

# Binding domain characterization of growth hormone secretagogue receptor

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## ABSTRACT

**Background and Objectives:** Activation of ghrelin receptor growth hormone secretagogue receptor (GHS-R) by endogenous or synthetic ligands amplifies pulsatile release of growth hormone (GH) and enhances food intake, very relevant to development and growth. GHS-R is a G-protein coupled receptor that has great druggable potential. Understanding the precise ligand and receptor interactions is crucial to advance the application of GHS-R. **Materials and Methods:** We used radiolabeled ligand-binding assay and growth hormone release assay to assess the binding and functional characteristics of GHS-R to synthetic agonists MK-0677 and GHS-25, as well as to endogenous peptide ligand ghrelin. We analyzed the ligand-dependent activity of GHS-R by measuring aequorin-based  $[Ca^{++}]_i$  responses. To define a ligand-binding pocket of GHS-R, we generated a series of human/puffer fish GHS-R chimeras by domain swapping, as well as a series of mutants by site-directed mutagenesis. **Results:** We found that the synthetic ligands have high binding affinity to GHS-R in the *in vitro* competitive binding assay. Remarkably, the *in vivo* GH secretagogue activity is higher with the synthetic agonists MK-0677 and GHS-25 than that of ghrelin. Importantly, the activity was completely abolished in GHS-R knockout mice. In GHS-R chimera analysis, we identified the C-terminal region, particularly the transmembrane domain 6 (TM6), to be critical for the ligand-dependent activity. Our site-directed mutagenesis study further revealed that amino acid residues D99 and W276 in GHS-R are essential for ligand binding. Interestingly, critical residues distinctively interact with different ligands, MK-0677 activation depends on E124, while ghrelin and GHS-25 preferentially interact with F279. **Conclusion:** The ligand-binding pocket of human GHS-R is mainly defined by interactive residues in TM6 and the adjacent region of the receptor. This novel finding in GHS-R binding domains advances the structural/functional understanding of GHS-R, which will help to select/design better GHS-R agonists/antagonists for future therapeutic applications.

**Key words:** ghrelin, growth hormone secretagogue, growth hormone secretagogue receptor, receptor binding

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## INTRODUCTION

The growth hormone (GH) axis, including the endocrine signaling of GH and insulin-like growth factor-1 (IGF-1), plays important roles in human development and growth. Aging is associated with declined activity of the GH-IGF-1 axis. In order to develop drugs for the treatment of hormone deficiencies in the elderly, a reverse pharmacology approach was

taken to identify small molecules that can restore the amplitude of GH pulsatility.<sup>[1,2]</sup> We elucidated the action of a class of small synthetic growth hormone releasing peptides (GHRPs) and used this knowledge to develop non-peptide mimetics,<sup>[3-5]</sup> such as Merck compounds MK-0677 and GH secretagogue (GHS)-25.<sup>[6,7]</sup> Daily treatment with MK-0677 reversed the GH-IGF-1 axis of 70–90-year-old subjects and produced GH and IGF-1 levels typical to those of

young adults.<sup>[8,9]</sup> In 1996, we expression-cloned an orphan G-protein coupled receptor (GPCR), which we named the GHS receptor (GHS-R) from a pituitary DNA library based on specific activation by the synthetic GH-releasing ligands MK-0677 and GHRP-6.<sup>[10]</sup> GHS-R1a with all seven transmembrane domains (TMs) was proven to be the functional receptor of ghrelin, and its ligands potentially offer an oral form of alternative therapy instead of injectable GH for GH deficiency.<sup>[11,12]</sup>

In 2000, we cloned the puffer fish *GHS-R* gene, which shares 58% identity to human ortholog. In common with the human GHS-R1a, it is activated by the structurally distinct ligands GHRP-6 and MK-0677.<sup>[13,14]</sup> The ligand activation domain of GHS-R1a has been evolutionarily conserved from puffer fish to humans, in support of the notion that the GHS-R1a and its natural ligand play a fundamentally important role in biology.

The physiological relevance of GHS-R1a has been confirmed by identification of two natural ligands, ghrelin and adenosine, by fractionating and assaying animal tissue extracts in cell lines engineered to express GHS-R1a.<sup>[15-17]</sup> Ghrelin plays an important role in modulating feeding patterns, insulin secretion, and glucose metabolism.<sup>[18,19]</sup> In contrast to ghrelin, a 28-amino acid octanoylated peptide,<sup>[15]</sup> adenosine is a purinergic nucleotide and it activates GHS-R1a through a binding domain distinct from that of ghrelin and MK-0677.<sup>[16,17]</sup> Even though they both activate GHS-R1a, when administering ghrelin and adenosine to rats, only ghrelin stimulates GH release.<sup>[16,20]</sup> The structure of synthetic agonist MK-0677 is different from that of the endogenous peptide ghrelin. Although molecular modeling studies developed from proton nuclear magnetic resonance (NMR) that compared the structural features of MK-0677 and other synthetic GHS-R1a ligands illustrated certain similarities with ghrelin, these studies did not precisely predict the ligand–receptor binding sites.<sup>[21]</sup> Our previous studies showed that ghrelin, not adenosine, functions as a competitor of [<sup>35</sup>S]MK-0677 binding,<sup>[17]</sup> suggesting ghrelin and MK-0677 share similar binding sites on GHS-R1a.

GHSs increase the activity of hypothalamic arcuate nucleus neurons involved in controlling the release of GH. GHS-25 is another small molecule mimetic similar to MK-0677. In common with other GHSs, GHS-25 induces significant amounts of Fos immunoreactivity in the arcuate nucleus of conscious male rats.<sup>[7]</sup> However, unlike other GHSs, GHS-25 also induces Fos immunoreactivity in the supraoptic nucleus, which suggests that in addition to its actions on the GH axis, GHS-25 may also regulate the release of neurohypophysial hormone.<sup>[7]</sup> Therefore, it raised the question whether there is a different subtype receptor for GHS-25.

Ghrelin analogs, as either agonists or antagonists, have been suggested to have important clinical impact. Several studies have been done on ghrelin and other GHSs,<sup>[2]</sup> but the binding pocket of GHS-R1a has not been fully defined. To determine the ligand-binding domains on GHS-R1a, we used receptor-binding assay, site-directed mutagenesis, and chimeric receptor approaches to study the binding and activity of GHS-R1a in response to ghrelin, MK-0677, and GHS-25.

## MATERIALS AND METHODS

### Materials

MK-0677, [<sup>35</sup>S]MK-0677, and GHS-25 were kindly provided by Merck Research Laboratories. Human ghrelin was purchased from Phoenix Pharmaceuticals. Glutathione was from Sigma, and coelenterazine was from Molecular Probes. The rest of the chemicals used in culture were all from Invitrogen.

### Construction of wild-type GHS-R1a expression clones

An EcoRI and NotI fragment (1.1 kb) of human and puffer fish (*Spherooides nephelus*) GHS-R cDNA, which includes the complete open reading frame, was generated as described previously.<sup>[10,13]</sup> This fragment was cloned in the mammalian expression vector pcDNA3 (Invitrogen).

### Mutagenesis of human GHS-R clones

Point mutants (double nucleotide changes) were constructed using QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Nucleotide sequence analysis of all mutants verified that nucleotide sequence errors had not occurred.

### Transfection of 293-AEQ17 cells

The lipofectamine procedure (Invitrogen) was used for transient transfections according to the manufacturer's instructions. Transfections were performed in 60-mm dishes (80% confluent cells) with 2 μg GHS-R1a plasmid DNA. Receptor expression was allowed to proceed for 48 h.

### Ligand binding assay

Binding of [<sup>35</sup>S]MK-0677 to crude membranes prepared from HEK293 cells that stably express GHS-R was performed as previously described.<sup>[10,22]</sup> Twenty-five micrograms of membrane protein was used to incubate with [<sup>35</sup>S]MK-0677 (0.1 nmol/L) + ligands at 20°C for 60 min. The membrane was then isolated and counted for [<sup>35</sup>S]MK-0677 incorporation. Specific binding (>90% of the total) was defined as the difference between total binding and nonspecific binding conducted in the presence of 50 nmol/L unlabeled MK-0677.

### GH release assay

To measure GH, mice were injected intraperitoneally (i.p.) with pentobarbital (50 mg/kg body weight); 15 min later, 100  $\mu$ L saline with or without 10  $\mu$ g of different ligands (ghrelin, MK-0677, or GHS-25) was i.p. injected. Blood was collected by retro-orbital bleeding at 0, 5, and 15 min after the injection. GH was measured in plasma samples using a rat GH EIA kit (ALPCO diagnostics).

### Aequorin bioluminescence assay

Measurement of GHS-R1a activity in the aequorin-expressing stable reporter cell line 293-AEQ17 was performed as previously described.<sup>[23,24]</sup> All testing constructs were transiently transfected into the cells for 48 h. Cells were charged for 2 h with coelenterazine under reduced conditions (30  $\mu$ mol/L reduced glutathione) in ECB buffer to generate  $\text{Ca}^{2+}$ -dependent luminescence.

### Statistical analysis

GraphPad Prism version 6.0 software (GraphPad Software, San Diego, CA, USA) was used. Two-way analysis of variance (ANOVA) with repeated measures or one-way ANOVA was used. Data are represented as mean  $\pm$  standard error of the mean (SEM), and  $P < 0.05$  was considered statistically significant.

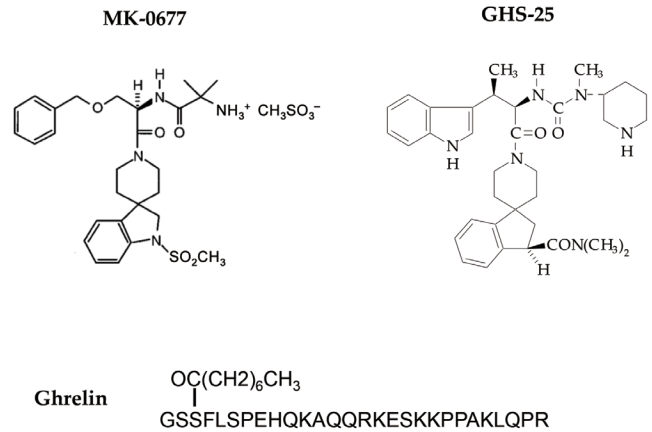
## RESULTS

### Ghrelin, MK-0677, and GHS-25 all bind to GHS-R1a

Ghrelin is an octanoylated peptide, whereas MK-0677 and GHS-25 are non-peptide synthetic mimetics. The structure of ghrelin is quite different from the small synthetic mimetics MK-0677 and GHS-25 (Figure 1A), but all activate GHS-R1a. Previously, using a receptor binding assay, we showed that the binding affinity of structurally diverse secretagogues is tightly correlated with GH secretory activity.<sup>[22]</sup> Using competition binding assay, we showed that MK-0677<sup>[13]</sup> and GHS-25<sup>[7]</sup> are potent synthetic agonists of GHS-R, and Kojima *et al.*<sup>[15]</sup> showed that ghrelin is the endogenous ligand of GHS-R1a. The binding affinity of ghrelin and GHS-25 is similar to that of MK-0677. Our binding assays using [<sup>35</sup>S]MK-0677 showed that MK-0677 and GHS-25 are competitive inhibitors, binding to human GHS-R1a with  $\text{IC}_{50}$  values at 0.3 and 5.6 nmol/L, respectively, which is consistent with the modestly lower functional activity of MK-0677 ( $\text{EC}_{50}$  at 1.1 nmol/L) compared to GHS-25 ( $\text{EC}_{50}$  at 7.7 nmol/L) in cells stably expressing hGHS-R1a and aequorin.<sup>[7]</sup>

### GHS-25's GHS activity is mediated by GHS-R1a

The functional relationship between GHS-R1a, ghrelin, and MK-0677 has been well established.<sup>[10,25]</sup> Our previous study showed that besides stimulating GH release, in comparison to ghrelin and MK-0677, GHS-25 induced



**Figure 1: Structure diagrams of MK-0677, GHS-25, and ghrelin. MK-0677 and GHS-25 are non-peptide small synthetic molecules, and ghrelin is an octanoylated peptide. GHS: growth hormone secretagogue.**

Fos immunoreactivity in hypothalamic regions beyond the arcuate nucleus.<sup>[7]</sup> To investigate whether there is a different receptor for GHS-25 other than GHS-R1a, we compared GHS-25-induced GH release in wild-type and *Ghsr* null mice. Similar to the effects of MK-0677 and ghrelin we reported previously,<sup>[25]</sup> GHS-25's GHS effect was only detected in wild-type mice, but not in *Ghsr* null mice (Figure 2), indicating that GHS-25's GHS action is mediated by GHS-R1a.

### Construction of chimeric human and puffer fish GHS-R clones

GHS-R is a member of the GPCR family, which has seven TM domains and extracellular/intracellular domains. The seven TM helices contain recognizable motifs that help to identify conserved structural elements used in the two-dimensional sequence alignments. Humans and puffer fish share a 58% identity in protein sequence homology.<sup>[13]</sup> We identified two restriction enzyme sites, *Apa*I and *Sca*I, which are in equivalent locations (at the same region of the receptor with same codon usage) in humans and puffer fish GHS-R cDNA clones, but not in the expression vector pcDNA3 itself. By using these restriction sites, we were able to swap equivalent fragments between human and puffer fish clones to generate two sets of chimeric clones: *Apa*I set and *Sca*I set (Figure 3). By further digestion of *Apa*I and *Sca*I, we were able swap fragments between sets of *Apa*I and *Sca*I clones to generate the *Apa*I–*Sca*I clones, either human clone with puffer fish insert or puffer fish clone with human insert.

To precisely define the binding pockets on GHS-R1a, we also used polymerase chain reaction (PCR)-based and oligonucleotide-directed mutagenesis to introduce unique restriction enzyme sites into the cDNAs to enable further

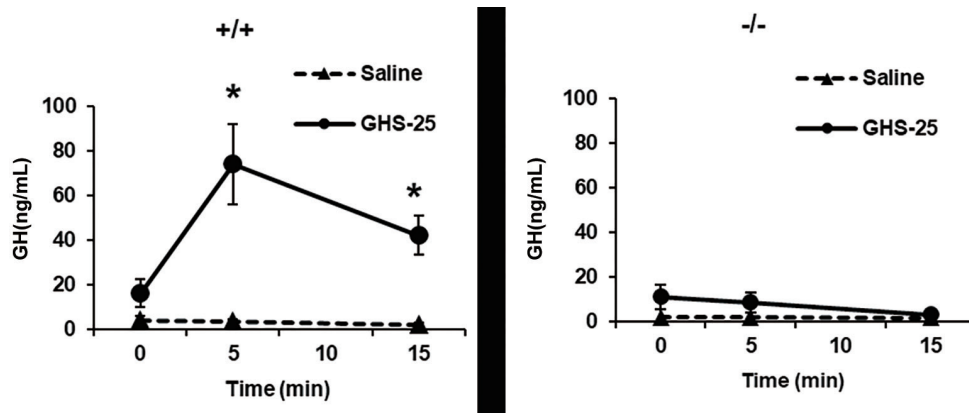


Figure 2: The GHS activity of GHS-25 in *Ghsr* wild-type (+/+) and *Ghsr* null (-/-) mice. GH was measured in plasma samples collected at 5 and 15 min after i.p. injection of 10 µg GHS-25 dissolved in 100 µL saline. *n* = 9, \**P* < 0.001. *Ghsr* wild-type (+/+) and *Ghsr* null (-/-). GH: growth hormone; GHS: growth hormone secretagogue; i.p.: intraperitoneal.

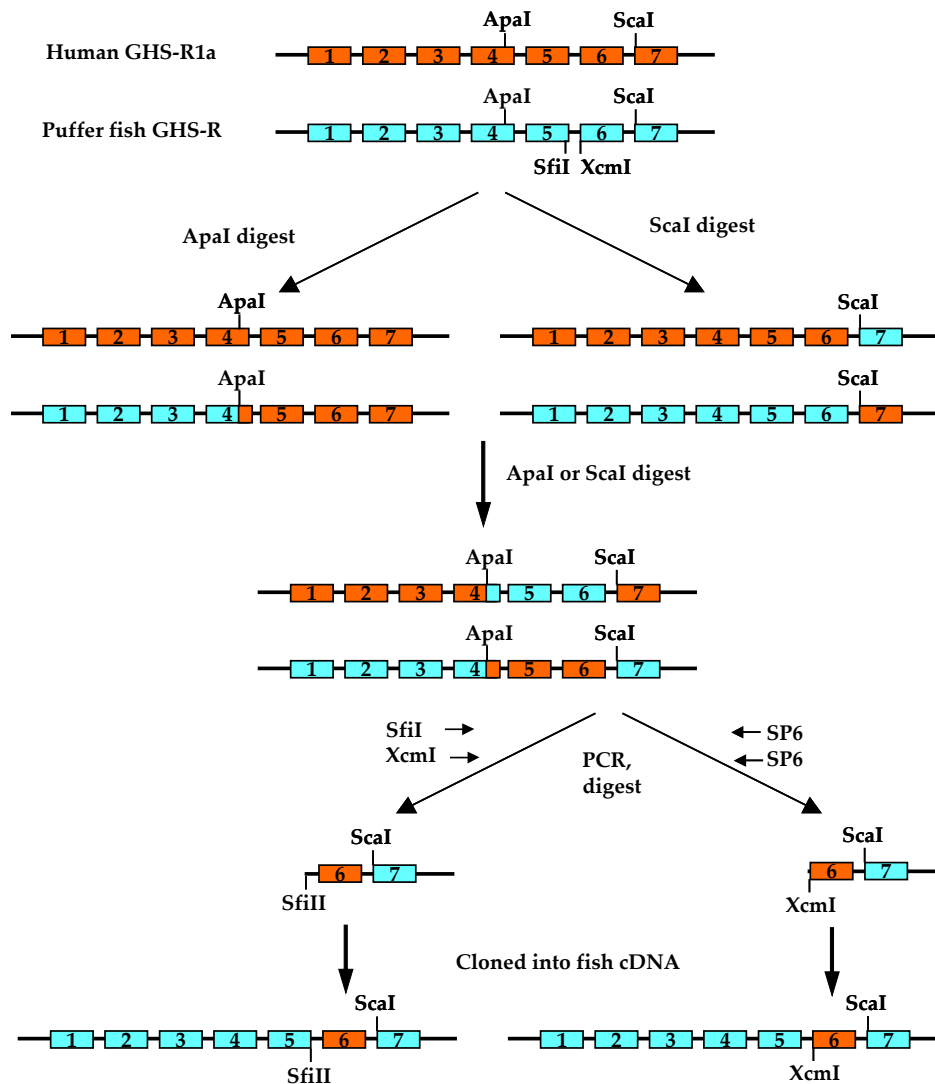
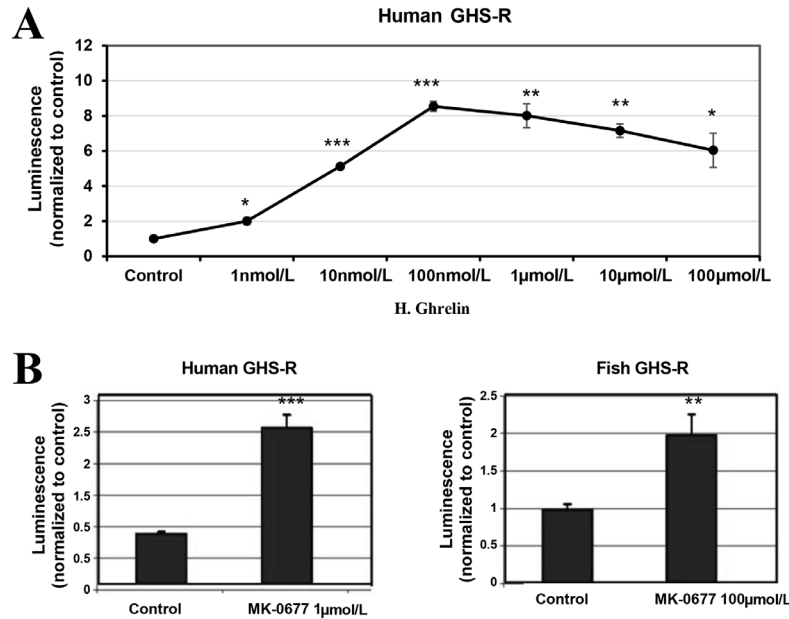


Figure 3: Schematic diagram of cloning strategy for generating human and puffer fish GHS-R chimeras. The rectangles show GHS-R TMs. Human GHS-R TMs are in orange color, and puffer fish GHS-R TMs are in blue color. The forward and reverse arrows indicate the PCR primers used. GHS-R: growth hormone secretagogue receptor; PCR: polymerase chain reaction; TM: transmembrane.



**Figure 4: The relative bioluminescence activity of WT human and fish GHS-R with the ligands, ghrelin and MK-0677. (A) Titration study of human GHS-R activation with various concentrations of ghrelin. (B) Required concentration of MK-0677 to activate WT human and fish GHS-R. Each data point was measured in triplicate and the experiment was repeated at least 3 times. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Control versus treatment of ghrelin or MK-677. GHS-R: growth hormone secretagogue receptor; WT: wild type.**

fragment swapping. SfiI site is present in puffer fish receptor, but not in human receptor pcDNA3 construct. By using a forward primer with SfiI binding sequences at the 5' end and a short sequence complementary to human GHS-R TM5 at the 3' end, a reverse primer complementary to SP6 in pcDNA3, and the fish/human/fish ApaI–ScaI clone as template, we are able to amplify a PCR fragment with the third intracellular loop, TM6/the third extracellular loop (ECL3) of human, and TM7 of puffer fish with a newly introduced SfiI site at the 5' end. The PCR fragment was further restriction enzyme digested with SfiI and NotI (with a pcDNA3 polylinker site at the 3' end of the cDNA), then swapped into an equivalent place of SfiI- and NotI-digested wild-type puffer fish clone to generate the fish/human/fish SfiI clone. The XcmI clone was generated similar to the SfiI clone. All chimeric receptor clones were sequenced to make sure that the cloning sites were accurate with no mutation introduced by PCR and had the correct replacement of equivalent coding sequences.

### Identification of ghrelin domain using human/puffer fish GHS-R chimeras











GHS-R1a activation leads to generation of inositol trisphosphate ( $IP_3$ ) and  $Ca^{2+}$  release through activation of the G protein subunit  $G\alpha_{11}$ .<sup>[10,23]</sup> It has been shown that mammalian cells which stably expressed jellyfish aequorin can be used to assess the activation of cell surface receptor-induced  $Ca^{2+}$  mobilization.<sup>[24]</sup> Thus, the aequorin

bioluminescence assay was carried out in aequorin-expressing stable reporter cell line HEK 293 AEQ-17.

To determine the optimal concentrations of the ligands, we conducted a titration study for human GHS-R1a with ghrelin at concentrations of 1 nmol/L, 10 nmol/L, 100 nmol/L, 1  $\mu$ mol/L, 10  $\mu$ mol/L, and 100  $\mu$ mol/L. We found that 10 nmol/L ghrelin can activate wild-type human GHS-R1a, and the receptor activation reaches plateau with 100 nmol/L ghrelin (Figure 4A). However, on the other hand, while 1  $\mu$ mol/L MK-0677 is sufficient to activate human GHS-R, 100  $\mu$ mol/L MK-0677 is required to activate fish GHS-R (Figure 4B). Because the human–fish chimeras are made of partially human and partially fish GHS-R, we used 1 and 100  $\mu$ mol/L to assess the activity of these human–fish chimera.

The relative activity of all the chimeras with MK-0677, ghrelin, and GHS-25 is shown in Table 1. Wild-type human and puffer fish receptors were used as controls for respective chimeric constructs. All ligands can bind on the wild-type human receptor, but only high concentration of MK-0677 can activate the wild-type puffer fish receptor. Ghrelin and GHS-25 are inactive ligands for the puffer fish receptor. The ApaI set of receptors showed that TM5, 6, and 7 regions define the specificity of binding pockets for MK-0677, ghrelin, and GHS-25, respectively. Interestingly, as shown by the ScaI set of receptors, TM7 appears to distinguish the activation between human and

**Table 1: Activity of human/puffer fish growth hormone secretagogue receptor chimeras in response to various ligands<sup>§</sup>**

GHS-R1a	Structure	Activation score			
		MK-0677	Ghrelin	GHS-25	Ligand dose (μmol/L)
Human (H) wt		++++	++++	++++	1
Fish (F) wt		++	-	-	100
H/F ApaI chimera		-	-	-	100
F/H ApaI chimera		++++	+++++	++++	1
H/F Scal chimera		++	++	++	1
F/H Scal chimera		++	-	+++	100
H/F/H ApaI–Scal chimera		+++	-	+++	100
F/H/F ApaI–Scal chimera		+++	+++	+++	1
		+++	+++++	+++	100
F/H/F SfiI chimera		+++	+++	+++	1
		+++++	+++++	+++++	100
F/H/F XcmI chimera		+++	+++	++	1
		+++++	+++++	+++	100

<sup>§</sup>Human TMs are colored in orange and puffer fish TMs are in blue. The relative bioluminescence activity of different chimeras under different concentrations of ligands is scored by the number of +s. Our score system is defined as: no activity as “-” and activity equals to 100% of WT human receptor activity as “+++++.” Thus “+” = 25% activity, “++” = 50% of activity, “+++” = 75% of activity of WT human receptor. In every assay, the activity of each construct was measured in triplicate, and each construct was tested at least 3 times. TM: transmembrane; WT: wild type.

puffer fish for GHS-25. As shown with the ApaI–Scal chimera set, ghrelin’s binding pockets are mainly defined by the residues proximate to TM5 and TM6. SfiI chimera further narrowed the binding domains to the residues proximate to human TM6. More interestingly, the XcmI chimera, containing only TM6 and the ECL3, was proven still functional, suggesting that ghrelin’s binding pockets can be further restricted to TM6 and ECL3.

### ***The activity of human GHS-R1a mutants and the identification of unique binding sites of different ligands on GHS-R1a***

Diversity in the TM region (especially the presence of a charge in the hydrophobic helices) can give clues to the location sites specifically tailored to interact with ligands.<sup>[26,27]</sup> MK-0677 and ghrelin activate human GHS-R1a through overlapping binding domains, as we illustrated with GHS-R1a human/puffer fish chimeras. To further identify the key amino acid residues that are critical for binding and activation by ligands, we have identified a number of amino acids which are distinctively different in human and puffer fish. Using site-directed mutagenesis, we first generated a whole series of human GHS-R1a mutants at the potential key residues and assessed their activity by aequorin bioluminescence assay. To further interrogate the specific binding site(s) of human GHS-R1a, we used structurally distinctive GHS-R1a agonists, ghrelin, MK-0677, and GHS-25, to assess the activation of the mutant human GHS-R1a generated by site-directed mutagenesis. A summary of the activity of all GHS-R1a mutants is shown in Table 2. The results revealed the specificity of the mutants for MK-0677, ghrelin, and GHS-25. D99N and E124Q damped the activation of MK-0677.

C116A and W276A eliminated the activation of both ghrelin and GH-25, but remained responsive to MK-0677. We also found the activity of MK-0677, ghrelin, and GHS-25; all required the amino acid residues D99 and W276. In addition, our data show that MK-0677 activity requires interaction with E124 and H280, ghrelin activity requires C116, M213, and F279, whereas GHS-25 activity is sensitive to changes in many residues in GHS-R1a. Overall, our findings suggest that each ligand has its unique requirement for the binding pockets, and the unique ligand-binding domains share at least two critical residues of D99 in TM2 and W276 in TM6.

## **DISCUSSION**

The ghrelin receptor GHS-R1a is a promising alternative pharmacologic target for treatment of GH deficiency, which has a significant impact on children’s development and growth, as well as a great relevance to adults’ lean mass and bone mineral density that have an influence on fracture risk and quality of life.<sup>[28,29]</sup> GHS-R1a is an important drug target for metabolic disorders including diabetes, obesity, and cachexia. The endogenous ghrelin level is dependent on circadian rhythm and fasting state. It has been shown that physical exercise can transiently increase ghrelin secretion.<sup>[30]</sup> Therefore, development of synthetic agonists or antagonists of GHS-R1a as pharmacological agents has been vigorously pursued, and some candidates have been tested in clinical trials.<sup>[6,31–34]</sup> While evaluation of these compounds or peptides are still ongoing, understanding of the molecular basis of GHS-R1a and ligand interactions, particularly the ligand-binding pocket structure, is essential for the design and development of new GHS-R mimetics.

**Table 2: Summary of mutant human growth hormone secretagogue receptor-1a activity in response to various ligands<sup>1</sup>**

Domain	Mutation	Activity score		
		MK-0677	Ghrelin	GHS-25
FL	Wild type	+++	+++	+++
TM2	D99N	-	++	-
TM2	R102H	+++	+++	++
TM3	C116A	+++	-	-
TM3	Q120H	+++	+++	+++
TM3	S123A	++	+++	++
TM3	E124Q	-	+++	+++
ICL2	Y142A	+++	++	++
ECL2	D191N	+++	+++	+
ECL2	D194N	+++	+++	+
TM5	M213K	+++	+	++
TM5	S217A	+++	++	++
TM5	S218A	+++	+++	+
TM6	W276A	++	-	-
TM6	F279L	+++	+	+
TM6	H280F	++	+++	++
TM6	H280Q	+++	+++	++
TM6	R283H	+++	+++	+
TM6	Y284F	+++	+++	+++

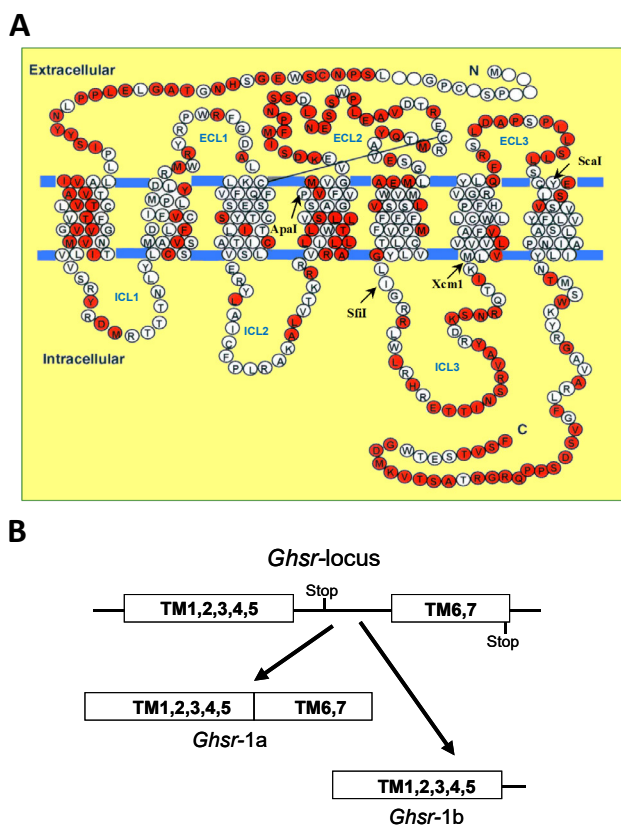
<sup>1</sup>For each mutant, the number indicates the amino acid location of the point mutation, the letter before the number indicates the original amino acid, and the letter after the number indicates what the amino acid was changed into. The relative bioluminescence activity of different mutants activated by different ligands was scored by the number of +s. The activity equivalent to full-length WT human receptor activity was denoted as "+++", each "+" is equivalent to 33% of WT activity, and no activity was denoted as "-". Also, 100 nmol/L of MK-677, Ghrelin, or GH-25 was used. ECL2: extracellular loop 2; FL: full length; GHS: growth hormone secretagogue; ICL2: intracellular loop 2; TM2: transmembrane domain 2; TM3: transmembrane domain 3; TM5: transmembrane domain 5; TM6: transmembrane domain 6.

In this study, we compared the binding characteristics of GHS-R1a peptide ligand ghrelin and two synthetic GHS-R1a agonists MK-0677 and GHS-25. We found that ghrelin and GHS-25 are competitors of MK-0677, although they are structurally different (Figure 1). Importantly, the GHS activity of all of them was completely abolished in *Ghsr* knockout mice (as GHS-25 shown in Figure 2 and MK-0677 shown in reference [25]), suggesting they all are specific GHS-R1a ligands. It is also noteworthy that while 10 µg of GHS-25 induced 70 ng/mL of GH release 5 min after its administration (Figure 2), 10 µg of ghrelin or MK-677 induced 24 or 48 ng/mL of GH release, respectively,<sup>[25]</sup> suggesting that GHS-25 is a more potent GHS than ghrelin and MK-677. The potent *in vivo* effect of GHS-25 could be attributed to many factors. In addition to its binding efficiency to GHS-R, it could also be due to its pharmacological half-life and/or its ability to cross the blood-brain barrier.

GHS-R1a protein sequence is evolutionarily conserved. Puffer fish GHS-R1a shares 58% amino acid identity with humans and is activated by the structurally distinct ligand peptide GHRP-6 and non-peptide MK-0677.<sup>[13,14]</sup> Surprisingly, in contrast to GHRP-6 and MK-0677, ghrelin is unable to activate the puffer fish GHS-R1a (Table 1). GPCRs exhibit considerable variation in the structural determinants of ligand recognitions. Agonists and antagonists may bind to GHS-R1a in vastly different manners, as they are competitive ligands for a common receptor. Chimeric receptor has been used as a major tool to study the structure-function relationship of the seven-helix GPCR family.<sup>[35]</sup> Hence, we used the human and fish GHS-R1a chimeric receptors to assess the entire structure-function relationship of GHS-R1a and to identify its specific recognition sites for various ligands.

Given the very high degree of sequence identity between the human and puffer fish receptors, especially the TMs, it is reasonable to predict that both human and puffer fish GHS-R1a receptors would bind to its common core in a consistent manner. However, surprisingly, ghrelin and GHS-25 were not able to activate the puffer fish receptor (Table 1). The human and puffer fish chimera allowed us to mix and match different binding pockets to identify the unique binding sites that distinguish puffer fish from human sequences. The *ScaI* and *ApaI-ScaI* sets of chimeras suggested that the selectivity of the ligands is mainly achieved through the favorable interactions with the structural elements proximate to TM5 and TM6 (Table 1), which distinguish the peptide ligand ghrelin from small synthetic ligands MK-0677 and GHS-25. With the *XcmI* chimera, the finding further narrowed the binding domain into a region of 40 amino acids between *XcmI* and *ScaI* that constitutes human GHS-R TM6 and ECL3 (Figure 5A), and this replacement is sufficient to rescue ghrelin activation in the puffer fish receptor backbone. Amazingly, with only 40 humanized amino acid residues, we were able to transform the inactive puffer fish receptor to an active "humanized" receptor with activity comparable to that of wild-type human receptor in ghrelin activation. However, it should be noted that any result from chimeric studies may not fully reflect the true structure-function relationship of the wild-type receptors. Nevertheless, our data offer a valuable insight for addressing the question of the structural basis of ligand binding and ligand selectivity.

Among GPCR ligands, the interaction of a small ligand with the receptor is very different from that of a peptide ligand. Small molecules primarily interact with the amino acid residues in the TMs, but peptide ligands have also been shown to interact with extracellular domains.<sup>[35]</sup> Our data showed that swapping GHS-R C-terminal regions affects the activity of ghrelin more than the activity of



**Figure 5: The predicted topology and the alternative splitting forms of GHS-R. (A)** The amino acid sequence homology between human and puffer fish GHS-R (modified from reference [13] with permission). The receptor structure is illustrated by a schematic representation of TM domains, ECL, and ICL. Conserved amino acid residues are shown in white circles, and the amino acid residues of puffer fish GHS-R different from those in humans are shown in red circles. The empty circles are the amino acid residues present in humans, but absent in puffer fish. The restriction enzyme sites used to construct the chimeras are pointed by the arrows. **(B)** The diagram of two GHS-R alternative transcripts. The receptor isoform GHS-R1a contains TM 1–7, and GHS-R1b only contains TM 1–5. ECL: extracellular loops; GHS-R: growth hormone secretagogue receptor; ICL: intracellular loops; TM: transmembrane.

MK-0677 and GHS-25 (Table 1). As shown in Figure 5A, the amino acid sequences of human and puffer fish receptors are highly conserved in TM6, but the residues on ECL3 are quite different between humans and puffer fish. Ghrelin has a very unique octanoylated modification of a hydroxyl group on its third serine residue different from small synthetic molecules, which is essential for ghrelin's binding to GHS-R1a.<sup>[15]</sup> It is known that human *GHS-R* gene genomic sequence contains an intron that divides the open reading frame into two exons, encoding TM 1–5 and TM 6–7, which generates two GHS-R subtypes: 1a and 1b.<sup>[10]</sup> GHS-R1a encodes a full-length isoform and is a functional receptor; in contrast, GHS-R1b is a shorter isoform with only TM 1–5 (Figure 5B), which is an inactive receptor.

Our site-directed mutagenesis data further demonstrated

that ghrelin, MK-0677, and GHS-25 bind to distinct, but partially overlapped pockets on GHS-R1a (Table 2). Particularly, the findings showed that W276A and F279L mutations in TM6 abolished ghrelin activity. Interestingly, a naturally occurring mutant allele of F279L has been identified in a family with obesity and short stature,<sup>[36]</sup> confirming that TM6 residues are critical to ghrelin's GH-stimulating function. Another identified human mutant GHS-R1a variant is A204E, which has been reported to be associated with short stature in two families.<sup>[37]</sup> Recently, a mouse model of GHS-R A203E mutation that ablates constitutive GHS-R activity and blunts GH release has been reported, and the phenotype showed short body and femur length in aged mice.<sup>[38]</sup> In addition to the important binding residues in GHS-R1a C-terminal region, we also observed the mutation D99N in TM2 that abolished the activity of all three ligands. Although our domain swapping study did not specifically check TM2, the assay of SfiI and XcmI chimeras showed a lower activity than wild-type human GHS-R1a at a ligand dose of 1  $\mu\text{mol/L}$  (Table 1), suggesting absence of human TM2 could partially affect the receptor activity in response to ligands.

Our finding collectively supports that TM6 and ECL3 are essential for the optimal activity of GHS-R1a in response to agonists in general, and specificity to natural ligand ghrelin.<sup>[13,39]</sup> This is also well in line with the recent elegant work from Kojima's group that elucidated the crystal structure of the ghrelin receptor bound to an antagonist.<sup>[40]</sup> The crystal structure of GHS-R bound to an antagonist confirmed that the binding pocket of GHS-R1a is bifurcated into two cavities by a salt bridge between E124 and R283.<sup>[39,40]</sup> A unique characteristic feature is a gap structure between TM6 and TM7. The extracellular side of the TM7 curves outward to a greater degree than that of corresponding structures of other class A GPCRs. In agreement with this observation, our finding revealed that humanizing region of ECL3 plays a major role in converting the puffer fish GHS-R1a to a structure that is no longer inert toward activation by ghrelin. In fact, recent multiple studies of GHS-R protein structure demonstrated that important ECL3 amino acid residues at the junction to TM6 contribute to form ligand-binding pocket, particularly the phenylalanine residues F286 and F290, which are located near the extracellular surface of the receptor and play a critical role in the entry of ghrelin into the receptor core.<sup>[40]</sup> Structure showed that F286 and F290 interact with ghrelin's fifth leucine residue<sup>[41]</sup> and F286 is also in close proximity to ghrelin's third serine side chain.<sup>[42]</sup> Mutating F286 and F290 to alanine or polar and charged amino acids displays dramatically diminished receptor activity.<sup>[41-43]</sup>

In conclusion, GHS-R1a is capable of binding different ligands in a variety of ways, depending on the selectivity



and chemical structure of ligands. The residues on TM6 and ECL3 are essential for the natural ligand ghrelin-dependent activation. Understanding of GHS-R1a and ligand interactions will facilitate the development of better ghrelin receptor agonists/antagonists for treatment of GH deficiency and neuronal developmental disorders.

## Author Contributions

Conceptualization: Sun Y and Smith RG; methodology and analysis: Sun Y, Kennedy H, and Smith AGA; writing of the original draft preparation: Sun Y and Smith RG; writing of revision and editing: Ye X and Sun Y; supervision and funding acquisition: Smith RG. All authors have read and agreed to the published version of the manuscript.

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## Conflict of Interest

The authors declare no conflict of interest.

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