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Cloning of a novel putative G-protein-coupled receptor (NLR) which is expressed in neuronal and lymphatic tissue

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A novel G-protein-coupled receptor was isolated from mouse and rat neuronal and lymphatic tissues. The amino acid sequence of the rat receptor (rNLR) shows an overall homology of 80% to a recently cloned receptor from Burkitt's lymphoma cells (BLR1) which is exclusively expressed in lymphatic tissues [(1992) Eur. J. Immunol. 22, 2795]. Much less homology between rNLR and BLR1 was observed at the N-terminus (about 40%), whereas rNLR and the mouse homologue mNLR show 92% amino acid identity. Northern blot analysis of NLR revealed a predominant 5.5 kb mRNA species in various brain regions and neuronal cell lines, whereas in the spleen a 3 kb transcript is predominant. This distribution suggests a role of NLR in the nervous and immune systems.

G-protein-coupled receptor; NLR; NG108-15 cell; Polymerase chain reaction; BLR1; Burkitt's lymphoma; Interleukin 8

1. INTRODUCTION

G-protein-coupled receptors are activated by hormones, neurotransmitters or neuropeptides and transduce these signals into the cell by specific interaction with GTP binding proteins. Recent evidence suggests that some members of the cytokine family also produce their effects by interacting with G-protein-coupled receptors [1]. In particular, interleukin-8 (IL-8), which belongs to a family of structurally related cytokines similar to platet factor 4, exerts its effect through a G-protein-coupled receptor [2,3]. Sequence homology between the cloned G-protein-coupled receptors indicates a high degree of similarity of the high- and low-affinity IL-8 receptors with the receptors for chemoattractants, such as fMet-Leu-Phe, C5a and, surprisingly, also for neuropeptide receptors, such as the delta opiate receptor (DOR-1) [4], neuropeptide Y-3 receptor [5] and the somatostatin-2 receptor [6]. Furthermore, a novel member of a G-protein-coupled receptor (BLR1) has been cloned from Burkitt's lymphoma cells with 37% homology to the low affinity IL-8 receptor and shown to be exclusively expressed in lymphatic tissue [7].

In the present study we describe the cloning of NLR, a putative G-protein-coupled receptor which exhibits a high homology to BLR1 and, in contrast to BLR1, is expressed not only in lymphatic tissues such as the spleen but also in various brain areas and in neuronal cell lines.

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2. MATERIALS AND METHODS

2.1. *Poly(A) RNA preparation and Northern blot analysis*

Total RNA was isolated by the acid guanidinium isothiocyanate method [8] and poly(A) RNA was selected by QuickPrep columns (Pharmacia, Freiburg, Germany). After electrophoresis the RNA was blotted onto Nytran membranes (Schleicher & Schüll, Germany) and hybridized with a [^{32}P]UTP-labeled cRNA probe according to standard methods [9].

2.2. *Polymerase chain reaction, cloning and sequencing*

Polymerase chain reaction (PCR) to amplify cDNA's encoding potential G-protein-coupled receptors was performed as follows: first strand cDNA for PCR reaction was generated from 1 μg NG108-15 cell poly(A) RNA using an oligo(dT) primer and MoMLV reverse transcriptase (SuperScript, BRL) in 20 μl final reaction volume. 1 μl of reaction was amplified by 30 cycles of PCR [10] using primers derived from transmembrane segments III and VI [11]. Cycle conditions were 1.5 min at 95°C, 2 min at 50°C and 2 min at 72°C. The resulting products were purified with PrimeErase columns (Stratagene), blunted with Klenow polymerase and cloned into pBluescript (Stratagene). Dideoxynucleotide sequencing was then performed by the chain termination method [12] with Sequenase-2.0 (USB). Four clones showing the motif of G-protein-coupled receptors were obtained (561-45, 561-51, 561-39, 561-8).

2.3. *Library screening and subcloning*

Clone 561-8 was ^{32}P-labelled by the random primer technique [13] and used as a probe to screen a rat genomic library constructed in phage λEMBL3 and a NG108-15 hexanucleotide-primed cDNA library in λZAP II (custom synthesis by Invitrogen, Heidelberg, Germany). Out of 5×10^5 genomic phage recombinants screened, three identical clones were isolated and subjected to restriction mapping and Southern blot analysis. A 7 kb *Bam*HI fragment containing the full coding region was subcloned in pBluescript. Out of 1×10^6 recombinant NG108-15 cDNA phages screened only one 3′ truncated clone, designated N18-10, was isolated.

In order to isolate the rat N-terminal Exon (EX1), primer extension and second strand synthesis was performed on 1 μg rat spleen mRNA with primer SPR-3 (Fig. 1B) according to the protocol of reverse transcriptase Superscript (BRL). The resulting products were cloned in pBluescript and verified by colony hybridization and sequencing.

2.4. In situ hybridization

A sagittal section of rat brain was hybridized with a [^{35}S]UTP-labelled antisense RC8 RNA probe and for control with a sense RNA probe, under conditions previously described [14].

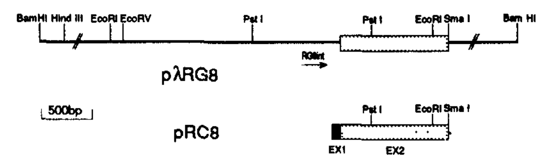
2.5. Stable expression and ligand binding

The cDNA clone RC8 was subcloned in pRc/CMV (Invitrogen, Heidelberg, Germany) and transfected into CHO-K1 cells by Lipofectin (BRL). Individual clones were isolated by limiting dilution in DMEM-F12-medium (BRL) with 600 μg/ml G418. Two clones expressing the highest levels of RC8 mRNA were used for binding experiments as described [17].

3. RESULTS AND DISCUSSION

Poly(A) RNA from NG108-15 cells, a hybrid cell line of mouse N18TG2 neuroblastoma and rat C6BU-1 glioma parent cell lines, was subjected to PCR amplification with degenerate primers to transmembrane domain III and VI of G-protein-coupled receptors as described [11]. Sequence determination of cloned PCR fragments revealed several putative receptor-encoding cDNA clones. Clone 561-45 was identical to rat endothelin 1A receptor [15] and clone 561-51 shows sequence identity to the mouse somatostatin 2 receptor [16]. Screening of a NG108-15 library with this partial cDNA resulted in the cloning of an isoform of the SST2 receptor (SSTR2B) [17]. In addition several novel putative receptor fragments were identified (data not shown). One cDNA fragment (561-8) was used as a hybridization probe to screen a rat genomic library. Restriction analysis of several positive phage clones led to the isolation of a 7 kb *Bam*HI fragment named λRG8 (Fig. 1A). This fragment contains the coding region from membrane domain I–VII plus the stop codon, but lacks a hydrophilic N-terminal extracellular domain and a start codon, indicating the existence of an intervening sequence. Therefore a cDNA library was constructed from rat spleen mRNA by primer extension using a →

A



B

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                                                     PCR-Ex1
                    ATG AAC TCC CCG ATA TCG CTA GAC ATG GGC GCC ATC     36
                     M   N   S   P   I   S   L   D   M   G   A   I      12
                            EX1|EX2
ACC TAC AAC ATG GAC GAT CTG TAC AAG GAA CTG GCC ATC TAC AGT AAC         84
 T   Y   N   M   D   D   L   Y   K   E   L   A   I   Y   S   N          28
                                                            CHO
AGC ACG GAG ATC CCC CTA CAG GAC AGT ATC TTC TGT TCT ACA GAG GAG        136
 S   T   E   I   P   L   Q   D   S   I   F   C   S   T   E   E          44

GGG CCC CTA CTG ACT TCC TTT AAG ACG ATA TTC ATG CCT GTG GCC TAC        180
 G   P   L   L   T   S   F   K   T   I   F   M   P   V   A   Y          60
                                I
AGC CTC ATC TTC CTC CTG GGT ATG ATG GGA AAC ATC CTC GTA CTG GTA        228
 S   L   I   F   L   L   G   M   M   G   N   I   L   V   L   V          76

ATC CTG GAG AGG CAC CGG CAC ACT CGG AGC TCA ACG GAG ACC TTC CTG        276
 I   L   E   R   H   R   H   T   R   S   S   T   E   T   F   L          92
                 II
TTC CAC CTG GCA GTA GCC GAC CTT CTC TTG GTC TTC ATC TTG CCT TTT        324
 F   H   L   A   V   A   D   L   L   L   V   F   I   L   P   F         108

GCA GTG GCT GAG GGC TCT GTG GGT TGG GTC CTA GGG ACC TTC CTC TGC        372
 A   V   A   E   G   S   V   G   W   V   L   G   T   F   L   C         124
                                III
AAA ACT GTG ATC GCC CTG CAC AAG ATC AAT TTC TAC TGC AGC AGC CTG        420
 K   T   V   I   A   L   H   K   I   N   F   Y   C   S   S   L         140

CTG CTG GCC TGC ATA GCT GTC GAC CGT TAT CTG GCC ATC GTC CAT GCG        468
 L   L   A   C   I   A   V   D   R   Y   L   A   I   V   H   A         156
                                                    PCR M3
GTC CAC GCC TAC CGC CGC CGC CGT CTC CTC TCT ATC CAC ATC ACC TGC        516
 V   H   A   Y   R   R   R   R   L   L   S   I   H   I   T   C         172
                                IV
TCG ACT ATT TGG CTA GCC GGC TTC CTA TTT GCC TTG CCA GAA CTC CTC        564
 S   T   I   W   L   A   G   F   L   F   A   L   P   E   L   L         188

TTT GCC AAG GTT GTC CAA CCT CAT AAC AAC GAG TCC TTA CCA CAG TGC        612
 F   A   K   V   V   Q   P   H   N   N   E   S   L   P   Q   C         204
                                    CHO
ATC TTC TCC CAA GAA AAT GAA GCC GAA ACT AGA GCC TGG TTC GCC TCC        660
 I   F   S   Q   E   N   E   A   E   T   R   A   W   F   A   S         220
                                V
CGG TTT CTC TAC CAC ACT GGG GGC TTC CTG CTA CCG ATG CTC GTG ATG        708
 R   F   L   Y   H   T   G   G   F   L   L   P   M   L   V   M         236

GCC TGG TGT TAT GTG GGA GTG GTA CAC AGG CTA TTG CAG GCC CAG CGG        756
 A   W   C   Y   V   G   V   V   H   R   L   L   Q   A   Q   R         252

CGC CCT CAG AGG CAG AAG GCA GTC AGG GTG GCC ATC TTG GTG ACA AGC        804
 P   P   Q   R   Q   K   A   V   R   V   A   I   L   V   T   S         268
                                VI
ATC TTC CTT CTC TGC TGG TCA CCC TAC CAC ATT GTC ATC TTC CTC GAT        852
 I   F   L   L   C   W   S   P   Y   H   I   V   I   F   L   D         284

ACA CTG GAA AGG CTG AAG GCT GTG AAC AGC AGT TGC GAA TTG AGT GGC        900
 T   L   E   R   L   K   A   V   N   S   S   C   E   L   S   G         300
                                CHO
TAC CTC TCA GTG GCT ATC ACC TTA TGT GAA TTC CTG GGC CTG GCT CAC        948
 Y   L   S   V   A   I   T   L   C   E   F   L   G   L   A   H         316
                                VII
TGC TGT CTC AAT CCC ATG CTC TAC ACC TTC GCT GGC GTA AAG TTC CGC        996
 C   C   L   N   P   M   L   Y   T   F   A   G   V   K   F   R         332

AGT GAC TTG TCT CGG CTT CTG ACC AAG CTG GGC TGT GCT GGC CCG GCC       1044
 S   D   L   S   R   L   L   T   K   L   G   C   A   G   P   A         348
             P               P               ◊
TCC CTT TGT CAG CTC TTT CCG GGC TGG CGC AAG AGC AGT CTC TCT GAG       1092
 S   L   C   Q   L   F   P   G   W   R   K   S   S   L   S   E         364
                                                 P       P
TCA GAG AAC GCT ACT TCT CTT ACC ACC TTC TAG ATC CCG GGG TCT CAG       1140
 S   E   N   A   T   S   L   T   T   F                                 374
                   SPR-3
CGGCTCTGTTTCTGTTTTCCTTGGGAGCATAAACGTGGTGTTAGAAACCTGTCTAACATAAGC
TGGGATTCTAAGAGCTATTGGGGCCAGTG                                         1236
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Fig. 1. (A) Organization and restriction map of λRG8 rat genomic clone and RC8 rat spleen cDNA clone. The thick line represents genomic sequences, the shaded box the exon 2, and the black box the exon 1 only present in RC8. RG8int represents an intron-specific primer 122 bp upstream of the exon 2 acceptor site used for PCR. (B) Nucleotide and amino acid sequence of rNLR as deduced from RC8 and λRG8 sequences downstream of the SPR-3 primer. The putative membrane-spanning domains are boxed and are assigned on the basis of Kyte and Doolittle hydrophobicity plot [25]. Potential N-linked glycosylation sites (CHO), potential phosphorylation recognition sites (P) [26] and the potential palmitoylation site at the C-terminus (◊) are indicated. Primers used for reverse transcription (SPR-3) and PCR (PCR-Ex1, PCR-M3) are marked by a solid line and dashed lines with arrows, respectively. The intron–exon junction is marked.

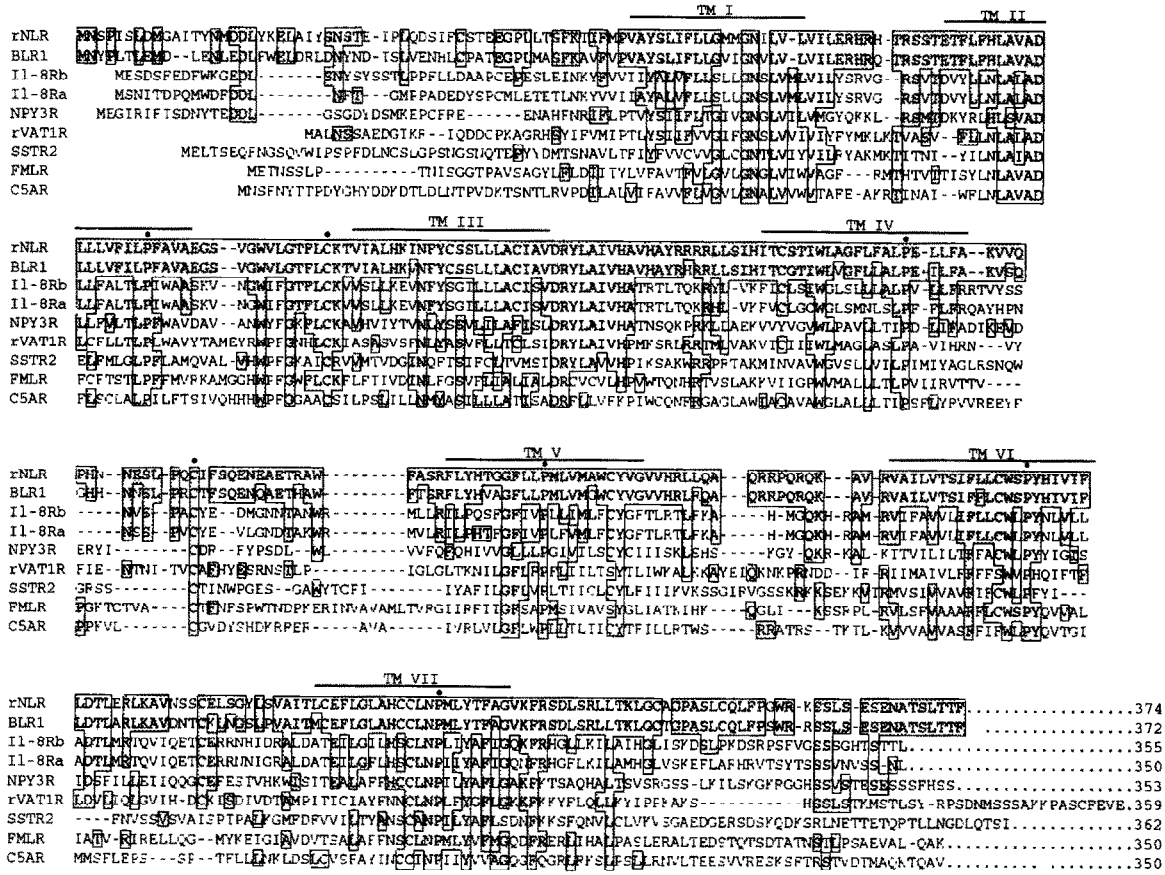

Fig. 2. Alignment of amino acid sequences of rNLR and of other G-protein-coupled receptors. Gaps, indicated by hyphens, have been introduced to obtain maximum homology. The approximate positions of the transmembrane regions are indicated by a bold line above the sequences, identical amino acids with respect to rNLR are boxed. Highly conserved receptor motifs throughout G-protein-coupled receptors are indicated by dots. The sequences are taken from [27] (IL-8Rb, low-affinity human interleukin 8 receptor), [19] (IL-8Ra, high-affinity human interleukin 8 receptor), [5] (NPY3-R, bovine neuropeptide Y receptor), [6] (rSSTR2, rat somatostatin 2 receptor), [20] (rVAT1R, rat vascular type 1 angiotensin II receptor), [21] (FMLR, human *N*-formylpeptide receptor), and [22] (C5AR, human complement 5a receptor).

λRG8-specific primer (SPR-3; Fig. 1B). Sequencing of λRG8-positive colonies revealed a cDNA clone RC8, which is identical to the transmembrane domains I–VII of lRG8 and has additional sequences at the N-terminus, including several methionines as putative start codons (Fig. 1A).

The longest open reading frame of RC8 cDNA specifies a sequence of 1,122 bp corresponding to a protein of 374 amino acids with seven highly hydrophobic regions (Fig. 1B), designated rNLR (rat neurolymphatic receptor). The protein sequence of rNLR predicts potential N-linked glycosylation sites in the N-terminal extracellular region as well as in the second and third extracellular loop. In addition, several serine and threonine residues are located between transmembrane domains III and IV and within the C-terminal region and may represent potential phosphorylation sites. A conserved cysteine residue (Fig. 1B, residue number 344) may function as an acceptor site for palmitoylation [18].

An homology search on protein databases reveals the highest similiarity of rNLR to BLR1 (80%), a G-protein-coupled receptor recently isolated from Burkitt's lymphoma cells [7] (Fig. 2). In addition, rNLR shows significant homologies to the low and high affinity receptors for IL8 (IL-8Rb, IL-8Ra, 37%, 35%)[19], a bovine neuropeptide Y receptor (NPY3, 35%) [5], the vascular type-1 angiotensin II receptor (rVAT II R, 30%) [20] and the rat somatostatin 2 receptor (rSST2R, 26%) [6]. rNLR also exhibits similarities to the receptors for fMet-Leu-Phe (FMLR, 25%) [21] and human C5a anaphylatoxin (C5AR, 24%) [22]. All these receptors contain common features, such as the conserved proline residues in transmembrane regions IV, V, VI, and VII, known to function as helix initiators and thought to surround the ligand binding pocket within the membrane; furthermore cysteine residues are present in the second and third extracellular loops, which may be involved in the formation of disulfide bridges [18].

In addition, we have isolated a 3′-truncated cDNA clone from a NG108-15 library, termed N18-10, encod-

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                 ⊖      ⊖⊖  ⊖          ⊖    ⊖      ⊖⊖                   ________TM I_________       ___
rNLR  MNSPISLDMGAITYNMDDLYKELAIYSNSTEIPLQDSIFCSTEEGPLLTSFKTIFMPVAYSLIFLLGMMGNILVLVILERHRHTRSSTET
mNLR  ..Y.LT....S.............F............N....V.........AV....................................
BLR1  ..Y.LT.E.--DLE.LE..FW..DRLD.YNDTS.VENHL.PAT....MA...AV.V...........VI..V..........Q.......
        .    ⊖   ⊖ ⊖  ⊖⊖    ⊖ ⊖  ⊖   ⊖    ⊖       ⊖

      ________TM II_______                ________TM III________                  ____TM IV__
rNLR  FLFHLAVADLLLVFILPFAVAEGSVGWVLGTFLCKTVIALHKINFYCSSLLLACIAVDRYLAIVHAVHAYRRRRLLSIHITCSTIWLAGF
mNLR  ...................................SA..........RM.............................TA......
BLR1  ........................................V........................H..........G....V..

      _________                                  ________TM V__________                ____TM VI__
rNLR  LFALPELLFAKVVQPHNNESLPQCIFSQENEAETRAWFASRFLYHTGGFLLPMLVMAWCYVGVVHRLLQAQRRPQRQKAVRVAILVTSIF
mNLR  ............G.....D.....T.............T......I..........G.................................
BLR1  .L....I.....S.G.H.N...R.T.....Q...H...T......VA.........G..........R......................
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Fig. 3. Amino acid sequence alignment of rNLR (rat), mNLR (mouse) and BLR1(human) [7] in the N-terminal region, including transmembrane domains TM1 to TM6. Identical residues with respect to rNLR are indicated by dots, negatively charged amino acids are marked by θ.

ing for the first 270 amino acids of mNLR, the mouse homologue of rNLR. Fig. 3 shows a high overall sequence identity between rNLR and mNLR (92%), whereas BLR1 revealed a relatively low homology of about 40% in the N-terminal extracellular region compared to both NLR species.

The distribution of mNLR and rNLR mRNA was examined by Northern blot analysis and PCR (Fig. 4). A predominant mRNA of about 5.5 kb along with minor transcripts of 3.8 and 3 kb were observed in poly(A) RNA from NG108-15 neuroblastoma × glioma hybrid cells and rat pituitary GH3 cells, in various mouse brain regions such as the cerebellum and cortex, as well as in the liver. In contrast, only a 3 kb mRNA species is found in the mouse spleen total RNA and in human Raji B cells (Fig. 4A). No signal could be detected in mouse thymus. This 3 kb transcript in B cells and lymphatic tissue is in line with the size of mRNA measured for BLR1 [7]

The expression of rNLR mRNA in various rat tissues determined by PCR confirms the expression pattern of mNLR mRNA (Fig. 4B). In addition to cerebellum and

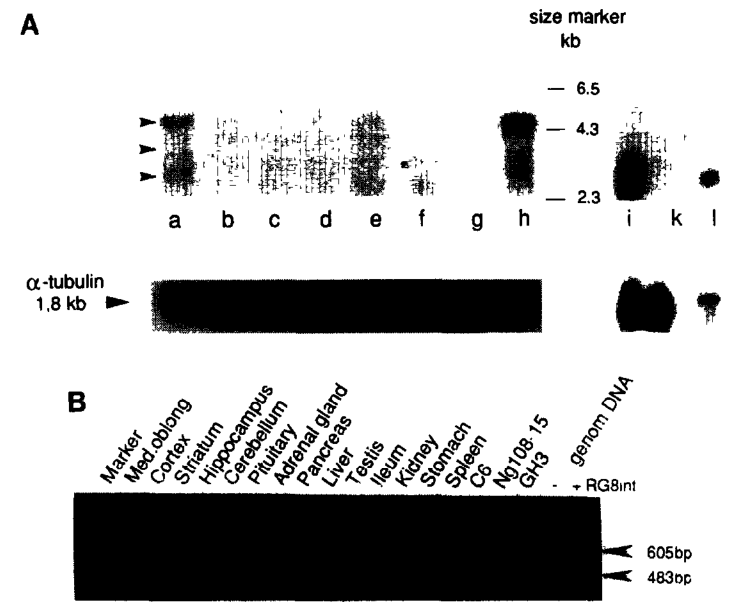

Fig. 4. (A) Northern blot analysis of mNLR receptor mRNA in several mouse tissues as well as NG108-15 and rat pituitary GH3 cell lines. 3.5 μg aliquots of poly(A) RNA, except 30 μg total mouse spleen and thymus RNA, were subjected to electrophoresis, transferred onto nylon membranes and hybridized with a ^{32}P-labelled N18-10 cRNA probe. mRNA bands are indicated by arrows. Equal loading and quality of RNA was shown by control hybridization with a ^{32}P-labelled mouse α-tubulin cRNA as shown on the bottom. *Hin*dIII-digested fragments of phage λ were used as size markers. Lanes: a, NG108-15 cells; b, liver; c, striatum; d, midbrain; e, cerebellum; f, cortex; g, ATt20 cells; h, GH3 cells; i, spleen; k, thymus; l, human Raji B cells. (B) PCR analysis of rNLR receptor transcripts in rat tissues. Total RNA was isolated, digested with RQ1 RNase-free DNase I (Promega) and converted to single-stranded cDNA by specific priming of 4 μg total RNA with primer SPR-3 (Fig. 2B) and Superscript reverse transcriptase (BRL). cDNAs were amlipified [10] using *Taq* polymerase (Pharmacia) and up- and downstream primers PCR-EX1 and PCR-M3 as indicated in Fig. 2B for 35 cycles (96°C for 1 min, 54°C for 1 min, and 72°C for 1 min), which results in a single cDNA species of the expected size (483 bp). Primers flanking the intron region were designed for amplifying cDNA sequences only. No amplification was observed using rat genomic DNA as a template in contrast to PCR with an intron-specific primer (RG8int: 5′ CATAGAATAGTGCAAACAAA 3′; for approximate position see Fig. 2A), yielding a 605 bp fragment. PCR products were run on 2% agarose gels and stained with ethidium bromide. Tissues and cells used are indicated above. *Hae*III-digested fragments of Φx174 phage was used as size markers (in bp: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118).

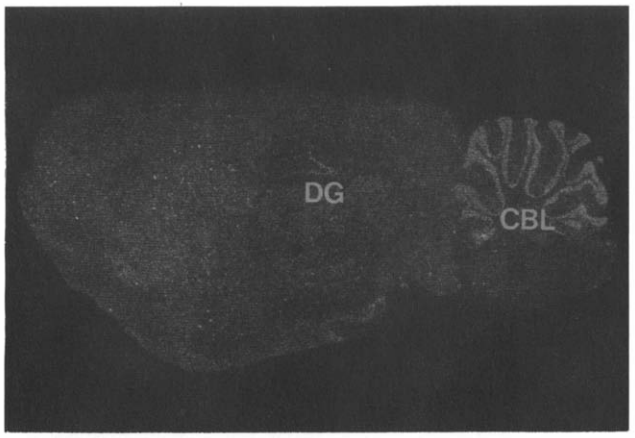

Fig. 5. Localization of NLR receptor mRNA by in situ hybridization. A sagittal section of rat brain was hybridized with an antisense RC8 cRNA probe, labelled with [^{35}S]UTP. The slices were exposed for 4 days at −70°C with an intensifying screen. Areas with specific mRNA expression are indicated. CBL, cerebellum; DG, dentate gyrus. Hybridization with the sense probe shows no specific signals (data not shown).

cortex rNLR is also present in the striatum, the pituitary and adrenal gland. Since rat C6 glioma cells do not express the NLR receptor, the mRNA present in NG108-15 mouse neuroblastoma × rat glioma hybrid cells appears to derive exclusively from the mouse neuroblastoma parent cell line, N18TG2.

The existence of NLR mRNA in the brain is also demonstrated by in situ hybridization experiments (Fig. 5). In the sagittal section of a rat brain, hybridization signals with an antisense probe were observed predominantly over cell layers of the cerebellum. In addition, a lower expression level is found over the granule cells of the dentate gyrus of the hippocampus. This localization of NLR mRNA is in good accordance with the observation that neuronal cells, but not glia cells, express the receptor in NG108-15 cells.

Taken together, rNLR and its mouse analogue represent putative G-protein-coupled receptors which are expressed in neuronal and lymphatic tissue. They share a high homology to a recently cloned receptor from Burkitt's lymphoma cells (BLR1) [7] and hybridize to the same 3 kb mRNA species in spleen. In other tissues, however, a 5.5 kb NLR transcript is predominant. The nature of the 5.5 kb mRNA is presently not known. The NLR gene, like the BLR1 gene, contains an intervening sequence between the N-terminus and the first transmembrane domain. In the BLR1 gene this intron comprises 9 kb, and a similar intron size has been found for the mouse analogue of the gene (M. Lipp, personal communication). The possibility of alternatively spliced mRNA species within this intron can be excluded, since a single amplification product was obtained by PCR with intron-flanking primers and cDNAs from rat tissues and NG108-15 cells. In addition, the N-terminus of rNLR which has been cloned from rat spleen, is almost identical to the N-terminus of its mouse analogue, which has been cloned from mouse neuroblastoma cells (N18TG2), indicating that spleen and neuronal cells may express a NLR mRNA with similar 5′ coding regions. We argue that the 5.5 kb mRNA may represent a 5′-extended species, which derives from different transcription initiation sites of the gene.

Although rNLR and its mouse analogue share a high overall sequence homology to the human BLR1, the homology at the N-terminus is much less pronounced (Fig. 3). A similar situation is observed in the case of the high- and low-affinity IL-8 receptors which exhibit a high homology except in the N-terminal region. Therefore, we suggest that this N-terminal divergence between rat/mouse NLR vs. human BLR1 may not reflect species differences. Instead NLR and BLR1 may represent homologous receptors that differ in their affinity for a common ligand or have structurally related ligands. This hypothesis is also supported by the observation that BLR1 has 11 negative amino acid residues in the extracellular N-terminus, whereas both rNLR and mNLR have 7 negatively charged amino acids (Fig. 3), thought to play an important role for the IL-8 receptor in binding of the basic ligand [3].

In order to determine the ligand for NLR we stably expressed the clone, RC8, under the control of the CMV promotor in CHO-K1 cells. Two independent clonal cell lines expressing the highest level of RC8 mRNA (data not shown) were tested for binding with the following ligands: [^{3}H]CCK-8 (cholecystokinin-8), [^{3}H]DADLE (D-Ala-D-Leu-enkephalin), [^{3}H]angiotensin II, [^{3}H]oxytocin and [^{125}I]11-Tyr-somatostatin. None of these ligands showed binding to either NLR-expressing clonal CHO lines.

The homology of the putative G-protein-coupled receptor NLR to both the cytokine and neuropeptide family of receptors raises the question of its biological importance in neuroimmune connections. Several neuropeptides, such as neuropeptide Y, calcitonin gene-related peptide, galanin and opiods, besides their wide distribution throughout the central and peripheral nervous system, have been demonstrated to be localized in nerve endings innervating lymphoid organs [23]. Interestingly, neuropeptide Y-positive nerve terminals were found in close apposition with lymphoid compartments of the rat spleen [24]. Thus, the precise localization of NLR expression in the lymphoid compartments of the spleen, as well as the identification of its ligand, may contribute to our understanding of neuroimmunomodulation.

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