A new family of orphan G protein-coupled receptors predominantly expressed in the brain

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Abstract The cloning of a cDNA encoding a G protein-coupled receptor homologous to the endothelin type B receptor, but unable to bind endothelin, was recently reported and termed ET_BR-LP. We report here the isolation of a human cDNA encoding a receptor that is highly related to ET_BR-LP and which was therefore termed ET_BR-LP-2. Comparison of the two amino acid sequences revealed 68% overall homology and 48% identity. As is the case for ET_BR-LP, the new receptor is strongly expressed in the human central nervous system (e.g. in cerebellar Bergmann glia, cerebral cortex, internal capsule fibers). Membranes of HEK-293 cells stably expressing ET_BR-LP-2 did not bind endothelin-1, endothelin-2, endothelin-3, bombesin, cholecystokinin-8 or gastrin-releasing peptide.

Key words: Endothelin; Orphan; G protein-coupled receptor

1. Introduction

Endothelin is the most potent vasoconstricting substance known to date [1]. The endothelin family includes three isopeptides (ET-1, ET-2 and ET-3) of 21 amino acid residues [2], which exert their pleiotropic effects through at least two related G protein-coupled receptors, termed ET_A and ET_B [3,4]. ET_A binds preferentially ET-1 and ET-2, is located on smooth muscle cells and is thought to be mainly responsible for the endothelin-induced vasoconstriction. ET_B on the other hand binds all three isopeptides with comparable affinities. It is present on endothelial cells, and induces a dilatation of smooth muscle cells through the release of prostacyclin, nitric oxide or adrenomedullin by endothelial cells. The existence of additional types of endothelin receptors has been suspected for a long time. Thus, an ET_C receptor with a higher affinity for ET-3 was pharmacologically distinguished [5,6]. A cDNA encoding such a receptor was indeed cloned from *Xenopus laevis* melanophore cells [7], but so far attempts to identify its mammalian counterpart have not been successful. In addition, vasoconstriction induced by ET_B agonists was believed to be due to an additional endothelin receptor, which was referred to as ET_{B2} [8]. We and others have recently shown that this receptor was the previously known ET_B receptor [9,10], which thus can induce both constriction and relaxation of smooth muscle cells. The only additional endothelin receptors that could be identified so far correspond to splice variants of the ET_B receptor [11,12].

Recently, Zeng et al. reported the existence of a new G protein-coupled receptor that was significantly homologous to the endothelin ET_B receptor and predominantly expressed in brain [13]. However, this receptor could not bind endothelins or other peptides such as bombesin and neuropeptide Y when expressed in cells. In the search for receptors homologous to the endothelin receptors, we have also detected and identified the same human receptor, which Zeng et al. named ET_BR-LP (ET_B receptor-like protein). In addition, we isolated a cDNA encoding a second human receptor, highly related to ET_BR-LP. We report here the identification and characterization of this novel receptor, which has been termed ET_BR-LP-2.

2. Materials and methods

2.1. cDNA cloning

A cDNA fragment corresponding to a human expressed sequence tag (EST; GenBank accession number HS771168) displaying a high similarity to the ET_B endothelin receptor sequence was amplified by PCR from random-primed brain cDNA and used as a probe to screen a human caudate nucleus cDNA library (Clontech). Library screening was carried out as previously described [14] on approximately one million phages. Twenty-five strong and three faint signals were obtained. The strong hybridization signals were shown by PCR to correspond to a cDNA including the EST fragment. Analysis of a clone weakly hybridizing to the probe revealed a different but highly related sequence. The insert of this clone was then used to screen the same caudate nucleus library. Twenty clones were obtained, the longest of which was sequenced to completion.

2.2. Northern blot analysis

Total RNA (10 μg) from HEK-293 cells was fractionated on a 1.2% (w/v) agarose gel containing 20 mM morpholinopropane sulfonic acid, 5 mM sodium acetate, 1 mM EDTA and 1.8% (v/v) formaldehyde and transferred overnight to Nylon N (Amersham) in 1×TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) using a Transphor electrophoresis unit (Hoefer Scientific Instruments). Two blots, each displaying poly-$(A)^+$ RNA from eight human tissues, were purchased from Clontech. The three membranes were hybridized with a ^{32}P-random-primed probe synthesized using the 1638 bp ET_BR-LP-2 cDNA. Hybridization (at 65°C for 12 h in ExpressHyb solution (Clontech) containing denatured herring sperm DNA (100 μg/ml) and 10^6 cpm/ml of the labelled probe) was followed by three steps of washing at 65°C for 15 min each in 2×SSC, 0.1% (w/v) SDS, in 1×SSC, 0.1% (w/v) SDS, and in 0.1×SSC, 0.1% (w/v) SDS. The blots were then exposed to an autoradiographic film for 12 h.

2.3. In situ hybridization histochemistry

An oligodeoxyribonucleotide probe complementary to the human ET_BR-LP-2 cDNA (corresponding to nucleotides 1444–1385) was la-

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Abbreviations: ET_BR-LP, ET_B receptor-like protein; EST, expressed sequence tag

The nucleotide sequence of the receptor cDNA reported in this paper has been submitted to the GenBank/EMBL Data Libraries with the accession number Y16280.

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belled at the 3′ end with terminal deoxynucleotidyl transferase (Life Technologies) and [^{35}S]dATP then purified. Cryostat sections of human cerebellum, caudate putamen and temporal cortex were mounted on Superfrost Plus slides and then fixed in phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde for 20 min followed by three 5 min washes in PBS. Sections were hybridized in 50 μl of 4×SSC, 20% dextran sulfate, 50% deionized formamide, 0.1 M dithiothreitol, 0.5×Denhardt's solution, containing denatured herring sperm DNA (0.25 mg/ml) and 3×10^5 dpm of the ^{35}S-labelled probe. Sections were covered with Fujifilm coverslips and incubated in a moist chamber at 43°C overnight. After removal of the coverslips, the sections were washed twice in 1×SSC, 10 mM dithiothreitol for 15 min at 55°C, then in 0.5×SSC, 10 mM dithiothreitol once for 15 min at room temperature. After a dip in double distilled water, sections were dehydrated in ethanol and exposed to Hyperfilm β-max (Amersham Corp.) or Ilford K5 nuclear research emulsion.

2.4. Expression of ET_BR-LP-2 in HEK-293 cells

A *Bam*HI/*Sap*I cDNA fragment containing a Kozak leader sequence (5′-GCCACC-3′) followed by the first 481 bp of the ET_BR-LP-2 coding sequence was synthesized by PCR, and ligated onto a 1112 bp *Sap*I/*Eco*RI fragment (excised from a phage obtained by the human caudate nucleus library screening) into the expression vector pCEP4 (Invitrogen), resulting in the plasmid pET$_B$R-LP-2. A human embryonic kidney cell line (HEK-293) was cultured in MEMα medium supplemented with 10% heat-inactivated fetal calf serum. HEK-293 cells were transfected with the pET$_B$R-LP-2 plasmid using lipofectamine (Life Technologies) and were then selected using 100 U/ml hygromycin. Resistant colonies were screened by Northern blotting.

2.5. Radioligand binding assays

Competition binding assays with membranes of stably transfected or control HEK-293 cells were performed using endothelin-1, endothelin-2, endothelin-3, bombesin, cholecystokinin-8 and gastrin releasing peptide as ^{125}I-labelled ligands. Microsomal membranes were prepared as described earlier [15], adding phenylmethylsulfonyl fluoride (17.4 mg/l) and phenanthroline (19.8 mg/l) to prevent proteolytic degradation. The assay mixture contained 250 μl of 50 mM Tris buffer (pH 7.4, 25 mM $MnCl_2$, 1 mM EDTA, 0.5% (w/v) BSA), 0.1–35 μg protein and 30–100 pM ^{125}I-labelled ligand (30 000–100 000 cpm). After incubation for 2 h at 22°C, bound and free ligand were separated by filtration. Each assay was performed three times in triplicates. Non-specific binding was assessed in the presence of 100–1000 nM of the corresponding unlabelled peptides.

3. Results and discussion

Searching for additional subtypes of endothelin receptors, we noticed the presence of an EST in the GenBank database that displayed a high homology with the ET_B receptor sequence. The information given by this EST was used to isolate a cDNA with an open reading frame of 1839 nucleotides from a human caudate nucleus cDNA library. The encoded 613 amino acid protein displayed the structure of a seven transmembrane domain receptor and shared modest but significant homology with the ET_B receptor (37% similarity, 27% identity). Cloning of the same receptor cDNA was recently reported by another group [13] who named the receptor ET_BR-LP (human ET_B receptor-like protein). A few differences, possibly due to polymorphisms (or to inaccurate sequencing), could be detected when comparing our sequence to that of Zeng et al. [13]. One of these differences represents a

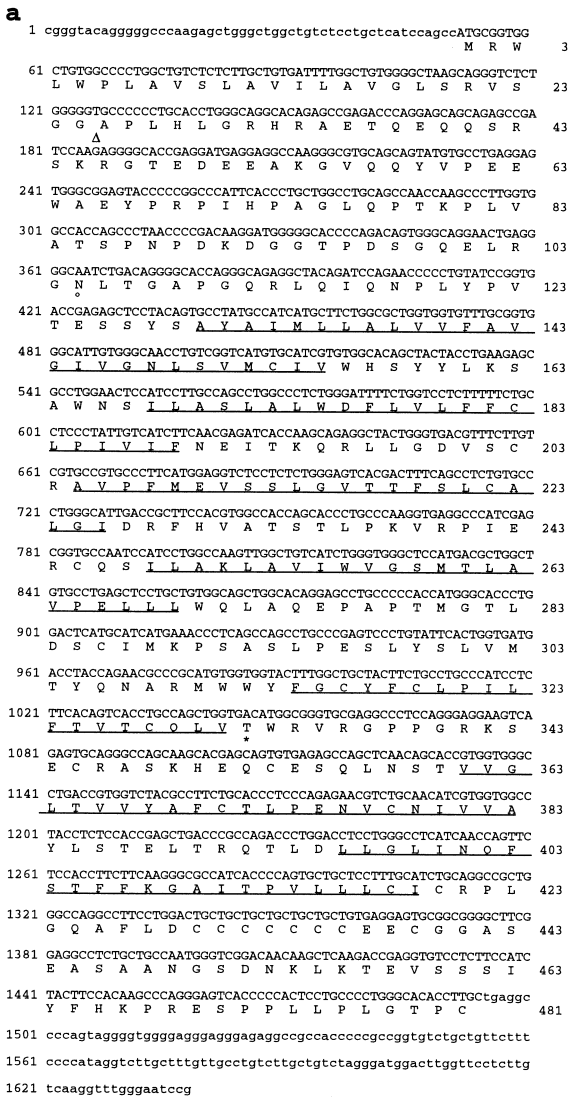

Fig. 1. a: cDNA sequence and deduced amino acid sequence of ET_BR-LP-2. The potential seven transmembrane domains are underlined. ° and * indicate putative sites for *N*-linked glycosylation and protein kinase C phosphorylation, respectively. b: Alignment of the amino acid sequences of ET_BR-LP (bottom sequence) and ET_BR-LP-2 (top sequence).

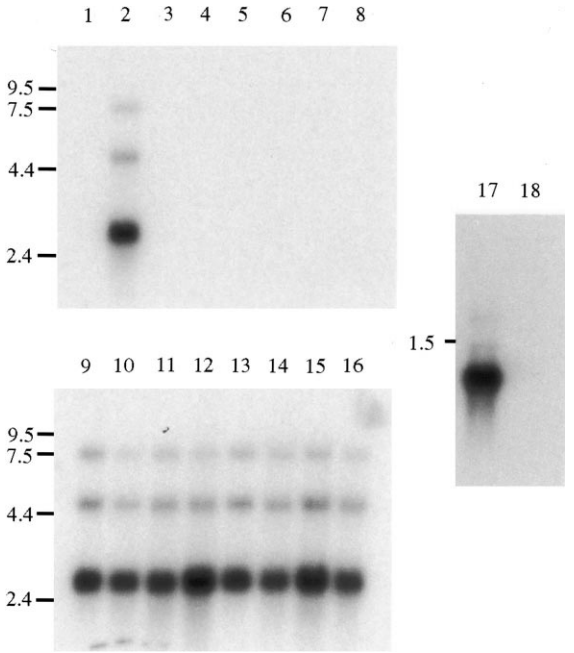

Fig. 2. Tissue expression of ET_BR-LP-2 mRNA analyzed by Northern blotting. Each sample contains 2 μg of poly(A)$^+$ RNA, except for lanes 17 and 18 (10 μg of total RNA). Markers of known molecular weight are indicated (in kbp). Lane 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, cerebellum; 10, cortex; 11, medulla; 12, spinal cord; 13, occipital lobe; 14, frontal lobe; 15, temporal lobe; 16, putamen; 17, HEK-293 cells stably transfected with human ET_BR-LP-2; 18, non-transfected HEK-293 cells.

silent mutation (C instead of T^{2825} or C/T^{2825}), while the rest of them change the amino acid content of ET_BR-LP: G/A^{2056} (G/D^{93}), G/A^{2094} (A/T^{106}), G/T^{2131} (G/V^{118}), G/T^{2257} (G/V^{160}), G/T^{2324} (W/C^{182}), G/T^{2471} (E/D^{231}), C/T^{2689} (A/V^{304}), C/G^{2763} (L/V^{329}), T/G^{3286} (F/L^{503}), T/G^{3287} (C/G^{504}) and A/G^{3570} (T/A^{598}). In agreement with Zeng et al. [13], we found high levels of the mRNA of ET_BR-LP mRNA in the brain, especially in the corpus callosum, and did not detect any binding of ET-1 or ET-3 to cells stably expressing the receptor.

In addition to strong hybridization signals corresponding to ET_BR-LP cDNA clones, a few very faint signals could be detected by screening the human caudate nucleus library. Purification and analysis of one of these weakly hybridizing clones revealed a sequence different but highly related to the sequence of ET_BR-LP. This incomplete cDNA was used as a probe to rescreen the cDNA library. The longest of the 20 clones detected (as assessed by PCR) was purified and thoroughly sequenced. It contained a sequence of 1638 bp with an open reading frame of 1443 nucleotides, encoding a putative protein of 481 amino acid residues (Fig. 1A). This protein displayed the common features of G protein-coupled receptors, including the presence of seven hydrophobic putatively transmembrane stretches. Like all the members of this receptor family, it also displays two cysteines in the first and second extracellular loops, which are believed to form a disulfide bridge, and several other conserved residues, such as an aspartic acid in the second transmembrane domain and the 'D/E-R-Y/F' motif in the second cytoplasmic loop. Due to its high homology (68% similarity and 48% identity, Fig. 1B) with ET_BR-LP, this new receptor was termed ET_BR-LP-2. Like ET_BR-LP and the endothelin receptors, ET_BR-LP-2 has a putative signal peptide. In addition, it displays a potential site for protein kinase C phosphorylation (third cytoplasmic loop) and one potential site for *N*-linked glycosylation (N-terminal extracellular domain). An interesting feature of ET_BR-LP-2, which is also shared by ET_BR-LP and by the two endothelin receptors, is the presence of a cluster of cysteines (residues 430–436 in Fig. 1) in its C-terminal extremity. Several G protein-coupled receptors have been shown experimentally to be palmitoylated on cysteine residues in their cytoplasmic tails; in particular, palmitoylation of ET_A and ET_B receptors is believed to modulate their coupling with G proteins [16,17].

The distribution of ET_BR-LP-2 mRNA in human tissues was assessed by Northern blotting, using a 1638 bp probe encompassing the whole coding sequence of ET_BR-LP-2 (Fig. 2). Hybridization of the probe to the blots failed to detect any signal in peripheral tissues (heart, placenta, lung, liver, skeletal muscle, kidney, pancreas). In contrast, one strong signal of 2.8 kbp and two weaker signals of 4.5 kbp and 7.5 kbp could be observed in brain. The presence of three

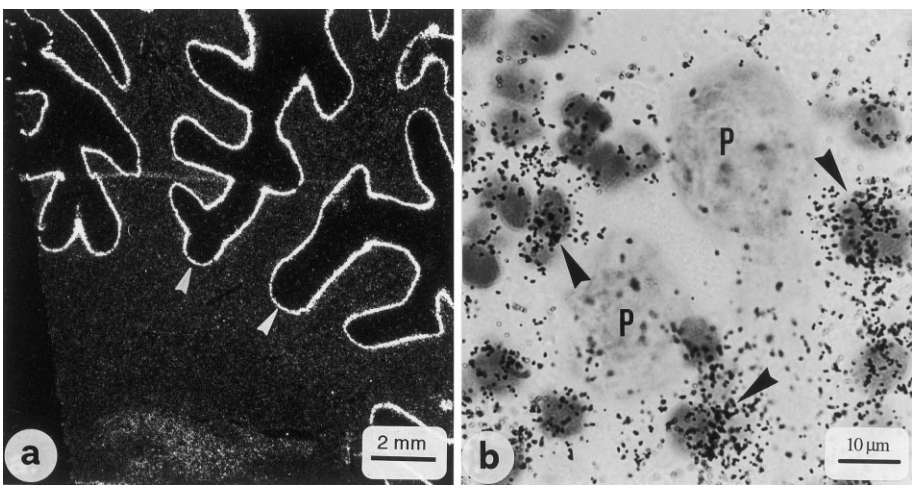

Fig. 3. Regional distribution of ET_BR-LP-2 transcripts (a) and cellular sites of synthesis (b) in human cerebellum, revealed by in situ hybridization histochemistry. Arrowheads identify the Bergmann glia of the Purkinje cell layer. Note the lack of hybridization signal in Purkinje neurons (P).

different signals could correspond to the existence of distinct polyadenylation sites in the ET_BR-LP-2 gene. The expression of ET_BR-LP-2 was further analyzed in the central nervous system. A similar pattern of three signals, with a strong signal of 2.8 kbp, was detected by Northern blot in cerebellum, cortex, medulla, spinal cord and putamen, as well as in the occipital, frontal and temporal lobes. In situ hybridization histochemistry was performed with an ^{35}S-labelled specific oligonucleotide to determine the sites of synthesis of ET_BR-LP-2 in human cerebellum, caudate putamen and temporal cortex. Extremely high levels of transcripts were detected in the cerebellar Bergmann glia of the Purkinje cell layer, whereas adjacent Purkinje cells and granule cells were not labelled (Fig. 3). This is in contrast to the reported expression of the related receptor ET_BR-LP in cerebellar Purkinje neurons [13]. A moderate to strong hybridization signal was also detected in the internal capsule fibers of the caudate putamen and in temporal cortex (not shown).

The ET_BR-LP-2 receptor was stably expressed in HEK-293 cells, as was confirmed by Northern blot analysis (Fig. 2). In competition binding assays performed on cell membranes, no significant difference was observed between ET_BR-LP-2 transfected and non-transfected HEK-293 cells for any of the tested ligands, i.e. ET-1, ET-2, ET-3, bombesin, cholecystokinin-8 and gastrin-releasing peptide. A weak binding of ET-1 could be attributed to a small number (18 fmol/mg protein) of ET_A receptors present in non-transfected HEK 293 cells. Similar results were obtained in our hands with HEK-293 cells expressing the related ET_BR-LP receptor, in agreement with Zeng et al. [13] who could not detect any binding with endothelin-1, endothelin-3 and bombesin.

In view of the strong homology that they share, ET_BR-LP and ET_BR-LP-2 receptors most likely bind the same ligand, or at least some closely related ligands. Their high levels of expression in human brain (preferentially, but perhaps non-exclusively, in neurons and glia, respectively) indicate their physiological importance. We speculate that they are part of a novel peptidergic system that remains to be identified.

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