Molecular cloning, genomic organization and selective expression of bombesin receptor subtype 3 in the sheep hypothalamus and pituitary

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ABSTRACT

The bombesin receptor subtype 3 (BRS-3) is considered an orphan receptor as it has a low affinity for bombesin-like peptides and no identified natural ligand. We have reported a novel form of gastrin-releasing peptide (GRP) present in high abundance in the pregnant uterus of women and sheep. As BRS-3 was originally cloned from guinea pig uterus, we postulated that the uterine GRP-like peptide may be its natural ligand. We have therefore cloned the gene for the sheep homologue of BRS-3 and determined its distribution. The sheep BRS-3 gene spans 4 kbp and comprises three exons with intron-exon borders at positions similar to those observed for the human and mouse BRS-3 genes. The predicted amino acid sequence of ovine BRS-3 has approximately 85% identity with the human, mouse and guinea pig receptors. Highly conserved amino acids important in mediating receptor G-protein coupling to second messengers and important in ligand binding were found to be conserved in ovine BRS-3. One potentially important deviation was noted: ovine BRS-3 possesses an arginine residue at position 294 instead of a histidine residue as found in all other BRS-3. His²⁹⁴

was previously identified as important in ligand-receptor interactions while Arg²⁹⁴ was implicated in high ligand affinity. Thus ovine BRS-3 may have binding characteristics different from those of the human, mouse and guinea pig BRS-3 receptors. In the ewe, BRS-3 mRNA expression was detected in pituitary and hypothalamus but not in tissues of the pregnant uterus (endometrium, myometrium, chorioallantois or amnion). Nor was BRS-3 expression detected in the non-pregnant uterus or in testis. This pattern of BRS-3 expression is similar to that observed in the mouse but different from that observed in the human, rat and guinea pig. We conclude that there is no local interaction between uterine GRP-like peptide and BRS-3. However, the high expression of BRS-3 in the pituitary coupled with elevated circulating levels of this GRP-like peptide during pregnancy suggests an alternate pathway. Cloning of the ovine BRS-3 gene will permit a detailed functional analysis of this receptor in the sheep and its role in the mediation of action of uterine GRP.

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INTRODUCTION

The bombesin-like peptide family is made up of three groups of related peptides. The prototype peptides of each family – bombesin, ranatensin, phyllolitorin – were initially isolated from frog skin but have also been found to be more widespread in mammals (Spindel *et al.* 1993). To date, two mammalian members of the bombesin-like peptide

family have been identified, isolated, cloned and characterized: gastrin-releasing peptide (GRP) and neuromedin B (NMB), which are the mammalian homologues of bombesin and ranatensin respectively (Spindel *et al.* 1993). No mammalian member of the phyllolitorin family has been identified so far.

GRP mRNA encodes a precursor of approximately 148 amino acids containing a signal sequence, GRP-(1–27) and a C-terminal extension

peptide of variable length (Reeve *et al.* 1988). Proteolytic cleavage and C-terminal amidation generate the two major biologically active GRP forms, GRP-(1–27) and GRP-(18–27) (Reeve *et al.* 1988). However, other forms of undefined biological activity are also present in tumours (Miyake *et al.* 1994) and in specific uterine tissues (Giraud *et al.* 1993). We have shown that the pregnant uterus of women (Whitley *et al.* 1996) and sheep (Giraud *et al.* 1996) produces a GRP-like peptide that is larger and biochemically distinct from the well-characterized GRP-(1–27) and GRP-(18–27) found in the gut and brain. This finding of a novel GRP-like peptide in the pregnant uterus has also been reported by others (Xiao *et al.* 1996).

There are three types of mammalian bombesin receptors: bombesin receptor subtype 3 (BRS-3), GRP receptor (GRP-R) and NMB receptor (NMB-R) (see Kroog *et al.* 1995 for review). Ligands have been identified for GRP-R and NMB-R (GRP and NMB respectively) but to date no naturally occurring high-affinity ligand has been identified for BRS-3 (Ryan *et al.* 1998). This observation and the detection of BRS-3 mRNA expression in the pregnant uterus of women (Gorbulev *et al.* 1994, Whitley *et al.* 1996) and guinea pigs (Gorbulev *et al.* 1992) make the larger form of GRP found in the pregnant uterus an attractive candidate as the naturally occurring ligand for the BRS-3 receptor.

Our extensive studies in sheep have revealed that at late pregnancy GRP mRNA is the major product of the endometrium, accounting for 20% of all mRNA synthesized (Whitley *et al.* 1994). Temporal expression has been demonstrated with 5- and 24-fold rises in peptide and mRNA respectively, observed during the luteal regression phase of the oestrous cycle (Whitley *et al.* 1998). During pregnancy, similar increases in peptide and mRNA are observed (Giraud *et al.* 1994). We hypothesized that BRS-3 was the cognate receptor for the uterine forms of GRP. In this study we have therefore characterized ovine BRS-3 in terms of its genomic organization, putative cDNA structure, distribution in the non-pregnant and pregnant uterus, pituitary, hypothalamus and testis. We have also compared these observations with studies in human, rat, mouse and guinea pig.

METHODS

Isolation and nucleotide sequencing of sheep genomic BRS-3

Half a million recombinant bacteriophages representing a library of EcoRI fragments of sheep

genomic DNA purified from white blood cells and cloned into the λ EMBL3 vector (supplied by Dr Tim Adams, Department of Veterinary Science, University of Melbourne) were screened by hybridization with full-length human BRS-3 cDNA (kindly supplied by J Battey, NIDHD, NIH, Bethesda, MD, USA and described in Fathi *et al.* 1993). Hybridization of 32 P-labelled probe (prepared using Rediprime kit, Amersham, Castle Hill, NSW, Australia) and recombinant phage DNA on nitrocellulose membranes (Hybond C extra, Amersham) proceeded at 42 °C overnight in 30% formamide, 50 mM sodium phosphate, 5 $\times$ SSC, 1 mM sodium pyrophosphate, 5 $\times$ Denhardt's solution, 50 μ g/ml herring sperm DNA, pH 7.0. Unbound probe was removed by rinsing the membranes twice in 2 $\times$ SSC, 0.1% SDS and then twice for 15 min in fresh buffer, followed by two 15 min washes in 0.1 $\times$ SSC, 0.1% SDS at 50 °C. Following autoradiography of the washed membranes with Kodak X-OMAT AR X-ray film (Kodak, Coburg, Victoria, Australia) and two intensifying screens at –80 °C for 7 days, 80 putative positive phage clones were observed. DNA from a single positive plaque was purified using the plate lysate method (Sambrook *et al.* 1989) and a restriction map constructed by Southern hybridization of restriction fragments using human BRS-3 cDNA as probe. A 4.4 kbp PstI fragment was isolated, purified and ligated with vector pGEM 3Zf+ (Promega, Annandale, NSW, Australia). Double-stranded DNA of this recombinant plasmid was purified using Qiagen-tip 20 (Qiagen, Clifton Hill, Victoria, Australia) and the nucleotide sequence of the sheep DNA determined by automated DNA sequencing (ABI Prism, Perkin-Elmer, Foster City, CA, USA) with dye-labelled dideoxynucleotides and scanning laser detection. The 4.4 kbp PstI fragment did not contain the last ten codons of the coding sequence. The corresponding DNA was subsequently isolated using a small SmaI–PstI fragment (nt 1254–1313, Fig. 1) derived from the 3' end of the 4.4 kbp PstI fragment as a probe in Southern hybridization of restriction fragments prepared from purified DNA of the original positive phage clone. A 0.8 kbp SmaI–BamHI overlapping fragment was identified, purified and sub-cloned, and the nucleotide sequence determined as described above.

RNA preparation

Trizol reagent (Gibco BRL, Mulgrave, Victoria, Australia) was used to isolate approximately 1 mg of total RNA from sheep tissue – non-pregnant endometrium, myometrium, hypothalamus, and pituitary; late pregnant (around 130 days) endometrium,

FIGURE 1. Genomic organization, nucleotide sequence, and predicted amino acid sequence of the sheep BRS-3 gene. Nucleotide sequences corresponding to codons are shown in upper case. Numbering in italics corresponds to the transcribed sequence. Putative transmembrane domains are in bold lettering. Bold numbering refers to amino acids. Stars show the conserved cysteine residues. Circles show the location of potential sites for N-linked glycosylation. Triangles show the location of potential PKC phosphorylation sites. Restriction sites for endonucleases SmaI (5' CCCGGG 3') and PstI (5' CTGCAG 3') are underlined. GenBank Accession numbers for genomic sequence encompassing exons 1, 2 and 3 are AF108207, AF108208 and AF108209 respectively. GenBank Accession number for mRNA sequence is AF108210.

myometrium, amnion, and chorioallantois; and ram testis – according to the manufacturer's instructions. PolyA+ RNA was purified from total RNA using biotinylated oligo dT complexed to magnetized streptavidin (PolyAT Tract; Promega).

cDNA preparation and reverse transcription (RT)-PCR

First-strand cDNA was synthesized from 250 ng polyA+ RNA using SuperScript Reverse Transcriptase according to the manufacturer's instructions (Gibco BRL). A portion (one-twentieth) was used in PCR for detection of BRS-3 and β -actin gene expression. PCR consisted of 2.5 U HotStarTaq Polymerase (Qiagen), 1 $\times$ HotStarTaq Polymerase buffer (supplied by the manufacturer and including 1.5 mM $MgCl_2$), 0.2 mM dNTPs and 2 ng/ μ l each of the forward and reverse primers in a total volume of 50 μ l. Primer pairs were designed to span an intron to ensure that PCR products were derived from cDNA and not contaminating genomic DNA. The primers used for detection of ovine BRS-3 gene expression were OUR37 (5' TTACCCTGTTTCTGAGAGGCTCCTGCA 3'; exon 2, nt 756–782 according to Fig. 1) and OUR38 (5' GTTTTGGCAATTCGCTTCCGGGATT 3'; exon 3, nt 956–932 according to Fig. 1). Primers used for the detection of ovine β -actin gene expression were 5' TGGGAGCACATGGAGAA GATCTG 3' and 5' CAGCACAGCCTGGAT GGCCACGTAC 3'. These were derived from partial coding sequence for *Ovis aries* β -actin mRNA (GenBank locus OAU39357) with exon boundaries deduced by comparison with the human β -actin gene sequence (GenBank locus HUMAC CYBB). Amplification consisted of a 15 min incubation at 95 °C followed by 30 (β -actin) or 40 (BRS-3) cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s (FTS-960 Thermal Sequencer, Corbett Research, Mortlake, NSW, Australia). One-fifth of each reaction was subjected to electrophoresis through a 2% agarose gel and products visualized by staining with 0.5 μ g/ml ethidium bromide prior to vacuum transfer to nylon membrane (Hybond N+; Amersham). 32 P-labelled oligonucleotides (BRS-3, 5' TGAGGAACAAAGGCATGCCCCG CA 3', exon 2, nt 900–922 according to Fig. 1; β -actin, 5' CCCAAGGCCAACCGTGAGAAGAT GA 3') were used for Southern hybridization of the blot using methods described in Whitley *et al.* (1996). When observed, the BRS-3 PCR product of approximately 200 bp was size fractionated in 1.2% low-melting agarose, purified away from the agarose using QIAEX silica slurry (Qiagen) and subject to ligation with pCR2.1 according to the manufac-

FIGURE 2. Genomic arrangement of the ovine BRS-3 gene. Position of recognition sites for restriction endonucleases AvaI (A), BamHI (B), EcoRI (E), HindIII (H), PstI (P) and XbaI (X) are shown. Exons are denoted as black boxes.

turer's instructions (Invitrogen, San Diego, CA, USA). Recombinant plasmid was purified and the nucleotide sequence of the insert determined as described above.

The putative cDNA sequence derived from the three exons observed in the BRS-3 gene sequence was confirmed by RT-PCR using primers designed to putative 5' and 3' untranslated sequence. The forward primer was OUR40 (5' GAGTATCTG AGTGTCTTGGATTG 3', nt 1–24 according to Fig. 1) and the reverse primer was OUR41 (5' CTACGCATAGCTCAGAGTTGAAC 3', nt 1383–1405 according to Fig. 1). A 1405 bp product was observed after two rounds of amplification and subcloned into vector pCR2.1 as described above. A consensus sequence was obtained from three recombinant molecules to combat the possible introduction of errors during amplification using HotStarTaq Polymerase, which does not possess proof-reading activity.

RESULTS

Isolation, restriction mapping and nucleotide sequence of ovine BRS-3

Figure 2 shows a partial restriction map of the ovine BRS-3 gene defined using exon-specific fragments of human BRS-3 cDNA as probes in Southern hybridization. Figure 1 shows the nucleotide sequence of the exons with some adjacent intron sequence. This confirmed that the ovine BRS-3 gene consisted of three exons separated by two introns of approximately 1.4 and 1.9 kbp. This arrangement is similar to that observed for the human (Gorbulev *et al.* 1994) and mouse (Ohki-Hamazaki *et al.* 1997a) BRS-3 genes except that the sheep BRS-3 gene introns are slightly larger (1.1 and 1.4 kbp for human gene, 1.2 and 1.5 kbp for mouse gene). Intron/exon boundaries were inferred by alignment of the ovine sequence with the human gene and confirmed by RT-PCR using forward and reverse primers designed to sequence within the 5' and 3' untranslated regions respectively. Intron 1 interrupts codon Arg¹⁴⁵ and intron 2 lies between

Gln²⁶² and Ile²⁶³. Both predicted splice junctions adhered to the general rule for donor and acceptor consensus sequences in which the 5' border of an intron starts with a GT and the 3' border terminates with an AG (Breathnach & Chambon 1981). The boundaries of the second intron were confirmed by analysis of the RT-PCR product generated by primers OUR37 (exon 2) and OUR38 (exon 3). The nucleotide sequence of the 201 bp product was determined in both directions and agreed with the predicted junction. No promoters for transcription initiation were found when the nucleotide sequence was analysed using a consensus-based weight matrix for POLII recognition sites (PROSCAN; Prestidge 1995). This is probably due to a lack of upstream sequence (273 bp from the ATG start site). Gorbulev *et al.* (1994) analysed approximately 1 kbp of upstream sequence and were still unable to clearly identify a putative promoter site for the human BRS-3 gene. Codon 1 (nt 142, Fig. 1) of the BRS-3 gene product was assigned on the basis of amino acid homology to the human, mouse and guinea pig BRS-3 proteins. The ATG within GAAGAATGT is a poor match to the GCC (A/G) CCATGG Kozak consensus sequence for translation initiation in vertebrates (Kozak 1987) but is almost identical to the human sequence GAAGAAATGG. No polyadenylation signal could be identified, probably due to insufficient downstream sequence.

Proposed amino acid sequence of ovine BRS-3

The coding region of the ovine BRS-3 receptor predicts a protein of 399 amino acids – identical in length to the human, mouse and guinea pig BRS-3 receptors (Fig. 3). Ovine BRS-3 has 86% identity with the human receptor and 84% identity with the mouse and guinea pig receptors. Hydropathy analysis clearly showed seven hydrophobic regions consistent with seven transmembrane domains, which are typical of the G-protein-coupled receptor superfamily (GPCRs).

Three potential sites for *N*-linked glycosylation (NXS/T) could be identified in the extracellular N-terminal domain of ovine BRS-3 (Asn¹⁰, Asn¹⁸, Asn²⁹) with a fourth site (Asn¹⁹⁷) located in the second extracellular domain (designated by circles in Fig. 1 and individually boxed in Fig. 3). A potential protein kinase C (PKC) phosphorylation site conserved amongst all bombesin receptors was also found in the third intracellular domain of ovine BRS-3 (designated by a triangle in Fig. 1 and a broken line box in Fig. 3). The C-terminal domain possessed a second potential PKC site (Ser³⁹¹, triangle in Fig. 1, broken line box in Fig. 3) as well

as 11 Ser or Thr residues which could be targets for phosphorylation by other kinases. Residues Asp⁹³, Cys¹²⁰, Arg¹⁴⁵, Cys²⁰³, Ala²⁶⁰ and Asn³²⁶ within the sequence NPX₂₋₃Y, are highly conserved among all GPCRs and were also present in ovine BRS-3 (cysteines designated by stars in Fig. 1 and all other residues vertically boxed in Fig. 3). A putative palmitoylation site is observed, Cys³⁴⁷Cys³⁴⁸ (shaded in Fig. 3).

Tissue distribution of ovine BRS-3 expression

RT-PCR of polyA⁺ purified RNA was used for detection of BRS-3 and β -actin mRNA expression (Fig. 4). β -Actin PCR product was observed for all cDNA samples tested and confirmed by Southern hybridization using an oligonucleotide to a sequence situated between the forward and reverse primers. No BRS-3 PCR product could be detected in sheep testis, pregnant or non-pregnant endometrium or myometrium, nor in the uteroplacental membranes – amnion and chorioallantois. However, BRS-3 PCR products were observed for cDNA prepared from pituitary and hypothalamus and confirmed by Southern hybridization using an internal oligonucleotide probe and by nucleotide sequence analysis.

DISCUSSION

BRS-3 was first reported in 1992 (Gorbulev *et al.* 1992) and despite extensive characterization of the receptor (Mantey *et al.* 1997, Ryan *et al.* 1998) a ligand has not yet been identified. Use of a newly described high-affinity synthetic ligand has shown that this receptor has a unique pharmacology compared with other receptors (Mantey *et al.* 1997). Renewed interest in BRS-3 has occurred recently with description of the BRS-3 knockout mouse (Ohki-Hamazaki *et al.* 1997b) and observation that deletion of the BRS-3 gene results in a host of disturbances in energy metabolism. Our interest in the bombesin family lies in our findings that expression of a GRP-related peptide in the endometrium is altered and apparently regulated during different reproductive states and cycles (Giraud *et al.* 1993, 1994, 1996, Whitley *et al.* 1994, 1996, 1998). Biochemical characterization of uterine GRP has revealed a molecule that is larger than, and distinct from, GRP-(1–27) (Giraud *et al.* 1996). We have hypothesized that this molecule may be the ligand for BRS-3 and that BRS-3 mediates the actions of this molecule in the uterus. The results presented here demonstrate that ovine BRS-3 is not expressed in the pregnant or non-pregnant uterus

and therefore could not mediate any local actions of uterine GRP.

Most features of the predicted amino acid sequence of ovine BRS-3 conform to those described generally for GPCRs and specifically for bombesin receptors and have been described in

detail elsewhere (Strader *et al.* 1994, Akeson *et al.* 1997, Ohki-Hamazaki *et al.* 1997a). There are four consensus sites for *N*-linked glycosylation in ovine BRS-3 which may be associated with ligand affinity and coupling to G-proteins as suggested for murine GRP-R by Kusui *et al.* (1994). Also important in

TMI									
oBRS-3	MSQRQPQSPNQTLIS	TTNDTESSSSVVPND	STNKRR	TGDN	SPGIE	ALCAIYITYAVIISV	60		
hBRS-3	-A---H---	I---	S---	N---	GWS---	---	60		
mBRS-3	---S---	I---	T---	S---	T-H-GW---	---	60		
cBRS-3	---K---	I---	S---	T---	GW---	---	60		
mGRP-R	MA--NCSHL	NLDVDPFL-CNDT	FN	OSLSPPKM--	WFHPG	FIYV-PAV-GL--VI	54		
i1 TMII e1									
oBRS-3	GILGNAILIKVFFKT	KSMQTV	PNIFITSLA	FGD	LLLLLTCVPVDV	THYLAEGWLFGRIGC	120		
hBRS-3	---	---	---	---	---	A---	120		
mBRS-3	---	---	---	---	---	A---	120		
cBRS-3	---	---	---	L---	---	A---	120		
mGRP-R	-LI-IT-	I-CTV	---RN---	L-S---	L---	V-A--A	SKY--DR-	114	
* TMIII i2 TMIV									
oBRS-3	KVLSFIRLT	SVGVSV	FTLTILSADRYKAVV	KPLERQPP	NAILKTC	AKAGCIWIMSMI	180		
hBRS-3	---	---	---	---	S---	V---V---F---	180		
mBRS-3	---	---	---	---	---	---	180		
cBRS-3	---	---	---	---	S---	---	180		
mGRP-R	-LIP-Q-	---	A-	---	I-	R-MDI-ASH-LM-I-	L--AL--V--LL-I	174	
* e2 TMV									
oBRS-3	PEAIFSNVYTFQDPD	KNVTFKAC	ASYPVSE	RLLQEIHSL	LCFLVF	YIIPLSIISVYYS	240		
hBRS-3	---	R--N	-M--ES-T---	K	---	---	240		
mBRS-3	---	Q--N	R---ES-N--I--	---	---	---	240		
cBRS-3	---	H-LR--N	-M-SEW--F----	K-----	A--S----	---	240		
mGRP-R	---V--DLHP-HVK-	T-Q-	IS--P--H-N	E-HPK--	MAS----	-V---A-----YF-	234		
i3 TMVI * e3									
oBRS-3	ARTLYKSTLNIPTEE	QRHARKQIESRKR	IA	KTVLVLVALFALCWL	PNHLLYLYRSF	TSTQT	300		
hBRS-3	---	S---	---	R---	---	---	300		
mBRS-3	---	S---	---	---	---	H---YES	300		
cBRS-3	---	S---	V---	---	---	N--H---HKA	300		
mGRP-R	--N-IQ-AY-L-V-G	NI-VK--	---	L---	---	F-G--F--	---	VI--R-YHYSE	294
* TMVII									
oBRS-3	YMSSTVHLFVTIIS	RILAFS	NSCVNPFAL	YWLSNTFQQH	FKAQL	FCCKAGRDPDPTA...	357		
hBRS-3	-V-P-AM-FIF--F-	-V---	---	---	KS--K----	---	E--E-PV...	357	
mBRS-3	-ANH-D-PFVII-F-	-V---	---	---	K----	C-L--EQ-E-PL...	357		
cBRS-3	-E---AI-FI--F-	-V---	---	---	K--KQ----	---	GEL-E-PL...	357	
mGRP-R	.V-T-ML-FVTS-CA	-L--T--	---	L--	KS-RKQ-NT--	L--QPGLMNRSHSTG	353		
oBRS-3	.ANTPLDNLAVMGRV	PGAASTQMSEISVSPFTGC	SVKKEDDRV	399					
hBRS-3	.-D-S-TT-----T-	--TG-I-----	TS----	QAE--F	399				
mBRS-3	.GDI--N--T-----	-ATG-AHV-----	TL-S-S	-A--GE-K-	399				
cBRS-3	.-A--NS-----	S-TEN-HI---G-AS-I-R	PM----	EN--	399				
mGRP-R	RST-CMTSFKSTNPS	ATFSLINRNICHEGYV		384

FIGURE 4. RT-PCR for detection of BRS-3 and β -actin mRNA in various ovine tissues. cDNA was prepared from pituitary (1), hypothalamus (2), testes (3), endometrium (4) and myometrium (5) from non-pregnant sheep; endometrium (6), myometrium (7), amnion (8) and chorioallantois (9) from late term pregnant sheep or from water (10). Products from RT-PCR for BRS-3 mRNA (panels a and b) and for β -actin mRNA (panels c and d) were detected by agarose gel electrophoresis and ethidium bromide staining (panels b and d) and by Southern hybridization using gene-specific oligonucleotide probes (panels a and c).

mediating receptor G-protein coupling to second messengers are other highly conserved amino acids: Ala²⁷⁰ within the third intracellular loop and Arg¹⁴⁵ within the motif DRY that is completely conserved amongst all bombesin receptors. The motif NPX₂₋₃Y within the seventh transmembrane domain is highly conserved amongst the GPCRs and the sequence NPFALY is completely conserved for all bombesin receptors. The Tyr residue within this motif has been shown to be required for internalization of the β 2-adrenergic receptor (Barak *et al.* 1994) but mutation of this Tyr had no effect

on internalization of GRP-R (Slice *et al.* 1994). A consensus sequence for PKC phosphorylation is Ser²⁶⁵ distal to the third intracellular loop in ovine BRS-3 and completely conserved amongst all bombesin receptors.

Highly conserved cysteine residues found within the first and second extracellular loops of ovine BRS-3 (Cys¹²⁰ and Cys²⁰³) are conserved amongst all bombesin receptors and may be important for the formation of the ligand-binding site as demonstrated for β -adrenergic receptor (Strader *et al.* 1994). Other cysteine residues conserved amongst all bombesin receptors are found within transmembrane domains II, VI and VII (Cys¹⁰⁰, Cys²⁸³ and Cys³²⁴) and Cys⁴⁸ and Cys¹⁶⁵ are conserved amongst all BRS-3.

A number of features have been identified in the cytoplasmic C-terminus of many GPCRs that appear to be involved in the mediation of receptor internalization (or sequestration). These include a putative palmitoylation site, PKC consensus sequence and multiple Ser and Thr residues (Strader *et al.* 1994). These can all be identified in the C-terminus of the predicted ovine BRS-3. There is a putative palmitoylation site (Cys Cys) at Cys³⁴⁷ that is present for all bombesin receptors. A site for phosphorylation by PKC is well conserved amongst GRP-R (Thr³⁶² within the consensus TSFK) and NMB-R and has been shown to be partially responsible for mediating internalization of GRP-R (Benya *et al.* 1993) but is absent for all BRS-3. For ovine BRS-3, the sequence SVKK near the C-terminus at Ser³⁹¹ fits the PKC phosphorylation consensus predicted by Kennelly & Krebs (1991), but is not well conserved amongst all BRS-3 receptors and is absent in guinea pig BRS-3 (Fig. 3). There are 11 Ser/Thr residues in the intracellular C-terminus of ovine BRS-3 similar to all other bombesin receptors and reported to be important in GRP-R internalization (Benya *et al.* 1993).

Despite the high amino acid sequence homology, there is one potentially important difference in ovine BRS-3 when compared with human, mouse and guinea pig BRS-3. Akesson *et al.* (1997) hypothesized

FIGURE 3. Comparison of BRS-3 protein sequences from sheep (oBRS-3), human (hBRS-3), mouse (mBRS-3) and guinea pig (cBRS-3) and mouse GRP-R (mGRP-R) was performed using the CLUSTAL W sequence alignment program (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html>, Thompson *et al.* 1994). Amino acids that differ from the ovine BRS-3 sequence are shown. Putative transmembrane (TM), extracellular(e) and intracellular(i) domains are indicated. The BRS-3 protein sequences were obtained from GenBank: loci Z97362, X76498, L08893 (human, all identical), AB010280 (mouse) and X67126 (guinea pig), as was mouse GRP-R at locus MUSGRPOM. NXT/S indicates putative sites for N-linked glycosylation. Amino acids conserved in most GPCRs are boxed. Consensus sequences for possible phosphorylation by PKC are surrounded by a dashed box. Putative palmitoylation sites are indicated by shaded CC. Four amino acids in GRP-R necessary for high affinity ligand binding are indicated * (Akesson *et al.* 1997).

that amino acids essential for high-affinity bombesin binding would be conserved amongst all known GRP-R, NMB-R and BB4 (an amphibian receptor for [Phe¹³]bombesin; Nagalla *et al.* 1995) and would diverge in the BRS-3 sequences. Transfection of mammalian cells with various mutated GRP-R revealed four amino acids in mouse GRP-R required for high-affinity agonist binding and conserved in all GRP-R but not BRS-3. One of these residues, Arg²⁹⁴, is found in the ovine BRS-3 amino acid sequence but not in human, mouse or guinea pig BRS-3, which possess His²⁹⁴. Akeson *et al.* (1997) constructed a mutated human BRS-3 with the H294R substitution (as well as R127Q) and showed increased affinity for GRP and bombesin. This suggests that ovine BRS-3 may have different binding characteristics compared with other BRS-3 and will be ascertained by mutagenesis and binding experiments.

Unlike GRP-R, the tissue-specific expression of BRS-3 mRNA appears to be highly species-dependent. Abundant expression is detected in the rat testis (Fathi *et al.* 1993) but barely detected in the mouse testis (Ohki-Hamazaki *et al.* 1997a) and not found in sheep testis (Fig. 4). BRS-3 mRNA expression has been detected in the pregnant uterus of guinea pig (Gorbulev *et al.* 1992) and women (Gorbulev *et al.* 1994, Whitley *et al.* 1996) but not in mouse (Ohki-Hamazaki *et al.* 1997a) or sheep (this study). Weak expression of BRS-3 mRNA was detected in guinea pig brain (area not specified) (Gorbulev *et al.* 1992), while in mouse brain it was localized to hypothalamic regions (Ohki-Hamazaki *et al.* 1997a) but expression was not detected in rat brain (Fathi *et al.* 1993). Expression of BRS-3 mRNA is restricted compared with other mammalian bombesin receptors. GRP-R and NMB-R are much more widely expressed in rat, mouse and human tissues as well as lung carcinoma cell lines (Fathi *et al.* 1996, Toi-Scott *et al.* 1996), cultured human bronchial epithelial cells (DeMichele *et al.* 1994), prostate carcinoma cell lines (Aprikian *et al.* 1996) and prostate carcinoma tissue (Bartholdi *et al.* 1998). The only reported exception appears to be individual nasal mucosa samples where the frequency of BRS-3 mRNA detection was equal to that of NMB-R and more than double the frequency of GRP-R mRNA detection (Ali *et al.* 1996).

The initial isolation of BRS-3 cDNA from guinea pig pregnant uterus and the observation that BRS-3 expression was restricted largely to the pregnant uterus led to the proposal that BRS-3 was a uterine-specific bombesin receptor (Gorbulev *et al.* 1992). Lack of a naturally occurring ligand for BRS-3 and detection of a novel GRP-like peptide in the pregnant sheep uterus (Fraser *et al.* 1992, Giraud *et al.*

1993) led to the attractive proposition that BRS-3 could mediate the function of uterine GRP. The findings presented in this study that BRS-3 is not expressed in the sheep uterus dispel this hypothesis. This does not mean, however, that the novel uterine GRP is not the natural ligand for BRS-3. It is possible that the pituitary (which is a major site of BRS-3 expression) is the target tissue for uterine GRP as very high blood levels are found in both the maternal and fetal plasmas (Giraud *et al.* 1993, Whitley *et al.* 1996). Several reports have indicated that bombesin-like peptides have a physiological role in the anterior pituitary with effects on the release of growth hormone and prolactin (Kentroti & McCann 1996), adrenocorticotrophic hormone (Au *et al.* 1997) and thyroid-stimulating hormone (Santos *et al.* 1995) – hormones known to be involved in energy metabolism and with secretion rates that change markedly during pregnancy. Interestingly, the major phenotypic effects observed in BRS-3 knockout mice are changes in energy metabolism and adiposity (Ohki-Hamazaki *et al.* 1997b). Potential interaction between the novel form of uterine GRP and the pituitary BRS-3 will be of particular interest with regard to possible metabolic effects. These studies are in progress.

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