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The Transcription Factor Bright Associates with Bruton’s Tyrosine Kinase, the Defective Protein in Immunodeficiency Disease

Carol F. Webb,2a‡ Yoshio Yamashita,‡ Neil Ayers,* Seth Evetts,* Yolene Paulin,* Mary Ellen Conley,§ and Elizabeth A. Smith*

Binding of the transcription factor Bright to Ig heavy chain loci after B cell activation is associated with increased heavy chain transcription. We now report that Bright coprecipitates with Bruton’s tyrosine kinase (Btk), the defective enzyme in X-linked immunodeficiency disease (xid). Furthermore, we observed Btk in the nucleus of activated murine B cells, and mobility shift assays suggest that it is a component of the Bright DNA-binding complex. While Bright protein was synthesized in activated spleen cells from xid mice, it did not bind DNA or associate stably with Btk. These data suggest that deficiencies in Bright DNA-binding activity may contribute to the defects in Ig production seen in xid mice. The Journal of Immunology, 2000, 165: 6956–6965.

Bright (B cell regulator of Ig heavy chain transcription) is a 70-kDa B cell-restricted protein that interacts with A+T-rich sequences in the intronic enhancer of the murine heavy chain locus and 5'-flanking sequences of some variable heavy chain (VH) promoters (1, 2). It was originally identified in an Ag-specific B cell line, BCg3R-1d, as a mobility-shifted complex induced after stimulation with IL-5 and Ag (3) that correlated directly with increased Ig heavy chain mRNA levels (1, 3). Transfection experiments confirmed Bright’s ability to up-regulate Ig transcription in mature B cell lines through interactions with the heavy chain intronic enhancer (2, 4). Bright expression during normal B cell differentiation appears to be tightly regulated and is not limited to mature or activated B cell stages, as was indicated by studies using cell lines (5). Indeed, Bright mRNA is expressed in the fetal liver and in early lymphoid progenitor cells from the bone marrow, where it is most abundant in pre-B cells. However, Bright mRNA is not detected in pro-B cells or immature B cells (5). Furthermore, unstimulated splenic B lymphocytes do not contain detectable levels of Bright, but can be induced with LPS stimulation for 2–3 days to produce active Bright mobility-shifted complexes (5). Bright presumably functions to up-regulate Ig heavy chain transcription in activated B cells, but its role in early B cell development is unclear.

Appropriate expression and regulation of Ig synthesis are critical for normal B cell development both during early stages of differentiation, where its absence may lead to blocks in B lymphocyte development (6–8), and at later stages, where maintenance of Ig expression appears to be critical for mature lymphocyte survival (9). X-linked immunodeficient (xid) mice have reduced numbers of B lymphocytes due to blocks in B cell differentiation at the pre-B to immature B cell stage, resulting in abnormally low levels of serum Ig (10). Both xid and the human immunodeficiency disease X-linked agammaglobulinemia (XLA) (11) are caused by mutations in the gene encoding Bruton’s tyrosine kinase, or btk, located on the X-chromosome (12–15). Btk is a member of the tec family of tyrosine kinases and contains an amino-terminal pleckstrin homology domain (PH), a Tec homology domain (TH), two Src homology domains (SH3 and SH2), and an SH1, or kinase domain, at the carboxyl end. It is found in the cytoplasm of B lymphocytes throughout differentiation, except in terminally differentiated plasma cells (16, 17). The primary defects observed in xid mice are in the B lymphocyte lineage, but monocytes, platelets, and erythrocytes also express Btk. It has been clearly shown in knockout mice that disruption of btk is sufficient to cause the xid phenotype (18–20), but it is not known how the defective kinase exerts its effects on B lymphocyte differentiation and Ig expression.

While a wide variety of mutations in btk have been observed in XLA patients (21–24), a single amino acid change in the PH domain of Btk results in the xid defect (14, 15). Interestingly, this mutation does not affect the in vitro kinase activity of the protein, but may interfere with the ability of Btk to translocate to the cell membrane and/or to interact with critical substrate(s) in vivo. In normal B lymphocytes, cross-linking of the IL-5 (25), or Ag receptors (26–28), leads to rapid translocation of Btk to the cell surface membrane, where it becomes autophosphorylated and activated through interactions with other kinases (29–31). The PH domain of Btk has been shown to bind phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P3) at the cell membrane, where it regulates calcium flux pathways in conjunction with the inositol phosphatase, Src homology 2 domain-containing inositol phosphatase (32–35). Xid Btk binds PtdIns-3,4,5-P3 poorly, and xid B cells do not sustain increased calcium influxes after signaling through the

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2 Address correspondence and reprint requests to Dr. Carol F. Webb, Oklahoma Medical Research Foundation, 825 NE 13th Street, Mail Stop 29, Oklahoma City, OK 73104. E-mail address: Carol-Webb@omrf.ouhsc.edu
3 Abbreviations used in this paper: xid, X-linked immunodeficient; XLA, X-linked agammaglobulinemia; PH, pleckstrin homology domain; TH, Tec homology domain; SH, Src homology domain; PtdIns-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; PI, phosphatidylinositol; TFII-I, transcription factor II-I; Nf145NR, nuclear factor-κ light chain enhancer, P1, Bruton’s tyrosine kinase; CDP, CAAT-displacing protein.
Ig receptor (32–34). While the important role Btk plays immediately after Ig receptor signaling is well documented, it may have additional functions. It associates with several proteins, including protein kinase C (36), G proteins (37, 38), c-Cbl (39), and a few Src-type kinases (40, 41). In addition, Btk phosphorylates the transcription factor BAP135, or transcription factor II-I (TFII-I), in activated B lymphocytes and may regulate its activity (42–44). Thus, Btk may participate in a number of complex pathways.

While studying the signaling events necessary to induce Bright expression, we noted that the tyrosine kinase inhibitor, genistein, reduced Bright complex formation. Furthermore, many of the stimuli that induced Bright EMSA complexes in splenic B cells (including IL-5, Ag, LPS, and Abs to CD38 and RP105) were among those reported by others to give abnormal responses in xid mice (10, 45, 46). In addition, Bright expression in sorted populations of normal B cells (5) coincided with stages in B cell differentiation when Btk was reported to be activated (10). Therefore, we investigated whether xid spleen cells could be stimulated to produce Bright DNA-binding complexes. We present evidence that Btk is physically associated with the transcription factor Bright in control B cells, and that this association is abnormal in xid B cells. Our data suggest a potential mechanism by which mutations in Btk can lead to defects in Ig synthesis. Furthermore, our results suggest that Bright functions downstream of Btk and that Btk may have previously unappreciated roles within the nucleus.

Materials and Methods

Mice and cell culture
Six- to 8-wk-old male CBA/CaHN-BTK(xid)/l (xid), CBA/J mice, female 129sv×Btktm1k (Btk+/−), and 129SV mice from The Jackson Laboratory (Bar Harbor, ME) were used by 12 wk of age. Whole spleens from several mice were teased into single-cell suspension, pooled, washed, and resuspended at 106 cells/ml in RPMI 1640 with l-glutamine, 20% heat-inactivated FBS, 100 μM penicillin, 100 μg/ml streptomycin, 5 × 10−5 M 2-ME, and 1 mM sodium pyruvate (47). Xid and CBA/J control animals were adjusted to contain the same total number of CD19+ B cells. LPS from Escherichia coli 0111:B4 (Sigma, St. Louis, MO) at 10 μg/ml or one wild-type or CD40 ligand-expressing baculovirus-infected SP9 cells per 10 B cells (48) were added and the cultures were maintained in 7% CO2 at 37°C for 1–4 days. In some cases spleen cells were enriched for B lymphocytes by anti-Thy-1.1 and complement-mediated depletion (gift from Dr. J. Kearney, Birmingham, AL) followed by separation over Nycodenz (Nycodem, Oslo, Norway). BCg3R-1d, EL-4, and CHO cells were maintained in RPMI 1640 or DMEM with 7% FCS as previously described (3). Stable transfectants were produced using modified pBK-Bright6 (2) with Cugene 6 (Roche, Indianapolis, IN) and were cloned by sorting one cell per well with a FACStarPlus (Becton Dickinson, Mountain View, CA). Genistein (ICN Biochemicals, Costa Mesa, CA) in DMSO was used at 25 μg/ml; solvent alone was used as a control. Cell viability, assessed by trypan blue exclusion, was >88% at this concentration.

Nuclear extracts and mobility shift assays

Nuclear extracts were prepared by hypotonic lysis, protein concentrations were determined with Bradford reagents (Bio-Rad, Richmond, CA), and EMSAs were performed in 4% nondenaturing acrylamide gels after incubation for 15 min at 37°C with γ-32P-labeled probes as previously described (49). The prototypic Bright binding site (bfl50) from the S107 V1 5′-flanking sequence (1), a 145-bp fragment of the BCL1 heavy chain promoter containing the octamer site (49), and an oligonucleotide from the κ intronic enhancer (5′-ATCTCAACAGAGGGGACTTTCCGAGAGCCA-3′) containing the −5′-B site were used as probes. Abs were added 5 min before incubation with DNA probes and were polyclonal rabbit anti-Bright (gift from Dr. P. Tucker, University of Texas, Austin, TX), affinity-purified goat anti-peptide (p29) serum reactive with the amino terminus of Bright (5), preimmune goat serum, mouse monoclonal anti-Btk ascites reactive with the PH-TH domain of human Btk (Upstate Biotechnology, Lake Placid, NY), polyclonal rabbit antisera reactive with the SH3-SH2 domains of Btk (50), control ascites SK7094, and polyclonal rabbit anti-CD19/Cux (gift from Dr. Ellis Neufeld, Yale University, New Haven, CT) reactive with nuclear factor-κ negative regulator (NFκNR) (4). Phosphatase-coated beads were used according to the manufacturer’s directions (Upstate Biotechnology).

Western blots and immunoprecipitations

Proteins were subjected to SDS-PAGE under standard denaturing conditions in 7.5% acrylamide, transferred to nitrocellulose membranes, and blocked with 5% BSA in 0.5% gelatin and 0.05% thimerosal as previously described (51). In vitro translated proteins were produced using TNT rabbit reticulocyte lysates (Promega, Madison, WI) as previously described (5, 49). Bright was detected with polyclonal rabbit anti-Bright and alkaline phosphatase-labeled goat anti-rabbit Ig (Southern Biotechnology Associates, Birmingham, AL). Btk was detected with monoclonal anti-Btk (PH-TH) as described above or with affinity-purified goat anti-peptide antisera (C20) reactive with the kinase domain (Santa Cruz Biotechnology, Santa Cruz, CA). Alkaline phosphatase-conjugated goat anti-mouse Ig or rabbit anti-goat IgG (both from Southern Biotechnology Associates) were used as secondary reagents and were preadsorbed against nuclear extracts from BCg3R-1d to increase specificity. Substrate was purchased from Bio-Rad (Hercules, CA). Immunoprecipitations were performed with Protein A/G Plus-agarose beads according to the manufacturer (Santa Cruz Biotechnology). Each of the anti-Bright and anti-Btk reagents described above was used for immunoprecipitation, but was developed with reagents that reacted with different domains than those used for immunoprecipitation to ensure specificity. Duplicate blots were developed with secondary reagents as described above. The anti-phosphotyrosine reagents used were monoclonal PY99 (Santa Cruz Biotechnology), BC20H anti-Pyr:HRP (Transduction Laboratories, Lexington, KY), and monoclonal IgG2a (Upstate Biotechnology).

Immunofluorescence, flow cytometry, and confocal microscopy

Cells in suspension (5 × 104 cells/200 μl) were incubated for 20 min on ice with tetramethylrhodamine isothiocyanate-anti-IgD, FITC-anti-IgM (Southern Biotechnology), or anti-CD19, 1D3 (PharMingen, San Diego, CA), with FITC-labeled goat F(ab′)2, anti-rat IgG (H+L), Propidium iodide (PI) was included as a gating parameter to identify dead cells. Rat IgG2a-biotin was used as an isotype-matched control for anti-CD19. Sorted cells were stained, and nuclear extracts were prepared as described above. For nuclear sorting, cells were first panned on anti-Mac-1-coated plates to remove monocytes (5) and showed <1% Mac-1+ cells by FACS analyses. Nuclei were prepared as described above and were stained with FITC-anti-κ and -λ to detect cytoplasmic Ig and with PI to identify individual nuclei. Cytoplasm-free nuclei were gated as FITC− and PI+ events using a FACStar Plus (Becton Dickinson) and resulted in >98% pure populations by postsort analyses. Sorted nuclei were centrifuged and resuspended directly in SDS-sample buffer at a concentration of 5 × 107/20 μl. Confocal microscopy was performed using a Leica TCS NT confocal system (Heidelberg, Germany) with four-laser excitation, and images were analyzed using Leica TCS software. Cells were fixed in 3.7% formaldehyde in PBS, permeabilized for 5 min with 0.05% Triton X-100, washed, stained with rabbit anti-IgM-Tritc and undiluted monoclonal ascites anti-Btk (PH-TH), washed twice, and stained with goat anti-mouse IgG1-FITC, Anti-T15 Id Ab, AB1.2, or control ascites were isotype-matched controls. Cells were mounted using Prolong Antifade (Molecular Probes, Eugene, OR), and consecutive sections were scanned at 2-μm intervals at >100 magnification. Fluorescence sorting and confocal microscopy were performed at the Flow Cytometry, Cell Sorting, and Confocal Microscopy Laboratory, Oklahoma Center for Medical Medicine, University of Oklahoma Health Sciences Center.

Results

Induction of Bright DNA-binding activity requires a tyrosine kinase

Our previous studies showed that Bright induction in the BCg3R-1d cell line required both Ag and IL-5 (3). Signaling through both these receptors initiates a series of intracellular events requiring tyrosine kinases. Fig. 1 shows that addition of genistein to BCg3R-1d cells in culture reduced levels of Bright mobility-shifted complexes (Fig. 1A). Binding of other transcription factors, including Oct-1, was not affected by genistein (Fig. 1B), suggesting that genistein treatment did not result in general protein degradation. Genistein has been reported to inhibit topoisomerase II activity as well as tyrosine kinases (52). Because previous studies showed serologic cross-reactivity between Bright

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The amount of nuclear extract was increased to 12 μg/lane (Fig. 2A) as an octamer-containing probe (B) and 5 μg of nuclear extract from uninduced (control) and IL-5- plus Ag-induced (IL-5 + Ag) BCg3R-1d cells maintained for 24 h with or without genistein (Gen). Labeled probe without extract was loaded in the first lane of each panel. Bright and Oct-1 are indicated by arrows, and Oct-2 is the mobility-shifted species below Oct-1. Results obtained using stimulated CBA/J spleen cells were similar. Addition of genistein to in vitro translated Bright (I.V.T. Bright) before EMSA did not inhibit Bright binding. Data are representative of three experiments.

FIGURE 1. The tyrosine kinase inhibitor, genistein, decreased Bright binding activity. EMSAs were performed with bfl50 (A) or an octamer-containing probe (B) and 5 μg of nuclear extract from uninduced (control) and IL-5- plus Ag-induced (IL-5 + Ag) BCg3R-1d cells maintained for 24 h with or without genistein (Gen). Labeled probe without extract was loaded in the first lane of each panel. Bright and Oct-1 are indicated by arrows, and Oct-2 is the mobility-shifted species below Oct-1. Results obtained using stimulated CBA/J spleen cells were similar. Addition of genistein to in vitro translated Bright (I.V.T. Bright) before EMSA did not inhibit Bright binding. Data are representative of three experiments.

and topoisomerase II (51), we considered that genistein might directly inhibit interactions of Bright with DNA. However, addition of genistein to previously isolated extracts containing Bright activity did not significantly affect binding of Bright to DNA (Fig. 1C). These data demonstrated that tyrosine kinase activity is required for the induction of functional Bright complexes.

Xid spleen cells respond to LPS and CD40 ligand, but fail to produce stable Bright mobility-shifted complexes

To determine whether B cells from mice producing the defective kinase, Btk, could produce Bright, normal adult spleen cells from either xid mice or CBA/J controls were cultured for 2–3 days with or without 10 μg/ml LPS or with wild-type or CD40 ligand-expressing SF9 insect cells (48). As expected, Bright DNA-binding activity was undetectable in unstimulated cultures (Fig. 2A). Both LPS and CD40 ligand stimulation induced the low abundance Bright EMSA complexes in spleen cells from control CBA/J mice (Fig. 2A), and Bright activity was routinely detected using <1 μg of these nuclear extracts. However, neither stimulus produced stable Bright mobility-shifted species from xid spleen cells, even if the amount of nuclear extract was increased to 12 μg/lane (Fig. 2A). Bright activity was evident by 24 h after LPS or CD40 ligand stimulation in CBA/J extracts, but was not detected in nuclear extracts prepared from xid mice at any time point from 24 h to 4 days after stimulation. No differences in induction of Bright DNA-binding activity were observed in spleen cells from males vs females or from any strain examined, including BALB/C, 129/SV, C57BL/6, and CBA/J mice. However, paralleling our results using xid mice, nuclear extracts from btk−/− mice, which do not produce Btk (19), also failed to show stable Bright mobility shifts (not shown). These data demonstrated that Btk is important for Bright function.

We asked whether other transcription factors that interact with the Ig gene loci and are induced after B cell activation (53–55) were induced in xid cells in our culture system. Others have shown normal induction of NF-κB in small resting B cells from xid mice within 4 h of treatment with LPS or anti-μ (56). The xid nuclear extracts shown in Fig. 2A exhibited abundant induction of both octamer binding proteins and NF-κB after stimulation with either LPS or CD40 ligand by EMSA (Fig. 2B) and are consistent with results reported by others (57). Oct-2 levels were increased relative to Oct-1 levels in both the control and xid splenic B cells in response to LPS, demonstrating that the xid spleen cells were sensitive to LPS. Therefore, other transcription factors that are induced upon B cell activation appeared to be induced normally in the xid cultures.

Xid cells synthesize Bright protein after stimulation

One possible explanation for the failure to observe Bright activity from xid cells is that the xid cells remain immature in phenotype even after LPS activation. Indeed, wild-type immature B cells do not express detectable levels of Bright mRNA (5). Therefore, we asked whether Bright protein synthesis had occurred in stimulated xid cells despite the absence of mobility-shifted complexes. Western blots performed with polyclonal anti-Bright serum identified one 70-kDa band using in vitro translated Bright and a triplet of bands that differed only slightly in size in extracts of the B cell line, BCg3R-1d (Fig. 2C), suggesting that Bright may undergo post-translational modification in B cells. Although Bright was not detected in unstimulated cells from any strain (as demonstrated for the btk−/− strain; Fig. 2C, lane 9), Western blots of extracts from both CBA/J and xid spleen cells stimulated with CD40 ligand (shown in Fig. 2C) revealed the same triplet of Bright proteins observed in the BCg3R-1d cell line. Bright was also evident in extracts from CD40 ligand-stimulated btk−/− spleen cells that totally lack Btk protein (Fig. 2C, lane 6). Although the amount of Bright protein may be slightly less in xid than in normal cells, the levels of Bright did not show significant differences among the strains compared with the relative levels of Btk present in the same samples (lower panel). These data show that Bright protein synthesis can occur in the absence of Btk, although Btk is required for production of Bright complexes capable of binding DNA.

To ensure that the abnormal Bright binding activity we observed in the xid cells was not merely a reflection of a lower percentage of xid spleen cells becoming activated, we sorted IgD−, IgMlow B cells from highly enriched B cell fractions of both xid and CBA/J spleen cells after stimulation with LPS or CD40 ligand. Earlier studies showed that the mature IgD− cells were highly enriched for Bright (5). FACS analyses indicated that both the stimulated xid and CBA/J cultures contained similar percentages of IgD−, IgMlow cells (Fig. 3A). However, only the nuclear extracts from sorted xid cells consistently failed to yield the Bright mobility-shifted complexes observed in sorted CBA/J extracts under standard conditions at pH 8.3 (Fig. 3B). Other nuclear proteins, including the octamer proteins shown in Fig. 3B, were readily detected in the sorted xid cells. Although inconsistent and weak mobility-shifted complexes of a faster mobility that contained Bright were observed when the assays were performed at pH 8.9 (not shown), Bright complexes from CBA/J extracts also showed enhanced binding at pH 8.9. Therefore, while Bright complexes may form under some conditions in xid B cells, they differed qualitatively and quantitatively from those observed using wild-type extracts.

Bright and Btk are associated in control, but not in xid, B cells

The above experiments demonstrated that production of wild-type Bright DNA-binding activity was dependent upon events that involved Btk, but did not address whether Bright and Btk interact directly or whether Bright activation occurred through interaction with intermediates that were activated by Btk. Therefore, immunoprecipitation experiments were performed to determine whether
Bright associates with Btk (Fig. 4A). Both affinity-purified anti-Bright (p29) peptide Abs and polyclonal anti-Btk (SH3-SH2) serum precipitated Bright from extracts of the BCg3R-1d cell line (Fig. 4A, lanes 1 and 2). As expected, anti-Btk (SH3-SH2) did not precipitate Bright from CD40 ligand-stimulated btk−/− extracts (Fig. 4A, lane 3) that contained Bright but lacked Btk, confirming that anti-Btk serum did not immunoprecipitate Bright directly. Similarly, Bright and Btk could be coprecipitated from CD40 ligand-stimulated CBA/J cells (Fig. 4B, lanes 3 and 4), but not from unstimulated cells that contained no detectable Bright protein.

**FIGURE 2.** Stable Bright mobility-shifted species were not produced in xid splenic B cells in response to LPS or CD40 ligand despite the presence of Bright protein. A, EMSAs were performed with the prototypic Bright binding sites (bf150) and 6 μg (except where indicated otherwise) of nuclear extracts prepared from CBA/J or xid spleen cells maintained for 48 h in medium alone, with 10 μg/ml LPS with CD40 ligand-expressing SF9 insect cells, or with wild-type virus-infected SF9 cells at a ratio of 1:10 B cells. The BCg3R-1d cell line treated with IL-5 + Ag was used as a positive control for Bright (arrow). B, EMSAs were performed exactly as described (A) with DNA probes containing the octamer consensus site or an oligonucleotide from the κ intronic enhancer. Binding of the octamer factors Oct-1, Oct-2, an Oct-1 dimer (Oct-1d) (76), and NF-κB are indicated. Data are representative of five separate experiments. C, In vitro translated Bright, human Oct-1, and nuclear extracts from the B and T cell lines, BCg3R-1d and EL-4, were Western blotted with polyclonal rabbit anti-Bright (lanes 1–4). Thirty-five micrograms of extract from xid, CBA/J, 129/SVJ, and Btk−/− spleen cells cultured for 48 h with CD40 ligand exhibited Bright protein (lanes 6–8 and 10), while unstimulated cells from Btk−/− mice did not (lane 9). A seroreactive triplet was evident in all stimulated spleen cell extracts and the BCg3R-1d positive control. A duplicate blot developed with anti-Btk (C20) revealed Btk in all samples except those from Btk−/− mice (bottom panel, lanes 5–10). Similar data were obtained in four separate experiments.
A octamer-containing fragment (BCg3R-1d) extracts were used as positive controls. These data are representative of three experiments.

In contrast, Bright and Btk did not coprecipitate in similar immunoprecipitation experiments using CD40 ligand-stimulated extracts from xid B cells (Fig. 4B). Anti-Btk antisera efficiently precipitated the mutated form of Btk from xid extracts (Fig. 4B, lane 2, lower panel), showing that the xid point mutation did not affect the ability of the anti-Btk antisera to react with the SH3-SH2 domains of Btk. Likewise, Bright was immunoprecipitated with anti-Bright from the same extracts (Fig. 4B, lane 1), but Btk did not coprecipitate with it. These data are consistent with the idea that the xid mutation prevents the formation of stable Btk complexes.

Btk is a component of the Bright complex

To determine whether Bright’s ability to bind DNA depended upon phosphorylation by Btk, we treated B cell extracts with tyrosine-specific phosphatase-coated beads before analysis by mobility shift. Phosphatase treatment failed to inhibit Bright binding activity, but did inhibit binding of the T cell complex NFκB.

FIGURE 3. Xid B cells stimulated with LPS give rise to IgD+ IgM− cells that also lack Bright binding activity. A, FACS profiles of IgM− vs IgD-stained splenic B lymphocytes from both CBA/J and xid mice are shown 48 h after stimulation with LPS. B, Mobility shifts of nuclear extracts prepared from the IgD+ IgM− sorted xid and CBA/J B cells in gate R2 shown in A using either the bf150 Bright binding site (left) or the octamer-containing fragment (right). BCg3R-1d extracts were used as positive controls. These data are representative of three experiments.

FIGURE 4. Bright and Btk coprecipitated from control B cells, but not from xid or Btk−/− cells. A, Immunoprecipitations performed with 350 μg of protein (lanes 1–3) and anti-Btk (SH3-SH2) serum precipitated Bright from IL-5–plus Ag-activated BCg3R-1d cells (lane 1), but not from Btk−/− cells stimulated with CD40 ligand (lane 3). Bright was also precipitated by affinity-purified BCg3R-1d cells (lane 2). Lane 4 contained 35 μg (1/10th) of the Btk−/− extract used for the precipitation in lane 3 and demonstrated the presence of Bright protein in the starting sample. Bright was detected with polyclonal rabbit anti-Bright serum. B, Protein extracts from CD40 ligand-stimulated xid and CBA/J cells (lanes 1–4), and from control stimulated (SF9) CBA/J cells (lanes 5 and 6) were immunoprecipitated (I. P. anti-Bright and I. P. anti-Btk) exactly as described in A, and duplicate blots were developed with polyclonal anti-Bright (top panel) or anti-Btk (C20; bottom panel). Extract from BCg3R-1d cells was loaded in lane 7 as a positive control. Btk and Bright are indicated by arrows, and molecular size markers are indicated on the left. Data are representative of four experiments.

[Image of FIGURE 3 and FIGURE 4]
sates also contain some endogenous Btk, as exhibited by reactivity with anti-Btk Abs (Fig. 5C). We reasoned that if Bright were dependent upon Btk for binding, addition of exogenous Btk to suboptimal levels of Bright should enhance Bright binding activity. Indeed, dilutions of in vitro translated Bright exhibited enhanced binding by mobility shift analyses after addition of E. coli-expressed Btk. Addition of identical quantities of BSA, GST, or GST-tagged Jun (not shown) did not affect Bright binding activity. These data suggest that Btk associates with Bright to form a DNA-binding complex.

To investigate whether Bright complexes could be formed from eukaryotic cell extracts in the absence of Btk, we isolated a number of stably transfected CHO cell lines that expressed varying levels of Bright protein by Western blotting (Fig. 5D). None of the nuclear extracts from the 10 cell lines examined (Fig. 5E) or from similar stable transfectants produced from COS cells (not shown) showed formation of stable EMSA Bright complexes. Furthermore, coexpression of Btk in these transfectants did not reproduce Bright binding activity (not shown). Although the reasons for our failure to reconstitute Bright binding activity in non-B cell lines remain obscure, the additional mobility-shifted complex observed in these cells comigrates with and shares Ab reactivity with NFκB in EL-4, a complex previously shown to have inhibitory effects on Bright activity (4). However, the ability to reconstitute Bright activity in vitro (Fig. 5D) suggests that Btk is critical for formation of the Bright DNA-binding complex and is consistent

FIGURE 5. Btk is a component of the DNA-bound Bright complex. A, EMSAs were performed with bfl50 and BCg3R-1d (B cell) or EL-4 (T cell) extracts after or without pretreatment with the tyrosine-specific phosphatase indicated. Data are representative of three assays. B, IL-5- plus Ag-stimulated BCg3R-1d extracts (left panel) or in vitro translated Bright (right panel) were incubated with Abs to either Bright or Btk before mobility shift analyses. The Abs used were polyclonal rabbit anti-Bright (α-Bright), affinity-purified goat anti-peptide 29 reactive with Bright (α-P29), anti-Btk monoclonal ascites (α-Btk(PH-TH)), polyclonal rabbit anti-Btk (α-Btk(SH3-SH2)), control ascites, preimmune goat serum, and rabbit anti-CDP (α-CDP). Bright complexes are indicated by the arrows. C, A Western blot of proteins from EL-4, BCg3R-1d, in vitro translated Bright (I.V.T. Bright), and in vitro translated green fluorescence protein-tagged Btk developed with anti-Btk demonstrated low levels of endogenous Btk in the rabbit reticulocyte lysates used for in vitro translation. D, Mobility shift assays were performed using diluted in vitro translated Bright alone or in combination with identical quantities of BSA or bacterial lysates containing Btk. E, Nuclear extracts from several stably transfected BrCHO clones were assayed by EMSA for Bright binding activity using bfl50 as probe (upper panel). Bright in BCg3R-1d cells is indicated with an arrow. The lower panel shows 10 μg of nuclear protein from the same transfectants in a Western blot developed with polyclonal anti-Bright. The lower m.w. species observed from in vitro translated Bright and one of the post-translationally modified forms were evident. Data for B–E are representative of six experiments.
with both our immunoprecipitation data and the failure to observe mobility-shifted complexes in xid or \( btk^{-/-} \) extracts.

**Btk translocates to the nucleus**

Btk had not been previously reported in the nucleus, a requirement if Btk is indeed a critical component of the **Bright** transcription complex. Therefore, we searched for Btk in nuclear fractions from resting and LPS-stimulated spleen cells. Although the primary source of Btk in splenic cultures is B lymphocytes, Btk is also produced by monocytes. Nuclei were prepared by hypotonic lysis from monocyte-depleted cells using our standard protocol. Nuclear pellets were then stained with PI to exclude any live cells, and with FITC-labeled anti-light chain Abs to identify nuclei or live cells that retained associated cytoplasmic contaminants. PI\(^+\), FITC\(^-\) nuclei were isolated by sorting, and a representative Western blot comparing the presorted and sorted nuclei is shown in Fig. 6A. Btk was evident in each of the CBA/J and xid samples, including those from sorted nuclei that were devoid of contaminating cytoplasmic Ig heavy chain.

These findings were confirmed by confocal microscopy with permeabilized LPS-stimulated CBA/J blasts stained with anti-IgM TRITC to identify IgM\(^+\) B lymphocytes and with anti-Btk (PH-TH) plus anti-IgG1-FITC to detect Btk. Consecutive sections through an IgM\(^+\) blast are shown in Fig. 6B. If Btk is absent from the nucleus, one might expect to find sections of nuclei that lack green staining. However, Btk was evident both within the cytoplasm and within the large nucleus of this cell in every section despite the absence of IgM in the same nuclear regions. The IgM\(^+\) plasma cell in Fig. 6C exhibited a dark nucleus that lacked FITC staining in any section. This cell provided a good internal negative control, as Btk production is halted at the plasma cell stage (16, 17). Cells stained with an isotype-matched control and anti-IgM TRITC (Fig. 6D) did not exhibit either cytoplasmic or nuclear staining with the FITC secondary reagent. Cells from LPS-stimulated xid cells appeared similar to those shown here. Thus, both biochemical studies using an Ab against the kinase domain of Btk and confocal microscopy with an Ab recognizing the PH-TH domain of Btk confirm the existence of Btk within the nuclei of LPS-stimulated blasts, where its presence may result in active **Bright** complexes capable of affecting heavy chain transcription.

**Discussion**

These data identify **Bright**, a DNA-binding protein that interacts with the Ig heavy chain locus, as a potential downstream mediator of the xid defect. We have shown that formation of **Bright** DNA-binding complexes requires activation through a tyrosine kinase. Xid splenic B cells were deficient in **Bright** complex formation, suggesting that Btk, the defective tec family tyrosine kinase in those mice, was necessary for **Bright** function. **Bright** and Btk were shown to associate in immunoprecipitated complexes, and Btk was identified as a component of the active **Bright** DNA-binding complex. Consistent with these findings, Btk was shown to be present within the nucleus as well as the cytoplasm. Stable transfectants that expressed **Bright** without Btk also failed to produce **Bright** DNA-binding complexes. These data suggest a new and direct potential role for Btk in Ig production.

Although **Bright** was originally proposed to interact with DNA as a simple tetramer (2), our attempts to purify **Bright** indicated that it was likely to be a heteromeric complex (our unpublished observations). Three anti-Btk and two anti- **Bright** Abs coprecipitated **Bright** and wild-type Btk from activated murine spleen cells. Addition of exogenous Btk to suboptimal levels of **Bright** enhanced **Bright** binding activity, supporting the supershift data in Fig. 5B and suggesting that Btk is a component of the active **Bright** complex.
complex. The stoichiometry of these interactions is currently unknown and may include multiple subunits of both Bright and Btk. In addition, our failure to reconstitute Bright complexes in transfected cell lines could indicate a requirement for a third protein component. Indeed, preliminary data now suggest that this is likely. The failure of xid Btk to coprecipitate with Bright indicates that stable interactions between Bright and Btk may require a wild-type Btk PH domain. It is not clear whether the PH domain interacts directly with Bright, or whether it is required to produce active forms of Btk that might then associate with Bright or additional proteins in this complex. The PH domain of Btk was previously shown to be necessary for appropriate interactions with PtdIns-3,4,5-P_3, and activation of Btk at the cytoplasmic membrane (33–35, 58, 59). Indeed, we found that even xid-Btk was present in nuclear fractions, where it presumably would be available for interactions with Bright (Fig. 6). Therefore, the autophosphorylated and/or active forms of Btk produced after interaction with the cell membrane may be required for association with the Bright complex.

The finding that Btk associated with Bright in a DNA-bound complex, as demonstrated by reactivity with Abs to both the PH-TH and SH3-SH2 domains of Btk (Fig. 5), was unexpected. The simplest explanation that might have accounted for both the absence of Bright binding in xid extracts and the association of Bright and Btk, would have been that Bright was a substrate for Btk. However, this was not the case. We failed to detect phosphorylated forms of Bright by immunoprecipitation and Western analyses. Furthermore, phosphatase treatment did not inhibit the ability of Bright to bind to DNA. However, we cannot rule out the possibility that Bright might be transiently phosphorylated by Btk at time points earlier than the 24–48 h at which extracts were harvested, or that phosphorylation could affect a very low percentage of Bright within cells. We have been unable to demonstrate that Bright binds to DNA in the absence of Btk. While we have no evidence that Btk interacts directly with DNA, crystallization of the amino terminus of Btk revealed an unpredicted zinc finger within the TH domain (60). The ability to bind zinc may be important for function, because mutations that disrupt the zinc finger in human Btk result in XLA, the human counterpart of xid (61).

Both biochemical studies and confocal microscopy demonstrated Btk within the nucleus of B lymphoblasts. The Tec family kinase Txk has recently been shown to translocate to the nucleus of Jurkat cells after PHA stimulation (62), and while these studies were in progress, data presented by others also appeared to show nuclear localization of Btk in transfectedants (63, 64). Both Txk and Btk contain internal nuclear localization consensus sequences, and mutation of the Txk consensus sequence inhibited its translocation to the nucleus (15, 62). Interestingly, the xid point mutation falls within the putative nuclear localization sequence in Btk (15), and we originally hypothesized that failure of xid Btk to enter the nucleus might explain the absence of Bright DNA-binding activity in xid cells. However, the sorted nuclei from both unstimulated and stimulated xid-Btk cells contained Btk by Western blotting (Fig. 6). Therefore, Btk must translocate to the nucleus with some nuclear localization sequence function. Further studies will be necessary to determine how Btk translocates to the nucleus.

Clearly, Btk plays an important role in modulating calcium signals in the cytoplasm of B cells (32), and recent data suggest that Btk may modulate NF-kB activity in response to signals through the Ig receptor (57, 65). The ability of Btk to translocate to the nucleus and form DNA-binding complexes with Bright is not inconsistent with those data. Interestingly, many of the factors shown to be important for Btk-mediated events in the cytoplasm may also be present in B cell nuclei, implying that similar activation processes could occur there. Nuclear phosphatidylinositol 4,5-bisphosphate regulates the association of a DNA-binding complex with chromatin after T cell activation (66), and nuclear matrix-bound phosphatidylinositol 3-kinase has also been reported to regulate granulocyte differentiation (67). Moreover, the presence of some of these signaling mediators has been correlated with changes in gene expression. Thus, Btk may share some functions in both the cytoplasm and the nucleus.

The loss of Bright activity in xid mice may contribute to the lower serum Ig levels observed in these animals. One of the first defects observed in xid mice was an inability to mount a good response to the Ag, phosphocholine, a response dominated by Abs using the S107 family V1 heavy chain gene (68, 69). Strikingly, the first binding sites identified for Bright were 5’ of the V1 gene promoter (1). Therefore, a possible explanation for the poor response of xid mice to phosphocholine is that appropriate maturation of cells expressing the V1 gene in vivo stringently requires Bright activity. Consistent with that explanation, Bright binding sites exist within the 5’-flanking sequences of some, but not all, VH genes (70). The role of Bright in the expression of these genes is currently unknown.

Our data are consistent with a model in which Bright acts to tether Btk to sites within the Ig heavy chain locus, thereby enhancing transcription. One of the proteins phosphorylated by Btk is contained in B cells is BAP135/TFII-I (42, 43, 71), a transcription factor that binds to initiator sequences and facilitates transcription of promoters that lack consensus TATA boxes (72, 73). TFII-I can also interact directly with upstream regulatory sequences to enhance transcription (74, 75). We have previously shown that initiator sequences may play an important role in transcription from murine heavy chain promoters, including the V1 gene (1, 76). Bright binding sites are located 250 and 500 bases upstream of the V1 transcription start site and putative TFII-I binding site. TFII-I was shown to activate the Vβ TCR promoter through its initiator element (73), and tyrosine phosphorylation was required for that activity (77). Therefore, we propose that Bright/Btk complexes facilitate the phosphorylation of TFII-I, which then results in enhanced Ig transcription.

The precise role Bright may play in B cell development is unclear and awaits production of the appropriate knockout mouse. Current data suggest that Bright increases Ig transcription, but is not essential for basal levels of Ig synthesis. Immature B lymphocytes express surface Ig without Bright mRNA (5), and xid mice produce serum Ig, albeit at lower levels than normal mice. Nonetheless, appropriate expression of the Ig locus, even in mature cells, is important for B cell survival (9), and Bright, like Oca-B and Oct-2 (78–80), may play its primary role in more mature B cells. For example, increases in transcription may be necessary for maintenance of critical levels of surface Ig expression during the differentiation process in the germinal center. Thus, while it is not clear that deficiencies in Bright/Btk activity within the Ig locus will explain all the defects associated with X-linked immunodeficiency disease, they may contribute to the inefficient production of Ig in these mice.

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