Isolation and characterisation of the marmoset gonadotrophin releasing hormone receptor: Ser¹⁴⁰ of the DRS motif is substituted by Phe

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Abstract

In order to facilitate the understanding of gonadotrophin-releasing hormone (GnRH) agonist and antagonist action in the primate animal model, the marmoset GnRH receptor (GnRH-R) was cloned and characterised. It was shown to have 95% and 85% sequence identity with the human and rat GnRH-Rs, respectively, and, when transiently expressed in COS-7 cells, it exhibited high-affinity des-Gly¹⁰, [D-Trp⁶]-GnRH binding, with a K_d value similar to those of both the rat and human forms, but with a greatly reduced B_{max} value. The ED₅₀ for production of GnRH-induced total inositol phosphate (IP) for the marmoset GnRH-R was also similar to those of the rat and the human, but the maximal response compared with the rat receptor was markedly reduced. In all mammalian forms of the GnRH-R cloned to date, the conserved DRY region of G-protein-coupled receptors is substituted with DRS. The most interesting feature of the marmoset GnRH-R was the substitution of this motif with DRF. In

order to investigate the DRS to DRF substitution, a Ser¹⁴⁰Phe rat GnRH-R mutant was generated. The mutant had a K_d value similar to that of the wild-type rat receptor, although the B_{max} value was slightly lower, indicating that expression of functional mutant receptor at the cell surface was reduced. The ED₅₀ value for IP production was also similar to that of the wild-type receptor, with a reduction in maximal response. The level of internalisation for the rat wild-type and mutant GnRH-R constructs was also assessed and the Ser¹⁴⁰Phe mutant was shown to have an increased rate of receptor internalisation, suggesting a role for this residue in regulating internalisation. These results show that the marmoset GnRH-R exhibits a substitution in the DRS motif and that this substitution may play a part in desensitisation and internalisation events.

Journal of Endocrinology (1999) **163**, 447–456

Introduction

The gonadotropin-releasing hormone receptor (GnRH-R) has been cloned from a number of mammalian species: mouse (Tsutsumi *et al.* 1992), rat (Eidne *et al.* 1992, Kaiser *et al.* 1992), cow (Kakar *et al.* 1993), human (Kakar *et al.* 1992, Chi *et al.* 1993), sheep (Brooks *et al.* 1993, Illing *et al.* 1993) and pig (Weesner & Matteri 1994) and, more recently, a non-mammalian species (catfish; Tensen *et al.* 1997), and has been shown to be a 7-transmembrane G-protein-coupled receptor (GPCR). The mammalian form has a number of unique features in that it is the only GPCR cloned to date that exhibits a complete lack of an intracellular C-terminal tail, a region associated with internalisation and desensitisation of GPCRs (Heding *et al.* 1998). The conserved Asp in transmembrane domain (TM) II and Asn in TMVII are substituted with Asn and Asp, respectively. The highly conserved region, DRY, of

the second intracellular region is also substituted, with DRS.

The marmoset (*Callithrix jacchus*) is an animal model for many reproductive biology studies and has been used extensively to study the effects of GnRH agonists and antagonists *in vivo* (Lunn *et al.* 1990). The marmoset, unlike most other species, exhibits maintained secretion of luteinising hormone (LH) and testosterone after treatment with GnRH agonist implants, but not after GnRH antagonist treatment. It has been shown that, although in these animals pituitary desensitisation does take place tonic, non-pulsatile secretion of LH takes place which stimulates the secretion of testosterone (Lunn *et al.* 1992). It has been suggested that there may be a species difference in the marmoset mechanism of pituitary desensitisation to GnRH and that this difference may be the result of an alternative structure for the marmoset receptor. In order to allow the more effective use of this animal model in

Table 1 Oligonucleotide primer structures for PCR cloning of the marmoset GnRH-R. The table gives the 5'-3' sequences and the annealing sites for each oligo. The orientation of the oligo (forward or reverse) primer is also given. Oligos 1 and 2 were used for cloning the full-length coding region, whereas oligos 5 and 6 were used to verify the region coding for the DRF sequence. The other oligos were used to verify the remaining areas of the receptor sequence. PCR primers were designed according to the human GnRH-R cDNA (Kakar *et al.* 1992)

	Sequence	Nucleotide position	Direction
Oligo			
1	5'-ATGGCAAACAGTGCCTCTCC-3'	1-20	Forward
2	5'-GCTTTGATCCACTTATCTATGGATATTTTCTCTGTGA-3'	951-987	Reverse
3	5'-CACTCTCACCTTGTCTGG-3'	88-105	Forward
4	5'-GGAAAGATCCGAGTGACTG-3'	104-122	Reverse
5	5'-GCTTTTCTCCATGTATGC-3'	364-381	Forward
6	5'-ACTACTGAGGATCCAGG-3'	489-504	Reverse
7	5'-GCTTCCTGGTATCACGA-3'	417-434	Reverse
8	5'-CCTGGCCTGGATCCTCAGTAG-3'	484-504	Forward
9	5'-CCCCACGAACATACT-3'	736-753	Reverse
10	5'-GCCACTTCATTACTGTCTGC-3'	818-838	Forward

understanding the mechanism of GnRH agonist and antagonist action, the marmoset GnRH-R was cloned and characterised. The receptor was found to share a high sequence identity with the human GnRH-R and, like the human GnRH-R, to be expressed at very low levels *in vitro*. The ability of the marmoset GnRH-R to bind a GnRH agonist and couple to the IP second messenger pathway was compared to the rat and human forms of the receptor. It was also found that, in the marmoset GnRH-R, the highly conserved DRY region was substituted with DRF, rather than the DRS motif observed in the other mammalian forms of the GnRH-R. The DRS region of the GnRH-R is known to be important for receptor internalisation (Arora *et al.* 1995, 1997). Mutating the Ser of the mouse GnRH-R to Tyr increases receptor internalisation (Arora *et al.* 1995), whereas mutating the Ser to Ala has no significant effect (Arora *et al.* 1997). The levels of expression for the marmoset GnRH-R were very low, so, in order to investigate this substitution, a Ser¹⁴⁰Phe mutant of the rat GnRH-R was generated and its effects on functionality compared with that of the wild-type rat receptor. The levels of receptor internalisation for the wild-type and Ser¹⁴⁰Phe mutant rat receptor constructs were also assessed.

Materials and Methods

Production of marmoset cDNA

Male marmoset pituitary tissue was obtained from a bank of stored tissues. These had been extracted from animals that were killed as part of unrelated experiments on gonadal function as described elsewhere (Young *et al.* 1997). The pituitary tissue samples (from six anterior pituitary glands, approximately 50 mg total weight) were

pooled and processed for total RNA extraction. The pituitary tissue from two independent batches of six marmoset pituitaries were processed on two separate occasions in order to verify the sequence of the marmoset GnRH-R. The tissue was homogenised in 1 ml tri-reagent (Sigma Chemicals Co., Poole, Dorset, UK) then shaken with 200 µl chloroform. Samples were centrifuged and the RNA-rich supernatant transferred to a fresh tube. RNA was purified by phenol-chloroform extraction and the contents and purity were estimated by u.v. absorbance at 260/280 nm. A 1 µg sample of pituitary RNA was reverse transcribed with a commercial kit (First Strand cDNA synthesis kit, Clontech, Palo Alto, CA, USA) using oligo-dT primers. Efficacy of reverse transcription was assessed by PCR with primers for GAPDH, a house-keeping gene.

PCR cloning of the marmoset GnRH-R

PCR was performed on two independent occasions using oligos 1 and 2 (Table 1) corresponding to the start and stop regions of the human GnRH-R, and marmoset cDNA obtained from two separate batches of pituitary tissue as a template. This yielded a band of approximately 1 kb, which was purified and cloned into the TA cloning vector, PCRII (Invitrogen, Leek, Netherlands). The DNA was sequenced on an Applied Biosystems 373A automated sequencer (Perkin Elmer, Warrington, Lancs, UK). Analysis of the sequence was performed by means of the program GeneJockey II (Biosoft, Cambridge, Cambs, UK). To verify certain regions of the marmoset GnRH-R sequence, seven further PCR reactions were performed on the cDNA using a range of PCR primers (oligos 3-10) specific to the human and marmoset GnRH-R sequences (Table 1). On each occasion, these PCR fragments were cloned into the PCRII vector and sequenced.

Site-directed mutagenesis

Site-directed mutagenesis of the rat GnRH-R was performed as described elsewhere (Cook & Eidne 1997). The Ser residue at position 140 was mutated to a Phe using the oligonucleotide sequence, 5'-CTGGATCGCTTTCTGGCCG-3'.

Cell culture and receptor expression

The full-length marmoset GnRH-R cDNA was subcloned into the expression vector, pcDNA3 (Invitrogen). COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (HIFCS), 0.3 mg/ml glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (all obtained from Sigma). Rat, human, marmoset and the Ser¹⁴⁰Phe mutant GnRH-R DNA (10 µg) were transiently transfected into monolayer cultures of COS-7 cells in 75 cm² flasks using the DEAE dextran method (Promega, Madison, WI, USA).

Receptor-binding assessment

The peptide des-Gly¹⁰,[D-Trp⁶]-GnRH was obtained from Sigma. Cell membranes were prepared 48 h after transfection. Membranes were resuspended in assay buffer (40 mM Tris-HCl, 2 mM MgCl₂, pH 7.2) and approximately 50 µg total protein added per tube. Saturation binding assays were performed using a radiolabelled GnRH agonist ¹²⁵I-des-Gly¹⁰,[D-Trp⁶]-GnRH (¹²⁵I-[GnRH-A]) with a specific activity of 26–57 µCi/µg, over a range of concentrations (0.25–6.5 nM). Assays were incubated at 4 °C before filtration onto Whatman GFB filter paper (BDH, Lutterworth, Leics, UK). Receptor dissociation constant (K_d) and receptor number (B_{max}) were calculated using a hyperbolic function fit with Sigma Plot (Jandel Scientific). All assay points were in duplicate, and experiments were performed at least three times.

Measurement of total inositol phosphate

The transfected cells were incubated at 37 °C for 24 h post-transfection and then trypsinised. The cells were transferred to 24-well plates and labelled with 1.0 µCi/well ³H-myo-inositol in inositol-free DMEM (Gibco, Paisley, Strathclyde, UK), dialysed HIFCS, 0.3 mg/ml glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were incubated for a further 48 h and then treated with GnRH (human, Sigma). Total inositol phosphate production was assessed as described elsewhere (Heding *et al.* 1998), and is expressed as a percentage of the maximal response to GnRH by the wild-type rat GnRH-R.

In situ hybridisation

Stored frozen pituitary tissue removed from male marmosets was cryostat-sectioned and thaw-mounted

onto poly-L-lysine-coated microscope slides. Antisense and sense riboprobes were prepared as described previously (Sellar *et al.* 1993). Hybridisation was performed as described by Whitelaw *et al.* (1995), with some modifications as described by Sellar *et al.* (1993). Photomicrographs were taken in both the dark and the light field.

Receptor-internalisation assay

Levels of GnRH-induced wild-type and Ser¹⁴⁰Phe rat GnRH-R internalisation were assessed by the methods described previously (Heding *et al.* 1998). To acquire further information on the effect of the Ser¹⁴⁰Phe substitution on GnRH-R internalisation kinetics, the four-compartment model described by Koenig & Edwardson (1997) was applied, in an attempt to model receptor intracellular trafficking. This model predicts that there are four major pathways of receptor movement within the cells: (i) receptor endocytosis from the surface of the cell into endosomes, (ii) recycling from endosomes back to plasma membrane, (iii) receptor movement from endosomes to lysosomes for degradation and (iv) the delivery of newly synthesised receptor to the plasma membrane. Utilising a numerical parameter-fitting routine, we calculated a complete set of rate constants for the receptor endocytosis (k_e), recycling (k_r), synthesis (k_s) and degradation (k_x).

Statistical analyses

The ability of the different receptor constructs to bind the ligand, stimulate second messenger function and internalise were analysed using *t*-tests. A *P* value of *P* < 0.05 was considered significant.

Results

PCR cloning of the marmoset GnRH-R

PCR using oligos 1 and 2 (Table 1), coding for the start and stop regions of the human GnRH-R, and marmoset cDNA as a template yielded a DNA fragment of 987 bp. These PCR reactions were performed on two separate samples of cDNA obtained from two independent batches of DNA. The sequence of the receptor is shown in Fig. 1. Sequence alignment of the marmoset GnRH-R showed that it had 95% and 85% sequence identity with the human and rat GnRH-R respectively (Fig. 2). As the primers used for the PCR reaction were human GnRH-R-specific, the first 20 and last 38 bases of the marmoset sequence are identical to those of the human sequence, and these sequences were excluded from the calculation of the sequence identity between the human and marmoset receptors. The oligo primer 2, coding for the C-terminal end of the receptor, corresponds to a very

	Met	Ala	Asn	Ser	Ala	Ser	Pro	Glu	Gln	Asn	Gln	Asn	His	Cys	Leu	Ala	Ile	17
1	<u>ATG</u>	<u>GCA</u>	<u>AAC</u>	<u>AGT</u>	<u>GCC</u>	<u>TCT</u>	<u>CCC</u>	GAA	CAG	AAT	CAA	AAT	CAC	TGT	TTA	GCC	ATC	
	Asn	Asp	Ser	Ile	Pro	Leu	Met	Gln	Gly	Asn	Leu	Pro	Thr	Leu	Thr	Leu	Ser	34
52	AAT	GAC	AGC	ATC	CCA	CTG	ATG	CAG	GGC	AAC	CTC	CCC	ACT	CTC	ACC	TTG	TCT	
	<u>Gly</u>	<u>Lys</u>	<u>Ile</u>	<u>Arg</u>	<u>Val</u>	<u>Thr</u>	<u>Val</u>	<u>Thr</u>	<u>Phe</u>	<u>Phe</u>	<u>Leu</u>	<u>Phe</u>	<u>Leu</u>	<u>Leu</u>	<u>Ser</u>	<u>Thr</u>	<u>Thr</u>	51
103	GGA	AAG	ATC	CGA	GTG	ACA	GTT	ACT	TTC	TTC	CTT	TTT	CTA	CTC	TCT	ACA	ACC	
	<u>Phe</u>	<u>Asn</u>	<u>Ala</u>	<u>Ser</u>	<u>Phe</u>	<u>Leu</u>	<u>Leu</u>	<u>Lys</u>	Leu	Gln	Lys	Trp	Thr	Gln	Lys	Lys	Glu	68
154	TTT	AAT	GCG	TCT	TTC	TTG	TTG	AAA	CTT	CAG	AAG	TGG	ACA	CAG	AAG	AAA	GAG	
	Lys	Gly	Lys	Lys	<u>Leu</u>	<u>Ser</u>	<u>Arg</u>	<u>Met</u>	<u>Lys</u>	<u>Leu</u>	<u>Leu</u>	<u>Leu</u>	<u>Lys</u>	<u>His</u>	<u>Leu</u>	<u>Thr</u>	<u>Leu</u>	85
205	AAA	GGG	AAA	AAA	CTC	TCA	AGA	ATG	AAG	CTG	CTC	TTA	AAA	CAT	CTG	ACC	TTA	
	<u>Ala</u>	<u>Asn</u>	<u>Leu</u>	<u>Leu</u>	<u>Glu</u>	<u>Thr</u>	<u>Leu</u>	<u>Ile</u>	<u>Val</u>	<u>Met</u>	Pro	Leu	Asp	Gly	Met	Trp	Asn	102
256	GCC	AAC	CTG	TTG	GAG	ACT	CTG	ATT	GTC	ATG	CCA	CTG	GAT	GGA	ATG	TGG	AAC	
	Ile	Thr	Val	Gln	Trp	Tyr	Ala	Gly	Glu	Phe	<u>Phe</u>	<u>Cys</u>	<u>Lys</u>	<u>Val</u>	<u>Leu</u>	<u>Ser</u>	<u>Tyr</u>	119
307	ATT	ACA	GTC	CAA	TGG	TAT	GCT	GGA	GAG	TTC	TTC	TGC	AAA	GTC	CTC	AGT	TAT	
	<u>Leu</u>	<u>Lys</u>	<u>Leu</u>	<u>Phe</u>	<u>Ser</u>	<u>Met</u>	<u>Tyr</u>	<u>Ala</u>	<u>Pro</u>	<u>Ala</u>	<u>Phe</u>	<u>Met</u>	<u>Met</u>	<u>Val</u>	<u>Val</u>	<u>Ile</u>	<u>Ser</u>	136
358	CTA	AAG	CTT	TTC	TCC	ATG	TAT	GCC	CCA	GCC	TTC	ATG	ATG	GTG	GTG	ATT	AGC	
	<u>Leu</u>	Asp	Arg	Phe	Leu	Ala	Ile	Thr	Arg	Pro	Leu	Ala	Leu	Lys	Ser	Asn	Arg	153
409	CTG	GAC	CGC	TTC	CTG	GCT	ATC	ACG	AGG	CCC	CTA	GCT	CTG	AAA	AGC	AAC	AGA	
	Lys	Leu	Gly	Gln	Ser	<u>Met</u>	<u>Val</u>	<u>Gly</u>	<u>Leu</u>	<u>Ala</u>	<u>Trp</u>	<u>Ile</u>	<u>Leu</u>	<u>Ser</u>	<u>Ser</u>	<u>Ile</u>	<u>Phe</u>	170
460	AAG	CTT	GGA	CAG	TCC	ATG	GTT	GGC	CTG	GCC	TGG	ATC	CTC	AGT	AGT	ATC	TTT	
	<u>Ala</u>	<u>Gly</u>	<u>Pro</u>	<u>Gln</u>	<u>Leu</u>	<u>Tyr</u>	<u>Ile</u>	<u>Phe</u>	<u>Arg</u>	<u>Met</u>	<u>Ile</u>	His	Leu	Ala	Asp	Ser	Ser	187
511	GCG	GGA	CCA	CAG	TTA	TAC	ATC	TTC	AGG	ATG	ATT	CAT	TTA	GCA	GAC	AGC	TCT	
	Gly	Gln	Thr	Lys	Val	Phe	Ser	Gln	Cys	Val	Thr	His	Cys	Ser	Phe	Pro	Gln	204
562	GGA	CAA	ACA	AAA	GTT	TTC	TCT	CAA	TGT	GTA	ACA	CAC	TGC	AGT	TTT	CCA	CAA	
	Trp	Trp	His	Gln	Ala	<u>Phe</u>	<u>Tyr</u>	<u>Asn</u>	<u>Phe</u>	<u>Phe</u>	<u>Thr</u>	<u>Phe</u>	<u>Ser</u>	<u>Cys</u>	<u>Leu</u>	<u>Phe</u>	<u>Ile</u>	221
613	TGG	TGG	CAT	CAA	GCA	TTT	TAT	AAC	TTT	TTC	ACC	TTC	AGC	TGC	CTT	TTC	ATC	
	<u>Thr</u>	<u>Pro</u>	<u>Leu</u>	<u>Leu</u>	<u>Ile</u>	<u>Met</u>	<u>Leu</u>	<u>Ile</u>	<u>Cys</u>	Asn	Ala	Lys	Ile	Ile	Phe	Thr	Leu	238
664	ACC	CCG	CTT	CTC	ATC	ATG	CTG	ATC	TGC	AAT	GCA	AAA	ATC	ATC	TTC	ACC	CTA	
	Thr	Arg	Val	Leu	His	Gln	Asp	Pro	His	Lys	Leu	Gln	Leu	Asn	Gln	Ser	Lys	255
71	ACA	CGG	GTC	CTT	CAT	CAG	GAC	CCG	CAC	AAA	CTA	CAA	CTG	AAT	CAG	TCC	AAG	
	Asn	Asn	Ile	Pro	Arg	Ala	Arg	Leu	Arg	<u>Thr</u>	<u>Leu</u>	<u>Lys</u>	<u>Met</u>	<u>Thr</u>	<u>Val</u>	<u>Ala</u>	<u>Phe</u>	272
766	AAC	AAT	ATA	CCA	AGA	GCA	CGG	CTG	AGG	ACT	CTA	AAA	ATG	ACG	GTT	GCA	TTT	
	<u>Val</u>	<u>Thr</u>	<u>Ser</u>	<u>Phe</u>	<u>Thr</u>	<u>Val</u>	<u>Cys</u>	<u>Trp</u>	<u>Thr</u>	<u>Pro</u>	<u>Tyr</u>	<u>Tyr</u>	<u>Val</u>	<u>Leu</u>	<u>Gly</u>	<u>Ile</u>	<u>Trp</u>	289
817	GTC	ACT	TCA	TTT	ACT	GTC	TGC	TGG	ACT	CCC	TAC	TAT	GTC	CTA	GGA	ATT	TGG	
	<u>Tyr</u>	<u>Trp</u>	<u>Phe</u>	<u>Asp</u>	<u>Pro</u>	<u>Glu</u>	<u>Met</u>	<u>Leu</u>	<u>Asn</u>	<u>Arg</u>	<u>Val</u>	<u>Ser</u>	<u>Asp</u>	<u>Pro</u>	<u>Val</u>	<u>Asn</u>	<u>His</u>	306
868	TAT	TGG	TTT	GAT	CCT	GAA	ATG	TTA	AAC	AGG	GTG	TCA	GAT	CCA	GTA	AAT	CAC	
	<u>Phe</u>	<u>Phe</u>	<u>Phe</u>	<u>Leu</u>	<u>Phe</u>	<u>Ala</u>	<u>Phe</u>	<u>Leu</u>	<u>Asn</u>	<u>Pro</u>	<u>Cys</u>	<u>Phe</u>	<u>Asp</u>	<u>Pro</u>	<u>Leu</u>	<u>Ile</u>	<u>Tyr</u>	323
919	TTC	TTC	TTT	CTC	TTT	GCT	TTC	TTA	AAC	CCA	<u>TGC</u>	<u>TTT</u>	<u>GAT</u>	<u>CCA</u>	<u>CTT</u>	<u>ATC</u>	<u>TAT</u>	
	<u>Gly</u>	<u>Tyr</u>	<u>Phe</u>	Ser	Leu	STOP												328
970	<u>GGA</u>	<u>TAT</u>	<u>TTT</u>	<u>TCT</u>	<u>CTG</u>	<u>TGA</u>												

Figure 1 Nucleotide and deduced amino acid sequences of the marmoset GnRH-R. The first 20 and last 38 base pairs of the marmoset sequence were obliged to be the same as the human sequence, because the oligonucleotide primers used for the PCR were human GnRH-R specific (shown in boxes). The Ser to Phe substitution is shown in bold. Potential N-linked glycosylation sites (*), and phosphorylation sites (●) are indicated. The putative transmembrane domains I–VII are underlined.

Figure 2 Alignment of the GnRH-R protein sequences from the marmoset, human, sheep, cow, pig, rat and mouse. The non-conserved regions of the receptors are shown in the open boxes. The substitution of DRS to DRF is shown in bold and the regions corresponding to the human GnRH-R specific oligonucleotides used for PCR of the marmoset coding region are underlined.

highly conserved region of the mammalian GnRH-R (this region is identical for all the mammalian forms of the GnRH-R cloned to date), so it is likely that the marmoset sequence closely resembles this primer (Fig. 2). However, the N-terminal region exhibits much greater variability in the region encoded by primer 1 (Fig. 2), and thus there may be mismatches in this portion of the marmoset GnRH-R sequence. A number of other oligonucleotides were also used to verify various internal regions of the marmoset coding region (oligos 3–10; Table 1). Because of the difficulty in obtaining marmoset tissue for this study, we were unable to resolve the 5' and 3' non-coding regions of the receptor.

Hydrophobicity analysis (Kyte & Doolittle 1982) of the marmoset GnRH-R showed the distinctive 7-TM domain pattern. Like the human GnRH-R, the marmoset receptor has an extra amino acid – a Lys in extracellular loop II – compared with the rat. The most interesting feature of the marmoset GnRH-R was the substitution of the Tyr in the DRY region in the second intracellular loop with a Phe residue. In all other mammalian forms of the GnRH-R cloned to date, this Tyr residue is replaced with a Ser. This site is highlighted in Figs 1 and 2. In order to investigate the importance of the Phe residue at this site, we generated a Ser¹⁴⁰Phe rat GnRH-R mutant. The rat GnRH-R was used in preference to either the marmoset or human GnRH-R to investigate the importance of this site because of the very low levels of expression of the human and marmoset homologues. To verify the uniqueness of the Ser to Phe substitution, we also PCR-cloned the stump-tailed macaque (*Macaca arctoides*)

GnRH-R. This sequence, in common with the other mammalian forms of the GnRH-R, encoded DRS (results not shown).

Ligand binding of the rat, human, marmoset and Ser¹⁴⁰Phe mutant receptors

The rat GnRH-R transiently expressed in COS-7 cells exhibited high-affinity GnRH binding, with a K_d value of 1.99 ± 0.18 nM and a B_{max} value of 7.99 ± 1.01 pmol/mg protein (Fig. 3, Table 2). Both the human and marmoset GnRH-Rs also had high-affinity GnRH agonist binding (K_d values 2.21 ± 0.05 and 2.09 ± 0.10 nM, respectively), but with significantly reduced B_{max} values (1.36 ± 0.29 and 0.62 ± 0.17 pmol/mg protein, $P < 0.05$) compared with that of the rat GnRH-R. The receptor affinity ($K_d = 2.06 \pm 0.05$ nM) and the B_{max} value (6.76 ± 1.01 pmol/mg protein) for the Ser¹⁴⁰Phe mutant were similar to those of wild-type rat GnRH-R.

GnRH-activated inositol phosphate production by the rat, human, marmoset and Ser¹⁴⁰Phe mutant receptors

The rat GnRH-R activated total inositol phosphate production with an ED_{50} of 1.19 ± 0.23 nM (Fig. 4, Table 2). The human form of the receptor activated inositol phosphate production with an ED_{50} value (1.21 ± 0.96 nM) similar to that of the rat, although the maximal response was significantly reduced compared with that of the rat GnRH-R ($28.4 \pm 4.60\%$, $P < 0.05$). The ED_{50} for the marmoset receptor was similar to those of the other

Figure 3 Saturation binding of ^{125}I -[GnRH-A] (0.25–6.5 nM) to COS-7 cell membranes (approximately 50 μg total protein) expressing wild-type (WT) rat GnRH-R, human GnRH-R, marmoset GnRH-R and Ser 140 Phe mutant rat GnRH-R. Data points are the mean of duplicate samples and the graph is a representative example from at least three experiments.

receptor constructs, although it had a significantly reduced ($P<0.05$) maximal inositol phosphate production compared with that of the rat. The Ser 140 Phe mutant receptor had a significantly reduced maximal inositol phosphate of $71.6 \pm 6.38\%$ response compared with that of the wild-type rat receptor, although the ED_{50} of 1.64 ± 0.50 nM was comparable to that of the wild-type.

In situ hybridisation

Whole marmoset pituitary gland sections probed with marmoset GnRH-R-specific riboprobes showed an even distribution of the GnRH-R mRNA in the anterior pituitary, most probably indicating the presence of the

Figure 4 GnRH-stimulated (0.1–1000 nM) total inositol phosphate (IP) production by COS-7 cells expressing rat wild-type (WT) GnRH-R, human GnRH-R, marmoset GnRH-R and Ser 140 Phe rat mutant GnRH-R. Data points are the mean of triplicate samples and the graph is a representative example from at least three experiments.

gonadotrophs (Fig. 5a,b). There was an absence of expression in the posterior pituitary.

Wild-type rat and Ser 140 Phe rat mutant receptor internalisation

GnRH receptors expressed in COS-7 cells have been shown to undergo ligand-induced internalisation (Loumaye & Catt 1983). A direct comparison between the wild-type rat and the Ser 140 Phe mutant receptor internalisation was made by calculating the percentage of bound radiolabelled ligand that was internalised over a range of

Table 2 Dissociation constants (K_d) and receptor number (B_{max}) were calculated from saturation binding assays. Total inositol phosphate (IP) production and maximum IP response from dose-dependent GnRH-stimulated total IP assays were also calculated

GnRH-R construct	Receptor binding K_d (nM)	B_{max} (pmol/mg protein)	Total IP production	
			ED_{50} (nM)	(% rat WT max)
Rat (WT)	1.99 ± 0.18	7.99 ± 1.01	1.19 ± 0.23	100
Human	2.21 ± 0.05	$1.36 \pm 0.29^*$	1.21 ± 0.96	$28.4 \pm 4.60^*$
Marmoset	2.09 ± 0.10	$0.61 \pm 0.17^*$	11.5 ± 11.2	$12.2 \pm 1.62^*$
Ser140Phe rat mutant	2.05 ± 0.05	6.76 ± 1.01	1.64 ± 0.50	$71.6 \pm 6.38^*$

Values represent the mean $\pm$ S.E.M. from at least three independent experiments. *Significant change in K_d , B_{max} , ED_{50} , maximum IP response or receptor expression ($P<0.05$) compared with those of the rat wild-type (WT) receptor.

Figure 5 Dark (a) and light (b) field photomicrographs showing expression of the GnRH-R mRNA in the marmoset whole pituitary gland. High levels of mRNA are expressed in the anterior pituitary (AP) with an absence of expression in the posterior pituitary (PP). Scale bar represents 200 μ m.

Figure 6 Time-dependent changes in wild-type (WT) GnRH-R and Ser¹⁴⁰Phe rat mutant GnRH-R. The time-dependent loss of the cell-surface GnRH-R was measured by changes in radioligand binding. The graph is a representative example from at least three experiments performed in duplicate. The amount of receptors on the cell surface as a function of time was fitted using the four-compartment model (Koenig & Edwardson 1997).

time intervals (Fig. 6). These data were further analysed according to Koenig & Edwardson's (1997) four-compartment model (Heding *et al.* 1998) and a set of rate constants calculated for receptor endocytosis (k_e), recycling (k_r), synthesis (k_s) and degradation (k_x). The endocytosis, recycling and degradation rate constants for the Ser¹⁴⁰Phe mutant receptor were all significantly altered ($k_e = 0.018 \pm 0.003$; $k_r = 0.069 \pm 0.001$; $k_x = 0.141 \pm 0.03$, $P < 0.05$) compared with those of the wild-type receptor ($k_e = 0.014 \pm 0.002$; $k_r = 0.057 \pm 0.005$; $k_x = 0.068 \pm 0.02$), indicating that the mutant receptor undergoes a slightly increased rate of internalisation, recycling and degradation. There was no significant difference between the synthesis rate constant (k_s) for the two receptors.

Discussion

In order to facilitate the understanding of GnRH agonist and antagonist action in the primate animal model, the marmoset GnRH-R was cloned and characterised. It exhibits a high degree of homology with the human form of the receptor and has the extra Lys in the second extracellular loop that is a characteristic of the human form of the receptor. Both the N- and C-terminal regions of the marmoset GnRH-R were obligated to be identical to those of the human receptor, as human specific oligonucleotide primers were used for the PCR reactions. The C-terminal portions of all the mammalian forms of the GnRH-R cloned to date are identical (Fig. 2); however,

there is a considerable amount of inter-species variability in the N-terminal portion of the receptor. Thus it may be that there are inaccuracies within the N-terminal area of the marmoset GnRH-R. We were unable to resolve the 5' and 3' untranslated regions of the marmoset receptor because of difficulties in obtaining sufficient marmoset pituitary tissue. However, we were able to perform *in situ* hybridisation, which showed that expression of GnRH-R mRNA in the marmoset was localised to the anterior portion of the pituitary, with an absence of expression in the posterior pituitary.

In transiently transfected cells, the marmoset GnRH-R exhibits low levels of expression, similar to that of the human. The reasons for this low expression compared with other species such as the rat or the mouse are unknown, but it has been demonstrated previously that receptor expression is unaffected by the presence or absence, in the cDNA construct, of the 5' and 3' non-coding regions of the receptor (Faccenda & Eidne 1997). The most interesting feature of the marmoset GnRH-R is the substitution of the Tyr in the highly conserved DRY motif of the second intracellular loop with Phe. In all the mammalian forms of the GnRH-R cloned to date, the Tyr in this region is substituted with Ser. The Asp and Arg residues of this region are highly conserved, and have been shown to have effects on receptor expression, activation and internalisation (Arora *et al.* 1997), in addition to being important for binding (Ballesteros *et al.* 1998). The Tyr/Ser residue is believed to be a potential phosphorylation site, acting as a substrate for a G-protein receptor kinase, and thus important for receptor desensitisation and internalisation (Palczewski 1997). This suggestion was supported by the findings of a study carried out by Arora and colleagues (1995), which demonstrated that mutating the Ser to a Tyr in the mouse GnRH-R increased the rate of receptor internalisation and agonist binding affinity, although no effect was observed on G-protein coupling. However, it has also been shown that generating a mouse Ser¹⁴⁰Ala mutation had no significant effects on ligand binding, receptor coupling or internalisation (Arora *et al.* 1997).

In order to investigate the importance of the substitution of Ser with Phe in this region, a Ser¹⁴⁰Phe rat GnRH-R mutant was generated. The rat GnRH-R was used for this part of the study because of the low levels of expression observed for both the human and marmoset receptor *in vitro*. The Ser¹⁴⁰Phe mutant bound GnRH with a receptor affinity identical to that of the wild-type, although the $B_{\max}$ value was slightly reduced. The Ser¹⁴⁰Phe mutant had significantly reduced maximal inositol phosphate response compared with the wild-type rat receptor, although the EC_{50} value for GnRH-induced inositol phosphate production was similar to that of the wild-type. These data suggest that the presence of the Phe at this site interferes slightly with the efficiency of receptor expression, and may also reduce receptor coupling.

As the DRS/F region is associated with GnRH-R internalisation, the ability of the rat Ser¹⁴⁰Phe mutant to internalise, compared with that of the wild-type rat receptor, was also assessed, and the rate constants calculated. The Ser¹⁴⁰Phe mutant exhibited an increased rate of receptor internalisation, recycling and degradation compared with the wild-type. A substitution of Ser to Tyr at this position has been previously shown to increase markedly the level of receptor internalisation (Arora *et al.* 1995). Tyrosine is an aromatic amino acid with a hydroxyl group, and serine also has a hydroxyl group. Phenylalanine is aromatic in nature, suggesting that the aromaticity of the residue at this site is more important in terms of regulating receptor internalisation. These data would suggest that phosphorylation of the Y/S/F site is not essential for receptor internalisation and desensitisation, as phenylalanine is not a phosphorylation site. In the recently cloned, non-mammalian catfish form of the receptor – the only GnRH-R cloned to date that has an intracellular C-terminal tail – this site is occupied by a histidine residue (Tensen *et al.* 1997), supporting the postulate that phosphorylation of this residue is not an important mediator of receptor internalisation. However, in the catfish receptor, it is possible that the presence of potential phosphorylation sites within the intracellular C-terminal tail may be more important for regulating receptor internalisation, as the catfish receptor exhibits faster internalisation than the rat GnRH-R (M Vrecl, personal communication). It is possible that the presence of the Phe in this position introduces a protease-specific site, as the mutant receptor is degraded at a slightly increased rate compared with the wild-type. However, it may be that the level of degradation is a result of the greater rate of receptor internalisation and is simply related to the increased number of receptors exposed to proteases inside the cell.

The male marmoset, along with the red deer and the bull, does not respond to GnRH agonists with a suppression of LH or testosterone secretion. It has been shown that although in these animals pituitary desensitisation to GnRH does occur, tonic, non-pulsatile secretion of LH takes place which stimulates the secretion of testosterone (D'Occhio & Aspdén 1996; Lunn *et al.* 1992). In order to investigate whether these differences in pituitary desensitisation were a result of an alternative structure for the marmoset GnRH-R, the receptor was cloned and sequenced. However, although we have shown that there are, indeed, differences between the marmoset GnRH-R and the other cloned mammalian forms of the receptor, we have been unable to account for the observed differences in receptor desensitisation in response to exogenous GnRH administration.

We have not investigated the effects of all the amino acid substitutions that are unique to the marmoset receptor, only the most interesting Ser¹⁴⁰Phe difference, thus it may be that any species differences are the result of one or more of these substitutions that have not been

studied here. It is also possible that there are differences between the species that would be detected if the levels of expression of marmoset GnRH-R were high enough to enable experiments to be performed on the receptor itself and not on a modified form of the higher-expressing rat GnRH-R.

In conclusion, we have cloned the marmoset GnRH-R, which has a high sequence identity with the human form of the receptor. Uniquely, the marmoset receptor has been shown to code for DRF, rather than the DRS found in all other mammalian forms of the GnRH-R, in the second intracellular loop, and the presence of this amino acid may result in slightly faster receptor internalisation rates.

Acknowledgements

We are grateful to Professor R. Millar for critical evaluation of the manuscript and Dr Milka Vrecl for expert technical assistance with receptor internalisation assays.

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Received 13 January 1999

Revised manuscript received 8 July 1999

Accepted 23 July 1999