskolin-treated) of cells which had been infected with the wild-type baculovirus. Data are representative of 2 independent experiments; error bars indicate the SD of two experiments performed in duplicate.

Guanylyl Cyclase/Adenylyl Cyclase Chimera. After the successful restoration of adenylyl cyclase activity from nonlinked particulate adenylyl cyclase halves, either M1C1 or M2C2 was replaced by a moiety from soluble guanylyl cyclase. There was one combination that reconstituted an active enzyme (Table 1): The simultaneous infection of insect cells with baculoviruses encoding sGC-α and the M2C2 half of adenylyl cyclase resulted in a cAMP-generating enzyme activity in the plasma membrane. This GC/AC chimera could be generated by the C-terminal halves of all three adenylyl cyclase subtypes (I, II, and V) tested. It is noteworthy that the activity of the type I chimera

Table 1: Activity of Cyclase Chimeras[a]

construct		AC activity[b,c]	K_M(ATP)
wild type	AC I	741 ± 75	30 ± 10
bisected	IM1C1−IM2C2	82 ± 15	50 ± 30
chimera	α-IM1C1	nd[c]	−
	α-IM2C2	86 ± 18	200 ± 100
	β-IM1C1	nd[c]	−
	β-IM2C2	nd[c]	−
wild type	AC II	184 ± 15	35 ± 10
bisected	IIM1C1−IIM2C2	10 ± 5	40 ± 15
chimera	α-IIM1C1	nd[c]	−
	α-IIM2C2	274 ± 10	160 ± 30
	β-IIM1C1	nd[c]	−
	β-IIM2C2	nd[c]	−
wild type	AC V	40 ± 10	7 ± 2
bisected	VM1C1−VM2C2	10 ± 5	25 ± 5
chimera	α-VM1C1	nd[c]	−
	α-VM2C2	130 ± 20	95 ± 20
	β-VM1C1	nd[c]	−
	β-VM2C2	nd[c]	−

[a] Insect cells were infected with baculoviruses encoding the designated halves of cyclase or wild-type adenylyl cyclase (AC I, AC II, AC V). Twenty micrograms of membranes was assayed for cAMP generation in the absence of any stimulator. [b] Specific activity is given in pmol of cAMP min^{-1} (mg of protein)$^{-1}$. [c] Data were corrected for the endogenous, particulate adenylyl cyclase activity of insect cells (10−30 pmol of cAMP min^{-1} (mg of protein)$^{-1}$) by subtracting the specific basal activity of cells which had been infected with the wild-type baculovirus. Values below double the background activity were denoted as nondetectable (nd). The absolute detection limit of the assay was 2−3 pmol of cAMP. Data are representative of at least 2 independent experiments performed in duplicate; variations indicate the SD.

(α-IM2C2) was in the range of the bisected adenylyl cyclase type I, whereas chimeras of types II and V exhibited higher activities than the bisected or even wild-type adenylyl cyclase types II and V. This phenomenon was caused by the low basal activities of type II and type V adenylyl cyclase, suggesting that M1C1 has an inhibitory effect on the M2C2 half. Our findings are in accordance with the previously described proteolytic activation of type II adenylyl cyclase (*17*). With the exception of the described α-M2C2 chimeras, no cyclic nucleotide generating activity could be restored by any of the other chimeric cyclase moieties; especially the inverse chimera (AC/GC chimera, M1C1-β) did not show any activity, not even the expected cGMP-forming one (not shown). The lack of enzyme activity is not due to an insufficient expression as protein levels of the cyclase moieties were not significantly changed by the simultaneous expression of any other cyclase domain (see Figure 2A).

Substrate Affinity. The GC/AC chimera provided functional evidence for the structural modeling experiments applied to different cyclases (*7*). Although not predicted by computer, the inverse chimera (M1C1-β1) was inactive, indicating some higher order requirements for functional complementarization of guanylyl cyclases. A measure of the degree of complementarization is the substrate affinity. As shown in Figure 4, the apparent ATP affinity significantly decreased just by dissecting the adenylyl cyclase. A further diminution of substrate affinity occurred when M1C1 was replaced by sGC-α. The calculated K_m values for ATP of the active constructs are listed in Table 1. Taken together, the substrate specificity was still exhibited in both the bisected adenylyl cyclases and the GC/AC chimera, but substrate affinities were reduced compared to wild-type adenylyl cyclases.

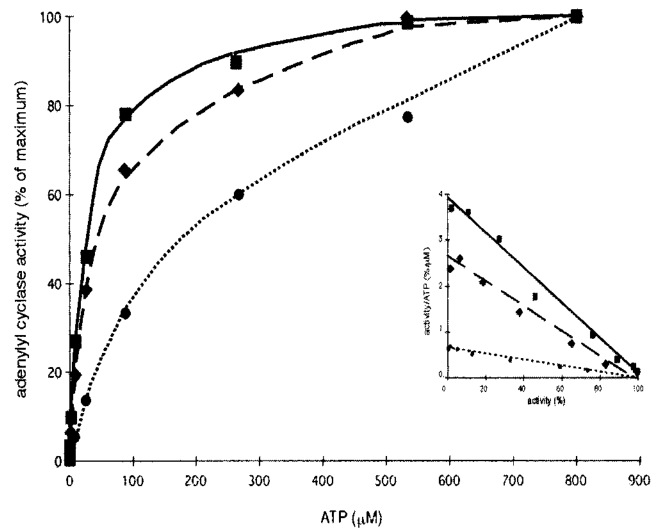

FIGURE 4: ATP dependency of the GC/AC chimera. Insect cells were infected with baculoviruses encoding wild-type adenylyl cyclase type I (squares), bisected adenylyl cyclase type I (diamonds), or the type I chimera α-IM2C2 (circles). Twelve milligrams of membranes was assayed for cAMP generation in the presence of the indicated substrate concentration. Maximal basal activities (100%) were 809 pmol of cAMP min^{-1} (mg of protein)$^{-1}$ for the wild type, 110 pmol of cAMP min^{-1} (mg of protein)$^{-1}$ for the bisected adenylyl cyclase, and 120 pmol of cAMP min^{-1} (mg of protein)$^{-1}$ for the GC/AC chimera. Data are corrected (see Table 1) and representative of 4 independent experiments. Duplicate determinations varied about 5% of the indicated values.

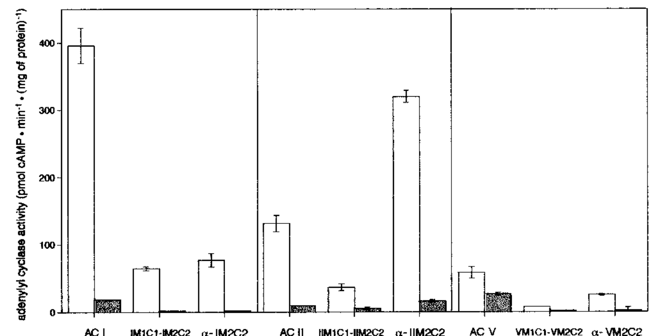

FIGURE 5: P-site inhibition on cyclase chimeras. Insect cells were infected with baculoviruses encoding the designated proteins. Twenty milligrams of membrane protein was assayed for basal cAMP generation in the presence of 100 μM sodium pyrophosphate with (dark bars) or without (open bars) 2′-deoxyadenosine 3′-monophosphate (200 μM). Data are corrected (see Table 1) and representative of 2 independent experiments; error bars indicate the SD of two experiments performed in duplicate.

Inhibition by P-Site Ligands. Another adenylyl cyclase-specific enzymatic feature is the inhibition by so-called P-site ligands. Recently, it was shown that these substances act in the presence of pyrophosphate noncompetitively with ATP at the catalytic site (*18*). This intrinsic feature was fully conserved in all cyclase constructs tested. Figure 5 shows that inhibition was retained in the bisected adenylyl cyclases and GC/AC chimera, and was as complete as in the respective wild-type adenylyl cyclase enzymes.

Regulation by Gα_s. Next, we investigated the contribution of M1C1 to activate adenylyl cyclase by Gα_s or forskolin. As all mammalian particulate adenylyl cyclases known to date can be activated by Gα_s or forskolin (except type IX), this question could not be answered by chimeras composed of different subtypes of adenylyl cyclases. However, the

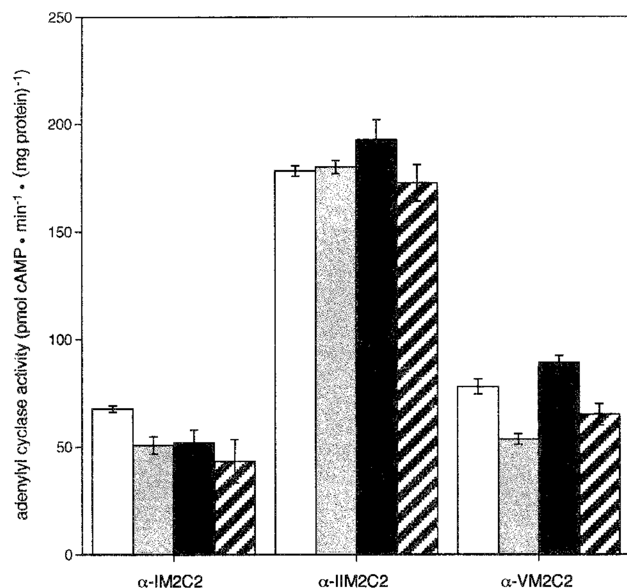

FIGURE 6: Activation of the GC/AC chimeras. Insect cells were infected with baculovirus encoding the α_1-subunit of soluble guanylyl cyclase (α) and baculoviruses encoding the designated C-terminal halves of adenylyl cyclase subtypes (IM2C2, IIM2C2, VM2C2). Ten milligrams of membranes was assayed for cAMP generation in the absence of any stimulator (open bars) or in the presence of 50 nM GTPγS-activated Gα_s (gray bars), 100 μM forskolin (black bars), or 50 nM GTPγS-activated Gα_s plus 100 μM forskolin (hatched bars). Data were corrected for the endogenous, particulate adenylyl cyclase of insect cells [basal, 7 pmol of cAMP min^{-1} (mg of protein)$^{-1}$; Gα_s-stimulated, 68 pmol of cAMP min^{-1} (mg of protein)$^{-1}$; forskolin-stimulated, 75 pmol of cAMP min^{-1} (mg of protein)$^{-1}$; doubly stimulated by Gα_s and forskolin, 306 pmol of cAMP min^{-1} (mg of protein)$^{-1}$]. Data are representative of at least 2 independent experiments; error bars indicate the SD of two experiments performed in duplicate.

preservation of Gα_s stimulation in the GC/AC chimeras would indicate that M2C2 is sufficient for the Gα_s binding and activation process. Figure 6 shows that the GC/AC chimera was not stimulated by Gα_s. The importance of the N-terminal half for adenylyl cyclase activation by Gα_s is supported by the recent finding that a point mutation in IC1 F293 (corresponding to F379 in VC1) abolishes the responsiveness of soluble adenylyl cyclase (IC1−IIC2) to Gα_s (*19*). The same amino acid was identified by an elegant screening system for Gα_s-insensitive mutants performed in yeast by Zimmermann et al. (*20*).

Regulation by Forskolin. Based on the crystal data from soluble adenylyl cyclase, the preservation of the forskolin response in the GC/AC chimera appeared more reasonable: Two duplicated ATP binding sites at the interface region of C1−C2 have been proposed in ancient adenylyl cyclase. These domains diverged during evolution; one site still binds the ATP substrate, and the other has been “cannibalized” by forskolin (*21*). The forskolin binding site motif is highly conserved throughout the adenylyl cyclases. The corresponding site in guanylyl cyclases is similarly conserved and homologous to adenylyl cyclases. From the basal activity of the GC/AC chimera, it was obvious that the ATP binding interface was intact. Accordingly, the duplicated counterpart may also have been intact. However, from experiments shown in Figure 6, it became evident that the forskolin binding site was not restored in the GC/AC chimera. Even site-directed mutagenesis in sGC-α to substitute for amino acids obviously involved in forskolin interaction of M1C1 (C534Y, C596D, F598W, G599S) did not render the chimeric protein sensitive to forskolin (not shown). As the GC/AC chimera was able to generate significant amounts of cAMP at a basal level, but could not be stimulated by Gα_s or forskolin, we concluded that sGC-α is significantly different from M1C1 outside of the catalytic cleft.

During preparation of the manuscript, data were published (*22*) showing that both highly conserved regions of the sGC-β (KV**E**TIG, PRY**C**L) and adenylyl cyclase IIC2 subunits (KI**K**TIG, PQY**D**I) are involved in substrate binding. From the two corresponding sites in their counterparts, only one did contribute to the substrate specificity in sGC-α (P**R**YCL) and none in adenylyl cyclase VC1. The emerging view is that the adenylyl cyclase catalytic cleft is mainly built by the C2 moiety and required only some complementary structures to restore enzymatic activity. This complementarization was curiously and uniquely achieved by sGC-α.

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