

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*

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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)¹ and the truncated

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 G_s and adenylyl 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 β ₂-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 (17, 27). 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 (29), calcitonin (30), and glucagon (31) receptors, whereas truncation increased

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¹ The abbreviations used are: GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; NIDDM, non-insulin-dependent diabetes mellitus; CT, carboxyl-terminal tail; VIP, vasoactive intestinal peptide; PTH, parathyroid hormone; PTH-RP, PTH-related peptide; DMEM, Dulbecco's modified Eagle's medium; BB, binding buffer.

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. Carboxyl-terminally truncated mutants of the receptor, produced by site-directed 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 adenylyl cyclase, and ligand-induced internalization. In addition, 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, utilizing the rat receptor cDNA (5) in the vector pcDNA 3 (Invitrogen Co., San Diego, CA) as template. The 5'-sense oligonucleotide primer corresponded to coding nucleotides -3 to +19 (5'-AGGATGCCCTGCG-GCTGTTGC-3'). Five 3'-antisense oligonucleotide primers were designed 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 sequence 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'-CTACAAAGGCACCGCCCGGGGGGC-3' (GIP-R-425); 1255-1236, 5'-CTACTGCCCCAGGTGCGGACGTG-3' (GIP-R-418); 1216-1196, 5'-CTAGAGACGCAGACGGCGGATCTC-3' (GIP-R-405); 1198-1175, 5'-CTAGATCTCCGACTGTACCTCTTTGTTGAT-3' (GIP-R-400); 5'-CTATGCTGCTGCTGCTGCGATCTCCGACTGTACCTCTT-TGTT3' (GIP-R-400A₃); and, 5'-CTATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTACCTCTTTGTTGATGAAGCA-3' (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 adenylyl cyclase, a double-stranded site mutagenesis strategy (Chameleon, Stratagene, La Jolla, CA) was used to substitute individually the 8 residues (394-401) extending from the predicted seventh transmembrane domain, with alanine residues. The resulting constructs were designated GIP-R-K394A, GIP-R-E395A, GIP-R-V396A, GIP-R-Q397A, GIP-R-S398A, GIP-R-E399A, GIP-R-I400A, and GIP-R-R401A. A deletion mutant lacking residues 397-400 (GIP-R-ΔQSEI), a mutant with cysteine 411 changed to alanine (GIP-R-C411A), and a series of alanine substitution mutations with CT serine residues mutated either individually (S398A, S406A, S426A, S427A, S440A, S453A), in multiples (S426A/S427A) or completely (S398A-S453A), were generated using the same methodology. The identities of all mutants were confirmed by sequence analysis (T₇-Sequencing, Amersham Pharmacia Biotech).

Cell Transfection and Tissue Culture—For transient expression studies, COS-7 cells (3 × 10⁶) were seeded in 10-cm dishes (Becton Dickinson, Lincoln Park, NJ) and cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Cansera, Roxdale, Ontario). Cells were transfected the following day with 5 μg (for cAMP assays) or 10 μg (for binding assays) of either the wild type receptor (GIP-R-455) cDNA or the appropriate mutant constructs by the DEAE-dextran method as described previously (5). All plasmid DNAs were prepared and purified using identical protocols, and the integrity of the DNA was checked before transfection. To minimize variability in day-to-day transfection efficiency, the various constructs for each set of experiments were generally all transfected at the same time using the same mixture. In all cases, the wild type receptor construct was transfected as a control to allow correction for

any small day-to-day variations in transfection efficiency. 15 h after transfection, the cells were passaged into either 6-well plates for cAMP studies, or 10-cm dishes for binding studies, and cultured for an additional 48 h. For production of permanent CHO-K1 cell lines expressing the mutant GIP receptor cDNAs, cells were grown in DMEM/F12 media (Life Technologies, Inc.) supplemented with 10% newborn calf serum (Cansera) on 10-cm dishes until ~80% confluent. Cells were transfected with 10 μg of the appropriate cDNA using the CaPO₄ co-precipitation method (5). As we have previously demonstrated that cloned CHO-K1 cell lines obtained by pooling of clones, and maintained under high stringency selection, express receptors at levels similar to high level expressing clones (33), pooled CHO-K1 cell lines expressing receptor constructs were isolated under high G418 (800 μg/ml) selection. The wild type GIP-R-455 cDNA and stable line have been described previously (5, 33).

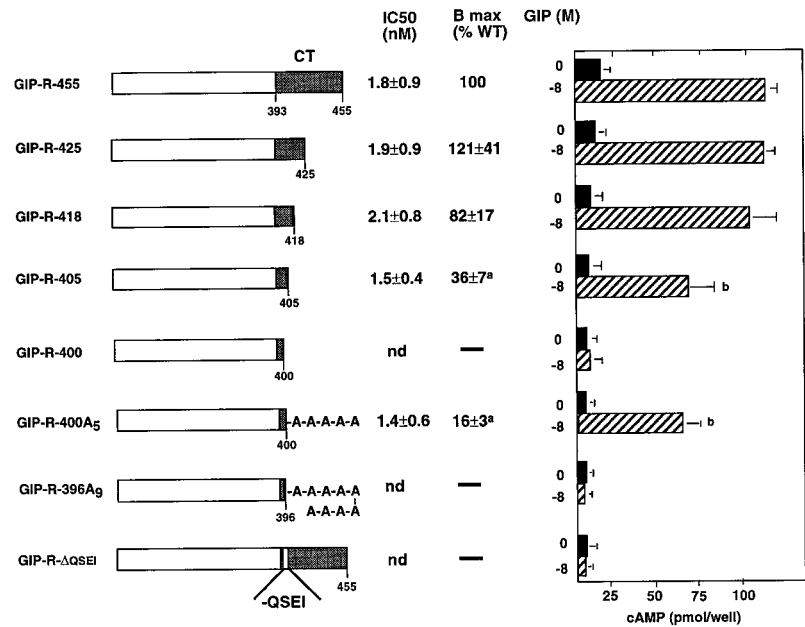
Iodination of GIP and Binding Analysis—Synthetic porcine GIP (5 μg) was iodinated by the chloramine-T method, and the ¹²⁵I-GIP further purified by reverse phase high pressure liquid chromatography to a specific activity of 250-350 μCi/μg, as described previously (5). Aliquots of tracer were lyophilized and stored at -20 °C until use. Binding analyses were performed as described previously (5) with minor modifications. Briefly, COS-7 cells were washed twice in binding buffer (BB; DMEM containing 20 mM HEPES and 0.1% bovine serum albumin, pH 7.4). Cells (5 × 10⁶/tube) were then incubated for 60 min at 37 °C with ¹²⁵I-GIP (50,000 cpm), in the presence or absence of unlabeled synthetic human GIP (Hukabel, Montreal, Canada or Bachem, Torrance, CA) in a final volume of 200 μl. (It has been shown previously that porcine and human GIP have identical binding affinities for the rat GIP receptor (5).) After incubation, cell suspensions were centrifuged at 12,000 × g, the cells washed once in ice cold BB, and cell-associated radioactivity in the pellet was measured in a gamma counter. CHO-K1 cells (1-5 × 10⁵/well), plated 2 days before use in 24-well plates, were washed twice at 4 °C in BB, consisting of DMEM/F12, 20 mM HEPES, and 0.1% bovine serum albumin, pH 7.4, and incubated for 12-16 h at 4 °C with ¹²⁵I-GIP (50,000 cpm) in the presence or absence of unlabeled GIP (each concentration tested in triplicate). Cells were then washed twice in ice cold buffer, solubilized with 0.1 M NaOH (1.0 ml), and transferred to test tubes for counting of cell-associated radioactivity. Nonspecific binding for both cell types was defined as that measured in the presence of an excess of human GIP (1 μM), and specific binding was expressed as a percentage of maximum binding (%B/B₀).

Measurement of cAMP Production—Receptor-expressing COS-7 cells (24 h after transfection) or CHO-K1 clones were passaged into multi-well plates and cultured for an additional 48 h. Cells were then washed in assay buffer containing 0.1% bovine serum albumin and preincubated for 60 min, followed by a 30-min stimulation period with GIP in the presence of 1 mM isobutylmethylxanthine (Research Biochemicals International, Natick, MA), as described previously (5, 33). Cells were extracted with ice-cold 70% ethanol, and cAMP levels were measured by radioimmunoassay (Biomedical Technologies, Stoughton, MA). Data were expressed as a percentage of maximal levels observed with the wild type receptor (GIP-R-455) for CHO-K1 cells, and picomoles of cAMP/well for COS-7 cells.

Internalization Studies—Internalization of receptor-bound ligand with wild type and truncated mutants was determined as described by Widmann *et al.* (32). Briefly, cells were washed twice with BB, and incubated in the presence of ¹²⁵I-GIP (50,000 cpm) for 0-120 min (in triplicate), as indicated in Fig. 6. Binding was stopped by two washes with ice-cold BB, followed by a 10-min wash in ice-cold stripping buffer (150 mM NaCl, 50 mM glycine, pH 3.0) to remove surface bound tracer. Acid-released ¹²⁵I-GIP was collected, and the cells solubilized in NaOH to determine internalized (acid resistant) radioactivity. Nonspecific binding in both fractions was determined for all time points by performing the identical experiments in the presence of 1 μM unlabeled GIP and subtracting corresponding values. The internalized ¹²⁵I-GIP for each time point was expressed as a percentage of total bound (acid resistant + acid stripable).

The internalization protocol used to study CHO-K1 cells stably expressing receptor constructs with serine to alanine mutations in the CT tail was based on that of Kallal *et al.* (34) and Petrou *et al.* (35). Cells were washed twice with BB at 37 °C, and then incubated with 100 nM GIP for 0-60 min in triplicate, as indicated in Fig. 9. Cells were then stripped of non-internalized bound GIP by incubating with ice-cold stripping buffer (as above) for 5 min, followed by two rinses with ice-cold BB. Cells were then incubated with ¹²⁵I-GIP (50,000 cpm) for 4 h at 4 °C to prevent further internalization or recycling, washed twice with ice-cold BB, and solubilized in 1 ml of NaOH for measurement of cell-associated radioactivity. Nonspecific binding was measured in the pres-

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 IC₅₀ 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).



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 Dunnett'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

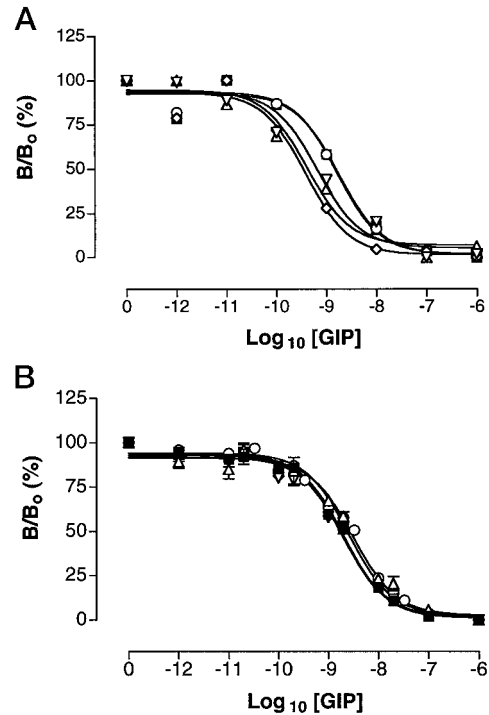


FIG. 2. Binding analysis of GIP-R truncation mutants in COS-7 and CHO-K1 cells. Binding of the GIP-R mutations were examined for ¹²⁵I-GIP binding in COS-7 cells expressing truncated receptor mutants (A) and in CHO-K1 cells stably expressing mutants (B). A summary of the results in CHO-K1 cells and statistical 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.

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-ΔQSEI). 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

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
Mean \pm S.E.; $n = 6$; * $p < 0.05$ or ** $p < 0.01$ compared with GIP-R-455.

Construct	^{125}I -GIP Binding IC_{50}	B_{max}	cAMP production	cAMP production EC_{50}
	<i>nM</i>	% wild type	% wild type max	<i>pM</i>
GIP-R-455	2.24 ± 0.35	100	100	69 ± 27
GIP-R-425	2.73 ± 0.23	126 ± 34	$55 \pm 13^{**}$	27 ± 20
GIP-R-418	2.28 ± 0.27	74 ± 16	$23 \pm 5^{**}$	$15 \pm 1^*$
GIP-R-405	2.16 ± 0.12	$29 \pm 2.9^*$	$8 \pm 2^{**}$	$11 \pm 1^*$
GIP-R-400	ND ^a	— ^b	—	ND
GIP-R-400A ₅	$3.80 \pm 0.21^*$	$12 \pm 1.1^*$	$31 \pm 7^{**}$	$1,163 \pm 320^{**}$
GIP-R-396A ₉	ND	—	—	ND
GIP-R- Δ QSEI	ND	—	—	ND

^a ND, not determined.

^b —, nondetectable.

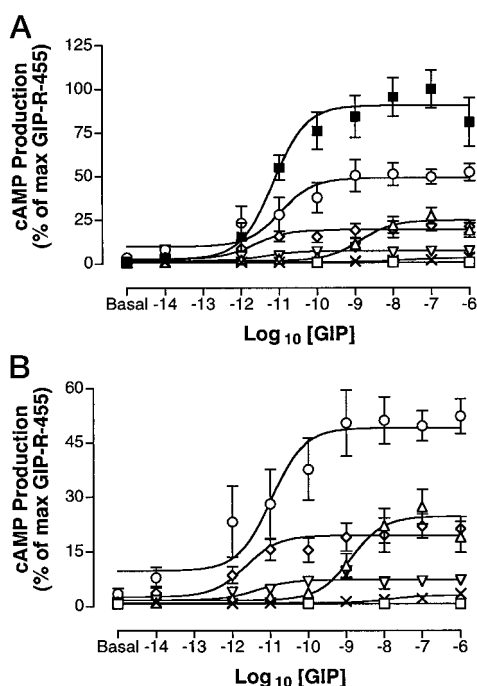


FIG. 3. Analysis of GIP-stimulated cAMP production in CHO-K1 cells expressing carboxyl-terminal truncated mutants. A concentration-dependent analysis of GIP-stimulated cAMP formation is shown in CT mutations compared with GIP-R-455 receptor (A), and a comparison among the mutant receptors (B). A summary of the results (% maximal response and EC_{50} values) and statistical analyses is shown in Table I. Data represent the mean \pm S.E. ($n \geq 3$). ■, GIP-R-455; □, GIP-R-400; X, GIP-R-396A₉; △, GIP-R-400A₅; ▽, GIP-R-405; ◇, GIP-R-418; ○, GIP-R-425.

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

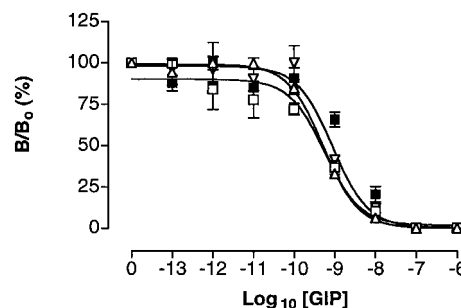


FIG. 4. Binding of carboxyl-terminal alanine substitution mutants expressed in COS-7 cells. A series of point mutations was generated to examine each of the amino acids 394–401 within the proximal CT with respect to GIP binding. Data represent mean \pm S.E.; $n \geq 3$. Summary statistics for all alanine substitution constructs shown in Fig. 5. ■, GIP-R-455; △, GIP-R-E395A; □, GIP-R-V396A; ▽, GIP-R-I400A.

(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- Δ QSEI) 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). EC_{50} 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 EC_{50} value for cAMP production ($1,163 \pm 320$ 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 stimulation with concentrations as high as 1 μM (Fig. 3, Table I).

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 ^{125}I -GIP varied among the other seven mutants, with IC_{50} 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-

FIG. 5. Summary of binding and GIP-stimulated cAMP accumulation in carboxyl-terminal alanine substitution mutants expressed in COS-7 cells. Receptor mutations with specific CT substitutions, shown *underlined* on the left, were examined for their ability to bind GIP in 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. Data are represented as mean \pm S.E.; $n =$ three independent experiments; **, $p < 0.01$ versus GIP-R-455.

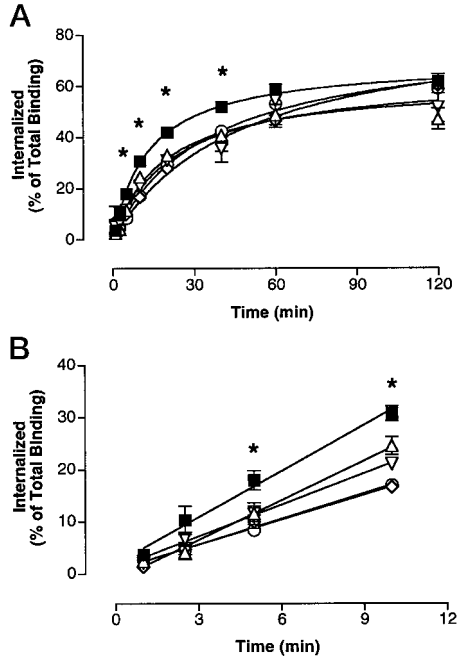
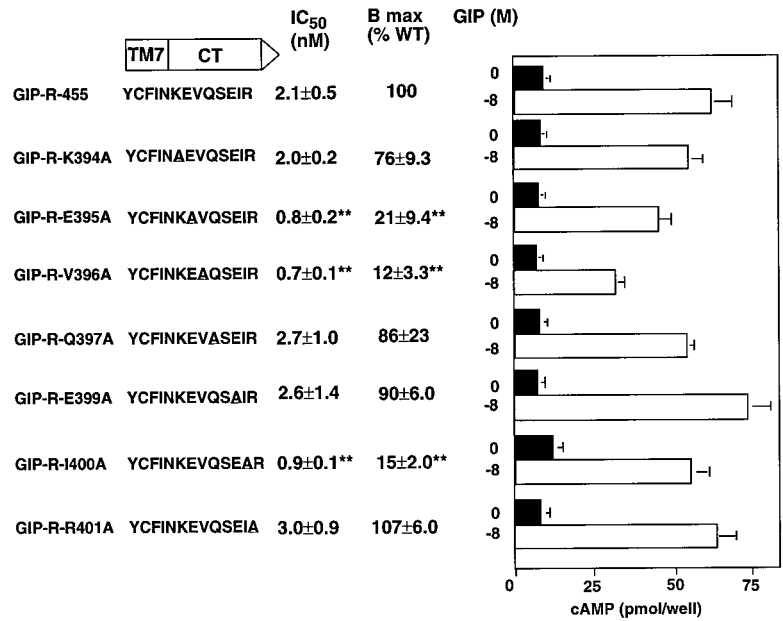


FIG. 6. ^{125}I -GIP internalization kinetics of carboxyl-terminal truncation mutants in CHO-K1 cells. Receptor mutants were examined in CHO-K1 cells for internalization over a 2-h stimulation period (A) and over a 10-min period (B); $n \geq$ three independent determinations. Data are summarized and statistical analyses presented in Table II. *, significant difference of GIP-R-455 rate from mutants, $p < 0.05$. ■, GIP-R-455; △, GIP-R-400A₅; ▽, GIP-R-405; ◇, GIP-R-418; ○, GIP-R-425.

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

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

Values are mean \pm S.E. of $n \geq$ three individual experiments.

Construct	Slope	Maximum internalization
	%/min	%
GIP-R-455	2.94 \pm 0.26	64.9 \pm 2.7
GIP-R-425	1.60 \pm 0.08**	69.8 \pm 1.0
GIP-R-418	1.64 \pm 0.16*	73.8 \pm 2.4
GIP-R-405	2.20 \pm 0.16*	60.3 \pm 3.3
GIP-R-400A ₅	2.53 \pm 0.24	61.8 \pm 2.9

* *, Significant difference from GIP-R-455, $p < 0.05$.

(64.9 \pm 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 IC_{50} 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

FIG. 7. Summary of binding and GIP-stimulated cAMP accumulation in carboxyl-terminal serine to alanine substitution mutants expressed in COS-7 cells. Receptors with specific serine mutations were examined for their ability to bind GIP in 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. Data are represented as mean \pm S.E.; $n =$ three independent experiments; *, $p < 0.05$ versus wild type (WT).

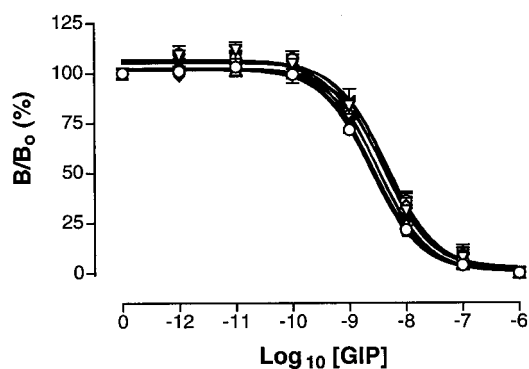
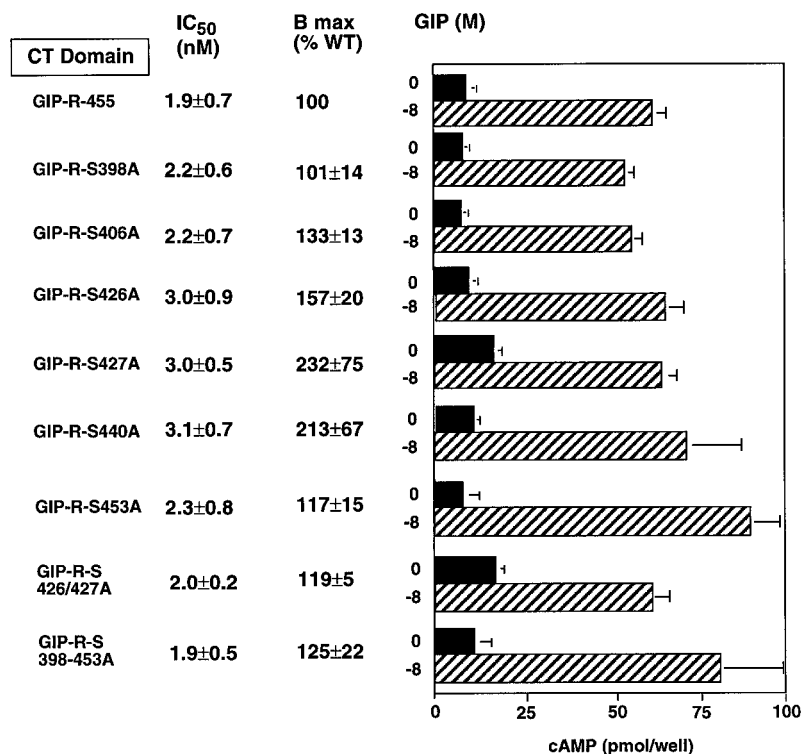


FIG. 8. Competitive binding studies on CHO-K1 cells stably transfected with carboxyl-terminal serine to alanine mutations, complete serine mutation, or a cysteine mutation. Data are presented as mean \pm S.E. ($n = 3-10$). Summary statistics are included in Table III. \circ , GIP-R-455; Δ , GIP-R-S398A; ∇ , GIP-R-S406A; \blacksquare , GIP-R-C411A; \diamond , GIP-R-S426A; *, GIP-R-S427A; \times , GIP-R-S440A; +, GIP-R-S453A; \blacklozenge , GIP-R-S426A/S427A; \bullet , GIP-R-S398A-S453A.

to binding affinity, although mutation of serines 427 or 440 resulted in a doubling of B_{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 ($0.75 \pm 0.08\%/min$) 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 NH_2 -terminal region of the human β_2 -adrenergic receptor CT was critical for coupling to G-proteins and activation of adenylyl cyclase, whereas shortening of the avian β -adrenergic receptor CT resulted in increased basal and agonist-stimulated cyclic AMP production, and reductions in agonist EC_{50} 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 (IC_{50} 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 adenylyl 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-

TABLE III

Binding and internalization characteristics of CHO-K1 cells stably transfected with GIP receptor carboxyl-terminal serine mutants

Data represent mean \pm S.E. ($n = 3-10$). IC_{50} and B_{max} values were not significantly different from wild type-GIP-R by analysis of variance or Dunnett's test. * and **, significant differences from GIP-R-455, $p < 0.05$ and 0.01 , respectively.

Construct	IC_{50}	B_{max}	Internalization rate over first 15 min	Maximum internalization
	<i>nM</i>	% wild type	%/min	%
GIP-R-455	2.88 \pm 0.26	100	1.64 \pm 0.17	36.4 \pm 1.4
GIP-R-S398A	2.75 \pm 0.22	102 \pm 10	1.60 \pm 0.05	34.6 \pm 2.3
GIP-R-S406A	3.75 \pm 0.37	89 \pm 41	1.63 \pm 0.06	36.5 \pm 0.3
GIP-R-C411A	2.41 \pm 0.26	70 \pm 16	1.70 \pm 0.14	36.7 \pm 3.0
GIP-R-S426A	3.88 \pm 0.67	106 \pm 39	1.14 \pm 0.09**	27.8 \pm 1.0**
GIP-R-S427A	4.21 \pm 0.84	89 \pm 45	1.22 \pm 0.06*	30.7 \pm 0.5*
GIP-R-S440A	4.30 \pm 0.92	80 \pm 31	1.32 \pm 0.06	30.0 \pm 0.8*
GIP-R-S453A	2.96 \pm 0.30	73 \pm 10	1.55 \pm 0.12	34.6 \pm 1.9
GIP-R-S426A/S427A	4.25 \pm 0.23	116 \pm 7	1.19 \pm 0.10*	30.1 \pm 1.6*
GIP-R-S398A-S453A	3.35 \pm 0.20	164 \pm 16	0.75 \pm 0.08**	28.3 \pm 1.0**

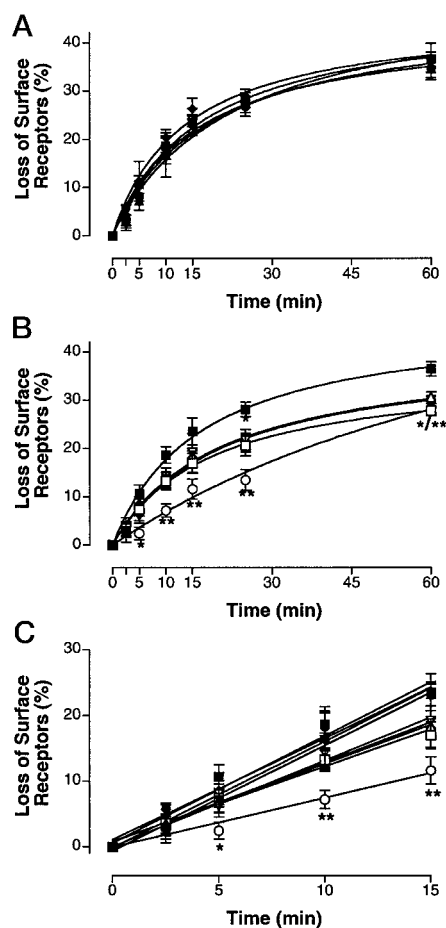


FIG. 9. Internalization kinetics of carboxyl-terminal serine to alanine mutant GIP receptors in transfected CHO-K1 cells over 1 h. A, mutants not differing from GIP-R-455; B, mutants showing altered internalization kinetics; C, examination of first 15 min of internalization. Data are represented as mean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

ing G-protein coupling is in agreement with studies on other heptahelical receptors. In mutational analysis experiments similar to those described here, progressive truncations of the CT of the opossum (28) and human (38) PTH/PTH-RP receptors resulted in no significant alterations in cyclic AMP production. Similarly, removal of the distal two-thirds of the thyrotropin

(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 G_s (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 B_{max} , 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 EC_{50} 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 G_s 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 these 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 indicates that specific amino acids in the proximal part of the CT are important for expression.

It therefore appears that although there is considerable redundancy in the specific amino acids in the proximal CT of the GIP receptor that can allow G-protein coupling, the length of the tail and specific amino acids are both important for insertion into the plasma membrane. Because the proximal CT of the glucagon receptor (403–415 NKEVQSELRRRW) is almost identical to that of the GIP receptor (393–405 NKEVQSEIR-RLRL), it is likely that a similar level of redundancy exists for this receptor. Specificity of G-protein binding in the secretin-VIP receptor family probably resides elsewhere within the intracellular domains, and evidence has been presented recently indicating a critical role for a single amino acid in the NH₂-terminal portion of the IC-3 loop of the GLP-1 receptor for efficient coupling to adenylyl cyclase (21). This region is highly conserved among the secretin-VIP family and will probably prove to be equally important for GIP receptor activation. However, because IC-3 probably lies in close proximity to the prox-

imal portion of the CT (17) the latter region may play a modulatory role.

The CT regions of a number of G-protein-coupled receptors, including those for yeast α -mating factor (23), calcitonin (41), and GLP-1 (32) have been shown to be phosphorylated on serine and threonine residues and to be involved in both receptor desensitization and internalization. There is variability, however, and the majority of the CT of the PTH/PTH-RP (25) and β -adrenergic (42) receptors are not required for receptor sequestration. Huang *et al.* (25) suggested that there may be both positively and negatively acting regions in the CT of the PTH/PTH-RP receptor. The results from the current study, targeted at determining a possible role for individual serines in the GIP receptor for sequestration, revealed that although their mutation had little effect on IC₅₀ values or the level of receptor expression, mutation of serine residues 426 or 427 to alanine, singly or in combination, resulted in decreased initial rates of internalization. This is in agreement with the truncation experiments, in which removal of serines in the CT by chain termination reduced receptor internalization. Because the level of reduction in sequestration was greatest with the complete receptor knockout, other serines, such as amino acid 440, may also play a role. In contrast to the finding of Tseng and Zhang (37), that substitution of alanine for cysteine 411 ablated rat GIP receptor desensitization in L293 cells resulting from 24 h of incubation with ligand, this mutation was found to have no effect on receptor internalization.

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.

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