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Topological characterization of the lymphoid-specific seven transmembrane receptor BLR1 by epitope-tagging and high level expression

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We have tagged the human lymphocyte-specific G-protein-coupled receptor BLR1 either with an amino-terminal or a carboxyl-terminal epitope-tag recognized by an anti-MYC monoclonal antibody. Flow cytometry was used to determine the efficiency of transient transfections and to establish human embryonic kidney 293 cell clones showing stable high level expression of BLR1. Analysis of permeabilized versus non-permeabilized transfected 293 cells demonstrated that BLR1 is an integral plasma membrane protein, topologically oriented therein as predicted for other members of this class of seven pass membrane receptors. In addition, BLR1 was expressed in 293 cells to high levels as a glycosylated membrane protein. The easily detectable and assayable expression of tagged G-protein-coupled receptors, as exemplified for BLR1 in 293 cells, provides a suitable system for further functional studies and offers an efficient screening tool for the identification of receptor-specific antibodies, ligands, or receptor-associated proteins.

The Burkitt's lymphoma receptor 1 (BLR1) originally identified in Burkitt's lymphoma cell lines by subtractive hybridization has all structural features common to the superfamily of G-protein-coupled receptors, including seven hydrophobic putative membrane spanning domains [1]. BLR1 shares striking homologies to chemokine receptors, including IL-8R [2,3] and the MIP-1α receptor [4] and to the neuropeptide Y receptor [5]. It is the first member of this receptor family expressed in lymphocytes and

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Abbreviations: BLR1, Burkitt's lymphoma receptor 1; IL-8R, Interleukin 8-receptor; MIP-1α, macrophage inflammatory protein 1α; AA, amino acids; BLR1-MYC$_C$, carboxyl-terminally tagged BLR1; BLR1-MYC$_N$, amino-terminally tagged BLR1.

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shows a differentiation-specific expression pattern; it is expressed in mature B cells but not in pre B-cells or plasma cells [1, 6].

In addition to the 3.0 kb mRNA detectable in lymphatic cells recent studies with the murine and rat homologues of human BLR1 revealed a further mRNA species of 5.5 kb in certain neuronal cell lines and in various brain areas [6, 7].

Heterologous expression of G-protein-coupled receptors in different eukaryotic cells has been used for functional studies [8-10]. In contrast to cell lines naturally expressing the protein of interest this approach yields positiv overexpressing transfectants and offers the advantage that non-transfected cells can be used as suitable negative controls. This is of particular importance for the analysis of unknown properties as it allows subtractive screening procedures for the identification of unknown ligands, receptor-specific antibodies, or proteins involved in receptor-mediated signal transduction.

Here we report the heterologous expression of human BLR1 in human embryonic kidney 293 cells. As the ligand for BLR1 is still unknown and antibodies against the receptor are not available we tagged it with a peptide derived from the human c-MYC protein to which a monoclonal antibody is available. Using amino-terminally and carboxyl-terminally tagged BLR1 constructs we demonstrate that BLR1 is a highly glycosylated plasma membrane protein integrated in the predicted orientation. Compared to other cell lines tested 293 cells were transfected very efficiently and showed also high levels of BLR1 expression. These cells not only offer a suitable system to further investigate BLR1 but may also serve as a general strategy for characterizing other G-protein-coupled receptors.

Materials and Methods

Construction of epitope-tagged BLR1. The cDNA encoding BLR1 was inserted into pGEM-4Z (Promega). Using the polymerase chain reaction the natural translation stop codon was deleted and the cDNA extended by a sequence coding for the amino acids PGG. To generate the carboxyl-terminally tagged BLR1 receptor (BLR1-MYC_C) a double-stranded oligonucleotide was inserted at the 3'-end of this mutated receptor cDNA. The oligonucleotide encodes a spacer of 3 amino acids (sequence: SGP), the 11-AA c-MYC-derived epitope (sequence: EQKLISEEDLL; Lit) and the translation stop codon. The amino-terminally tagged BLR1-protein (BLR1-MYC_N) was constructed by inserting an AA spacer (sequence: MAP) and the MYC-epitope sequence (sequence: EQKLISEEDLPG) in front of the second amino acid of BLR1 by a synthetic double-stranded oligonucleotide. The cDNA sequences of the mutant receptors were verified by dideoxynucleotide chain-termination sequencing of denaturated double-stranded plasmid templates with the T7 Sequencing kit (Pharmacia).

***In vitro* translation.** The cDNAs for both BLR1 and tagged BLR1 were subcloned into pGem-4Z (Promega). RNA was transcribed *in vitro* using SP6 DNA dependent RNA polymerase with linearized plasmids as template. Translations were performed with nuclease-treated lysates at 30°C for 60 min according to the manifacturer's instructions (Promega). Methionine-free amino acid mix (Promega) was used for labelling proteins during translation with [^{35}S]methionine (>1000 Ci/mmol; Amersham).

Cell culture. Human embryonic kidney 293 cells (ATCC CRL 1573) were cultured in Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 60 U/ml penicillin and 60 U/ml streptomycin. For glycosylation inhibition experiments cells were grown in methionine-deficient DMEM for 1 hour. Medium was then supplemented with 30 μCi/ml [^{35}S]methionine and 10 μg/ml tunicamycin (Sigma) and cells were harvested 4 hours later.

Expression of BLR1 in 293 cells. The cDNAs for both BLR1 and tagged BLR1 were subcloned into the mammalian expression vector Rc/CMV (Invitrogen). 293 cells were transfected with 15 μg plasmid DNA, using the $Ca_3(PO_4)_2$ precipitation method as previously described [11,12]. 40 h after transfection cells were examined for transient expression of BLR1. For selection of cells stably expressing BLR1 400 μg/ml G 418 (Gibco) were added to the culture medium after transfection and individual antibiotic-resistent clones were isolated and cloned 3 times by limiting dilution.

Immunodetection of BLR1. Crude membrane fractions were prepared from 293 cells by resuspending the cells in ice-cold TEM (25 mM Tris/HCl, 5 mM $MgCl_2$ 1 mM EDTA, pH 7.4). After 10 min on ice cells were dounced and the nuclei pelleted by centrifugation at 100g for 3 min. The supernatant was removed and centrifugated at 50000g at 4 °C for 20 min. Pelleted membranes were resuspended in sample buffer without prior heating of the probes and resolved by electrophoresis through 10% polyacrylamide/SDS gels [13]. Proteins were transferred electrophoretically to nitro-cellulose (BA85; Schleicher & Schuell) and immunoblotting was performed in TBST/5% non-fat dry milk powder using the tag-specific 9E10 mAb (Dianova) at 10 μg/ml and horseradish peroxidase-conjugated secondary antibody (Sigma) at 1:2000 dilution. Immunoreactive proteins were visualized by chemoluminescence according to the manufacturer's protocol (Amersham). Immunoprecipitations were carried out essentially as previously described [14] using the anti-MYC 9E10 mAb, crude cell membranes and PBS/1% Triton X-100 as washing buffer.

Immunocytochemistry was done according to standard techniques [15]. In brief 293 cells were grown on poly-L-lysine coated slides and after two washes slides were dried thorougly at 42°C for 1 hour. Cells were fixed in dry acetone (10 min, RT) and then sequentially rehydrated in grated ethanol. Slides were incubated first with the tag-specific 9E10 mAb (5μg/ml; 1h), then with a biotinylated goat-anti-mouse-conjugate (1:100; 45 min; Dianova) and finally with streptavidin-conjugated alkaline phosphatase (1:80; 45 min; Gibco). Reaction was visualized using Fast Red (Sigma) and photographs were taken with a kappa video camera (CF 15/2).

FACS analysis. For FACS analysis 2x10^6/ml. cells were washed once with cold staining buffer (PBS, 4% FCS, 5 mM EDTA, 0.1% NaN_3). Cells were stained at 4°C for 30 min with the 9E10 mAb at a final concentration of 500 ng/ml. For the detection of intracellular epitopes cells were fixed in paraformaldehyde (2.5% in PBS, 4°C, 15 min; Sigma) and permeabilized with digitonin (0.0025% in PBS, 4 min; Sigma) at RT prior to incubation with the antibody. Cells were washed in staining buffer and then incubated with a polyclonal rabbit-anti rat-FITC conjugate (1:160, 4°C; Dako). After two final washes cells were analysed on a FACScan flow cytometer (Becton Dickinson).

Results and Discussion

The blr1 gene isolated from BL64 cells encodes a receptor which shares features common to proteins of the superfamily of G-protein-coupled receptors [16,17], including an extracellular amino terminus, an intracellular carboxyterminal end and

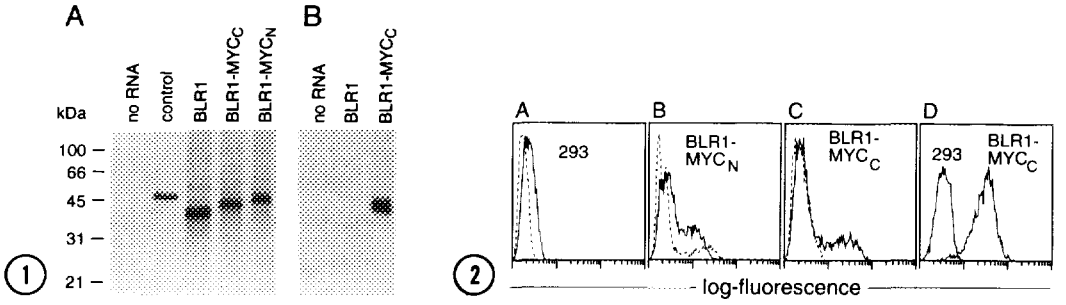

Figure 1. Expression of BLR1 and epitope-tagged BLR1 by rabbit reticulocytes.
A: *In vitro* transcribed mRNA's of untagged (BLR1), amino-terminally tagged (BLR1-MYC_N) and carboxyl-terminally tagged BLR1 (BLR1-MYC_C) were labeled with [^{35}S]-methionine during translation and resolved by SDS-PAGE as described in Materials and Methods.
B: Immunoblotting of *in vitro*-translated proteins using the tag-specific 9E10 mAb.

Figure 2. Expression of epitope-tagged BLR1 in 293 cells.
Human embryonic 293 cells were transiently transfected with amino-terminally tagged (BLR1-MYC_N; B) and carboxyl-terminally tagged BLR1 (BLR1-MYC_C; C) or vector alone (A) and analysed by flow cytometry as decribed in Materials and Methods. Dotted lines indicate staining of intact cells, whereas solid lines represent analysis of cells permeabilized by detergent. Specific expression of 293 cells stable transfected with BLR1-MYC_C compared to untransfected control 293 cells is shown in D.

seven hydrophobic putative membrane-spanning domains [1]. In order to specifically detect BLR1 *in vitro* and *in vivo* we fused a peptide to the C-terminal end of BLR1 coding for a flexible 6-AA spacer and the c-MYC derived epitope which is recognized by the 9E10 mAb [18]. It has been shown previously that the use of epitope tags does not alter the function of G-protein-coupled receptors [19-21]. Therefore, fusion of the epitope to the carboxyl terminus of BLR1, allowing the detection of full-length products, should not influence correct insertion into the cellular membrane and should allow functional studies of the expressed BLR1 protein.

In vitro expression of the unmodified and epitope-tagged BLR1-cDNAs by rabbit reticulocytes revealed 38 and 40 kDa proteins, respectively (Fig. 1A). Western blotting of the *in vitro*-translated receptor proteins using the tag-specific 9E10 mAb specifically detects only the MYC-epitope-tagged BLR1 protein but not the untagged receptor (Figure 1B). The observed molecular masses are in agreement with those predicted from their amino acid sequences and suggest the absence of posttranslational modifications of the expressed receptors, although there are two putative N-linked glycosylation sites in the first and third extracellular domains [1]. Interestingly the addition of dog pancreas microsomal membranes [22], which has been shown to be essential for the expression of glycosylated β-2 adrenergic receptor *in vitro* did not influence the efficiency of expression or the expression pattern ([23]; data not shown). To exclude the possibility of truncated BLR1 proteins being expressed from internal methionines as translation starts [24] we expressed *in vitro* a BLR1 protein modified

with the epitope-tag at the amino-terminus. As demonstrated in Figure 1A this protein too has an apparent molecular mass of about 41 kDa as observed for the carboxyl-terminal tagged protein, indicating the expression of unmodified full-length products by reticulocytes.

To analyse BLR1 *in vivo* we transiently expressed the epitope-tagged BLR1 protein under the control of a CMV promotor in different eukaryotic cell lines. Efficiency of transfection and levels of BLR1 expression were analysed by flow cytometry. Transfection efficiency never exceeded 1% in experiments using NIH 3T3, CHO or x63 Ag8.653 cells, which all do not express endogenous BLR1-specific mRNAs, or NG 108C15 cells, which express the murine homologue of BLR1. In contrast, human embryonic kidney 293 cells could be transfected with an efficiency of up to 50% expressing high levels of BLR1 as demonstrated in Figure 2. BLR1 expression of 293 cells transfected with BLR1-MYC_C was only observed after cells had been permeabilized by detergent prior to antibody staining (Fig. 2C), demonstrating that the carboxyl terminus of the receptor is located in the cytoplasm. In contrast 293 cells transiently transfected with amino-terminally tagged BLR1, allowed staining with the tag-specific antibody of both intact and permeabilized cells (Fig. 2B). This observation is consistent with the presence of receptors on the cell surface and the proposed topology of an extra-cellular amino terminus.

Using limiting dilution we established some 20 independent G 418-resistent BLR1-expressing 293 cell clones. These cell lines show different levels of BLR1 expression, as

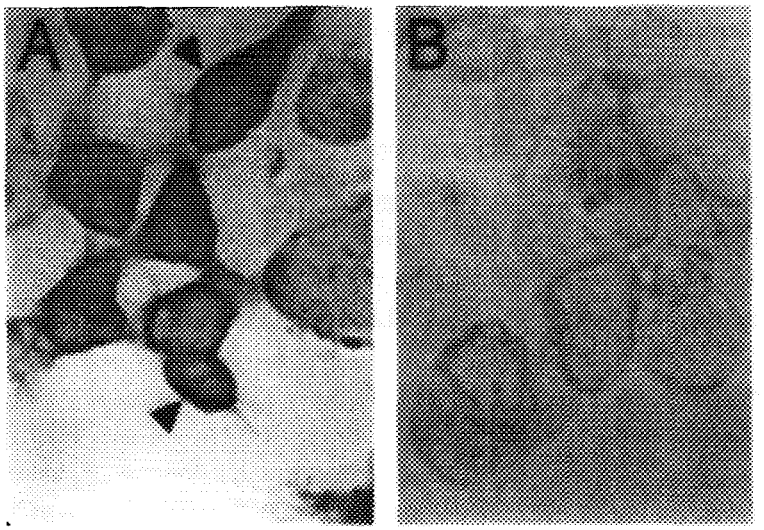

Figure 3. Immunocytochemistry of 293 cells stably transfected with BLR1.
293 cells stably transfected with BLR1-MYC_C (A) and untransfected 293 control cells (B) were stained with the tag-specific mAb as described in Materials and Methods. Only positive transfected 293 cells were specifically stained at the cellular membrane (arrowheads).

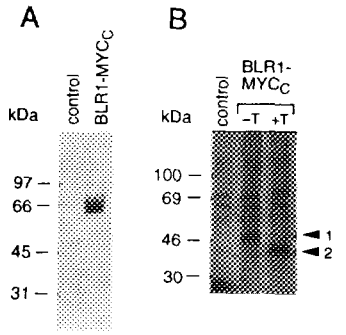

Figure 4. Detection of epitope-tagged BLR1 in cellular membranes.
A: Immunoblotting of crude membranes of 293 cells stably expressing carboxyl-terminally tagged BLR1 (BLR1-MYC_C) using the MYC-tag mAb 9E10.
B: Immunoprecipitation of metabolically labeled crude membranes of stably BLR1-MYC_C expressing cells without (-T; 1) and after (+T; 2) tunicamycin treatment.

easily tested by flow cytometry using the tag-specific mAb (data not shown). The same assay was also used frequently to control the reversion frequency and expression level of the established cell lines during long time culture. One of the 293 clones which expressed BLR1 very efficiently (Fig. 2D) was used for further characterization of the BLR1 protein. Immunocytochemistry of this stable BLR1-expressing cell line demonstrated that BLR1 is inserted into the cellular membrane (Fig. 3A). This observation conforms with the results obtained by flow cytometry using 293 cells transfected with BLR1-MYC_N, and both observations strongly supports the notion that BLR1 is a protein integrated into the membrane.

Interestingly, the molecular mass of BLR1 expressed in 293 cells differs from that of the protein expressed *in vitro*. As shown in Figure 4 A, a prominent band of about 60 kDa was observed in crude membrane preparations (Fig. 4A) indicating the possible presence of posttranslational modifications. The idea that the protein is modified posttranslationally is also supported by the remarkable width of the protein band in SDS-PAGE which is typical for glycosylated proteins. To analyse the nature of the differences of the molecular masses of BLR1, we cultured BLR1-transfected cells in the presence of tunicamycin, an antibiotic agent preventing glycosylation by the enzyme N-acetylglucosamine-1-phosphatetransferase and thereby blocking the formation of the first lipid linked intermediate GlcNac-P-P-dolichol [25]. As depicted in Figure 4 B a protein of about 55 kDa is immunoprecipitated by the tag-specific antibody from crude membranes of BLR1-expressing cells metabolically labeled by [^{35}S]-methionine. However, tunicamycin treatment of the cells resulted in a reduction of the apparent molecular mass from about 55 kDa to 40 kDa (Fig. 4B), which is also observed by the *in vitro* expressed unmodified receptor as shown before in Figure 1A. This finding indicates the presence of N-linked glycosylation at BLR1 expressed *in vivo* by 293 cells.

The data presented show that BLR1 is expressed in human 293 cells as a glycosylated protein integrated into the cellular membrane in an orientation characteristic for type III

membrane proteins. The heterologoes overexpression of tagged G-protein-coupled receptors in 293 cells may offer a unique method for the identification of ligands and receptor-related proteins and might provide a suitable system for the generation and identification of specific mAbs which are rarely reported for G-protein-coupled receptors.

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