The Signal Transfer Regions of Gαs

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The crystal structure of soluble functional fragments of adenylyl cyclase complexed with Gα and forskolin, shows three regions of Gα on the activities of full-length adenylyl cyclases 2 and 6. A peptide encoding the Switch II region (amino acids 222–247) stimulated both adenylyl cyclases partially inhibited Gα stimulation. Corresponding Switch II region peptides from Gα and Gα did not stimulate adenylyl cyclase. A peptide encoding the Switch I region (amino acids 199–216) also stimulated AC2 and AC6. The stimulatory effects of the two peptides at saturating concentrations were non-additive. A peptide encoding the third contact region (amino acids 268–286) located in the α3-β5 region, inhibits basal, forskolin, and Gα-stimulated enzymatic activities. Since this region in Gα interacts with both the central cytoplasmic loop and C-terminal tail of adenylyl cyclases this peptide may be involved in blocking interactions between these two domains. These functional data in conjunction with the available structural information suggest that Gα activation of adenylyl cyclase is a complex event where the α3-β5 loop of Gα may bring together the central cytoplasmic loop and C-terminal tail of adenylyl cyclase thus allowing the Switch I and Switch II regions to function as signal transfer regions to activate adenylyl cyclase.

Signaling through heterotrimeric G proteins involves non-covalent protein-protein interactions. Among the best studied of these is the regulation of adenylyl cyclase by Gα, (1, 2). There is considerable molecular information about the interactions between Gα and adenylyl cyclase (3). The crystal structure of the Gα complexed with the functional fragments of adenylyl cyclase has been solved (4) as has been the structure of the Gα by itself (5). There is reasonably good agreement between the mutagenesis studies both on Gα (6) and on adenylyl cyclase (7, 8) itself and the contact points observed in the co-crystal structures. Nevertheless, little is known about the role of these regions in signal transfer from Gα to adenylyl cyclase. We have previously used peptides encoding regions of Gβ to identify the functions of these regions in transfer of signal from Gβ to phospholipase C-β (9, 10). Two types of effects were observed. Some peptides encoding regions known to directly contact PLCβ were able to stimulate PLCβ, and these are thought to encode signal transfer regions. Other peptides that are also thought to contact PLC-β were able to inhibit Gβγ stimulation of PLC-β but by themselves had no effect on PLC activity. These regions are termed general binding domains, which are involved in the protein-protein interactions and contribute to the overall affinity but by themselves do not transfer signal information. Since three distinct regions in Gα interact with adenylyl cyclase we determined which of these contact sites were involved in signal transfer. For this we synthesized peptides encoding these regions and tested their ability to modulate basal Gα and forskolin-stimulated adenylyl cyclase activities.

EXPERIMENTAL PROCEDURES

Materials—Amino acids and reagents for peptides were from Bachem. [γ-32P]ATP was from PerkinElmer Life Sciences. Tissue culture reagents and fetal calf serum were from Life Technologies, Inc. Protease inhibitors were from Sigma. All other reagents were the highest analytical grade that was commercially available.

Peptide Synthesis—Peptides were synthesized on an Applied Biosystem peptide synthesizer (model 431A) and purified by high pressure liquid chromatography on 1–75% acetonitrile gradients. Purified peptides were lyophilized and stored at –20 °C, and each peptide container was filled with nitrogen for long-term storage. When required peptides were dissolved in water to a final concentration of 1–3 mM. For each assay peptides stock solutions were freshly prepared. Identity and purity of the peptides was verified by mass spectrometry.

Expression of G Protein Subunits and Adenylyl Cyclases—HexaHis Q213L-Gαq (kind gift of Dr. T. Patel, University of Tennessee) was expressed in JM109 (DE3) cells. The protein in cell lysates was purified on Ni-NTA columns according to a protocol kindly provided by the Patel laboratory. This protocol is essentially similar to the method described by Graziano et al. (11). AC2 and AC6 were expressed in Hi5 cells by infection with recombinant baculovirus. Membranes were prepared from infected cells and used for the assays (12).

Adenylyl Cyclase Assays—Enzymatic activity was measured by conversion of [γ-32P]P to [γ-32P]cAMP. AC2 and AC6 assays have been described (13, 14). When required, the peptides were mixed with adenylyl cyclase containing membranes and held on ice for 10 min prior to assays. Approximate 1–2 μg of AC2 Hi5 cell membranes and 3–5 μg of AC6 membrane per assay tube were used. Concentration of activated Gαq was 2 μM and that of forskolin 30 μM. All assays contained a mixture of protease inhibitors. Final concentration of protease inhibitors was leupeptin 3.2 μg/ml, aprotinin 2 μg/ml, phenanthroline 1.0 mM, and phenylmethylsulfonyl fluoride 1.0 mM. The protease inhibitor mixture was always freshly prepared. All experiments were repeated three or more times with qualitatively similar results. Typical experiments are shown. Values are means ± S.D. of triplicate determinations.

RESULTS

The Switch II regions of Gα subunits undergo substantial conformational changes upon exchange of GDP for GTP (15, 16), and the GDP-bound Gα subunit is known to have at least a 10-fold lower affinity for adenylyl cyclase than the GTPγS bound form (17). Since the Switch II region is also involved in physical contact with adenylyl cyclase it appeared likely that

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‡ The abbreviation used is: PLC, phospholipase C.
this region might be involved in signal transfer, we synthesized a 26-amino acid peptide encoding the Switch II region and tested its effect on the stimulation of AC2 and AC6 expressed in Hi5 cells. The $\text{G}_q$-(222–247) (Switch II) peptide stimulated both enzymes 2- to 3-fold (Fig. 1A and B). Although this stimulation is extensive, it is typically about 30–50% of that observed with saturating concentrations of activated $\text{G}_q$ under these assay conditions. As controls we used peptides encoding the Switch II region of $\text{G}_{q\alpha}$ (amino acids 199–224) or $\text{G}_{q\beta}$ (amino acids 204–229). The Switch II regions of $\text{G}_{q\alpha}$ or $\text{G}_{q\beta}$ are very similar to the Switch II region of $\text{G}_q$ except for some of the contact residues and the last Asp in the a2 helix. Neither the $\text{G}_{q\alpha}$ or $\text{G}_{q\beta}$ peptides stimulated either adenylyl cyclase (Fig. 1, A and B). The Switch II peptide stimulated on top of the forskolin both AC2 and AC6 activities. Stimulation was about 3-fold while the observed EC$_{50}$ did not change. The $\text{G}_{q\alpha}$ or $\text{G}_{q\beta}$ peptides also did not further stimulate the activity in the presence of forskolin (Fig. 1, C and D). We next tested the effect of $\text{G}_q$-(222–247) peptide on the $\text{G}_q$-stimulated activity of AC2 and AC6. In both cases the $\text{G}_q$-stimulated activity was inhibited by about 30–35% (Fig. 1, E and F). For neither adenylyl cyclase did the inhibition reach to a level equal to the stimulation observed by the peptide alone. Thus although the $\text{G}_q$-(222–247) peptide behaves as a “partial agonist” in a qualitative sense it does not appear to do so in a strict quantitative sense. The peptides encoding the $\text{G}_{q\alpha}$ or $\text{G}_{q\beta}$ did not affect the $\text{G}_q$-stimulated activity of either adenylyl cyclase.

We next tested the effects of an 18-mer peptide (199–216) encoding the Switch I region from $\text{G}_{q\alpha}$. The Switch I peptide behaved similarly to the Switch II peptide. The peptide by itself stimulated both AC2 and AC6 (Fig. 2, A and B). As a control we used an 18-mer with the following substitutions: G206P, I207D, E209K, and K211A. Residue 207 contacts with adenylyl cyclase, while the 209 and 211 substitutions would disrupt local conformation. The substituted peptide had no effect on either AC2 or AC6 activities. The Switch I peptide stimulated both AC2 and AC6 on top of forskolin-stimulated activities (Fig. 2, C and D). As with the Switch II peptide forskolin does not appear to shift the position of the peptide concentration-effect curves. The Switch I peptide also inhibited the $\text{G}_q$-stimulated activity of both AC2 and AC6 by about 30% (Fig. 2, E and F). Thus the Switch I peptide also qualitatively behaved as a partial agonist.

Since both the Switch I and Switch II peptides stimulated adenylyl cyclase we tested if the effects of the two peptides were additive. This was done both in the absence and presence of forskolin. At each concentration, the peptides were tested individually or in combination of equal amounts. For AC2, at lower concentrations, the effects of the peptides were additive, giving the appearance of a left-shifted curve. However, at saturating concentrations the effects were not additive. The total stimulation appears to plateau at levels seen with the Switch I peptide (Fig. 3A). In contrast, for AC6 it appears that the effect of Switch II peptide predominates. Even when both peptides were present the total stimulation was very similar to that seen with the Switch II peptide alone (Fig. 3B). In the presence of forskolin, the responses of both AC2 and AC6 appear to be similar. For both adenylyl cyclase isoforms the maximal activity plateaus close to what is observed with the Switch I peptide (Fig. 3, C and D). These results suggest that there may be complex inter-regulation between forskolin interactions and $\text{G}_q$ interactions with adenylyl cyclases.

We also tested the effects of a peptide encoding amino acids 268–286, which contains the contact site in the $\alpha_3$-$\beta_3$ region of $\text{G}_{q\alpha}$ that interacts with both the C-terminal tail as well as the
FIG. 3. Effect of simultaneous addition of varying concentrations of the Switch I and Switch II encoding peptides of Go_s on the basal- (top panels) and forskolin-stimulated (bottom panels) activities of AC2 (left panels) and AC6 (right panels) isoforms. Indicated concentrations are for the individual peptides. All values are means of triplicate determinations and coefficient of variance is less than 10%.

FIG. 4. Effect of the varying concentrations of a peptide encoding the αββ5 region regions of Go_s on the basal- (top panels), forskolin-stimulated (middle panels), and activated Go_s-stimulated activities of AC2 (left panels) and AC6 (right panels) isoforms. As a control a substituted (W277R, W280K) peptide was used. All values are means of triplicate determinations and coefficient of variance is less than 10%.

central cytoplasmic loop of adenyl cyclase. As a control we used a peptide where the Trp-277 and -280 were replaced with Arg or Lys, respectively. This peptide inhibited basal- and forskolin-stimulated activity by about 30% (Fig. 4, A–D) while it very substantially (~ 75%) inhibited the Go_s-stimulated activity (Fig. 4, E and F). The effect of this peptide suggests that it may interact with either the central cytoplasmic loop or the C-terminal tail and thus inhibit all activities. The greater extent inhibition of Go_s inhibition may arise because appropriate α3-β2 contact is required to appropriately orient the Switch I and/or Switch II region to stimulate enzymatic activity.

DISCUSSION

Both the Switch I and Switch II peptides show a stimulatory effect on basal adenyl cyclase activity and further stimulation in the presence of forskolin. This is not entirely surprising, when one considers the crystal structure. Both the Switch I and II regions of Go_s bind to the region within the α1-α2' domain of the C-terminal tail of adenyl cyclase, and the contact residues are only a few residues apart. It should be noted though that only a single residue in Switch I contacts the α1'-α2' region of the cytoplasmic tail of adenyl cyclase. Hence we cannot definitively rule out the possibility that its stimulatory ability arises from its interactions with regions of the native enzyme that are not present in the crystal structure. Although one might intuitively expect that there may be a single signal transfer region, this is the second protein in which we have found multiple signal transfer regions, Gβ being the other (10). Thus it is possible that there are built-in redundancies within signaling components to maximize signal flow.

The Gso3β5 peptide blocks Go_s stimulation, and to a lesser extent basal- and forskolin-stimulated activities as well. Since the Gso3β5 peptide had the interaction residues for both the central cytoplasmic loop as well as the C-terminal tail of adenyl cyclase, we expected it may mimic the effect of forskolin. A reasonable explanation for the observed inhibitory effects is that the peptide binds only to the central cytoplasmic loop or C-terminal tail and thus prevents them from interacting with one another. We added several residues on both C-terminal and N-terminal regions of the peptide and did not see any reduction of the inhibitory effects. Adding more residues greatly reduced the solubility of the peptide and making experiments with the longer peptides not feasible.

An intriguing aspect of our findings is that of the combined effects of the Switch I and Switch II peptides on adenyl cyclase activities. As expected at low concentrations the effects of the peptides was additive for AC2. A different effect was observed for AC6 where the presence of the Switch II peptide appears to block the effect of the Switch I peptide (Fig. 3, B). Thus it appears that there may be some differences in the mode of interactions between Go_s and the different adenyl cyclase isoforms. This is an area that requires further study in a systematic fashion. In the presence of forskolin the effects of the Switch I peptide appears to predominate for both AC2 and AC6 since the activity with saturating concentrations of both peptides appears to be closer to the activity seen with the Switch I peptide rather than the Switch II peptide (Fig. 3, C and D). These results suggest that there may be dynamic interactions between the forskolin binding sites and the interaction sites for Switch I and II resulting in preferential interactions of one or another of the signal transfer regions. Further studies focusing on the dynamics of the contacts and local conformational changes are required to critically ascertain the molecular mechanisms involved in forskolin regulation of interactions of the signal transfer regions of Go_s with adenyl cyclases.

An interesting question arises from these studies: why have multiple signal transfer regions if only one is used. One possibility is that both are used since in the crystal structure residues in both the Switch I and Switch II region make contact with adenyl cyclase. However it should be noted that crystal
structure contains only part of the central cytoplasmic loop of
AC5 and the C-terminal tail of AC2 and hence is not an accu-
rate representation of a native enzyme. Further structural
studies with native proteins should clarify this issue. Another
possibility is that depending on the initial interactions either
the Switch I or Switch II region contacts the adenylyl cyclases
and each of these is capable of inducing the local changes
necessary to activate the enzyme. Thus the Goαs-adenylyl cy-
clase complex may contain multiple conformational species.
Dynamic conformational studies will be needed to sort through
these alternative scenarios. Irrespective of the molecule spe-
cific details that emerge from future studies, this study along
with our previous on Goβ (9, 10) indicate that it is possible to
assign distinct functions to the different regions of G protein
subunits that contact their direct effectors. How the signal
transfer regions and the other regions of G proteins cooperate
to achieve reversible signal flow with high specificity remains
to be determined.

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Page 45752, Fig. 1: The description of symbols at the top of this figure contains a typographical error. The Gαi peptide is 199–224 (not 119–224). The correct figure is shown following.

**Fig. 1.** Effect of the varying concentrations of peptides encoding the Switch II regions of Gαs, Gαi, and Gαq on the basal (top panels), forskolin-stimulated (middle panels), and activated Gαs-stimulated activities of AC2 (left panels) and AC6 (right panels) isoforms. All values are means of triplicate determinations and coefficient of variance is less than 7%.