A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue

Ane M. Kvingedal*, Erlend B. Smeland

Department of Immunology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway

Received 14 February 1997

Abstract cDNA encoding a novel putative G-protein-coupled receptor, named LyGPR (lymphocyte derived G-protein-coupled receptor) was cloned using a reverse transcription-PCR approach. The LyGPR amino acid sequence is 375 residues long and shows similarity (about 30–35% identity) both to the angiotensin receptors and members of the chemokine receptor family. Northern blot analysis revealed a 3.1-kb LyGPR transcript expressed predominantly in lung, heart and lymphoid tissues. LyGPR expression was also detected in the pre-B acute lymphoblastoid leukemia cell lines Reh and Nalm-6, in the Burkitt's lymphoma line Daudi, and in hematopoietic progenitor cells from bone marrow, as well as in B cells, T cells and monocytes from peripheral blood.

Key words: G-protein-coupled receptor; Lymphocyte; Hematopoietic progenitor cell

1. Introduction

G-protein-coupled receptors (GPRs) are integral membrane proteins responding to a wide range of extracellular signals including neurotransmitters, hormones, chemokines and regulatory peptides [1]. Stimulation of these receptors mediates activation of GTP-binding proteins (G-proteins), which regulate various cellular effectors, like specific enzymes and ion channels [2]. Molecular cloning of GPR genes has revealed a superfamily of structurally related proteins containing an extracellular N-terminus and seven hydrophobic membrane-spanning α-helices [3]. Novel members of this expanding family are frequently isolated by use of PCR strategies or homology cloning.

The identification of receptors for IL-8 and some of the chemoattractants has led to an increased understanding of the role of GPRs in the biology of granulocytes and monocytes. In lymphocytes, however, GPRs have so far gained less attention. Lymphocytes are known to express, often at low levels, receptors for various neuropeptides and opioid peptides playing roles in immune modulation (neuroimmunomodulation). They also express several chemokines and chemokine receptors involved in lymphocyte adhesion and migration [4]. Recent findings have demonstrated important roles for chemokines in the regulation of growth and differentiation of lymphocytes [5,6], and this will hopefully accelerate the identification of ligands to some of the novel chemokine orphan receptors showing predominant expression in lymphoid tissue.

In this paper we report the isolation of a cDNA sequence encoding LyGPR, a novel putative GPR-coupled receptor expressed at highest levels in lung, heart and lymphocyte-enriched tissues.

2. Materials and methods

2.1. Isolation of LyGPR cDNA

Degenerate oligonucleotide primers corresponding to conserved amino acid motifs NLA(V/L)AD in the second and (C/F)W(S/L)PY in the sixth transmembrane (TM) segment of G-protein-coupled receptors were constructed and used in PCR to amplify cDNA from the human B-cell line Daudi. The primer sequences, which include flanking restriction enzyme sites, were: 5′-GGAATTCAA(C/T)CT(C/G)GC(C/G)(C/G)T(C/G)GC(A/C/G/T)GA(C/T) (forward) and 5′-GCTCTAGAT(C/G)(A/G)TA(A/C/G/T)GG(C/G)(A/G)(A/G)CCAG-(A/C)A (reverse). cDNA was made as follows: Poly(A)$^+$ RNA was isolated from about 1×10^6 Daudi cells using 300 μg Dynabeads oligo(dT)$_{25}$ (Dynal, Norway) essentially as described by the manufacturer. However, instead of being eluted, the purified mRNA was kept annealed to the beads and was washed twice in RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$). A first-strand cDNA synthesis reaction (30 μl) was then performed directly in RT buffer containing 10 mM DTT, 0.5 mM dNTP, 15 U RNasin (Promega) and 200 U Superscript II (BRL) for 1 h at 42°C, 10 min at 46°C and 10 min at 50°C, keeping the beads in suspension. After removal of the RT mix, the beads were washed once in TE (pH 8.0) [7] and incubated for 5 min with 0.2 M NaOH, then washed once in SSPE [7] containing 0.1% Tween 20, and finally stored in 100 μl TE at 4°C. 1 μl was used as template in a 25 μl PCR reaction containing Taq polymerase and buffer from Boehringer Mannheim, 0.2 mM dNTP, and 10 pmol of each primer. Thirty-five cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) of PCR were carried out. PCR products were cloned in pUC19 [7] and sequenced using Sequenase (Amersham) and the manufacturer's protocols. A 550-bp fragment showing similarity to GPR sequences was ^{32}P-labelled by random priming and used as probe in library screening: about 5×10^5 pfu of a λgt11 cDNA library prepared from human fetal liver (Clontech) was screened using the manufacturer's protocols.

2.2. Northern blot analysis

Cell lines used were: the pro-B cell line Tom 1 [8]; the pre-B cell lines Reh (CRL 8286; ATCC) and Nalm-6 (ACC 128; DSM); the Burkitt's lymphoma cell line Daudi (CCL 213; ATCC); the T cell line Jurkat (TIB 152; ATCC), the myeloblastoid cell line KG-1a (CCL 246.1; ATCC) and the early erythroid cell line K562 (CCL 243; ATCC). The cells were grown in RPMI 1640 with 10% fetal bovine serum at 37°C. Poly(A)$^+$ RNA was isolated from about 15×10^6 cultured cells using oligo(dT)$_{25}$ magnetic beads (Dynal, Norway). Following electrophoresis in a 1.2% agarose/formaldehyde gel, the RNA was blotted onto a Hybond-N membrane (Amersham) and subjected to hybridization with ^{32}P-labelled DNA [7].

2.3. RT-PCR expression analysis

Normal lymphocytes, granulocytes and monocytes were all isolated from buffy coats of normal donors. B cells were isolated by use of Dynabeads-M450 CD19 (Dynal, Norway) as previously described

*Corresponding author. Fax: (47) 22500730.
E-mail: a.m.kvingedal@labmed.uio.no

Abbreviations: PCR, polymerase chain reaction; BLR1, Burkitt's lymphoma receptor 1; IL-8, interleukin 8; TM, transmembrane; ATCC, American Type Culture Collection; DSM, German Collection of Microorganisms and Cell Cultures

PII S0014-5793(97)00278-0

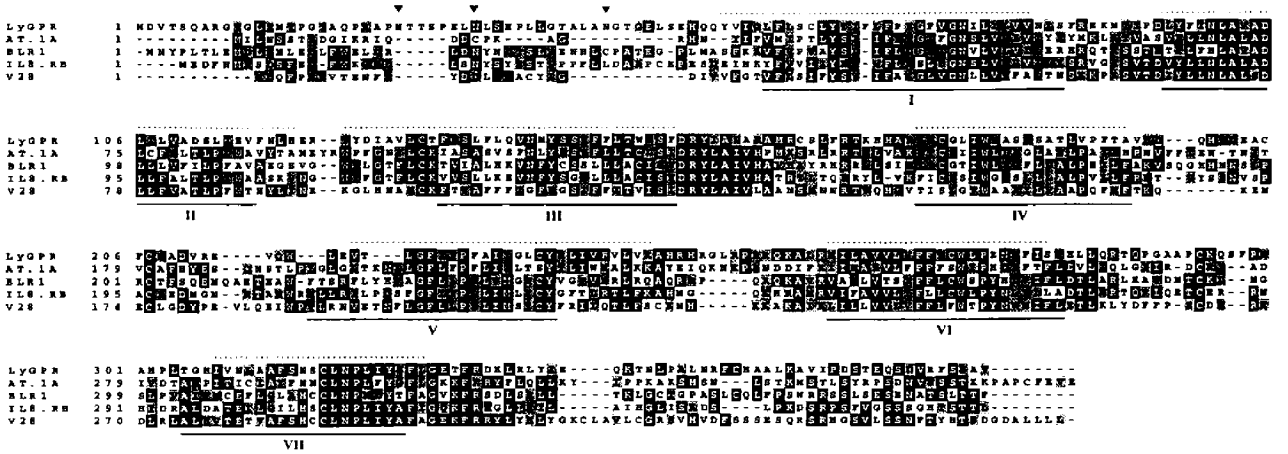

Fig. 1. Sequence alignment of LyGPR with related G-protein-coupled receptors. Multiple amino acid sequence alignment (ClustalW 1.6) [14] of LyGPR with the angiotensin II receptor 1A, AT.1A (SwissProt P30556); the orphan chemokine receptor BLR1 (P32302), the low-affinity IL-8 receptor IL8.RB (P25025) and the orphan chemokine receptor V28 (P49238). Amino acids identical or similar in at least two of the sequences are indicated by black or shaded areas, respectively. Potential sites for *N*-linked glycosylation in LyGPR are marked by inverted triangles. Putative transmembrane regions in LyGPR predicted by the program TMpred (ISREC) are indicated by dotted lines above the sequences. The solid lines below the sequences show the putative transmembrane regions in IL8.RB (information obtained from SwissProt). The cDNA sequence of LyGPR is deposited in the EMBL Nucleotide Sequence Database and has the accession number X98510.

[9,10], granulocytes and monocytes by use of Dynabeads-M450 CD15 and CD14, respectively, following the same procedure as for B cells, except that beads were removed after cell lysis. T cells were isolated by depletion of HLA class II expressing cells using Dynabeads-M450 pan human HLA class II.

Hematopoietic progenitor cells were isolated from human bone marrow obtained by iliac crest aspiration from adult volunteers. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Nycomed, Norway), and $CD34^+$ cells and $CD19^+$ cells were obtained from these by positive selection using immunomagnetic beads [11]. Subpopulations of $CD34^+$ cells and $CD19^+$ cells were isolated by flow cytometric cell sorting (FACStar Plus cell sorter; Becton Dickinson). $CD34^+CD38^-$ cells were obtained after costaining with phycoerythrin (PE)-conjugated anti-CD34 (HPCA-2, Becton Dickinson, San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD38 (IOB6, Immunotech, Marseilles, France). Mature bone marrow B cells ($CD19^+IgM^+$) and pro- and pre-B cells ($CD19^+IgM^-$) were obtained after staining with PE anti-CD19 (HD37; DAKO, Denmark) and FITC anti-IgM (DAKO, Denmark).

Poly(A)$^+$ RNA was isolated from about 3×10^5 cells using 100 μg oligo $(dT)_{25}$ beads. The RNA was eluted in 10 μl H_2O. First strand cDNA was made from 5 μl in a 15 μl RT reaction using Superscript II (BRL) and similar conditions as described previously, except that now the cDNA was in solution. A mock reaction without transcriptase was performed on the rest of the mRNA and used as control in subsequent PCR experiments to check for contaminating genomic DNA.

For detection of LyGPR transcripts 1 μl of the cDNA was amplified in a 25 μl PCR (40 cycles: 94°C for 1 min, 58°C for 1 min, 72°C for 1 min) with primers LyGPR.forw. (5′- GCACGAGCGGTACTACGAC) and LyGPR.rev. (5′- GACATCCGCGAAACAGAAG). These primers specify a 277-bp fragment located in the protein-coding part (between TM II and TM V) of the transcript.

Primers for β-actin were: 5′-GTGAATTCCTTCTACAATGAGCTGCGTG (forward) and 5′-CGTCTAGAGCCATCTCTTGCTCGAAGTC (reverse) (located at nt 307 and nt 705, respectively, in sequence X00351 [12]. PCR was performed as for LyGPR, except that only 35 cycles were run, and the amount of template was reduced to 0.1 μl.

3. Results and discussion

A PCR approach based on degenerate primers corresponding to TM II and TM VI sequences common to GPRs was used in order to identify novel GPR genes expressed in B cells. Amplification of Daudi cDNA, as described, resulted in products appearing as one major band of about 550 bp when analyzed by agarose gel electrophoresis. After cloning and sequencing, one of the cDNAs showing characteristics of GPR sequences was chosen for further characterization. To obtain full-length cDNA, a λgt11 cDNA library from human fetal liver was screened using the 550-bp fragment as probe. A positive clone containing a 2.4-kb insert was sequenced. This cDNA has a 1125-bp open reading frame flanked by 770-bp 5′- and 510-bp 3′-sequences. The deduced amino acid sequence is 375 residues long and specifies a putative GPR, designated LyGPR (lymphocyte derived G-protein-coupled receptor. It has three potential *N*-glycosylation sites (Asn-25, -32 and -44) located in the N-terminal end, and is predicted by hydrophobicity analysis [13] and the computer program TMpred (ISREC) to contain seven transmembrane segments (Fig. 1).

The sequence shows some similarity to both angiotensin and chemokine receptor sequences (Fig. 1). It is 32% and 35% identical to the angiotensin II receptor AT1A [15] and IL-8 receptor B [16], and 33 and 35% identical to the orphan chemokine receptors BLR1 [17] and V28 [18], respectively. The sequence motif DRY, located at the end of TM III, is conserved in many GPRs and has been shown to be important for G-protein-mediated signalling [19]. In chemokine receptors an extended motif, DRYLAIV, is particularly conserved. LyGPR lacks this motif, as well as a conserved cysteine residue, which is located close to TM I in the N-terminal end of most chemokine receptors. Thus, LyGPR seems to be only distantly related to the typical chemokine receptors, and it is presently unclear whether its putative ligand is a chemokine or perhaps a neuropeptide. A recent report describes the sequence of a protein which is identical to LyGPR, and is encoded by a gene localized to chromosome 7p22 [20]. When a panel of chemokines was tested on transfected cells expressing this protein, no response could be detected [20].

Northern blot analysis of LyGPR expression in various human tissues (Fig. 2A,B) reveals a single, 3.1-kb transcript, which is most abundant in lung, heart and lymphoid tissues.

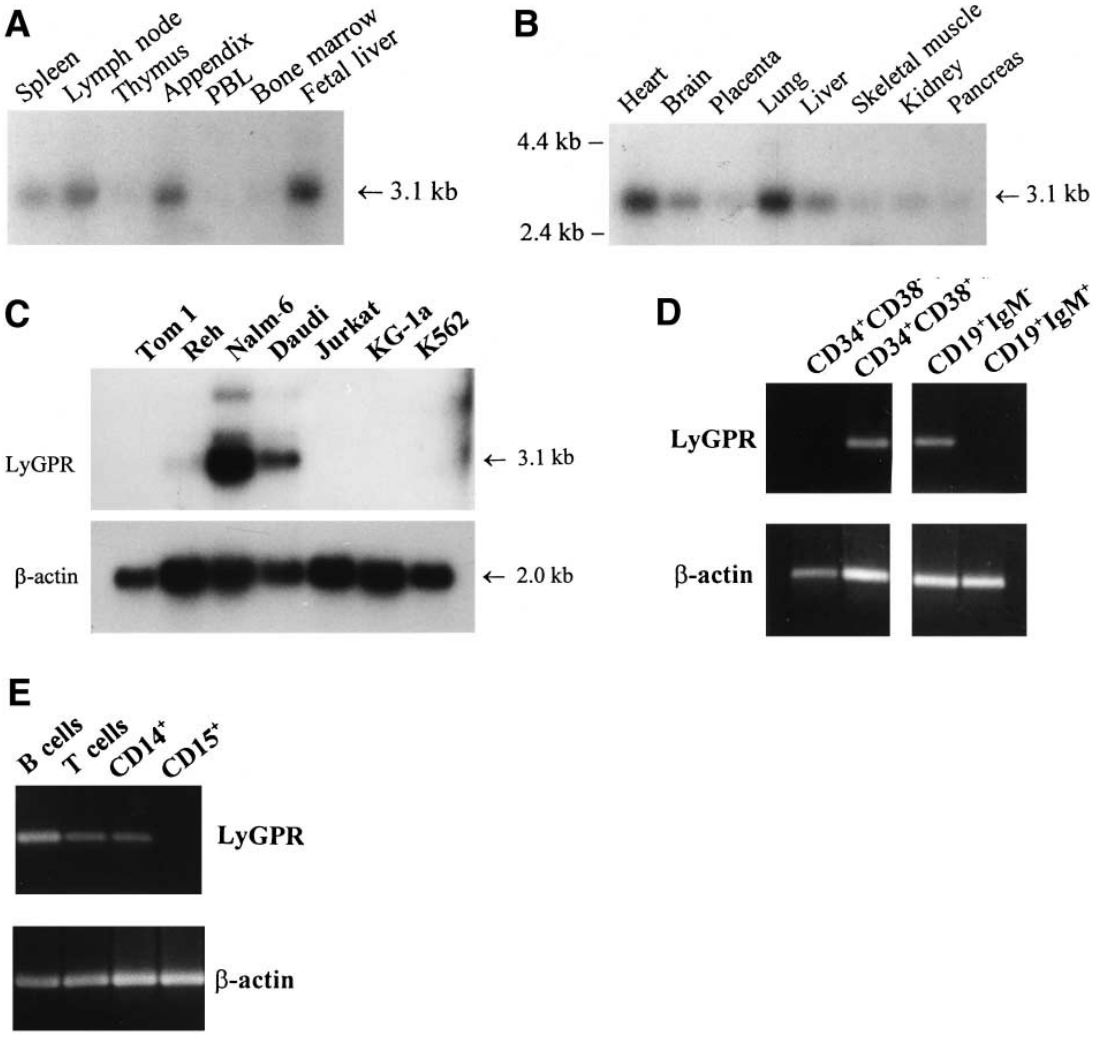

Fig. 2. Expression of LyGPR in human tissues and cells. Tissue distribution of LyGPR mRNA (A) in tissues important in the immune system and (B) in various tissues. Northern blots containing 2 μg poly(A)$^+$ RNA per lane were obtained from Clontech (Palo Alto, CA, USA) and hybridized using a labelled 550-bp LyGPR cDNA fragment (PBL: peripheral blood leukocytes). C: LyGPR expression in hematopoietic cell lines. The Northern blot contains approximately 3 μg poly(A)$^+$ RNA per lane. RT-PCR analysis of LyGPR expression in purified hematopoietic cells from (D) bone marrow and (E) peripheral blood. Amplification of β-actin was performed with fewer cycles and less template (1/10) than the amplification of LyGPR. The DNA was stained with SYBR Green I (Molecular Probes, Inc.).

Within the immune tissues tested, LyGPR mRNA is readily detected in spleen, lymph node and appendix (adult tissue), and a relatively high level of LyGPR expression in seen in fetal liver (Fig. 2A). When mRNA from cultured cells representing various hematopoietic linages was analyzed, LyGPR expression was found in the pre-B cell lines Reh and Nalm-6 and in the mature B cell line Daudi (Fig. 2C). In Nalm-6 and Daudi minor transcripts of 3.8 and ~8 kb are detectable in addition to the predominant 3.1-kb transcript. No LyGPR mRNA was detected in the pro-B cell line Tom 1, the early T cell line Jurkat, the myeloblast cell line KG-1a or in the early erythroid cell line K562 (Fig. 2C). Except from a 0.7-kb difference in estimated transcript size, these expression data are largely in line with what is reported by Owman et al. [20], who in addition demonstrated ubiquitous expression within the central nervous system.

The expression pattern of LyGPR in purified cell populations from bone marrow (Fig. 2D) and peripheral blood (Fig. 2E) was examined using RT-PCR. With this sensitive technique LyGPR mRNA could not be detected in the most immature hematopoietic cells (CD34$^+$CD38$^-$), but was found in CD34$^+$CD38$^+$ cells. This population contains most hematopoietic precursor cells capable of in vitro colony formation, including cells with multilineage potential. Among bone marrow B lineage cells LyGPR mRNA was found in CD19$^+$IgM$^-$ cells, the population containing pro- and pre-B cells. In the experiment presented in Fig. 2E, no LyGPR transcripts are detectable in mature, IgM$^+$ B cells from bone marrow. This result is, however, not consistent, as LyGPR expression in CD19$^+$IgM$^+$ cells has sometimes been observed. In peripheral blood B cells LyGPR mRNA is readily detected by RT-PCR analysis (Fig. 2E). We were, like Owman et al. [20], unable to detect LyGPR transcripts in purified peripheral blood B cells by Northern blot analysis (data not shown), indicating a relatively weak expression of LyGPR in resting B lymphocytes. Circulating T cells and CD14$^+$ monocytes also show expression of LyGPR, albeit at a lower level than B cells, whereas no LyGPR mRNA was detected in CD15$^+$ cells (neutrophils and eosinophils) (Fig. 2E). In hematopoietic cells the pattern of LyGPR expression shows some similarity

to that of BLR1, an orphan receptor most abundantly expressed in B cells [17], but also present in peripheral T cells [21] and monocytes [22]. BLR1 is quite closely related to the chemokine receptors and is suggested to function in the regulation of lymphocyte traffic and in neuroimmune communication. In peripheral blood BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells [21].

LyGPR is expressed at various stages of B cell differentiation, including pro- and pre-B cells. The high level of LyGPR expression in the pre-B cell line Nalm-6 is intriguing. It is not due to gene amplification, indicated by Southern blot analysis of Nalm-6 genomic DNA (data not shown). Whether the variable expression of LyGPR in B cell lines and various lymphoid tissues reflects differences in activation and/or differentiation stages during B cell development, remains to be clarified. However, we are currently in the process of generating LyGPR-specific monoclonal antibodies, which will be useful for a more precise identification and phenotypic characterization of LyGPR expressing cells, and for the elucidation of the role of LyGPR in B cell development.

Acknowledgements: We thank Inger-Beate Foelstad for skilful technical assistance in the molecular biology work, and June Myklebust and Nina Kristin Maasvaer for purification of bone marrow and blood cell populations. This work was supported by The Norwegian Cancer Society.

References

[1] H.G. Dohlman, J. Thorner, M.G. Caron, R.J. Lefkowitz, Annu Rev Biochem 60 (1991) 653–688.
[2] E.J. Neer, Cell 80 (1995) 249–257.
[3] W.C. Probst, L.A. Snyder, D.I. Schuster, J. Brosius, S.C. Sealfon, DNA Cell Biol 11 (1992) 1–20.
[4] J.A. Hedric, A. Zlotnic, Curr Opin Immunol 8 (1996) 343–347.
[5] T. Nagasawa, S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, T. Kishimoto, Nature 382 (1996) 635–638.
[6] K.B. Bacon, B.A. Premack, P. Gardner, T.J. Schall, Science 269 (1995) 1727–1730.
[7] Sambrook J, Fritch EF, Maniatis T. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
[8] M. Okabe, S. Matsushima, M. Morioka, M. Kobayashi, S. Abe, K. Sakurada, M. Kakinuma, T. Miyazaki, Blood 69 (1987) 990–998.
[9] S. Funderud, B. Erikstein, H.-C. Aasheim, K. Nustad, H.K. Blomhoff, H. Holte, E.B. Smeland, Eur J Immunol 20 (1990) 201–206.
[10] A.-M. Rasmussen, E.B. Smeland, B. Erikstein, L. Caignault, S. Funderud, J Immunol Methods 146 (1992) 195–202.
[11] L.S. Rusten, S.E.W. Jacobsen, O. Kaalhus, O.P. Veiby, S. Funderud, E.B. Smeland, Blood 84 (1994) 1473–1481.
[12] P. Ponte, S.Y. Ng, J. Engel, P. Gunning, L. Kedes, Nucleic Acids Res 12 (1984) 1687–1696.
[13] J. Kyte, R.F. Doolittle, J Mol Biol 157 (1982) 105–132.
[14] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res 22 (1994) 4673–4680.
[15] C.A. Mauzy, O. Hwang, A.M. Egloff, L.-H. Wu, F.-Z. Chung, Biochem Biophys Res Commun 186 (1992) 277–284.
[16] P.M. Murphy, H.L. Tiffany, Science 253 (1991) 1280–1283.
[17] T. Dobner, I. Wolf, T. Emrich, M. Lipp, Eur J Immunol 22 (1992) 2795–2799.
[18] C.J. Raport, V.L. Schweickart, R.L. Eddy, T.B. Shows, P.W. Gray, Gene 163 (1995) 295–299.
[19] C.M. Fraser, F.-Z. Chung, C.-D. Wang, J.C. Venter, Proc Natl Acad Sci USA 85 (1988) 5478–5482.
[20] C. Owman, P. Blay, C. Nilsson, S.J. Lolait, Biochem Biophys Res Commun 228 (1996) 285–292.
[21] R. Föerster, T. Emrich, E. Kremmer, M. Lipp, Blood 84 (1994) 830–840.
[22] L. Barella, M. Loetscher, A. Tobler, M. Baggiolini, B. Moser, Biochem J 309 (1995) 773–779.