Mutations in the DNA-binding Domain of the Transcription Factor Bright Act as Dominant Negative Proteins and Interfere with Immunoglobulin Transactivation*

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Bright, for B cell regulator of immunoglobulin heavy chain transcription, binds A+T-rich sequences in the intronic enhancer regions of the murine heavy chain locus and 5′-flanking sequences of some variable heavy chain promoters. Most resting B cells do not express Bright; however, it is induced after stimulation with antigen or polyclonal mitogens. Bright activation results in up-regulation of μ transcription; however, it is not clear whether Bright function is critical for normal B cell development. To begin to address Bright function during B cell development, seven mutated forms of Bright were produced. Five of the seven mutants revealed little or no DNA binding activity. Furthermore, because Bright binds DNA as a dimer, two of the mutants formed complexes with wild type Bright and acted in a dominant negative fashion. Dominant negative Bright prevented the up-regulation of μ transcription in transfected Chinese hamster ovary cells transfected with wild type Bright. These data identify regions within Bright that are required for the DNA binding activity of Bright and for its function as a transcription factor.

The transcription factor Bright (or B cell regulator of IgH transcription) is a B cell-specific protein that was first discovered in a mature mouse B cell line, BCg3R1-d, as a mobility-shifted protein complex that caused 3–6-fold increases in μ heavy chain mRNA levels in response to stimulation with a T-dependent antigen and interleukin-5 (1, 2). Bright binds to A+T-rich regions of the intronic heavy chain enhancer that have been identified as matrix association regions and regions 5′ of some VH promoters, including the V1 S107 family gene (3, 4). The cDNA for Bright was isolated in 1995, and the protein was shown to interact with DNA as a multimeric complex that includes multiple copies of Bright (4).

Recent studies suggest that Bruton’s tyrosine kinase (Btk),1 the defective enzyme in xid (X-linked immunodeficiency disease) mice, is a component of the Bright DNA-binding complex (5). xid mice exhibit deficiencies in B cell development and abnormalities in immunoglobulin production (6, 7). Initial evidence indicated that the Bright DNA-binding complex was supershifted by antibodies that reacted with several different domains of Btk (5). Furthermore, Bright co-precipitated with Btk from wild type, activated splenic B cells, but Btk and Bright did not co-precipitate from xid B cells despite both proteins being present. These data suggested that Bright and Btk are functionally linked. However, the role of Bright in normal B cell development is unknown.

Expression of Bright in both mouse and human tissues is tightly regulated at the level of transcription (8, 9). In the mouse, Bright mRNA was detected in all embryonic tissues, but became B-lymphocyte restricted in the adult. Bright mRNA is apparent at the pre-B and activated germinal center stages of B cell differentiation (8). The human Bright homologue (10) shows a similar expression pattern in the adult where mRNA expression is primarily limited to B lymphocytes of the pro-B cell, germinal center centroblasts, and mature recirculating subpopulations (9). Thus, in both the adult mouse and human, Bright expression is predominantly B cell-specific.

Bright belongs to a growing family of proteins that bind DNA through an A+T-rich interacting domain (ARID) (11). ARID-containing proteins are highly conserved throughout evolution where many of them play important roles as developmental regulators (11). ARID-containing proteins occur in all eukaryotic organisms and include 13 sequences in the human data base (11, 12). Bright is the only member of the ARID family for which gene target sequences have been identified. One of the best characterized members of this family is the Dri (Drosophila homologue Dead Ringer) gene, first identified in 1996 (13). The ARID region of Dri is essential for anterior-posterior patterning and for muscle development in the fly embryo (14–16). The crystal structure of the ARID region of Dri has been solved and revealed a unit of eight α-helices with a short two-stranded anti-parallel β-sheet (17). NMR analysis predicted that helices 5 and 6 together with the intervening loop would contact DNA within the major groove (18). Because of the greater than 90% homology of Bright and Dri within the ARID region (19), the crystal structures may be quite similar. The crystal structure of Dri suggests regions of Bright that interact with DNA.

To learn how Bright regulation of the μ heavy chain is important for B cell development, we sought to produce mutations in Bright that were predicted to interfere with its function. Three important functions of Bright were targeted for our studies: DNA binding activity, protein interaction, and nuclear localization. Several point mutations made within the ARID
Dominant Negative Bright

phenylmethylsulfonyl fluoride (5 × 10−4 M), leupeptin (1 × 10−2 mg/ml), and aprotinin (5 × 10−2 mg/ml), as previously described (22), and the protein concentrations were determined using Bradford reagent (Bio-Rad). The proteins were incubated with a γ-32P-labeled DNA probe at 37 °C for 15 min, and the EMSAs were performed in 4% non-denaturing acrylamide gels, as previously described (2). The DNA probe was a 150-bp BamH1-FokI fragment from the S107 V 1·flanking sequence (bf150) (2) containing the prototypic Bright binding site. For supershift assays, antibodies and proteins were preincubated 5 min prior to probe addition. The antibodies used were: polyclonal rabbit anti-Bright (bf150) (2), polyclonal rabbit anti-CDP/Cux (gift of E. Neufeld, Yale University, New Haven, CT), and preimmune serum.

**Confluent Microscopy and Flow Cytometry**—The cells were harvested and washed in phosphate-buffered saline with 3% fetal calf serum. After fixation in 1% paraformaldehyde for 15 min at 37 °C, the cells were stained with polyclonal antibody purified goat and mouse Bright against the peptide ALHSGVLVLAGHAE from the amino-terminus domain of Bright. Preimmune sera were collected prior to immunization, and IgG was isolated as a negative control. Incubation with primary antibody was followed by rabbit anti-goat IgG-Alexa568 (Molecular Probes, Eugene, OR) for 15 min on ice. 4′,6′-diamidino-2-phenylindole dihydrochloride (Molecular Probes) was added to the cells to a final concentration of 0.5 μg/ml prior to drying. The cells were stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (Molecular Probes). The cells were then washed twice in phosphate-buffered saline containing 0.1% Tween 20 for 1.25 h at room temperature with 0.1 M Tris, 0.5 M NaCl, and 0.1% Tween 20 before suspension in SDS sample buffer. The samples were heated for 5 min at 95 °C and centrifuged briefly, and the supernatants were analyzed by Western blotting.

**Real Time and Reverse Transcription-PCR**—RNA was isolated using Trizol according to manufacturer’s protocol (MRC, Cincinnati, OH), treated with DNase (2 units) (Ambion, Austin, TX) for 30 min at 37 °C, subjected to phenol-chloroform extraction, and used to generate cDNA with the SuperScript II RNase H reverse transcriptase kit (Invitrogen). Immuno globulin transcription was measured by real time quantitative reverse transcriptase PCR using TaqMan Universal PCR Master Mix (Applied Biosystems) with 250 nm of specific primers and 5 pmol of TaqMan probe designed using the PrimerExpress software (Applied Biosystems) and obtained from Applied Biosystems or Integrated DNA Technologies (Washington, D.C.). TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase control reagents (Applied Biosystems) were used to ensure cDNA integrity. Expression of the V1 reporter construct was measured using the following primers and probe: 5′-CAAGTCGAAGCCCATCATCCTTG-3′ and 5′-CACTGGACGACACTGAGTTCGAGGAC-3′. The amplified products were examined by ethidium bromide gel electrophoresis, and the sequences were confirmed by DNA sequencing using the Oklahoma Medical Research Foundation sequencing core. Amplification reactions (25 μl) were performed in triplicate in 96-well plates under the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min and analyzed with the ABI Prism 7700 SDS (PE Applied Biosystems, Foster City, CA). V1 transcript expression was quantified from the average of triplicate samples using a standard curve derived from a V1 containing plasmid. Samples without reverse transcriptase were also analyzed to ensure that the amplified products did not result from the transcribed vector DNA. Calculations for V1 expression were performed according to the equation 2−ΔΔCT as suggested by the manufacturer. For

**Wild type and mutant Bright** were tagged on the carboxyl terminus with the myc-His sequence using the vector pcDNA4/TO/myc-His B (Invitrogen). Briefly, Bright was PCR-amplified with oligonucleotides that added a 5′ EcoRI site and a 3′ XbaI site while deleting the endogenous stop codon and was ligated into the EcoRI- and XbaI-digested vector fragment. Sequences were further subcloned into the MigR1 retroviral vector, allowing GFP expression from an internal enhancer in the retroviral vector. The amplified products were examined by ethidium bromide gel electrophoresis, and the sequences were confirmed by DNA sequencing using the Oklahoma Medical Research Foundation sequencing core.

**Western Blotting and Immunoprecipitation**—In vitro translated proteins were produced with TNT rabbit reticulocyte lysates (Promega, Madison, WI). The proteins were subjected to SDS-polyacrylamide gel electrophoresis under standard denaturing conditions through a 7.5% acrylamide gel and transferred to nitrocellulose membranes as previously described (5). Bright was detected with rabbit anti-Bright, and Btk was detected with goat anti-Btk (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by alkaline-phosphatase-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (Southern Bio-technology, Birmingham, AL), respectively. The blots were developed with alkaline-phosphatase substrate (Bio-Rad). Preimmune goat sera or mouse anti-Sp1 (Santa Cruz Biotechnology) were used as isotype-matched controls.

**Experimental Procedures**

**Cell Lines and Transfections**—Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 7% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 × 10−3 M 2-mercaptoethanol, and 1 mM sodium pyruvate. The M12g3Ri and BCg3R-iId cell lines, transfected derivatives of the murine B cell lines M12.4 and BC11B1, respectively, contained coding sequences for a T15 idotype immunoglobulin (1) and were maintained in RPMI 1640 with the same supplements. CHO cells were transfected using FuGENE (Roche Applied Science) according to the manufacturer’s directions. M12g3Ri cells were transfected by electroporation at 0.24 kV with a Gene Pulser (Bio-Rad). Transfected cells were maintained in complete RPMI 1640 with 10% fetal calf serum. In some cases, the cells were stimulated for 48 h with 10 mg/ml LPS (Sigma) to induce endogenous Bright expression after transfection.

**Transfection**—Cells were transfected with the retroviral vector allowing GFP expression from an internal enhancer in the retroviral vector. The MigR1 retroviral vector, allowing GFP expression from an internal enhancer in the retroviral vector. The MigR1 retroviral vector, allowing GFP expression from an internal enhancer in the retroviral vector.

**Mutant Construction and Expression Vectors**—A full-length mouse Bright cDNA clone in the plBCKMV (4) was used as a template to introduce single amino acid changes or small deletions in the Bright coding sequence through site-directed mutagenesis with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The PCR conditions used were followed by 17 cycles of 95 °C for 40 s, 60 °C for 1 min, and 68 °C for 16 min with an additional 20 min at 68 °C to complete the reaction. The mutations produced were: GGC to GCC, at amino acid position 286 (P286A), TGG to GGC, W299A; TTC to GCA, F317A; TAT to GCA, Y330A; REKLES at amino acids 455−460 was changed to AEAELA; and the nucleotides encoding the amino acids IRK at positions 402−404 were deleted. A double mutant (DP) was also generated that contained both the W299A and Y330A mutations. All of the sequences were verified using the Oklahoma Medical Research Foundation sequencing facility and Vector NTI software.

**Electrophoretic Mobility Shift Assays (EMSAs)**—The nuclear extracts were prepared by hypotonic lysis with the protease inhibitors

**Experimen...
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Intracellular Localization of Bright—Endogenous Bright exists in both the cytoplasm and nucleus in B lymphocytes where it associates with nuclear matrix proteins (24). To determine whether mutant Bright proteins exhibited altered intracellular localization, CHO cells were transfected with a construct that co-express GFP and His-Myc-tagged Bright proteins. Transfected cells were identified by GFP expression, and Bright was identified using an anti-Bright antibody and a secondary antibody conjugated to Alexa-568 (Fig. 3). The stained cells were viewed by confocal microscopy. Comparisons of cells transfected with wild type Bright or with mutant Bright containing the ARID mutations showed similar staining patterns with Bright protein most abundant in the nucleus. This observation indicates that mutation of the ARID domain does not adversely affect the ability of Bright to translocate to the nucleus. In contrast, the Bright location in cells expressing the K(---) mutant showed higher levels of cytoplasmic staining than was observed with either the wild type or ARID mutants. However, these cells also exhibited Bright staining within nuclear regions. Therefore, sequences other than the KIKK consensus may also contribute to the nuclear localization of Bright. Nevertheless, the mutant proteins largely behaved as predicted from the structure of Dri.

**RESULTS**

Creation of Bright Mutants—Bright has been subdivided into five protein domains according to predictions from amino acid homology and analysis of deletion mutants (4). The domains are depicted in Fig. 1 and include an acidic amino terminus of unknown function, the ARID domain predicted to be important for DNA binding activity, a putative activation domain containing a consensus nuclear localization sequence, a protein-protein interaction domain with a helix-turn-helix structure (23), and a short carboxyl terminus. Deletions of the acidic and carboxyl-terminal domains did not affect DNA binding or transcription activity of Bright (4). Site-directed mutagenesis was used to create amino acid changes hypothesized to affect the DNA binding function of Bright. Specifically, four large, bulky amino acids within the ARID domain predicted to be involved in DNA interactions (17) were independently mutated to alanine (Fig. 1). Two of these mutations were introduced simultaneously to create a fifth mutant called DP for double mutant. In addition, the putative nuclear localization sequence KIKK was altered by deleting the last three amino acids of that sequence K(---) (Fig. 1). Finally, because several ARID family members, including Bright, exhibit extended regions of amino acid homology within the putative protein interaction domain (4, 17), mutations were also made in the protein interaction domain to change amino acids in the sequence REKLES to the sequence AEALEA.

**Dominant Negative Bright**

![Fig. 1. Bright protein domain structure and mutations. Schematic diagram of Bright showing domain structure and the targeted mutations within the DNA-binding domain (shaded boxes).](image)

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efficiently inhibit the DNA binding activity of the native protein because extracts from cells expressing W299A with native Bright retained efficient DNA binding. On the other hand, the Y330A and DP mutants inhibited the majority of Bright DNA binding activity in this experiment. Further experiments showed no difference between the DP and Y330A mutants (not shown). These data show that dimers containing W299A mutant Bright can bind DNA, whereas dimers containing the Y330A mutation do not bind DNA efficiently. Because both the Y330A and wild type Bright were expressed at equivalent levels in these experiments (Fig. 5a, lower panel), the data show that dimers containing Y330A act in a dominant negative fashion and inhibit Bright DNA binding activity.

**Dominant Negative Bright Mutants Fail to Activate Immunoglobulin Transcription**—The data in Figs. 4 and 5 indicate that the Bright mutants are capable of forming heterodimers with native Bright and that they can interfere with DNA binding activity in EMSAs. We tested the ability of such dimers to function as dominant negative proteins by examining their ability to inhibit transcription induction by native Bright complexes. For these studies, native Bright and the various mutants were transfected into CHO cells along with a V1 immunoglobulin reporter gene (2). The V1 reporter contains the Bright recognition sequence such that enhanced expression of the V1 gene is dependent on co-expression of Bright. Real Time PCR analyses of V1 immunoglobulin transcripts indicated that wild type Bright increased transcription of the V1 heavy chain immunoglobulin locus by 5–7-fold (Fig. 6). This level of transcription induction is consistent with previous observations of Bright activity (2, 4). In contrast, co-expression of the DP mutant with wild type Bright reduced V1 heavy chain transcription by 85% relative to wild type Bright alone. These findings are consistent with the interpretation that the Bright mutants act in a dominant way to prevent transcription of the V1 target gene.

**Mutants Inhibit Endogenous Bright DNA Binding Activity in B Cells**—Our data show that heterodimers containing wild type and mutant Bright suppress Bright transcription activation in transfected cell lines of nonhematopoietic origin. To determine whether these mutants functioned as dominant neg-
atives by inhibiting endogenous Bright function in B lymphocytes, the Myc-His-tagged proteins were expressed in M12g3Ri cells. This cell line constitutively expresses low levels of endogenous Bright. The cells were stimulated with LPS after transfection to induce new production of native Bright protein. Transfectants expressing Bright mutants were isolated by flow cytometry based on GFP expression. Nuclear proteins were isolated from the sorted cells, assessed by Western blot, and analyzed by EMSA for Bright DNA binding activity (Fig. 7). Transfected Bright proteins (upper bands) were expressed at slightly higher levels than endogenous Bright as demonstrated by Western blot (Fig. 7a). Therefore, Bright expression is not grossly altered by this protocol.

The M12g3Ri cell line expresses two complexes that interact with the Bright binding site, NF\(\mu\)NR, which contains CDP and Bright as shown by EMSA for the mock-transfected cells (Fig.
The upper NFκB complex was previously described by our lab and others as a protein complex that competes with Bright by binding to DNA sequences that overlap the Bright motif (2, 4, 25). Anti-Bright supershifted all of the Bright bands but did not affect the CDP-containing NFκB complex. Conversely, anti-CDP abrogated binding of the upper complex without affecting Bright. Thus, the NFκB complex serves as an internal control. Antibodies to the Myc tag were used to specifically detect Bright complexes containing the DP and wild type Bright mutant proteins. The addition of anti-Myc antibody did not affect the mock-transfected complexes but supershifted the Bright bands from the wild type transfected cells. This indicates that the transfected, tagged Bright formed heterodimers with endogenous Bright in the B cells. The DP-transfected extracts exhibited reduced levels of Bright complexes relative to the mock-transfected populations (Fig. 7b, compare first and ninth lanes) consistent with the idea that heterodimeric complexes containing DP Bright were incapable of binding DNA. The low intensity bands remaining in the ninth, eleventh, and twelfth lanes did not react with anti-Myc antibodies and likely reflect endogenous Bright dimers formed prior to LPS stimulation. These experiments suggest that DP Bright functions as a dominant negative protein in B cells by interfering with newly expressed endogenous Bright DNA binding activity.

The DP Mutation within Bright Does Not Alter the Ability of Bright to Associate with Btk—Our previous data showed that wild type Bright interacts with Btk and that Btk is present within the DNA binding complex of Bright (5). Therefore, we asked whether the mutations we produced in the ARID and activation domains affected this association. Wild type Btk and carboxyl-terminal Myc-His-tagged Bright proteins were co-expressed in CHO cells. Immunoprecipitations with anti-Myc antibodies were performed, and the precipitated protein was blotted for Bright and Btk (Fig. 8). In each case, Btk was pulled down with Bright. The data reveal that these mutations within Bright do not interfere with the ability to interact with Btk. Furthermore, these results suggest that DNA binding activity of DP Bright is critical for its function as a transcription activator.

DISCUSSION

To increase our understanding of the function of the B cell-restricted transcription factor Bright, seven mutants were gen-
FIG. 8. Bright mutants associate with Btk. Proteins from CHO cells co-transfected with Myc-His-tagged Bright and wild type Btk were immunoprecipitated with anti-Myc antibody and developed with anti-Bright (top row) and anti-Btk (bottom row). The left panel (Load) represents 10 μg of whole cell extract from each transfection, and the right panel (IP) represents the immunoprecipitations, both developed with anti-Bright or anti-Btk (C-20). The data are representative of three experiments.

Dominant Negative Bright

The ARID domain of Dri, the homologue of Bright, is predicted to contact DNA. To assess the dominant negative activity of the DP mutant expressing endogenous Bright, we first determined whether the DP Bright mutant functions as a dominant negative protein. To this end, we performed experiments in which dominant negative Bright was introduced into LPS-stimulated B cells by retroviral transduction concomitantly with induction of native Bright, allowing transduction with retroviral vectors. Although several of the ARID mutants failed to bind DNA when expressed in non-B cells, only a few of those mutants effectively inhibited binding of wild type Bright to DNA. In addition, our data also implicate specific sequences, such as the ARID domain, in the ability of Bright to form heterodimers as supported by the ability of the mutants to co-purify with native Bright. Furthermore, the data presented here show that the DP Bright mutant interferes with native Bright function in a transcription assay using an immunoglobulin promoter reporter assay. Thus, the DP mutant functions as a dominant negative protein.

Assessing the dominant negative activity of the DP mutant in B cells was more complicated. In a transfected B cell line that expressed constitutive Bright protein, no effect was observed unless the cells were first stimulated with LPS to induce enhanced levels of new endogenous Bright synthesis prior to transfection with dominant negative Bright. In this case, heterodimer formation of endogenous and tagged mutant Bright was possible. However, endogenous Bright dimers that were already present in the cell line did not appear to be affected by introduction of the dominant negative protein. These data imply that once formed, Bright dimers are relatively stable, but that the ARID mutants do not compete well for binding with native Bright that already exists as a dimer. To fully understand the effects of the dominant negative protein on wild type Bright function, the two proteins may need to be expressed simultaneously at equal levels.

Unstimulated splenic B cells do not express Bright protein (8). However, Bright activity is induced in splenic B cells stimulated with LPS in culture. LPS stimulation also induces B cell proliferation, allowing transduction with retrovirual vectors. Although we performed experiments in which dominant negative Bright was introduced into LPS-stimulated B cells by retroviral transduction concomitantly with induction of native Bright, LPS also induces plasma cell differentiation and isotype
switching (28–30). Deletion of the \( \mu \) locus during isotype switching made it impossible to determine whether DN Bright interfered with endogenous \( \mu \) transcript expression in these stimulated B cells. Indeed, RNA from DP transduced cells analyzed by real time PCR for \( \mu \) heavy chain transcripts showed no significant difference in \( \mu \) heavy chain transcription in three of four experiments compared with control virus transduced cells (data not shown). Thus, assessing the activity of DP Bright in native B cells in vitro has not been possible.

Bright is also expressed in pre-B cells in the mouse (8), and its function in those cells is unknown. Our data confirm the ability of DP Bright to interfere with transactivation of an immunoglobulin reporter gene, suggesting that it acts as a dominant negative protein in vitro. Production of a transgenic model expressing this mutant where dominant negative Bright can be expressed earlier than the endogenous wild type protein should allow future studies to determine whether DP Bright interferes with \( \mu \) heavy chain transcription in normal B cells. Such mice will ultimately provide important information regarding Bright function in B cell differentiation.

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