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$G_{i\alpha1}$ Selectively Couples Somatostatin Receptor Subtype 3 to Adenylyl Cyclase: Identification of the Functional Domains of this α Subunit Necessary for Mediating the Inhibition by Somatostatin of cAMP Formation

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SUMMARY

A major cellular action of the neuropeptide somatostatin (SRIF) is the inhibition of adenylyl cyclase activity. SRIF induces this effect after its interaction with membrane-bound receptors. Five SRIF receptors (SSTRs), which differ in their functional coupling to adenylyl cyclase, have recently been cloned. The third SSTR cloned, SSTR3, effectively mediates the inhibition of adenylyl cyclase by SRIF. The molecular mechanism by which SRIF modulates intracellular cAMP synthesis via SSTR3 was investigated by initially identifying which G_α subunits are involved in coupling SSTR3 to adenylyl cyclase. SRIF did not inhibit cAMP formation in Chinese hamster ovary cells stably expressing SSTR3 and $G_{i\alpha2}$ or $G_{i\alpha3}$ but lacking $G_{i\alpha1}$. However, SRIF did inhibit forskolin-stimulated cAMP formation in Chinese hamster ovary cells stably expressing SSTR3 and $G_{i\alpha1}$, indicating that $G_{i\alpha1}$ selectively couples SSTR3 to adenylyl cyclase. To investigate the functional domains of $G_{i\alpha1}$ necessary for interaction with SSTR3, a chimeric α subunit ($G_{i\alpha2}/G_{i\alpha1}$) was constructed, consisting of the amino-terminal two thirds of $G_{i\alpha2}$ ligated to the carboxyl-terminal third of $G_{i\alpha1}$. SRIF inhibited cAMP formation in cells expressing SSTR3 and the $G_{i\alpha2}/G_{i\alpha1}$ chimera. These findings indicate that the carboxy-terminal third of $G_{i\alpha1}$ interacts with SSTR3 and is important in transmitting the signal of SSTR3 activation to adenylyl cyclase. In contrast, a similar $G_{i\alpha2}/G_{i\alpha3}$ chimera did not couple SSTR3 to adenylyl cyclase, further indicating that $G_{i\alpha3}$ does not contribute to SRIF inhibition of adenylyl cyclase activity. These findings demonstrate that $G_{i\alpha1}$ selectively couples SSTR3 to adenylyl cyclase, and they indicate that the carboxyl-terminal region of this α subunit is involved in mediating SRIF inhibition of adenylyl cyclase activity.

A major intracellular action of the neuropeptide SRIF is the inhibition of adenylyl cyclase activity. SRIF induces this effect by binding to its receptors, which then activate pertussis toxin-sensitive G proteins. The G_α subunit $G_{i\alpha}$ can be modified by pertussis toxin and was first identified for its ability to mediate neurotransmitter and hormone inhibition of adenylyl cyclase activity (1–4). SSTRs from rat brain, the cell line AtT-20, and pancreatic tumor cells associate with $G_{i\alpha1}$ and $G_{i\alpha3}$ but not $G_{i\alpha2}$, even though similar levels of each $G_{i\alpha}$ subtype are present in these tissues (5–7). Using an antibody inactivation approach, it was observed that $G_{i\alpha1}$ selectively couples SSTRs to the inhibition of adenylyl cyclase activity (8). Although $G_{i\alpha3}$ does not functionally couple SSTRs to adenylyl cyclase, reconstitution studies have reported that $G_{i\alpha3}$ can couple SSTRs to K^+ channels (9). Because $G_{i\alpha1}$ and $G_{i\alpha3}$ share 94% amino acid sequence identity, small amino acid differences in these $G_{i\alpha}$ forms must be responsible for directing SSTRs to distinct signaling pathways.

The carboxyl terminus of G_α is thought to be critical in the association of this subunit with receptors. Recent studies have shown that $G_{o\alpha}$ selectively couples SSTRs to Ca^{2+} channels and that $G_{o\alpha2}$ but not $G_{o\alpha1}$ is involved in this coupling (10, 11). These two forms of $G_{o\alpha}$ are derived from differential splicing of a common $G_{o\alpha}$ transcript and differ only in their carboxyl-terminal regions (12–14). This indicates that differences in the carboxyl terminus of these splice variants are responsible for determining their ability to functionally couple SSTRs to Ca^{2+} channels.

Five SSTRs have recently been cloned from human, rat, and mouse genomic libraries (15–18). The receptors differ in their

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ABBREVIATIONS: SRIF, somatostatin; SSTR, somatostatin receptor; HEK, human embryonic kidney; CHO, Chinese hamster ovary; bp, base pair(s); GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

pharmacological specificities (19, 20) and some couple to adenylyl cyclase (SSTR2B, SSTR3, and SSTR4) (16, 17, 21), whereas others do not (SSTR1, SSTR2A, and SSTR5) (16, 19). Of the cloned receptors, SSTR3 is unique, in that it has low affinity for clinically used SRIF analogs such as Sandostatin and MK-678 and is the predominant SSTR type expressed in pancreas. SRIF is a potent inhibitor of insulin and glucagon release from pancreatic islets, and this effect may be selectively mediated by SSTR3 (22). One mechanism by which SRIF has been shown to inhibit hormone release is through a blockade of adenylyl cyclase activity, and SSTR3 effectively mediates the inhibition of adenylyl cyclase activity by SRIF.

To investigate the molecular mechanisms by which SRIF modulates intracellular cAMP synthesis, we first identified which $G_{i\alpha}$ protein is responsible for coupling SSTR3 to adenylyl cyclase and then determined whether the carboxyl-terminal region of $G_{i\alpha}$ is critical in specifying these functional associations. Previous studies have shown that, after transient expression of SSTR3 in HEK 293 cells, which express $G_{i\alpha1}$ and $G_{i\alpha3}$, SRIF is able to inhibit adenylyl cyclase activity (23). In contrast, SSTR3 stably expressed in a CHO cell line, which has undetectable levels of $G_{i\alpha1}$ but relatively high levels of $G_{i\alpha3}$, does not mediate inhibition by SRIF of adenylyl cyclase activity (16). This suggests that $G_{i\alpha1}$ is critical for coupling SSTR3 to adenylyl cyclase. In this study, it is demonstrated that $G_{i\alpha1}$ selectively couples cloned SSTR3 to adenylyl cyclase and that the carboxyl-terminal third of this α subunit is important for functional association.

Experimental Procedures

Materials. G418 and α-minimal essential medium with ribonucleosides and deoxyribonucleosides were obtained from GIBCO. The Western blotting alkaline phosphatase Protoblot kit was purchased from Promega. GTPγS was obtained from Boehringer Mannhiem and the cAMP radioimmune assay kit was purchased from DuPont. SRIF was obtained from Peninsula and CGP 23996 was a gift from Barbara Petrack (Ciba Geigy).

G protein constructs. The cDNAs encoding $G_{i\alpha1}$ and $G_{i\alpha3}$ were excised from the plasmids pGEM2αi1 and pGEM2αi3, which were provided by Dr. R. Reed (Johns Hopkins University). The $G_{i\alpha2}$ cDNA was contained in the plasmid pYSK136 (24). For expression in mammalian cells, the cDNAs were cloned into the vectors pDzbapaI or pCN. For the construction of pCNαi2, pYSK136αi2 was digested with *Sma*I and *Eco*RI. The 1300-bp fragment containing the $G_{i\alpha2}$ coding region was then inserted into *Eco*RV/*Eco*RI-digested pCN. For construction of pCNαi3, pGEM2αi3 was digested with *Hpa*I and *Eco*RI. The 1839-bp fragment containing the $G_{i\alpha3}$ coding region was inserted into *Hpa*I/*Eco*RI-digested pCN. The $G_{i\alpha2}/G_{i\alpha3}$ chimera was created at a common *Bam*HI site, located at amino acid 211. pCNαi2 and pCNαi3 were digested with *Bam*HI and *Xho*I. The 1500-bp fragment from the pCNαi3 digestion, containing the portion of $G_{i\alpha3}$ cDNA between the *Bam*HI site and the 3′ end, was ligated to the 5000-bp fragment from the pCNαi2 digestion, containing the portion of the corresponding cDNA between the 5′ end and the *Bam*HI site. For the construction of the corresponding $G_{i\alpha2}/G_{i\alpha1}$ chimera, the *Bam*HI restriction site was introduced in $G_{i\alpha1}$ (it is absent in $G_{i\alpha1}$ due to a single-base, silent mutation) by polymerase chain reaction mutagenesis. pGEM2αi1 was used as the template with the following primers: GCGGAAGAAGTGGATCCACTGCTTTGAAGGCG (in which the base that was mutated to introduce the *Bam*HI site is underlined) and ATAAATAAAGCTGATCAAAACTGCC (in which the *Bcl*I restriction site was introduced in the 3′ untranslated region of the gene). The 880-bp polymerase chain reaction product was digested with *Bam*HI and *Bcl*I and ligated with the *Bam*HI/*Bcl*I fragment of pCNαi2, containing the amino-terminal two thirds of $G_{i\alpha2}$. The identity of the plasmid constructs was confirmed by restriction enzyme digestion and partial sequence analysis.

Isolation of stable transformants. CHO cells (DG44 variant) were grown to 50% confluency in α-minimal essential medium with ribonucleosides and deoxyribonucleosides, in T25 (25-cm^2) flasks. The cells were transfected, by a Ca_2PO_4 precipitation method (16, 25), with 7 μg of the *Bst*EII-*Xba*I fragment of the mouse SSTR3 gene in the vector pCMV6c and 7 μg of one of the following; pDzbapaI, $G_{i\alpha1}$pDzbapaI, $G_{i\alpha2}$pCN, $G_{i\alpha2}/G_{i\alpha1}$pCN, or $G_{i\alpha2}/G_{i\alpha3}$pCN. The cells were then selected and maintained in 200 μg/ml G418.

SSTR3 binding. Stable colonies of CHO cells obtained by selection with G418 were screened for the presence of SSTR3 by a binding assay with the SRIF-specific agonist ^{125}I-CGP23996 (specific activity, 505 Ci/mmol) (26). The membrane binding assay was performed by incubating CHO cell membranes with 0.5 nM ^{125}I-CGP23996 and varying concentrations of SRIF or GTPγS. The binding assay was terminated by rapid vacuum filtration and the filters were washed with 12 ml of Tris·HCl buffer, pH 7.8, and counted in a γ counter (80% efficiency). Specific binding was defined as total binding of ^{125}I-CGP23996 minus binding in the presence of 1 μM SRIF-14.

Western analysis. Conditions for analyzing G proteins via Western blotting have been described previously (27). The primary antibodies bound to proteins on the nitrocellulose membranes were detected by the alkaline phosphatase Protoblot kit from Promega. The G protein-directed antisera used were obtained after injection of synthetic peptides corresponding to specific regions of the $G_{i\alpha}$ subunit. Antisera 3646 (anti-$G_{i\alpha1}$) and 1521 (anti-$G_{i\alpha2}$) are selective on Western blots and in detecting recombinant $G_{i\alpha}$ subtypes (28). A 1/250 dilution was used for immunoblotting with antisera 3646 and 1521.

cAMP measurement. The measurement of cAMP accumulation in CHO cells was performed as described previously (16). Briefly, cells were exposed for 30 min to Dulbecco's modified Eagle medium with 50 μM isobutylmethylxanthine, at 37° in 10% CO_2. The medium was removed and replaced with similar medium containing 10 μM forskolin, forskolin and 1 μM SRIF, or vehicle control, for 30 min. At the end of this time, the medium was removed, 500 μl of 1 N HCl were added to the cells, and the cell fragments were sonicated. The samples were evaporated in a Speed Vac concentrator and the cAMP content was measured using a commercially available cAMP radioimmunoassay (DuPont).

Results

$G_{i\alpha1}$ couples SSTR3 to adenylyl cyclase. Previous studies have shown that, after transient expression of SSTR3 in HEK 293 cells, which express $G_{i\alpha1}$ and $G_{i\alpha3}$, SRIF was able to inhibit adenylyl cyclase activity (23). However, in CHO cells expressing the mouse SSTR3 gene, SRIF could not inhibit cAMP formation (16, 23). Western blotting revealed that these CHO cells express $G_{i\alpha3}$ but very little if any $G_{i\alpha1}$ or $G_{i\alpha2}$, suggesting that $G_{i\alpha3}$ does not couple SSTR3 to adenylyl cyclase (23, 25). To investigate SSTR3/$G_{i\alpha}$/adenylyl cyclase coupling, SSTR3 was expressed with either $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha2}/G_{i\alpha1}$, or a $G_{i\alpha2}/G_{i\alpha3}$ chimera in CHO cells. Fig. 1 illustrates these chimeric α subunits. After the selection of stable transformants, each clonal cell line was tested for the presence of SSTR3 by using the specific SSTR agonist ^{125}I-CGP23996 in a binding assay and for the newly expressed G proteins by immunoblotting. Specific binding of ^{125}I-CGP23996 to membranes from cells expressing SSTR3 and $G_{i\alpha1}$, $G_{i\alpha2}$, or the chimeric G proteins was approximately 5.6 fmol/mg of protein for each cell line and was inhibited by SRIF with similar IC_{50} values (approximately 100 nM in all cases). GTPγS reduced agonist binding by 35–40%, values similar to those reported by Yasuda *et al.* (16). All of the newly expressed

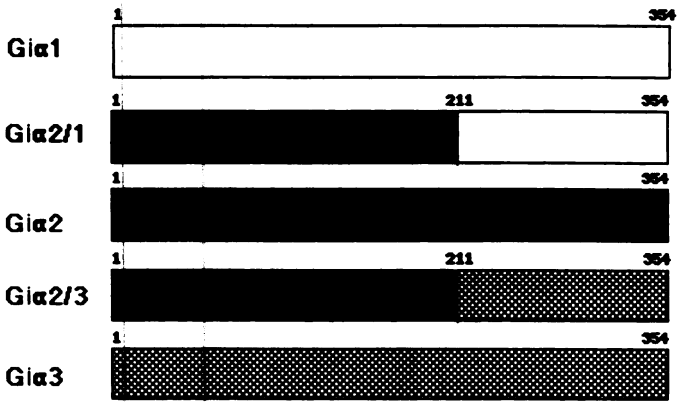

Fig. 1. G protein constructs.

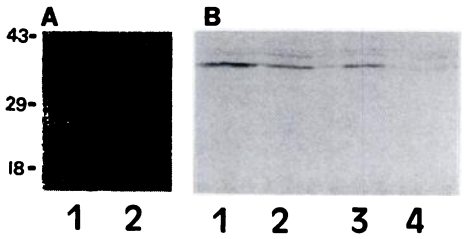

Fig. 2. Expression of the $G_{i\alpha}$ constructs in CHO cells, as detected by immunoblotting. CHO cells stably expressing $G_{i\alpha1}$ (A, *lane 1*) were analyzed by immunoblotting and screened with the $G_{i\alpha1}$-selective antiserum 3646. Nontransfected CHO cells lacking $G_{i\alpha1}$ were analyzed in the adjacent lane (A, *lane 2*). CHO cells stably expressing $G_{i\alpha2}$ (B, *lane 1*), the $G_{i\alpha2}/G_{i\alpha1}$ chimera (B, *lane 2*), or the $G_{i\alpha2}/G_{i\alpha3}$ chimera (B, *lane 3*) or nontransfected cells (B, *lane 4*) were screened with the $G_{i\alpha2}$-selective antiserum 1521.

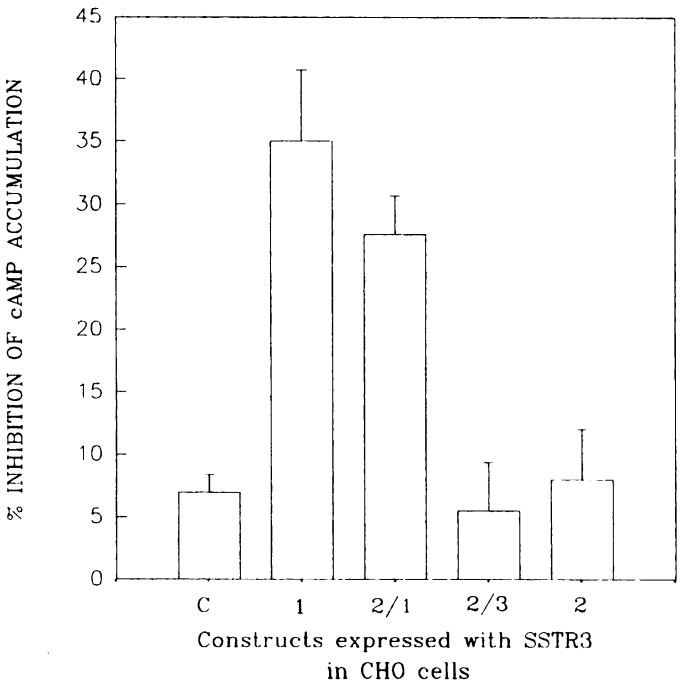

Fig. 3. Coupling of SSTR3 to adenylyl cyclase in CHO cells expressing the $G_{i\alpha}$ constructs. CHO cells stably expressing SSTR3 and $G_{i\alpha1}$ (*1*), $G_{i\alpha2}/G_{i\alpha1}$ (*2/1*), $G_{i\alpha2}/G_{i\alpha3}$ (*2/3*), or $G_{i\alpha2}$ (*2*) or cells expressing only SSTR3 (*C*) were tested for SRIF inhibition of forskolin-stimulated cAMP formation. The cells express a similar basal level of cAMP accumulation and a similar fold stimulation of cAMP accumulation in response to forskolin. Plotted is the percent inhibition of forskolin-stimulated cAMP formation induced by 1 μM SRIF.

G proteins were detected in representative clones by Western blotting (Fig. 2).

In cells expressing SSTR3 and $G_{i\alpha1}$, SRIF inhibited forskolin-stimulated cAMP accumulation by 25–30% (Fig. 3), which is similar to the level of SRIF inhibition of cAMP formation observed in COS-7 cells or HEK 293 cells transiently expressing SSTR3 (23). Similar results were obtained in other clones expressing SSTR3 and $G_{i\alpha1}$. In contrast, in cells expressing SSTR3 with $G_{i\alpha2}$ SRIF did not inhibit forskolin-stimulated cAMP formation (Fig. 3). This finding, together with previous results (16) showing that the endogenously expressed $G_{i\alpha3}$ does not couple SSTR3 to adenylyl cyclase, indicates that $G_{i\alpha1}$ selectively couples SSTR3 to adenylyl cyclase.

The carboxyl terminus of $G_{i\alpha1}$ is important for the functional coupling of SSTR3 to adenylyl cyclase. To investigate the regions of $G_{i\alpha1}$ needed for functional association with SSTR3, SSTR3 was expressed with a $G_{i\alpha2}/G_{i\alpha1}$ chimera in which the carboxyl-terminal third of $G_{i\alpha2}$ was replaced with the corresponding sequence of $G_{i\alpha1}$ (see Fig. 1). SRIF was able to inhibit forskolin-stimulated cAMP formation in CHO cells expressing SSTR3 and the $G_{i\alpha2}/G_{i\alpha1}$ chimera (Fig. 3). This effect was observed in multiple clones expressing SSTR3 and the $G_{i\alpha2}/G_{i\alpha1}$ chimera. Furthermore, the inhibition was similar to that observed in cells expressing SSTR3 and $G_{i\alpha1}$. In contrast, SRIF did not inhibit cAMP formation in clones expressing SSTR3 and the $G_{i\alpha2}/G_{i\alpha3}$ chimera (Fig. 3). These findings demonstrate that the carboxyl-terminal third of $G_{i\alpha1}$ is important for the functional coupling of SSTR3 with adenylyl cyclase.

Discussion

This study demonstrates that $G_{i\alpha1}$ selectively couples cloned SSTR3 to adenylyl cyclase in CHO cells. This is consistent with previous results showing that $G_{i\alpha1}$-directed antiserum specifically blocks SRIF inhibition of adenylyl cyclase activity in AtT-20 cells (8). These findings, together with the biochemical (5–7, 23) and electrophysiological results (9–11), suggest that the different G proteins couple SSTRs to distinct cellular effector systems.

An important finding of this study is the demonstration that the carboxyl-terminal region of $G_{i\alpha1}$ is involved in functionally coupling SSTR3 to adenylyl cyclase. This was shown by the ability of the $G_{i\alpha2}/G_{i\alpha1}$ chimera to mediate coupling of SSTR3 to adenylyl cyclase. In contrast, a similar $G_{i\alpha2}/G_{i\alpha3}$ chimera did not allow SRIF to inhibit cAMP formation. The finding that the carboxyl-terminal region of $G_{i\alpha1}$ is important for functional coupling of SSTR3 to adenylyl cyclase is consistent with the results obtained for $G_{s\alpha}$ (29, 30). Most chimeric G proteins made to investigate the regions involved in contacting receptors and interacting with effectors are $G_{s\alpha}/G_{i\alpha}$ combinations (29). In contrast, the results obtained with the $G_{i\alpha2}/G_{i\alpha1}$ versus the $G_{i\alpha2}/G_{i\alpha3}$ chimera show that subtle differences between the $G_{i\alpha}$ subtypes can determine functional interactions.

SSTR3 in CHO cells is maintained in a high affinity state for agonist binding, although it cannot functionally couple to adenylyl cyclase. SSTR3, then, potentially associates with $G_{i\alpha3}$ or $G_{o\alpha2}$, because CHO cells express these subunits but lack $G_{i\alpha1}$ or $G_{i\alpha2}$ (23, 25). Interestingly, the corresponding carboxyl-terminal regions of $G_{i\alpha1}$ and $G_{i\alpha3}$ have very few differences in amino acid sequence. Site-directed mutagenesis of these resi-

dues may reveal their importance in the selective coupling of SSTR3 to adenylyl cyclase by $G_{i\alpha 1}$.

In summary, the results of this study show that $G_{i\alpha 1}$ couples SSTR3 to adenylyl cyclase. Furthermore, because all subtypes of $G_{i\alpha}$ have the potential to inhibit adenylyl cyclase (3, 4), the fact that the SSTR mediates its inhibition of adenylyl cyclase activity through a particular $G_{i\alpha}$ subtype indicates the selective coupling and/or activation of that subtype by SSTR3.

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