Downstream Activation of a TATA-less Promoter by Oct-2, Bob1, and NF-κB Directs Expression of the Homing Receptor BLR1 to Mature B Cells*

(Received for publication, July 22, 1998)

Ingrid Wolf‡‡, Veniamin Pevzner‡‡, Edelgard Kaiser§, Günter Bernhardt‡, Estefania Claudio‡‡, Ulrich Siebenlist‡‡, Reinhold Förster‡, and Martin Lipp‡§ §§

From the ‡Department of Tumorogenetics and Immunogenetics, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, D-13122, Berlin-Buch, Germany, the §Institut für Biochemie, Ludwig Maximilians Universität München, Feodor-Lynen-Strasse 25, D-81377, Munich, Germany, and the §§Laboratory of Immunoregulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892

The chemokine receptor, BLR1, is a major regulator of the microenvironmental homing of B cells in lymphoid organs. In vitro studies identify three essential elements of the TATA-less blr1 core promoter that confer cell type- and differentiation-specific expression in the B cells of both humans and mice, a functional promoter region (−36 with respect to the transcription start site), a NF-κB motif (+44), and a noncanonical octamer motif (+157). The importance of these sites was confirmed by in vivo studies in gene-targeted mice deficient of either Oct-2, Bob1, or both NF-κB subunits p50 and p52. In all of these animals, the expression of BLR1 was reduced or absent. In mice deficient only of p52/NF-κB, BLR1 expression was unaffected. Thus our data demonstrate that BLR1 is a target gene for Oct-2, Bob1, and members of the NF-κB/Rel family and provides a link to the impaired B cell functions in mice deficient for these factors.

BLR1/CXCR5 is the receptor of the recently identified CXC chemokine BCA-1/BLC (1, 2). The cDNA of human BLR1 was originally isolated from a Burkitt’s lymphoma cell line (3), and its homologues were cloned from murine and rat tissues (4, 5). Within the hematopoietic system, the expression of BLR1 is restricted to mature resting B cells and a subset of T helper memory cells (6). Disruption of the blr1 gene in mice revealed an essential role of this receptor in directing lymphocyte migration (7). Blr1−/− mice lack inguinal lymph nodes and possess either none or a significantly reduced number of morphologically altered Peyer’s patches. The migration of B cells to Peyer’s patches and splenic follicles was severely impaired in these animals, resulting in significant morphological alterations of the primary lymphoid follicles and lack of functional germinal centers.

The regulatory mechanisms directing cell type- and differentiation-specific expression of genes to mature B cells have not been resolved. Candidate transcription factors are members of the NF-κB/Rel family that includes several factors; among these a heterodimer of p50 (NFκB1) and p65 (RelA) is one of the prevailing forms in mammalian cells. All members of the NF-κB family share a common amino-terminal Rel homology domain, which is required for dimerization as well as for DNA binding. Upon induction by various stimuli, NF-κB factors activate a wide array of genes, including those involved in the early immune response (for review see Ref. 8).

The octamer consensus sequence, 5′-ATGCAAAT-3′, was initially identified as an essential regulatory element in promoters and enhancers of the immunoglobulin genes but is also present in promoters of numerous ubiquitous genes (for review see Ref. 9). The octamer motif is recognized by the ubiquitously expressed transcription factor Oct-1 and by the B lymphocyte-restricted factor Oct-2. Both are highly homologous in their bipartite DNA-binding POU domain (for review see Ref. 10). As both Oct-1 and Oct-2 bind equally well to the octamer sequence, it remains unclear whether Oct-2 specifically directs the expression of octamer-dependent genes in B lymphocytes. Several observations suggest the existence of additional B cell-restricted cofactors essential for an octamer-dependent gene activation in B cells. Recently, a novel B cell-specific coactivator of octamer binding factors termed Bob1 (OBF-1/OCA-B) was identified and cloned from human (11–13) and murine (14, 15) tissues. Bob1 interacts with the POU domains of either Oct-1 or Oct-2 forming a ternary complex on DNA. Upon the binding of Bob1, Oct-1 is converted from an ubiquitous to a lymphocyte-specific factor and shows increased specificity of DNA binding (16).

Although in gene-targeted mice Oct-2 is dispensable in the antigen-independent stage of B lymphocyte development, B cell maturation seems to be severely impaired in these animals (17). Recently, two Oct-2-regulated genes, CD36 (18) and CRISP-3 (19), have been identified. As their function in the immune system is not clear, there might be yet undiscovered targets of Oct-2 regulation that are relevant for B cell maturation. Data presented here demonstrate that in B cells regulation of the BLR1 gene is cooperatively controlled by NF-κB, Oct-2, and Bob1. The observation that the B cell homing chemokine receptor, BLR1, is a target for Bob1 and Oct-2 links these factors to the molecule with an essential function in the immune system.

EXPERIMENTAL PROCEDURES

Genomic Cloning—Human genomic clones were isolated from a library made from genomic DNA of the Burkitt’s lymphoma cell line BL64 in the vector EMBL3A (20); murine clones were isolated from...
Cell Culture, DNA Transfection, and Reporter Assays—B cell lines (Raji, lymphoblastoid line IARC549, and Abl/Oct2-ER) and HeLa cells were transfected with 5–20 μg of various reporter constructs using the calcium phosphate method. Raji and Abl/Oct2-ER cells were transfected by electroporation (1070 microfarads and 250–300 V). Cells were harvested 24 h after transfection and lysed with reporter lysis buffer (Promega). The amount of expressed CAT was determined by a CAT enzyme-linked immunosorbent assay system (Boehringer Mannheim). Luciferase activity was determined in a LB 9501 luminometer (Berthold) using a luciferase assay system (Promega). All transfections were normalized to the level of total cellular protein (Bradford assay, Bio-Rad) and repeated at least three times.

Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear proteins were extracted as described previously (23). Peripheral blood cells depleted of BLR1-expressing lymphocytes were isolated using a MACS magnetic cell separator as described before (6). The double-stranded oligonucleotides were labeled by filling in the overhangs with T4 polymerase (Stratagene) in the presence of radioactive NTPs at 54 °C. 30 fmol were used per lane in electrophoretic mobility shift assay. Oligonucleotides for electrophoretic mobility shift assay had the following sequences (top strand): Oct, 5′-TCGCTATTTTGGCTTATATTTTC-3′; Oct-2, 5′-ATTCAGAAAGCTCACTGATACTAAGAGGAAGCTGCAAGCTTAGC-3′; Oct-2 variant, 5′-ATTCAGAAAGCTCACTGATACTAAGAGGAAGCTGCAAGCTTAGC; Oct-m, 5′-ATTCAGAAAGCTCACTGATACTAAGAGGAAGCTGCAAGCTTAGC; E-box, 5′-TGCAGACCGACCCCGGACG.

Flow Cytometry Analysis—Lymphocytes were isolated from the spleen of mice at 4–6 weeks except for progenies of Oct-2−/− mice that were isolated within 1 h after delivery. The cells were incubated with either anti-B220-fluorescein isothiocyanate (Caltac) and anti-BLR1-biotin monoclonal antibodies (7) or with anti-IgM-fluorescein isothiocyanate, B220-phycocerythrin, and anti-BLR1-biotin for 30 min at 4 °C. After two washes, the biotinylated antibody was revealed with streptavidin-phycocerythrin (Jackson ImmunoResearch) or with streptavidin-Cy-Chrome™ (Pharmingen) staining. The cells were subjected to the flow cytometry analysis using a FACSScan flow cytometer (Becton Dickinson).

RESULTS

The TATA-less Human BLR1 Promoter Directs RNA Initiation to a Single Start Site—Following the determination of the BLR1 gene structure (3, 4), the 5′-flanking region of the first exon was subcloned and sequenced. As shown in Fig. 1A, a specific start site of the BLR1 transcript was observed using RNA from Burkitt’s lymphoma cell line, Raji. The localization of the start site could be confirmed by PCR analysis using primers flanking the initiation site (Fig. 1B). Only primer pair 1 (O+1, sequence +1 to +20 bp) generated a PCR product on the RNA template, whereas primer pair 2 (O−56, sequence −56 to −36 bp) did not. As a control, PCR was done on a DNA template using both primer pairs (Fig. 1B).

Alignment of the nucleotide sequences of the human BLR1 promoter (EMBL accession no. X83755) and its murine counterpart (EMBL accession no. X83756) shows highly conserved regions that include consensus sequences for potential regulatory elements such as API, LEF-1, NF-xB, a purine-rich sequence (Pu.box), a noncanonical octamer, and an E-box motif (Fig. 2A). Both promoters lack TATA and CCAAT boxes as well as initiator-like sequences (24).

Cell Type and Differentiation Stage Specificity of BLR1 Promoter Activity—To define the sequences responsible for cell type- and differentiation-specific BLR1 expression, a series of 5′-deletion mutants of the human promoter region starting at position −1097 was linked to the bacterial chloramphenicol acetyltransferase gene and transfected into Raji, IARC549, or HeLa cell lines (data not shown). CAT expression driven from the BLR1 promoter was only detectable in Raji cells and not in IARC549 or HeLa cells. Similarly, a murine blr1 promoter cloned into the luciferase reporter vector mblr1-Luc was consistently active in Raji but not in HeLa cell lines (data not shown). The minimal 5′-deletion construct p−78/+215 retained cell type-specific activity. These results suggest that regulatory elements lying within positions −78 and +215 are sufficient to confer basal activity as well as cell type specificity of the BLR1 promoter.

To identify the regulatory motifs essential for promoter activity, we introduced a variety of mutations into the wild-type
construct p–612/+215. Each mutation destroyed a single consensus sequence for a putative regulatory element. Among all tested mutations, only three dramatically decreased the level of CAT expression in Raji cells (Fig. 2B). All of them lie within the region identified as essential for promoter activity and specificity (278 to 1215). Mutation B6 reduced promoter activity by 80%. However, we could not specify the proteins binding to this site and named it a “functional promoter region” (FPR), implying that it may serve as a functional substitution for the TATA box within the BLR1 promoter. Mutation of the NF-kBIII binding site (Fig. 2B, pN7) caused a decrease of the promoter activity by 60%. Using the electrophoretic mobility shift assay, we could detect binding of proteins of the NF-kB/rel family to this site (data not shown). Mutation of the noncanonical octamer site (Fig. 2B, pE8) reduced promoter activity by more than 90%.

To clarify the role of factors binding the octamer site, we analyzed the expression of luciferase driven by the murine blr1 promoter in the Oct-2 conditional Abl/Oct2-ER cell line. This murine pre-B cell line is characterized by a homozygous mutation of the endogenous oct-2 gene and is stably transformed with a construct coding for a chimera between Oct-2 and the hormone-binding domain of the human estrogen receptor (25).

FIG. 2. Structural and functional analyses of the human and murine BLR1 promoter. A, sequence comparison between binding sites of different transcription factors in human BLR1 and murine blr1 promoters. Changed nucleotides in the mutated constructs are indicated. B, on the left are schematic drawings of the mutated BLR1-CAT fusion constructs. Each mutation is indicated by a black symbol. The wild-type p–612/+215 and mutated constructs (20 μg) were transfected into Raji cells. CAT expression was measured, and the level of p–612/+215 CAT expression was set to 100%. C, Oct-2-, NF-kB-, and Bob1-dependent activation of the murine blr1 promoter. 20 μg of mblr-Luc plasmid or constructs m-E8 and m-E8–2 with a mutated octamer sequence were transfected or cotransfected with NF-kB p50/p65 (5 μg each) and Bob1 (10 μg) in Abl/Oct2-ER cells. Each transfection was split in half, and 4-OHT was added to one part of the final concentration of 1 μM where indicated. Activity of mblr1-Luc, m-E8, or mE8–2 alone was set to 1 in all experiments, and the other bars present data of six independent experiments as -fold activation of luciferase activity above that. D, cotransfection of 20 μg of mblr-Luc with 10 μg of pOEV1, 10 μg of pCATCH-Bob1, or with both vectors into Abl/Oct2-ER cells. E, activation of the blr1 promoter in HeLa cells. mblr1-Luc (10 μg) was cotransfected with 5–10 μg of constructs expressing transcription factors Oct-2, NF-kB, and Bob1. Luciferase activity observed with mblr1-Luc was set to 1.
though the levels of activation differed and increased 8- and 4-fold, respectively. However, the other mutated construct, m-E8–2, did not respond to NF-κB and remained completely inactive. Bob1 could only very slightly add to the activation by NF-κB alone, but the combinatory action of Bob1 and NF-κB complemented with the induction of Oct-2 by 4-OHT produced an over 45-fold activation of the promoter. In both cases, neither of the mutated vectors showed any response. The construct, m-E8, bears a single nucleotide substitution at position 5 within the octamer sequence. As reported recently, this substitution still permits very weak residual binding activity to the resulting site (26). However, 4 of 8 nucleotides are substituted in mutation m-E8–2, thus completely eliminating any binding to this region. In our experiments, the oligonucleotide corresponding to mutation m-E8 was able to weakly compete for Oct-1 and Oct-2 binding to the octamer sequence from the bllr1 promoter, whereas the oligonucleotide corresponding to mutation m-E8–2 did not compete under the same conditions (data not shown). Therefore, little residual activity of Oct-1 possibly binding to the mutation m-E8 could allow moderate activation of the promoter, whereas the oligonucleotide corresponding to mutation m-E8–2 did not compete under the same conditions (data not shown).

As mentioned above, no expression of the reporter gene was detected in nonlymphoid cells. We tested whether the bllr1 promoter exerts activity in the HeLa cell line upon ectopic expression of Oct-2A, NF-κB, and Bob1 (Fig. 2E). Neither of the latter factors could strongly activate the reporter expression on its own. However, significant transactivation patterns were mediated by various combinations of NF-κB, Oct-2A, or Bob1. The most striking induction was achieved with all three factors together. Even in this case, however, the level of reporter expression was still much lower than that in lymphoid cells (data not shown). Therefore, additional unidentified B cell-specific activators seem to be required for full promoter function.

Binding of Oct-2 Correlates with the Expression of BLR1—As shown above, the octamer motif plays a crucial role in the expression of the BLR1 gene. We examined the binding of the Oct proteins to the noncanonical octamer site. Incubation of an oligonucleotide probe with Raji nuclear extracts produced several octamer-specific nucleoprotein complexes (Fig. 3, lane 1). These complexes were formed by the octamer binding factor Oct-1 and two alternatively spliced variants of Oct-2, Oct-2A and Oct-2B, and could be specifically competed (Fig. 3, lanes 2–9). We also tested nuclear extracts from lymphoblastoid IARC549 B cells, peripheral blood lymphocytes negative for BLR1 expression (PBL BLR1–), and nonlymphoid HeLa cells (Fig. 3, lanes 10–12). The binding of Oct-2A and Oct-2B was very low in lymphoblastoid IARC549 B cells, which represent an activated stage and do not express BLR1, and was absent in PBL BLR1+ and HeLa cells. Other nucleoproteins, which bind to the determined consensus sequences within the BLR1 promoter, showed no differences in their DNA binding activity in nuclear extracts from Raji or IARC549 cells (data not shown).

BLR1 Expression Is Highly Reduced in Lymphatic Organs of Oct-2, Bob-1, and p50/p52 NF-κB Knockout Mice—We were interested in testing whether the expression of bllr1 would be affected in mice deficient for Oct-2. These mice die within a few hours after delivery for unknown reasons (17). The expression of BLR1 in spleen lymphocytes from newborn progeny of Oct-2–/– mice was analyzed by flow cytometry. BLR1 expression was only barely detectable on B220 positive cells from null mutant mice, whereas cells isolated from wild-type littermates expressed levels of BLR1 usually observed in newbons (Fig. 4, A and B). As the in vitro data suggest that the bllr1 promoter is fully active only in the presence of Bob1 and NF-κB, we further tested the expression of BLR1 in mice deficient for these factors. Expression of BLR1 in B220+ cells derived from lymph nodes or spleen was unaffected both in wild-type and heterozygous animals; however, it was more than 50% reduced in Bob1 null mutant mice (Fig. 4C, data shown for lymph nodes only). Furthermore, expression of BLR1 was virtually absent in mice lacking both p50 and p52 NF-κB proteins (Fig. 4D). Interestingly, mice lacking p52 only showed regular levels of BLR1.

### DISCUSSION

We isolated and characterized the promoter for the BLR1 gene. BLR1 transcription initiation starts at a single site, but the promoter sequence lacks classical features like a TATA box or an initiator-like sequence. The core promoter sequence required for basic and tissue-specific expression was found to reside in a fragment encompassing a region from position −78 to +215 with respect to the transcriptional start site. By mutational analysis, it was found that this fragment harbors three regions essential for promoter activity: 1) a site termed FPR located at position −36, which suggests that it may be important for the formation of the preinitiation complex; 2) a NF-κB site; and 3) a noncanonical octamer sequence. The latter is the key determinant for BLR1 promoter activity, because a mutation abrogating binding of Oct factors resulted in a complete promoter inactivation. Available evidence suggests that NF-κB...
NF-κB, Bob1, and Oct-2 factors act in a synergistic fashion to regulate BLR1 promoter activity (see Fig. 2). It was reported that the binding of NF-κB p50/p65 subunits to DNA introduces a bend into the DNA molecule (27). Taking into consideration the location of the essential binding sites for NF-κB, Oct, and the FPR in BLR1 promoter, it is tempting to speculate that this NF-κB-mediated DNA bending brings FPR and the octamer element into close proximity to each other. This would enable the interaction of octamer-bound factors to the protein(s) bound to FPR. The importance of this interaction is substantiated by the evidence that Oct-2 may facilitate the formation of the preinitiation complex (28). Furthermore, Oct-2 can interact with the TATA box-binding protein TBP (29). The described regulation of the BLR1 promoter activity is the first example for the cooperative regulation via NF-κB and octamer binding factors from a position downstream of the transcriptional start site.

We demonstrated that the B cell-specific coactivator Bob1 potentiates Oct-2-mediated activation of the b1r1 promoter. Bob1 has its own activation domain (11), but the mechanism of activation is not fully understood. It is suggested that Bob1 acts as a bridge, connecting the octamer-bound transcription factor to components of the TFIIID complex (12). Therefore, this interaction may also contribute to the regulation of the BLR1 promoter function.

The ternary complex of Bob1 with octamer factors shows higher selectivity in recognition of the octamer sequences, as Oct-1 or Oct-2 alone (16). The octamer site in the BLR1 promoter is a noncanonical motif, with a substitution T/A in the third position. It was shown that the fifth base in the octamer site is most critical for the formation of the ternary complex, and it should be occupied by adenosine (16). It was suggested that the first three nucleotides of the octamer sequence are also involved in the recognition of the octamer sequence. However, our data indicate that the b1r1 promoter is activated by Bob1, implying that within the context of this gene the second and the third bases of the octamer motif are not necessarily required for the activation.

Analysis of mice with targeted deletion of Oct-2, Bob1, or NF-κB provides further evidence for the critical requirement of these factors for the expression of BLR1. Like Oct-2-deficient mice, Bob1 knockout mice (30–32) show unaffected immunoglobulin gene expression but have severely reduced numbers of mature B cells and lower levels of secondary antibody isotypes in serum. Most important is the observation that Bob1−/− mice completely fail to develop germinal centers following immunization with thymus-dependent antigen. The same phenotype is shared by mice with the targeted disruption of p52/NF-κB2 (33, 34) and with double knockout of p50/p52 genes (35). Adaptively transferred p52-deficient lymphocytes can form germinal centers (33, 34), and consistent with this observation, we detected unaffected expression of BLR1 on these cells. In p50/p52 double knockout mice development of B cells is halted at stage B220low, IgM+, CD23+. Cells with this phenotype are also found in normal spleens, although at low frequency. In wild-type animals, these cells express BLR1 at the same level as observed for mature B cells, but we could not detect BLR1 on corresponding cells derived from double knockout mice. The absence of germinal centers is also a hallmark of the phenotype of b1r1-deficient mice. Following a T cell-dependent antigenic challenge, B cells become activated but fail to migrate into B cell follicles of the spleen. As a consequence, germinal centers do not develop (7). Thus, the severely reduced expression of BLR1 in both Bob1 and p50/p52 null mice may contribute to the absence of germinal center reactions in these animals as a consequence of impaired B lymphocyte migration.

Acknowledgments—We are grateful to Thomas Wirth for providing the Abb/Oct2-ER cell line, Lynn Corcoran for providing Oct-2−/− mice, Peter J. Nielsen for Bob1−/− mice, and Anita Mattis for the 129SV b1r1 genomic library clone. We thank Melanie Fischer, Dagmar Breitfeld, and Oliver Mück for excellent technical assistance.

REFERENCES
Oct-2-dependent Expression of BLR1
