# Mutational analysis of the glucagon receptor: similarities with the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (PACAP)/secretin receptors for recognition of the ligand's third residue

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Receptor recognition by the Asp[3] residues of vasoactive intestinal peptide and secretin requires the presence of a lysine residue close to the second transmembrane helix (TM2)/first extracellular loop junction and an ionic bond with an arginine residue in TM2. We tested whether the glucagon Gln[3] residue recognizes the equivalent positions in its receptor. Our data revealed that the binding and functional properties of the wild-type glucagon receptor and the K188R mutant were not significantly different, whereas all agonists had markedly lower potencies and affinities at the I195K mutated receptor. In contrast, glucagon was less potent and the Asp[3]-, Asn[3]- and Glu[3]-glucagon mutants were more potent and efficient at the double-mutated K188R/I195K receptor. Furthermore, these alterations were selective for position 3 of glucagon, as shown by the functional properties of the mutant Glu[9]- and Lys[15]-glucagon. Our results suggest that although the Gln[3] residue of glucagon did not interact with the equivalent binding pocket as the Asp[3] residue of vasoactive intestinal peptide or secretin, the Asp[3]-glucagon analogue was able to interact with position 188 of the K188R/I195K glucagon receptor. Nevertheless, the Gln[3] side chain of glucagon probably binds very close to this region in the wild-type receptor.

Key words: glucagon analogue, receptor point mutation.

## INTRODUCTION

Glucagon belongs to the same peptide family as glucagon-like peptides 1 and 2 (GLP1 and GLP2), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), growth-hormone-releasing factor (GRF) and secretin, which are characterized by a Phe[6]Thr[7] 'signature' (Figure 1). Despite their marked sequence homologies, glucagon and GLP cross-react only weakly at the receptor level, and GRF, VIP, PACAP and secretin do not recognize the glucagon receptor and vice versa. This selectivity can be attributed to several differences in the peptide sequences, including the presence of an uncharged Gln[3] in glucagon, as opposed to the acidic Asp[3] or Glu[3] residues found in the other peptides.

The glucagon receptor belongs to a subfamily of seven transmembrane G-protein-coupled receptors, characterized by a large N-terminal domain containing highly conserved cysteine residues and by conserved transmembrane helices. This subfamily includes, in addition to the receptors for the glucagon peptide family cited above, the receptors for gastric inhibitory peptide (GIP), corticotrophin-releasing factor (CRF), parathyroid hormone (PTH), amylin, calcitonin-gene-related peptide (CGRP) and calcitonin [1].

We demonstrated previously that the Asp[3] residues of secretin [2,3] and VIP interact with two basic residues (Arg and Lys) within the second transmembrane helix of their respective receptors [4,5]. The same charged residues are also found in the GLP1 and GLP2 receptors, but are replaced in the glucagon receptor by Lys and Ile residues, respectively.

In the present work, we tested the hypothesis that Gln[3] in glucagon might interact with the equivalent receptor positions by replacing the corresponding second transmembrane domain (TM2) amino acids, Lys[188] and Ile[195], with Arg and Lys respectively, as in the GLP1, GLP2, VIP, PACAP and secretin receptors (Figure 2) and testing against glucagon analogues mutated in position 3. Our results suggest strongly that both mutations are simultaneously necessary to switch the Gln[3]-preferring receptor properties to Asp[3]-preferring receptor properties.

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            3       9      15      21
Gluc    HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH
Glp1    HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2
Secr    HSDGTFTSELSRLREGARLQRLLQGLV-NH2
VIP     HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH2
PACAP   HSDGIFTDSYSRYRKQMAVKKYLAAVL-NH2
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**Figure 1 Amino acid sequence alignment of glucagon (Gluc), GLP1 (truncated active form 7–36), secretin (Secr), VIP and PACAP**

The sequence alignment was generated using the PileUp algorithm from the Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, WI, U.S.A. In glucagon, the positions that were mutated in the present work, 3, 9, 15 and 21, are boxed.

## MATERIALS AND METHODS

### Construction of the mutated receptors

Generation of the mutated receptors was achieved using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) essentially according to the manufacturer's instruc-

Abbreviations used: CHO, Chinese hamster ovary; EC1, first extracellular loop; GRF, growth-hormone-releasing factor; TM2, second transmembrane domain; GLP, glucagon-like peptide; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; VPAC, VIP/PACAP receptor; CRF, corticotrophin-releasing factor; PTH, parathyroid hormone.

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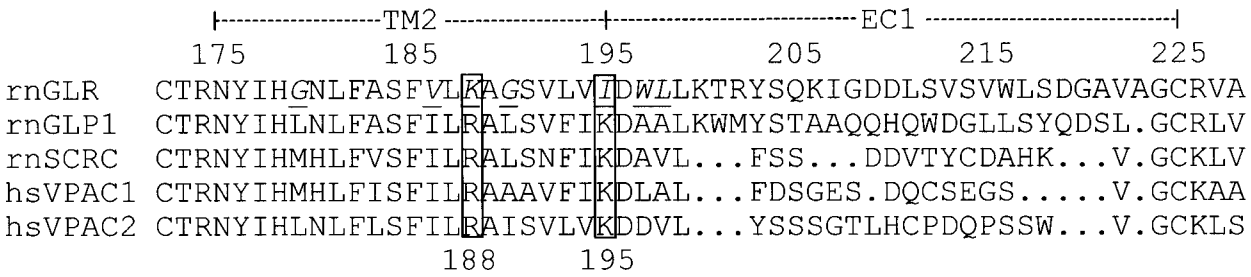


**Figure 2 Amino acid sequence alignment of TM2 and EC1 regions of the receptors for glucagon (rnGLR), GLP1 (rnGLP1), secretin (rnSCRC) and VIP (hsVPAC$_1$ and hsVPAC$_2$)**

The residues that differ in the glucagon receptor from the other receptors in TM2 and at the TM2/EC1 junction are in italic and underscored. The boxes indicate the residues in the glucagon receptor that were mutated in this study. Numbering is relative to the protein sequence of the rat glucagon receptor (Swiss-Prot accession number P30082 [6]). Sequence alignment was generated using the PileUp algorithm from the Wisconsin Package Version 10.2. rn, *Rattus norvegicus*; hs, *Homo sapiens*.

**Table 1 Binding studies: $-\log IC_{50}$**

The unlabelled peptide concentration necessary for 50% tracer binding inhibition ($IC_{50}$) was determined by non-linear regression (GraphPad Prism software). The results are reported on a logarithmic scale ($pIC_{50} = -\log IC_{50}$), and the errors are S.D. N.D., not determined, due to insufficient specific binding; Gl, glucagon.

| | $pIC_{50}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Glucagon receptor | Glucagon | Asp$^3$-Gl | Asn$^3$-Gl | Glu$^3$-Gl | Glu$^9$-Gl | Asp$^3$/Glu$^9$-Gl | Lys$^{15}$-Gl | Lys$^{21}$-Gl |
| Wild-type | 8.1 ± 0.1 | 6.7 ± 0.1 | 7.1 ± 0.1 | 6.6 ± 0.1 | 7.6 ± 0.1 | 7.0 ± 0.1 | 6.0 ± 0.1 | 7.4 ± 0.1 |
| K188R | 8.1 ± 0.1 | 6.7 ± 0.1 | 7.5 ± 0.2 | 6.7 ± 0.3 | 7.4 ± 0.1 | 6.8 ± 0.1 | 6.6 ± 0.1 | 8.3 ± 0.1 |
| I195K | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| K188R/I195K | 8.1 ± 0.1 | 8.2 ± 0.1 | 7.6 ± 0.1 | 8.2 ± 0.1 | 7.4 ± 0.1 | 6.8 ± 0.1 | 6.0 ± 0.1 | 7.5 ± 0.2 |

tions. Briefly, the rat glucagon-receptor coding region [6], inserted into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.), was submitted to 22 cycles of PCR (95 °C for 30 s, 54 °C for 1 min and 68 °C for 14 min) in a reaction volume of 50 $\mu$l. The forward and reverse primers were complementary and contained the desired nucleotide changes (shown here as lower-case letters), flanked on either side by 13–15 perfectly matched nucleotides (shown as upper-case letters; only the forward primers are shown): K188R, 5′-GTCCTTCGTGCTCAgGGCTGGCTCTGTGC-3′; I195K, 5′-GCTCTGTGCTGGTCAagGATTGGCTGCTCAAG-3′; K188R/I195K, 5′-GTCCTTCGTGCTCAgGGCTGGCTCTGTGCTGGTCAagGATTGGCTGCTCAAG-3′.

Following PCR, 10 $\mu$l of the reaction mixture was removed and analysed by agarose-gel electrophoresis, and the remaining 40 $\mu$l was digested by 1 $\mu$l (10 units) of *Dpn*I restriction enzyme (Stratagene) for at least 2 h at 37 °C to remove the parental methylated DNA. The digested PCR products were transformed into TOP10 One Shot competent *Escherichia coli* cells (Invitrogen). Miniprep plasmid DNA was prepared from several colonies and verified by agarose-gel electrophoresis [7], of which three were retained and purified further on Qiaquick PCR purification spin columns (Qiagen, Hilden, Germany). The mutations were then screened using an automated DNA-sequencing apparatus with the BigDye Terminator Sequencing Prism kit from ABI (PerkinElmer, Foster City, CA, U.S.A.). Plasmid DNA from one clone, presenting the correct mutation, was prepared using a midiprep endotoxin-free kit (Stratagene), of which 20 $\mu$g was electroporated (Electroporator II; Invitrogen) into wild-type Chinese hamster ovary (CHO) K1 cells. The complete nucleotide sequence of the receptor coding region was established by DNA sequencing. Selection was carried out in culture medium [Ham's F12/Dulbecco's modified Eagle's medium (1:1), containing 10% fetal calf serum, 1% penicillin (10 m-units/ml), 1% streptomycin (10 $\mu$g/ml) and 1% L-glutamine (200 mM)] (Life Technologies, Paisley, Scotland, U.K.), supplemented with 700 $\mu$g of G418/ml of culture medium. After 10–15 days of selection, isolated colonies were transferred to 24-well microtitre plates, grown until confluence, trypsinized and expanded further in 6-well microtitre plates. For each mutation, 24 recombinant cell clones were screened by testing the ability of 10 $\mu$M glucagon or the Asp$^3$-glucagon mutant to stimulate membrane adenylate cyclase activity.

### Membrane preparation

Membranes were prepared from scraped cells, pelleted and then lysed in 1 mM $NaHCO_3$ solution followed by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 4 °C for 10 min at 400 ***g*** and then the supernatant was centrifuged for a further 15 min at 20000 ***g***. The pellet was resuspended in 1 mM $NaHCO_3$ and used immediately as a crude membrane fraction.

### Binding studies

Binding studies were performed as described previously [8], using [$^{125}$I]glucagon (receptor grade with a radiospecific activity of 81.4 TBq/mmol; NEN Life Science, Boston, MA, U.S.A.). In all cases, the non-specific binding was defined as residual binding in the presence of 1 $\mu$M glucagon. Binding was performed at 25 °C in a total volume, per assay, of 120 $\mu$l, containing buffer [20 mM Tris/maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin and 1% BSA (pH 7.4)] and 3–30 $\mu$g of protein. Bound and free radioactivity were separated by filtration through glass-fibre GF/C filters pre-

soaked for 24 h in 0.01 % polyethyleneimine and rinsed three times with a 20 mM sodium phosphate buffer (pH 7.4) containing 1 % BSA.

### Adenylate cyclase activity

Adenylate cyclase activity was determined by the procedure of Salomon et al. as described previously [9]. Membrane proteins (3–15 $\mu$g) were incubated in a total volume of 60 $\mu$l containing 0.5 mM [$\alpha$-$^{32}$P]ATP, 10 $\mu$M GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phosphoenolpyruvate, 30 $\mu$g/ml pyruvate kinase and 30 mM Tris/HCl at a final pH of 7.8.

### Peptide synthesis

The peptides used were synthesized in our laboratory as described in [10,11]. Peptide purity was assessed by capillary electrophoresis, and conformity by electrospray MS.

### Statistics

All competition curves and dose–response curves were analysed by non-linear regression (using GraphPad Prism software). The differences between the $IC_{50}$, $EC_{50}$ and efficacy values were tested for statistical significance by Student's $t$ test; $P < 0.05$ was accepted as being significant.

## RESULTS

### Interaction of glucagon and mutants with the rat wild-type recombinant glucagon receptor expressed in CHO cells

The cell line used expressed $10.0 \pm 3.2$ pmol of glucagon receptors/mg of protein, as assessed by analysis of [$^{125}$I]glucagon/glucagon competition curves assuming that glucagon and [$^{125}$I]glucagon have the same affinity for the receptors [12]. As shown previously [13], the glucagon concentration necessary for half-maximal inhibition of [$^{125}$I]glucagon binding was significantly lower than the concentration necessary for half-maximal activation of adenylate cyclase (Tables 1 and 2, Figures 3A and 4A). This may have been due to a reduction of glucagon's affinity by GTP, present in the adenylate cyclase incubation medium but not in the binding-assay buffer [13]. Replacement of glucagon's Gln$^3$ by Glu$^3$, Asn$^3$ or Asp$^3$ [14] and of Asp$^9$ by Glu$^9$ [15] decreased the affinity, potency and efficacy of the analogues for the wild-type receptor (Tables 1 and 2, Figures 3A and 4A). The double mutant Asp$^3$/Glu$^9$-glucagon [14] behaved as a glucagon antagonist on the wild-type receptor (Tables 1 and 2, Figures 3A and 4A). The Lys$^{15}$-glucagon analogue probably behaved as a full glucagon-receptor agonist (as extrapolated by non-linear regression analysis, see Figures 3A and 4A), albeit with a much lower affinity/potency than glucagon, whereas the Lys$^{21}$-glucagon analogue had a normal efficacy and potency at the wild-type glucagon receptor (Tables 1 and 2).

### Analysis of the mutated K188R, I195K and K188R/I195K glucagon receptors

Mutations were performed on residues located in the second transmembrane helix (Figure 2) of the rat glucagon receptor. The CHO cells expressing the mutated receptors were selected by screening Geneticin-resistant clones for the capacity of 10 $\mu$M glucagon or 10 $\mu$M Asp$^3$-glucagon to increase adenylate cyclase activity. We then performed [$^{125}$I]glucagon-binding assays and dose–response curves of adenylate cyclase stimulation in the

**Table 2 Functional studies: agonist $pEC_{50}$ or antagonist $pIC_{50}$ and efficacy as a percentage of the glucagon $E_{max}$**

Transposition of the adenylate cyclase dose–response curves in $pEC_{50}$ ($-\log EC_{50}$) or $pIC_{50}$ ($-\log IC_{50}$). Values were generated by the GraphPad Prism analysis program. The maximum over-basal adenylate cyclase activation ($E_{max}$; the maximum amount of cAMP produced after the basal level has been subtracted) for each peptide is also reported (in parentheses), considering in each experiment the maximal over-basal effect of glucagon as 100 %. Asp$^3$/Glu$^9$-glucagon did not activate adenylate cyclase through these sites, and so antagonist inhibition constants ($pIC_{50}$) are given. GI, glucagon. All errors are S.D.

| | $pEC_{50}$ or $pIC_{50}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Glucagon receptor | Glucagon | Asp$^3$-GI | Asn$^3$-GI | Glu$^3$-GI | Glu$^9$-GI | Asp$^3$/Glu$^9$-GI | Lys$^{15}$-GI | Lys$^{21}$-GI |
| Wild-type | $7.4 \pm 0.1$ (100 %) | $6.1 \pm 0.2$ ($12 \pm 5$ %) | $6.2 \pm 0.1$ ($25 \pm 4$ %) | $6.0 \pm 0.3$ ($22 \pm 4$ %) | $7.3 \pm 0.1$ ($30 \pm 6$ %) | $6.5 \pm 0.3$ (0 %)* | $5.3 \pm 0.2$ ($56 \pm 8$ %) | $6.9 \pm 0.1$ ($100 \pm 4$ %) |
| K188R | $7.1 \pm 0.2$ (100 %) | $5.8 \pm 0.1$ ($18 \pm 4$ %) | $6.2 \pm 0.2$ ($45 \pm 5$ %) | $5.5 \pm 0.3$ ($20 \pm 7$ %) | $7.2 \pm 0.5$ ($10 \pm 6$ %) | $6.7 \pm 0.3$ (0 %)* | $5.6 \pm 0.3$ ($85 \pm 20$ %) | $7.3 \pm 0.1$ ($95 \pm 5$ %) |
| I195K | $6.0 \pm 0.2$ (100 %) | $5.6 \pm 0.1$ ($70 \pm 15$ %) | $5.5 \pm 0.6$ ($60 \pm 18$ %) | $6.5 \pm 0.3$ ($45 \pm 18$ %) | $6.9 \pm 0.5$ ($12 \pm 8$ %) | $5.0 \pm 0.3$ (0 %)* | < 5.0† | $6.3 \pm 0.6$ ($100 \pm 15$ %) |
| K188R/I195K | $6.6 \pm 0.1$ (100 %) | $6.9 \pm 0.1$ ($110 \pm 17$ %) | $6.8 \pm 0.2$ ($100 \pm 14$ %) | $6.8 \pm 0.1$ ($100 \pm 16$ %) | $5.6 \pm 0.2$ ($20 \pm 10$ %) | $5.7 \pm 0.2$ ($36 \pm 10$ %) | $4.6 \pm 0.6$ ($63 \pm 60$ %) | $6.1 \pm 0.1$ ($95 \pm 7$ %) |

* $pIC_{50}$ values are given.
† Inactive up to 10 $\mu$M.

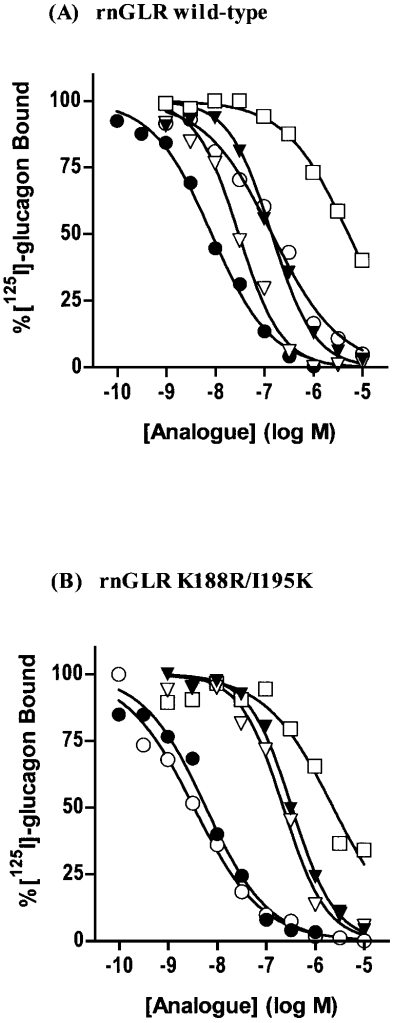


**Figure 3 Competitive binding curves**

Competitive binding curves, with [$^{125}$I]glucagon as a tracer, by glucagon (●), Asp$^3$-glucagon (○), Glu$^9$-glucagon (▽), Lys$^{15}$-glucagon (□) and Asp$^3$/Glu$^9$-glucagon (▼) to the wild-type glucagon receptor (**A**) or the double mutant K188R/I195K glucagon receptor (**B**). Data are expressed as the percentage of specific [$^{125}$I]glucagon bound in the absence of a competing peptide. Non-linear regression curves were generated by GraphPad Prism software. rnGLR, *Rattus norvegicus* glucagon receptor.

presence of glucagon and the various mutants. The selected cell lines used expressed $9.2 \pm 5.9$ pmol of K188R mutant glucagon receptors/mg of protein, and $9.5 \pm 4.9$ pmol of K188R/I195K double-mutant glucagon receptors/mg of protein, as assessed by analysis of [$^{125}$I]glucagon/glucagon competition curves.

As summarized in Tables 1 and 2, and in Figure 4(B), the binding and functional properties of the K188R glucagon receptor were not significantly different from those of the wild-type receptor. In contrast, the I195K mutation yielded a significantly lower potency for glucagon, affecting to a lesser extent the modified ligands Asp$^3$-, Asn$^3$- and Glu$^3$-glucagon. Furthermore, their efficacies increased with respect to glucagon (Tables 1 and 2, Figure 4C). Although [$^{125}$I]glucagon binding to the I195K glucagon receptor was detectable, it was insufficient to allow valid analysis of competition curves (non-specific binding represented 70–80 % of the bound radioactivity). This was probably due to the marked decrease in the affinity of this construct for glucagon, as the peptide's $EC_{50}$ value at the mutated receptor was at least 20-fold greater than at the wild-type receptor (Table 2).

Remarkably, however, the double mutant K188R/I195K glucagon receptor recognized [$^{125}$I]glucagon with a high affinity (Tables 1 and 2, Figure 3B). This mutant receptor was not only activated with a higher affinity by Asp$^3$- and Glu$^3$-glucagon than by the natural Gln$^3$-glucagon peptide, but Asp$^3$-, Glu$^3$- and Asn$^3$-glucagon behaved towards it as full agonists and Asp$^3$/Glu$^9$-glucagon acted as a partial agonist (Tables 1 and 2, Figure 4D). Furthermore, the significant changes in the receptor's functional profile were specific for the mutations in position 3 of the ligand. Indeed the efficacies of the other analogues Glu$^9$-, Lys$^{15}$- and Lys$^{21}$-glucagon were similar on both the wild-type and the mutated receptors, with the affinities of Glu$^9$-, Lys$^{15}$- and Lys$^{21}$-glucagon unaffected by the double mutation of the receptor (Tables 1 and 2).

## DISCUSSION

The specificities of glucagon and GLP1 for their respective receptors are carried by the glucagon N-terminal and the GLP1 C-terminal sequences, respectively: a glucagon/GLP1 hybrid peptide recognizes both receptors with high affinity [16]. Functional studies of a chimaeric glucagon/GLP1 receptor indicated that high-affinity glucagon recognition requires the transmembrane glucagon receptor domains [and, in particular, the TM2 and first extracellular loop (EC1) region], whereas high-affinity GLP1–receptor binding necessitates the N-terminal extracellular GLP1-receptor domain [17]. Altogether, these results suggest that, like PACAP, VIP and secretin, the C-terminal glucagon region anchors the peptide on the N-terminal receptor domain and facilitates the interaction between the N-terminal peptide sequence and the transmembrane receptor domain, thereby triggering receptor activation.

In solution, glucagon adopts a predominantly extended, flexible conformation [18]. Formation of an $\alpha$-helix in the central and C-terminal peptide region is nevertheless highly probable [19], and indeed observed in the crystal structure [20], as well as in contact with lipid micelles [21]. It is therefore plausible that glucagon, like PACAP [22], forms in contact with its receptor a $\beta$-coil structure, followed by an $\alpha$-helix.

Most members of the secretin-receptor family share two basic residues in the TM2 and EC1 regions. An arginine or lysine residue is found in the second transmembrane helix of all but the calcitonin receptor, and, with the exception of the receptors for glucagon, GRF and CRF, all the secretin-receptor family possess a second basic residue (lysine, arginine or histidine) close to the TM2/EC1 junction.

We concluded from our previous work on the secretin receptor [2,23] and the VIP/PACAP 1 and 2 receptors ($VPAC_1$ and $VPAC_2$) [4,5] that the TM2 arginine residue (equivalent to Lys$^{188}$ in the glucagon receptor) undoubtedly has the ability to interact directly with the agonist's Asp$^3$ side chain. We also suggested that the lysine residue found at the TM2/EC1 boundary (equivalent to Ile$^{195}$ in the glucagon receptor) facilitates this interaction, probably by stabilizing the activated receptor conformation that requires an Asp$^3$ residue for high-affinity agonist recognition [4,5].

Glucagon differs from VIP, PACAP and secretin at position 3, with a Gln$^3$ instead of an Asp$^3$. Therefore, we investigated whether the Gln$^3$ residue of glucagon is in contact with Lys$^{188}$ and Ile$^{195}$ in the glucagon receptor by converting them into either one or both of the equivalent basic residues found in the $VPAC_1$, $VPAC_2$ and secretin receptors.

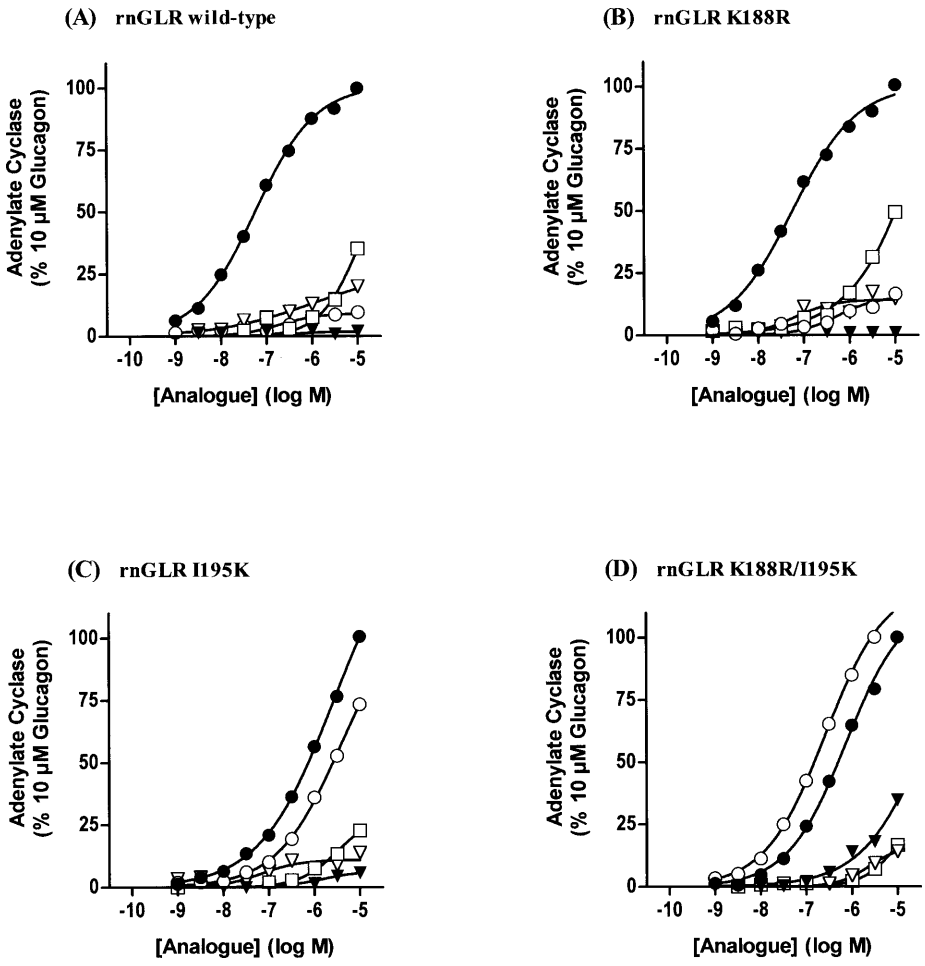


**Figure 4 Adenylate cyclase activation dose–response curves**

Dose–response curves are shown for glucagon (●), Asp$^3$-glucagon (○), Glu$^9$-glucagon (▽), Lys$^{15}$-glucagon (□) and Asp$^3$/Glu$^9$-glucagon (▼) activation of adenylate cyclase through the wild-type glucagon receptor (**A**), and the K188R (**B**), I195R (**C**) and K188R/I195K (**D**) mutant glucagon receptors. Data are represented as the percentage of the response obtained in each experiment by 10 $\mu$M glucagon. Non-linear regression curves were generated by GraphPad Prism software. rnGLR, *Rattus norvegicus* glucagon receptor.

It is not obvious from the present data that the glucagon Gln$^3$ residue recognizes the 'equivalent' binding site in its cognate receptor. Indeed, an arginine residue in the VIP and secretin receptors is essential for recognition of the Asp$^3$ side chain of the respective ligands, whereas, in contrast, introducing an arginine residue into the glucagon receptor did not affect the mutant receptor's pharmacological properties. On the other hand, in the I195K mutant glucagon receptor (as in the secretin, $VPAC_1$ and $VPAC_2$ receptors), the introduction of an arginine residue two helix turns below the lysine residue (I195K/K188R) improved principally the recognition of Asp$^3$-glucagon: in this mutated glucagon receptor, the arginine residue did seem able to interact preferentially with an Asp$^3$ residue.

It is interesting to note that the introduction of a positive charge at the TM2/EC1 boundary in the glucagon receptor (I195K) had the same effect as its removal from the secretin, $VPAC_1$ and $VPAC_2$ receptors (K195I $VPAC_1$ receptor, K179I $VPAC_2$ receptor and K173I secretin receptor): the natural ligand's potency decreased markedly (Figure 4C). This single mutation may be the reason for the very low affinity of glucagon for the two glucagon/GLP1 receptor chimaerae with a GLP1-receptor EC1 domain [17]. The receptor's ability to discriminate between glucagon analogues with uncharged and negatively charged residues in position 3 decreased markedly (Table 2 and Figure 4C). To ensure that the mutated I195K receptor did retain its ability to discriminate high- from low-affinity ligands as well as full from partial agonists, we tested the binding and functional properties of three other glucagon analogues: Lys$^{21}$-glucagon, which behaved like glucagon at the wild-type receptor, Lys$^{15}$-glucagon, a full agonist with a significantly decreased affinity for the wild-type receptor, and Glu$^9$-glucagon, with a markedly decreased efficacy at the wild-type receptor. As the effects of mutating glucagon's Asp$^9$ to Glu$^9$, Asp$^{15}$ to Lys$^{15}$ and Asp$^{21}$ to Lys$^{21}$ on the affinity and efficacy on the wild-type and I195K mutant receptor were similar (Table 2 and Figure 4C), the hypothesis that this mutation did selectively affect the recognition of position 3 of glucagon was strengthened further. Taken together, these results therefore suggest that I$^{195}$ is in very close proximity to the glucagon Gln$^3$ side chain. The receptors' ability to discriminate Gln$^3$- and Asn$^3$-glucagon decreased in the mutated I195K receptor, suggesting that the I$^{195}$ side chain interacted with the alkyl (-$CH_2$-$CH_2$-) part of the Gln$^3$ side chain.

The K188R and I195K double mutation did not affect the receptors' affinity for glucagon, but increased markedly the

affinity, potency and efficacy of the $Asp^3$-, $Glu^3$- and $Asn^3$-glucagon analogues. In contrast, the effects of glucagon mutations at positions 9, 15 and 21 on the peptide's affinity and efficacy were similar at both the wild-type and K188R/I195K mutant receptors (Table 2 and Figure 4C): the effect of the double mutation seemed restricted to the recognition of the glucagon side chain found at position 3. The effects of the two single mutations were not additive, suggesting that the two amino acids were close to one another, and that interaction of glucagon with the Lys or Arg at position 188 depended on the nature (either Ile or Lys) of the amino acid in position 195. It therefore seems plausible that a positively charged residue in position 195 is necessary to form a primary interaction with negatively charged side chains ($Asp^3$ or $Glu^3$), and subsequently facilitate their transit down to the first TM2 basic residue (Lys or Arg at position 188).

It is interesting to note that the efficacy of $Asp^3$-glucagon (i.e. its maximal response) increased significantly in the I195K and K188R/I195K mutated receptors. The equivalent basic amino acids have indeed been implicated in the activation of the PTH [24], secretin [2,23], $VPAC_1$ [4], and $VPAC_2$ [5] receptors. The PTH1 receptor TM2 residue $Arg^{233}$ (equivalent to the glucagon receptor $Lys^{188}$ position) is thought to interact in the activated receptor with a highly conserved Gln residue in TM7, $Gln^{451}$, this interaction being essential for receptor activation. We therefore suggest that, in the wild-type glucagon receptor, $Ile^{195}$ shields $Lys^{188}$ from the extracellular medium, thereby forcing it indirectly to interact with its correct partner in the activated receptor. In the I195K mutant receptor, the $Lys^{195}$ side chain facilitated the introduction of the glucagon side chain at position 3 down into the transmembrane region. As a result, $Lys^{188}$ was able to 'choose' between the peptide and its legitimate partner in the receptor, and the glucagon–receptor complex assumed an inappropriate conformation. In contrast, in the K188R/I195K mutant receptor, the two-pronged arginine residue was capable of interacting simultaneously with the peptide and its legitimate receptor partner, thereby allowing the receptor to retain its normal active conformation, and allowing efficient receptor activation by $Asp^3$- and $Glu^3$-glucagon derivatives.

In conclusion, our results suggest that, in the glucagon receptor, $Ile^{195}$ interacted with the $Gln^3$ side chain, and shielded the positive charge of $Lys^{188}$. In the double mutant K188R/I195K, the Lys side chain facilitated the transit of negatively charged residues in position 3 of glucagon down to $Arg^{188}$, and was therefore essential for efficient and potent receptor activation by $Asp^3$- and $Glu^3$-glucagon derivatives. Moreover, we suggest also that two residues in the TM2/EC1 region form a key domain for ligand recognition and receptor activation in most members of the class B (or secretin) subfamily of G-protein-coupled receptors.

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