The C_2 cytosolic loop of adenylyl cyclase interacts with the activated form of Gαs

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Abstract **Using the yeast two-hybrid system, we studied the physical interaction between the complete C_1 and C_2 cytosolic domains of *Xenopus laevis* type 9 (xl9C_1, xl9C_2) and the C_2 domain of rat type 6 (r6C_2) adenylyl cyclase (AC). Heterodimerization between xl9C_1 and xl9C_2 and homodimerization between C_2 (but not C_1) domains was observed. Interaction between C_2 and human Gαs (hGαs) was also detected and was dependent on Gαs activation. In contrast *X. laevis* Gαs (xlGαs), which is 92% identical to hGαs, was unable to interact with any of the three AC cytosolic domains tested, corroborating previous findings that showed no effector activation. Through the construction of chimeras, we demonstrated that the amino-terminal half of xlGαs was responsible for the lack of interaction with AC. Chimeras between mouse Gαi2 and Gαs (N-mGαi2/C-Gαs), that have previously shown to activate AC to a higher extent than wild-type Gαs, also interacted with the C_2 cytosolic domain and with a higher affinity. Interestingly, N-mGαi2/C-xlGαs chimera was not only able to interact with C_2 but also with the C_1 cytosolic domain.**

Key words: Adenylyl cyclase; G protein; Yeast two-hybrid system

1. Introduction

Adenylyl cyclase (AC) is a widely distributed G protein coupled receptor (GPCR) regulated effector system which catalyzes the synthesis of cAMP, a ubiquitous second messenger that mediates diverse cellular responses by activating cAMP-dependent protein kinases. To date, nine different mammalian isoforms have been cloned and characterized from different sources [1–3]. AC isoenzymes differ in their tissue distribution and regulatory properties. With the exception of AC type 9, all of them are stimulated by the active GTP-bound form of Gαs and the diterpene forskolin (Fk) [3–7]. Almost all AC (except type 4) are regulated directly or indirectly by Ca^{2+} [3,8,9]. Types 1, 3 and 8 are activated by Ca^{2+}-calmodulin, types 5 and 6 are inhibited by Ca^{2+} levels and types 2 and 5 are activated by protein kinases A and C (PKA, PKC) [10–14]. Gβγ heterodimers also modulate the activity of some AC isoforms, activating AC types 2 and 4 and inhibiting type 1 [15–17].

All known mammalian AC isozymes share a predicted topological structure designated as $NM_1C_1M_2C_2$, containing a short amino-terminal region (N), two highly hydrophobic domains (M_1 and M_2) that are postulated to contain six transmembrane helixes, and two cytoplasmic domains (C_1 and C_2). The amino-terminal halves of each cytoplasmic domain (C_{1a} and C_{2a}) share a high homology and are 50–90% similar to the corresponding domains of other AC isoforms and only 20–25% similar to the catalytic domains of guanylyl cyclases [3,18]. Recently, expression of a soluble form of a mammalian AC has been achieved, allowing for important biochemical and structural studies of the enzyme [19,20]. When the C_{1a} domain of AC type 1 and the C_2 domain of AC type 2 isoforms (joined by a small linker) were expressed, a soluble and active enzyme was obtained. This enzyme was activated by GTP-Gαs and Fk, and inhibited by P-site inhibitors and Gβγ dimers. Expression of the individual domains (C_1 or C_2) exhibited little or no enzymatic activity [20,21]. Interestingly, coexpression of the two AC cytosolic domains as separate entities showed enzymatic activity and regulatory properties of the native enzyme, indicating that non-covalent interactions between C_1 and C_2 are important for catalytic activity [22,23]. Consistent with this idea, crystallographic studies performed by different groups have shown that fragments of both soluble AC cytosolic domains can interact in the presence of Fk, and that only C_2 can form homodimers and physically interact with Gαs [24–27]. By using the two-hybrid system, Scholich et al. [28] have identified a 10-amino acid (aa) region within the C_2 domain in AC type 5 that was able to interact with a 112-aa peptide of C_{1b} and showed that this interaction was modulating the stimulation by Gαs.

Recently, we reported the cloning and characterization of a *X. laevis* type 9 AC [29], which is activated by the human Gαs, but not by its homologous xlGαs, even though both proteins share 92% of identity [30]. In order to try to understand this difference, we have evaluated the direct intermolecular interaction between the human and *Xenopus* Gαs and the AC cytosolic domains C_1 and C_2. Using the yeast two-hybrid system we show that only the activated form of the hGαs, but not the xlGαs, was able to interact with the C_2 domain of the enzyme. This is in agreement with the lack of activation of AC by the *Xenopus* Gαs subunit, that we had previously reported [30].

2. Materials and methods

2.1. Plasmids construction for the yeast two-hybrid system

Yeast cloning vectors containing the Gal4 DNA-binding domain (pAS2-1) and the GAL4 activating domain (pACT2) were obtained from Clontech. cDNAs containing the entire coding sequence of xlGαs, xlGαs(Q/L), hGαs, and hGαs(Q/L) were excised from their

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respective recombinant vectors with *Nco*I and *Sal*I and inserted in-frame into pAS2-1. Chimera 10 (ch10) was constructed by ligation of a mouse Gαi2 *Nco*I-*Tth*111I cDNA fragment (aa 1–193) to a human Gαs *Tth*111I-*Sal*I cDNA fragment (aa 201–379) and subcloned into pAS2-1 vector. ch10(Q/L) was obtained the same way as ch10, but using the human Gαs(Q/L) *Tth*111I-*Sal*I cDNA fragment. Chimera 9 (ch9) and ch9(Q/L) were synthesized as ch10 and ch10(QL) by using the xlGαs *Tth*111I-*Sal*I or the xlGαs(Q/L) *Tth*111I-*Sal*I cDNA fragments. Chimera 2 (ch2) was originated by ligation of an hGαs(Q/L) *Nco*I-*Bgl*II fragment (aa 1–280) to a xlGαs *Bgl*II-*Sal*I fragment (aa 281–379) and subcloned into the pAS2-1 vector. Chimera 1 (ch1) was constructed by ligation of an xlGαs(Q/L) *Nco*I-*Bgl*II fragment (aa 1–280) to an hGαs *Bgl*II-*Sal*I fragment (aa 281–379) and subcloned into the pAS2-1 vector. AC C_1 and C_2 domains were amplified by PCR using the *X. laevis* AC type 9 (xlAC9) and rat AC type 6 (rAC6) full-length cDNAs as template. The following oligonucleotides were used for the amplification of the different AC fragments: xlAC9C_1 (aa 346–832), forward primer containing an *Nco*I site 5′-CTTTTCACCATGGCTGAGGTCCGA-3′ and a reverse primer containing a *Bam*HI site 5′-AAGGAGTGGATCCTGCAAGGA-3′; xlAC9C_2 (aa 1037–1355), forward primer containing a *Bam*HI site 5′-CTGGTGTGGATCCTGAACCGT-3′ and a reverse primer containing an *Eco*RI site 5′-GATCCGAATTCTGCCTCACCG-3′; rAC6C_2 (aa 911–1135), forward primer containing a *Bam*HI site 5′-TATCTACGGATCCAACAGGTG-3′ and a reverse primer with an *Eco*RI site 5′-CACTGCTGGAATTCGCTAACTGCT-3′. After digestion with the appropriate restriction enzymes, the PCR fragments were subcloned into the pACT2 vector. The same *Nco*I-*Bam*HI xlAC9C_1 PCR fragment was subcloned into pAS2-1, but PCR fragments xlAC9C_2 and rAC6C_2 were first subcloned into the pCEV-29 vector, then excised with *Bam*HI and *Sal*I, and finally subcloned into the pAS2-1 vector. All constructs were sequenced to confirm the correct reading frames and fusion protein expression was analyzed by Western blot using specific antibodies against GAL4 activating domain, GAL4 DNA-binding domain and Gαs (from Santa Cruz) (data not shown).

2.2. Two-hybrid assay

The yeast two-hybrid assay was performed using the Y190 yeast strain (from Clontech), which was transformed with the appropriate plasmids, using the lithium acetate procedure and grown on SD plates in the absence of Trp and Leu. Protein interaction analysis was performed on SD plates without Leu, Trp and His (SD/Leu$^-$,Trp$^-$,His$^-$). After 3 days at 30°C, individual colonies were straked out and grown on SD/Leu$^-$,Trp$^-$ liquid medium and tested for β-galactosidase activity.

2.3. Quantitative β-galactosidase assay

Liquid culture assay for β-galactosidase was performed according to the instructions from the 'Luminescent β-gal Genetic Reporter System II' kit (from Clontech). Data for quantitative assays were corrected for yeast cell number and are the mean ± S.E.M. of triplicate assays.

3. Results

In order to study the physical interaction between the AC cytosolic domains, we subcloned the full-length fragments that codify for C_1 and C_2 of xlAC9 and for C_2 of rAC6 into appropriate two-hybrid system vectors (Fig. 1). After transformation of the Y190 yeast cells with the corresponding recombinant plasmids, β-galactosidase activity was measured. As shown in Fig. 2, a very strong interaction between C_2 domains was observed, the highest occurring between homologous domains (rC_2-rC_2 and xlC_2-xlC_2). The C_1 domain, on the contrary, showed no β-galactosidase activity stimulation thus indicating no homodimerization between these cytosolic domains. As expected, heterodimerization between the C_1 and C_2 xlAC cytosolic domains was also detected.

After proving by Western analysis that the AC cytosolic domains were expressed (data not shown) and able to interact in our two-hybrid system, we continued to look at their interaction with Gαs. To perform this assay, we used the wild type and constitutively activated Q^{212}/L mutant of the short form of hGαs and xlGαs. Interaction with the C_2 domain was observed, when the hGαs was tested, and this association was dependent on the activated state of the α subunit (Fig. 3). No interaction with C_1 was detected, confirming previous crystal-

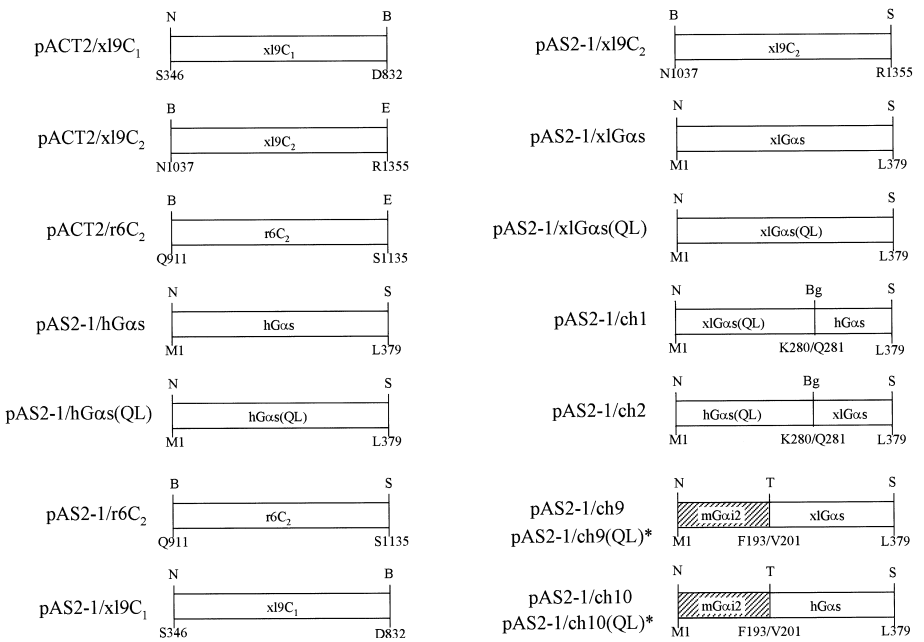

Fig. 1. Schematic representation of the constructions used in the yeast two-hybrid system analysis. Letters above each diagram represent the restriction enzymes utilized to subclone the different PCR amplified cDNAs into pACT2 and pAS2-1 vectors. B = *Bam*HI, Bg = *Bgl*I, E = *Eco*RI, N = *Nco*I, S = *Sal*I, T = *Tth*111I. Letters and numbers below indicate the amino acid and its corresponding position in the cDNA, respectively. pAS1-ch1(Q/L) and pAS1-ch2(Q/L) correspond to pAS-ch1 and pAS-ch2 constructions with the Q^{212}L mutation in the Gαs region. P53 and SV40Tag were used as a positive two-hybrid system control.

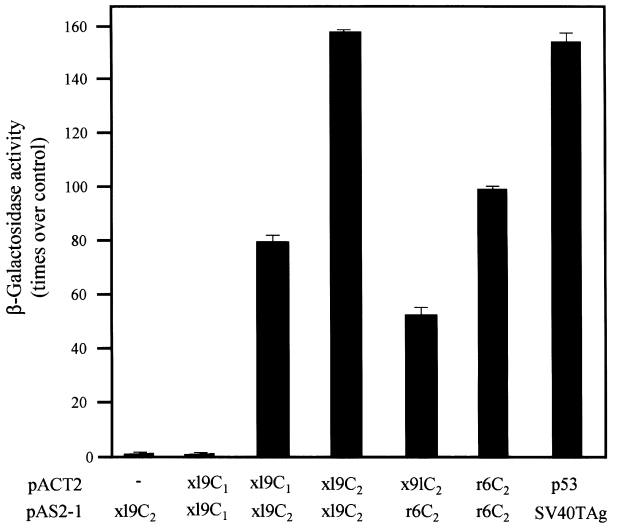

Fig. 2. Interaction of C_1 and C_2 domains of xl9AC and r6AC. Y190 were co-transformed with the indicated cDNA fragments subcloned in the two-hybrid vectors written on the left side of the figure. Transformants were grown in synthetic SD liquid medium without leucine and tryptophan at 30°C for three days and β-galactosidase activity was measured as described in Section 2.

lographic and biochemical studies performed with purified AC cytosolic fragments [19–23]. With the xlGαs, on the contrary, neither the wild type nor the activated mutant were able to interact with any of the cytosolic domains (data not shown). This result is in agreement with our previous findings which showed that the xlα subunit was not able to activate the S49 cyc$^-$ AC in an 'in vitro' reconstituted system [30]. In that study we demonstrated, by the construction of chimeras between human and *Xenopus* Gαs, that a region within the N-terminal half, which constitutes the helical domain of the protein, was responsible for the lack of activation [30]. For this reason, we decided to test out the constitutively activated (Q/L) chimeric proteins for their physical interaction with the xlAC cytosolic domains. When coexpressed with the C_2 domain, ch2 (N-hGαs/C-xlGαs) but not ch1 (N-xlGαs/C-hGαs)

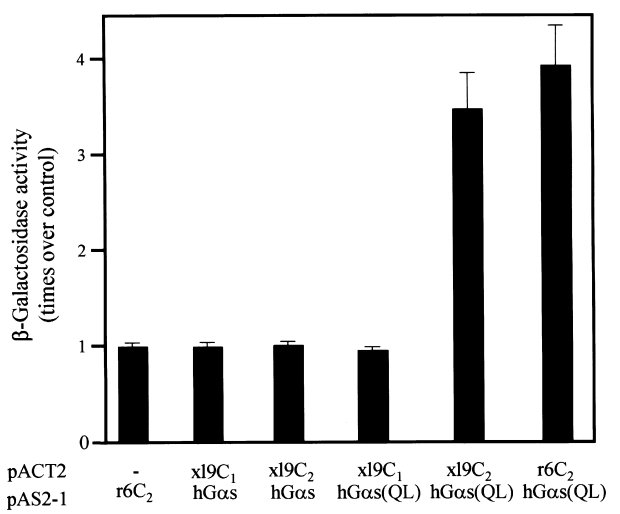

Fig. 3. Interaction of AC C_1 and C_2 domains with wild-type hGαs and mutant Gαs(Q/L). Experimental conditions are similar to those described in Fig. 2.

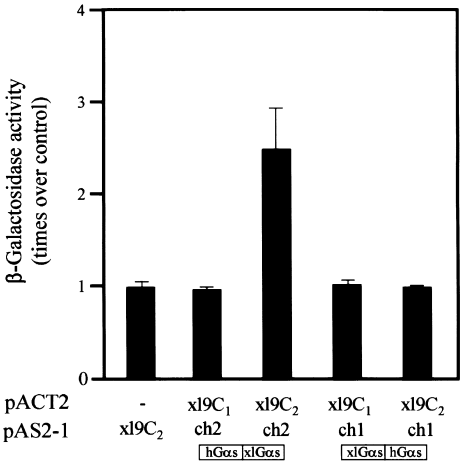

Fig. 4. Interaction of AC C_1 and C_2 domains of AC with Gαs chimeras ch1, ch1(Q/L), ch2 and ch2(Q/L). Experimental conditions are similar to those described in Fig. 2.

was able to stimulate the β-galactosidase reporter gene (Fig. 4), confirming the inability of ch1 to activate AC. In addition, since Gαi/Gαs chimeras were shown to activate AC to a higher extent than wild-type Gαs [31–34], we decided to also test them in the two-hybrid assay. As shown in Fig. 5, both Q/L mutated chimeras (ch9 and ch10) were not only able to interact with C2, but also with a substantially higher affinity (compare Fig. 3 with Fig. 5), confirming the AC activation studies mentioned above. Surprisingly, ch9 (N-mGαi2/C-xlGαs) but not ch10 (N-mGαi2/C-hGαs) was able to interact very strongly with the C_1 AC cytosolic domain.

4. Discussion

We have expressed the full-length AC cytosolic domains C1 and C2 fused to the GAL4-DNA binding or activating domains and tested 'in vivo' for their capacity to interact. Protein-protein interaction was quantitated by measuring β-galactosidase activity with a highly sensitive chemiluminescent

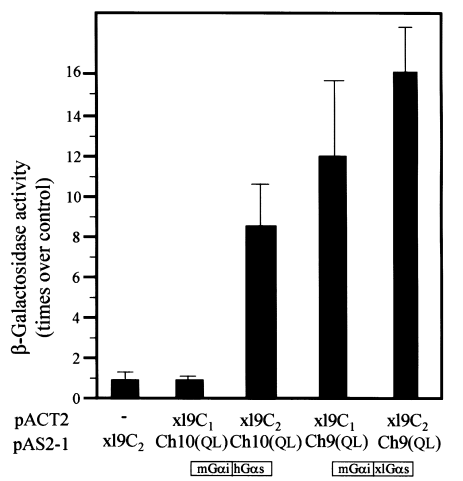

Fig. 5. Interaction of AC C_1 and C_2 domains of AC Gαs with Gαi-Gαs chimeras. Chimera 9 and chimera 10 were constructed as indicated in Fig. 1 and co-transformed in Y190 with AC C_1 or C_2 domains as indicated in the bottom of the figure. Experimental conditions are similar to those described in Fig. 2.

assay. The two-hybrid system employed in this study has the advantage of allowing us to differentiate between homologous or heterologous interactions and also to estimate the relative interaction affinities that are proportional to the β-galactosidase activity.

Our results show that the full-length C_2 cytosolic domain of xlAC9 was able to homodimerize very strongly (similar to p53 with SV40TAg) and also to heterodimerize with a lower affinity to the C_1 domain of the same AC. This finding corroborates previous biochemical and crystallographic studies performed with fragments of C_1 and C_2 domains of heterologous AC [19–23]. Unfortunately, with the two-hybrid system used in this study, we were unable to look at the effect of Fk or Gαs on C_1-C_2 interaction that has been previously reported to substantially increase their affinity [26]. The xlAC9-C_1 domain, on the contrary, showed no capacity to homodimerize which contradicts the experiments reported by Sunahara et al. [26] performed with gel filtration, where it was shown that both cytosolic fragments were eluting as dimers. We believe that the reason for this discrepancy is the difference in size of the AC domains. In our study we used the complete C1 cytosolic domains that is 486 aa long, whereas Sunahara et al. expressed only a fragment of it, that is half the size (227 aa) of the whole domain.

Using the same approach, we were also able to measure a weak interaction between the hGαs(Q/L) and the C_2 domain, but no interaction with C1. This finding is also in good agreement with crystallographic data, which showed that Gαs was predominantly interacting with C_2 [27]. In addition, gel filtration and equilibrium sedimentation studies also corroborated the same results as well as the requirement of Gαs to be in an active conformation in order to interact with C_2. On the other hand, the lack of interaction observed with the xlGαs again corroborates and explains the inability of this α subunit to activate AC. We now believe, based on trypsin protection experiments (unpublished results), that the reason for this anomalous behavior is due to the incapacity of this protein to adopt the active conformation state necessary to interact with C_2 and to activate AC.

Interaction studies performed with Gαi/Gαs chimeras, that have previously shown to be better activators of AC than wild-type Gαs, confirm these results. Since the hybrid chimeric proteins showed a considerably stronger interaction with C_2 than the normal Gαs, this possibly indicates that these proteins tend to have a more permanent activated conformation.

Based on the pseudosimetric structure adopted by the C_1-C_2 heterodimer and to the simultaneous activating and inhibitory effect produced by Gαs and Gαi on AC, Tesmer et al. [27] have proposed a Gαi interaction site in C_1 equivalent to the Gαs binding site in C_2. This hypothesis has now been confirmed by demonstrating the formation of a stable complex between GTPγS-Gαi and the C_1 (but not C_2) domain of type 5 AC [35]. Now that a C_1-Gαi interaction has been proven, we can better understand the behavior of ch9 which showed the capacity to interact with C_2 and C_1. Since ch9 is a hybrid between Gαi and Gαs it is possible to speculate that the chimeric protein could adopt a conformation capable of binding to both domains. We do not know the reason why ch10 does not interact with C_1, and further analyses are needed in order to understand these differences.

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