Characterization of the Carboxyl-terminal Domain of the Rat Glucose-dependent Insulinotropic Polypeptide (GIP) Receptor

A ROLE FOR SERINES 426 AND 427 IN REGULATING THE RATE OF INTERNALIZATION

(Received for publication, March 15, 1999, and in revised form, June 3, 1999)

Michael B. Wheeler‡, Richard W. Gelling§, Simon A. Hinke‡, Ba Tu‡, Raymond A. Pederson§, Francis Lynn§, Jan Ehses§, and Christopher H. S. McIntosh§

From the ‡Departments of Medicine and Physiology, University of Toronto, Toronto, Ontario MSS 1A8, Canada and the §Department of Physiology, University of British Columbia, Vancouver V6T 1Z3, British Columbia, Canada

Glucose-dependent insulinotropic polypeptide (GIP) is a gastrointestinal hormone involved in the regulation of insulin secretion. In non-insulin-dependent diabetes mellitus, insulin responses to GIP are blunted, possibly due to altered signal transduction or reduced receptor number. Site-directed mutagenesis was used to construct truncated GIP receptors to study the importance of the carboxyl-terminal tail (CT) in binding, signaling, and receptor internalization. Receptors truncated at amino acids 425, 418, and 405, expressed in COS-7 or CHO-K1 cells, exhibited similar binding to wild type receptors. GIP-dependent cAMP production with the 405 mutant was decreased in COS-7 cells. Maximal cAMP production in CHO-K1 cells was reduced with all truncated forms. Binding was undetectable with a receptor truncated at amino acid 400; increasing tail length by adding 5 alanines restored binding and signaling. Mutants produced by alanine scanning of residues 394–401, adjacent to transmembrane domain 7, were all functional. CT truncation by 30 or more amino acids, mutation of serines 426/427, singly or combined, or complete CT serine knockout all reduced receptor internalization rate. The majority of the GIP receptor CT is therefore not required for signaling, a minimum chain length of ~405 amino acids is needed for receptor expression, and serines 426 and 427 are important for regulating rate of receptor internalization.

Incretins are peptide hormones released from the gastrointestinal tract into the circulation in response to a meal that potentiate glucose-stimulated insulin secretion. There are two established incretins: gastric inhibitory polypeptide/glucose-dependent insulinotropic polypeptide (GIP) and forms of glucagon-like peptide-1 (GLP-1). In non-insulin-dependent diabetes mellitus (NIDDM), the incretin effect following oral glucose is reduced or absent (1, 2), and the insulin response to intravenously administered GIP, but not GLP-1, has been reported to be severely blunted (2, 3). Possible explanations for a decreased responsiveness to GIP include a defective signal-transduction system and a reduction in the number of functional pancreatic islet receptors due to altered expression, mutation, or degree of desensitization and/or internalization. To elucidate which of these could be involved in reduced responsiveness, it is necessary to develop a greater understanding of the functional roles played by the different structural components of the GIP receptor.

The receptor for GIP (4–6) is a member of the seven transmembrane G-protein-coupled secretin-vasoactive intestinal peptide (VIP) family, which includes the receptors for glucagon (7), glucagon-like peptide-1 (8), secretin (9), VIP (10), parathyroid hormone/parathyroid hormone-related peptide (PTH/PTH-RP) (11), and calcitonin (12). The GIP receptor has been shown to stimulate adenylyl cyclase in pancreatic islet α- and β-cells (13), islet tumor cells (14–16), and cell lines transfected with pancreatic GIP receptor cDNAs (4–6). However little is known regarding the specific components of the GIP receptor, which are important for G-protein coupling or regulation of desensitization and internalization.

Extensive mutagenesis and chimeric receptor studies on the β-adrenergic and cholinergic receptors have indicated that all of the intracellular loops of the seven transmembrane class of receptors can play a role in G-protein binding, although the NH₂ and COOH termini of the third intracellular loop are considered to be of primary importance for both G-protein binding and conferring specificity of action (17–20). In recent studies on the GLP-1 receptor, which is very closely related to that for GIP, sequences in the proximal and distal portions of third intracellular loop were shown to be required for coupling to Gs and adenyl cyclase (21, 22). In contrast to the extensive studies on the role of the intracellular loops in G-protein-coupled receptor function, fewer functional studies on the role of the COOH-terminal tail (CT) have been performed. This region has been implicated in receptor desensitization and endocytosis (23–26), and the NH₂-terminal region of the β₂-adrenergic receptor CT has been shown to be important for G-protein coupling (17). Additionally, the CT has been suggested to play a role in routing transport of receptors to the plasma membrane (27, 28) and restricting lateral movement within this membrane (27, 28). Studies on COOH-terminally truncated members of the secretin-VIP family of receptors showed that reduction in the length of the CT resulted in increased binding affinity of the PTH/PTH-RP family of receptors, whereas truncation increased...
Characterization of the GIP Receptor COOH-terminal Domain

maximal cyclic (c)AMP production with the PTH/PTH-RP receptor (29) but decreased production with the calcitonin receptor (30). Recently phosphorylation of the CT of glucagon (26) and GLP-1 (32) receptors has been shown to be required for both receptor desensitization and sequestration and, in the case of the GLP-1 receptor, a series of three serine doublets exerts a dose-dependent influence on both events (32).

The current studies were directed at defining the functional importance of the CT of the rat GIP receptor. Carboxy-termi- nally truncated mutants of the receptor, produced by site-determined mutagenesis and expressed in COS-7 and CHO-K1 cells, were studied with a view to determining the effect of such mutations on ligand binding, stimulation of G-protein-coupling to adenyl cyclase, and ligand-induced internalization. In ad- dition, studies were made on the effects of partially replacing the CT of the shortest, inactive, mutant form with a poly- alanine tail, and of mutating individual amino acids within the proximal CT and all serines throughout the CT. It is concluded that the majority of the GIP receptor CT is not required for signaling, a minimum length of the tail, rather than the specific constituent amino acids, is important for transport and insertion of the receptor into the plasma membrane, and that Ser-426 and Ser-427 are involved in receptor internalization.

EXPERIMENTAL PROCEDURES

Preparation of Rat GIP Receptor Truncation Mutants—A polymerase chain reaction strategy was used to generate truncation mutants, uti- lizing the rat receptor cDNA (5) in the vector pCNA 3 (Invitrogen Co., San Diego, CA) as template. The 5′-sense oligonucleotide primer corre- sponded to coding nucleotides –3 to +19  (5′-AGGATGCCCCTGGG-GGCTTTGTC–3′). Five′-antisense oligonucleotide primers were de- signed such that a stop codon (underlined) was introduced at desired positions to generate the mutants GIP-R-425, GIP-R-418, GIP-R-405 and GIP-R-400. Additionally, to examine the effect of nonspecific se- quence extension of the COOH-terminal tail, primers were designed to extend residues 400 and 396 with poly-alanine tails of 5 and 9 residues. These are designated GIP-R-405A5 and GIP-R-396A9.

To examine more exactly the contribution of the most proximal residues in the CT for receptor expression and G-protein coupling to adenyl cyclase, double-stranded site mutagenesis (Chame- leon, Stratagene, La Jolla, CA) was used to substitute individually the 8 residues (394–401) extending from the predicted seventh transmem- brane domain, with alanine residues. The resulting constructs were designated GIP-R-405A5 and GIP-R-396A9.

The respective primers corresponded to coding nucleotide bases: 1255–1276, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-425); 1255–1236, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-418); 1236–1216, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-405); and 1216–1196, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-400).}

Characterization of the GIP Receptor COOH-terminal Domain

maximal cyclic (c)AMP production with the PTH/PTH-RP receptor (29) but decreased production with the calcitonin receptor (30). Recently phosphorylation of the CT of glucagon (26) and GLP-1 (32) receptors has been shown to be required for both receptor desensitization and sequestration and, in the case of the GLP-1 receptor, a series of three serine doublets exerts a dose-dependent influence on both events (32).

The current studies were directed at defining the functional importance of the CT of the rat GIP receptor. Carboxy-termi- nally truncated mutants of the receptor, produced by site-determined mutagenesis and expressed in COS-7 and CHO-K1 cells, were studied with a view to determining the effect of such mutations on ligand binding, stimulation of G-protein-coupling to adenyl cyclase, and ligand-induced internalization. In ad- dition, studies were made on the effects of partially replacing the CT of the shortest, inactive, mutant form with a poly- alanine tail, and of mutating individual amino acids within the proximal CT and all serines throughout the CT. It is concluded that the majority of the GIP receptor CT is not required for signaling, a minimum length of the tail, rather than the specific constituent amino acids, is important for transport and insertion of the receptor into the plasma membrane, and that Ser-426 and Ser-427 are involved in receptor internalization.

EXPERIMENTAL PROCEDURES

Preparation of Rat GIP Receptor Truncation Mutants—A polymerase chain reaction strategy was used to generate truncation mutants, uti- lizing the rat receptor cDNA (5) in the vector pCNA 3 (Invitrogen Co., San Diego, CA) as template. The 5′-sense oligonucleotide primer corre- sponded to coding nucleotides –3 to +19  (5′-AGGATGCCCCTGGG-GGCTTTGTC–3′). Five′-antisense oligonucleotide primers were de- signed such that a stop codon (underlined) was introduced at desired positions to generate the mutants GIP-R-425, GIP-R-418, GIP-R-405 and GIP-R-400. Additionally, to examine the effect of nonspecific se- quence extension of the COOH-terminal tail, primers were designed to extend residues 400 and 396 with poly-alanine tails of 5 and 9 residues. These are designated GIP-R-405A5 and GIP-R-396A9.

The respective primers corresponded to coding nucleotide bases: 1255–1276, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-425); 1255–1236, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-418); 1236–1216, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-405); and 1216–1196, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-400).

To examine more exactly the contribution of the most proximal residues in the CT for receptor expression and G-protein coupling to adenyl cyclase, double-stranded site mutagenesis (Chame- leon, Stratagene, La Jolla, CA) was used to substitute individually the 8 residues (394–401) extending from the predicted seventh transmem- brane domain, with alanine residues. The resulting constructs were designated GIP-R-405A5 and GIP-R-396A9.

The respective primers corresponded to coding nucleotide bases: 1255–1276, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-425); 1255–1236, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-418); 1236–1216, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-405); and 1216–1196, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-400).

To examine more exactly the contribution of the most proximal residues in the CT for receptor expression and G-protein coupling to adenyl cyclase, double-stranded site mutagenesis (Chame- leon, Stratagene, La Jolla, CA) was used to substitute individually the 8 residues (394–401) extending from the predicted seventh transmem- brane domain, with alanine residues. The resulting constructs were designated GIP-R-405A5 and GIP-R-396A9.

The respective primers corresponded to coding nucleotide bases: 1255–1276, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-425); 1255–1236, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-418); 1236–1216, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-405); and 1216–1196, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-400).

To examine more exactly the contribution of the most proximal residues in the CT for receptor expression and G-protein coupling to adenyl cyclase, double-stranded site mutagenesis (Chame- leon, Stratagene, La Jolla, CA) was used to substitute individually the 8 residues (394–401) extending from the predicted seventh transmem- brane domain, with alanine residues. The resulting constructs were designated GIP-R-405A5 and GIP-R-396A9.

The respective primers corresponded to coding nucleotide bases: 1255–1276, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-425); 1255–1236, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-418); 1236–1216, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-405); and 1216–1196, 5′-CTATAGCGACGACGGGAGGCTGGCTG-3′ (GIP-R-400).
ence of 1 μM GIP. Binding data were then expressed as percentage loss of surface receptors.

Data Analysis—Data are expressed as means ± S.E., with the number of individual experiments presented in brackets. Receptor binding and cAMP data were analyzed using the nonlinear regression analysis program PRISM (GraphPad, San Diego, CA). All data were tested for significance using analysis of variance analysis and, when appropriate, by Dunnet’s test for multiple comparisons as a post-hoc test for significance.

RESULTS

Initially, a number of truncated forms of the GIP receptor were constructed to examine the effect of progressive CT deletion on receptor expression and G-protein activation. Receptors truncated at residues 405, 418, and 425 exhibited high affinity binding in competition binding experiments, similar to that seen in cells expressing the 455-amino acid wild type receptor (GIP-R-455) in both transient studies with COS-7 cells (Figs. 1 and 2A) and in stable cell lines generated in CHO-K1 cells (Fig. 2B, Table I). Receptor expression levels, as determined from B max values, indicated that GIP-R-425 was expressed at least as efficiently as the wild type receptor in both cell systems (121 ± 41% in COS-7; 126 ± 34% in CHO-K1) (Table I, Fig. 1). However, cells expressing GIP-R-418 exhibited 82 ± 17% (COS-7)/74 ± 16% (CHO-K1) and those expressing GIP-R-405 36 ± 7% (COS-7)/29 ± 3% (CHO-K1) of the maximal binding obtained with the wild type receptor. This indicated that these truncated receptors were not as efficiently expressed as the longer forms of the receptor at the plasma membrane level. When the receptor was truncated at amino acid 400 (GIP-R-400) no detectable 125I-GIP binding was observed. Cells expressing GIP-R-425, GIP-R-418, and GIP-R-405 displayed similar binding affinities to that of the full-length receptor in both COS-7 and CHO-K1 cells (Figs. 1 and 2; Table I).

The lack of detectable binding with GIP-R-400 suggested that either specific residues that had been deleted or a minimum CT tail length were important for receptor expression or binding. To determine whether the length of the tail was the determining factor, a sixth construct was prepared consisting of GIP-R-400 to which 5 alanine residues were added (GIP-R-400A5) to produce a similar chain length to GIP-R-405. Extension of the CT, with these nonspecific amino acid residues, restored binding in both transient and stable expression systems to levels approximating 50% of those seen for GIP-R-405 (Figs. 1 and 2; Table I). Although the level of expression of this receptor was low, compared with that of the wild type receptor, both COS-7 and CHO-K1 cells expressing GIP-R-400A5 specifically bound 125I-GIP with similar affinity to the wild type receptor (Figs. 1 and 2; Table I).

To examine the role of the more proximal residues of the CT, two further constructions were designed, one with residues 397–405 replaced with 9 alanine residues (GIP-R-396A9), and the other with residues 397–400 deleted (GIP-R-DQSEI). Neither of these constructs, when transfected into COS-7 or CHO-K1 cells, displayed 125I-GIP binding in competition experiments (Fig. 1; Table I). These data suggested that the CT tail

Fig. 1. Examination of GIP-R truncation mutations for binding and cAMP stimulation in COS-7 cells. Receptor mutations shown diagrammatically on the left were examined for their ability to bind GIP in binding displacement assays (summarized as IC 50 values and relative receptor expression (%B max of GIP-R-455)), and for basal and GIP-stimulated (10 nM) cAMP accumulation. Mean ± S.E.; n = three independent experiments. Significant differences from GIP-R-455 in B max (a) and cyclic AMP production (b) p < 0.05. (nd, not determined; †, nondetectable).

Fig. 2. Binding analysis of GIP-R truncation mutants in COS-7 and CHO-K1 cells. Binding of the GIP-R mutations were examined for 125I-GIP binding in COS-7 cells expressing truncated receptor mutants (A) and in CHO-K1 cells stably expressing mutants (B). A summary of the results in CHO-K1 cells and statistical analyses is shown in Table I. COS-7 cell statistics included in Fig. 1 are mean ± S.E.; n ≥ 3. ■, GIP-R-455; △, GIP-R-400A5; ⊳, GIP-R-405; ○, GIP-R-418; ○, GIP-R-425.
Characterization of the GIP Receptor COOH-terminal Domain

Table I

Summary of binding data and cyclic AMP responses with carboxyl-terminal tail-truncated forms of the GIP receptor expressed in CHO-K1 cells

| Construct   | 125I-GIP Binding | cAMP production | cAMP production |
|-------------|------------------|-----------------|-----------------|
|             | IC50 (nM)       | Bmax % wild type | EC50 pmol/well | EC50 pmol/well |
| GIP-R-455   | 2.24 ± 0.35     | 100             | 100             | 69 ± 27        |
| GIP-R-425   | 2.73 ± 0.23     | 126 ± 34        | 55 ± 13**       | 27 ± 20        |
| GIP-R-418   | 2.85 ± 0.27     | 74 ± 16         | 23 ± 5**        | 15 ± 1*        |
| GIP-R-405   | 2.19 ± 0.12     | 29 ± 2.9*       | 8 ± 2**         | 11 ± 1*        |
| GIP-R-400   | ND**            | —               | ND**            | ND             |
| GIP-R-400A5| 3.80 ± 0.21*    | 12 ± 1.1*       | 31 ± 7**        | 1,163 ± 320**  |
| GIP-R-396A9| ND              | —               | ND              | ND             |
| GIP-R-ΔQSEI| ND              | —               | ND              | ND             |

*ND, not determined.
**—, nondetectable.

Fig. 3. Analysis of GIP-stimulated cAMP production in CHO-K1 cells expressing carboxyl-terminal truncated mutants.

A concentration-dependent analysis of GIP-stimulated cAMP formation was generated to examine each of the amino acids 394–401 within the proximal CT with respect to GIP binding. Data represent mean ± S.E.; n = 6; *p < 0.05 or **p < 0.01 compared with GIP-R-455.

length was important for efficient expression, but that specific residue(s) within the region 397–400 may also be functionally important.

To assess the influence of the CT on receptor coupling to G-proteins and adenylyl cyclase, cAMP responses to GIP were determined in transfected COS-7 and CHO-K1 cells. Maximal cAMP production was greatest with GIP-R-455 in both systems. In COS-7 cells there were no significant differences in cAMP accumulation between the truncated receptor mutants GIP-R-425 (100 ± 6.5 pmol/well) or GIP-R-418 (93.4 ± 14.6 pmol/well) and the GIP-R-455 expressing cell line (104 ± 17 pmol/well) (Fig. 1, p > 0.05, n = 3). However, COS-7 cells expressing GIP-R-405 displayed significantly decreased cAMP production (60.3 ± 12.7 pmol/well) in response to GIP (Fig. 1, n = 3, p < 0.05). As would be expected from the binding experiments, GIP-R-400 did not respond to 10 nM GIP (5.3 ± 0.7 pmol/well). Of particular importance was the observation that extension of the receptor tail length to 405 amino acids (GIP-R-400A5) restored cAMP production to levels equivalent to GIP-R-405 (Fig. 1). Cells expressing the longer poly-alanine construct (GIP-R-386A9) or the construct with residues 397–400 deleted (GIP-R-ΔQSEI) failed to respond to GIP, indicating that these receptors were either not expressed or that regions important for G-protein coupling had been changed or deleted (Fig. 1). In light of the binding data, the former is more likely.

Although similar results were obtained with the stable CHO-K1 cell system, none of the truncated receptors displayed maximal cAMP production equivalent to that seen with the GIP-R-455 cell line (Fig. 3; Table I). EC50 values for GIP-R-418 and GIP-R-405 were decreased 4–6-fold (Table I, Fig. 3). Surprisingly, although there was only a small decrease in the binding affinity of GIP-R-400A5 for GIP (3.8 ± 0.2 nM) compared with the wild type receptor (2.2 ± 0.4 nM), there was a large increase in the EC50 value for cAMP production (1,163 ± 320 pm) compared with the full-length receptor (69 ± 27 pm) (Fig. 3, Table I). In agreement with the COS-7 cell experiments, CHO-K1 cells transfected with GIP-R-396A9 were not responsive to GIP-R-455 (Fig. 1). Cells expressing the longer poly-alanine construct (GIP-R-386A9) or the construct with residues 397–400 deleted (GIP-R-ΔQSEI) failed to respond to GIP, indicating that these receptors were either not expressed or that regions important for G-protein coupling had been changed or deleted (Fig. 1). In light of the binding data, the former is more likely.

Given the fact that deletions of the receptor beyond residue 400 were not tolerated with respect to expression, an alanine scan approach was adopted to examine the importance of residues between 394 and 401 for binding and G-protein coupling. These mutants were examined in COS-7 cells only (Figs. 4 and 5).

All seven of the alanine-substituted mutants displayed specific GIP binding and cAMP activation (Figs. 4 and 5). High affinity binding of 125I-GIP varied among the other seven mutants, with IC50 values ranging from 0.7 to 3.0 nM. However, substitutions at positions Glu-395, Val-396, and Ile-400 resulted in receptor binding affinities increased 2–3-fold (Fig. 5). These same mutants were also expressed at significantly re-
duced levels. GIP-R-K394A, GIP-R-Q397A, GIP-R-E399A, and GIP-R-R401A displayed expression levels similar to GIP-R-455 (Fig. 5). Cyclic AMP responses with GIP-R-V396A were approximately 50% of those with GIP-R-455 (Fig. 5).

Because the CT has been shown to influence sequestration of some G-protein-coupled receptors, the effect of truncation on receptor internalization was determined. All constructs that were expressed in CHO-K1 cells were found to internalize over time as assessed by an increase in the acid-resistant pool (Fig. 6). The wild type receptor clone displayed a rapid increase in acid-resistant binding over time, reaching maximal levels (64.9 ± 2.7% of total bound) within 120 min (Fig. 6, Table II). Maximum internalization of the truncated receptors did not differ significantly from that seen for the full-length GIP-R-455 receptor at 60–120 min. Further incubation times, of up to 4 h, failed to reveal any differences in maximal uptake among the different receptor constructs in CHO-K1 cells (data not shown).

Analysis of the initial linear uptake period showed that truncation of the tail to 425 or 418 amino acids resulted in significant decreases in the rate of internalization over the first 10 min, when compared with the wild type receptor (Fig. 6; Table II). Further truncation of the CT by 50 amino acids (GIP-R-405) partially restored the rate of uptake to wild type values, and uptake of the construct GIP-R-400A5 was not significantly different from the wild type receptor.

To examine a possible role for specific CT amino acids in the regulation of internalization, serine residues were targeted, because phosphorylation of serine or threonine residues normally precedes internalization of G-protein receptors (reviewed in Ref. 36). Each of the serine residues in the CT was therefore mutated to alanine, either singly or in multiples, and IC50 values, cyclic AMP production, and internalization determined. Additionally, because Tseng and Zhang (37), using truncation and alanine scan protocols similar to those described in this study, recently provided evidence that substitution of alanine for cysteine 411 completely ablated rat GIP receptor desensitization, the effect of this mutation (GIP-R-C411A) on receptor internalization was also examined. When expressed in COS-7 (Fig. 7) or CHO-K1 cells (Fig. 8, Table III) none of the mutants differed significantly from the wild type receptor with respect

---

**Table II**

Maximum receptor internalization and the rate of receptor uptake over the initial 10 min with carboxyl-terminal tail-truncated forms of the GIP receptor expressed in CHO-K1 cells

| Construct       | Slope     | Maximum internalization |
|----------------|-----------|-------------------------|
|                | %/min     | %                       |
| GIP-R-455      | 2.94 ± 0.26 | 64.9 ± 2.7              |
| GIP-R-425      | 1.60 ± 0.08 | 69.8 ± 1.0              |
| GIP-R-418      | 1.64 ± 0.16 | 73.5 ± 2.4              |
| GIP-R-405      | 2.20 ± 0.16 | 60.3 ± 3.3              |
| GIP-R-400A5    | 2.53 ± 0.24 | 61.8 ± 2.9              |

*, Significant difference from GIP-R-455, p < 0.05.
to binding affinity, although mutation of serines 427 or 440 resulted in a doubling of $B_{\text{max}}$ in transiently transfected cells (Fig. 7). There were no significant differences in cAMP responses to 10 nM GIP between the wild type and any of the mutant receptors (Fig. 7). In agreement with the reduced internalization rate observed with the CT truncated receptors, mutation of serine residues 426 or 427 to alanine resulted in decreases in initial rates of internalization (Fig. 9, Table III). GIP-R-S440A also demonstrated a decrease in its mean rate of internalization, although this did not reach significance. Maximum internalization of all three mutants was also reduced. A double mutant, GIP-R-S426A/S427A, and a complete CT serine knockout mutant, GIP-R-S398A-S453A in which serines 398, 406, 426, 427, 440, and 453 were all mutated to alanine residues, both exhibited reduced rates of internalization and maximum internalization. The latter mutant was the most profoundly affected with an internalization rate (0.75 ± 0.08%/min) only 46% of that of the wild type receptor (Table III). Mutation of cysteine 411 to alanine had no effect on internalization.

**DISCUSSION**

The intracellular loops of the heptahelical receptors have been implicated in G-protein recognition, coupling, and activation (19), but there is no consensus as to the importance of the CT with regard to these functions. Indeed, there appears to be considerable variability in the importance of this region among the different G-protein-coupled receptor types. For example, O’Dowd et al. (17) showed that the NH2-terminal region of the human $\beta_2$-adrenergic receptor CT was critical for coupling to G-proteins and activation of adenylyl cyclase, whereas shortening of the avian $\beta$-adrenergic receptor CT resulted in increased basal and agonist-stimulated cyclic AMP production, and reductions in agonist EC50 values (27). There have been few studies on the importance of the CT of the secretin-VIP receptor family, and the only relatively consistent finding has been an increase in affinity for agonists with CT-truncated mutants, as reported for the PTH/PTH-RP (29), calcitonin (30), and glucagon (31) receptors. In the case of the GIP receptor, removal of up to 50 amino acids from the CT had no significant effect on receptor binding affinity (IC50 values). This is similar to the human glucagon receptor, for which 62 of the amino acids in the CT were shown not to be required for binding (26). Interestingly, however, with the GIP receptor, three separate alanine substitution mutations within the proximal part of the CT (E395A, V396A, I400A; Figs. 4 and 5) resulted in increased receptor affinity. The cause of such increases is unclear, but one possibility, suggested by Iida-Klein et al. (29) in studies on the PTH/PTH-RP receptor, is that sequences in the CT lower the affinity of the wild type receptor for agonist, and that structural changes to the tail can reduce this effect. Changes in the interaction of the CT with intracellular structural components could be involved (27).

It is evident from the GIP-induced cyclic AMP responses of the truncation mutants that the majority of the COOH terminus of the receptor is not essential for coupling to adenyl cyclase, because a mutant consisting of as few as 13 of the 63 amino acids was capable of increasing cAMP production. The ability to remove a substantial portion of the CT while retain-
Characterization of the GIP Receptor COOH-terminal Domain

Table III

| Construct                      | IC50 (nM) | % wild type | Bmax (% of wild) | Internalization rate over first 15 min | Maximum internalization |
|-------------------------------|-----------|-------------|------------------|----------------------------------------|------------------------|
| GIP-R-455                     | 2.88 ± 0.26| 100         | 1.64 ± 0.17      | 36.4 ± 1.4                             |
| GIP-R-S398A                   | 2.75 ± 0.22| 102 ± 10    | 1.60 ± 0.05      | 34.6 ± 2.3                             |
| GIP-R-S406A                   | 3.75 ± 0.37| 89 ± 41     | 1.63 ± 0.06      | 36.5 ± 0.3                             |
| GIP-R-C411A                   | 2.41 ± 0.26| 70 ± 16     | 1.70 ± 0.14      | 36.7 ± 3.0                             |
| GIP-R-S426A                   | 3.88 ± 0.67| 106 ± 39    | 1.14 ± 0.09**    | 27.8 ± 1.0**                           |
| GIP-R-S427A                   | 4.21 ± 0.84| 89 ± 45     | 1.22 ± 0.06**    | 30.7 ± 0.5**                           |
| GIP-R-S440A                   | 4.30 ± 0.92| 80 ± 31     | 1.32 ± 0.06      | 30.0 ± 0.8**                           |
| GIP-R-S453A                   | 2.96 ± 0.30| 73 ± 10     | 1.55 ± 0.12      | 34.6 ± 1.9                             |
| GIP-R-S426A/S427A             | 4.25 ± 0.23| 116 ± 7     | 1.19 ± 0.10**    | 30.1 ± 1.6**                           |
| GIP-R-S398A-S453A             | 3.35 ± 0.20| 164 ± 16    | 0.75 ± 0.08**    | 28.3 ± 3.0**                           |

Fig. 9. Internalization kinetics of carboxyl-terminal serine to alanine mutant GIP receptors in transfected CHO-K1 cells over 1 h. A, mutants not differing from GIP-R-455; B, mutants showing altered internalization kinetics; C, examination of first 15 min of internalization. Data are presented as mean ± S.E.; n = 3–10; *, p < 0.05; **, p < 0.01 (S426A, S427A and S426A/S427A significantly different from GIP-R-455, p < 0.01). See Table III for statistical analysis. I, GIP-R-455; ▲, GIP-R-S398A; ▼, GIP-R-S406A; ▽, GIP-R-C411A; ●, GIP-R-S440A; □, GIP-R-S426A; △, GIP-R-S427A; ▽, GIP-R-S440A; ○, GIP-R-S426A/S427A; □, GIP-R-S398A-S453A.

(39) and luteinizing hormone/chorionic gonadotropin receptors (40) also resulted in no change in ligand-induced activation of adenylyl cyclase. In contrast, COOH-terminally truncated forms of both the rat PTH/PTH-RP (29) and avian β-adrenergic (27) receptors were found to couple to adenylyl cyclase with much higher efficacy than the wild type receptors. Evidence was presented suggesting that the CT decreases PTH/PTH-RP receptor affinity for Gs (29). The situation with the PTH/PTH-RP is probably complicated, however, because pertussis toxin-sensitive inhibitory effects of PTH on adenylyl cyclase were observed only in wild type receptors, and it was proposed that the CT plays a crucial role in interactions between receptors and inhibitory G-proteins. In contrast to the PTH/PTH-RP receptor (29), decreased maximal cAMP production was observed with truncated GIP receptors. Although these decreases, as well as those in Bmax, could have resulted from variability in transfection efficiency, such a possibility was minimized by performing all transfections for a set of experiments at the same time and using the wild type construct as a control for transfection variability. As discussed further below, a more likely explanation is a reduction in plasma membrane expression levels. Interestingly, significantly lower EC50 values (4–6-fold) were obtained for GIP-R-418 and GIP-R-405. One possible interpretation of this result is that CT shortening removes specific amino acids that induce less efficient receptor-induced Gs coupling to adenylyl cyclase.

When the GIP receptor was truncated by 50 or more amino acids, the level of measurable receptor binding decreased dramatically. Truncation probably decreases the efficiency of receptor insertion in the plasma membrane, and the reduced maximal cyclase stimulation with GIP-R-405 reflects this reduced membrane expression. Cells transfected with the truncated mutant GIP-R-400 exhibited neither binding nor the ability to stimulate adenylyl cyclase. Although this could be due to either lack of receptor expression in the plasma membrane or dramatically reduced agonist binding to an expressed receptor, the former is more likely. There is probably a minimum length for efficient folding of a heptahelical receptor and its translocation from the endoplasmic reticulum and insertion into the plasma membrane. Such a lack of expression explains the inability to detect biological responses with severely truncated mutants. Similar suggestions were made to explain the lack of detectable binding with extensively truncated PTH/PTH-RP receptors (28) and, more recently, with the human glucagon receptor (26). In the latter study, using CT mutation and alanine substitution techniques similar to those used here, it was shown conclusively that, as with the GIP receptor, the majority of the CT of the glucagon receptor could be deleted without compromising membrane insertion. Furthermore, a
glucagon receptor mutant (RT410) equivalent to GIP-R-400 (apart from 1 less amino acid), was shown by immunostaining not to be expressed in the plasma membrane, whereas the mutant RT415, almost equivalent to GIP-R-405, was expressed. Unfortunately, the absence of a GIP receptor antibody precluded us from performing immunostaining studies similar to those of Buggy et al. (26). Taken together, the two studies suggest that a COOH-terminal peptide length of between 10 and 15 amino acids is necessary for membrane expression of this receptor sub-family. However, while the current studies were nearing completion, Tseng and Zhang (37) reported on similar CT-truncated rat GIP receptor mutants expressed in L293 cells. They were unable to detect binding with a mutant truncated at position 395, but found that a receptor truncated at amino acid 399 was expressed almost as efficiently as the wild type receptor and exhibited unchanged binding affinity. This is in contrast to our observation that binding with GIP-R-400 was undetectable. The reason for this discrepancy is unclear but could be related to differences in processing and targeting in the different cell lines. Further support for a minimal chain length of greater than 400 amino acids for receptor expression in CHO-K1 and COS-7 cells resulted from our CT extension studies.

Two possibilities were considered regarding the structure of the CT necessary for membrane expression. Either the specific sequence RLRL (amino acids 402–405) was required, or the chain length itself was the determining factor, and the specific amino acids were immaterial. A mutant receptor was therefore prepared, which extended the COOH-terminal chain with 5 alanines to produce a 405-amino acid protein (GIP-R-405A5). The level of receptor binding and the maximal level of cyclic AMP production with this receptor mutant were similar to those produced with GIP-R-405. However, there was a large increase in the EC₅₀ value for cAMP production (1,163 ± 320 pm) compared with the full-length receptor (69 ± 27 pm) indicating that specific amino acids within the 400–405 region influence the efficiency of G-protein coupling. Interestingly, neither cells transfected with a GIP-R-396 construct extended with 9 alanine residues to a length of 405, nor those with a receptor in which amino acids 397–400 were deleted demonstrated any binding. This suggested that specific residues in the proximal part of the CT may be important for expression.

Because it was not possible to test this hypothesis using the truncation paradigm, amino acids in the region 394–401 were individually mutated to alanines. Although all were expressed in COS-7 cells, the observation that the E395A, V396A, and I400A mutants were expressed with greatly reduced efficiency in L293 cells, the observation that the E395A, V396A, and I400A mutants were expressed with greatly reduced efficiency in COS-7 cells, the observation that the E395A, V396A, and I400A mutants were expressed with greatly reduced efficiency in CHO-K1 and COS-7 cells resulted from our CT extension studies.

In conclusion, the current studies demonstrated that the majority of the GIP receptor CT is not required for signaling but that a minimum chain length of approximately 405 amino acids is needed for receptor expression. Specific serine residues within the CT, particularly serines 426 and 427, play an important role in regulating the rate of receptor internalization.

Acknowledgements—We thank Xinfang Li and Cuiyan Nian for expert technical assistance with the studies described in this manuscript.

REFERENCES
1. Nauck, M., Stockman, R., Ebert, R., and Creutzfeldt, W. (1986) Diabetologia 29, 46–52
2. Holst, J. J., Gromada, J., and Nauck, M. A. (1997) Diabetologia 40, 984–986
3. Nauck, M. A., Himmsaat, M. M., Osvald, C., Holst, J. J., Ebert, R., and Creutzfeldt, W. (1990) J. Clin. Invest. 91, 301–307
4. Uden, T. B., Messey, E., Button, D. C., Brownstein, M. J., and Bonner, T. I. (1993) Endocrinology 133, 2870–2877
5. Wheeler, M. B., Gelling, R. W., McIntosh, C. H. S., Georgiou, J., Brown, J. C., and Pederson, R. A. (1995) Endocrinology 136, 4629–4639
6. Gribkoff, S., Purret, A., Hoffer, E. H., Cherif, D., Viennet, N., Froegel, P., and Thorens, B. (1995) Diabetes 44, 1202–1208
7. Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kupfer, J. L., Sheppard, P. O., Sprecher, C. A., O'Hara, P. J., Foster, D., Walker, K. M., Chen, L. H. J., McKernan, P. A., and Kindsvogel, W. (1993) Science 259, 1614–1616
8. Thorens, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8641–8645
9. Ishihara, T., Nakamura, S., Kario, Y., Takahashi, K., and Nagata, S. (1991) EMBO J. 10, 1635–1641
10. Sreedharan, S. P., Robichon, A., Peterson, K. E., and Goetzl, E. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4986–4990
11. Jippner, H., Abo-Shamra, A.-B., Freeman, M., Kong, X. F., Shipani, E., Richards, J., Kolakowski, J. L. F., Hock, J., Potts, J. T., Kronenberg, H. M., and Segre, G. V. (1991) Science 254, 1024–1026
12. Lin, Y., Harris, T. L., Flannery, M. S., Arufo, A., Kaji, E. H., Gorn, A., Kolakowski, L. F., Jr., Lodish, H. F., and Goldring, S. R. (1991) Science 254, 1022–1024
13. Moose, K., Heimberg, H., Flamez, D., Huygen, P., Quartier, E., Ling, Z., Pipiereza, D., Gremlich, S., Thoren, B., and Schuit, F. (1996) Diabetes 45, 257–261
14. Amirraff, B., Vaucin-Jacques, N., and Laburthe, M. (1984) Biochem. Biophys. Res. Commun. 128, 671–676
15. Maletti, A., Altman, J. J., Hoa, D. H. B., Carquist, M., and Rosselin, G. (1987) Diabetes 36, 1336–1340
16. Lu, M., Wheeler, M. B., Beng, X.-H., and Boyd, A. E., II (1993) Endocrinology 132, 94–100
17. O'Dowd, E. F., Hnatowich, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. (1988) J. Biol. Chem. 263, 15985–15992
18. Liggett, S. B., Caron, M. G., Lefkowitz, R. J., and Hsuan, W. S. (1991) J. Biol. Chem. 266, 4816–4821
19. Hedin, K. E., Duersken, K., and Clapham, D. E. (1983) Cell. Signal. 5, 505–518
20. Busenitz, E. S., Spalding, T. A., Hill-Eubanks, D., and Brann, M. R. (1995) Biochem. Biophys. Res. Commun. 202, 1365–1371
Characterization of the GIP Receptor COOH-terminal Domain

J. Biol. Chem. 270, 3141–3146
21. Takhar, S., Gyorkey, S., Su, R.-C., Mathi, S. K., Li, X., and Wheeler, M. B. (1996) Endocrinology 137, 2175–2177
22. Mathi, S. K., Chan, Y., Li, X., and Wheeler, M. B. (1997) Mol. Endocrinol. 11, 424–432
23. Renzeke, J. E., Blumer, K. J., Courchesne, W. E., and Thorner, J. (1988) Cell 55, 221–234
24. Hausdorf, W., Caron, M., and Lefkowitz, R. (1990) FASEB J. 4, 2881–2889
25. Huang, Z., Chen, Y., and Nissenson, R. A. (1995) J. Biol. Chem. 270, 151–156
26. Buggy, J. J., Heurich, R. O., MacDougall, M., Kelley, K. A., Livingston, J. N., Yoo-Warren, H., and Rossomando, A. J. (1997) Diabetes 46, 1400–1405
27. Parker, E., and Ross, E. (1991) J. Biol. Chem. 266, 9987–9996
28. Huang, Z., Chen, Y., Pratt, S., Chen, T.-H., Bambino, T., Sholback, D. M., and Nissenson, R. A. (1995) Mol. Endocrinol. 9, 1240–1249
29. Iida-Klein, A., Guo, J., Xie, L. Y., Juppner, H., Potts, J. T., Jr., Kronenberg, H. M., Bringhurst, F. R., Abou-Samra, A. B., and Segre, G. V. (1995) J. Biol. Chem. 270, 8458–8465
30. Findlay, D. M., Housami, S., Lin, H. Y., Myers, D. E., Brady, C. L., Darcy, P. K., Ikeda, K., Martin, T. J., and Sexton, P. M. (1994) Mol. Endocrinol. 8, 1691–1700
31. Unson, C. G., Cypess, A. M., Kim, H. N., Goldsmith, P. K., Carruthers, C. J. L., Merrifield, R. B., and Sakmar, T. P. (1995) J. Biol. Chem. 270, 27720–27727
32. Widmann, C., Dolci, W., and Thorens, B. (1997) Mol. Endocrinol. 11, 1094–1102
33. Gelling, R. W., Wheeler, M. B., Xue, J., Gyorkey, S., Nian, C., Pederson, R. A., and McIntosh, C. H. S. (1997) Endocrinology 138, 2640–2643
34. Kallal, L., Gagnon, A. W., Penn, R. B., and Benevite, J. L. (1998) J. Biol. Chem. 273, 322–328
35. Petrov, C., Chen, L., and Tashjian, A. H., Jr. (1997) J. Biol. Chem. 272, 2326–2333
36. Bohm, S. K., Grady, E. F., and Bunnett, N. W. (1997) Biochem. J. 322, 1–18
37. Tseng, C.-C., and Zhang, X.-Y. (1998) Mol. Cell. Endocrinol. 139, 179–186
38. Schneider, H., Feyen, J. H. M., and Seuwen, K. (1994) FEBS Lett. 351, 281–285
39. Caizanhalk, G. D., Nagayama, Y., Russo, D., Wadsworth, H. L., and Rapaport, B. (1999) J. Biol. Chem. 265, 29970–29975
40. Rodriguez, M. C., Xie, Y.-B., Wang, H., Collison, K., and Segaloff, D. L. (1992) Mol. Endocrinol. 6, 327–336
41. Nygaard, S. C., Kuestner, R. E., Moore, E. E., and Stroop, S. D. (1997) J. Bone Miner. Res. 12, 1681–1690
42. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. (1994) Annu. Rev. Biochem. 63, 101–132