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Molecular cloning of a new bombesin receptor subtype expressed in uterus during pregnancy

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The homology screening approach has been used to clone a new member of the guanine-nucleotide-binding-protein-coupled receptor superfamily from guinea pig uterus. The cloned cDNA encodes a 399-amino-acid protein and shows the highest amino acid similarity to members of the bombesin receptor family; 52% and 47% similarity to the gastrin-releasing-peptide (GRP) receptor and the neuromedin-B receptor, respectively. Binding experiments with the stably transfected LLC-PK$_1$ cell line expressing the new receptor protein confirmed the bombesin-like nature of the cloned receptor. The relative order of ligand affinity, GRP = neuromedin C $\gg$ neuromedin B, suggests that the cloned cDNA represents the GRP subtype rather than the neuromedin-B subtype of bombesin receptors. Northern-blot analysis of mRNA species from several guinea-pig tissues showed that the mRNA for the new bombesin receptor subtype is expressed mainly in uteri of pregnant animals.

Two related peptides, ranatensin and bombesin, found in frog skin (Nakajima et al., 1970; Anastasi et al., 1971) were the first members of a large family of regulatory peptides, named later bombesin-like peptides. To date, more than 20 peptides of this family are known to share a common amino acid sequence at their C-terminus. The peptides can be classified into three subfamilies according to their C-terminal tripeptide; the bombesin, the ranatensin/litorin and the phyllolitorin subfamilies (Erspamer, 1988). To date, there are only three bombesin-like peptides identified in mammalian tissues; gastrin-releasing peptide (McDonald et al., 1979), neuromedin B (Minamino et al., 1983) and neuromedin C (Minamino et al., 1984). Gastrin-releasing peptide (GRP) and neuromedin C (C-terminal decapeptide of GRP) belong to the bombesin subfamily and neuromedin B belongs to the ranatensin/litorin subfamily (Fig. 1). No mammalian counterpart to phyllolitorin peptide has been yet described.

Bombesin-like peptides demonstrate a wide range of biological activities in mammals, such as secretion of gut hormones and the regulation of smooth-muscle contraction. They are thought to play an important role in the regulation of homeostasis and thermoregulation, as well as in cell proliferation. These peptides can act as autocrine growth factors for small-cell lung carcinoma (for review see Lebacq-Verheyden et al., 1990).

Bombesin-like peptides exert their effects via receptors coupled by pertussis-toxin-insensitive guanine-nucleotide-binding proteins to phospholipase C (Secar et al., 1991). The detailed binding studies with a number of bombesin analogues resulted in distinguishing two different subtypes of bombesin receptors, the GRP-preferring receptor and the neuromedin-B-preferring receptor (von Schrenck et al., 1989; von Schrenck et al., 1990). The best characterized bombesin receptors are those from gastrointestinal tissues and from some established cell lines (Sinnett-Smith et al., 1990; Cardona et al., 1992). A GRP receptor subtype from fibroblasts (Spindel et al., 1990; Battey et al., 1991) and a neuromedin-B receptor subtype from rat esophagus (Wada et al., 1991) have been recently cloned. Some observations indicate that other subtypes of mammalian bombesin receptor may exist (Shapira et al., 1991; Coriay et al., 1991).

The rodent uterus is known to have very high sensitivity to bombesin-like peptides which induce smooth-muscle contraction (Erspamer, 1988; Mukai et al., 1991). Until now, the uterine bombesin receptor has not been identified. In this paper, we report the molecular cloning of the uterine bombesin receptor from guinea pig that represents a new subtype of bombesin receptors.

MATERIALS AND METHODS

Materials

GRP (porcine), neuromedin B and neuromedin C were obtained from Bachem. Nylon filters were purchased from Amersham and Sartorius. Plasmid pWLneo was from Stratagene and pGEM-7Zf(+) was from Promega.

mRNA preparation

Total RNA was isolated from frozen guinea-pig tissues by the guanidinium thiocyanate method (Chirgwin et al., 1979)

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Abbreviations. GRP, gastrin-releasing peptide; G-protein, guanine-nucleotide-binding protein; IC_{50}, concentration giving half-maximal inhibition.

Note. The novel nucleotide sequence data published here have been submitted to the EMBL sequence data bank(s) and are available under accession number X67126.

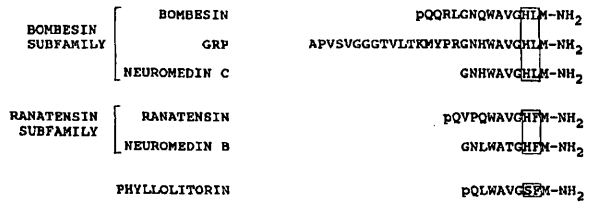


Fig. 1. The amino acid sequences of amphibian bombesin-like peptides and their mammalian counterparts, GRP, neuromedin C and neuromedin B. Boxed amino acid residues distinguish three subfamilies of bombesin-related peptides.

and subjected to oligo(dT)-cellulose chromatography. cDNA was prepared using, as a template, mRNA isolated from the uterus of a pregnant guinea pig taken at day 50 post conception.

Construction and screening of the cDNA library

Oligo(dT)-primed cDNA was synthesized using Amersham cDNA synthesis kit as recommended by the manufacturer. It was ligated to *Eco*RI arms of λgt 10 (Stratagene), packaged with Gigapack Plus packaging extract (Stratagene) and transfected into *Escherichia coli* C600 Hfl. λgt 10 cDNA library was screened with the ^{32}P-labelled degenerate oligonucleotide 5′-GG(G/C)A(A/G)CCAGCAGA(G/T)(A/G)GC(G/A)AA-3′. Duplicate filters were hybridized in 6× NaCl/Cit. (NaCl/Cit., 0.15 m NaCl, 15 mM sodium citrate, pH 7.0), Denhardt's solution [0.1% (mass/vol.) bovine serum albumin, 0.1% polyvinilpyrrolidon (mass/vol.) and 0.1% Ficoll (mass/vol.)], 0.05% sodium pyrophosphate at 40°C and washed in 6× NaCl/Cit. containing 0.05% sodium pyrophosphate at 52°C.

Isolation and sequencing of cDNA clones

Bacteriophage DNA from positive plaques was isolated according to Sambrook et al. (1989) and cDNA inserts were recloned into pUC19 and pGEM-7Zf(+) for further analysis. Sequencing was performed by the dideoxy-chain-termination method using a Sequenase version 2.0 kit (United States Biochemicals) and utilising both the exonuclease III/mung bean nuclease approach (Henikoff, 1984) and appropriate oligonucleotide primers.

Cell culture and transfection experiments

The PU18 cDNA insert was subcloned into the eukaryotic expression vector pCDM8, carrying a cytomegalovirus promoter (Seed, 1987). Porcine kidney cells, LLC-PK$_1$, were transfected with this plasmid by the calcium-phosphate-precipitation method according to Graham and van der Eb (1973). Plasmid pWLneo was used for cotransfection and geneticin (Gibco BRL) was added to the growth medium at a concentration of 1 mg/ml for selection. After a 3-week selection period, eight geneticin-resistant clones were picked out and subjected to binding studies.

Binding assay

Stably transfected cells were grown to 70–80% confluency, rinsed with 0.14 M NaCl, 5 mM KCl, 0.01 M Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.2 (NaCl/P$_i$), treated with 0.02% EDTA in NaCl/P$_i$ for 30 min at 37°C, then harvested. The cells were washed once with NaCl/P$_i$ and resuspended at a concentration of about 1.2×10^6 cells/ml in ice-cold Dulbecco's Modified Eagle's Medium/Hepes, pH 7.3 containing 0.01% bovine serum albumin and 1 mg/ml bacitracin. The saturation analysis was performed by incubation of 200 µl cell suspension with different concentrations of ^{125}I-bombesin (2200 Ci/mmol, NEN). Unspecific binding was determined in the presence of a 1000-times excess of nonradioactive bombesin. The displacement study was carried out by incubation of 200 µl cell suspension with 0.4 nM ^{125}I-bombesin in the presence of different concentrations of nonradioactive GRP, neuromedin B or neuromedin C. All incubations were performed for 30 min at 30°C in duplicate. Bound ligand was separated from free ligand by filtering the incubation mixture through Whatman GF/F filters presoaked in 0.3% poly(ethylenimine). Each displacement experiment was carried out three times. The binding data were analysed using the Ligand program (Munson, 1983).

Northern-blot analysis

Approximately 7.5 µg each mRNA sample from several guinea-pig tissues was resolved on a 1% formaldehyde/agarose gel, transferred to Hybond-N membrane (Amersham) and fixed by cross-linking using ultraviolet irradiation (UV Stratalinker, Stratagene). Hybridization was carried out at 42°C in 50% formamide, 5× solution A (0.18 m NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA; solution A), 5× Denhardt's solution and 0.5% SDS, using a ^{32}P-labelled fragment of a PU61 cDNA insert as a probe. The filters were washed to a final stringency of 0.25× solution A and 0.1% SDS at 65°C.

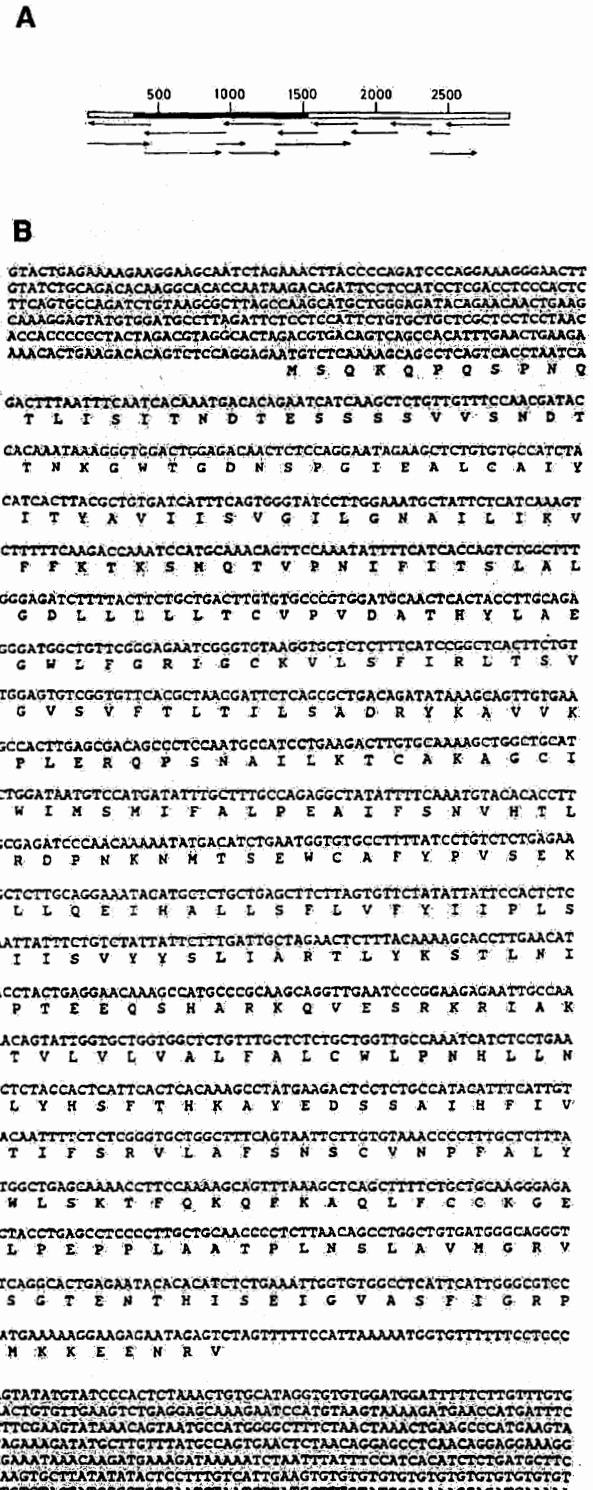
RESULTS

Isolation of PU18 cDNA

A degenerate 20-bp oligonucleotide (see Materials and Methods) was designed to be complementary to one of the most conserved parts of the guanine-nucleotide-binding (G)-protein-coupled receptors, to a region within the sixth transmembrane domain. After subjecting the λgt10 cDNA (about 200000 recombinant phages) to two screening rounds, two independent positive plaques were isolated with inserts of 1.5 kb (clone PU11) and 2 kb (clone PU61), respectively. These inserts gave no cross-hybridization and were recloned in pUC19 for sequence analysis. The PU11 insert was shown to represent the guinea-pig substance-P receptor (Gorbulev et al., 1992). The partial sequencing of PU61 cDNA showed similarity to G-protein-coupled receptors; however, it seemed not to be a full-length copy of mRNA. Using a fragment of PU61 cDNA as a probe, we screened 300000 recombinant phages from the same λgt10 cDNA library and found two additional clones, one of them, PU18, with an insert of about 3 kb was subjected to further analysis.

Nucleotide and deduced amino acid sequence of PU18

The sequencing strategy and nucleotide sequence of PU18 cDNA are shown in Fig. 2. PU18 cDNA consists of 2930 bp and has an open reading frame for 399 amino acids, representing a protein with a relative molecular mass of 44341. The 5′-untranslated region is 328 bp long and the 3′-untranslated region contains 1383 bp with two polyadenyla-



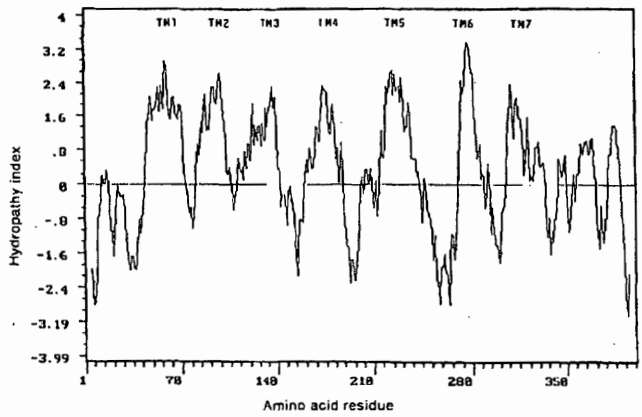


Fig. 3. Hydropathy plot of the predicted PU18 amino acid sequence by the method of Kyte and Doolittle (1982) with a window size of 9 amino acids. Positive values represent hydrophobic regions. Position of putative transmembrane domains (TM1–TM7) is indicated.

tion signals, AATAAA, located 16-bp and 5-bp upstream of a poly(A) tail. A hydropathy-plot analysis of the deduced amino acid sequence, performed according to Kyte and Doolittle (1982), shows seven hydrophobic regions typical for the seven transmembrane domain structure of G-protein-coupled receptors (Fig. 3). Within the putative N-terminal extracellular part of PU18 protein, there are three potential *N*-glycosylation sites at positions Asn10, Asn18 and Asn29. This suggests that the cloned receptor, like other G-protein-coupled receptors, may be a glycoprotein. The amino acid sequence contains several sites for phosphorylation, one of them, Ser265 followed by three basic amino acids, within the putative third intracellular loop is very likely the target for protein-kinase-C action (Kishimoto et al., 1985). In the C-terminal region, there are also two cysteine residues (Cys347 and Cys348), which can be palmitoylated (Ovchinnikov et al., 1988). In the case of the human β_2-adrenergic receptor, this post-translational modification has been shown to play a crucial role in the functional coupling of the receptor to its effector system (O'Dowd et al., 1989). The protein encoded by PU18 cDNA shares most of the other structural features common for G-protein-coupled receptors. They include the tripeptide Asp-Arg-Tyr, located just after the third transmembrane domain, two cysteine residues (Cys120 and Cys203) in the first and the second extracellular loops that are thought to form a disulfide bridge (Strader et al., 1989), and a conserved aspartate residue (Asp93) in the second transmembrane domain.

The comparison of the predicted amino acid sequence of the PU18 receptor with that of other known G-protein-coupled receptors revealed the highest similarity of the PU18 protein to the bombesin-receptor family; 52% identity to mouse gastrin-releasing-peptide receptor (Battey et al., 1991) and 47% identity to rat neuromedin-B receptor (Wada et al., 1991). Fig. 4 presents the alignment of the three receptors.

Fig. 2. Characteristics of PU18 cDNA. (A) Outline of PU18 cDNA and the sequencing strategy. The black box indicates an open reading frame. The nucleotide numbering (in bp) begins from the first nucleotide of cDNA. Arrows show direction of sequencing and extension of the determined sequence. (B) Nucleotide and deduced amino acid sequence of PU18 cDNA. Two polyadenylation signals are underlined.

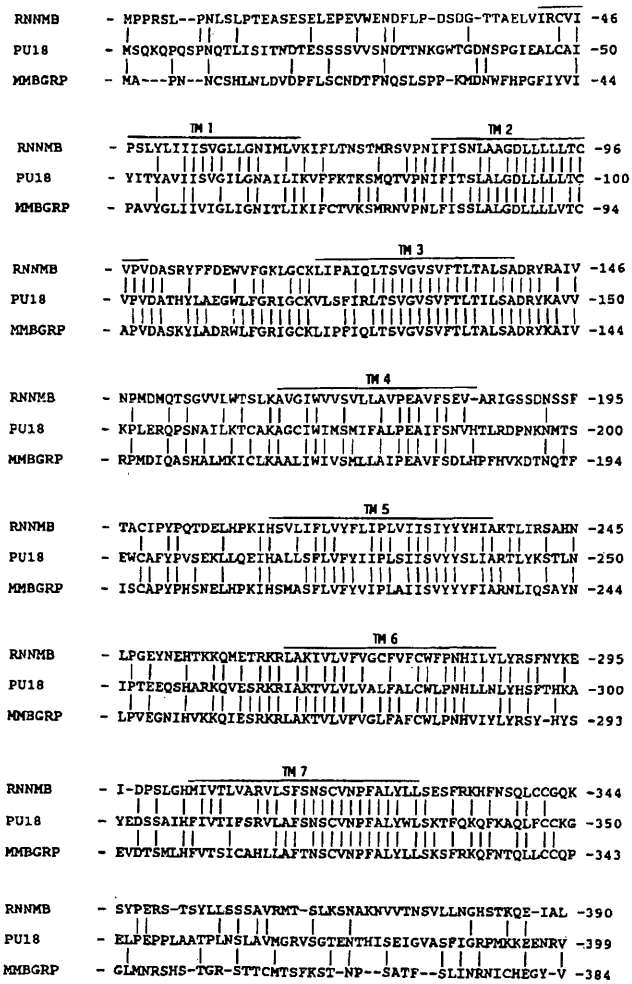


Fig. 4. Amino acid sequence alignment of PU18, mouse GRP receptor (MMBGRP; Battey et al., 1991) and rat neuromedin B receptor (RNNMB; Wada et al., 1991). Identical residues are shown by the vertical lines. Putative transmembrane domains (TM1–TM7) are overlined. The numbering of the amino acids is indicated on the right.

The similarity is higher (59% and 54%, respectively) if we exclude the N-terminal extracellular part and the C-terminal cytoplasmic part of the polypeptides. These regions of the three receptors differ completely from each other. It is noteworthy that the PU18 receptor has, in its C-terminal region, considerably less potential sites for phosphorylation than both the cloned bombesin receptors described previously. When individual transmembrane domains are compared, the second domain exhibits the highest degree of conservation and the first and fourth domains are the most divergent ones. The identity with other G-protein-coupled receptors is significantly less. The sequence similarity with the GRP receptor and the neuromedin-B receptor suggests that the cloned receptor is a member of the bombesin receptor family.

Functional study of PU18 cDNA

To determine the functional specificity of the new receptor, we recloned PU18 cDNA into the eukaryotic expression vector pCDM8, which contains a cytomegalovirus promoter (Seed, 1987). After cotransfection of renal epithelial LLC-PK$_1$ cells (Hull et al., 1976) with this plasmid and the plasmid

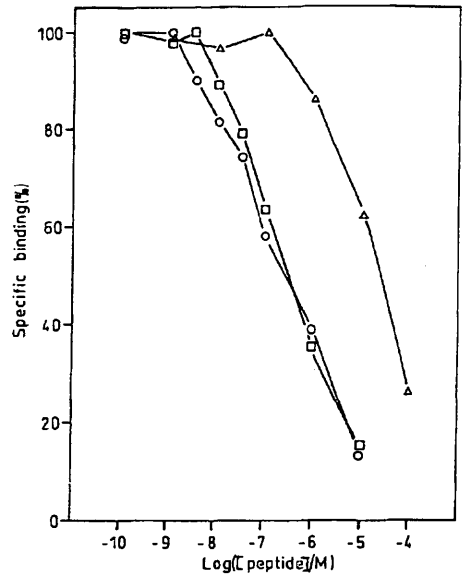


Fig. 5. Displacement of ^{125}I-bombesin binding by bombesin-like peptides, GRP, neuromedin B and neuromedin C. Points are the means of three determinations each conducted in duplicate. Experimental details are described in the text (see Materials and Methods). (○) GRP; (□) neuromedin C; (△) neuromedin B.

pWLneo bearing the gene for resistance to geneticin, we selected several stably transfected cells and subjected eight of them to binding assays with ^{125}I-bombesin. The cell line with the highest level of specific binding was chosen for a more detailed analysis.

Binding experiments with increasing concentrations of ^{125}I-bombesin demonstrated the existence of a bombesin-binding site on the transfected cell line (data not shown). The expressed receptor was further characterized by displacement studies using ^{125}I-bombesin as radiolabelled ligand and GRP, neuromedin C and neuromedin B as competitors. As seen in Fig. 5, GRP and neuromedin C inhibit ^{125}I-bombesin binding to cells expressing PU18 much more effectively [half-maximal inhibition (IC_{50}) of 290 nM and 320 nM, respectively] than neuromedin B that shows a very low affinity for the cloned receptor (IC_{50} of 20 μM). These results characterize the new receptor as a GRP-preferring type of bombesin receptor.

Expression of PU18 mRNA in different tissues

We examined the expression of mRNA for the new bombesin receptor subtype in tissues derived from several organs of guinea pig; in the brain, the kidney, the lactating mammary gland and the uterus under different physiological states, from the pregnant and nonpregnant animals. Northern-blot analysis revealed that PU18-corresponding mRNA is expressed mainly in the uteri of pregnant animals, resulting in a band of hybridization of about 3.6 kb (Fig. 6). A much less intensive band of 2.9 kb is observed in brain mRNA. To prove that this hybridization pattern does not reflect the loss of mRNA from other tissues, the filter used in this experiment was washed completely and subjected to another hybridization with the cDNA for the guinea-pig substance-P receptor. The results of this hybridization showed the expression of substance-P-receptor mRNA in all tissues tested, but predominantly in nonpregnant uterus (Gorbulev et al., 1992).

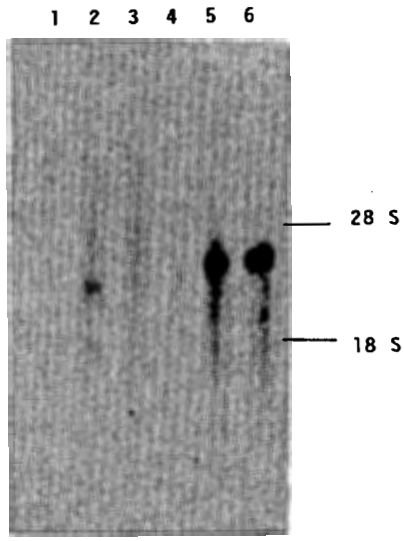


Fig. 6. Northern-blot analysis of mRNA isolated from several guinea pig tissues. Lanes contain 7.5 μg mRNA from kidney (lane 1), brain (lane 2), nonpregnant uterus (lane 3), lactating mammary gland (lane 4) and pregnant uterus, 57 d and 50 d after conception (lanes 5 and 6, respectively). ^{32}P-labelled fragment of PU61 cDNA was used as a probe for hybridization. The positions of 18S and 28S ribosomal RNA species are indicated.

DISCUSSION

The aim of our study was to identify neuropeptide receptors expressed in the uterus. The structural similarity of G-protein-coupled receptors has been recently used to design degenerate oligonucleotides for cloning new members of this superfamily using the polymerase-chain-reaction technique (Libert et al., 1989) or by homology screening (Eva et al., 1990). The last approach led us to the cloning of a guinea-pig uterine cDNA encoding a new receptor that belongs to the bombesin receptor family. The similarity between this receptor and two other cloned rodent bombesin receptors, the GRP receptor and the neuromedin-B receptor, is 52% and 47%, respectively. The sequence divergence is too extensive to be explained as a difference between the same receptor subtypes from different species, especially if one takes into account their taxonomic relationship. As a rule, such receptors show about 90% identity. The similarity between the new cloned receptor and two other bombesin receptors lies in the range usually observed for receptor subtypes within the same family; 54% for human β_1-adrenergic and β_2-adrenergic receptors, 43% for porcine M_1 and M_2 receptors (Lefkowitz and Caron, 1988), 47–57% for three rat tachykinin receptors (Shigemoto et al., 1990). Thus, we conclude that the cloned receptor represents a new member of the bombesin receptor family.

To confirm this assumption, we studied the transient and permanent expression of PU18 cDNA placed in the eukaryotic vector pCDM8 using different eukaryotic cell systems. COS cells were found to have their own quite high level of bombesin-like receptor expression, therefore we failed to detect any increase of ^{125}I-bombesin-binding to COS cells transfected with PU18 cDNA. Renal epithelial LLC-PK$_1$ cells showed no significant bombesin-like receptor expression and were therefore suitable for establishing a stably transfected cell line. Binding analysis of the stably expressed PU18 receptor demonstrated an affinity for ^{125}I-bombesin and a relative ligand affinity in the order GRP = neuromedin C ≫ neuromedin B. These results proved the bombesin-like nature of the cloned receptor from guinea-pig uterus and excluded this receptor as being a neuromedin-B-preferring subtype of the bombesin receptors. Our results concerning the ligand specificity of the new receptor are in accordance with the structure/activity relationship of neuromedin-B and neuromedin-C peptide analogues in the contraction of the rat uterus. These pharmacological studies demonstrated the presence of a neuromedin-C-preferring receptor in the rat uterus (Mukai et al., 1991).

The binding affinity of the new receptor to GRP and neuromedin C is too low (IC_{50} approximately 300 nM) to prove unequivocally that either GRP or neuromedin C are the natural ligands for this receptor. Until now, there have been only three mammalian bombesin-related peptides discovered. We cannot rule out the possibility that some other members of the bombesin family (for example, members of the phyllolitorin group) exist in mammals not identified yet.

Another explanation for the expression of a low-affinity bombesin receptor subtype in LLC-PK$_1$ cells would be that these cells do not produce the G-protein α-subunit, which is coupled to this receptor in myometrial cells. The generally accepted model suggests that high-affinity binding of agonists to G-protein-coupled receptors depends on the formation of a ternary complex between the agonist-bound receptor and the corresponding G protein (or at least its α-subunit; DeLean et al., 1980). Bombesin receptors couple to phosphoinositide turnover via pertussis-toxin-insensitive G-proteins (Secar et al., 1991), which probably belong to the so called G_q class (for review see Simon et al., 1991). Sometimes, when the specific G-protein α subunit is absent, the receptor is able to interact with another nonoptimal G-protein α subunit, but less effectively (Kurose et al., 1991). In this case, the receptor will have a low affinity in binding experiments. For example, tachykinin NK_3 receptor expressed in the transfected Chinese hamster ovary cells was of low affinity (IC_{50} of 240 nM) compared to the high affinity (IC_{50} of 8 nM) of this receptor transiently expressed in COS-7 cells (Gether et al., 1992). LLC-PK$_1$ cells could cause the low-affinity state of the PU18 receptor also by an altered pattern of post-transcriptional modification of the protein as compared to myometrial cells. Both these possibilities can be examined by expressing the PU18 receptor in other cell lines.

The receptor encoded by the cloned cDNA is the first example of a cloned bombesin receptor from reproductive tissues. The observation that its expression in guinea-pig uterus is restricted to the course of pregnancy is of interest and it will be fruitful to study the regulation of the bombesin-receptor expression during pregnancy in more detail. The cloned receptor is a good tool for such investigations.

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