Identification of the Subunits of GTP-binding Proteins Coupled to Somatostatin Receptors*

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Somatostatin (SRIF) induces its biological effects by interacting with membrane-bound receptors that are linked to cellular effector systems via G proteins. We have studied SRIF receptor-G protein associations by solubilizing the SRIF receptor from rat brain and AtT-20 cells and immunoprecipitating the receptor-G protein complex with peptide-directed antisera against the different subunits of the G protein heterotrimer. Antiserum 8730, which selectively interacts with all Gi subtypes, maximally and specifically immunoprecipitated SRIF receptor-Gi complexes. To identify the subtypes of Gi that are coupled to SRIF receptors, the subtype-selective antisera 3646, 1521, and 1518, respectively, were used to immunoprecipitate SRIF receptor-Gi complexes. Antiserum 3646 immunoprecipitated SRIF receptor-Gi1 complexes from both brain and AtT-20 cells. Antiserum 1521 immunoprecipitated Gi2 and Gi3 complexes from both brain and AtT-20 cells but did not immunoprecipitate SRIF receptors from these tissues. Antiserum 1518 immunoprecipitated AtT-20 cell SRIF receptors but uncoupled brain SRIF receptor-Gi protein complexes. This result was confirmed with another peptide-selective antiserum, SQ, directed against Gi3. The findings from these studies indicate that Gi1 and Gi2 are coupled to SRIF receptors, whereas Gi3 is not. Even though brain and AtT-20 cell SRIF receptors were both coupled to Gi, the receptors from these tissues differed in their coupling to Gi. Antiserum 2353, which is directed against Gi,S immunoprecipitated SRIF receptors from AtT-20 cells, but did not immunoprecipitate or uncouple SRIF receptor-Gi protein complexes from rat brain. To determine the β subunits associated with the SRIF receptor, antisera directed against Gβ3 and Gβ2 were used to immunoprecipitate SRIF receptor-Gi protein complexes from brain. Peptide-directed antisera against Gβ3 selectively immunoprecipitated solubilized brain SRIF receptors. However, antisera directed against the Gβ3 subunit did not immunoprecipitate brain SRIF receptors, suggesting that brain SRIF receptors may preferentially associate with Gβ3. In addition to immunoprecipitation with Gi and Gβ, SRIF receptors coimmunoprecipitated the Gi protein γ subunits, Gγ2 and Gγ3. These results provide the first evidence that SRIF receptors are coupled to different subunits of G proteins and suggest that selectivity exists in the association of different G protein subunits with the SRIF receptor.

Somatostatin (SRIF)1 is a peptide expressed in the nervous system that inhibits hormone secretion from the anterior pituitary and is involved in the control of neuronal activity and neurotransmitter release in the brain (Brazeau et al., 1972; Epelbaum, 1986; Pittman and Siggins, 1981; Chesselet and Reisine, 1983). SRIF induces its physiological actions by interacting with membrane-bound receptors. These receptors mediate the inhibitory effects of SRIF on adenylate cyclase activity (Jakobs and Schultz, 1983) and Ca2+

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1 The abbreviations used are: SRIF, somatostatin-14; G protein, guanine nucleotide-binding regulatory protein; Gi, stimulatory regulator of adenylate cyclase; Gi, inhibitory regulator of adenylate cyclase; Gi, G protein isolated from brain; PTX, pertussis toxin; GTPγS, guanosine 5'-O-(thiotriphosphate); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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subunit (Gαx and Gγ3) and at least four forms of γ subunit (Gγ1, Gγ2, Gγ5, and Gγ6) are expressed in mammalian cells. Little information is available concerning the physiological roles of the β/γ subunits or their interaction with neurotransmitter receptors. β/γ subunits of G proteins have been reported to reconstitute high affinity agonist binding to partially purified muscarinic cholinergic receptors (Florio and Sternweis, 1989) and to couple with yeast mating factor receptors (Blumer and Thorner, 1990). Certain β/γ subunits of G proteins have also been reported to regulate phospholipase A2 activity (Kim et al., 1989). Furthermore, β/γ subunits have been postulated to mediate SRIF’s inhibition of basal adenylcyclase activity, possibly by sequestering Gαs (Hildebrandt and Kohnke, 1990).

Since both α, β, and γ subunits of PTX-sensitive G proteins all may have a role in mediating the biological actions of SRIF on neurons, it is important to determine which G protein subunits are directly coupled to brain SRIF receptors. While previous studies have proposed that G proteins couple to SRIF receptors and may mediate some of the biological effects of SRIF, no direct evidence has been reported on the exact nature of these interactions or which G protein subtypes may be involved in mediating SRIF’s physiological actions. In the present study, we have attempted to determine whether SRIF receptors are coupled to specific α, β, and γ subunits of G proteins. For these studies, rat brain SRIF receptors and SRIF receptors from the pituitary cell line AtT-20 were solubilized in an active, G protein-coupled form and immunoprecipitated with antisera generated against the different subunits of G proteins. The findings of our study indicate that active SRIF receptors are complexed with Gαx, Gαx, Gγ3, and possibly with Gγ5 and/or Gγ6. Furthermore, differences exist in the coupling of brain- and pituitary-derived SRIF receptors with Gαs.

MATERIALS AND METHODS

RESULTS

SRIF Receptors from Rat Brain and AtT-20 Cells Are Coupled to Gαs—In order to identify the G proteins that are coupled to solubilized rat brain and AtT-20 cell SRIF receptors, we have utilized peptide-directed antisera generated against the different α subunits of G proteins to immunoprecipitate SRIF receptor-G protein complexes. For the studies on rat brain SRIF receptors, solubilized proteins were subfractionated by gel exclusion chromatography. This partial purification of the SRIF receptor (3–5-fold) was necessary for the successful immunoprecipitation of SRIF receptor-G protein complexes. For the studies on rat brain SRIF receptors, solubilized proteins were subfractionated by gel exclusion chromatography. This partial purification of the SRIF receptor (3–5-fold) was necessary for the successful immunoprecipitation of SRIF receptor-G protein complexes. This fractionation was not necessary for the immunoprecipitation of the solubilized AtT-20 cell SRIF receptor-G protein complex with G protein-directed antisera. Initially, it was important to determine which α subunits were present in the solubilized sample used for immunoprecipitation. Therefore, a Western analysis was performed on solubilized brain and AtT-20 cell proteins with antisera that could selectively detect Gαx and Gγ3 subtypes. The results of the Western blotting indicate that all subtypes of Gαx, and Gγ3 were present in both rat brain (Fig. 1) and AtT-20 cell (not shown) solubilized samples, indicating that the receptor has the potential to couple to any or all of the PTX-sensitive Gα subunits.

To determine if Gαs is coupled to the solubilized SRIF receptor, antisera 8730, which was generated against a peptide corresponding to the C-terminal region of Gαs, was employed (Carlson et al., 1989). Antiserum 8730 has been previously reported to specifically interact with Gαx, but shows a preference for Gαx and Gγ3 (Carlson et al., 1989; Williams et al., 1990). Antiserum 8730 selectively detects and immunoprecipitates brain Gαs. This was shown by solubilizing rat brain proteins, subjecting them to 10% SDS-PAGE and immunoblotting. Antiserum 8730 selectively detected brain proteins of approximately 41 kDa (Fig. 1). Furthermore, 8730 immunoprecipitated pertussis toxin-sensitive α subunits of G proteins. For these studies, solubilized, fractionated brain proteins were immunoprecipitated by 8730, and the immunoprecipitate was reacted with pertussis toxin and [32P]labeled NAD. The [32P]ADP-ribosylated Gαs was subjected to SDS-PAGE and autoradiography. As shown in Fig. 2A, a significantly greater level of [32P]ADP-ribosylated 41-kDa protein was present in the 8730 immunoprecipitate as compared with preimmune controls. To further show that 8730 immunoprecipitates Gαs from rat brain, solubilized Gαs proteins were reacted with [3P]labeled NAD and PTX and the [32P]ADP-ribosylated Gαs, was immunoprecipitated with 8730. The immunoprecipitate was subjected to SDS-PAGE and autoradiography. As shown in Fig. 2B, 8730 immunoprecipitated significantly greater levels of [32P]ADP-ribosylated Gαs than preimmune controls. These studies indicate that 8730 can immunoprecipitate Gαs.

Antiserum 8730 immunoprecipitated specific, high affinity 125I-labeled MK 678 binding activity from the solubilized brain proteins. This is shown by a significant increase in the level of specific binding of 125I-labeled MK 678 to SRIF receptors in the 8730 immunoprecipitates in contrast to preimmune controls (Fig. 3A). This represents a specific precipitation of approximately 40% of high affinity SRIF receptor-G protein complexes, as indicated by the loss of SRIF receptor-binding activity in the supernatant of the antiserum 8730 immunoprecipitates when compared with preimmune controls (Fig. 3B). The amount of specific 125I-labeled MK 678 binding sites lost from the supernatant following 8730 immunoprecipitation is similar to the amount of SRIF receptors detected in the immunoprecipitate, suggesting that 8730 immunoprecipitated SRIF receptor-G protein complexes rather than uncoupling the SRIF receptor from the G protein. The immunoprecipitation of the SRIF receptor by antiserum 8730 was specific since it could be blocked by the peptide to which 8730 was generated (Fig. 3). In contrast, an unrelated peptide did not prevent 8730 from immunoprecipitating brain SRIF receptors (Fig. 3). Neither peptide nor antiserum 8730 interfered with the 125I-labeled MK 678 binding assay (not shown). 125I-labeled MK 678 binding to the antiserum 8730 immunoprecipitate is selectively inhibited by the SRIF analog [D-Trp9]SRIF but not by the inactive peptide somatostatin-28 (1–14) (Fig. 4A). Furthermore, it was also selectively abolished by the nonhydrolyzable GTP analog GTPγS, indicating that 125I-labeled MK 678 specifically labels G protein-coupled SRIF receptors in the immunoprecipitate (Fig. 4B). These studies demonstrate that brain SRIF receptors with high affinity and specificity for SRIF agonists can be selectively immunoprecipitated with antiserum directed against Gαs.

Antiserum 8730 also immunoprecipitated Gαs from the pituitary cell line AtT-20, as indicated by the analysis of the PTX-catalyzed [32P]ADP ribosylation α subunits in the 8730 immunoprecipitates by SDS-PAGE and autoradiography (data not shown). It also immunoprecipitated SRIF receptors from AtT-20 cells (data not shown). This effect was as selec-
Antisera 3646, 1521, and 1518 detect 40-41-kDa proteins from rat brain (Fig. 1) and AtT-20 cells as assessed by Western blotting. Antisera 3646, 1521, and 1518 were able to immunoprecipitate relatively similar levels of PTX-sensitive G protein. This was shown by analyzing the pertussis toxin-catalyzed [32P]ADP-ribosylated a subunits from rat brain in the immunoprecipitates of each antisera by SDS-PAGE and autoradiography (Fig. 5). The same results were obtained in studies using Gm-specific antisera to identify G subunits in immunoprecipitates from solubilized AtT-20 cells (not shown). Interestingly, despite the abilities of antisera 3646 and 1521 to immunoprecipitate similar amounts of Gm, only antisera 3646 significantly immunoprecipitated solubilized SRIF receptors from both rat brain (Fig. 6) and AtT-20 cells (Fig. 7). Maximally, 40% of the solubilized SRIF receptor-G protein complex was immunoprecipitated from rat brain proteins by antisera 3646, as indicated by the loss of specific 125I-labeled MK 678-binding sites in the supernatants of antisera 3646 immunoprecipitates (Fig. 6B). Similar results were obtained with SRIF receptors from AtT-20 cells. The concentration of antiserum 1521 used in these studies was

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 3.** Anti-Gm antisera immunoprecipitates soluble, rat brain SRIF receptors. Soluble SRIF receptors coupled to Gm from rat brain were immunoprecipitated with 8730 alone or in the presence of 140 µg of KNNLKDCGFL, the peptide to which the antisera was generated (8730p) or 140 µg of RPPGFSFR, an irrelevant peptide (Contp). The presence of SRIF receptors in the immunoprecipitate was detected using the 125I-labeled MK 678 binding assay as described under "Materials and Methods." Specific 125I-labeled MK 678 binding to the immunoprecipitates is depicted in A. Values are presented as the amount of specific 125I-labeled MK 678 binding (cpm) and are the average of the means ± S.E. of six different experiments done in duplicate tubes. PI, preimmune control. As an indication of the relative amount of solubilized SRIF receptor immunoprecipitated, the loss of specific high affinity 125I-labeled MK 678-binding sites in the supernatants following the immunoprecipitation of the receptors with 8730 is presented in B. The values presented are the means ± S.E. of high affinity 125I-labeled MK 678-binding sites in the supernatants of the immunoprecipitates described in A. *, means that are significantly different (paired t test) from preimmune controls.

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 6.** Ability of Gm subtype-selective antisera to immunoprecipitate or uncouple SRIF receptor-G protein complexes from rat brain. Solubilized and subfractionated brain SRIF receptor-Gm complexes were reacted with antisera (1:20 final dilution) 3646 (anti-Gm1), 1521 (anti-Gm2), 1518 (anti-Gm3), and SQ (anti-Gm4) or preimmune serum (PI) and immunoprecipitated. The immunoprecipitates and supernatants were separated and analyzed for the presence of high affinity SRIF receptor-G protein complexes with the 125I-labeled MK 678 binding assay. A, the immunoprecipitates were assayed for specific 125I-labeled MK 678 binding. B, the decrease in specific 125I-labeled MK 678-binding sites in the supernatant after the immunoprecipitation. These are the means ± S.E. of six different experiments done in duplicate assay tubes. *, means that are statistically different from preimmune controls.
that which immunoprecipitated equivalent amounts of PTX-labeled Gα subunit as antiserum 3646 (shown in Fig. 5). The lack of ability of antisera 1521 to immunoprecipitate SRIF receptors was, therefore, not due to its inability to immunoprecipitate G↓i. These findings indicate that, in both tissues, the SRIF receptor is coupled to G↓i, and not G↓o.

Interestingly, antisera 1518, which is generated against a peptide corresponding to a unique sequence of G↓i, reduced the amount of rat brain SRIF receptor detected in the supernatant (Fig. 6B) but did not cause a corresponding increase in the level of SRIF receptor binding activity in the immunoprecipitate (Fig. 6A). Antiserum 1518 did not interfere in the specific binding of 125I-labeled MK 678 to soluble brain SRIF receptors. These findings suggest that antisera 1518 may have disrupted the coupling of the solubilized brain SRIF receptor to G↓i. In contrast, antisera 1518 did immunoprecipitator SRIF receptors from AtT-20 cells (Fig. 7). This indicates that the SRIF receptor from AtT-20 cells is also coupled to G↓i, and that its association with G↓i may be different from that of the rat brain SRIF receptor. To further investigate the interaction of G↓i with the solubilized brain SRIF receptor, another antisera, generated against a peptide corresponding to a different sequence of G↓i than antisera 1518 was tested for its ability to immunoprecipitate SRIF receptors. SQ was generated against a peptide corresponding to a region of G↓i that is slightly different from the epitope of antisera 1518, but to a region of the G protein similar to that of 3646 and 1521 (see Table I). SQ has been reported to selectively react with recombinant G↓i and specifically detects immunoreactive G proteins in rat brain (Goldsmith et al., 1988; Cortes et al., 1988). It is able to immunoprecipitate PTX-sensitive G↓i from rat brain (Fig. 5). Like antisera 1518, SQ caused a reduction in 125I-labeled MK 678 binding activity in the rat brain supernatant but did not immunoprecipitate SRIF receptors (Fig. 6). SQ did not interfere with the specific binding of 125I-labeled MK 678 to the solubilized SRIF receptor (not shown), suggesting that it also may have uncoupled SRIF receptors from G↓i to reduce affinity of the SRIF receptors for agonists. SQ, however, was able to immunoprecipitate the SRIF receptor from AtT-20 cells as was observed for 1518 (Fig. 7). Therefore, although their exact interactions may differ, both brain and AtT-20 cell SRIF receptors are coupled to G↓i.

Since antisera 3646 and 1518 are directed against different α subunits, it would be expected that they immunoprecipitate different SRIF receptor-G↓i complexes. This hypothesis is supported by the finding that the abilities of antisera 3646 and 1518 to immunoprecipitate AtT-20 cell SRIF receptor-G↓i complexes were nearly additive. For these studies, solubilized SRIF receptors were immunoprecipitated by either antisera 3646, 1518, or their combination, and the presence of SRIF receptors in the immunoprecipitate was detected with the 125I-labeled MK 678 binding assay. No binding activity was present in the preimmune control pellet and 446 ± 56 and 398 ± 81 cpm of 125I-labeled MK 678 binding was detected in either the antisera 3646 or 1518 immunoprecipitates, respectively. Treatment of solubilized receptors with both antisera resulted in 656 ± 57 cpm of 125I-labeled MK 678 binding in the pellet.

**TABLE I**

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Peptide sequence</th>
<th>Specificity no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8730</td>
<td>KNNLKDGCGLF</td>
<td>α↓i</td>
</tr>
<tr>
<td>3646</td>
<td>CLDRIAQPNYI</td>
<td>α↓i</td>
</tr>
<tr>
<td>1521</td>
<td>CLERIAQDYI</td>
<td>α↓o</td>
</tr>
<tr>
<td>1518</td>
<td>CIDFGEARADDAR</td>
<td>α↓i</td>
</tr>
<tr>
<td>SQ</td>
<td>CLDRISQSNYI</td>
<td>α↓i</td>
</tr>
<tr>
<td>2353</td>
<td>CEGYDKERKAD</td>
<td>α↓i</td>
</tr>
<tr>
<td>8135</td>
<td>CDPVRGQRMTTRTLR</td>
<td>β↓i&lt;β↓o</td>
</tr>
<tr>
<td>8132</td>
<td>CEGNVRVSRELAGTGY</td>
<td>β↓i</td>
</tr>
<tr>
<td>8129</td>
<td>CEGNVRVSRELPGHTY</td>
<td>β↓i</td>
</tr>
<tr>
<td>BP1-16</td>
<td>TVGFAGHSDVMISLS</td>
<td>β↓i</td>
</tr>
<tr>
<td>γ↓1</td>
<td>MPVINEPLTDDKDL</td>
<td>γ↓1</td>
</tr>
<tr>
<td>γ↓2</td>
<td>MASNTASISAQRL</td>
<td>γ↓2</td>
</tr>
<tr>
<td>γ↓3</td>
<td>MKGETPVNSTMGG</td>
<td>γ↓3</td>
</tr>
</tbody>
</table>

a Based on sequences described in Jones and Reed (1987).

b Based on sequences described in Fong et al. (1987).

c Based on sequences described in Amatruda et al. (1988).

d Based on sequences described in Gautam et al. (1990).

**FIG. 7** G↓i subtype-selective antisera immunoprecipitate SRIF receptors from AtT-20 cells. Solubilized AtT-20 cell proteins were immunoprecipitated with the same G↓i subtype-selective antisera and preimmune sera (PI) as described in the legend to Fig. 6. The immunoprecipitates were washed and analyzed for the presence of SRIF receptors with the 125I-labeled MK 678 binding assay. Values are presented as specific 125I-labeled MK 678 binding and are the means ± S.E. of five different experiments done in duplicate assay tubes. *, means that are significantly (paired t test) different from preimmune controls.

**TABLE I** Antisera raised against peptides corresponding to predicted sequences of G protein subunits

- Antisera: 3646, 1518, 2353, 8135, 8132, 8129, BP1-16, γ↓1, γ↓2, γ↓3
- Peptide sequence: KNNLKDGCGLF, CLDRIAQPNYI, CLERIAQDYI, CIDFGEARADDAR, CLDRISQSNYI, CEGYDKERKAD, CDPVRGQRMTTRTLR, CEGNVRVSRELAGTGY, CEGNVRVSRELPGHTY, TVGFAGHSDVMISLS, MPVINEPLTDDKDL, MASNTASISAQRL, MKGETPVNSTMGG
- Specificity: α↓i, α↓o, α↓i, α↓i, α↓i, α↓i, α↓i, β↓i<β↓o, β↓i, β↓i, β↓i
To study the association of \( \beta \) subunits with brain SRIF receptor-\( \alpha \) subunits, attempts were made to immunoprecipitate solubilized SRIF receptors with antisera directed against \( \alpha \). Solubilized, fractionated rat brain SRIF receptors were reacted with anti-\( \alpha \) antisera 8730 (1:20 final dilution), anti-\( \alpha \) antisera 2353 (1:20 final dilution), or preimmune serum (PI) and immunoprecipitated. The immunoprecipitate and supernatant were separated and assayed separately for high affinity SRIF receptors using the \( ^{125}\text{I} \)-labeled MK 678 binding assay. This result suggests that 8730 could immunoprecipitate \( \alpha \) and \( \beta \) and is supported by the following results. AtT-20 cells were metabolically labeled with \( ^{35}\text{S} \) methionine, and the proteins were solubilized. Antiserum 8730 was found to coimmunoprecipitate radiolabeled proteins that migrated with similar mobilities as \( \alpha \) and \( \beta \) (Fig. 12A). Furthermore, analysis of the 8730 immunoprecipitates of rat brain proteins by Western blotting using anti-\( \alpha \) antisera 8132 revealed the presence of \( \beta \) immunoreactivity (Fig. 12B). Since 8730 does not directly interact with \( \alpha \) as indicated in Fig. 1, it appears to coimmunoprecipitate \( \alpha \) and \( \beta \) complexes as well as the SRIF receptor.

To study the association of \( \beta \) subunits with brain SRIF receptor-\( \alpha \) complexes, attempts were made to immunoprecipitate solubilized SRIF receptors with antisera directed against \( \alpha \). Solubilized, fractionated rat brain SRIF receptors were screened with the \( \alpha \) and \( \beta \) antisera and both were present in the sample (Fig. 13). Antiserum 8132, which is directed against \( \alpha \), immunoprecipitated solubilized, high affinity, G protein-coupled brain SRIF receptor, as indicated by the increase in specific \( ^{125}\text{I} \)-labeled MK 678 binding sites in the antisera 8132 immunoprecipitate in contrast to preimmune controls (Fig. 14A). Antiserum 8132 immunoprecipitated approximately 60% of high affinity soluble SRIF receptor-\( \alpha \) protein complexes, as indicated by the loss of SRIF receptor-binding activity in the supernatant of the 8132 immunoprecipitates in comparison with preimmune controls (Fig. 14B). Immunoprecipitation of the SRIF receptor by 8132 is specific, since it is blocked by the peptide to which 8132 was generated (Fig. 14). In contrast, an unrelated peptide did not block the effect of 8132 (Fig. 14). Neither peptide interfered with the specific binding of \( ^{125}\text{I} \)-labeled MK 678 to the solubilized SRIF receptor (not shown). To further investigate the nature of the \( \alpha \) coupled to the SRIF receptor-\( \alpha \) complex, we tested a different peptide-generated anti-\( \alpha \) antisera (8135) for its ability to immunoprecipitate SRIF receptor-\( \alpha \) protein complexes. Antiserum 8135 is generated against a peptide corresponding to amino acid sequences found common in both \( \alpha \) and \( \beta \).
and \( G_{\beta 5} \). It interacted with the same size of solubilized brain proteins as 8132, as assessed by immunoblotting (Fig. 13) and, like 8132, immunoprecipitated high affinity solubilized brain SRIF receptor-G protein complexes (Fig. 14).

Antiserum 8129 was used in the immunoprecipitation protocol to determine whether \( G_{\beta 5} \) associates with the solubilized brain SRIF receptor. Antiserum 8129 is generated against a unique epitope of \( G_{\beta 5} \) and detects proteins from brain of comparable electrophoretic mobility as \( G_\beta \) as assessed by immunoblotting (Fig. 13). Furthermore, it immunoprecipitates \([\text{35S}]\)methionine-labeled proteins with similar electrophoretic mobility as \( G_\beta \) (data not shown). Although antiserum 8129 is capable of immunoprecipitating \( G_{\beta 5} \), it did not coimmunoprecipitate solubilized SRIF receptors from brain under the same conditions that antiserum 8132 was able to immunoprecipitate SRIF receptors (Fig. 14). Additionally, 8129 did not disrupt SRIF receptor-G protein coupling, since no change in specific \( ^{125}I \)-labeled MK 678 binding to the solubilized SRIF receptors in the supernatants of 8129 immunoprecipitates could be detected (Fig. 14). Antiserum 8129 and 8132 are directed against the same region of \( G_\beta \) whose amino acid sequence differs in \( G_{\beta 5} \) and \( G_{\beta 6} \). Thus, the inability of 8129 to immunoprecipitate SRIF receptors from brain is not likely to be due to its epitope being hidden under the conditions employed. To further test whether SRIF receptors preferentially associate with \( G_{\beta 6} \) rather than \( G_{\beta 5} \), another \( G_{\beta \gamma} \) antiserum, BP1-16, which is generated against the N-terminal region of \( G_{\beta 5} \) and specifically interacts with \( G_{\beta 5} \) (Amatruda et al., 1988) was tested for its ability to immunoprecipitate SRIF receptor-G protein complexes. Like antiserum 8129, BP1-16 was not able to immunoprecipitate soluble SRIF receptors (Fig. 14).
The finding that 8132 coimmunoprecipitated G_{636} and SRIF receptor-binding activity and that 8730 coimmunoprecipitates G_{i,6} complexes, as well as the SRIF receptor, may indicate that SRIF receptors are associated with the G_{i,6}-G_{636} complex. This hypothesis is supported by the finding that antisem 8132 coimmunoprecipitated pertussis toxin-sensitive G_{i,6} as well as the SRIF receptor. Thus, the reaction of the 8132 immunoprecipitates with pertussis toxin and \(^{32}\)P-labeled NAD revealed the presence of significantly greater amounts of \[^{32}\]P ADP ribosylated, 41-kDa proteins in the immunoprecipitate compared with preimmune controls, suggesting that coinmoprecipitation of G_{i,6} and G_{636} occurred (Fig. 15). 8132 appeared to immunoprecipitate similar levels of pertussis toxin-sensitive G_{i,6} as antisem 8730, which is specifically directed against G_{i,6} (Fig. 15A). The coinmoprecipitation of G_{i,6} and G_{636} could be due to 8132 selectively interacting with and immunoprecipitating \(\beta_{6}\) subunits that were complexed with G_{i,6} or conceivably, 8132 directly interacts with and immunoprecipitates G_{i,6}, under the conditions employed. This latter possibility seems unlikely since 8132 does not directly interact with G_{i,6} based on the results of Western blotting (Fig. 13) and does not immunoprecipitate solubilized \[^{33}\]S-labeled G_{i,6} from HEL cells under conditions in which G_{i,6} and G_{636} are dissociated (data not shown). However, to further test these two possibilities, we \[^{32}\]P ADP-ribosylated solubilized G protein \(\alpha\) subunits from rat brain in the presence of pertussis toxin and then treated the labeled G proteins with the nonhydrolyzable GTP analog GTPyS to promote the dissociation of the radiolabeled \(\alpha\) subunits from G_{636}. The samples were then immunoprecipitated with either 8132 or 8730, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography. In samples pretreated with GTPyS, 8132 did not immunoprecipitate \[^{32}\]P ADP-ribosylated \(\alpha\) subunits of G proteins, whereas 8730 was able to immunoprecipitate radiolabeled 41-kDa proteins (Fig. 15B). These findings indicate that antisem 8132 does not directly interact with G_{i,6} but instead is able to immunoprecipitate G_{i,6} only when it is complexed to G_{636}.

The results described above indicate that the G_{i,6} that is complexed with SRIF receptors is selectively associated with G_{636} since antisem directed against G_{636} immunoprecipitated SRIF receptor-G_{i,6}-G_{636} complexes, whereas antisera against G_{636} do not. However, G_{i,6} appears to be able to interact with both G_{636} and G_{636} because antisem 8730, which is able to immunoprecipitate both free G_{i,6} as well as a proportion of total G_{i,6} that is bound to SRIF receptors, is able to commoimmoprecipitate both G_{636} and G_{636} immunoreactivity. This is shown by the presence of antisem 8132, 8135, 8129, and BP1-16 immunoireactive material in the 8730 immunoprecipitate (Fig. 16). Therefore, either the association of G_{i,6} with SRIF receptors induces G_{i,6} to selectively couple with G_{636} or the linkage of G_{i,6} with G_{636} promotes G_{i,6} to selectively interact with SRIF receptors.

The finding that SRIF receptors are associated with G_{i,6} suggests that the receptor is also associated with G_{i,6}. At least four different G_{i} (G_{i,1}, G_{i,2}, G_{i,3}, and G_{i,4}) are expressed in mammalian cells (Gautam et al., 1989, 1990). Recently, three peptide-directed antiseras against G_{i,1}, G_{i,2}, or G_{i,3} have been described (Gautam et al., 1990). Using these antiseras, we observed G_{i,2} and G_{i,3} but not G_{i,1} immunoreactivity in solubilized, fractionated proteins from rat brain by immunoblotting (data not shown). This finding is consistent with the results of Gautam et al. (1990), who reported that G_{i,1} is selectively expressed in retina, whereas both G_{i,2} and G_{i,3} are expressed in brain. Analysis of 8730 immunoprecipitates revealed the presence of both G_{i,2} and G_{i,3} immunoreactivity but not G_{i,1} immunoreactivity (Fig. 17). Much greater levels of G_{i,1} immunoreactivity were detected in the 8730 immunoprecipitate than G_{i,1} immunoreactivity. Since 8730 also immunoprecipitated SRIF receptors, these findings suggest that G_{i,2} and/or G_{i,3} may be associated with the solubilized brain SRIF receptor along with G_{636} and G_{i,6}.

The results of the present study indicate that the solubilized brain SRIF receptor associates with G_{i,6}-G_{636} complexes and possibly G_{i,2} and G_{i,3}. Interestingly, agonist binding to the receptor dissipates the \(\beta_{6}\) subunit from the receptor-G_{i,6} complex. This is shown in studies where the solubilized SRIF receptor was treated with 10 \(\mu\)M \[^{3}\]H Trp-8-SRIF for 1 h at room temperature. Under these conditions, the binding of the SRIF analog to the receptor reaches equilibrium (He et al., 1990). After the pretreatment, attempts were made to immunoprecipitate the SRIF receptor with either 8730 (against G_{i,6}) or 8132 (against G_{636}). Following the immunoprecipitation, \[^{3}\]H Trp-8-SRIF was washed away from the immunoprecipitate and a \[^{3}\]H-labeled MK 678 binding assay was performed to detect the presence of high affinity SRIF receptors in the immunoprecipitate. The extensive washing procedure by itself did not alter SRIF receptor-G protein complexes in the immunoprecipitate but was needed in these studies to try to remove any excess \[^{3}\]H Trp-8-SRIF remaining in the immunoprecipitates that could interfere with the \[^{3}\]H-labeled MK 678 binding assay. Antiserum 8730 immunoprecipitated SRIF receptors that had been pretreated with \[^{3}\]H Trp-8-SRIF to a similar extent as receptors that had not been exposed to the SRIF agonist (Fig. 18). These findings suggest that G_{i,6} remains coupled to SRIF receptors following their interaction with the agonist. However, 8132 did not immunoprecipitate SRIF receptors that had been pretreated with agonist, whereas it did immunoprecipitate SRIF receptors not treated with \[^{3}\]H Trp-8-SRIF (Fig. 18). This finding suggests that, under the

![Fig. 18. Agonist binding to the solubilized SRIF receptors selectively uncouples G_{636} but not G_{i,6} from the receptor-G protein complex.](image-url)

To determine the effect of agonist binding to the SRIF receptor on the receptor’s association with G protein subunits, solubilized SRIF receptors were treated for 1 h at room temperature with 10 \(\mu\)M \[^{3}\]H Trp-8-SRIF and then immunoprecipitated with anti-G_{i,6} antisem (8730) or anti-G_{636} antisem (8132). The immunoprecipitates were washed extensively to remove excess \[^{3}\]H Trp-8-SRIF and then analyzed for the presence of high affinity SRIF receptors using the \[^{3}\]H-labeled MK 678 binding assay. Control samples treated in the same manner except that they were exposed to an aliquot of Buffer A rather than \[^{3}\]H Trp-8-SRIF were tested for the ability of antiserum 8730 and 8132 to immunoprecipitate SRIF receptor-G protein complexes. Presented is the mean ± S.E. of specific high affinity \[^{3}\]H-labeled MK 678 binding to the immunoprecipitates in three different experiments. * means that are significantly different (using a paired t test) from preimmune controls.
assay conditions employed, agonist treatment can dissociate $G_{i36}$ from soluble SRIF receptor-G$_{i}$ complex. Therefore, it is possible to have high affinity agonist binding to the soluble SRIF receptor when it is coupled to G$_{i}$ in the absence of $\beta/\gamma$ subunits.

**DISCUSSION**

SRIF receptors are believed to couple to different cellular effector systems via G proteins. This hypothesis is based on the finding that high affinity agonist binding to SRIF receptors is abolished by GTP analogs and that the SRIF inhibition of adenyllylcylase activity and its regulation of voltage-dependent $Ca^{2+}$ and $K^+$ currents is GTP-dependent (Jakobs and Schultz, 1983; Mahy et al., 1988; Yatani et al., 1987; Wang et al., 1989, 1990; Hildebrandt and Kohnken, 1990; Epelbaum, 1986). Pretreatment of a variety of cells with pertussis toxin has been reported to abolish the biological actions of SRIF and uncouples SRIF receptors from G proteins (Reisine et al., 1985; Reisine and Guild, 1985; Mahy et al., 1988; Koch et al., 1985). These findings suggest that SRIF receptors couple to either $G_i$ and/or $G_o$. In the present study, we have attempted to further specify the nature of the pertussis toxin-sensitive G proteins coupled to brain SRIF receptors, as well as SRIF receptors from the pituitary cell line AtT-20.

Several different approaches have been previously employed to identify the G proteins linked to various hormone and neurotransmitter receptors. Cerione et al. (1985) were able to reconstitute purified $\beta$-adrenergic receptors with purified $G_i$. Furthermore, Florio and Sternweis (1989) reconstituted partially purified muscarinic receptors with purified $G_o$. Recently, Sengoles et al. (1990), reported that purified dopamine receptors from bovine pituitary were reconstituted with purified and reconstituted forms of $G_o$. These authors reported that the dopamine receptor preferentially associated with $G_o$. This was the first study showing selectivity of a neurotransmitter or hormone receptor for a subtype of $G_o$.

In the past, reconstitution with purified components was the method used to explore the selectivity of interactions between individual receptors and G proteins (Cerione et al., 1985, 1986; Ohara et al., 1988; Haga et al., 1985, 1986; Kurose et al., 1986; Florio and Sternweis, 1989). The resolution of the structurally similar G protein subtypes proved difficult; therefore, the conclusions from these experiments remain uncertain. Recently, the synthesis of recombinant G proteins in *Escherichia coli* has allowed the isolation of $G_o$ subunits free of contamination by other subunits. Reconstitution experiments utilizing recombinant G proteins provide a clearer understanding of receptor-G protein associations, but these experiments are still limited by the artificial environment created when pure components are placed into phospholipid vesicles. Also, it has not been shown that the reconstituent forms of $G_o$ are processed in a manner that allows for appropriate coupling to receptors.

Another approach to identify G proteins coupled with neurotransmitter receptors is through the use of antisera directed against $\alpha$ subunits of G proteins to immunoprecipitate receptor-G protein complexes. Matesic et al. (1989) recently reported that brain muscarinic receptors coupled to G proteins could be immunoprecipitated with peptide-directed antisera against $G_o$. We have used a similar approach to detect the species of $G_o$ and $G_{i36}$ coupled to SRIF receptors.

In preliminary studies, we had previously reported that an antiserum directed against the C-terminal region of $G_{i36}$, antiserum 8730, could immunoprecipitate the brain SRIF receptor (He et al., 1990). We have confirmed this finding and have shown the specificity of the immunoprecipitation reaction.

The ability of this antiserum to immunoprecipitate the SRIF receptor-G$_{i36}$ complex suggests that coupling of the receptor to G$_{i36}$ is maintained despite the interaction of the antisera with the C-terminal of G$_{i36}$. This finding is of interest since recent studies have shown that the C-terminal region of G$_{i36}$ is involved in coupling to $\beta$-adrenergic receptors (Masters et al., 1988). Furthermore, it is believed that the C-terminal region of G$_{i36}$ is involved in coupling to receptors since this region of the subunit contains the receptor site for pertussis toxin-catalyzed ADP-ribosylation and pertussis toxin causes the uncoupling of G$_{i36}$ from receptors. However, the ability of antiserum 8730 to immunoprecipitate SRIF receptor-G$_{i36}$ complexes suggests that the antiserum does not interfere with G$_{i36}$-SRIF receptor coupling and does not compete with the receptor for sites on G$_{i36}$.

Studies employing antisera selective for the different subtypes of G$_o$ revealed that G$_{i36}$ but not G$_{i336}$ is coupled to brain and AtT-20 cell SRIF receptors. This finding is of interest since Sengoles et al. (1990) recently reported that D2 dopamine receptors from bovine pituitary preferentially associate with G$_{i336}$, and G$_{i36}$ is coupled to brain SRIF receptors from AtT-20 cells. However, they both appeared to uncouple solubilized brain SRIF receptors from G$_{i336}$ since they reduce specific high affinity $^{125}I$-labeled MK 678 binding to the solubilized brain SRIF receptors, but did not immunoprecipitate $^{125}I$-labeled MK 678-binding sites. Since neither antiserum interfered with specific $^{125}I$-labeled MK 678 binding to the solubilized brain SRIF receptor, it is likely that they reduced high affinity $^{125}I$-labeled MK binding by uncoupling the SRIF receptors from G$_{i336}$. The differing effects of G$_{i336}$ selective antisera in uncoupling or immunoprecipitating solubilized brain and AtT-20 cell SRIF receptors support the hypothesis that physical variations exist between brain and AtT-20 cell SRIF receptors. These physical differences might be subtle in nature since purified brain and AtT-20 cell SRIF receptors have a similar size as analyzed by SDS-PAGE (He et al., 1989).

In addition to G$_{i36}$, G$_{i336}$ may also have a role in coupling SRIF receptors to cellular effector systems. Antiserum 2353, which is directed against an internal segment of G$_{i336}$, immunoprecipitated solubilized SRIF receptors from AtT-20 cells, suggesting that G$_{i336}$ is coupled to SRIF receptors in these cells. Furthermore, its ability to immunoprecipitate AtT-20 cell SRIF receptors was additive with antiserum 8730, suggesting that the antisera interact with and immunoprecipitate different populations of AtT-20 cell SRIF receptor-G$_{i336}$ complexes. In contrast, 2353 did not immunoprecipitate brain SRIF receptors despite being able to immunoprecipitate soluble PTX-sensitive G$_o$, from brain. This finding suggests that either brain SRIF receptors are not coupled to G$_{i336}$ or that the antiserum 2353 epitope on G$_{i336}$ is not accessible when G$_{i336}$ is associated with the brain SRIF receptor. In either case, these findings, as well as the differing effects of G$_{i336}$ antisera to immunoprecipitate or uncouple brain and AtT-20 cell SRIF receptor-G protein complexes suggest that differences exist in the coupling of brain and AtT-20 cell SRIF receptors with G proteins. The functional consequences of such differences in coupling remain to be elucidated.
SRIF receptors appear to associate with the \( G_a - G_y \) complex. This is indicated by the ability of antisera directed against \( \beta \) subunits of G proteins to immunoprecipitate solubilized brain SRIF receptors and the appearance of \( G_y \) and \( G_y \) subunit immunoreactivity in the SRIF receptor immunoprecipitates. Interestingly, using a similar approach as employed in the present study, Matesic et al. (1989) were able to immunoprecipitate muscarinic receptor-G protein complexes with antisera directed against a common region of \( G_y \) and \( G_y \). Matesic et al. (1989) reported that muscarinic receptors did not coinmunoprecipitate with the \( \beta \) subunit of G proteins since screening of the muscarinic receptor immunoprecipitates with antisera against \( G_y \) did not detect immunoreactive material. Furthermore, antisera directed against G protein \( \beta \) subunits did not immunoprecipitate solubilized muscarinic receptor (Matesic et al., 1989). These authors reported that the presence of agonist was needed during the solubilization procedure in order for muscarinic receptors to remain coupled to G proteins. In contrast, brain SRIF receptors can be solubilized in the absence of agonist (He et al., 1990). The presence of agonist does not disrupt the coupling of brain SRIF receptor with G, but does cause the release of \( G_y \). These latter results indicate that our findings are consistent with those of Matesic et al. (1989) and suggest that agonist binding to some neurotransmitter receptors dissociates \( G_y \) from the receptor while maintaining coupling to the \( \alpha \) subunit. Interestingly, Florio and Sternweis (1989) have reported that both purified \( G_y \) and \( \beta / \gamma \) subunits were needed to reconstitute high affinity agonist binding to the partially purified brain muscarinic receptor, suggesting that, as with the brain SRIF receptor, muscarinic receptors are complexed with \( \alpha \), as well as \( \beta \) subunits of G proteins. These findings taken together may indicate that the \( G_y \) complex is needed for the association of \( G_y \) with receptors but that, upon agonist binding to the receptor, in the absence of GTP, the \( G_y \) can dissociate from the complex while \( G_y \) remains coupled.

Although it has been suggested that many different neurotransmitter and hormone receptors couple to \( \alpha \) subunits of G proteins, relatively little information is available concerning the interaction of \( \beta \) subunits with receptors. In addition to the findings of Florio and Sternweis (1989), Fung (1983) reported that \( \beta \) subunits of transfducin couple with rhodopsin. Furthermore, Blumer and Thorner (1990) recently reported that \( \beta \) subunits of yeast G proteins are needed for the maintenance of \( \alpha \)-factor mating receptors in a high affinity agonist state. The mechanisms by which \( \beta \) subunits maintain receptors in a high affinity agonist state are not known. They may be necessary for anchoring \( \alpha \) subunits to cell membranes so the \( \alpha \) subunit can couple with receptors as proposed by Sternweis (1986) or they may be necessary for maintaining \( \alpha \) subunits in a conformation that promotes coupling to receptors. A role for anchoring \( \alpha \) subunits to cell membranes is not supported by the results of several direct studies. Hofmann and Thorner (1990) showed that \( \beta \) subunits of yeast G proteins are not needed for \( \alpha \) subunit association with yeast cell membranes and it has also been reported that \( \beta \) subunits are not needed to anchor the \( \alpha \) subunits of transducin to the cell membrane for coupling to rhodopsin (Fung, 1983). Therefore, it is more likely that \( \beta \) subunits maintain \( \alpha \) subunits in a conformation that facilitates receptor coupling.

At least two forms of \( \beta \) subunits are expressed in mammalian cells that are encoded by different genes but which share over 90% sequence similarities (Amatruda et al., 1988). Antiserum 8132 used in the present study was generated against a peptide fragment found in the \( \beta_3 \) subunit and selectively interacts with \( G_{56} \). Antiserum 8135 is directed against both \( G_{56} \) and \( G_{56} \). In contrast, antisera 8129 is directed against a unique fragment of the \( \beta_5 \) subunit. Antisera 8129, 8132, and 8135 are able to immunoprecipitate \( \beta \) subunits of G proteins. However, only antisera 8132 and 8135 immunoprecipitated solubilized SRIF receptors. Antisera 8132 and 8132 are directed to the same region of the \( \beta \) subunit, which differs in amino acid sequence in \( G_{56} \) and \( G_{56} \). Since they are directed against analogous epitopes, their differing abilities to immunoprecipitate SRIF receptors are unlikely to be due to differences in accessibility of the epitopes but more likely to result from SRIF receptors preferentially coupling to \( G_{56} \). Furthermore, antisera BP1-16, which is selectively directed against \( G_{56} \), did not immunoprecipitate SRIF receptors, further indicating that SRIF receptors are preferentially coupled to \( G_{56} \). The \( G_{56} \) subunit appears to associate with the SRIF receptor and \( G_{56} \). This is indicated by the ability of \( G_{56} \)-directed antisera to immunoprecipitate pertussis toxin-sensitive \( \alpha \) subunits as well as the SRIF receptor. Furthermore, antisera directed against \( G_{56} \) can immunoprecipitate \( G_y \) immunoreactivity. Interestingly, antisera directed against \( G_{56} \), immunoprecipitated both \( G_{56} \) and \( G_{56} \) immunoreactivity from solubilized rat brain. These findings indicate that \( G_{56} \) may be able to couple with both \( G_{56} \) and \( G_{56} \), but that the \( G_{56} \) that is associated with solubilized SRIF receptors preferentially interacts with \( G_{56} \). Conceivably, the interaction with the SRIF receptor induces in \( G_{56} \), a conformation such that it selectively couples with \( G_{56} \). Alternatively, \( G_{56} \) may induce in \( G_{56} \), a conformation that promotes its coupling with SRIF receptors. These findings suggest that specificity exists in the interaction of the G protein subunits with each other and the SRIF receptor.

The \( \beta \) subunits of G proteins are known to be closely associated with \( \gamma \) subunits. \( \gamma \) subunits may serve an important role in promoting coupling of \( \beta \) subunits to \( \alpha \) subunits and receptors, as suggested by the findings of Blumer and Thorner (1990). Recent studies have reported that at least four different \( G_y \) subunits are expressed in mammals. The finding in both antisera 8730 and antisera 8132 immunoprecipitates that PTX catalyzed \( [32P] \)ADP ribosylation of 41-kDa proteins indicates that one or more of these \( \gamma \) subunits are present in the immunoprecipitates and are associated with SRIF receptors since PTX-catalyzed ADP-ribosylation requires the association of both \( \beta \) and \( \gamma \) subunits with \( G_{56} \) (Huff and Neer, 1996; Gao et al., 1987a, 1987b). In agreement with the findings of Gautam et al. (1990), it was observed that \( G_{56} \), \( G_{56} \) immunoactivity is expressed in solubilized brain proteins but not \( G_{56} \), immunoactivity, which is selectively expressed in retina. Analysis of antisera 8730 immunoprecipitates, which contain the SRIF receptor-\( G_{56} \)-\( G_{56} \) complex, for the presence of \( G_{56} \) revealed \( G_{56} \) immunoactivity and a small but detectable level of \( G_{56} \) immunoactivity. Differences in the levels of \( G_{56} \) and \( G_{56} \) that were immunoprecipitated could be due to a preferential association of \( G_{56} \) with SRIF receptor-\( G_{56} \)-\( G_{56} \) complexes. Studies are in progress to determine whether the \( \gamma \) subunit-directed antisera can specifically immunoprecipitate \( \gamma \) subunits as well as SRIF receptor-\( G_{56} \)-\( G_{56} \) complexes. Such studies are necessary to clearly establish whether \( G_{56} \) and/or \( G_{56} \) are associated with brain SRIF receptors.

In conclusion, the present study provides the first direct evidence that brain SRIF receptors are specifically coupled to \( G_{56} \)-\( G_{56} \) complexes. The immunoprecipitation approach employed in this study has proven useful in identifying the array of G protein subtypes coupled to SRIF receptors. Such information could allow an elucidation of the molecular mechanisms underlying the diverse physiological actions induced by
SRIF in the central nervous system.

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Materials and Methods

Materials - For these studies, frozen rat brains were obtained from Pel-Freeze. ACH501 utropin was obtained from Zeb Biochemica and Centiprep 30 was purchased from Amicon. MK 576 was obtained from Dr. H. Gruber at Manchester (UK). The solubilization buffer (Raynor and Reisine, 1989) contained as previously described (He et al., 1989, 1990) except that 1.5 M of the following additions were included: 20 mM glycerol, 0.5 mM aspartylglycine, 0.01 mM propionamidc acid in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 5 mM MgCl2, 100 μg leupeptin, 2 μg pepstatin and 200 μg bacitracin. The sample was homogenized with a Potter-Elvehjem (stirring for 15 sec) and then placed on ice for 15 min with constant stirring. The sample was centrifuged at 100,000 x g for 45 min at 4°C. The soluble supernatant was collected and placed on ice for 15 min and then centrifuged at 100,000 x g for 45 min at 4°C. The supernatant was collected and placed on ice for 15 min with constant stirring. The sample was centrifuged at 100,000 x g for 45 min at 4°C. The supernatant was then dialyzed against a 10 mM Tris-HCl (pH 7.8) buffer containing 50 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 100 μg leupeptin, 2 μg pepstatin and 200 μg bacitracin (buffer B). The supernatant was then dialyzed against a 10 mM Tris-HCl (pH 7.8) containing 50 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, 100 μg leupeptin, 2 μg pepstatin and 200 μg bacitracin (buffer B). The supernatant was used to solubilize SRIF receptors from rat brain. The supernatant was concentrated by filtration and counted using a 1000 μM [35S]SRIF binding assay. The presence of solubilized SRIF receptors was detected using the [125I]-MK 678 assay as described previously (Raynor and Reisine, 1989; Goldsmith et al., 1989; Antiserum 8127 is directed against a C-terminal region of G4a and selectively detects G4a-like receptors. In a third protocol, solubilized SRIF receptors were exposed to IgG antibodies to determine whether the SRIF receptor is associated with G4a or G4d. The samples were then placed on ice for 15 min followed by the addition of 10 μM [35S]-SRIF. Next, the samples were incubated for 3 hr at room temperature on a rotator and immunoprecipitated with G4a-directed antibodies as described in Materials and Methods. The immunoprecipitated proteins were subjected to SDS-PAGE and autoradiography to visualize the presence of G4a bound SRIF receptors. Specificity of the [125I]-MK 678 binding to immunoprecipitated SRIF receptors for these studies, solubilized SRIF receptors coupled G4a proteins were immunoprecipitated with anti-G4a antibodies. The supernatant was removed and the precipitate was washed twice with buffer A and then washed twice again with a buffer containing 10 mM HCl and 0.05% Triton X-100, and the precipitate was washed with 100 μM [35S]-SRIF. The reaction was terminated with cold buffer as described previously and the samples subjected to vacuum filtration and counted in a gamma counter.

125I-Methionine labeling of proteins in AtT-20 cells - For these studies, AtT-20 cells (approximately 10 million) grown and subcultured as previously described (He et al., 1989, 1990) were incubated with 50 μl of DMEM (dulbecco's modified Eagle medium) containing 45 μM [35S]-methionine, 45 μM [35S]-cysteine and 45 μM [35S]-cysteine, overnight at 37°C in a humidified cell incubator with 10% CO2. Cells were washed once with buffer A and then solubilized as previously described (He et al., 1990). The solubilized proteins were immunoprecipitated with G4a protein directed antibodies, after which the supernatant was removed and the precipitate washed twice. Protein G-Sepharose was then washed twice with buffer B and then added to the supernatant. The reaction was terminated with cold buffer as described previously and the samples subjected to vacuum filtration and counted in a gamma counter.
The reaction mixture was constantly shaken on a rotator. A 1:120 dilution of either antiserum 8730 or 8132 was added to the sample which was rotated at 4°C for 4 h. 100 μl of 50% (w/v) protein A sepharose was added and mixture incubated overnight under the same conditions as described above. The sample was centrifuged at 10,000 rpm for 2 min in an Eppendorf microcentrifuge and the supernatant removed. The immunoprecipitate was resuspended in buffer A and incubated on a rotator at room temperature for 15 min. The sample was then centrifuged again at 10,000 rpm for 2 min and the supernatant removed. The immunoprecipitate was resuspended and incubated in the same manner as described above. This cycle of washing was repeated one more time so that the immunoprecipitate was washed a total of 3 times to completely remove free D-TRP-SRI F from the immunoprecipitate and then the immunoprecipitate was assayed for the presence of SRI F receptors using the [125I] MK 678 binding assay. Similar studies were performed on samples not pretreated with D-TRP-SRIF as controls.

**Figure 1**: Presence of G_{15} and G_{16} immunoreactivity in solubilized, fractionated rat brain proteins.

Solubilized and subfractionated rat brain proteins were subjected to 10% SDS-PAGE and Western blotting using antisera 8730 (anti-G_{15} antiserum), 2953 (anti-G_{16} antiserum), 3646 (anti-G_{15/16} antiserum), 1521 (anti-G_{15} antiserum), and 1518 (anti-G_{16} antiserum). All antisera were used at dilutions of 1:250. Arrows point to proteins migrating with similar electrophoretic mobility as purified G_{15/16}.

**Figure 2**: Antiserum 8730 is able to immunoprecipitate G_{15} from rat brain.

To determine whether 8730 is able to immunoprecipitate G_{15} from rat brain, solubilized rat brain proteins were reacted with 8730 (1:20 final dilution) and immunoprecipitated with protein A sepharose beads. The immunoprecipitate was treated with pertussis toxin and 32P-NAD to 32P-GDP-ribosylates G_{15}. The proteins were then subjected to 10% SDS-PAGE and autoradiography. The autoradiogram is presented in part A and compares the amount of 32P-GDP-ribosylated 41 kDa protein in preimmune (PI) and antisera 8730 (8730) immunoprecipitates. Equal amounts of protein were added to each lane. To further establish that 8730 can immunoprecipitate G_{15}, solubilized rat brain proteins were first reacted with 32P-NAD and pertussis toxin and immunoprecipitated with 8730 or exposed to preimmune controls (PI) (Part B). The immunoprecipitates were subjected to 10% SDS-PAGE and autoradiography. Size markers for the gels are presented to the left of each autoradiogram.

**Figure 3**: Specificity of [125I] MK 678 binding to SRIF receptors in 8730 immunoprecipitates.

To characterize the binding of [125I] MK 678 binding sites in 8730 immunoprecipitates, soluble rat brain SRIF receptors were immunoprecipitated with 8730. The immunoprecipitates were washed extensively and then subjected to the [125I] MK 678 binding assay. The ability of varying concentrations of D-TRP-SRIF (A) to inhibit [125I] MK 678 binding to immunoprecipitated SRIF receptors is presented in (A) as in the inability of a high concentration of the inactive SRIF analog somatostatin 28 [114] (B). The IC_{50} value for D-TRP-SRIF's inhibition of [125I] MK 678 binding is 4 ± 2 nM which is similar to its ability to inhibit [125I] MK 678 binding to solubilized SRIF receptors as previously reported (He et al., 1990). To determine whether the immunoprecipitated SRIF receptor remains coupled to D proteins, the ability of the GTP analog GppNHp (A) or ATP (B) to inhibit specific [125I] MK 678 was examined and those results are presented in (B). These are the averages results of three different experiments.

**Figure 4**: Anti-G_{15} subtype antiserum immunoprecipitate G_{15} from rat brain.

Rat brain proteins were solubilized, subfractionated and immunoprecipitated with antisera 8730 (lane 1), 2466 (lane 2), 1521 (lane 1), 1518 (lane 4), Gd (lane 5) or preimmune serum (PI) at final dilutions of 1:20. The immunoprecipitates were washed and incubated with PTK and 32P-NAD. The 32P-GDP-ribosylated G_{15}'s were subjected to 10% SDS-PAGE and visualized by autoradiography. Arrow points to proteins migrating with similar mobility as purified G_{15/16}.

**Figure 5**: Anti-G_{16} subtype antiserum immunoprecipitate G_{16} from rat brain.

Rat brain proteins were solubilized, subfractionated and immunoprecipitated with either antisera 8730 or 2353 (both at 1:20 dilution) or preimmune sera (PI). The immunoprecipitates were treated with PTK and 32P-NAD and the 32P-GDP-ribosylated G_{16}'s were subjected to 10% SDS-PAGE and visualized by autoradiography. Arrow points to proteins migrating with similar mobility as purified G_{15/16}.

**Figure 6**: Specificity of [125I] MK 678 binding to SRIF receptors in 8730 immunoprecipitates.

To determine whether 8730 may immunoprecipitate with G_{16}, the mouse anterior pituitary cell line ACT-20 was metabolically labeled with 2-3H-methionine, the proteins solubilized under non-denaturing conditions and immunoprecipitated with 8730. The immunoprecipitate was subjected to 10% SDS-PAGE and autoradiography. Two major protein bands were detected with one migrating with the same electrophoretic mobility as G_{16} (a) and the other migrating with the same electrophoretic mobility as G_{15} (b). B: To further test whether 8730 immunoprecipitated G_{16} along with G_{15}, 8730 immunoprecipitates from rat brain were subjected to Western blotting and screened with 8132, which selectively detects G_{16} immunoreactivity. Antiserum 8132 immunoreactivity was detected in the 8730 immunoprecipitate but not in preimmune controls (PI).

**Figure 7**: Specificity of [125I] MK 678 binding to SRIF receptors in 8730 immunoprecipitates.

To determine whether the immunoprecipitated SRIF receptor remains coupled to D proteins, the ability of the GTP analog GppNHp (A) or ATP (B) to inhibit specific [125I] MK 678 was examined and those results are presented in (B). These are the averages results of three different experiments.
**Figure 13:** Detection of alpha and beta subunits of solubilized G proteins from rat brain by Western blotting.

Solubilized, fractionated rat brain proteins were subjected to 10% SDS-PAGE and Western blotting using preimmune sera (lane 1), 8730 (lane 2), 8122 (lane 3), 8129 (lane 4), BPI-16 (lane 5) and 8125 (lane 6). Equal amounts of protein were added to each lane. Arrows point to either the migration of 40-41 kDa proteins (a) or 35-36 kDa proteins (b).

**Figure 16:** Anti-Giα antisera can coimmunoprecipitate Gα36 and Gα15 immunoreactivity.

To determine whether both Gα36 and Gα15 coimmunoprecipitate with Giα, solubilized rat Giα was immunoprecipitated with 8730 and the immunoprecipitate was subjected to Western blotting and screened with the anti-Giα antisera 8112 (lane 1) and antisera 8115 which recognizes both Gα36 and Gα33 (lane 2) or with anti-Gα15 antisera 8129 (lane 3) and BPI-16 (lane 4). Preimmune controls (PI) are also presented. Immunoreactive material comigrated with purified Gα subunit and is indicated by the arrow B.

**Figure 17:** Coimmunoprecipitation of Gα immunoreactivity using anti-Giα antisera.

To determine whether Gα subunits coimmunoprecipitate with Giα, solubilized rat brain proteins were immunoprecipitated with 8730 (anti-Giα) and the immunoprecipitates were subjected to 15% SDS-PAGE, Western blotting and screened with antisera directed against Gα1 (PI), Gα2 (PI) and Gα3 (PI).