Isolation and characterisation of the marmoset gonadotrophin releasing hormone receptor: Ser^{140} 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-Gly^{10},[D-Trp^{6}]-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 ED_{50} 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 Ser^{140}Phe 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 ED_{50} 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 Ser^{140}Phe 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.

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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

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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)

Oligo	Sequence	Nucleotide position	Direction
1	5′-ATGGCAAACAGTGCCTCTCC-3′	1–20	Forward
2	5′-GCTTTGATCCACTTATCTATGGATATTTTTCTCTGTGA-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′-CCCCCACGAACTACAACT-3′	736–753	Reverse
10	5′-GCCACTTCATTTACTGTCTGC-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 Ser^{140}Phe 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 Ser^{140}Phe 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 housekeeping 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′-CTGGATCGCT TTCTGGCCG-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 Ser^{140}Phe mutant GnRH-R DNA (10 μg) were transiently transfected into monolayer cultures of COS-7 cells in 75 cm^2 flasks using the DEAE dextran method (Promega, Madison, WI, USA).

Receptor-binding assessment

The peptide des-Gly^{10},[D-Trp^6]-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 $MgCl_2$, pH 7·2) and approximately 50 μg total protein added per tube. Saturation binding assays were performed using a radiolabelled GnRH agonist ^{125}I-des-Gly^{10},[D-Trp^6]-GnRH (^{125}I-[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 ^{3}H-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 Ser^{140}Phe rat GnRH-R internalisation were assessed by the methods described previously (Heding *et al.* 1998). To acquire further information on the effect of the Ser^{140}Phe 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

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    Met Ala Asn Ser Ala Ser Pro Glu Gln Asn Gln Asn His Cys Leu Ala Ile  17
  1 ATG GCA AAC AGT GCC TCT CCC 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
    Gly Lys Ile Arg Val Thr Val Thr Phe Phe Leu Phe Leu Leu Ser Thr Thr  51
103 GGA AAG ATC CGA GTG ACA GTT ACT TTC TTC CTT TTT CTA CTC TCT ACA ACC      I
    Phe Asn Ala Ser Phe Leu Leu Lys 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 Leu Ser Arg Met Lys Leu Leu Leu Lys His Leu Thr Leu  85
205 AAA GGG AAA AAA CTC TCA AGA ATG AAG CTG CTC TTA AAA CAT CTG ACC TTA      II
    Ala Asn Leu Leu Glu Thr Leu Ile Val Met 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 Phe Cys Lys Val Leu Ser Tyr 119
307 ATT ACA GTC CAA TGG TAT GCT GGA GAG TTC TTC TGC AAA GTC CTC AGT TAT
    Leu Lys Leu Phe Ser Met Tyr Ala Pro Ala Phe Met Met Val Val Ile Ser 136  III
358 CTA AAG CTT TTC TCC ATG TAT GCC CCA GCC TTC ATG ATG GTG GTG ATT AGC
    Leu 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 Met Val Gly Leu Ala Trp Ile Leu Ser Ser Ile Phe 170
460 AAG CTT GGA CAG TCC ATG GTT GGC CTG GCC TGG ATC CTC AGT AGT ATC TTT      IV
    Ala Gly Pro Gln Leu Tyr Ile Phe Arg Met Ile 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 Phe Tyr Asn Phe Phe Thr Phe Ser Cys Leu Phe Ile 221
613 TGG TGG CAT CAA GCA TTT TAT AAC TTT TTC ACC TTC AGC TGC CTT TTC ATC      V
    Thr Pro Leu Leu Ile Met Leu Ile Cys 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 Thr Leu Lys Met Thr Val Ala Phe 272
766 AAC AAT ATA CCA AGA GCA CGG CTG AGG ACT CTA AAA ATG ACG GTT GCA TTT      VI
    Val Thr Ser Phe Thr Val Cys Trp Thr Pro Tyr Tyr Val Leu Gly Ile Trp 289
817 GTC ACT TCA TTT ACT GTC TGC TGG ACT CCC TAC TAT GTC CTA GGA ATT TGG
    Tyr Trp Phe Asp Pro Glu Met Leu Asn Arg Val Ser Asp Pro Val Asn His 306
868 TAT TGG TTT GAT CCT GAA ATG TTA AAC AGG GTG TCA GAT CCA GTA AAT CAC
    Phe Phe Phe Leu Phe Ala Phe Leu Asn Pro Cys Phe Asp Pro Leu Ile Tyr 323
919 TTC TTC TTT CTC TTT GCT TTC TTA AAC CCA TGC TTT GAT CCA CTT ATC TAT      VII
    Gly Tyr Phe Ser Leu STOP                                            328
970 GGA TAT TTT TCT CTG TGA
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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.

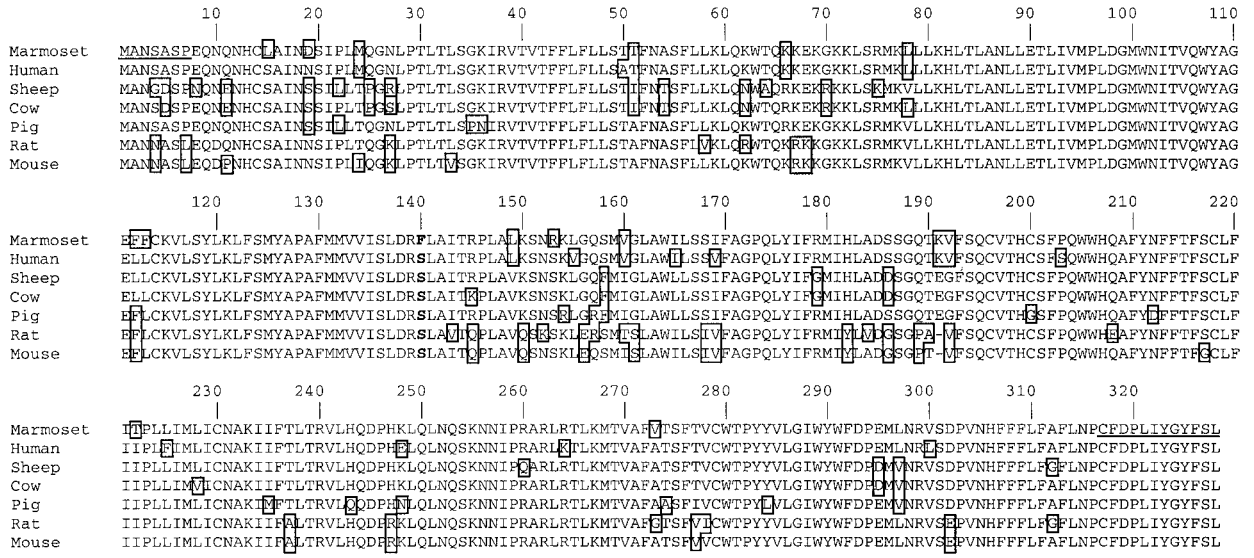

Figure 2 Alignment of the GnRH-R protein sequences from the marmoset, human, sheep, cow, pig, rat and mouse. The non-conserved regions of the receptors are shown in the open boxes. The substitution of DRS to DRF is shown in bold and the regions corresponding to the human GnRH-R specific oligonucleotides 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 Ser^{140}Phe 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 Ser^{140}Phe mutant were similar to those of wild-type rat GnRH-R.

GnRH-activated inositol phosphate production by the rat, human, marmoset and Ser^{140}Phe 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 ± 4·60%, $P<0·05$). The ED_{50} for the marmoset receptor was similar to those of the other

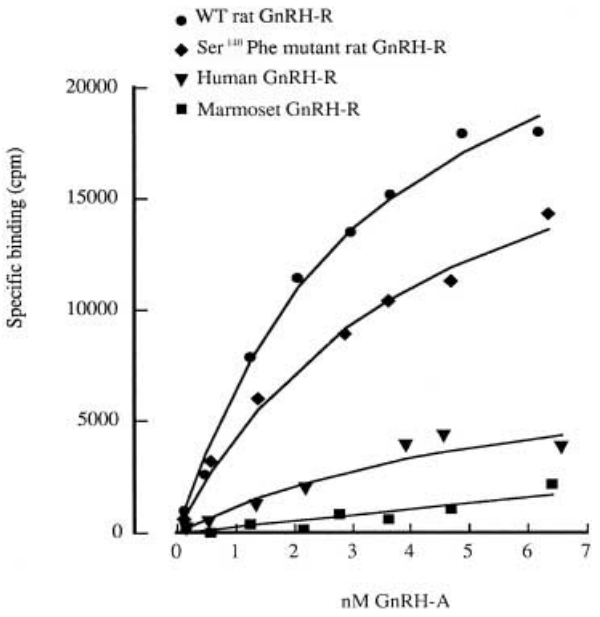

Figure 3 Saturation binding of ^{125}I-[GnRH-A] (0·25–6.5 nM) to COS-7 cell membranes (approximately 50 µg total protein) expressing wild-type (WT) rat GnRH-R, human GnRH-R, marmoset GnRH-R and Ser^{140}Phe mutant rat GnRH-R. Data points are the mean of duplicate samples and the graph is a representative example from at least three experiments.

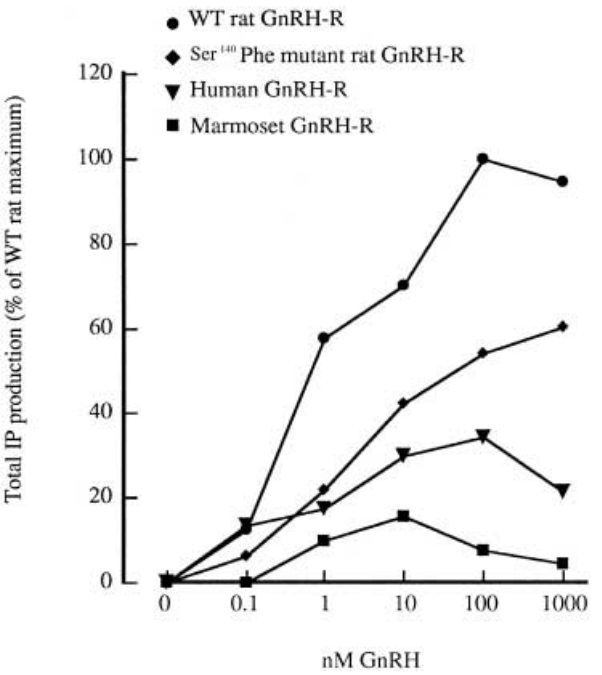

Figure 4 GnRH-stimulated (0·1–1000 nM) total inositol phosphate (IP) production by COS-7 cells expressing rat wild-type (WT) GnRH-R, human GnRH-R, marmoset GnRH-R and Ser^{140}Phe rat mutant GnRH-R. Data points are the mean of triplicate samples and the graph is a representative example from at least three experiments.

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 ± 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 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

	Receptor binding K_d (nM)	B_{max} (pmol/mg protein)	Total IP production	
			ED_{50} (nM)	(% rat WT max)
GnRH-R construct				
Rat (WT)	1·99 ± 0·18	7·99 ± 1·01	1·19 ± 0·23	100
Human	2·21 ± 0·05	1·36 ± 0·29*	1·21 ± 0·96	28·4 ± 4·60*
Marmoset	2·09 ± 0·10	0·61 ± 0·17*	11·5 ± 11·2	12·2 ± 1·62*
Ser140Phe rat mutant	2·05 ± 0·05	6·76 ± 1·01	1·64 ± 0·50	71·6 ± 6·38*

Values represent the mean ± 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.

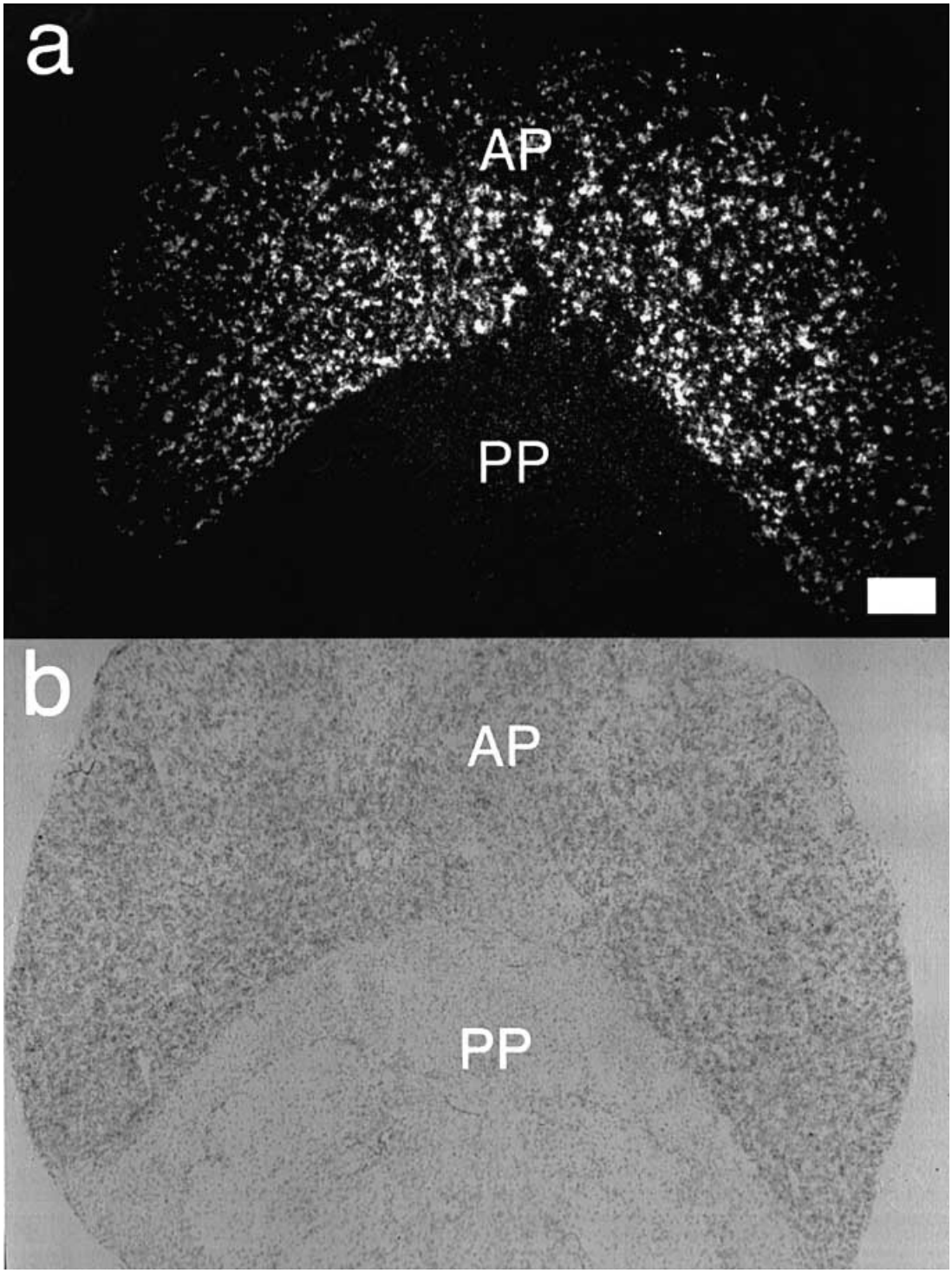

Figure 5 Dark (a) and light (b) field photomicrographs showing expression of the GnRH-R mRNA in the marmoset whole pituitary gland. High levels of mRNA are expressed in the anterior pituitary (AP) with an absence of expression in the posterior pituitary (PP). Scale bar represents 200 µm.

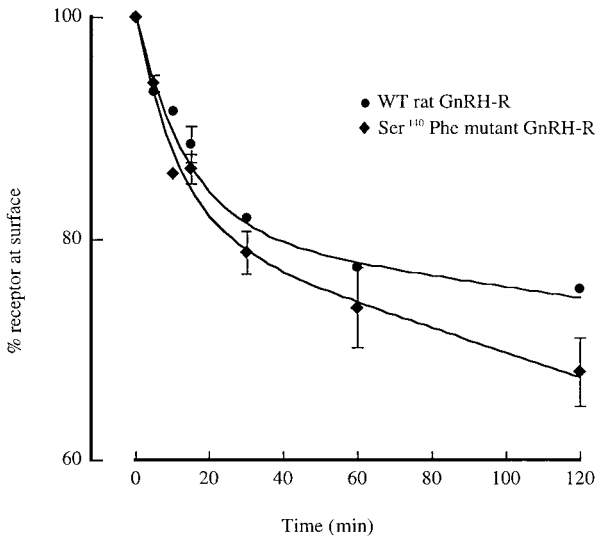

Figure 6 Time-dependent changes in wild-type (WT) GnRH-R and Ser^{140}Phe rat mutant GnRH-R. The time-dependent loss of the cell-surface GnRH-R was measured by changes in radioligand binding. The graph is a representative example from at least three experiments performed in duplicate. The amount of receptors 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 Ser^{140}Phe mutant receptor were all significantly altered ($k_e = 0{\cdot}018 \pm 0{\cdot}003$; $k_r = 0{\cdot}069 \pm 0{\cdot}001$; $k_x = 0{\cdot}141 \pm 0{\cdot}03$, $P<0{\cdot}05$) compared with those of the wild-type receptor ($k_e = 0{\cdot}014 \pm 0{\cdot}002$; $k_r = 0{\cdot}057 \pm 0{\cdot}005$; $k_x = 0{\cdot}068 \pm 0{\cdot}02$), 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 Ser^{140}Ala 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 Ser^{140}Phe 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 Ser^{140}Phe mutant bound GnRH with a receptor affinity identical to that of the wild-type, although the B_{max} value was slightly reduced. The Ser^{140}Phe 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 Ser^{140}Phe mutant to internalise, compared with that of the wild-type rat receptor, was also assessed, and the rate constants calculated. The Ser^{140}Phe 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 & Aspden 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 Ser^{140}Phe 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.

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