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Human–*Xenopus* chimeras of $G_s\alpha$ reveal a new region important for its activation of adenylyl cyclase

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Abstract

G proteins are heterotrimeric GTPases that play a key role in signal transduction. The α subunit of G_s bound to GTP is capable of activating adenylyl cyclase. The amino acid sequences derived from two *X. laevis* cDNA clones that apparently code for $G_s\alpha$ subunits are 92% identical to those found in the short form of human $G_s\alpha$. Despite this high homology, the *X. laevis* $G_s\alpha$ clones expressed in vitro, yielded a protein that are not able to activate the adenylyl cyclase present in S49 cyc^- membranes in contrast with human $G_s\alpha$ similarly expressed. This finding suggested that the few amino acid substitutions found in the amphibian subunit are important in defining the functionality of the human $G_s\alpha$. The construction of chimeras composed of different fractions of the cDNAs of the two species was adopted as an approach in determining the regions of the molecule important in its functionality in this assay. Four pairs of chimeras were constructed using reciprocal combinations of the cDNAs coding for human and *Xenopus* $G_s\alpha$. These eight constructs were expressed in vitro and equivalent amounts of the resulting proteins were assayed in the activation of adenylyl cyclase with GTPγs and isoproterenol. The results obtained here clearly indicate that the Gα sequence that extends from amino acid 70 to 140, is important for the functionality of human $G_s\alpha$ in activating adenylyl cyclase.

Key words: G-protein; $G_s\alpha$ subunit; Signal transduction; Oocyte; *Xenopus laevis*

1. Introduction

The G proteins are heterotrimeric GTPases that play a crucial role in the transduction of many extracellular signals [1–3]. These proteins are composed of α, β and γ subunits which are complexed with each other when the α subunit is bound to GDP. Activation by an occupied receptor stimulates the exchange of GDP for GTP and the dissociation of βγ from the α–GTP complex. The Gα subunits, which are encoded in a family of more than 20 analogous genes, carry out many functions. In addition to their capacity to bind guanine nucleotides and their intrinsic GTPase activity, these subunits determine the specificity of interaction between the G protein and the receptor. In most cases, the Gα–GTP complex interacts with the effector protein and regulates its activity.

The α subunit of G_s ($G_s\alpha$) has been found to function in the stimulation of adenylyl cyclase and in the regulation of membrane ion channels [4] and possibly in protein transport [5]. One of the signal transduction systems studied in great detail involves the activation of adenylyl cyclase by β-adrenergic receptors. In this system, all the components have been identified, purified and reconstituted in a synthetic membrane [6,7]. The finding that S49 cyc^- cells have a mutation that lacks the $G_s\alpha$ subunit but contains all the other components necessary for β-adrenergic regulation of adenylyl cyclase has been very helpful in the studies on the function of the subunit [8].

Significant information has been obtained regarding the structural features of $G_s\alpha$ that are relevant for its function. These studies have been greatly helped by the cloning and expression of the cDNAs coding for $G_s\alpha$ from several species.

The elucidation of the three-dimensional structure of two related GTPases, bacterial elongation factor Tu [9] and the p21 product of the *ras* proto oncogene [10] has also been very useful in the analysis of the Gα structural motifs. Using this information together with natural and induced mutants of $G_s\alpha$ and chimeric proteins composed of portions of Gα subunits with different specificities, it has been possible to define regions of $G_s\alpha$ involved in the binding and hydrolysis of GTP and in its interaction with the receptor [11–13], with the βγ subunits [14] and with the adenylyl cyclase [15].

For a number of years we have been interested in the regulation of adenylyl cyclase that participates in the hormonal induction of amphibian oocyte maturation [16–19]. As part of these studies we have cloned and sequenced 5 different *X. laevis* oocyte cDNAs coding for Gα subunits [20,21]. Two of these cDNA clones (6A1 and 12B2) were found to be highly analogous to the mammalian $G_s\alpha$ short form [22]. In vitro expression of

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these two *X. laevis* cDNAs into proteins of the correct M_r was attained. However, the resulting proteins were found to be essentially inactive when assayed for their capacity to stimulate the adenylyl cyclase of S49 cyc$^-$ membranes in the presence of GTPγS, isoproterenol, or AlF_4^-.

The inactivity of the $G_s\alpha$-like proteins of *X. laevis*, suggested that this finding could be used to explore further the regions involved in the functionality of the human $G_s\alpha$. This communication describes the results obtained with this approach. Chimeras of the human and *X. laevis* cDNAs were constructed containing 4 pairs of reciprocal combinations of fractions of the genes from the two species. These chimeric cDNAs were expressed in vitro and the resulting proteins were assayed in the S49 cyc$^-$ system. The results obtained clearly indicate that the region of the human $G_s\alpha$ protein encompased between amino acids 70 and 140 plays an important role in the capacity of this subunit to activate adenylyl cyclase. When this region from the *X. laevis* protein, containing 23 amino acid substitutions with only 10 non-conservative changes, is introduced into the human protein, the resulting chimera is inactive. Conversely, substitution of this region from the human into the *X. laevis* protein, is enough to confer activity to the rest of *X. laevis* protein.

2. Materials and methods

2.1. Radiochemicals and biochemicals

[α-^{32}P]ATP (200–400 Ci/mol) was enzymatically synthetized according to Walseth and Johnson [26]. [^{35}S]methionine (>1,400 Ci/mmol) were from Du Pont-New England Nuclear. RNasin, RNase-free dithiothreitol and reticulocyte lysate were purchased from Promega Biotec. T7 RNA polymerase, RNase-free DNase Q and RNase-free ribonucleoside triphosphates were from Stratagene. *Xba*I, *Nco*I, *Sal*I, *Tth*111I, *Pvu*II, *Bgl*II and *Pst*I, restrictions enzymes and buffers were from Bethesda Research Laboratories. All other reagents, chemicals and biochemicals, and enzymes were of the highest commercially available purity.

2.2. cDNAs

Human $G_s\alpha$ comes from human liver [22] and *Xenopus* $G_s\alpha$ from a λgt10 oocyte cDNA library [20]. $G_{i2}\alpha$ cDNA fragment was kindly given to us by Drs. K. Sullivan and H. Bourne (UCSF) and the *Nco*I–*Tth*111I fragment was used to construct the $G_{i2}\alpha/G_s\alpha$ chimeras.

2.3 Construction of $G_s\alpha$ chimeras

$G_s\alpha$ chimeras were constructed from *Xenopus laevis* (XL) oocyte $G_s\alpha$ cDNA [21] and human liver $G_s\alpha$ cDNA [22]. **Ch1** was generated by ligation of a XL $G_s\alpha$ *Nco*I–*Bgl*II cDNA fragment (codons 1–280) to a human $G_s\alpha$ *Bgl*II–*Sal*I cDNA fragment (codons 281–379 and 3′ untranslated region). **Ch2** was generated by ligation of a human $G_s\alpha$ *Nco*I–*Bgl*II cDNA fragment (codon 1–280) to a XL $G_s\alpha$ *Bgl*II–*Xba*I cDNA fragment (codons 281–379 plus 3′ untranslated region). **Ch3** corresponds to the ligation of a XL $G_s\alpha$ *Nco*I–*Tth*111I cDNA fragment (codons 1–200) to a human $G_s\alpha$ *Tth*111I–*Sal*I cDNA fragment (codons 201–372 plus 3′ untranslated region). **Ch4** was originated by ligation of a human $G_s\alpha$ *Nco*I–*Tth*111I cDNA fragment (codons 1–200) to a XL $G_s\alpha$ *Tth*111I–*Xba*I cDNA fragment (codons 201–379 plus 3′ untranslated region). **Ch5** was constructed by replacing the XL $G_s\alpha$ *Pst*I–*Tth*111I cDNA fragment (codons 31–200) by a *Pst*I–*Tth*111I cDNA fragment from human $G_s\alpha$ (same codons). **Ch6** was constructed as Ch5 but in this case replacing the human Gα cDNA fragment by the XL $G_s\alpha$ cDNA fragment. **Ch7** was constructed by replacing the XL $G_s\alpha$ *Pst*I–*Pvu*II cDNA fragment (codons 31–157) by a human $G_s\alpha$ *Pst*I–*Pvu*II cDNA fragment (same codons). **Ch8** was constructed as Ch7, but in this case replacing the human cDNA fragment by the XL $G_s\alpha$ cDNA fragment. **Ch9** was constructed by ligation of a *Nco*I-*Tth*111I (created by site directed mutagenesis) cDNA fragment from mouse $G_{i2}\alpha$ (codons 1–193) to a human $G_s\alpha$ *Tth*111I–*Xba*I cDNA fragment (codons 201 379). **Ch10** was originated as Ch9, but in this case the $G_{i2}\alpha$ fragment was ligated to a *Tth*111I–*Sal*I cDNA fragment from XL $G_s\alpha$ (codons 201–379).

2.4. In vitro transcription of mRNA

All $G_s\alpha$ cDNA constructs were subcloned in the *Nco*I–*Sal*I site or *Nco*I–*Xba*I site of pAGA vector [23] and 10 μg of linearized recombinant plasmid with *Hin*dIII was transcribed with the T7 RNA polymerase (Promega) as described [24] with some modifications. Normally the reaction mixture (200 μl) contained 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl 1 mM of each αdNTP, 30 mM DTT, 1 unit/μl of the RNase inhibitor RNasin, and 100 units of T7 RNA polymerase. The transcription reaction mixture was incubated 1 h at 37°C and the DNA template was eliminated by the action of DNase Q at a concentratoin of 1 unit/μg of DNA template. Finally the mRNA was resuspended in steril RNase-free treated water at a concentration of 1 μg/μl and stored at −20°C. Quantification of mRNA was done measuring O.D at 260 nm

2.5. In vitro translation of a $G_s\alpha$ protein

The in vitro translation was done as described [24] with some modifications. Normally the translation mixture (10 μl) contained 1 μg of the $G_s\alpha$ mRNA, 20 mM KCl, 20 μM of a non radioactive amino acid mixture without methionine, 7 μl of reticulocyte lysate and 20 μM [^{35}S]methionine (16.7 μCi). The mixture was incubated 60 min at 30°C and labeled proteins were analyzed on 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The amount of $G_s\alpha$ proteins synthesized was quantified by thrichloroacetic acid precipitation of the proteins labeled with [^{35}S]methionine and the pmol of protein was calculated as described [25].

2.6 Reconstitution of adenylyl cyclase activity and assay

The cyc-reconstitution assays were done essentially as described [24] with some modifications. Normally 50 fmol of $G_s\alpha$ protein was added to 10 μg of cyc-membranes in a final volume of 50 μl that contained 0.12 mM ATP (1,500–2,000 cpm/pmol) and 10 mM $MgCl_2$ as described previously [16] in the absence or presence of the variables indicated in the figures. Unless indicated otherwise, when present, GTP was 10 μM, GTPγS was 100 μM and isoproterenol was 10 μM. Incubations were at 32°C for 40 min. cAMP formed in the assays was isolated and quantified according to modifications [16] of the original method [26].

3. Results

Fig. 1 presents a comparison of derived amino acid sequences of the *Xenopus* $G_s\alpha$ clone 6A1 short form [21] with the cDNA clone from human liver [22] and with the cDNA $G_{i2}\alpha$ clone from mouse. The figure summarizes the few amino acid changes between both $G_s\alpha$ subunits and also marked in Fig. 1 are the regions of the protein that have been assigned to play different roles. It can be seen that there is a total of only 29 amino acid substitutions out of 379 residues, 13 of them being non-conservative alterations. The amino acid changes are clustered in the amino terminal half of the molecule.

Despite this high degree of homology, the proteins coded by the two *X. laevis* cDNAs when expressed in vitro were essentially inactive in their capacity to activate the S49 cyc$^-$ adenylyl cyclase in constrast to the human protein similarly expressed.

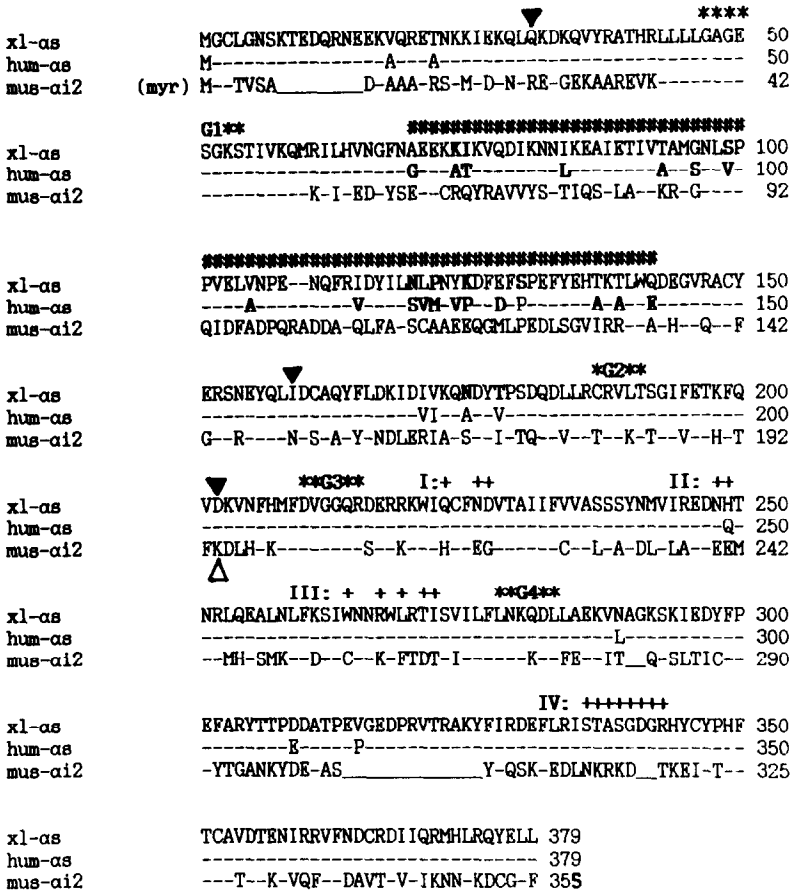

Fig. 1. Comparison of deduced amino acid sequences of *Xenopus laevis* $G_s\alpha$ (xl-αs, clone 6A1), human $G_s\alpha$ (hum-αs, short form without serine) and murine $G_{i2}\alpha$ (mus-αi2). The latter two sequences are compared to that of the first. Identical amino acids are denoted by broken lines and gaps by solid lines. Identity between hum-αs and xl-αs (clone 6A1) is 92% (29 amino acid differences out of 379). Clone 12B2 from *X. laevis* is identical to clone 6A1 except for the following substitutions: V18A; T22A; V105A; Y122H; E126D; E139D; Q140H; E142D; S178T; E284D; N287L; V313F; G314N. The susceptibility of mus-αi2 to be myristoylated on glycine 2 is denoted by (myr). ****, regions G1 through G4 involved in guanine nucleotide binding; ++++, regions I through IV proposed by Berlot and Bourne to interact with adenylyl cyclase in the basis of studies with $G_s\alpha/G_i\alpha$ chimeras [15]; ####, region between $G_s\alpha$-70 and $G_s\alpha$-140 shown in this report to confer adenylyl cyclase stimulating activity to xl-αs; bold amino acids in xl-αs, non-conservative amino acid substitutions between xl-αs (70–177) based in the following conservative grouping of amino acids: C; S, T, P, A and G; N, D, E and Q; H, R, and K; M, I, L, and V; F, Y and W; bold amino acids in hum-αs, amino acids of hum-αs that confer adenylyl cyclase stimulating activity to xl-αs; ▼, boundaries of fragments used to construct hum-αs/xl-αs chimeras Ch5, Ch6, Ch7 and Ch8 (see section 2 and Fig. 2); △, boundary of mus-αi2 fragments used to construct mus-αi2/xl-αs and mus-αi2/hum-αs chimeras Ch9 and Ch10.

As shown in Fig. 2A,B, the human $G_s\alpha$ yields a very significant activation when assayed with either isoproterenol plus GTP or with GTPγS. The *X. laevis* protein from cDNA clone 6A1, on the other hand had practically no activity over the basal when assayed with isoproterenol and GTP although some slight activation could be detected with GTPγS. Identical results were obtained with the protein expressed from *X. laevis* cDNA clone 12B2 (not shown) which differs from the human also in 29 amino acids and from *X. laevis* 6A1 clone in 13 positions as indicated in the legend of Fig. 1A. For this reason all further work was carried out with the clone 6A1 from *X. laevis*. Four pairs of reciprocal chimeras (Ch1–Ch8) containing various portions of the human and *X. laevis* cDNA clones were constructed and these chimeric genes were expressed in vitro as detailed in section 2.

The activity of equivalent amounts of the chimeric $G_s\alpha$ proteins was tested in the same system (Fig. 2A,B). The results obtained are clear cut. Exchanging the carboxyl half of the proteins seems to have only a slight influence on the activity of the chimeric proteins, while the origin of the amino terminal portions does determine the capacity of the chimeras to activate the S49 cyc^- adenylyl cyclase. The construct containing the human amino terminal being active, while the *X. laevis* amino terminal produces an inactive chimera. Ch5–Ch8 further narrow down the critical area to the region between amino acid 31 and 157. Fig. 2B also contains an experiment with chimeras involving the amino terminal half of $G_{i2}\alpha$ from mouse with the carboxyl terminal of both *X. laevis* and human $G_s\alpha$ (Ch9 and Ch10). The results with Ch10 confirm those reported by Osawa] [14] with $G_{i2}\alpha$ and $G_s\alpha$ chimeras from human which establish that the carboxyl

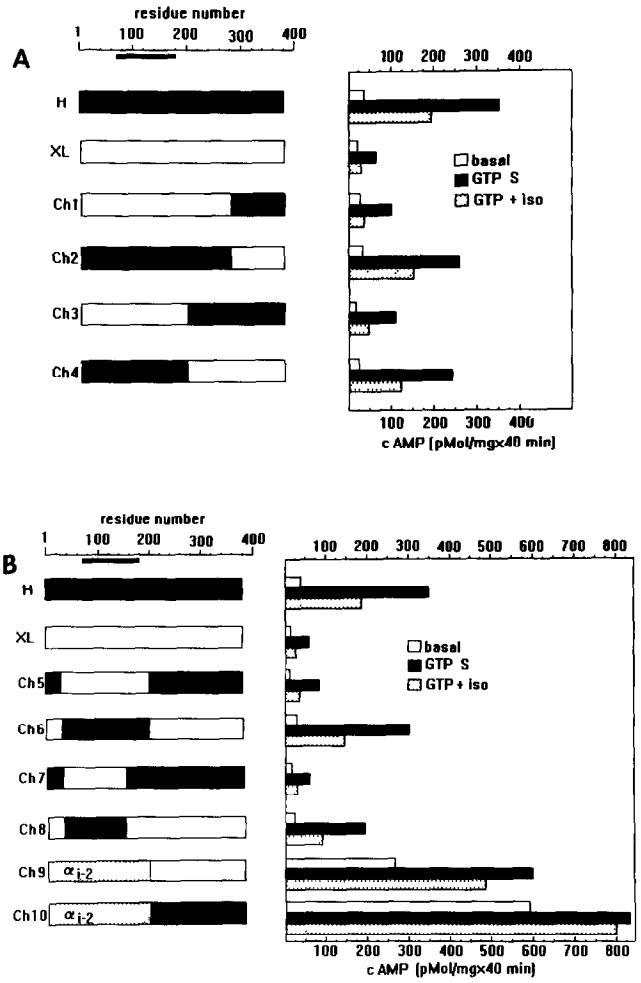

Fig. 2. Effect of Gα chimeras on cAMP synthesis by cyc⁻ membranes. (A) The left part shows a map of the different $G_s\alpha$ proteins and chimeras Ch1 to Ch4 used to reconstitute cyc⁻ membranes. The upper scale indicates the position of amino acid residues and the black filled bar below the scale indicates the region containing the cluster of different amino acids between human and *Xenopus* $G_s\alpha$. The right part shows the cAMP synthesis by cyc⁻ membranes reconstituted by the different $G_s\alpha$ chimeric proteins. Values represent the mean of duplicate determinations with less than 5% of variation. Cyclic AMP values (pmol/mg membrane protein in 40 min) are shown above and below the figure. (B) The same as in (A) but in this case using chimeras Ch6 to Ch10.

terminal portion of $G_s\alpha$ is capable of determining the capacity of this chimeric protein to activate adenylyl cyclase. All the $G_s\alpha$ chimeric proteins were also tested with AlF_4^- as an activator of this protein. The results obtained (not shown) were qualitatively identical to those shown for GTPγs.

4. Discussion

We have found that despite the high degree of homology between *Xenopus* and human $G_s\alpha$ proteins (close to 92%) when expressed in vitro their behavior was essentially different. The Xenopus protein was inactive in their capacity to activate the S49 cyc⁻ adenylyl cyclase in constrast to the human protein expressed under the same conditions. The inactivation of the human $G_s\alpha$ protein observed by introducing the *X. laevis* fragment corresponding to amino acids 31–157 is interesting because this section concentrates 19 of the 29 amino acid changes between the two proteins (Fig. 1). The differences in this section are restricted to a cluster that starts in amino acid 70 and ends in amino acid 140 (Figs. 1 and 3). The importance of this region is corroborated by the results obtained with the reciprocal chimera (Ch8) that show that this human portion is capable of rendering the *Xenopus* protein active.

Masters and Bourne [28] had previously noted the variability in the sequences in this region among the different G proteins and had, on this basis, suggested that it may play a role in their specific functions. Only 8 out of the 19 amino acid substitutions in this region are non-conservative (Fig. 1). Within this region the stretch that goes from amino acid 118–128 is very drastically mutated with 7/11 changes. The elegant work carried out by Osawa] [14] and by Berlot and Bourne [15] using $G_{i2}\alpha$

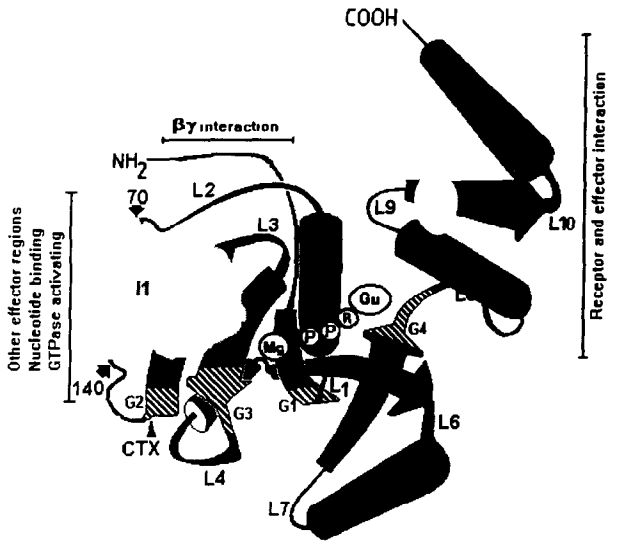

Fig. 3. Hypothetical three dimensional structure of $G_s\alpha$ (adapted from Masters et al. [28], Berlot and Bourne [15] and Olate and Allende [3]). α helices are indicated by grey cylinders, β-strands are represented by grey arrows, non-structured regions by black lines. β-strands, α-helices and loops are numbered starting from the NH2-terminus. Cross-hatched areas G1 to G4 are the regions involved in the binding of GTP. Loops and turns are indicated by letters L1 to L10. Arg^{184} (short form of Gsα), modified by cholera toxin (CTX), is indicated by a thin arrow. The $G_s\alpha$ molecule is shown bound to $GDP{\cdot}Mg^{2+}$. Gu means guanine base, R means ribose ring and P means phosphate groups. The light grey area represent the i1 region containing the cluster of 19 amino acid differences analyzed by us (position 70 to 140) and is limited by thick black head arrows. Vertical and horizontal black bars indicate different regions of the $G_s\alpha$ molecule implicated in the interaction and regulation of other signal transducing components

and $G_s\alpha$ chimeras and scanning mutagenesis demonstrated that a segment in the carboxyl end of the $G_s\alpha$ protein comprising residues 236–356 is required for activation of adenylyl cyclase [15]. Within this segment, Berlot and Bourne identified 4 short regions that are essential for this function of the $G_s\alpha$ protein (Fig. 1, + symbols). In addition, it is also clear from the results obtained with Ch9 that the *X. laevis* $G_s\alpha$ carboxyl portion is also able to confer $G_s\alpha$ activity to these types of chimeras. It is noteworthy that the basal activity of these chimeras involving $G_{i2}\alpha$ amino terminal portion is very high indicating possibly that these proteins tend to have a more permanent activated conformation. Our results described above are not necessarily contradictory with these findings, although a different region of $G_s\alpha$ important for this activity has been found. The $G_s\alpha$ and $G_{12}\alpha$ clones used by Osawa et al. [14] and by Berlot and Bourne [15] to prepare their chimeras contain 51 amino acid differences in the region that goes from amino acid 70 to 140. However, it is possible that these substitutions, which are mainly different from those found in the $G_s\alpha$-like clones of *X. laevis*, may preserve the structural features required for the stimulation of adenylyl cyclase. Berlot and Bourne [15] state explicitly that their results do not rule out the possibility that other regions of the $G_s\alpha$ molecule may also be important in adenylyl cyclase activation. It must also be noted that our recombinant proteins were tested in vitro, while these authors assayed their chimeric activities in vivo in transfected COS-1 and COS-7 cells.

Berlot and Bourne [15] proposed a 3-dimensional model for the structure of $G_s\alpha$ on the basis of its analogy with $P21^{ras}$. Since the region from amino acid 70 to 195 of $G_s\alpha$ is absent from $P21^{ras}$, this section which contains the fragment of the molecule which has been shown by our studies to play an important role in $G_s\alpha$ function, was not depicted in their model. Recently, a more exact 3D structure for the $G\alpha$ subunit has been structured, where region i1, that is highly variable among $G\alpha$ subunits is shown [29]. Conklin and Bourne [29] have proposed that the i1 region, encompasing from residue 82 to residue 217 (long form of $G_s\alpha$), forms a distinc structural domain folded independently from the GTPase core and could have other important roles for G protein function. This hypothesis has been now confirmed by Markby et al. [30] through an elegant work in which region i1, called now the 'gail region', is a separate domain that acts as a GAP. Fig. 3 shows a hypothetical model of the small form of the $G_s\alpha$ subunit, where the variable region that we have found to be important for human $G_s\alpha$ activation of adenylyl cyclase goes from residue 70 to 140 and in our model (short form of $G_s\alpha$) correspond exactly to the 'gail region'.

It is possible the *X. laevis* $G_s\alpha$-like protein and the chimeras containing this 70–140 region from that origin are less efficient in one or several of the general functions of G proteins such as GTP binding, guanine nucleotide exchange or the conformational change that accompanies their interaction with the guanosine triphosphate, GTPase activity or even more its interaction with adenylyl cyclase. These possibilities are presently being explored in our laboratory.

Regarding to the physiological function of the $G_s\alpha$-like proteins coded by the two cDNA clones found in *X. laevis*, the present experiments do not provide much information. Although the proteins coded by these clones are practically inactive when tested in a heterologous system, the S49 cyc^- membranes, these proteins may be able to activate the homologous *X. laevis* adenylyl cyclase. In this respect, it is pertinent to note that $G_s\alpha$ proteins coded by Drosophila [31] and by rat olfactory epithelium [32,33] when assayed for their capacity to stimulate s49 cyc^- membranes in the presence of GTPγS or GTP and isoproterenol, demonstrated activities that were at least 10-fold lower than the control $G_s\alpha$ protein. The discovery of the existence of several species of adenylyl cyclases [34] with different specificities may mean that various $G_s\alpha$ proteins may interact specifically with a particular species of this enzyme.

On the other hand, it is also possible that both of these *X. laevis* $G_s\alpha$-like proteins are involved in signal transduction with a different effector molecule. Recently, Murphy et al [35] have described the activation of a calcium channel by a $G_s\alpha$-type protein in *X. laevis* oocytes. In the last few years it has become frequent to discover new variants of $G\alpha$ subunits that are specific for different effector molecules.

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