Genomic organization and functional characterization of the mouse *GalR1* galanin receptor

Suke Wang*, Chaogang He, Maureen T. Maguire, Anthony L. Clemmons, Robert E. Burrier, Mario F. Guzzi, Catherine D. Strader, Eric M. Parker, Marvin L. Bayne

Department of CNS/CV Biological Reseach, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

Received 27 May 1997

Abstract Galanin mediates diverse physiological functions in digestive, endocrine, and central nervous systems through G-protein-coupled receptors. Two galanin receptors have been cloned but the gene structures are unknown. We report genomic and cDNA cloning of the mouse *GalR1* galanin receptor and demonstrate that the coding sequence is uniquely divided into three exons encoding the N-terminal portion through the fifth transmebrane domain, the third intracellular loop, and the sixth transmembrane domain through the C-terminus. Functional analysis of the encoded cDNA revealed active ligand binding and intracellular signaling. The expression is detected in brain, spinal cord, heart and skeletal muscle.

Key words: Galanin; G-protein-coupled receptor; Gene structure; Central nervous system

1. Introduction

Galanin is a widely distributed 29–30 amino acid peptide involved in diverse regulatory effects in many tissues [1,2]. Galanin modulates the release and secretion of many neurotransmitters and hormones in both the central nervous system and peripheral tissues, such as acetylcholine, gastrin, insulin, dopamine, growth hormone and prolactin. In peripheral tissues, galanin inhibits insulin release by beta cells of the pancreas [3,4], as well as gastrin and somatostatin release from rat stomach. In addition, intracerebraventrical injection of galanin patently stimulates feeding in satiated animals [5,6].

The action of galanin is mediated through specific receptors. The receptor is a glycoprotein with an apparent molecular weight of 54 kDa [7] and a signaling pathway is linked through G-proteins to the inhibition of adenylyl cyclase through a pertussis toxin-sensitive mechanism [8], as well as to the modulation of other effectors such as ATP-dependent K^+ channel and Ca^{2+} channels. Physiological studies suggest that the discovery of potent and specific galanin receptor antagonists may provide novel treatments for Alzheimer's disease [9,10] and morbid obesity [5,11]. Use of peptide agonists and antagonists in pharmacological analysis suggests the existence of multiple galanin receptor subtypes [12–15] which was confirmed by molecular cloning of human and rat *GalR1* [12–14] and rat *GalR2* receptors [15,16]. These studies revealed that the receptors are members of the G-protein-coupled receptor (GPCR) superfamily, characterized by seven hydrophobic transmembrane domains (TM) [17]. The human *GalR1* receptor has been mapped to chromosome 18q23 [18]. Despite the cDNA cloning of *GalR1* and *GalR2* receptors, the genomic organizations of these genes have not been described. In this report we describe the genomic and cDNA cloning, exon/intron organization, and functional analysis of the mouse galanin receptor.

2. Materials and methods

2.1. Materials

Rat [^{125}I]galanin (2200 Ci/mmol) and α-[^{32}P]dATP (5000 Ci/mmol) were purchased from Du Pont-NEN (Boston, MA). Freshly frozen mouse tissues (Swiss-Webster) were obtained from Pel-Freeze Biologicals (Rogers, AR). Mouse multiple tissue Northern blots (MTN) were obtained from Clontech (Palo Alto, CA, cat. #7762-1). Rat galanin, rat galanin 1–16, M40, C7, and M35, and M15 were purchased from Peninsula Laboratories (Belmont, CA). Rat galanins 2–29 and 10–29 and oligonucleotides used in this study were custom-synthesized by Bio-synthesis, Inc. (Lewisville, TX). Since rat and mouse galanins are identical, rat [^{125}I]galanin will be referred to as mouse [^{125}I]galanin, and rat galanin and peptides as mouse galanin and peptides. Sequence determinations were performed with the fluorescent dye termination method (Perkin Elmer, Branchburg, NJ) on an automated DNA sequencer (Model 373, Applied Biosystems, Inc.). Sequencing data were analyzed with DNA* software package (DNAstar, Inc. Madison, WI).

2.2. Isolation of mouse galanin receptor genomic clones

The human*GalR1* cDNA, used as a probe to obtain mouse galanin genomic clones, was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of human melanoma cells and cloned in vector pCRII (Invitrogen, San Diego, CA). The sequence was identical to the published sequence [12]. The full-length human *GalR1* cDNA was excised out of the pCRII vector by restriction digestion and purified by agarose gel electrophoresis. The cDNA was labeled with ^{32}P and used as probe to screen a mouse embryonic stem cell (129SvEv stem embryonic cell) genomic library in bacterial artifacial chromosomes (BAC) at high hybridization stringency (65°C) (Genome System, Inc., St. Louis, MO). The BAC mouse *GalR1* clones were analyzed by restriction enzyme digestion and Southern blotting. Fragments containing exonic sequences were subcloned into Bluescript (ks-vector, Stratagene) for sequencing analysis.

2.3. Isolation of mouse GalR1 cDNA and tissue distribution

PCR primers were designed based on the nucleotide sequences in the 5′ and 3′ untranslated regions of mouse *GalR1* gene and used in PCR to obtain mouse *GalR1* cDNA. Total RNA of mouse brain was extracted with the Tri Reagent kit (Molecular Research Center, Cincinnati, OH). Poly A^+ RNA from the total RNA was purified with an mRNA purification kit which employs oligo(dT)-cellulose chromatography (Pharmacia, Piscataway, NJ). Double-stranded cDNA was synthesized from the poly A^+ RNA with a Marathon cDNA amplification kit (Clontech). Mouse *GalR1* cDNA was obtained by PCR with the mouse brain cDNA as template (0.1 μg) by using an Advantage PCR kit (Clontech) for the amplification. The reaction (40 μl) was driven by the KlenTaq polymerase (5 U) in the presence of a forward primer (oligo 57, 5′-tagccaggacaggcaggaggatagt-3′) and a reverse primer (oligo 58, 5′-gggaggctcccgcaaaccttcacac-3′) with a thermal cycling profile of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min (40

*Corresponding author. Fax: (908) 298-2383.
E-mail: suke.wang@spcorp.com

PII S0014-5793(97)00695-9

cycles) preceded with heating at 94°C for 5 min and followed by an extension at 72°C for 7 min. Agarose gel analysis of the reaction product showed a single band at the expected size of about 1.3 kb. The PCR product (3 μl) was ligated into a bi-directional TA cloning vector, pCR3 (Invitrogen). For Northern blot analysis, mouse MTN blots with 2 μg poly A+ RNA in each lane were hybridized for 1.5 h at 68°C in an ExpressHyb solution (Clontech) using ^{32}P-labeled full-length mouse *GalR1* cDNA as a probe (Random priming kit, Life Technology, Gaithersburg, MD). After hybridization, the blots were washed first with wash solution I (2× SSC, 0.05% SDS) for 10 min. at room temperature then with wash solution II (0.1× SSC, 0.1% SDS) for approximately 30 min at 45°C.

2.4. Transfection of COS-1 cells and [^{125}I]galanin binding assay

COS-1 cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) were split 1:6 into 150 mm dishes (Nunc) 3 days prior to transfection. Transfection and membrane preparations were performed as previously described [16]. Protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) (Sigma, St. Louis, MO) as standard. Binding of mouse [^{125}I]galanin to the mouse *GalR1* receptor cells was performed in a buffer containing 10 mM HEPES (pH 7.4), 0.05% BSA (RIA grade), 0.1% bacitracin, and 0.5 mM EDTA. Ligand saturation plots were performed in triplicate with 2.5 μg of the membrane protein in a total volume of 50 μl using 1 μM cold galanin to determine non-specific binding. Peptide competition studies were performed in duplicate in a total volume of 200 μl, containing 5 μg of membrane protein and 0.1 nM [^{125}I]galanin. Incubations were at room temperature for 1 h and were terminated by rapid vacuum filtration through Multiscreen FB Filter Plates (Millipore, Bedford, MA) pretreated with 0.3% polyethylenimine. The filters were then washed 3 times with 100 μl of phosphate-buffered saline (pH 7.4). All data were analyzed using non-linear regression software (Prism GraphPad, San Diego, CA) to fit one- and two-site binding and the K_i calculated according to the method of Cheng and Prusoff [19].

2.5. Cyclic AMP analysis

COS-1 cells grown in 150 mm plates were transfected as described above. After an overnight post-transfection growth, the cells were trypsinized off plates and subcultured into wells of 24 well plates at a density of 2.5×10^5 cells/well and allowed to grow for 3 more days. Cells were washed with PBS and incubated in DMEM for 1.5 h at 37°C then incubated in 1 ml of DMEM containing 0.1 mM forskolin, 0.2 mM 3-isobutyl-1-methylxanthine and mouse galanin at indicated concentrations for 1 h at 37°C. The measurements were performed in triplicate and intracellular cAMP was assayed with a [^{3}H]cAMP detection kit (Amersham, Arlington Heights, IL). The data were analyzed with the Prism non-linear regression program to obtain maximum inhibition and EC_{50} values.

3. Results and discussion

3.1. Cloning and genomic organization of murine GalR1 gene

A murine genomic BAC library was screened with a ^{32}P-labeled probe encoding the entire length of the human *GalR1* cDNA under high stringency hybridization conditions. Two positive clones with insert sizes ≥25 kb were obtained. Restriction enzyme digestion and Southern blot analysis indicated that the two clones were identical; thus only one of the clones was further analyzed by sub-cloning and sequencing. Fig. 1 shows the restriction map and Fig. 2 the genomic sequence of the mouse *GalR* gene. An open reading frame (ORF) of 1047 bp was identified and found to be disrupted by two introns (Figs. 1A and 2A). The first intron, approximately 3.4 kb in length, is located between nucleotides (nts) 663 and 664 (intron I) and the second, with a length of about 8.0 kb, between nts 729 and 730 (intron II) in the ORF. The disruptions resulted in three separated exons in the ORF. These exons, when translated to amino acid sequences, are separated in frame and the resulting amino acid sequences

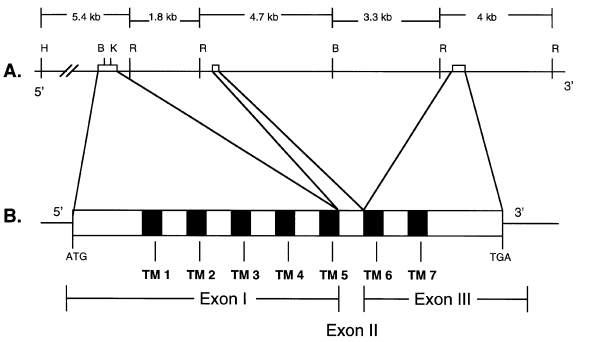

Fig. 1. Genomic organization of the mouse *GalR1* receptor gene. A: Restriction map of the gene. The line represents the gene with exons (boxes) interrupted by introns (lines between boxes). Empty boxes denote coding sequences and only the portions of exon I and exon III within the coding region are shown. Restriction sites are: R, *Eco*RI; B, *Bst*XI; H, *Hin*dIII; and K, *Kpn*I. The lengths of the restriction fragments are shown above the map. B: Detailed exon assignment within the coding region. The coding sequence is represented as one open box with the seven putative TMs shown as dark boxes.

correspond to amino acids 1–221 (N-terminus to TM5, exon I), amino acids 222–243 (third intracellular loop, exon II) and amino acids 244–348 (TM6 to C-terminus, exon III) (Fig. 2). The sequences of the two exon/intron junctions and the two intron/exon junctions closely match the doner and acceptor splice site consencus sequences, respectively [20]. Fig. 1B shows the spliced transcript of the gene containing the putative start and stop codons.

The putative mouse *GalR1* cDNA in the ORF consists of 1047 bp encoding a protein of 348 amino acids with a predicted molecular weight of 39.1 kDa. The receptor is highly homologous to human [12] and rat [14] *GalR1* receptors (91% and 94%, respectively) and possesses only 39% homology to rat *GalR2* [15,16]. The most variable regions are the N- and C-termini and the second extracellular loop. Comparison of the mouse *GalR1* amino acid sequence with other GPCRs and a hydrophathy analysis [21] suggest that the protein contains seven membrane spanning regions (TM) and an extracellular N-terminus and a cytoplasmic C-terminus. Features conserved in other members of the super family of GPCRs are also well conserved in mouse *GalR1* receptor. These include C108 and C186, a pair of cysteine residues that may form a disulfide bond between extracellular loops 1 and 2 (E_1 and E_2), two consensus sites for N-linked glycosylation in the N-terminal region, three serine residues for potential protein kinase C phosphorylation, two potential cAMP/cGMP protein kinase phosphorylation sites, and three C-terminal cysteine residues that can serve as potential palmitoylation site (Fig. 2B). Amino acid residues corresponding to H264, H267, F282 and E271 in the human *GalR1* receptor, which have been determined to be essential for the binding of galanin [22], are all conserved (Fig. 2B).

3.2. Comparison of GalR1 gene with other GPCR genes

The structure of the mouse *GalR1* gene is different from the known genomic structures of other GPCRs. Most of these receptors are intronless in the coding regions and only a small number of them contain one or more introns in the coding regions. Examples of single-intron genes are the neuropeptide Y Y-1 receptor, with a small intron (~80–97 bp) inserted

after the fifth TM and before intracellular loop 3 (I_3) [23,24]. The human α-1b-adrenergic receptor [25] and rat A_3 adenosine receptor [26] also have a single intron located at the proximal end of extracellular loop 4 (E_4) and in the middle of intracellular loop 2 (I_2), respectively. The bombesin receptor gene family contains two introns located in the central I_2 and I_3 regions, respectively [27]. The *GPCR* genes that possess more than two introns, such as the endothelin B receptor family with seven exons [28], opsin and rhodopsin receptor family with five to six exons [29,30], the tachykinin receptor

A

```
                -321  cctagacccgtacctctgtgttctggagcctgccgcccccgcacgggaaaggcttagctcgggacttgcagcaccgcctcctctttagccaggccagg
cacgaggatagtgtgatcgggcacagccagggtcgctcttccaggctttcttgcgggttgcgggaggtactagttggagacgcgcgcgctcgctctcgccgctctgtcctgg
gccactccgtgatcctaggctacctccagagccagttttccctggctggcacaactctccagggcgctccggtccgttgcacagcgccccaaggggtatcccagtaagtg

ATGGAACTGGCTATGGTGAACCTCAGTGAAGGGAATGGGAGCGACCCAGAGCCGCCAGCCCCGGAGTCCAGGCCGCTC                                  78
Met Glu Leu Ala Met Val Asn Leu Ser Glu Gly Asn Gly Ser Asp Pro Glu Pro Pro Ala Pro Glu Ser Arg Pro Leu                26

TTCGGCATTGGCGTGGAGAACTTCATTACGCTGGTAGTGTTTGGCCTGATTTTCGCGATGGGCGTGCTGGGCAACAGC                                 156
Phe Gly Ile Gly Val Glu Asn Phe Ile Thr Leu Val Val Phe Gly Leu Ile Phe Ala Met Gly Val Leu Gly Asn Ser                52

CTGGTGATCACCGTGCTGGCGCGCAGCAAACCAGGCAAGCCGCGCAGCACCACCAACCTGTTTATCCTCAATCTGAGC                                 234
Leu Val Ile Thr Val Leu Ala Arg Ser Lys Pro Gly Lys Pro Arg Ser Thr Thr Asn Leu Phe Ile Leu Asn Leu Ser                78

ATCGCAGACCTGGCCTACCTGCTCTTCTGCATCCCTTTTCAGGCCACCGTGTATGCACTGCCCACCTGGGTGCTGGGC                                 312
Ile Ala Asp Leu Ala Tyr Leu Leu Phe Cys Ile Pro Phe Gln Ala Thr Val Tyr Ala Leu Pro Thr Trp Val Leu Gly               104

GCCTTCATCTGCAAGTTTATACACTACTTCTTCACCGTGTCCATGCTGGTGAGCATCTTCACCCTGGCCGCGATGTCT                                 390
Ala Phe Ile Cys Lys Phe Ile His Tyr Phe Phe Thr Val Ser Met Leu Val Ser Ile Phe Thr Leu Ala Ala Met Ser               130

GTGGATCGCTACGTGGCCATTGTGCACTCGCGGCGCTCCTCCTCCCTCAGGGTGTCCCGCAACGCACTGCTGGGCGTG                                 468
Val Asp Arg Tyr Val Ala Ile Val His Ser Arg Arg Ser Ser Ser Leu Arg Val Ser Arg Asn Ala Leu Leu Gly Val               156

GGCTTCATCTGGGCGCTGTCCATCGCCATGGCCTCGCCGGTGGCCTACCACCAGCGTCTTTTCCATCGGGACAGCAAC                                 546
Gly Phe Ile Trp Ala Leu Ser Ile Ala Met Ala Ser Pro Val Ala Tyr His Gln Arg Leu Phe His Arg Asp Ser Asn               182

CAGACCTTCTGCTGGGAGCAGTGGCCCAACAAGCTCCACAAGAAGGCTTACGTGGTGTGCACTTTCGTCTTTGGGTAC                                 624
Gln Thr Phe Cys Trp Glu Gln Trp Pro Asn Lys Leu His Lys Lys Ala Tyr Val Val Cys Thr Phe Val Phe Gly Tyr               208

CTTCTGCCCTTACTGCTCATCTGCTTTTGCTATGCCAAG gtgagtgaggagcgctggcc------> intron I, 3.4 kb <-------cat                663
Leu Leu Pro Leu Leu Leu Ile Cys Phe Cys Tyr Ala Lys                                                              221
ccggttatgttttgcagGTCCTTAATCATCTGCATAAAAAGCTGAAAAACATGTCAAAAAAGTCTGAAGCATCCAAGAAAAAG                             729
                  Val Leu Asn His Leu His Lys Lys Leu Lys Asn Met Ser Lys Lys Ser Glu Ala Ser Lys Lys Lys         243
 gtaaattcacacacagatgc----------------------------------------------> intron II, 8 kb <----------
------------------------tccattactctctcttccag ACTGCACAGACCGTCCTGGTGGTCGTTGTAGTATTTGGCATATCCTGGCTG             780
                                              Thr Ala Gln Thr Val Leu Val Val Val Val Val Phe Gly Ile Ser Trp Leu 260

CCCCATCATGTCGTCCACCTCTGGGCTGAGTTTGGAGCCTTCCCACTGACGCCAGCTTCCTTCTTCTTCAGAATCACC                                 858
Pro His His Val Val His Leu Trp Ala Glu Phe Gly Ala Phe Pro Leu Thr Pro Ala Ser Phe Phe Phe Arg Ile Thr         286

GCCCATTGCCTGGCATACAGCAACTCCTCAGTGAACCCCATCATATATGCCTTTCTCTCAGAAAACTTCCGGAAGGCG                                 936
Ala His Cys Leu Ala Tyr Ser Asn Ser Ser Val Asn Pro Ile Ile Tyr Ala Phe Leu Ser Glu Asn Phe Arg Lys Ala         312

TACAAGCAAGTGTTCAAGTGTCATGTTTGCGATGAATCTCCACGCAGTGAAACTAAGGAAAACAAGAGCCGGATGGAC                                1014
Tyr Lys Gln Val Phe Lys Cys His Val Cys Asp Glu Ser Pro Arg Ser Glu Thr Lys Glu Asn Lys Ser Arg Met Asp         338

ACCCCGCCATCCACCAACTGCACCCACGTGTGAaggtttgcgggagcctcccgacttccagctcccatgtgtgttagagagaggagggcggagcgaa             1111
Thr Pro Pro Ser Thr Asn Cys Thr His Val •                                                                        348
ttatcaagtaacatggcagcttattctccacagcaattcctatcgatccaactacattccacagtggtaaaaggacgttgattgttcagggaactcgtgggtctactgaag
atcattttccaatttcattttactctataattgtatatatgagacaaaagaaacttctgtatagtttctagctctcaaggaatgaaagtcctacagcactctgcaaatgttttg
atctccatggtgtaaagtaagttctgtaaagcagagaataatatttgaaatccacattttcaggccagt*taatata*gggaaaaagttctttttaaaaaaaaatttgagagg
agaggtaatagtctagtctgtgctttgaaatacaactgtgggcacagaatagtgatttttatttttattgaacctttgtggaccggcacctcatctgagtcgtgggtgtccata
agtctgtctggggatgtgagacc                                                                                          1583
```

Fig. 2. The mouse *GalR1* sequences. A: Nucleotide and deduced amino acid sequences. Nucleotide numbering starts with 1 at the translation initiation site *A*TG. Nucleotides within introns 1 and 2 are not counted in the numbering system. Nucleotides in the coding region are shown in upper case letters. Introns and non-coding sequences are shown in lower case letters. Only ~20 nucleotides at each intron/exon junction are shown. Amino acids constituting the predicted TMs and nucleotide sequences corresponding to the 5′ (forward) and 3′ (reverse) primers used in cDNA cloning by RT-PCR are underlined. The underlined '*taatata*' denotes a putative polyadenylation signal. B: Alignment of the amino acid sequences of mouse, human [12] and rat [13] *GalR1* receptors. Amino acids in the human and rat receptors that are different from the mouse are shown. Gaps (−) are introduced to optimize the alignment. Putative TMs are overlined and indicated by 'TM' and Roman numerals. The potential glycosylation sites (*), phosphorylation sites (◇) for cAMP/cGMP protein kinase, cysteine residues (●) presumed to form a disulfide bond, protein kinase C phosphorylation sites (#) and palmitoylation sites (△) are indicated above the mouse *GalR* 1 amino acid sequence.

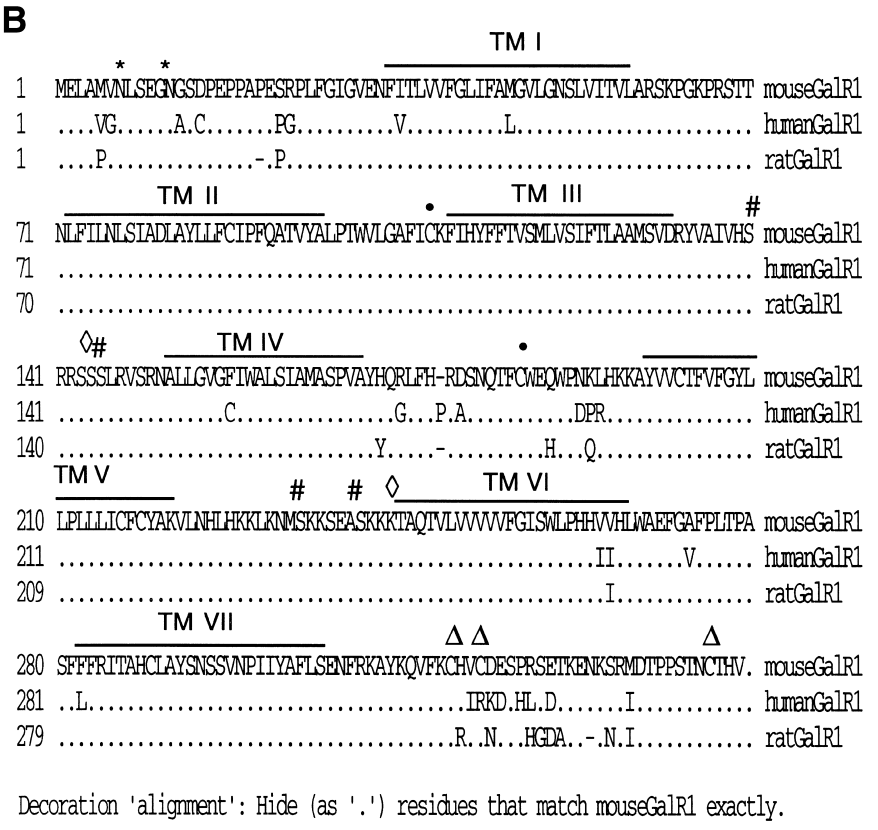

Fig. 2. (*continued*)

family with five exons [31,32], and the glucogan and GLP receptor family with approximately 12 exons, all have one single intron in I_3 regions. Since exons often encode functional domains of proteins, the observation that I_3 of nearly all of the intron-containing *GPCR* genes possesses a single intron is consistent with the suggestion that I_3 and/or TM6 forms a specific domain which at least partly determines the specificity of interaction with G-proteins. The genomic structure of the mouse galanin receptor is different from above receptors in that the two introns embrace exon II in-frame and are located at the proximal and distal ends of loop I_3 (Fig. 2). Thus exon II encodes 22 amino acids that span nearly the entire I_3. This region is highly basic, containing eight Lys and two His residues, and a comparison of the sequence with proteins in the data bank showed no appreciable homology of this region to any other known protein. Thus, the splicing pattern of intron I and intron II suggests that that the domain may have structurally evolved by 'exon-shuffling' [33] and this intracellular loop may be functionally involved in signaling [34,35].

3.3. *cDNA cloning and characterizaton of the murine GalR1 receptor*

To determine whether the genomic clone encodes an active galanin receptor, the cDNA was obtained by RT-PCR with primers annealing to the 5′ and 3′ flanking regions of the mouse genomic clone (forward primer [nt (−222)–(−246)] and reverse primer (nt1044–1068), Fig. 2A) and total mouse brain RNA as template. A PCR product of the expected size of ∼1.3 kb was generated and cloned into the mammalian expression vector pCR3. Sequencing of the mouse *GalR1* cDNA revealed a sequence identical to that of the ORF iden-

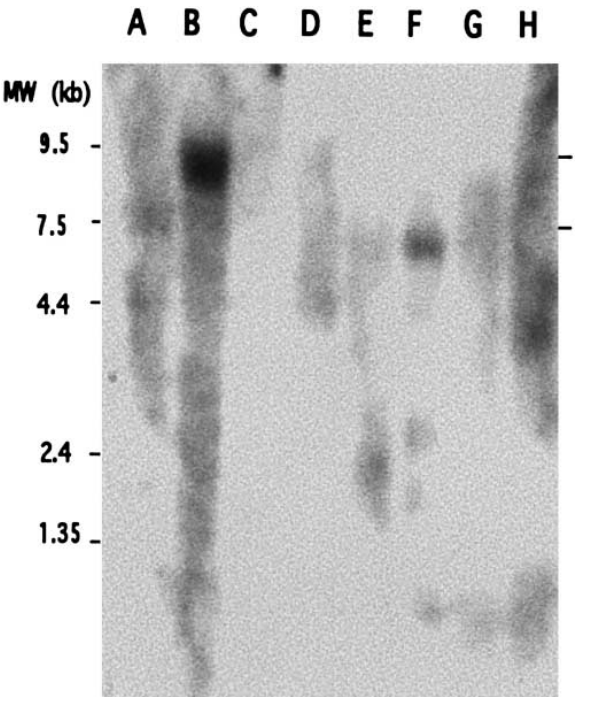

Fig. 3. Tissue distribution of mouse *GalR1* mRNA by Northern blot analysis. The graph shows one representative autoradiograph produced by 1 week exposure of the filter to Kodak BioMax film. Lanes indicate poly A^+ RNA from mouse heart (lane A), brain (lane B), spleen (lane C), lung (lane D), liver (lane E), skeletal muscle (lane F), kidney (lane G) and testis (lane H), respectively. The two small bars on the right indicate the size of the bands found in brain (∼9 kb) and skeletal muscle (∼7 kb), respectively.

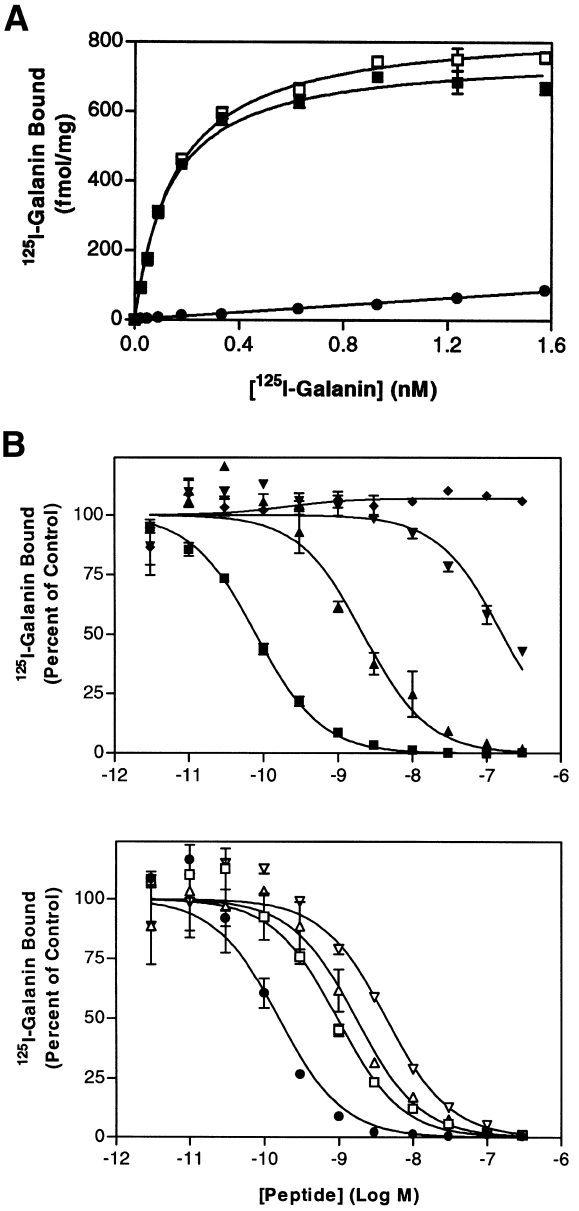

Fig. 4. Ligand binding of the mouse *GalR1* receptor. A: Saturation of mouse [^{125}I]galanin binding to mouse *GalR1* expressed in COS-1 cell membranes. Total (□), non-specific (●) and specific (■) binding are shown as the average ± SEM ($n = 3$). The curves represent the best fit to single-site binding isotherms. B: Competition for [^{125}I]galanin binding to COS-1 cell membranes containing the mouse *GalR1* receptor. The data are expressed as the percent of control specific binding (6659 ± 187 cpm, mean ± SEM, $n = 16$). Non-specific binding was determined to be 128 ± 8 cpm (mean ± SEM, $n = 8$). The peptides assayed included galanin (■), galanin 1–16 (▲), galanin 2–29 (▼), galanin 10–29 (◆), M15 (▽), M40 (□), C7 (△), and M35 (●).

tified in the genomic sequence (Fig. 2). No sequences from either intron I or intron II were found.

Northern blot analysis and RT-PCR were performed to determine mouse *GalR1* expression in a number of tissues. Full-length mouse *GalR1* cDNA labeled with ^{3}P was hybridized to Northern blots containing poly A^{+} RNA isolated from different mouse tissues (Fig. 3). Mouse brain showed abundant expression of *GalR1*, as evidenced by the presence of a strong 9 kb mRNA band. Skeletal muscle and heart showed moderate levels of expression of a 7–7.5 kb mRNA. No mouse *GalR1* mRNA was detected in liver, kidney, testis, lung and spleen. The size differences of the transcripts in various tissues may result from heterogeneous processing of the large 5′ and 3′ untranslated regions in different tissues. RT-PCR analysis showed high levels of mouse *GalR1* mRNA in brain and spinal cord, and a somewhat reduced level of expression in heart. No detectable signal was found in pituitary, stomach, small intestine, liver, testis, ovaries, pancreas, spleen, kidney and lung (not shown). The distribution is consistent with the Northern blot analysis (Fig. 3 and [14]) and in contrast to the wide tissue-distribution of GalR2 [16]. The finding that skeletal muscle expresses the mouse *GalR1* receptor relatively abundantly suggests additional roles of galanin in this tissue.

The ability of the receptor to bind galanin was examined by the radioligand binding assay. Shown in Fig. 4A is the binding of mouse [^{125}I]galanin to membranes prepared from transfected COS-1 cells. Analysis of the data using non-linear regression methods revealed a K_d for mouse [^{125}I]galanin of 0.134 ± 0.014 nM and a B_{max} of 767 ± 20 fmol/mg protein. The data were best fit to single-site model ($r = 0.996$), suggesting a single population of high-affinity receptors in these membranes. Mouse [^{125}I]galanin did not bind to COS-1 cell membranes derived from cells transfected with vector pCR3 alone. The pharmacological properties of the mouse *GalR1* receptor are shown in Fig. 4B. Mouse galanin has high affinity for the mouse *GalR1* receptor ($K_i = 0.047$ nM). Truncation of the C-terminus of the peptide produced moderate reduction in affinity for the receptor as shown with galanin1-16 ($K_i = 1.02$ nM). In contrast, deletion of the N-terminal amino acid Gly markedly reduces the affinity (galanin 2–29, $K_i = 84.6$ nM),

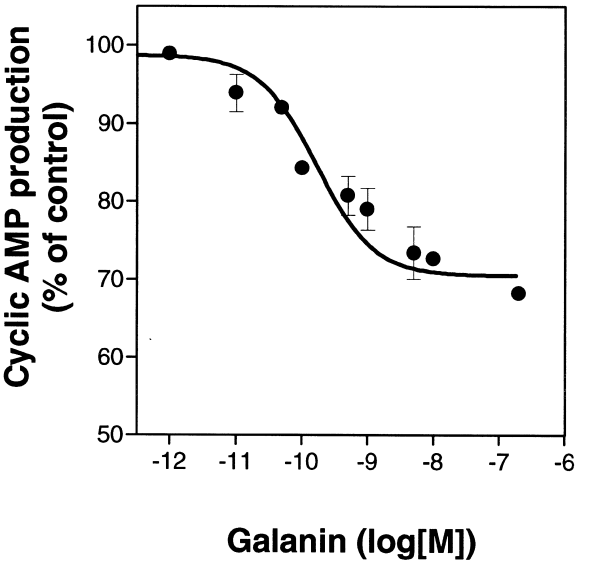

Fig. 5. Inhibition of forskolin-stimulated cAMP production by mouse galanin in COS-1 cells expressing the mouse *GalR1* recceptor. Transfected cells were incubated in the presence of 0.1 mM forskolin alone (control) and mouse galanin at the indicated concentrations. Results shown are mean ± SD ($n = 3$ wells). Error bars are smaller than the size of the symbols when not shown. The curve indicates the best fit to all the data points by one site non-linear regression analysis. The basal and forskolin-stimulated cAMP production levels were estimated at 5.0 ± 0.06 ($n = 3$) and 59.0 ± 1.2 ($n = 3$) pmol/10^{6} cells, respectively.

while removal of the first nine amino acids produces a peptide that has no affinity for the galanin receptor (galanin 10–29). Deletion of the Gly only resulted in slight loss of the affinity to GalR2 [16]. The chimeric peptides M35, M40, C7 and M15 bound to the receptor with K_i values of 0.067, 0.43, 0.96, and 2.24 nM, respectively. All of the peptides tested fit best to a single class of sites using non-linear regression methods.

Mouse galanin caused a concentration-dependent inhibition of forskolin-stimulated cAMP production in COS-1 cells expressing the mouse *GalR1* receptor. Non-linear regression analysis of the data gave a maximum inhibition of $67.0 \pm 2.4\%$ and EC_{50} of 0.19 ± 0.07 nM (Fig. 5). Thus, the cloned mouse receptor is functional in intracellular signaling and activation of the receptor leads to an inhibition of forskolin-stimulated cAMP formation, presumably via interaction with pertussis toxin-sensitive Gi/Go proteins.

In summary, we have isolated and characterized a gene encoding an active mouse *GalR1* receptor. The genomic organization of the mouse galanin receptor is unique among the GPCRs. Of particular interest is the observation that exon II encodes exclusively the third intracellular loop of the receptor, suggesting the potential for differential G-protein coupling by exon shuffling within this functionally important region. Examples of inactive GPCRs that occurs only in certain species have been observed, e.g. both mouse and human NPY PP2 receptors have been cloned, but only the mouse cDNA encodes an active receptor [36]. The expressional analysis of the mouse *GalR1* receptor demonstrate that the mouse gene encodes an active receptor. The amino acid sequence, tissue distribution, and pharmacological profile of the receptor are similar to those of other *GalR1* receptors [12–14], but distinct from those of *GalR2* receptor [16]. Both receptors are linked to inhibition of cAMP. The elucidation of the gene structure should enhance our understanding of the gene regulation and structure/function relationship of the *GalR1* receptors. The cloning and characterization of the mouse receptor should aid in studies of galanin functions, such as feeding and cognition, in mouse models.

References

[1] Tatemoto, K., Rokaeus, A., Jornwall, H., McDonald, T.J. and Mutt, V. (1983) FEBS Lett. 164, 124–128.
[2] Merchanthaler, I., Lopez, F.J. and Negro-Vilar, A. (1993) Prog. Neurobiol. 40, 711–769.
[3] Bartfai, T., Hokfelt, T. and Langel, U. (1993) Crit. Rev. Neurobiol. 7, 229–274.
[4] Hermansen, K., Yanaihara, N. and Ahren, B. (1989) Acta Endocrinol. (Copenh.) 121, 545–550.
[5] Tempel, D.L., Leibowitz, K.L. and Leibowitz, S.F. (1988) Peptides 9, 309–314.
[6] Crawley, J.N. et al. (1990) J. Neurosci. 10, 3695–3700.
[7] Chen, Y., Couvineau, A., Laburthe, M. and Amiranoff, B. (1992) Biochemistry 31, 2415–2422.
[8] Lagny-Pourmir, I., Amiranoff, B., Lorinet, A.M., Tatemoto, K. and Laburthe, M. (1989) Endocrinology 124, 2635–2641.
[9] Crawley, J.N. (1996) Life Sci. 58, 2185–2199.
[10] Ogren, S.O., Hokfelt, T., Kask, K., Langel, U. and Bartfai, T. (1992) Neuroscience 51, 1–5.
[11] Crawley, J.N., Robinson, J.K., Langel, U. and Bartfai, T. (1993) Brain Res. 600, 268–272.
[12] Habert-Ortoli, E., Amiranoff, B., Loquet, I., Laburthe, M. and Mayaux, J.F. (1994) Proc. Natl. Acad. Sci. USA 91, 9780–9783.
[13] Burgevin, C.M., Loquet, I., Quarteronet, D. and Habert-Ortoli, E. (1995) J. Mol. Neurosci. 6, 33–41.
[14] Parker, E.M., Izzarelli, D.G., Nowak, H.P., Mahle, C.D., Iben, L.G., Wang, J. and Goldstein, M.E. (1995) Mol. Brain Res. 34, 179–189.
[15] Howard, A.D. et al. (1997) FEBS Lett. 405, 285–290.
[16] S. Wang, T. Hashemi, C. He, C. Strader, and M. Bayne, Mol. Pharm. (1997) in press.
[17] Burbach, J.P. and Meijer, O.C. (1992) Eur. J. Pharmacol. 227, 1–18.
[18] Nicholl, J., Kofler, B., Sutherland, J., Shine, J. and Lismaa, T.P. (1995) Genomics 30, 629–630.
[19] Cheng, Y. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099–3108.
[20] Breathnach, R. (1981) Annu. Rev. Biochem. 50, 349–383.
[21] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[22] Kask, K., Berthold, M., Kahl, U., Nordvall, G. and Bartfai, Y. (1996) EMBO J. 15, 236–244.
[23] Herzog, H., Baumgartner, M., Vivero, C., Selbie, L., Auer, B. and Shine, J. (1993) J. Biol. Chem. 268, 6703–6707.
[24] Eva, C., Oberto, A., Sprengel, R. and Genazzani, E. (1992) FEBS Lett. 314, 285–288.
[25] Ramarao, C.S., Denker, J.M., Perez, D.M., Gaivin, R.J., Riek, R.P. and Graham, R.M. (1992) J. Biol. Chem. 267, 21936–21945.
[26] Murrison, E.M., Goodson, S.J., Edbrooke, M.R. and Harris, C.A. (1996) FEBS Lett. 384, 243–246.
[27] Corjay, M.H., Dobrzanski, D.J., Way, J.M., Viallet, J., Shapira, H., Worland, P., Sausville, E.A. and Battey, J.F. (1991) J. Biol. Chem. 266, 18771–18779.
[28] Mizuno, T., Saito, Y., Itakura, M., Ito, F., Ito, T., Moriyama, E.N., Hagiwara, H. and Hirose, S. (1992) Biochem. J. 287, 305–309.
[29] Nathans, J., Thomas, D. and Hogness, D.S. (1986) Science 232, 193–202.
[30] Nathans, J. and Hogness, D.S. (1983) Cell 34, 807–814.
[31] Graham, A., Hopkins, B., Powell, S.J., Danks, P. and Briggs, I. (1991) Biochem. Biophys. Res. Commun. 177, 8–16.
[32] Takahashi, K., Tanaka, A., Hara, M. and Nakanishi, S. (1992) Eur. J. Biochem. 204, 1025–1033.
[33] Rogers, J. (1985) Nature 315, 458–459.
[34] Selbie, L.A., Hayes, G. and Shine, J. (1989) DNA 8, 683–689.
[35] Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J. and Sealfon, S.C. (1992) DNA Cell Biol. 11, 1–20.
[36] Gregor, P., Feng, Y., DeCarr, L.B., Cornfield, L.J. and McCaleb, M.L. (1996) J. Biol. Chem. 271, 27776–27781.