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# The Extracellular Domain of the Epstein-Barr Virus BZLF2 Protein Binds the HLA-DR β Chain and Inhibits Antigen Presentation

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**The Epstein-Barr virus BZLF2 gene encodes a glycoprotein that associates with gH and gL and facilitates the infection of B lymphocytes. In order to determine whether the BZLF2 protein recognizes a B-cell-specific surface antigen, a soluble protein containing the extracellular portion of the BZLF2 protein linked to the Fc portion of human immunoglobulin G1 (BZLF2.Fc) was expressed from mammalian cells. BZLF2.Fc was used in an expression cloning system and found to bind to a β-chain allele of the HLA-DR locus of the class II major histocompatibility complex (MHC). Analysis of amino- and carboxy-terminal deletion mutants of the BZLF2.Fc protein indicated that the first 90 amino acids of BZLF2.Fc are not required for HLA-DR β-chain recognition. Site-directed mutagenesis of an HLA-DR β-chain cDNA and subsequent immunoprecipitation of expressed mutant β-chain proteins using BZLF2.Fc indicated that the β1 domain, which participates in the formation of peptide binding pockets, is required for BZLF2.Fc recognition. The addition of BZLF2.Fc to sensitized peripheral blood mononuclear cells in vitro abolished their proliferative response to antigen and inhibited cytokine-dependent cytotoxic T-cell generation in mixed lymphocyte cultures. Flow-cytometric analysis of Akata cells induced to express late Epstein-Barr virus antigens indicated that expression of BZLF2 did not result in reduced surface expression levels of MHC class II. The ability of BZLF2.Fc to bind to the HLA-DR β chain suggests that the BZLF2 protein may interact with MHC class II on the surfaces of B cells.**

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus whose natural host range is restricted to humans. It is the causative agent of infectious mononucleosis and is thought to play an important role in Burkitt's lymphoma, nasopharyngeal carcinoma, and some cases of Hodgkin's disease. The virus is transmitted primarily by saliva and lytically infects epithelial cells of the oropharynx and salivary glands. These infected epithelial cells are thought to transmit EBV to circulating B cells, which in turn support a persistent infection and likely function as the primary reservoir of virus within the host (23, 45). Attachment of EBV to susceptible cells occurs as a result of the binding of a viral glycoprotein, gp350, to cell surface CR2 (CD21) (12, 15, 33, 34, 44). The entry of the virus into cells appears to be a complex event that, by analogy with other herpesvirus systems, is mediated in part by the glycoproteins gH (gp85) and gL (gp25), which form a complex on the surfaces of infected cells and on extracellular virions (36, 46).

In EBV, the gH and gL proteins form a stable complex with a third glycoprotein, termed gp42, the product of the BZLF2 open reading frame, that appears to play a role in the infection of specific cells by EBV. A monoclonal antibody (MAb) directed against the BZLF2 protein has been shown to inhibit the infection of peripheral blood B lymphocytes but not the infection of the EBV-susceptible epithelial cell line SVK-CR2 (27). The specificity of the interaction between the BZLF2 protein and B cells suggests that this protein might bind to a molecule expressed preferentially on these lymphoid cells. In an attempt to investigate further the binding characteristics and functions of the BZLF2 protein, a recombinant chimeric form of the BZLF2 gene was constructed, expressed in mammalian cells, and then used to identify a ligand for the BZLF2 protein.

In this paper, we show that the extracellular domain of BZLF2 binds specifically to a β-chain allele of the major histocompatibility complex (MHC) class II HLA-DR locus. These class II molecules exhibit restricted expression patterns and are found predominantly, although not exclusively, on specialized antigen-presenting cells, such as macrophages, dendritic cells, and B cells, in which they function to present exogenously acquired antigen to the T-cell receptors on $CD4^+$ lymphocytes. We also identify the regions of a soluble protein containing the extracellular portion of the BZLF2 protein linked to the Fc portion of human immunoglobulin G1 (IgG1), designated BZLF2.Fc, and of the HLA-DR β chain that are necessary for the interaction of these two proteins and show that BZLF2.Fc abolishes MHC class II-directed T-cell proliferation. Finally, we show that the induction of BZLF2 protein expression on the surfaces of Akata cells does not reduce surface expression levels of the HLA-DR β chain.

## MATERIALS AND METHODS

**Construction of BZLF2.Fc.** The $CH_2$ and $CH_3$ domains of human IgG1, which correspond to amino acids 241 to 470 (9), were inserted downstream of the murine interleukin-7 (IL-7) leader sequence (amino acids 1 to 22) (32). This construct was subcloned into Bluescript (Stratagene, La Jolla, Calif.), and the resulting plasmid was digested with *Nsi*I and *Not*I. This digestion removed the last 15 amino acid codons from the Fc portion of IgG1. Complementary oligonucleotides that contain a 5′ *Nsi*I site, the deleted Fc codons followed by a $[(Gly_4)Ser]_2$ repeat (which serves as a flexible linker domain), and a 3′ *Spe*I site were constructed. The extracellular domain of the BZLF2 protein from amino acids 34 to 223 followed by an in-frame termination codon was amplified by PCR from genomic DNA with primers that contained a 5′ *Spe*I site and a 3′ *Not*I site.

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After digestion with these enzymes, the PCR fragment, complementary oligonucleotides, and plasmid vector were ligated, and the resultant plasmid construct, termed BZLF2.Fc, was sequenced in its entirety. The BZLF2.Fc fusion gene was excised from Bluescript and subcloned into the mammalian expression vector pDC406 for expression in CV-1/EBNA cells (29) or in COS-7 cells.

**Expression cloning.** Protein A-Sepharose CL-4B (LKB Biochemical, Piscataway, N.J.)-purified BZLF2.Fc protein was used to screen a mammalian expression library derived from the human T-cell clone PL-1, which had been cultured for 16 h in the presence of immobilized CD3 MAb (OKT3; American Type Culture Collection, Rockville, Md.). The expression vector, CV-1/EBNA cells, and screening procedure have been described previously (2, 29).

**EBV-infected cells.** Productive EBV infection was induced in Akata cells by cross-linking surface Ig through the addition of anti-human Ig antibody (Jackson ImmunoResearch Labs, Inc., Westgrove, Pa.) to the culture medium as previously described (43). Induction time was typically 16 h.

**Flow cytometry and immunoprecipitation.** A peptide representing amino acids 192 to 207 of the BZLF2 protein sequence was conjugated to ovalbumin, combined with TiterMax adjuvant (Vaxcel, Inc., Norcross, Ga.), and used to immunize BALB/c mice on day 0. Animals were given booster injections on day 14 with conjugated peptide in incomplete Freund's adjuvant, and sera were collected on day 28. The specificities of these sera against the BZLF2.Fc and control proteins were determined by Western blot (immunoblot) analysis. Western blots were developed by the enhanced chemiluminescence Western Blotting Analysis System from Amersham (Arlington Heights, Ill.). Immunoprecipitations were performed on extracts labeled for 20 min with [$^{35}$S]cysteine-methionine. All experiments were performed with protein A-Sepharose (Pharmacia, Piscataway, N.J.). Immunoprecipitations of BZLF2.Fc or immunoprecipitations using BZLF2.Fc as the detection reagent were performed without second-step antibodies directly onto protein A-Sepharose (Pharmacia). All other reactions used an HLA-DR α-chain-specific MAb (Dakopatts, Glostrup, Denmark), after which rabbit anti-mouse serum (Cappel Research Products, Durham, N.C.) and protein A-Sepharose were added. For flow cytometry, we used HLA-DR-specific MAbs that recognize the αβ heterodimer (clone L243; Becton Dickinson, San Jose, Calif.), the glycosylated form of the β chain (ALVA-42; Genzyme, Cambridge, Mass.), and the glycosylated and nonglycosylated forms of the β chain (catalog no. 0462; AMAC, Inc., Westbrook, Maine). BZLF2.Fc protein was detected in flow-cytometric analyses using a phycoerythrin-conjugated goat anti-human IgG MAb (Jackson ImmunoResearch Labs).

**Construction of BZLF2.Fc deletion mutants.** N-terminal (ΔN) and C-terminal (ΔC) deletion mutants were generated with a cDNA fragment containing the BZLF2.Fc coding region cloned into pBluescript (Stratagene). Unique restriction endonuclease sites within and at the borders of the sequence of the BZLF2 domain were used for the construction of most of these mutants. The internal *Hpa*I site was used to generate mutants ΔN-58 and ΔC-160, the *Bsm*I site was used to generate mutants ΔN-154 and ΔC-63, and the *Nde*I site was used to generate mutants ΔN-190 and ΔC-28. Portions of the BZLF2 extracellular sequence were released from the plasmid with one of the internal restriction endonuclease sites mentioned above and the 5′ *Spe*I site at the junction between the Fc and the BZLF2 domain for the N-terminal truncations or with the *Not*I site at the 3′ end of the BZLF2 domain for the C-terminal truncations. Oligonucleotide adapters were used to restore the correct reading frame and close the gap in the construct. The deletion mutants ΔN-90, ΔN-122, ΔC-96, and ΔC-129 were generated by PCR with specific oligonucleotide primers. All deletion mutants were sequenced after cloning into the expression vector pDC406 (29) as *Sal*I-*Not*I fragments.

**Site-directed mutagenesis.** Mutations were created in a cDNA encoding the β chain of an HLA-DR allele by the technique of PCR SOEing (19). Plasmids were constructed such that amino acids 44 to 108 inclusive were deleted in order to remove the $\beta_1$ domain, and amino acids 146 to 202 inclusive were deleted in order to remove the $\beta_2$ domain from the expressed proteins. All DNA constructs were sequenced in their entirety to confirm the mutations.

**Proliferation assays.** Peripheral blood mononuclear cells (PBMC) were purified as previously described (11). For the measurement of proliferation, cultures were performed in round-bottom 96-well microtiter plates in RPMI medium plus 10% fetal bovine serum. PBMC ($10^5$ in 200 μl) from selected donors known to be responsive to the particular antigen were cultured for 7 days with purified tetanus toxoid (1,000 Lf/ml; Connaught Laboratories, Inc., Swiftwater, Pa.) or purified *Lolium perenne* antigen derived from perennial ryegrass (Greer Laboratories, Inc., Lenoir, N.C.). Cells were pulsed with 1 μCi of [$^3$H]thymidine for the final 16 h of culture, and incorporated counts per minute were determined by tritium-sensitive avalanche gas ionization detection on a Matrix 96 Direct Beta Counter (Packard, Meriden, Conn.). For the cultures containing saturating amounts of human IgG1, the PBMC were cultured for 7 days in the presence or absence of tetanus toxoid (1:2,000 dilution of stock) and 1 μg of BZLF2.Fc per ml or 1 μg of a control Fc protein per ml. The control protein used in these assays was a chimeric Fc protein containing the extracellular portion of the p35 protein of vaccinia virus (Copenhagen strain) or a chimeric Fc protein containing the extracellular portion of a tyrosine kinase receptor.

**CTL assays.** Generation of cytotoxic T lymphocytes (CTL) was assessed by the release of $^{51}$Cr from target cells into culture medium as described previously (1). The control protein used in these assays was a mouse IgG1 antibody or a chimeric Fc protein containing the extracellular portion of a tyrosine kinase receptor.

## RESULTS

**The BZLF2 protein has homology with the C-type lectin family of proteins.** We searched for homologs to the predicted amino acid sequence of the full-length BZLF2 protein in the SWISS-PROT database (4) using the FASTA algorithm (17, 35) with a word size of 1. Protein sequences with the 80 highest scores were analyzed from the top 1,000 matches. Among the matches were several members of the C-type lectin family. Sequence alignment and comparison with family motifs (10) confirmed that the putative extracellular domain of the BZLF2 protein contained several features characteristic of the family, including six conserved cysteine residues and the WIGL motif, in which the isoleucine and leucine residues have been replaced with valine residues.

The C-type lectin family contains type II membrane-bound proteins that have an amino-proximal signal, an anchor domain, and a carboxy-terminal extracellular domain. The lymphoid cell surface antigens CD23, NKR-P1, and CD72 are all C-type lectins (Fig. 1). CD23 and CD72 are expressed on B cells, whereas NKR-P1 is expressed on natural killer cells (5, 24, 37, 47). The ligands for these antigens are present on lymphoid cells, suggesting that the BZLF2 protein may also have a ligand that is expressed on the surfaces of lymphoid cells.

**Identification of a ligand for the BZLF2 protein.** To search for a ligand for the BZLF2 protein, a soluble chimeric molecule in which a portion of the human IgG1 heavy chain was fused to the predicted extracellular domain of the BZLF2 protein was constructed (Fig. 2A). Since the BZLF2 protein is predicted to be a type II membrane protein, BZLF2.Fc was constructed such that the extracellular domain of the BZLF2 protein was positioned carboxy proximal to optimize the potential for binding to a specific ligand. The predicted molecular mass of BZLF2.Fc is approximately 45 kDa. Recombinant BZLF2.Fc, obtained from the supernatant of cells transfected with BZLF2.Fc and immunoprecipitated with protein A-Sepharose, had an apparent molecular mass of approximately 69 kDa under reducing conditions (Fig. 2B), suggesting that at least some of the four putative N-linked glycosylation sites found in the extracellular domain are utilized. BZLF2.Fc is believed to dimerize as a result of disulfide bonding within the Fc portion of the protein, and consistent with this, BZLF2.Fc has an apparent molecular mass of 145 kDa under nonreducing conditions (data not shown).

Purified BZLF2.Fc was used to identify a cell source for its ligand by flow-cytometric analysis of lymphoid cell lines. BZLF2.Fc-specific staining was observed on the human CD4$^+$ T-cell clone PL-1 after being cultured for 2 h in the presence of immobilized CD3 MAb. A cDNA library was generated from activated PL-1 cells, expressed in CV-1/EBNA cells, and then screened for binding to the BZLF2.Fc protein as described previously (29). One hundred twenty pools of 2,000 cDNAs were each screened prior to the identification of a single pool that was positive for BZLF2.Fc binding. This pool was sequentially broken down, and a single cDNA clone encoding a protein that bound BZLF2.Fc was isolated. The nucleotide sequence of this cDNA was determined by conventional techniques, and comparison of the deduced amino acid sequence with sequences in the GenBank database indicated that it encoded an HLA-DR (MHC class II) β-chain allele.

The detection of cell surface expression of the MHC class II β chain with BZLF2.Fc was surprising, since previous work had indicated that although the class II αβ heterodimer is transported to the surfaces of transfected cells, the β chain is not transported in the absence of the α chain (30). In these prior studies, surface expression was determined by flow-cytometric analysis using MAbs against the individual HLA-DR chains.

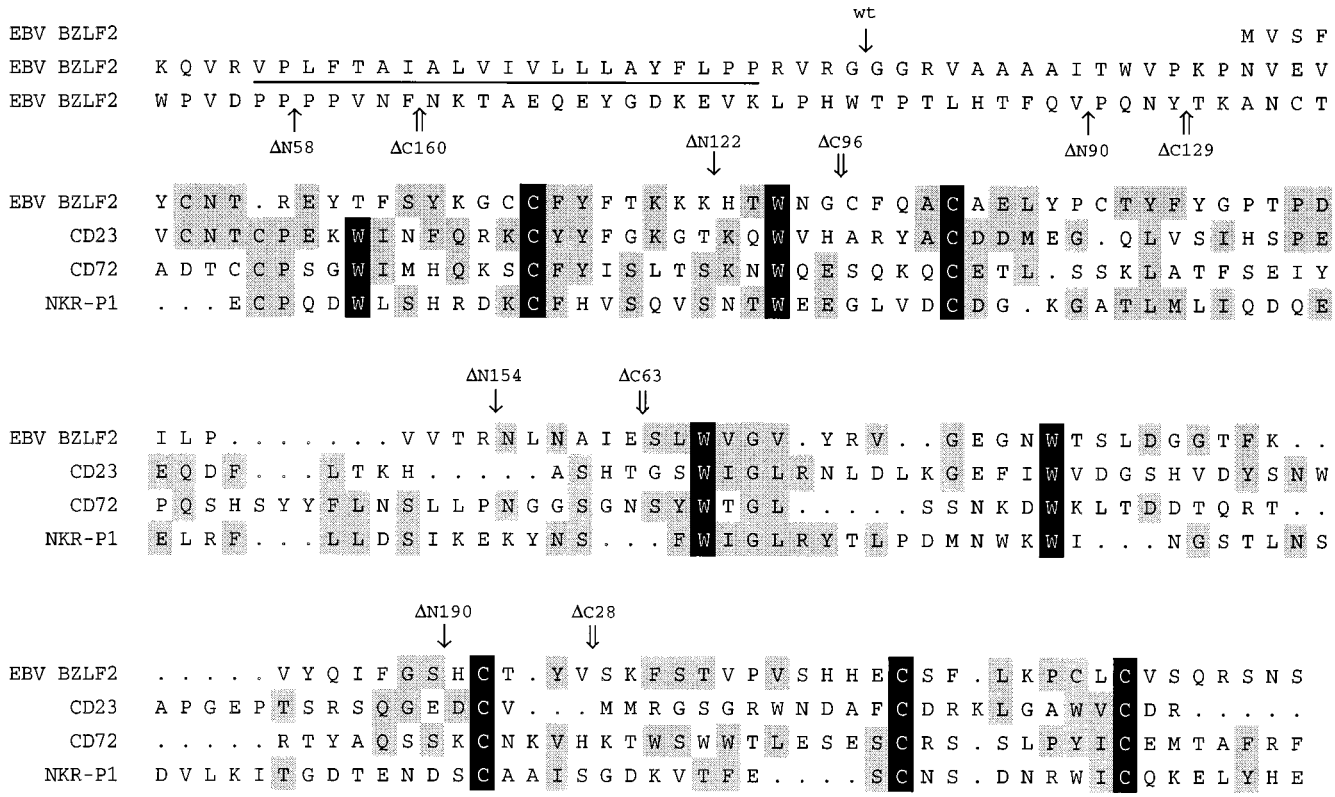


FIG. 1. Alignment of the predicted amino acid sequence of BZLF2 protein with those of several members of the C-type family of lectins. The BZLF2 protein sequence contains an amino-terminal signal-anchor sequence (underlined) and a carboxy-terminal extracellular domain. Cysteine and tryptophan residues typically conserved throughout the family are shaded in black; residues that are identical or similar are shaded in gray. Single arrows indicate sites of N-terminal truncations within the BZLF2.Fc protein; double arrows indicate sites of C-terminal truncations; wt indicates the beginning of the extracellular domain used to construct the fusion protein.

The discrepancy between our results and those reported earlier is most likely a result of enhanced expression by the plasmids used in our experiments, combined with the sensitive detection methods employed during expression cloning (16, 29).

Consistent with the results reported previously (30), cotransfection of cells with HLA-DR α- and β-chain-containing plasmids resulted in enhanced cell-surface expression of the β chain, as determined by BZLF2.Fc binding. As a control, cells transfected with the α chain alone showed no BZLF2.Fc binding (data not shown). The transport of the individual HLA-DR α or β chains in transfected cells was confirmed with MAbs against these molecules and by detection methods similar to those employed during expression cloning (29).

To determine whether BZLF2.Fc could bind to class II alleles expressed intracellularly, immunoprecipitation experiments were performed. Plasmids containing the β chain or an HLA-DR α chain were transfected into COS-7 cells either separately or together. Transfected cells were metabolically radiolabeled, and the lysates were precipitated with BZLF2.Fc or a MAb directed against the HLA-DR α chain and then with protein A-Sepharose and analyzed by polyacrylamide gel electrophoresis (PAGE) (Fig. 2C). These experiments indicate

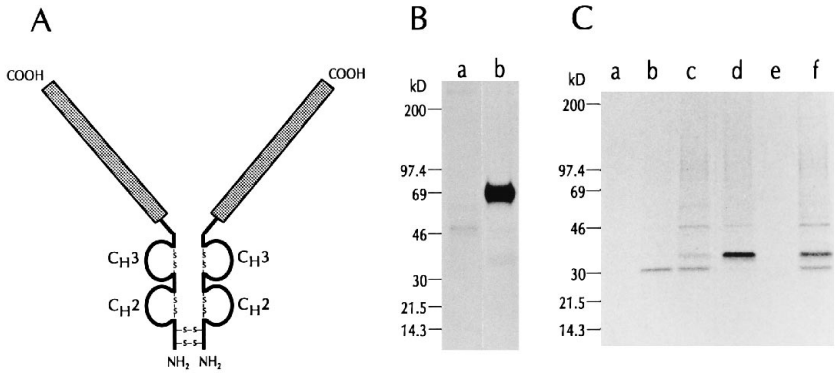


FIG. 2. (A) Diagrammatic representation of the BZLF2.Fc protein. The BZLF2 extracellular domains are indicated by the shaded bars, and the IgG1 Fc domains are indicated by heavy black lines. (B) Protein A-Sepharose precipitation of recombinant BZLF2.Fc protein. CV-1/EBNA cells transfected with either an empty vector (lane a) or BZLF2.Fc expression plasmids (lane b) were labeled for 1 h with 100 μCi of [$^{35}$S]Met-Cys, and the cell supernatants were precipitated with protein A-Sepharose. Samples were analyzed by PAGE under reducing conditions on 8 to 16% gradient gels. Molecular mass markers are indicated on the left. (C) Immunoprecipitation of the wild-type HLA-DR β chain using BZLF2.Fc. COS-7 cells were transfected with plasmids carrying the α chain (lanes a and d) or β chain (lanes b and e) or cotransfected with plasmids carrying both the α and β chains (lanes c and f) and labeled for 1 h with 200 μCi of [$^{35}$S]Met-Cys per ml, and the cell lysates were immunoprecipitated with BZLF2.Fc (lanes a to c) or antibody raised against the HLA-DR α chain (lanes d to f) and then with protein A-Sepharose. Immunoprecipitates were analyzed on 8 to 16% gradient polyacrylamide–sodium dodecyl sulfate gels under reducing conditions. Molecular mass markers are indicated on the left.

that BZLF2.Fc binds to the HLA-DR β chain but not the α chain (Fig. 2C, lanes a and b) and that BZLF2.Fc recognition of the αβ heterodimer is a result of β-chain binding. The ability of BZLF2.Fc to precipitate both α and β chains from lysates of cotransfected cells (Fig. 2C, lane c) also indicates that BZLF2.Fc binds to the β chain in such a fashion that it does not detectably interfere with the intracellular association of the α and β chains. As a control, antibody directed against the HLA-DR α chain immunoprecipitated the α chain or the α and β chains (Fig. 2C, lanes d and f) but not the β chain alone.

**The first 90 amino acids of BZLF2 are not required for β-chain recognition.** To identify the regions within the extracellular domain of the BZLF2 protein that are important for its interaction with the β chain, BZLF2.Fc mutants were constructed, in which the BZLF2 domain was truncated from either its N-terminal (ΔN mutants) or C-terminal (ΔC mutants) end. The mutant proteins were expressed in transiently transfected COS-7 cells and metabolically radiolabeled. Each of the mutant BZLF2.Fc proteins was detected in the culture medium of transfected cells (Fig. 3A). The apparent molecular masses of the deletion mutants ranged between approximately 66 and 40 kDa. Individual mutants showed substantial differences in their levels of expression, as judged by the amount of protein detected in the medium. Most significantly, the deletion of only 28 or 63 amino acids from the C-terminal end of BZLF2.Fc resulted in a large decrease in recoverable protein compared with that of full-length BZLF2.Fc (Fig. 3A, ΔC-28 and ΔC-63).

Mutant proteins were tested for binding in immunoprecipitation experiments using transfected cell culture medium that contained mutant BZLF2.Fc protein and metabolically labeled lysates derived from cells transfected with the β-chain cDNA. Only two deletion mutants, ΔN-58 and ΔN-90, immunoprecipitated the HLA-DR β chain in these assays (Fig. 3B). Thus, the first 90 amino acids of the BZLF2 protein are dispensable for recognition of the class II β chain. All truncations except ΔN-58 and ΔN-90 involved the loss of at least 1 of the 11 cysteine residues in this protein, suggesting that proper folding of BZLF2 may be critical to its function.

**BZLF2 requires the $\beta_1$ domain for binding.** Extensive structural and functional analyses of MHC class II β-chain-allelic proteins have indicated that the amino-proximal ($\beta_1$) Ig-like domain is highly polymorphic and participates in the formation of peptide binding pockets (6, 21, 22). The membrane-proximal ($\beta_2$) domain exhibits little polymorphism and is critical for CD4 recognition (7, 26).

In order to localize the β-chain binding site of the BZLF2.Fc protein, site-directed mutagenesis (19) was used to create mutant forms of the HLA-DR β-chain cDNA isolated during the above-described expression cloning. Plasmids containing the wild-type β chain or mutants in which either the $\beta_1$ or the $\beta_2$ domain was deleted were transfected into CV-1/EBNA cells alone or cotransfected with plasmids containing a cDNA encoding a wild-type HLA-DR α chain. Transfected cells were metabolically radiolabeled, and the lysates were immunoprecipitated with a MAb directed against the HLA-DR α chain or BZLF2.Fc and analyzed by PAGE (Fig. 4). Cotransfection of the HLA-DR α-chain plasmid and a β-chain plasmid with a deletion in either the $\beta_1$ (lane b) or the $\beta_2$ (lane c) domain followed by immunoprecipitation with antibody directed against the α chain indicated that either β-chain mutant could associate with the α chain. Immunoprecipitation of cell lysates transfected with the β-chain deletion mutants indicated that removal of the $\beta_2$ domain did not prevent BZLF2.Fc binding. Two forms of the $\beta_2$-domain deletion were recognized by BZLF2.Fc (Fig. 4, lane f). Because the single N-linked glycosylation site on the β chain is found in the $\beta_1$ domain, these two forms may represent differentially glycosylated forms of the mutant. In contrast, removal of the $\beta_1$ domain resulted in a protein that was not immunoprecipitated by BZLF2.Fc (Fig. 4, lane e), demonstrating that BZLF2.Fc recognition requires the portion of the β chain that participates in the formation of peptide binding pockets.

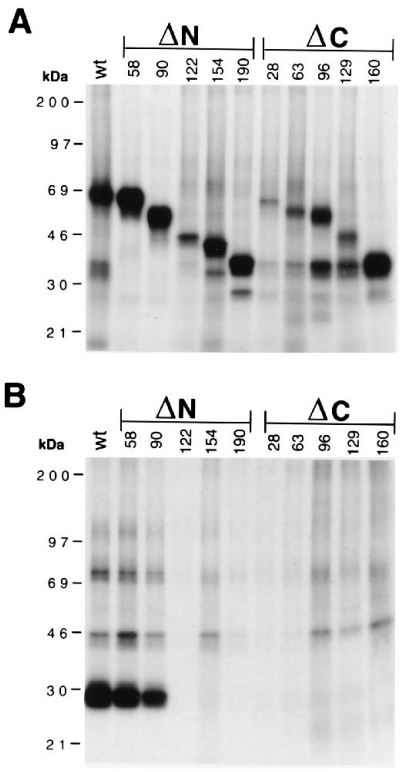


FIG. 3. (A) Expression of mutant BZLF2.Fc proteins with deletions in the BZLF2 domain. CV-1/EBNA cells were transfected with plasmids containing BZLF2.Fc cDNAs that had been truncated as described in Materials and Methods. Cells were labeled for 3 h with 100 μCi of [$^{35}$S]Cys-Met, and supernatants were precipitated with protein A-Sepharose and analyzed under reducing conditions on sodium dodecyl sulfate–8 to 16% polyacrylamide gels. The nomenclature used to describe the N-terminal mutants refers to the number of amino acids deleted when the initiating methionine is counted as 1 (i.e., ΔN-58 refers to an N-terminal truncation of 58 BZLF2 amino acids). Nontruncated BZLF2.Fc (lane wt) and the N-terminal deletion mutants ΔN-58, ΔN-90, ΔN-122, ΔN-154, and ΔN-190 are shown in the first six lanes. The numbering in the nomenclature describing the C-terminal deletion mutants refers to the lengths of the truncations if the C-terminal amino acid of wild-type BZLF2 is counted as 1 (i.e., ΔC-28 has a 28-amino-acid C-terminal deletion). (B) The first 90 amino acids of the BZLF2 protein are not required for HLA-DR β-chain recognition. CV-1/EBNA cells were transfected with plasmids containing a β-chain allele, labeled for 3 h as described above, and immunoprecipitated with transfected cell supernatants containing mutant BZLF2.Fc protein. The amount of BZLF2.Fc culture supernatants added was adjusted to reflect differences in expression levels. Molecular mass markers are noted at the left. wt, wild type.

**BZLF2.Fc abolishes MHC class II-directed antigen presentation.** BZLF2.Fc recognition of the $\beta_1$ domain suggested that BZLF2.Fc protein might function to alter the ability of MHC class II to present antigen in a biologically relevant fashion. To test this hypothesis, the effects of BZLF2.Fc protein on antigen-specific proliferation of PBMC in vitro were examined. PBMC from donors previously sensitized to either tetanus toxoid or ryegrass allergen were cultured with the appropriate antigen (11) in the presence or absence of BZLF2.Fc. The exogenously added antigen was taken up by the antigen-presenting cells in the culture, processed, and presented in the context of MHC class II to responding T cells. As indicated in

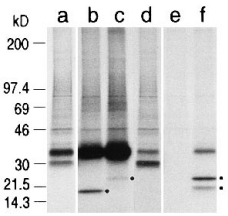


FIG. 4. Immunoprecipitation of mutant forms of the HLA-DR β chain using BZLF2.Fc. CV-1/EBNA cells were cotransfected with the wild-type α and β chains (lanes a and d), the α chain and the β chain with the $\beta_1$ domain deleted (lanes b and e), or the α chain and the β chain with the $\beta_2$ domain deleted (lanes c and f) and radiolabeled, and lysates were prepared. Lysates were immunoprecipitated with a MAb directed against the HLA-DR α chain (lanes a to c) or with BZLF2.Fc (lanes d to f). The positions of the protein with the $\beta_1$ domain deleted and the two forms of the protein with the $\beta_2$ domain deleted are indicated by small dots in lanes b, c, and f, respectively. Molecular mass markers are indicated on the left.

Fig. 5A and B, the addition of BZLF2.Fc to such cultures resulted in the complete inhibition of proliferation by sensitized PBMC whereas the addition of a heterologous, recombinant Fc chimeric protein had no effect.

Similar assays were used to test the biological activities of three of the N-terminal deletion mutants of BZLF2.Fc. The inclusion of purified ΔN-58, ΔN-90, and ΔN-122 mutant proteins in tetanus toxoid-driven PBMC proliferation assays showed that only mutant proteins that were able to bind the HLA-DR β chain (ΔN-58 and ΔN-90) blocked proliferation while the mutant ΔN-122 (a nonbinder) did not (3).

**BZLF2.Fc activity is not Fc receptor mediated.** The recombinant BZLF2.Fc protein used in these studies is greatly diminished (>10,000-fold) in its ability to bind Fc receptors compared with human IgG1. Nonetheless, it was of interest to determine if the effects of the BZLF2.Fc protein were in part mediated by attachment of the Fc portion of the molecule to Fc receptors on PBMC. Tetanus toxoid-specific PBMC proliferation assays were performed with BZLF2.Fc in the presence or absence of saturating amounts of human IgG1. In cultures containing a 50-fold excess of IgG1, BZLF2.Fc protein still abolished antigen-specific proliferation (Fig. 5C) whereas the control Fc protein again had no effect.

**BZLF2.Fc inhibits CTL generation.** To extend these findings, we examined the ability of BZLF2.Fc to inhibit the generation of functionally active CTL within a responding cell population in human mixed lymphocyte cultures. The formation of CTL in these cultures is dependent on the release of soluble growth factors (such as IL-2) from $CD4^+$ T cells that have been activated through recognition of MHC class II alloantigens on the irradiated stimulating cells. In these mixed lymphocyte cultures, the responding cell-stimulating cell combinations used to test the effects of BZLF2.Fc were selected for a strong allogeneic response in the absence of exogenous growth factors. The addition of BZLF2.Fc protein to these cultures completely inhibited the generation of antigen-specific CTL, whereas the addition of a control protein had no effect (Fig. 6A). Mixed lymphocyte cultures were also established from selected donors that exhibited a weak allogeneic response in the absence of exogenous cytokines. The low-level responses in these cultures are likely due to the absence of $CD4^+$ helper-T-cell cytokine production, which is necessary for the amplification of alloreactive CTL precursors. This weak response can be enhanced by the addition of IL-2. As anticipated, the presence of BZLF2.Fc in cultures containing exogenous IL-2 had no effect on CTL generation (Fig. 6B).

**BZLF2 does not reduce surface expression of MHC class II on Akata cells.** Many viruses, including herpesviruses, have evolved mechanisms for interfering with the expression of MHC class I molecules on the surfaces of infected cells (reviewed in references 28 and 41). The possibility that BZLF2 functions during infection to reduce surface expression of HLA-DR was addressed by flow-cytometric analyses of an EBV-positive Burkitt's lymphoma cell line (Akata) that had been induced to allow productive infection and by comparing these results to those obtained from uninduced cells.

Akata cells can be induced from latent to productive EBV infection by the addition of antibodies to human Ig (43). Cells were induced and examined with a BZLF2-specific antipeptide antiserum or a MAb directed against HLA-DR. Induction of productive EBV infection was monitored by metabolically radiolabeling parallel cultures and immunoprecipitating EBV gp350 (data not shown). gp350 is an abundant viral glycoprotein that is expressed in Akata cells only during productive infection. Analyses of bulk populations of uninduced cells (Fig. 7A) or induced cells (Fig. 7B) using anti-BZLF2 serum indicated that BZLF2 protein could be detected on cell surfaces following induction. A MAb that recognized the HLA-DR αβ heterodimer on these cells, however, indicated no difference in

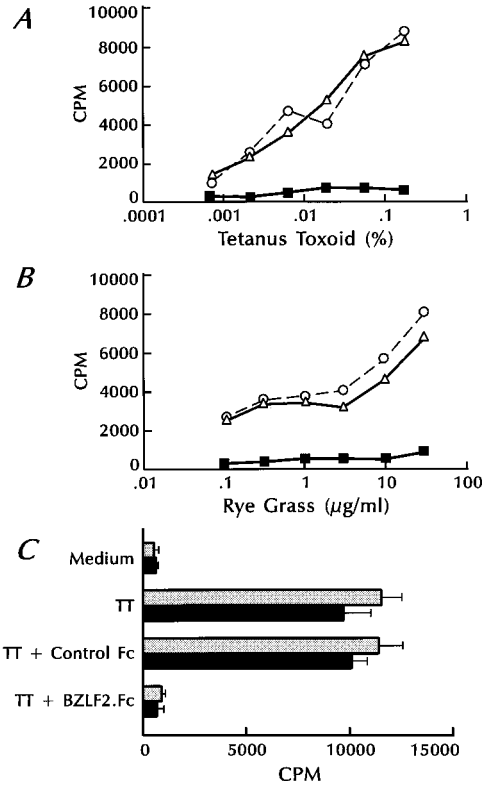


FIG. 5. BZLF2.Fc inhibition of antigen-driven PBMC proliferation. PBMC from tetanus toxoid- or ryegrass-sensitized individuals were cultured as described in Materials and Methods. (A) PBMC proliferative responses to titrated tetanus toxoid antigen in the absence (○----○) or presence (■——■) of 10 μg of BZLF2.Fc protein per ml or in the presence of 10 μg of a control Fc protein per ml (△——△). (B) Proliferative responses of PBMC to titrated ryegrass antigen and 10 ng of IL-2 per ml in the absence (○----○) or presence (■——■) of BZLF2.Fc or in the presence of 10 μg of a control Fc protein per ml and 10 ng of IL-2 per ml (△——△). Subsequent experiments showed identical results when BZLF2.Fc was used at concentrations as low as 1 μg/ml (data not shown). (C) Proliferative responses of PBMC in the presence of IgG1 saturation of Fc receptors. Assays were performed in the presence (■) or absence (▨) of 50 μg of human IgG1 per ml. All results are expressed as the mean counts per minute of [$^3$H]thymidine incorporated by triplicate cultures during the final 16 h. TT, tetanus toxoid.

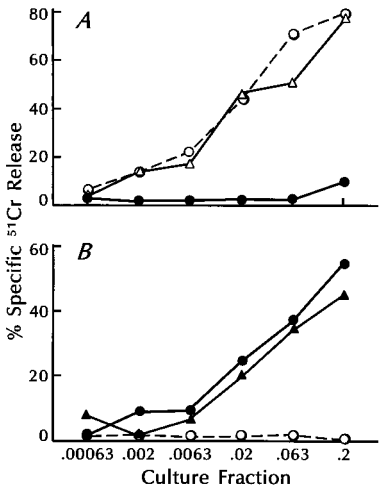


FIG. 6. Inhibition of T helper cell-dependent CTL generation by BZLF2.Fc. Generation of CTL was assessed by the release of $^{51}Cr$ from target cells into the culture medium as previously described (1). (A) BZLF2.Fc abolishes the formation of antigen-specific CTL. Mixed lymphocyte cultures contained no Fc protein (○----○), 2 μg of a control Fc-containing protein per ml (△——△), or 2 μg of BZLF2.Fc per ml (●——●). (B) BZLF2.Fc does not inhibit CTL generation in the presence of exogenous IL-2. Mixed lymphocyte cultures contained no protein (○----○), 10 ng of IL-2 per ml (▲——▲), or 10 ng of IL-2 per ml plus 2 μg of BZLF2.Fc per ml (●——●). Results are plotted on a linear-log graph indicating the specific $^{51}Cr$ release from a constant number of target cells as a result of incubation in the presence of the indicated dilution of the effector cell culture.

levels of cell surface class II expression following induction. These data indicate that in Akata cells, expression of BZLF2 does not reduce the expression of surface HLA-DR.

## DISCUSSION

The experiments described here indicate that the extracellular domain of the BZLF2 protein is capable of binding to the β chain of HLA-DR. The binding of BZLF2.Fc to PBMC abolishes antigen-driven proliferation of these cells; BZLF2.Fc also inhibits generation of CTL in mixed lymphocyte cultures. The ability of BZLF2.Fc to diminish T-cell proliferation is likely due to its ability to inhibit T-cell recognition of antigen presented in the context of MHC class II.

It is interesting that EBV infects B cells that express MHC class II on their surfaces and that the virus contains two genes, BCRF1 and BZLF2, that affect MHC class II molecules. BCRF1, the homolog of IL-10, downregulates gamma interferon production as well as MHC class II expression on monocytes (31, 42). The extracellular domain of the BZLF2 protein binds MHC class II and disrupts its function in vitro.

The ability of low concentrations of recombinant BZLF2.Fc to abolish antigen-driven PBMC proliferation and CTL generation in an alloreaction suggests that BZLF2.Fc may be useful as a therapeutic agent. Other chimeric Fc molecules, such as TNFR.Fc, tend to have relatively long half-lives in vivo (20), likely owing, in part, to their size. Thus BZLF2.Fc may be useful in autoimmune or allograft models. The formation of host antibodies against a virus-derived protein is of some concern; however, experiments with mice lacking MHC class II have shown that T-cell-dependent antigen responses require MHC class II expression (8, 18). Thus, BZLF2 inhibition of MHC class II-directed antigen presentation may diminish BZLF2-specific antibody formation. Consistent with this hypothesis, reduced antibody production against an Fc-containing immunosuppressive protein has been previously reported (14).

Our studies do not allow firm conclusions to be drawn regarding the specificity of BZLF2 protein binding to the wide variety of MHC class II gene products. Further studies are necessary to examine the binding of BZLF2 to HLA-DP, HLA-DQ, or HLA-DM molecules and to determine whether the BZLF2 protein exhibits differential binding characteristics within a given locus (i.e., allelic specificity). Although binding specificity may exist, the observation that BZLF2.Fc abolished antigen-driven PMBC proliferation from a variety of randomly selected donors that were tested in this study implies that the differences in binding may not be biologically important.

The true role of the BZLF2 protein during EBV infection remains uncertain. In herpes simplex virus, varicella-zoster virus, and cytomegalovirus, the gH and gL glycoproteins are involved in fusion and/or penetration of cells by the virus (13, 38–40). Since EBV gH and gL form a complex with the BZLF2 protein (27), BZLF2 protein may also be important for fusion or penetration of EBV into the B cell.

Li et al. (27) reported that MAb directed against BZLF2 protein inhibits infection of peripheral blood B cells, but not of epithelial cells, by EBV. Because B cells express MHC class II on their surface, the ability of BZLF2 to bind to MHC class II HLA-DR suggests that this interaction may play some role in facilitating EBV infection of B cells. Recent observations that MHC class II is present on the surface of the EBV virion further suggest that MHC class II may influence the cell tropism of EBV or may be important for intracellular pathways by which the virus exits cells (25). The presence of MHC class II on the surface of the virion may be due to its ability to bind the BZLF2 protein. Although we found that induction of EBV replication with expression of the BZLF2 protein on the surfaces of Akata cells did not reduce surface expression of MHC class II, intracellular BZLF2 protein may affect processing of class II molecules. Furthermore, Akata cells are derived from a Burkitt's lymphoma and may not faithfully mimic a newly infected primary B cell in vivo. The creation of single-gene deletion mutants of EBV, in which the BZLF2 gene is disrupted, will be useful in elucidating the function of the BZLF2 protein during infection.

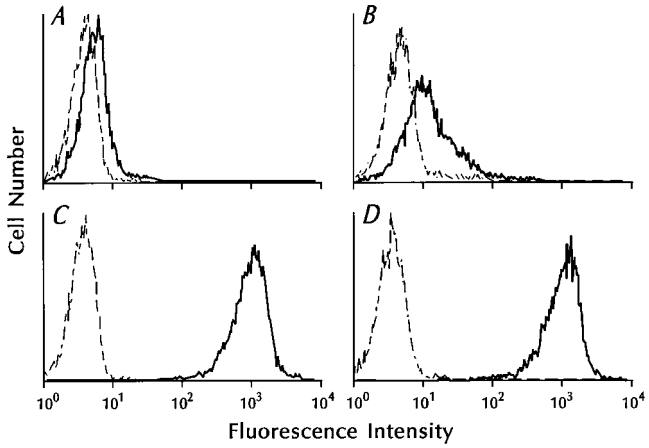


FIG. 7. Expression of BZLF2 on the surfaces of EBV-infected B cells does not diminish HLA-DR expression. Akata cells were analyzed by flow cytometry with a BZLF2-specific antipeptide serum (A and B) or a pan-HLA-DR MAb (C and D) under uninduced (A and C) or induced (B and D) conditions (in the latter case to allow productive EBV infection). The dotted lines represent the levels of staining seen with nonimmune serum (A and B) or an isotype-matched control MAb (C and D).

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