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Cloning and characterization of bonnet monkey GnRH receptor

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Gonadotrophin releasing hormone (GnRH) plays an important role in the reproductive processes of both males and females. It is synthesized by the hypothalamus and binds to a specific receptor on the pituitary to bring about the release of the gonadotrophins, lutineizing hormone and follicle stimulating hormone, which in turn bring about the release of the gonadal steroids. Although the structure of the GnRH receptor (GnRHR) has been elucidated from a number of sources, no information is available about the receptor from the non-human primate species. Here we report the cloning and characterization of the receptor from the pituitary of the bonnet monkey. Antiserum to a bacterially expressed recombinant fragment was used in Western blot analysis and fluorescence microscopy to demonstrate the presence of GnRHR in both human and monkey placentae and pituitary.

Key words: antibody/cloning/gonadotrophin releasing hormone receptor/monkey/pituitary

Introduction

Gonadotrophin releasing hormone (GnRH) is a decapeptide synthesized by the hypothalamus, which plays a key role in the regulation of mammalian reproductive processes (Hazum and Conn, 1988; Clayton, 1989; Conn *et al*., 1995). It is secreted by the hypothalamic neurons in a pulsatile manner, from where it is transported to the pituitary by means of the hypothalamo-hypophyseal portal system. It regulates the synthesis and secretion of the gonadotrophins LH and FSH, from the anterior pituitary gonadotrophs, which in turn regulate the hormonal and gametogenic functions of the gonads (Fink, 1988). This effect of GnRH is brought about by the binding of this peptide to high affinity receptors located on the surface of the gonadotrophs (Clayton, 1989).

The GnRH receptor (GnRHR) belongs to the extremely large and diverse family of proteins known as the G-protein coupled receptors (GPcR), which are characterized by the presence of seven transmembrane segments (Huckle and Conn, 1988) linked together by extracellular and intracellular domains. Although the *GnRHR* gene has been cloned in the mouse (Reinhart *et al*., 1992; Tsutsumi *et al*., 1992), the rat, (Eidne *et al*., 1992; Kaiser *et al*., 1992) and the sheep pituitary (Brook *et al*., 1993), among primates, information is available only about the human *GnRHR* (Kakar *et al*., 1992; Chi *et al*., 1993). There is a high degree of homology between the cloned GnRHR, particularly between mice and rats, in which 93% of the nucleotide sequences and 95% of the amino acid sequences are conserved (Kaiser *et al*., 1992). The GnRHR is composed of 327 (rat and mouse) and 328 (human) amino acids with seven apparent transmembrane regions, characteristic of the family of GPcR. An unusual structural feature of the GnRHR is the lack of the characteristically long intracellular C-terminal tail which is reported to be involved in mediation of down-regulation and desensitization of some GPcR (Dohlman *et al*., 1991). The GnRHR has also been found in a variety of other reproductive tissues such as placenta (Currie *et al*., 1981; Lin *et al*., 1995), as well as several non-reproductive tissues such as human liver, heart and kidney (Kakar and Jennes, 1995), where its functions are not established. Of the extrahypothalamic sites, the presence of the *GnRHR* mRNA in the human placenta has attracted considerable interest, in view of the reported regulation of synthesis and secretion of human chorionic gonadotrophin (HCG), by GnRH in the placenta (Khodr and Siler Khodr, 1978; Mathialagan and Rao, 1986). Recent studies have established the presence of both GnRH and its receptor mRNA in both cyto- and syncytiotrophoblast cells of human placenta (Lin *et al*., 1995; Wolfahrt *et al*., 1998). Using human placental cells (JEG-3) transfected with a construct containing the human GnRH upstream promoter region, it has been demonstrated (Chen *et al*., 1998) that both oestradiol and cortisol may be physiologically involved in the regulation of GnRH gene expression in the human placenta. Based on these results it is suggested that the paracrine and autocrine regulation of HCG secretion by placental GnRH is mediated through the regulation of *GnRHR* message (Lin *et al*., 1995). Thus while there are several reports regarding the role of GnRH in regulation of HCG in the human placenta, relatively much less information is available on the presence of *GnRHR* mRNA in the human placenta. It is only very recently that the presence of *GnRHR* mRNA in cyto- and syncytiotrophoblasts has been demonstrated using the technique of in-situ reverse transcription–polymerase chain reaction (RT–PCR) (Wolfahrt *et al*., 1998). In addition, all the published reports so far deal with the demonstration of *GnRHR* mRNA and not the protein in the placenta (Wolfahrt *et al*., 1998). Also considerable

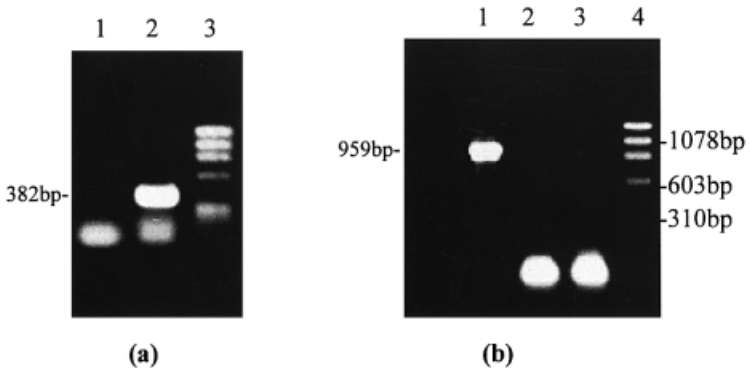

Figure 1. Reverse transcription–polymerase chain reaction (RT–PCR) to demonstrate the *GnRHR* in monkey pituitary. Two different sets of primers (based on the human sequence) were used for (**a**) and (**b**). (**a**) 1: no RT control; 2: 382bp RT–PCR product; 3: PhiX174 DNA ladder. (**b**) 1: 959bp RT–PCR product; 2: no RT control; 3: no template control; 4: PhiX174 DNA ladder.

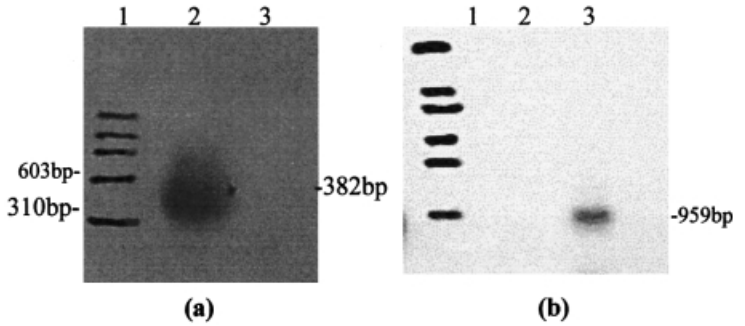

Figure 2. Southern blot analysis of reverse transcription–polymerase chain reaction (RT–PCR) product from monkey pituitary. (**a**) 1: PhiX174 DNA ladder; 2: RT–PCR product from monkey pituitary; 3: no RT control. (**b**) 1: 1 kb ladder DNA marker; 2: no RT control; 3: RT–PCR product from monkey pituitary. Human pituitary *GnRHR* cDNA was used as the probe.

controversy exists regarding the stage at which the *GnRHR* mRNA is present in the placenta (Boyle *et al*., 1998). It has also been reported by several investigators that the human placental GnRHR has lower affinity to the ligand compared to the pituitary receptor (Currie *et al*., 1981; Belisle *et al*., 1984) and therefore it will be of interest to compare the GnRHR from the pituitary and the placenta of the same species. Earlier studies from our laboratory have established that the pregnant bonnet monkey can be used as an experimental model system to investigate the role of placental GnRH in regulation of mCG (monkey chorionic gonadotrophin) (Rao and Moudgal, 1984; Rao and Chakraborti, 1990). Thus the characterization of the GnRHR from the pituitary and the placenta of the same species, in which both in-vivo and in-vitro studies can be carried out, will be of importance. As a first step towards this, we report the cloning and characterization of the *GnRHR* mRNA from the bonnet monkey pituitary and localization of GnRHR protein in the placenta and pituitary using an antiserum raised to a fragment of the monkey pituitary GnRHR.

Materials and methods

Materials

Tri reagent, Freund's complete reagent, Ficoll, formamide, salmon sperm DNA, protease inhibitors [phenylmethylsulphonyl fluoride (PMSF), soya bean trypsin inhibitor, *p*-amino benzamidine, leupeptin and aproteinin], bovine serum albumin (BSA), Tween 20, Ponceau, goat anti-rabbit gammaglobulin conjugated to fluorescein isothiocyanate (FITC) were obtained from Sigma, St Louis, MO, USA. Enhanced chemiluminescence (ECL) kit, Megaprime DNA labelling kit, Hybond nitrocellulose, Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase, nylon membrane, and Sephadex G-50 were obtained from Amersham–Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK. Goat anti-rabbit gammaglobulin conjugated to horseradish peroxidase was obtained from Bangalore Genei, Peenya, Bangalore, India. [α^{32}P]dCTP (specific activity: 3000 Ci/mmol) was obtained from NEN, Life Science Products Inc., Boston, MA, USA. The expression vector pGEX 5X-2 was obtained from Pharmacia-biotech, UK. Triton X-100 was obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

cDNA cloning

Pituitaries from adult bonnet monkeys which were killed following reproductive toxicity studies at the Primate Research Laboratory, IISc, Bangalore, were collected in TRI reagent. Total RNA was isolated by the protocol provided by the manufacturer, estimated, and subjected to reverse transcription by random hexamer priming using MMLV reverse transcriptase. PCR was carried out using specific sets of primers. Two primer sets that were based on the human pituitary *GnRHR* sequence were used for PCR amplification. Set I was expected to give an amplification product of 382 bp corresponding to the position 436–798 of the coding sequence of the GnRHR (amino acids 146–266), and set II, an amplification product of 959 bp corresponding to the position 1 to 949 of the coding sequence of the GnRHR (amino acids 1–316). The primers used for PCR were: set I, sense 5′ GCGGATCCCCTAGCTTTGAAAAGCAAC3′ and antisense 5′ CGAATTCTTATAGAGTCTTCAGCCGTGCTCT3′; set II: sense 5′ CTGGGAAAATATGGCAAACAGT 3′ and antisense 5′ ATGGGTTTAAAAAGGCAAAGA 3′.

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1 ATGGCAAACA GTGCTTTACC TGAACAGAAT CAAAATCACT GTTCAGTCAT CAACAACAGC

61 ATCCCACTAA TGCAGGGCAA CCTCCCCACT CTGACCTTGT CTGGAAAGAT CCGAGTGACA

121 GTTACTTTCT TCCTTTTTCT ACTCTCTGCG ACCTTTAATG CTTCTTTCCT GCTGAAACTT

181 CAGAAGTGGA CACAGAAGAA AGAGAAAGGG AAAAAGCTGT CAAGAATGAA GCTGCTCTTA

241 AAACATCTGA CCTTAGCCAA CCTGTTGGAG ACTCTGATTG TCATGCCACT GGATGGGATG

301 TGGAACATTA CAGTCCAATG GTATGCTGGA GAGTTCCTCT GCAAAGTTCT CAGTTATCTA
    Hind III
361 AAGCTTTTCT CCATGTATGC CCCAGCTTTC ATGATGGTGG TGATCAGCCT GGACCGCTCC

421 CTGGCTATCA CGAGGCCCCT AGCTTTGAAA AGCAGCAGCA AGCTCGGACA GTCCATGGTT
                BamH I
481 GGCCTGGCCT GGATCCTCAG TAGTGTCTTT GCAGGACCAC AGTTATACAT CTTCAGGATG
                                                              Pst I
541 ATTCATCTAG CAGACAGCTC CGGACAGACA AAAGTTTTCT CTCAATGTGT AACACACTGC

601 AGTTTTCCAC AATGGTGGCA TCAAGCATTC TATAACTTTT TCACCTTCAG CTGCCTCTTC

661 ATCATCCCTC TTCTCATCAT GCTGATCTGC AATGCAAAAA TCATCTTCAC CCTGACACGG
                 BamH I
721 GTCCTTCATC AGGATCCCCA CAAACTACAA CTGAATCAGT CCAAGAACAA TATACCAAGA

781 GCACGGCTGA AGACTCTAAA AATGACAGTT GCATTTGCCA CTTCATTCAC TGTCTGCTGG

841 ACTCCCTACT ATGTCCTAGG AATTTGGTAT TGGTTTGATC CTGAAATGTT AAACAGGGTG

901 TCAGACCCAG TAAATCACTT CTTCTTTCTC TTTGCCTTTT TAAACCCAT
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Figure 3. Nucleotide sequence of the 959 bp reverse transcription–polymerase chain reaction product from monkey pituitary. Sequencing was carried out by cycle sequencing on an ABI Prism genetic analyser. The open reading frame starts from position 1 of the reported sequence and extends till the position 949 of the sequence.

The conditions used for PCR were 94°C for 2 min, 52°C for 1 min, 72°C for 90 s for 40 cycles, for set I and 94°C for 2 min, 54°C for 1min and 72°C for 90 s, for set II using an MJ Research thermal cycler (MJ Research Inc., Waterdown, MA, USA).

The 382 bp product was then subjected to restriction digestion using *Bam*HI and *Eco*RI since the primers have been synthesized with *Eco*RI and *Bam*HI linkers to facilitate cloning. The expression vector pGEX 5X-2 was subjected to restriction digestion using the above-mentioned restriction endonucleases, following which both the 382 and the 959 bp products were agarose gel purified and ligation reaction was carried out using the required molar concentrations of the vector and the insert. *E.coli* DH5α cells were then transformed with the ligation mix and the presence of the insert was ascertained by restriction digestion of the plasmid DNA from different colonies (Sambrook *et al*., 1989).

Southern hybridization

The PCR products were analysed on a 1% agarose gel and the products were transferred onto nylon membrane. The human pituitary *GnRHR* cDNA (a kind gift from Dr Stuart C.Sealfon, Mount Sinai Medical Centre, New York, NY, USA) labelled with [α^{32}P]dCTP by the random primer method (using the Megaprime DNA labelling kit), was used as the probe. Twenty-five nanograms of the human pituitary cDNA was labelled using the manufacturer's protocol for a period of 30 min and the labelled DNA was separated from unlabelled deoxynucleotides using a Sephadex G-50 column. Hybridization was carried out in hybridization solution containing Ficoll, polyvinylpyrrolidone (PVP) and BSA (5×Denhardt's solution containing 0.5 g Ficoll 400, 0.5 g PVP and 0.5 g BSA), 6×SSC (1×SSC contains 8.765 g NaCl, 4.41 g sodium citrate per litre), 50% formamide and 100 μg of salmon sperm DNA. Hybridization was carried out at 42°C according to a published protocol (Sambrook *et al*., 1989).

Sequencing of the PCR products

DNA sequencing was performed on an ABI prism 310 automated genetic analyser using Dye-terminator chemistry. The sequencing reactions were performed using a Big Dye terminator cycle sequencing kit from Perkin Elmer Applied Biosystems.

Expression of the GnRHR *fragment and production of antibodies*

The PCR product of 315 bp (64 bp corresponding to position 436–491 of the coding sequence of the *GnRHR* were lost during cloning, because digestion of the PCR product with *Bam*HI restriction endonuclease resulted in the loss of 64 bp due to presence of an internal *Bam*HI site in the PCR product) was cloned into the expression vector pGEX 5X-2 and the protein was expressed as a GST (glutathione-*S*-transferase) fusion protein of 37 kDa (corresponding to amino acids 165 to 265 of the *GnRHR* sequence). The over-expressed protein was purified from *E.coli* BL 21 cells, by subjecting the *E.coli* cell lysate to preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), excising the band of interest and eluting the protein from the gel pieces by electroelution. This protein was then used to immunize rabbits. Rabbits were immunized with a primary dose of 250 μg of the purified protein in Freund's complete adjuvant. This was followed by booster doses of 100 μg and 50 μg of the purified protein, at weekly intervals, following which the rabbits were bled 7 days after the last booster and the serum tested for the presence of antibody by enzyme-linked immunosorbent assay (ELISA) using the purified protein (corresponding to 315 bp, which was expressed in *E.coli*) as the antigen.

Membrane preparation and Western blotting

Tissue (rat pituitary, monkey pituitary and human placenta) was homogenized in 2 ml of homogenization buffer containing 10 mmol/l

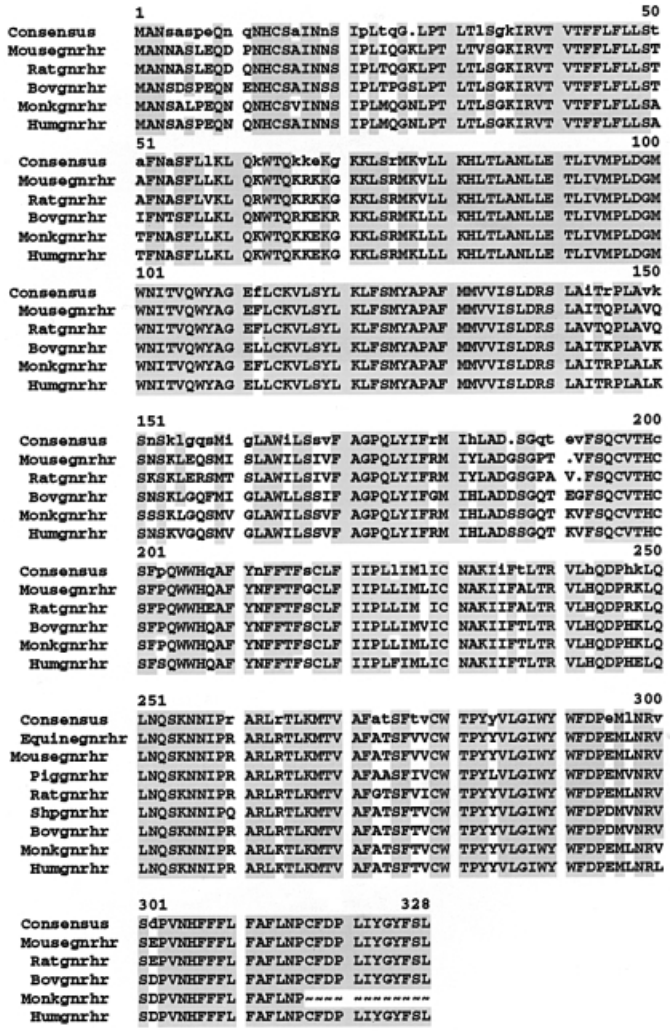

Figure 4. The predicted amino acid sequence of the monkey pituitary GnRHR and its homology with that from other species. Amino acids in the shaded areas are conserved between all species. The marked amino acids in the monkey GnRHR are those that differ from all other species (6, 16 and 152), those that differ only from the human (155, 203, 225 and 300) and those that differ from a few other species (112 and 248).

Tris–HCl pH 7.4, 250 mmol/l sucrose and protease inhibitors (2 mmol/l soya bean trypsin inhibitor, 2 mmol/l PMSF, 2 mmol/l *p*-aminobenzamidine, 5 µg/ml leupeptin, 2.5 µg/ml aproteinin), and was centrifuged at 400 *g* for 10 min at 4°C; the supernatant was centrifuged at 30 000 *g* for 1 h at 4°C in a Beckman J2-21M/E centrifuge using a JA-20 rotor to obtain a crude enriched membrane preparation with minor modifications to a published protocol (Nett *et al*., 1981). The supernatant was discarded and the pellet was resuspended in buffer containing 10 mmol/l Tris–HCl, pH 7.4, 0.1 mol/l NaCl, 0.2 mol/l $MgSO_4$. The protein content was estimated by Lowry's method (Lowry *et al*., 1951) using BSA as standard. Crude membrane protein (250 µg) was analysed by SDS–PAGE (10%) following which proteins were transferred onto nitrocellulose membrane (Towbin *et al*., 1979) for 2 h at 250 mA. The membrane was stained with Ponceau to check for the transfer (Sambrook *et al*., 1989) and the membrane was incubated with 5% non-fat milk for a period of 2 h. This was followed by incubation with the GnRHR antiserum at dilution of 1:1000 for a period of 2 h. The membrane was washed extensively with Tris-buffered saline (TBS) pH 7.4 containing 0.05% Tween 20, followed by washes with TBS. The blot was then incubated in 1:15 000 dilution of goat anti-rabbit gammaglobulin conjugated to horseradish peroxidase for 1 h and was washed extensively before developing using an ECL chemiluminiscence kit.

Processing of placental tissue for confocal microscopy

Fresh human placental tissue collected at medical termination of pregnancy or following Caesarean delivery was washed extensively to get rid of blood contamination. In every case, consent of the patient was obtained by the physician concerned before collection of the placenta by medical termination of pregnancy, Caesarean delivery or amniocentesis. Individual villi were then fixed overnight in 4% formalin and this was then followed by wash with phosphate-buffered saline (PBS), pH 7.3 to remove all traces of formalin. Villi were incubated in a 1% solution of bovine serum albumin in PBS, for 1 h to take care of non-specific adsorption. Following four washes with PBS, the villi were incubated with the appropriate dilution of antiserum to GnRHR fragment or normal rabbit serum for a period of 2 h at 37°C. The tissue was then washed extensively with PBS containing 0.2% Triton X-100, followed by a minimum of 10 washes with PBS and then incubated in a solution containing 1:200 dilution of goat anti-rabbit gammaglobulin conjugated to FITC for 1 h. Interference due to non-specific binding was eliminated by washing extensively with PBS containing 0.2% Triton X-100 followed by PBS. The tissue was then mounted onto glass slides (Fisher, USA) and visualized using a confocal microscope (Leica, Germany).

Results

RT–PCR

The results presented in Figure 1a and b, indicate that fragments of 382 and 959 bp are amplified upon RT–PCR from the bonnet monkey pituitary with the specific set of primers used (set I and set II respectively), and this is the size expected following genuine amplification from *GnRHR* mRNA, with the primers employed, based on the human pituitary *GnRHR* cDNA sequence. The absence of any amplification in the control lanes where the template was omitted establishes the purity of the reagents used for PCR.

Southern hybridization

It can be seen that the signals corresponding to 382 and 959 bp are obtained following hybridization to human *GnRHR* cDNA probe and no signal was seen in the no RT control lane, thus establishing the specificity of the PCR reaction (Figure 2a and b).

Sequencing of the PCR product

Final confirmation of the PCR product was carried out by sequencing of the 959 bp PCR product on an ABI prism machine (Genebank accession No. AF 156930) (Figure 3). The open reading frame starts at the very first base of the reported sequence and continues until the position 949. We have reported only a partial cDNA sequence and the sequence beyond position 949 is not available. The predicted amino acid sequence so obtained was subjected to a homology search using the FASTA program. The sequence showed 97% identity with the available published human pituitary sequence and showed a high degree of homology to the *GnRHR* sequences from other species such as sheep, mouse and rat. The alignment of bonnet monkey *GnRHR* and its sequence is presented to

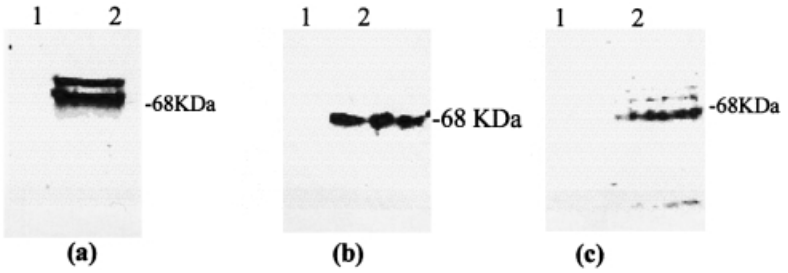

Figure 5. Western blotting of rat pituitary, human placenta and monkey pituitary with the GnRHR antiserum. (**a**) 1: rat pituitary membranes probed with normal rabbit serum; 2: rat pituitary membranes probed with GnRHR antiserum (1:1000). (**b**) 1: human placental membranes probed with normal rabbit serum; 2: human placental membranes probed with GnRHR antiserum (1:1000). (**c**) 1: monkey pituitary membranes probed with normal rabbit serum; 2: monkey pituitary membranes probed with GnRHR antiserum (1:1000).

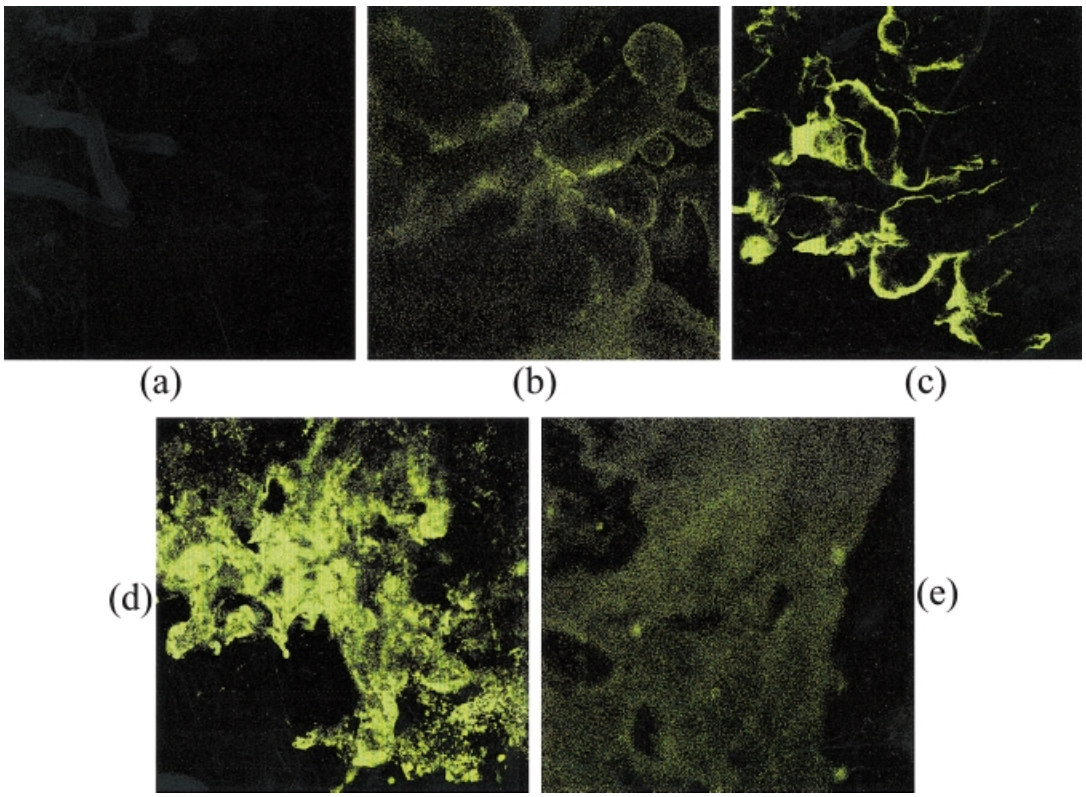

Figure 6. Immunofluorescent localization of the GnRHR protein in pituitary and placenta. (**a**) Normal rabbit serum-treated first trimester placenta. (**b**) First trimester human placenta. (**c**) Term human placenta. (**d**) Monkey placenta. (**e**) Human pituitary. Primary antibody used was GnRHR antiserum, which was followed by an anti-rabbit fluorescein isothiocyanate conjugate that is indicated by the green fluorescence. The samples were visualized at ×200 magnification using a Leica microscope.

indicate the homology of the translated protein sequence to the GnRHR from other species (Figure 4).

Western blotting

A signal corresponding to 68 kDa was observed with extracts from rat, monkey pituitary and human placenta, and no signal was seen in the normal rabbit serum-treated controls. In the case of rat and monkey pituitary, another signal of approximate molecular weight of 70 kDa was observed though the signal corresponding to 68 kDa was the most predominant signal (Figure 5a–c).

Immunofluorescence

The tissue, after incubation with antibody, was mounted onto a glass slide and visualized using a confocal microscope at a magnification of ×200. The staining pattern indicated that the expression of the GnRHR protein was localized to the cell membrane in both the first trimester and term placenta as evidenced by the fact that the fluorescence was restricted to the periphery (Figure 6b and c). The specificity of the staining was ascertained using the normal rabbit serum as a negative control, wherein no staining could be detected with any of the tissue samples (Figure 6a). Staining with the GnRHR antiserum was visible in the whole villi of monkey placenta (Figure 6d) as well as human pituitary (Figure 6e) sections. In the case of pituitary sections the staining was found to be localized to cell clusters, a fact which is in agreement with the published reports (Karande *et al*., 1995). Both the first trimester and term human placenta showed peripheral staining, whereas in the monkey placenta the staining was found to be associated

throughout the tissue. A similar staining pattern was observed in the monkey placenta samples with peptide antiserum raised to rat pituitary gonadotrophin releasing hormone receptor (data not shown), which was a kind gift from Dr Terry Nett, Colorado State University, Ft Collins, USA.

Discussion

Recent studies (Lin *et al*., 1995; Boyle *et al*., 1998; Wolfahrt *et al*., 1998) have demonstrated the presence of *GnRHR* mRNA in human placenta using in-situ hybridization and RT–PCR. Although results of these studies indicate the presence of *GnRHR* mRNA in human placenta, controversy still exists as to the stage at which it is present or predominantly expressed, or the cell type in which it is present. Thus, it has been reported that *GnRHR* mRNA is present in cytotrophoblast and syncytiotrophoblast cell layers and that the intensity of signal varied with gestation ages, being abundant at 6 weeks, peaking at 9 weeks, declining at 12 and 20 weeks and undetectable at term (Lin *et al*., 1995). In contrast, the message was detected by a modified RT–PCR protocol in 6 week and 8 week placental samples but not in 5 week and 7 week samples (Boyle *et al*., 1998). However, using in-situ RT–PCR and soluble phase RT–PCR, *GnRHR* gene expression was detected in all first and third trimester placentae with abundant signals for *GnRHR* message, both in cyto- and syncytiotrophoblasts, although the *GnRHR* message was at the limit of detection in cultured trophoblasts (Wolfahrt *et al*., 1998). It has been suggested that the reason for the variation in the expression of the *GnRHR* mRNA in the human placenta is that the message is extremely low (Boyle *et al*., 1998). Alternatively, *GnRHR* mRNA may have a short half-life or the mRNA may be translated as rapidly as it is transcribed with the result that it does not accumulate in the cell. It is also suggested that more than one form of *GnRHR* mRNA may be present at different stages of gestation (Boyle *et al*., 1998). It is pertinent to note in this connection that, in addition to our failure to demonstrate *GnRHR* mRNA by Northern analysis, we have not been able to demonstrate the *GnRHR* mRNA even by application of RT–PCR in the human placentae despite using several sets of primers which were designed based on the human pituitary sequence, suggesting the possibility that the human placenta may have more than one form of the *GnRHR* mRNA. Any one of the above reasons alone or in combination may be responsible for the difficulties associated with detection of *GnRHR* message in human placentae of different gestational stages. Considering these problems, we felt that an immunological approach using antibodies to larger fragment of the receptor to demonstrate the presence of GnRHR protein may yield more reliable results. In order to obtain specific antiserum to GnRHR, we have cloned and expressed a part of the GnRHR protein from the bonnet monkey. Our results establish that the nucleotide sequence of the bonnet monkey pituitary GnRHR has 97% homology with the human pituitary GnRHR although the 3′ sequence is not complete. The results of restriction map analysis and Southern hybridization provide additional evidence for the conclusion that there is extensive homology.

Although the use of antiserum to the full-length protein would have been more appropriate for immunological studies, we were unsuccessful in expressing the full-length fragment. However, expression of the small fragment of 382 bp was achieved as a GST-fusion protein and this corresponds to amino acids which encompass one transmembrane region, one extracellular loop and one intracellular loop.

Antibodies were raised to the fusion protein in rabbits and one of the rabbits produced high-titre antibodies indicating that the region expressed is highly immunogenic. Significant binding of the antigen by the antiserum was noticed even at a dilution of 1 in 80 000 in an ELISA. Though studies have been reported on the production of antibodies to peptide fragments [antiserum has been raised to amino acids 23–36 in the extracellular region of the murine GnRHR (Nett *et al*., 1981); antiserum has been raised to amino acids 1–29 in the extracellular region of the human pituitary GnRHR sequence (Karande *et al*., 1995)], this is the first report of production of antibodies to the transmembrane and extracellular loop. In addition to the ELISA, the ability of the antiserum to recognize the antigen used for immunizing the rabbits was ascertained in Western blot analysis. This antiserum has been successfully employed to demonstrate the presence of the GnRHR in rat pituitary and the human placental extracts. Before employing the antiserum for Western blot studies with pituitary and placental extracts, and to localize the GnRHR in placental villi and pituitary sections, we have also ascertained that the signals obtained by Western analysis are not due to GST protein by subjecting the rat pituitary extracts to Western blot analysis using antiserum to GST. It was observed that when antiserum to GST was used to probe rat pituitary extracts in a Western blot, we were able to detect only a lower molecular weight signal of ~53 kDa (data not shown) corresponding to the reported molecular weight of GST (Motoyamat and Dauterman, 1979) and when probed with an antiserum raised by us, no signal corresponding to 53 kDa was detected. While with the rat pituitary extract we observed two signals corresponding to approximate sizes of 68 and 70 kDa, with placental extracts we were able to observe only one signal of 68 kDa. It is of significance to note that in all the three cases, i.e. rat, monkey pituitary and placental extracts, the predominant signal obtained corresponded to the molecular weight of 68 kDa. Although the estimated molecular weight of the human GnRHR based upon predicted amino acid sequence is 37 kDa, a range of molecular weights (from 40 to 63 kDa) have been reported for rat pituitary GnRHR (Iwashita *et al*., 1985), despite the high degree of sequence homology at the nucleotide level between rat and human (Stojilkovic *et al*., 1994). One obvious explanation for the discrepancy could be the extent of glycosylation (Iwashita *et al*., 1985) although no data are available on this. Glycosylation of G protein-coupled receptors has been known to play a role in cell surface expression, protein folding, ligand recognition as well as receptor effector coupling (Wheatley and Hawtin, 1999). However, the fragment that we have expressed does not contain any potential glycosylation sites. Our estimate of molecular weight of 68 kDa for rat pituitary and human placental GnRHR is in close agreement with 70 kDa reported by Perrin *et al*. (1993) from cloned rat pituitary and mouse pituitary tumour cell line GnRHR using

a photo affinity labelling approach. Upon preincubation of the antiserum with rat pituitary membrane extracts there was also a reduction in the intensity of the signal in case of rat pituitary, thus further establishing that the antiserum indeed recognized the GnRHR. However, the results obtained using immunofluorescence and confocal microscopy were more uniform in that both the tissues tested, namely first trimester and term human placenta, fluorescence was observed in the periphery of the villi which is in agreement with the observation that the GnRHR is membrane bound. In this connection, *GnRHR* mRNA has been detected in human placental villi regardless of gestational age (Wolfahrt *et al*., 1998), results supported in the present paper. Our study is different from the rest in that we consistently found the presence of the GnRHR protein in the first trimester and term placenta in contrast to the reports based on in-situ hybridization referred to earlier (Lin *et al*., 1995) and in agreement with other observations (Wolfahrt *et al*., 1998). Also our studies did not reveal any difference in the intensity of the signal, suggesting that at the protein level there is no difference.

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