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Insights into agonist-elicited activation of the human glucose-dependent insulinotropic polypeptide receptor

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Abstract

Glucose-dependent insulinotropic polypeptide (GIP) and its receptor (GIPR) are part of the incretin system that regulates insulin release and glucose homeostasis. The GIPR is a class B1 G protein-coupled receptor (GPCR). In this study, we mutated a series of GIPR residues putatively important for ligand binding and receptor activation. These mutations were pharmacologically evaluated using GIPR selective agonists in cAMP accumulation, ERK1/2 phosphorylation (pERK1/2) and β-arrestin 2 recruitment assays. The impact of mutation on ligand efficacy was determined using operational modelling of pharmacological data for each mutant, with results mapped onto a full-length, active-state, GIPR model. The data revealed two key interaction networks, comprising transmembrane (TM) 7, TM1 and TM2, and extracellular loop (ECL) 3, TM5 and ECL2, respectively, which were important for peptide efficacy. Equivalent networks have previously hypothesized to be important for efficacy and biased agonism of related class B1 GPCRs. Both networks were critical for Gs-mediated cAMP signaling and the recruitment of β-arrestin 2, however, cAMP signaling was more broadly sensitive to mutation, with most residues displaying reduced signaling. Unlike the other two assays, activation of ERK1/2 was largely independent of the network between ECL3/TM5/ECL2 indicating that pERK1/2 is at least partially distinct from Gs or β-arrestin signaling pathways and indicating that this network is also critical for potential biased agonism of GIPR agonists. Collectively, our work advances understanding of structure-function of GIPRs and provides a framework for the design and/or interpretation of GIP analogues with novel signaling and regulation.

Keywords: Glucose-dependent insulinotropic polypeptide receptor, G protein-coupled receptor, GPCR structure-function, ERK, cAMP, arrestin

1. Introduction

Glucose-dependent insulinotropic polypeptide (GIP) and its receptor (GIPR) are part of the incretin system that controls insulin secretion following meal ingestion to regulate blood glucose. GIP is now recognized as having broad actions beyond the pancreas, including fat tissues, the central nervous system and bone [1-3]. As such, there has been substantive interest in the potential to target GIPRs to treat metabolic diseases. However, GIPRs are downregulated in diabetic patients and there is controversy in how to best pharmacologically target the receptor with both agonism and antagonism of GIPR having been claimed as beneficial to treat metabolic diseases [4, 5]. To date, dual agonists that target both the GIPR and the glucagon-like peptide-1 receptor (GLP-1R) have shown the greatest promise in clinical trials [5, 6]. Despite the importance of the GIPR, knowledge on molecular mechanisms of peptide binding and receptor activation is still limited.

As a member of the class B1 (secretin) GPCR subfamily, activation of GIPR is presumed to follow a two-step binding and activation mechanism [7]. In this model, the peptide ligand C-terminus forms initial interactions with the N-terminal extracellular domain (ECD) of the receptor that subsequently orient the N-terminal residues of the ligand to enable productive engagement with the receptor core. This in turn facilitates conformational changes in the receptor required for transducer binding and activation of downstream signaling [8]. Agonist binding to GIPRs promotes recruitment and activation of Gs protein leading to adenylate cyclase mediated cAMP production that activates protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC), key signaling intermediates for GIP-mediated insulin secretion [9]. In pancreatic β-cells, PKA also

activates mitogen-activated protein kinase (MAPK) cascades, including phosphorylation of ERK1/2 that can regulate expression of genes involved in proliferation and apoptosis [10, 11]. GIPR activation may also initiate recruitment of β-arrestin 2 and β-arrestin 1 [12, 13] that can play key roles in receptor desensitization, trafficking and scaffolding of alternate signaling proteins for class B1 GPCRs [14, 15].

Despite the physiological, and potentially therapeutic, importance of GIPR, there is only limited structural or structure-function data available to support mechanistic understanding of how GIP binds and activates the receptor. While crystal structures of the isolated N-terminal ECD of human GIPR with GIP(1–42) [7], human GIPR with Gipg013 Fab [16] and mouse GIPR with monoclonal antibodies [17] have been solved, no full-length structures have been determined. In contrast, multiple structures of the related glucagon receptor (GCGR), GLP-1R and GLP-2R have been determined [18-25]. Similarly, only limited GIPR mutagenesis studies have been performed, with those designed before full-length structures of related class B1 GPCRs became available [26, 27].

In this study, using recent structural data for active GLP-1R [21] and GCGR [18] as a guide, we have performed site-directed alanine mutation of residues predicted to be involved in GIP binding to the receptor core, encompassing the extracellular loops (ECLs) and transmembrane domain (TMD) of human GIPR to explore their importance for ligand binding and receptor activation.

2. Materials and methods

2.1 Peptides

Human GIP(1–42), GIP(1–30)NH_2 and mouse GIP (**Figure 1A**) that were used in the cAMP

accumulation and pERK1/2 assays were synthesized by GL Biochem (Shanghai, China). Human GIP(1–42) used in the β-arrestin 2 recruitment and competition binding assays was purchased from CASLO ApS (Lyngby, Denmark).

2.2 Mutagenesis, cell culture and transfection

The human GIPR incorporating an N-terminal FLAG epitope tag and 12 glycine linker in the pEF5/FRT/V5-Dest destination vector [12] was used as the template (wildtype (WT) receptor). This receptor had equivalent cAMP responses to untagged receptors (pEC_{50} untagged; GIP(1-42), 11.6±0.1, GIP(1-30)NH_2, 11.7±0.1, mouse GIP, 11.1±0.1; FLAG-tagged; GIP(1-42), 11.3±0.1, GIP(1-30)NH_2, 11.3±0.2, mouse GIP, 10.7±0.1; n=4). Mutagenesis was carried out with oligonucleotides incorporating the desired mutation (Genewiz, Suzhou, China) using a Muta-direct site-directed mutagenesis kit (Intronbio, Beijing, China) and confirmed by sequencing (Genewiz). WT and mutant GIPRs were isogenically integrated into FlpIn-human embryonic kidney 293 (HEK293) according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The stably transfected cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin, and 200 μg/mL hygromycin B (Invitrogen), in a humidified incubator at 37°C in 5% CO_2. These cell lines were measured for cell surface receptor expression level and were used for cAMP and pERK1/2 studies.

For the β-arrestin 2 recruitment assay, the HEK293 cells (ATTC; Manassas, VA, USA) were cultured in DMEM (ThermoFisher Scientific), containing 1% GlutaMAX™, and supplemented with 10% FBS and 1% penicillin (180 units/mL)/streptomycin (45 μg/mL), and incubated at 37°C, 10%

CO_2 and 95% air humidity. One day before the transient transfection, HEK293 cells were seeded in tissue culture-treated six-well plates ($0.75 \times 10^6 – 1 \times 10^6$ cells/well), before a polyethylenimine (PEI) transfection was performed. The pcDNA3.1+ plasmids encoding a C-terminally Venus fused construct of human GIPR or mutant GIPR were purchased from GenScript (Piscataway, NJ, USA). The next day, cells were transfected with 0.33 µg human WT or mutant GIPR, 0.042 µg Rluc8-Arrestin3-SP1, 0.8 µg mem-citrine-SH3 and 0.8 µg G protein-coupled receptor kinase 2 (GRK2). The DNA constructs were then mixed with PEI in a ratio of 1:2 (DNA:PEI), non-supplemented DMEM, and incubated at room temperature (RT) for 15 min, before added dropwise to the cells. The transfection was stopped after 24 h by replacing the medium with fresh DMEM medium supplemented as described above.

2.3 cAMP accumulation

HEK293 WT and mutant GIPR expressing cells were grown overnight, harvested and plated in 384-welll plates (3,100 cells/well). Peptide stimulation was performed as previously described [12]. The cAMP response was determined using a LANCE cAMP detection kit (PerkinElmer, Boston, MA, USA) and the data were first converted to absolute cAMP levels using a standard curve before normalized to the response of 100 µM forskolin (Sigma-Aldrich, St. Louis, MO, USA).

2.4 ERK1/2 phosphorylation

HEK293 WT and mutant GIPR cells were grown for 24 h in 96-well culture plates coated with poly-D-lysine (40,000 cells/well) followed by overnight serum deprivation. Optimal ERK stimulation time for each ligand of each mutant was first determined by a 20 min time-course experiment using 1 µM ligand concentration. None of the mutant receptors significantly altered

the kinetic profile of the pERK1/2 response. Concentration-response experiments were then performed at the peak pERK1/2 response time (~10 min). ERK1/2 phosphorylation was detected with an AlphaScreen SureFire ERK1/2 (p-Thr202/Tyr204) assay kit (PerkinElmer) as previously described [12]. Data were normalized to the maximal response elicited by 10% FBS determined at 7 min.

2.5 β-arrestin 2 recruitment

One day post transfection, the transiently transfected HEK293 cells were resuspended in PBS with 1% glucose (5 mM), after which they were divided (85 µL/well) into a white CulturPlate-96 (PerkinElmer). Next, coelenterazine h (Nanolight Technologies, Pinetop, AZ, USA) was added in a final concentration of 5 µM, and the reaction was started after the addition of ligands (concentrations ranging from 0.1 nM to 1 µM). After incubation at RT for 30 min, the luminescence (Rluc 485/40 nm and YFP 530/25 nm) was measured by a LB 940 Mithras Multimode Microplate Reader (Berthold Technologies GmbH & CO. KG, Bad Wildbad, Germany). Data were first baseline-corrected to themselves, after which they were normalized to the maximal response (E_{max}) elicited by WT GIPR.

2.6 Receptor expression

The cell surface expression level of GIPR was determined by anti-FLAG antibody binding to HEK293 cells stably expressing WT or mutant receptor using flow cytometry. Stable WT and mutant expressing HEK293 cells were grown overnight at a density 8×10^5 cells/well of 6-well culture plates. The cells were prepared according to the method of Chang and colleagues [23] and stained with mouse monoclonal anti-FLAG M2 antibody (F3165, 1:300, Sigma-Aldrich) as

primary antibody and Alexa Fluor 488 rabbit anti-mouse antibody (A11059, 1:300, Invitrogen) as the secondary antibody. For each data point, approximately 100,000 cellular events were collected with a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA).

2.7 Radioligand competition binding

2.7.1 Cell culture and transient transfection

COS-7 cells were maintained in DMEM 1885 medium, containing 3.9 g/L $NaHCO_3$, and supplemented with 10% FBS, 1% L-glutamine and 1% penicillin (180 units/mL)/streptomycin (45 µg/mL). One day before transfection, the COS-7 cells (1.2–1.5 $\times$ 10^6 cells) were seeded in 25 cm^2 flasks before a calcium phosphate transfection was performed. Ten µg of human WT or mutant GIPR (or pcDNA3.1 as control) in 120 µL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 2 mM EDTA-Na_2, pH 7.5) was mixed with 15 µL of $CaCl_2$ and then titrated into 120 µL 2 $\times$ HEPES buffered saline (HBS) buffer (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.2). The mixture was then incubated for 45 min at RT. Next, this transfection mixture and 2 mg/mL chloroquine were added to cells. After approximately 5 h, the transfection was terminated by replacing the medium with fresh supplemented DMEM 1885 $NaHCO_3$ medium.

2.7.2 Homologous competitive binding assay

One day before the experiment, the transiently transfected COS-7 cells were seeded in a white 96-Culturplate (5,000-12,500 cells/well for WT GIPR and 45,000 cells/well for mutants). After approximately 24 h, the COS-7 cells were washed two times with binding buffer (50 mM HEPES buffer, pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% (w/v) BSA) and then incubated for 15 min at 5°C. After the addition of 0.1 nM to 1 µM cold GIP(1–42) and 12.8 ± 0.8 pM ^{125}I-GIP(1–42), the plate

was incubated at 5°C for 3 h. The reaction was stopped by washing the plate two times with ice-cold binding buffer. Subsequently, the cells were lysed with 200 mM NaOH containing 1% SDS. The γ radiation intensity was measured with a PerkinElmer 2470 Wizard^2 Automatic Gamma Counter.

2.8 Data analyses

Functional data were analyzed using Prism 6 and 8 (GraphPad, San Diego, CA, USA). The normalization was performed to the maximum response of the respective assay system (forskolin for cAMP or FBS for ERK) and corrected by the vehicle background before presented as a percent of response elicited by WT. The negative log molar concentration eliciting a 50% response (pEC$_{50}$) and E_{max} values were obtained using a three-parameter logistic equation. In addition, efficacy of each mutant was quantified by Black-Leff operational model of agonism modified to directly estimate the transduction ratio [28-30].

$$Y = \frac{E_{max} \times (\tau/K_A) \times [A]}{[A] \times (\tau/K_A) + (1 + (\tau/K_A))}$$

E_{max} is the maximum response of the system, [A] is the concentration of agonist, K_A is the dissociation constant and τ is the operational value for efficacy, a term that incorporates agonist efficacy, receptor density and coupling within the system. With this model, efficacy, directly estimated as the transduction ratio (τ/K_A), can be quantified as a composite of the operational affinity and efficacy of agonist. For cAMP and pERK1/2 assays, estimated τ/K_A values were then corrected to cell surface expression, giving τ/K_Ac, and errors were propagated from both τ/K_A and cell surface expression [28].

Competitive binding data were normalized to the maximum specific response for each peptide, for each individual experiment (B-N)/(B_0-N) and expressed as a percentage for grouped analysis, where B is the bound radioactivity, B_0 is the bound radioactivity in the absence of competing peptide and N is non-specific binding (determined at 1 μM of GIP(1-42)). Data were fit to a three-parameter logistic equation to determine the 50% inhibitory concentration, expressed in log form as the errors are log normally distributed.

Statistical significance of the effect of GIPR mutation was determined using one-way ANOVA with Dunnett's post-test in comparison to the WT control, and significance was accepted at $P<0.05$.

2.9 Full-length, active-state GIPR homology model

The GIPR and GIP sequences were retrieved from the UniProt database [31] (entry P48546 and P09681, respectively) and modelled on GLP-1R:GLP-1 complex 5VAI [21] (sequence similarity ~64%) and the PDB entry 2QKH (GIP in complex with GIPR extracellular domain). Twenty homology models were generated with Modeller 9.19 [32], and the one with the higher DOPE score was energy minimized using Chimera [33] first, and then Maestro (Schrödinger Release 2020-1: Maestro, Schrödinger, LLC, New York, NY, USA, 2020) to re-optimize the geometry of planar side chains. The Gs protein was added by superimposition to the PDB entry 6E3Y [34] and energy minimized as reported above.

3. Results

A full-length, active-state, homology model of the GIPR was generated from GLP-1R structures

available at commencement of this study [21] and 34 residues predicted to be proximal to GIP, located within either TM helices or ECLs, were selected for mutation (**Figure 1B-1D**). Residues are labeled based on their location number in the protein sequence with Wootten class B numbering in superscript [35]. To enable parallel evaluation of the effects of mutations on cell surface expression of GIPR and function, an N-terminally FLAG-tagged form of the receptor was used (referred to as WT GIPR). WT and mutant receptors were stably expressed, with isogenic integration into FlpIn-HEK293 cells, for assessment of radioligand competition binding, cAMP accumulation and ERK1/2 phosphorylation. β-arrestin 2 recruitment assays were performed using WT or mutant GIPR-Venus constructs transiently transfected into HEK293 cells. The operational model of Black & Leff [29] was used to calculate transduction ratios (log τ/K_A) to quantify the relative effect of mutations on GIPR function for each of the pathways assessed.

3.1 Effect of binding pocket mutation on cell surface expression of GIPR

The effect of mutation on cell surface expression of GIPR was determined by FACS analysis of anti-FLAG antibody binding to the N-terminal FLAG epitope on the GIPR. All mutants were trafficked to the cell surface, with most having either no effect or <50% reduction in surface expression. Greatest impact was seen for $Y145^{1.47}A$ in TM1, $R183^{2.60}A$ and $R190^{2.67}A$ in TM2, P195A in ECL1 and $T223^{3.36}A$, $V227^{3.40}A$ and $N230^{3.43}A$ in TM3 that each exhibited >60% reduction in cell surface expression (**Figure 2A-2C, Table 1**).

3.2 Effect of GIPR binding pocket mutation on GIP(1-42) binding affinity

The effect of putative binding pocket mutations on GIP(1-42) affinity was determined by competitive inhibition of ^{125}I-GIP(1-42) binding to WT or mutant receptors in whole cell binding

assays (**Figure 3A, 3B, Table 2**). Despite WT levels of cell surface expression, no estimate of affinity was obtained for either the Y141$^{1.43}$A or I187$^{2.64}$A mutant due to very low specific binding. Most other mutants had only limited (<3-fold reduction in *p*IC_{50}) or no significant effect on GIP(1-42) affinity. Mutated residues that impacted affinity (**Figure 3B, Table 2**) localized to three main clusters in 3-dimensional (3D) space (**Figure 3C-3E**). The TM1/TM2 interface that included Y141$^{1.43}$A, Y145$^{1.47}$A, I187$^{2.64}$A and R190$^{2.67}$A, the ECL2 interaction face with the peptide, E288^{ECL2}A, R289^{ECL2}A and N290^{ECL2}A, and the glutamic acid residues of ECL3, E362^{ECL3}A and E363^{ECL3}A that interconnect with R300$^{5.40}$A. In addition, there was reduced affinity with mutants deep in the binding pocket, H353$^{6.52}$A, S381$^{7.46}$A and Q384$^{7.49}$A that contribute to a relatively conserved class B1 GPCR central polar network observed in inactive state structures [27, 36]. Discontinuous from these networks, one of the mutations with the largest reduction in affinity was Y231$^{3.44}$A (**Figure 3C-3E**). While not evident in the homology model, it is expected that this residue would form polar interactions with N-terminal peptide residues, including Y1^{G} and potentially E3^{G} (the superscript “G” refers to amino acids in the GIP peptide).

3.3 Effect of GIPR binding pocket mutation on peptide-mediated cAMP accumulation

The GIPR is canonically coupled to Gs-mediated production of cAMP and GIP peptides have highest potency for this pathway. We have previously reported that both GIP(1-42), the prohormone convertase 2 (PC2) cleaved form of proGIP, GIP(1-30)NH_2 and mouse GIP are all potent agonists of GIPR-mediated cAMP accumulation [12]. As such, we investigated the effect of putative binding pocket mutations on cAMP responses for all 3 peptides (**Figure 4, Figure 5A-5C, Table 1, Table 2**).

To quantify the relative effects of mutations on receptor function, the concentration-response data were fit to an operational model of agonism [29] with the resultant transduction ratios (log τ/K_A) corrected for cell surface expression (log τ/K_Ac) and analyzed for significant differences from the WT receptor (**Figure 5A-5C, Table 2**). The pattern of effect of mutations on cAMP signalling was essentially equivalent for all three GIP peptides (**Figure 5A-5C, Table 2**), and as such only the effects on GIP(1-42) are described below.

With the exception of GIPR residues in ECL1 (P195A, R196A) and selected residues in TM6 (E354$^{6.53}$A) and TM7 (S381$^{7.46}$A, Q384$^{7.49}$A), all putative binding pocket mutations had detrimental effects on GIP(1-42)-mediated cAMP production, ranging from <10-fold to >100-fold (**Table 2**). The greatest reductions (>100-fold) were observed for two networks of residues that reside in close proximity in 3D space. The first network was comprised of mutants in TM1 (Y141$^{1.43}$A) and TM2 (R183$^{2.60}$A, I187$^{2.64}$A, R190$^{2.67}$A) that interconnect TM1 and TM2, with latter residues extending to the base of the peptide binding pocket. The second network comprised residues that extended from TM7/ECL3 (L374$^{7.39}$A, R370$^{7.35}$A, E362^{ECL3}A, E363^{ECL3}A) to the top of TM5/ECL2 (N290^{ECL2}A, W296$^{5.36}$A, I299$^{5.39}$A, R300$^{5.40}$A) (**Figure 6A-6D**). Moderate to large reductions (>10-fold) in transduction ratios were observed for Y145$^{1.47}$A, V227$^{3.40}$A, N230$^{3.43}$A, Y231$^{3.44}$A, R289^{ECL2}A, F357$^{6.56}$A, F371$^{7.36}$A, K373$^{7.38}$A, E377$^{7.42}$A and I378$^{7.43}$A, while 3- to 10-fold decreases were seen for Q138$^{1.40}$A, L194$^{2.71}$A, T223$^{3.36}$A, Q224$^{3.37}$A, E288^{ECL2}A, I303$^{5.43}$A and H353$^{6.52}$A (**Figure 5A-5C, Figure 6B-6D, Table 2**).

3.4 Effect of GIPR binding pocket mutation on GIP(1-42)-mediated phosphorylation of ERK1/2

GIP(1-42) concentration-response curves for pERK1/2 were established at the peak response time following acute stimulation (~10 min) which was not different between the WT and mutant GIPRs. Four of the mutants Y145$^{1.47}$A, P195^{ECL1}A, T223$^{3.36}$A and V227$^{3.40}$A that had very low cell surface expression (**Figure 2**) did not display a robust response even at the highest concentration of 1 μM, while R190$^{2.67}$A and R300$^{5.40}$A had low potency responses that could not be confidently fit to the model and were not quantified (**Figure 7, Table 1, Table 2**).

Of the remaining mutants, only Y141$^{1.43}$A, R183$^{2.60}$A, I187$^{2.64}$A, R290^{ECL2}A and F357$^{6.56}$A had significant effects, exhibiting ~10-fold reductions in transduction ratios (**Figure 5D, Table 2**). Changes in signaling of less than 10-fold from WT were not significant due to the higher variance in the pERK1/2 assay, compared to cAMP data, although trends of reduced signaling were observed for residues in TM5, while the E354$^{6.53}$A mutant trended towards higher efficacy, despite lower cell surface expression compared to WT (**Figure 5D, Figure 7, Table 1, Table 2**).

3.5 Effect of GIPR binding pocket mutation on GIP(1-42)-mediated β-arrestin 2 recruitment

Peptide-mediated recruitment of β-arrestin 2 to WT or mutant GIPR was monitored by bioluminescence resonance energy transfer (BRET). To enhance the signal to noise of the response, GRK2 that increases C-terminal GIPR phosphorylation and arrestin recruitment [37] was co-transfected with the GIPR-Venus and β-arrestin 2-Rluc8 constructs into HEK293 cells. Unlike cAMP accumulation and pERK1/2 assays, the arrestin recruitment assay has no amplification. Moreover, the ligand-induced change in BRET should be independent on the absolute expression of receptor. To confirm that this was the case, pilot experiments with the WT receptor and I303$^{5.43}$A mutant were performed where the level of receptor transfected was titrated

4-fold (1 ×, 0.5 ×, 0.25 ×). In both cases, despite marked differences in the induced BRET signal for the WT and mutant receptors, there was no effect from reduced expression (**Figure 8A**). Consequently, no correction was applied to the operationally derived signal transduction ratios.

Multiple mutants led to either an abrogation of specific signal or data of low signal that was insufficiently robust to confidently quantify the response. With the exception of Q138$^{1.40}$A and L194$^{2.71}$A that were not different from WT, this included all mutants in TM1 and TM2, as well as N290^{ECL2}A, and all TM5 residues except for I303$^{5.43}$A that was equivalent to WT (**Figure 5E, Figure 8B, Table 2**). These mutants were among those most detrimental to signaling in cAMP accumulation assays (**Figure 5A**). There were also significant reductions in transduction ratios for T223$^{3.36}$A, V227$^{3.40}$A and Y231$^{3.44}$A in TM3, and for peptide proximal residues in TM6 (F357$^{6.36}$A), ECL3 (E362A, with E363A reduced but not significantly different), and TM7 (R370$^{7.35}$A, L374$^{7.39}$A). The effect of the I378$^{7.43}$A mutant could not be quantified due to high variability in the data, and all other mutants lacked significant effects on β-arrestin 2 recruitment, albeit that E354$^{6.53}$A trended towards increased response, similar to the effect in pERK1/2 assays (**Figure 5D, Figure 8B, Table 2**).

4. Discussion

The GIPR plays a key role in metabolism and is increasingly recognized as a potential therapeutic target for type 2 diabetes and obesity, particular for co-agonists of GIPR and the GLP-1R or GLP-1R and GCGR [5, 38, 39]. As such, understanding how the GIPR binds and is activated by peptide ligands is fundamental to advancing next generation therapeutics. However, currently there are no structures of the TM core of GIPR that is the allosteric conduit for GIP signaling. Similarly,

there is very limited structure-function mutagenesis work that can provide insight into how the GIPR translates GIP binding to different signaling and regulatory sequalae. Recent progresses in structural determination of the related GLP-1R and GCGR allowed us to probe the function of 34 GIPR residues predicted to be proximal to the N-terminal activation domain of GIP peptides. We have interpreted our results in the context of a 3D model of the GIPR built from the related GLP-1R. However, GIP uniquely contains Tyr at the N-terminus (His in GLP-1 or glucagon peptides) and this likely limits the accuracy of the homology model with respect to interactions deep within the receptor core. As such, we have concentrated on interpretation of patterns of mutational effects that are linked to conserved regions of the peptides and receptors.

Like all class B1 GPCRs, Gs-mediated production of cAMP is the most efficiently coupled second messenger pathway downstream of GIPR activation, critical to the physiological signaling of the receptor [9, 40]. In the current study, this pathway is highly amplified providing robust responses even for mutants with low cell surface expression. To quantify the effect of mutations on GIPR function, we have applied operational modelling [29] to derive the transduction ratio τ/K_A, where τ is the operational value for efficacy and K_A is the functional affinity of the ligand for the pathway under investigation. Importantly, the transduction ratio can be directly derived from model fitting of concentration-response data and provides a measure of receptor response that is normalized for effects on affinity [30]. The transduction ratio can also be corrected for differences in cell surface receptor expression to enable comparison of mutant effects even where there are varying levels of receptor expression [28], as is the case in the current study. Validation of this approach can be seen with the $P195^{ECL1}A$ mutant that is among the most poorly expressed but has a corrected transduction ratio that is equivalent to WT. GIP is a highly conserved peptide (**Figure**

1A) with mouse GIP differing only by $R18^{mGIP}$ substitution for $H18^{hGIP}$, $R30^{mGIP}$ for $K30^{hGIP}$ and $S34^{mGIP}$ for $N34^{hGIP}$ (the superscript "mGIP" and "hGIP" refer to amino acids in the mouse and human GIP peptides, respectively), but GIP can circulate in 2 forms, GIP(1-42) that is the most abundant active form, and GIP(1-30)NH_2, a product of PC2 processing of proGIP that is equivalent in length to incretin GLP-1 peptides [41]. Not surprisingly, the mutations in the core of the GIPR that engage the N-terminus of the peptides had an equivalent pattern of effect on cAMP signaling efficacy for all 3 ligands, with most mutants leading to at least a small reduction in peptide response (**Figure 5A-5C**). A small number of these mutants have been previously studied in assays of GIP(1-30)NH_2-mediated cAMP production [26, 27], with similar effects after consideration of differences in the relative expression of mutant receptors and system reserve. The single exception to this was $Y231^{3.24}A$ that was reported to have similar potency to WT GIPR in the previous study [26], but had reduced cAMP signaling in the current work and had the greatest loss of affinity in competitive binding assays (**Figures 3B-3E, Table 1, Table 2**). The reason for this discrepancy is unclear.

The most detrimental effects (>100-fold reduction in efficacy) were seen with GIPR mutants that comprised 2 clusters. The first of these involved residues in TM7, TM1 and TM2: $Y141^{1.43}$, $R190^{2.67}$, $I187^{2.64}$, $R183^{2.60}$ and $L374^{7.39}$ (**Figure 5, Figure 6, Figure 9A**). Recent work from high-resolution structures of the GLP-1R have linked this network to full and partial agonism where aromatic functional groups, e.g. phenylalanine in peptides, of full agonists coordinate the position of $Y^{1.43}$ in the receptor, enabling it to form H-bonds with $D^{2.68}$ of TM2 with an extended H-bonded network that also includes $K/R^{2.67}$. GLP-1R agonists that are unable to coordinate the location of $Y^{1.43}$ are partial agonists and TM1 and TM2 have conformations equivalent to inactive GLP-1R

[42]. In the active GIPR homology model, an equivalent network is predicted where $F6^{G}$ coordinates the location of $Y141^{1.43}$ allowing it to form interactions with TM2 ($D191^{2.68}$ and $R190^{2.67}$) (**Figure 9A**). It is likely that $I187^{2.64}$ is important for the coordination of the side chain rotamers of the higher positioned TM2 residues. $L374^{7.39}$ supports the location of $F6^{G}$, providing a rationale for the large detrimental effect of alanine mutation of this residue.

The second major cluster of mutations, with >100-fold decreases in peptide efficacy, comprised residues predicted to bridge ECL3 and the top of TM5/ECL2 (**Figure 5, Figure 6, Figure 9B**), suggesting that this contiguous network of residues plays a key role in the efficiency of coupling to Gs-mediated signaling. While it does not directly contribute to interactions with TM5, $R370^{7.35}$ is predicted to interact with $D9^{G}$ and this is likely important in the positioning of ECL3 to allow interaction between $E362^{ECL3}$ and/or $E363^{ECL3}$ with $R300^{5.40}$ that in turn stabilizes the rotamer position of $W296^{5.36}$ and $N290^{ECL2}$ that influence the conformation of ECL2. Residues at position 5.40 in class B1 GPCRs also form H-bond interactions, either directly or via water, with conserved polar residues in peptide agonists [43]. In the current model, $R300^{5.40}$ could form a water-mediated bond to $T5^{G}$. The stability of the ECL3/TM5/ECL2 network likely contributes to the stable interactions of the peptide N-terminal activation domain within the receptor core. Previous work with GLP-1R has linked the location and conformation of ECL3 to biased agonism [44]. Furthermore, correlative studies of small molecule GLP-1R agonist pharmacology and structure of active GLP-1R complexes with these agonists have provided insight into the conformational requirements for the receptor-agonist complex to mimic the spectrum of actions of GLP-1 [22, 42]. Intriguingly, maintenance of interactions between ECL3/TM5/ECL2 of the GLP-1R appeared to be critical to ligand efficacy for arrestin recruitment, receptor trafficking and activation of ERK1/2,

but was not required for high efficacy in cAMP production for small molecule agonists [42]. In the current study, β-arrestin 2 recruitment was principally affected by mutations to the two key clusters involved in cAMP efficacy (**Figure 5E, Figure 6F**), consistent with the importance of the ECL3/TM5/ECL2 network in maintaining efficient coupling of arrestins and also a potential role for this domain in receptor trafficking. In contrast, activation of ERK1/2 required the TM1/TM2 receptor activation network (**Figure 6E**), but was largely independent of the ECL3/TM5/ECL2 network, albeit that interactions of the peptide with N300$^{5.40}$ are likely still required as alanine mutation also reduced pERK1/2 efficacy (**Figure 5D, Figure 6E**).

Remarkably, the only significant effects from mutation were reductions in ligand binding or function. Although it did not achieve statistical significance, a potential outlier to this pattern was the E354$^{6.53}$A mutant that trended to higher efficacy, selectively in the pERK1/2 and β-arrestin 2 recruitment assays (**Figure 5A-5E**). Position 354$^{6.53}$ is the site of a naturally occurring polymorphism (E/Q354) that has been linked to risk of cardiovascular disease [45], diabetes [46] and bone fracture [47]. In vitro assessment of the impact of the polymorphism did not reveal any differences in assays of binding affinity, cAMP production or β-arrestin 2 recruitment, but did alter ligand residence time and GIPR trafficking (increased internalization, decreased recycling) [37, 48]. Within class B1 GPCRs, polar residues at position 6.53 form part of a conserved central polar network that undergoes reorganization upon agonist binding and receptor activation [49, 50]. Collectively, the data suggest that further experiments probing the role of E354$^{6.53}$, and other residues of the central polar network, in ligand-mediated receptor trafficking and signaling are warranted.

In addition to assessment of the effect of predicted GIPR binding pocket mutations on receptor function, we assessed GIP(1-42) affinity by competition for iodinated GIP. Affinity in this setting is a composite of the micro-affinity states associated with complexes of the agonist bound active receptor with transducer and regulatory proteins, but is principally influenced by the most prominent state, presumably that bound to Gs protein. There was reduction in affinity for mutant residues involved in formation of the fully active state (TM1-TM2 interface), consistent with the requirement for G protein binding for high affinity binding. Binding was also reduced with mutation of ECL2, particularly $E288^{ECL2}A$ and $R289^{ECL2}A$ that are predicted to form direct interactions with $S8^{G}$ and $D15^{G}$ of GIP (**Figure 3D**). Nonetheless, these ECL2 residues had almost no role in receptor activation in any of the pathways studied (**Figure 5A-5E**). Mutation of the glutamic acid residues in ECL3 also reduced peptide affinity, although it is unclear if this is mediated by direct interaction with $T5^{G}$ or due to indirect effects through changes to the conformation of the loop. Intriguingly, while $R370^{7.35}$ is predicted to form a polar interaction with $D9^{G}$, alanine mutation of this residue did not alter measured affinity. As such, while it appears to be critical for the conformation of ECL3, it does not appear to a major contributor to overall strength of ligand interaction, perhaps suggesting that this is a more transient interaction in the active receptor. Recent analysis of conformational variance of peptide-bound GLP-1R revealed that the stability of peptide-ECL3 interactions could vary substantially, even where there was high sequence conservation in the interaction segment of the peptide [42], providing evidence in support of this hypothesis.

In the current study, the use of isogenic recombination to generate stable cell lines also enabled us to assess the impact of mutations on receptor expression. While most alanine mutations were

well tolerated and had cell surface expression levels similar to the WT receptor, a number of mutants dramatically reduced expression. The most detrimental mutants were primarily those deep in the TM bundle (TM1, TM2 and TM3), suggesting that these residues contribute to stable packing and conformational stability of the receptor in the apo state. Alanine mutation of P195 in ECL1 also caused marked attenuation of receptor expression, however, this is most likely due to reduction in the efficiency of proper folding of the receptor, as the $P195^{ECL1}A$ mutant had a WT phenotype in all assays after correction for receptor expression.

Conclusion

Mutation of residues predicted to be in proximity of the N-terminal activation domain of GIP revealed important patterns of effects that correspond to two key interaction networks previously hypothesized to be important for efficacy and biased agonism of related class B1 GPCRs. Both networks were critical for Gs-mediated cAMP signaling and the recruitment of β-arrestin 2, however, cAMP signaling was broadly sensitive to mutation, with most residues reducing signaling, consistent with the dynamics of interaction with the N-terminus of GIP being important in efficiency of signaling. Unlike the other two assays, activation of ERK1/2 was largely independent of the network between ECL3/TM5/ECL2 indicating that pERK1/2 is at least partially distinct from Gs or β-arrestin signaling pathways, and indicating that this network is also critical for potential biased agonism of GIPR agonists. Collectively, our work advances understanding of structure-function of GIPRs and provides a framework for the design and/or interpretation of GIP analogues with novel signaling and regulation.

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Author contributions

P.M.S. and D.W. designed and oversaw the project. E.Y., V.L., A.T.D., F.H.Z., S.D. and T.Y.X. performed mutagenesis and signaling experiments; W.J.C.V.D.V. conducted binding and arrestin assays with the guidance of M.M.R.; G.D. generated the GIPR homology model; D.H.Y., M.-W.W., D.W. and P.M.S. supervised the research. E.Y., D.H.Y., M.-W.W., W.J.C.V.D.V., M.M.R., D.W. and P.M.S. analyzed data. E.Y., M.-W.W., D.W. and P.M.S. wrote the manuscript with input from all co-authors.

Competing interests

The authors declare no conflict of interests.

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Abbreviations

cAMP, cyclic adenosine monophosphate

BRET, bioluminescence resonance energy transfer

DMEM, Dulbecco's modified Eagle's medium

EC_{50}, half maximal effective concentration

ECD, extracellular domain

ECL, extracellular loop

E_{max}, maximum response

ERK1/2, extracellular signal-regulated kinase1/2

FBS, fetal bovine serum

F_{mut}, fold change between mutants and wildtype receptor

GCGR, glucagon receptor

GIP, glucose-dependent insulinotropic polypeptide

GIPR, glucose-dependent insulinotropic polypeptide receptor

GLP-1, glucagon-like peptide-1

GLP-1R, glucagon-like peptide-1 receptor

GPCR, G protein-coupled receptor

GRK2, G protein-coupled receptor kinase 2

HEK293, human embryonic kidney 293

MAPK, mitogen-activated protein kinase

PDB, protein data bank

pEC_{50}, negative logarithm of EC_{50}

PEI, polyethylenimine

pERK1/2, ERK1/2 phosphorylation

PKA, protein kinase A

RT, room temperature

TM, transmembrane

WT, wildtype

Figure legends

Figure 1. ***GIP family peptide sequences and full-length, active-state, homology model of GIPR bound to GIP and Gαsβ1γ2 protein highlighting the residues selected for mutation.*** **A**. Amino acid sequences of the human and mouse GIP peptides used in the current study in single letter format. **B**. Homology model of the active GIPR. The complex is presented in ribbon format with the receptor colored from N-terminus (blue) to C-terminus (red), GIP (dark red), with side chains displayed in x-stick format, Gαs (cyan), Gβ1 (dark blue) and Gγ2 (dark slate blue). GIPR residues mutated in the current study are displayed in space fill format. **C**. Magnified side view of the GIPR. **D**. Top-down view of the receptor core, with GIP displayed in x-stick format where the N-terminal extracellular domain (ECD) has been omitted for clarity. Transmembrane helices are numbered with roman numerals. ECL, extracellular loop.

Figure 2. ***Cell surface expression of alanine mutants of the GIPR. Receptor expression is normalized to the expression of the wildtype (WT) receptor and expressed as a percentage.*** **A**. Mean data from individual experiments from HEK293 cells stably expressing the WT or mutant GIPR, performed in duplicate, are displayed, along with the mean and 95% confidence intervals. **B**. Homology model of the active GIPR (grey, ribbon format), with mutated residues displayed in space fill format. GIP (dark red) is displayed in ribbon format with side chains shown in x-stick format. Top-down view with the receptor extracellular domain omitted for clarity. Transmembrane helices are labelled with Roman numerals. ECL, extracellular loop. **C**. Effect of mutants on cell surface receptor expression. Expression values that were different from 100% with 95% confidence were deemed statistically significant and colored according to the expression relative

to WT as annotated on the Figure. Grey, no significant effect. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35].

Figure 3. ***GIP(1-42) binding affinity at mutant GIPRs.*** **A**. Competition of GIP(1-42) for ^{125}I-GIP binding to wildtype (WT) or mutant GIPR transiently expressed in COS-7 cells, presented as mean ± SEM of 3-6 (mutant receptors) or 17 (WT) separate experiments performed in duplicate. Data are expressed as specific binding (B-N), corrected for total specific binding in the absence of unlabeled GIP (B_0-N) in each individual experiment, and displayed as a percentage. Data were fit to a 3-parameter logistic equation. **B**. Log IC_{50} values for WT and mutant receptors. **C**. Effect of mutants on GIP binding affinity mapped onto the GIPR homology model with mutated residues depicted in space fill format and the receptor backbone in grey ribbon format (top down view with the receptor ECD omitted for clarity). **D-E**. Side views of the GIP binding pocket with mutated receptor residues in x-stick format and GIP residues in wire format colored by atom with the backbone in dark red. The receptor backbone is omitted for clarity. Significantly different effects are colored by the magnitude of change from the WT receptor, as annotated on the Figure. Grey, no significant effect. Black, mutated residues where the effect could not be quantified due to insufficient signal. Data were analyzed by one-way ANOVA, with a Dunnett's post-test (mutant versus WT). Statistical significance was accepted at $P<0.05$. Significantly different values are colored according to the fold change from WT. ND, not determined due to insufficient radioligand binding window. The green dashed line is the mean affinity at the WT GIPR. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35]. Shaded areas denote transmembrane domains, labelled with Roman numerals.

Figure 4. ***Effect of GIPR mutants on cAMP accumulation in response to GIP peptides*****.** Wildtype (WT) and mutant receptors were stably expressed in HEK293 cells and stimulated for 40 min. Left panels, responses to GIP(1-42). Middle panels, responses to GIP(1-30)NH_2. Right panels, responses to mouse GIP. Data from individual experiments were normalized to the maximal response of cells expressing the WT receptor (expressed as a percentage) and fit to a 3-parameter logistic equation. Curve fits for the WT receptor are displayed as a dashed line. Data are mean ± SEM of 4 (mutant receptors) or 22 (WT receptor) individual experiments performed in duplicate. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35].

Figure 5. ***Effect of GIPR mutations on cAMP accumulation, pERK signaling, β-arrestin 2 recruitment and efficacy of GIP peptides.*** Efficacy (log τ/K_A) was determined using the operational model of Black and Leff [29], and corrected for cell surface expression for second messenger signaling pathways (log τ/K_Ac). **A-C**. Peptide efficacy for GIP(1-42) (**A**), GIP(1-30)NH2 (**B**) and mouse GIP (**C**) in cAMP accumulation assays. **D**. GIP(1-42) efficacy in pERK1/2 assays. **E**. GIP(1-42) efficacy in β-arrestin 2 recruitment assays. The effect of mutation was determined using one-way ANOVA, with a Dunnett's post-test (versus wildtype (WT) control), with $P<0.05$ deemed to be significant. Efficacy values significantly different for WT GIPR are colored according to the magnitude of effect, as annotated on the figure. The green dashed line represents the mean efficacy value for the WT receptor. ND, not determined due to insufficiently robust signal to quantify the response. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35]. Shaded areas denote transmembrane domains, labelled with

Roman numerals.

Figure 6. ***3D map of the effect of GIPR mutations on peptide efficacy.*** **A**. Homology model of the active GIPR (grey, ribbon format), with mutated residues displayed in space fill format. GIP (dark red) is displayed in ribbon format with side chains shown in x-stick format. Top-down view with the receptor extracellular domain omitted for clarity. Transmembrane helices are labelled with Roman numerals. ECL, extracellular loop. **B-D**. Effect of GIPR mutants on GIP(1-42) (**B**), GIP(1-30)NH_2 (**C**) and mouse GIP (**D**) efficacy in cAMP accumulation assays. **D**. Effect of GIPR mutants on GIP(1-42) efficacy in pERK1/2 assays. **E**. Effect of GIPR mutants on GIP(1-42) efficacy in β-arrestin 2 recruitment assays. Significantly different effects are colored by the magnitude of change from the wildtype (WT) receptor, as annotated on the figure. Grey, no significant effect. Black, mutated residues where the effect could not be quantified due to insufficient signal. Two major networks contributing to receptor function were identified. Network 1 (green dashed oval) comprised residues in TM1, TM2 and TM7. Network 2 (blue dashed oval) connected ECL3, the top of TM5 and ECL2.

Figure 7. ***Effect of GIPR mutations on GIP(1-42) mediated phosphorylation of ERK1/2.*** Wildtype (WT) and mutant receptors were stably expressed in HEK293 cells and stimulated for ~10 min. Data from individual experiments were normalized to the maximal response of cells expressing the WT receptor (expressed as a percentage) and fit to a 3-parameter logistic equation. Curve fits for the WT receptor are displayed as a dashed line. Data are mean ± SEM of 4 (mutant receptors) or 17 (WT receptor) individual experiments performed in duplicate. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35].

Figure 8. ***Effect of GIPR mutations on GIP(1-42) mediated β-arrestin 2 recruitment.*** Wildtype (WT) or mutant GIPRs C-terminally fused to Rluc8, β-arrestin 2-Venus and GRK2 were transiently transfected into HEK293 cells and GIP-induced recruitment was monitored by BRET. **A**. Titration of receptor expression did not alter the magnitude of BRET response or potency of GIP. B. Concentration-response curves to GIP(1-42) at WT and mutant GIPRs. Data were normalized to the maximum response of the WT receptor and expressed as a percentage. Data are mean ± SEM of 27 (WT), 6 ($E377^{7.42}A$) or 3 (other mutants) individual experiments performed in duplicate. Data are fit to a 3-parameter logistic equation. Curve fits for the WT receptor are displayed as a dashed line. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35]. βarr-2, β-arrestin 2.

Figure 9. ***Critical GIPR networks involved in peptide-mediated cAMP efficacy.*** **A**. Network 1 comprising residues in TM1, TM2 and TM7. **B**. Network 2 comprising residues in ECL3, TM5 and ECL2. The effect of GIPR mutations on the GIP(1-42) efficacy has been mapped onto the 3D active GIPR homology model. Only key residues in each of the networks are displayed for clarity. Mutated receptor residues are displayed in x-stick format, colored according the magnitude of effect from Figure 5A. The receptor backbone is displayed in thin grey ribbon format. TM helices are numbered using Roman numerals. ECL, extracellular loop. Superscript numbers for mutant residues refer to the Wootten class B GPCR number scheme [35]. GIP is displayed in wire format colored according to atom type with the backbone carbon colored dark red. Peptide amino acid labels are in single amino acid format, colored dark red, with a superscript “G” to indicate the peptide, GIP. The black dashed line in (**A**) depicts the interaction between $F6^{G}$ and $Y141^{1.43}$ that

coordinates the side chain rotamer allowing putative interactions with D191$^{2.68}$ and R190$^{2.67}$ in TM2 schematically illustrated with green dashed lines. The steric positioning of L374$^{7.39}$ that putatively stabilizes the location of F6^{G} is illustrated by the dark red dashed arc.