REKLES Is an ARID3-restricted Multifunctional Domain*

Received for publication, January 16, 2007, and in revised form, March 23, 2007 Published, JBC Papers in Press, March 29, 2007, DOI 10.1074/jbc.M700397200

Dongkyoon Kim, Loren Probst, Chhaya Das, and Philip W. Tucker

From the Section of Molecular Genetics and Microbiology and Institute of Cell and Molecular Biology, University of Texas, Austin, Texas 78712-0162

Bright/Dril1/ARID3a is a B cell-specific, matrix association (or attachment) region-binding transcriptional regulator of immunoglobulin heavy chain genes and of E2F1-dependent cell cycle progression. Bright contains a central DNA binding domain termed ARID (AT-rich interacting domain) and a C-terminal region termed REKLES (for a conserved amino acid motif). The ARID domain has been identified in seven highly conserved families of metazoan proteins (ARID1–5 and JARID1–2), whereas REKLES is found only in the ARID3 subfamily (composed of Bright/ARID3a, Bdp/ARID3b, and Bright-like/ARID3c). REKLES consists of two subdomains: a modestly conserved N-terminal NREKLESα and a highly conserved (among ARID3 orthologous proteins) C-terminal RREKLESβ. Previously we showed that Bright undergoes nucleocytoplasmic shuttling and that RREKLESα and -β were required, respectively, for nuclear import and Crm1-dependent nuclear export. Here we show that Bright further requires RREKLESβ for self-association or paralogue association and for nuclear matrix targeting. REKLES promotes and regulates the extent of Bright multimerization, which occurs in the absence or presence of target DNA and is necessary for specific DNA binding. RREKLESβ-mediated interaction of Bright with Bdp, which localizes strictly to the nucleus, traps Bright within the nucleus via neutralization of its nuclear export activity. These results identify REKLES as a multifunctional domain that has co-evolved with and regulates functional properties of the ARID3 DNA binding domain.

Bright is a B cell-specific trans-activator that regulates the immunoglobulin heavy chain (IgH) locus by binding to nuclear matrix association (or attachment) regions (MARS)2 upstream of several V(H) promoters and flanking the IgH intronic enhancer (Eμ) (1–3). Bright is expressed only in small pre-B and mature B cells, implying a need for its regulated expression during lineage development (4). Within B cells or when ectopically introduced into non-B cells, Bright shuttles between the nucleus and the cytoplasm in a Crm1- and cell cycle-dependent manner (5). Indeed, Bright can control cell cycle progression, as it or its human orthologue, Drill, was shown to rescue natural senescence or oncogenic RASV12-induced senescence when expressed in primary mouse embryonic fibroblasts (2). Drill1 can immortalize and transform mouse embryonic fibroblasts through its regulation of cyclin E1 and subsequently E2F1 activity (6).

Bright is the founder of the ARID transcription factor family, which contains 13 human genes (7). Bright/ARID3a and the other two members (Bdp/ARID3b and Bright-like/ARID3c) have been described as the “extended” or e-ARID subfamily, having additional conserved sequences at both the N and C termini of the core ARID domain (7). ARID3 subfamily proteins consist of an acidic N-terminal region of unknown function, the central e-ARID (AT-rich interacting domain) MAR-DNA binding domain, and a region termed REKLES (for a conserved amino acid motif) in the C-terminal third of the molecule (Fig. 1A) (8, 9).

Although the ARID domain has been extensively analyzed in several subfamily members, including ARID3/Dri, ARID5/Mrf-2, and ARID1/SWI/SNF (10–13), REKLES is far less characterized. In vitro Bright was shown to bind to IgH MARs as a large complex, and the region spanning (what is now defined as) the REKLES domain was required for MAR binding (1). Sequence alignments suggested (8, 9) that the REKLES domain could be divided into two subdomains, RREKLESα and RREKLESβ, separated by a more variable spacer region (Fig. 1A). Functional analyses of the mouse (Bright) and Drosophila (Dri) ARID3a orthologous proteins conflicted in the requirement of REKLES for self-association (8, 14). However, the sequences spanning REKLES were sufficient to mediate mouse Bright’s association with components of nuclear dot domains (Sp100 and Lysp100) that modulated its transactivation of an IgH reporter gene (15). Recently, we showed that RREKLESα and -β are required, respectively, for nuclear entry and export of Bright during its nucleocytoplasmic shuttling (5).

Here we show that, in addition to its function in nuclear export, the REKLESβ subdomain regulates the binding of Bright to MARs, its self- and paralogue (Bdp) association and stoichiometry, and its retention within the nuclear matrix. We further demonstrate that RREKLESβ-mediated Bright-Bdp interaction blocks nucleocytoplasmic shuttling of Bright by interfering with its nuclear export activity. We conclude that the RREKLESβ subdomain is a highly conserved, specialized multifunctional domain that co-evolved with the eARID of the ARID3 subfamily after the advent of the primordial ARID domain.

*This work was supported by the National Institutes of Health and the Mary Betzner Morrow Endowment. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Molecular Genetics and Microbiology, the University of Texas, 1 University Station A5000, NM51.124, Austin, TX 78712-0162. Tel.: 512-475-7705; Fax: 512-475-7707; E-mail: phltucker@mail.utexas.edu.

2 The abbreviations used are: MAR, matrix association (or attachment) region; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; NMTS, nuclear matrix targeting sequence; NLS, nuclear localization signal; NES, nuclear export signal; PIPES, 1,4-piperazinediethanesulfonic acid.
**EXPERIMENTAL PROCEDURES**

*Cell Culture and Transfections—* COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum under standard conditions. Mouse mature B cell lines, M12.4 and BCL1, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

COS-7 cells (1–3 × 10^5) were plated in a well of a 6-well plate 12 h before transfection. The cells were transiently transfected using 0.5 μg of DNA and FuGENE 6 transfection reagent (Roche Diagnostics) following the manufacturer’s directions. Co-transfection, we equalized the total DNA amounts by adding the appropriate amount of pCR3.1 empty circular vector.

*Plasmids—* pCG was constructed as described previously (5). The pCG open reading frame was deleted from pCG (pCE). A version of pCE containing a translation initiation codon was constructed by annealing two DNA primers (forward, 5′-TCGA-GTCCAAGCTGTCATTGAGTTTCTGCTCCA-3′; and reverse, 5′-AATTTCATGCACGCTTGGGAC-3′; complementary sequences are underlined and a starting codon is in boldface) and inserting the double-stranded DNA into the XhoI and EcoRI sites of pCE. Two Myc primers, Myc(+) 5′-AATTGGAGCAGAAAATCATCTCTGAGAGGATCTG-3′ and Myc(−) 5′-AATTGGAGCAGAAAATCATCTCTGAGAGGATCTGCTCCA-3′ (complementary sequences are underlined) were annealed and inserted into the EcoRI site of pCE. Two additional Myc tags were inserted into the EcoRI site as described above to generate pCEM. Bright cDNA was then inserted into the EcoRI and SacII sites of pCG (5), pCE1, and pCEM. Bright deletion and substitution mutants were generated by digestion of existing restriction enzyme sites and/or by standard PCR-based mutagenesis. Sequences of all constructs were verified by sequencing and then inserted into pCG, pCE1, and/or pCEM. A human Bdp cDNA was obtained from Dr. P. Claudio (Kemmel Cancer Institute, Thomas Jefferson University), amplified, and inserted into pCG and pCEM and then confirmed by DNA sequencing.

*Co-immunoprecipitation and Western Blotting—* We followed the method of Kim et al. (16) with modifications. At 48 h post-transfection, COS-7 cells, grown to confluence in a well of a 6-well plate, were lysed in 0.3 ml of lysis buffer (50 mM HEPES-KOH, pH 7.4; 200 mM KCl; 10% glycerol; 1% Nonidet P-40; 1 mM EDTA, and 1 mM DTT) supplemented with 0.3 μl of protease inhibitor mixture (Sigma) and incubated on ice for 5 min. After centrifugation at 12,000 rpm (5417R, Eppendorf), protein and supernatant was transferred to a fresh 1.5-ml tube after centrifugation at 2,000 rpm for 2 min (model 59-A, Fisher) and 30 μl of the supernatant was saved for the input samples. Three μl of anti-Myc antibody (Babco) was incubated with the lysate for 1 h with constant agitation. Then 30 μl of 50% protein-A-Sepharose beads (Amersham Biosciences) was added to the lysate and incubated for 2 h as above. Beads were washed three times by rounds of agitation (in 1 ml of lysis buffer for 5 min) and centrifugation. After the final wash, the beads were suspended in 37.5 μl of 1× SDS sample buffer and boiled for 5 min. The input samples were mixed with 7.5 μl of 5× SDS sample buffer and boiled for 5 min. Most procedures were performed at 4 °C. Proteins were separated by 9% SDS-PAGE and transferred to Protran nitrocellulose membranes (PerkinElmer Life Sciences). The membranes were incubated with anti-Myc monoclonal antibody (Babco) and anti-GFP monoclonal antibody (Santa Cruz Biotechnology). Membranes were then incubated with goat anti-mouse IgG peroxidase-conjugated antibodies (Amersham Biosciences), and bands were visualized with ECL Western blotting detection reagents (Amersham Biosciences) on Amersham Biosciences Hyperfilm ECL.

*In Vitro Transcription/Translation—* Bright, GFP-Bright, or myc-Bright was expressed using the T7 coupled Transcription/Translation Systems (Promega) following the manufacturer’s instructions. Protein integrity and level were assessed by anti-Bright Western blotting.

*Native Gel Electrophoresis—* Continuous native polyacrylamide gels (5–6%) were pre-run for 30 min at 40 V at 4 °C. After washing wells, 5–10 μg of protein, produced by in vitro transcription/translation or by subcellular fractionation of transfected COS-7 cells, was loaded (in <10 μl), and separation was achieved at 40 V for 6 h at 4 °C with an electrode buffer change at 3 h. Proteins were transferred onto nitrocellulose membranes, and Bright was detected with anti-Bright rabbit antiserum (1) or anti-Myc mouse antibody (Babco) followed by goat anti-rabbit IgG antibody or goat anti-mouse-IgG antibody conjugated with peroxidase (Amersham Biosciences).

*Electrophoretic Mobility Shift Assays (EMSA)—* In vitro DNA-binding reactions were conducted in a total volume of 25 μl of binding buffer (10 mM HEPES, pH 7.9; 10% glycerol (v/v); 50 mM NaCl; 0.5 mM EDTA; 0.02% Tween 20; 80 ng/μl poly(dI/dC), and 0.25 mM PMSF), containing 2 μg of nuclear extract and ~80,000 cpm of Bright-specific probe as follows: 5′-end-labeled, gel-purified fragments spanning either of the V_{H} S107 promoter-associated MARs (Bf150 or Tx125) as described previously (17). Samples were incubated for 20 min at room temperature and then resolved on 4% polyacrylamide gels.

For the supershift assays, anti-Bright antiserum (1) was added to the above binding reaction following probe incubation. Samples were placed on ice for 30 min before loading onto gels.

*Nuclear Protein Extraction—* Cytoplasmic and nuclear extracts were prepared according to the method of Johnson et al. (18). B cells (~1 × 10^7) were collected and washed twice with phosphate-buffered saline, and pellets were suspended in 100 μl of sucrose buffer I-A (100 mM Tris-Cl, pH 8.0; 0.32 M sucrose; 3 mM CaCl_2; 2 mM magnesium acetate; 0.1 mM EDTA; 1 mM DTT; 0.5 mM PMSF, and 0.5% Nonidet P-40) supplemented with 0.1 μl of protease inhibitor mixture (Sigma). Nuclei were separated from the soluble cytoplasmic fraction by centrifugation at 2,000 rpm for 2 min (model 59-A Fisher). Nuclei were suspended in 20 μl of low salt buffer (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM MgCl_2; 20 mM KCl; 0.2 mM EDTA; 0.5 mM DTT, and 0.5 mM PMSF) and 20 μl of high salt buffer (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM MgCl_2; 800 mM KCl; 0.2 mM EDTA; 1% Nonidet P-40; 0.5 mM DTT, and 0.5 mM PMSF) were then added dropwise. Then the extract was diluted with 100 μl of diluent (25 mM HEPES, pH 7.6; 25%
glycerol; 0.1 mM EDTA, and 0.5 mM PMSF). The mixture was centrifuged at 12,000 rpm for 15 min at 4 °C (5417R, Eppendorf), and the supernatant was used for EMSA.

Subnuclear Fractionation into Nuclear Matrix—Fractionations were performed according to the method of Reyes et al. (19). Following transfection with GFP-Bright wild type and mutant constructs, COS-7 cells (in 6-well plates) were scraped, washed, and suspended in 80 μl of CSKT (10 mM PIPES, pH 6.8; 100 mM NaCl; 300 mM sucrose; 3 mM MgCl2; 1 mM EGTA; 1 mM DTT, and 0.5% Triton X-100 supplemented with 0.5 mM protease inhibitor mixture). The suspension was incubated for 5 min and centrifuged at 7,500 rpm (5415C, Eppendorf) for 3 min. The supernatant was used for the soluble fraction, and the pellet was washed with CSK (CSKT minus Triton X-100), centrifuged, and suspended in 60 μl of CSK supplemented with 6 μl of RNase-free DNase I (Invitrogen). The suspension was incubated at 37 °C for 2 h and then 20 μl of 1 M ammonium sulfate (NH₄)₂SO₄/CSK was added to a final concentration of 0.25 M (NH₄)₂SO₄. After incubation at 4 °C for 5 min, the suspension was centrifuged at 7,500 rpm for 3 min. The supernatant contained the chromatin fraction. The pellet, containing the nuclear matrix fraction, was suspended in 80 μl of 2 M NaCl/CSK and centrifuged again at 7,500 rpm for 3 min. The pellet was suspended in 80 μl of 8 M urea buffer (8 M urea and 10 mM Tris-Cl, pH 8.0), and 5 μl of each fraction was analyzed by SDS-PAGE/anti-Bright Western blotting.

Cytological Studies—The GFP-transfected COS-7 cells were grown on coverslips for 24 h and fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with phosphate-buffered saline, coverslips were air-dried and mounted with mounting media (Vectashield H-1000, Vector Laboratories, Inc.) supplemented with 10 ng/ml of 4,6-diamidino-2-phenylindole and covered with glass slides. GFP, GFP-Bright, or GFP-Bdp localization was examined with an Axioplan fluorescence microscope (Zeiss). Based on the major localization of the signal, GFP cells were grouped into three categories as follows: nuclear accumulation (N=N), nuclear and cytoplasmic localization (N=N), and cytoplasmic accumulation (N=N). Percentages of cells within each group were calculated. Each localization experiment was repeated at least three times.

RESULTS

Self-association of Bright Requires REKLESβ—To define minimal sequences required for Bright self-association, we co-expressed tagged wild type and various mutant forms of Bright (Fig. 1B) in COS-7 cells. We then assayed for protein-protein associations via co-immunoprecipitation and Western blotting 24 h post-transfection. In accordance with previous observa-
Bright self-associates through the REKLESβ subdomain. Wild type myc-Bright was co-expressed with wild type or the indicated GFP-Bright mutants in COS-7 cells. Co-immunoprecipitations (IP) were performed with either anti-GFP (top panel) or anti-Myc (bottom panel) monoclonal antibodies. The asterisk denotes heavy chains of anti-Myc antibodies used for immunoprecipitation. WT, wild type Bright.

FIGURE 2. Bright self-associates through the REKLESβ subdomain. Wild type myc-Bright was co-expressed with wild type or the indicated GFP-Bright mutants in COS-7 cells. Co-immunoprecipitations (IP) were performed with anti-Myc antibody and whole cell extracts as described under “Experimental Procedures.” Western blotting (WB) was performed with either anti-GFP (top panel) or anti-Myc (bottom panel) monoclonal antibodies. The asterisk denotes heavy chains of anti-Myc antibodies used for immunoprecipitation. WT, wild type Bright.

Bright self-associates through the REKLESβ subdomain. Wild type myc-Bright was co-expressed with wild type or the indicated GFP-Bright mutants in COS-7 cells. Co-immunoprecipitations (IP) were performed with either anti-GFP (top panel) or anti-Myc (bottom panel) monoclonal antibodies. The asterisk denotes heavy chains of anti-Myc antibodies used for immunoprecipitation. WT, wild type Bright.

Next we examined the MAR DNA binding activity of Bright requires the REKLESβ domain. It has been reported previously that the elimination of multimerization of Bright coincided with its loss of DNA binding activity (1). Therefore, we examined whether the wild type REKLESβ domain is required for Bright to bind its target MARs in vitro. Using in vitro transcription/translation in reticulocyte lysates, we produced GFP-tagged wild type and mutant forms of Bright. All lysates used as sources of protein for these experiments contained approximately equal amounts of Bright, as confirmed by Western analysis (data not shown). We performed EMSAs with the previously established (20), Bright binding-specific V_th1 promoter-associated MARs (Bf150 or Tx125) as probes (Fig. 3). Wild type Bright (Fig. 3, lane 4) but not tag (GFP)-only bound to the Tx125 probe, confirming that in vitro translated GFP-Bright maintains DNA binding activity. Binding specificity was confirmed by supershift of the DNA-protein complex with anti-Bright antiserum (Fig. 3, lane 5). The 1–541 mutant, which contains a complete ARID and REKLES domain and interacts with Bright in co-immunoprecipitation assays, bound DNA specifically. However, further C-terminal deletion (mutant 1–493) eliminated binding, implying that residues 494–541 are required for Bright to bind MARs. An internal deletion (d500–521) that spans the spacer between the REKLESα and -β domains, which is still capable of Bright-Bright interaction (Fig. 2, lane 10), produced a slowly migrating DNA complex that retained binding specificity (Fig. 3, lane 10). This indicated (re-addressed below) that spacer deletion either altered multimerization stoichiometry or DNA bending, as wild type Bright inflicts a severe (~90°) conformational change on bound DNA (2). Mutants 1–493 (Fig. 3, lane 6) and d521–541 (lane 12), which lack critical REKLESβ sequences and did not interact with wild type Bright (Fig. 2), did not bind to the MAR probe despite the presence of the ARID domain. These results imply that the MAR DNA binding activity of Bright requires the self-association domain as well as the ARID domain.

REKLESβ-mediated Self-association Is Necessary but Not Sufficient for DNA Binding—Next we examined the MAR DNA-binding properties of REKLESβ point mutants produced in vitro by transcription/translation in reticulocyte lysates (Fig. 4A). Even though point mutations G532A (Fig. 4, lane 14–16), G532P (lane 17–19), or Y535A (lane 11–13) did not impair the self-association of Bright (Fig. 2 and data not shown), suggesting that these residues play a concerted role in Bright-Bright interaction and/or contribute to a different REKLESβ-mediated function(s).

DNA Binding Activity of Bright Requires the REKLESβ Domain—It has been reported previously that the elimination of multimerization of Bright coincided with its loss of DNA binding activity (1). Therefore, we examined whether the wild type REKLESβ domain is required for Bright to bind its target MARs in vitro. Using in vitro transcription/translation in reticulocyte lysates, we produced GFP-tagged wild type and mutant forms of Bright. All lysates used as sources of protein for these experiments contained approximately equal amounts of Bright, as confirmed by Western analysis (data not shown). We performed EMSAs with the previously established (20), Bright binding-specific V_th1 promoter-associated MARs (Bf150 or Tx125) as probes (Fig. 3). Wild type Bright (Fig. 3, lane 4) but not tag (GFP)-only bound to the Tx125 probe, confirming that in vitro translated GFP-Bright maintains DNA binding activity. Binding specificity was confirmed by supershift of the DNA-protein complex with anti-Bright antiserum (Fig. 3, lane 5). The 1–541 mutant, which contains a complete ARID and REKLES domain and interacts with Bright in co-immunoprecipitation assays, bound DNA specifically. However, further C-terminal deletion (mutant 1–493) eliminated binding, implying that residues 494–541 are required for Bright to bind MARs. An internal deletion (d500–521) that spans the spacer between the REKLESα and -β domains, which is still capable of Bright-Bright interaction (Fig. 2, lane 10), produced a slowly migrating DNA complex that retained binding specificity (Fig. 3, lane 10). This indicated (re-addressed below) that spacer deletion either altered multimerization stoichiometry or DNA bending, as wild type Bright inflicts a severe (~90°) conformational change on bound DNA (2). Mutants 1–493 (Fig. 3, lane 6) and d521–541 (lane 12), which lack critical REKLESβ sequences and did not interact with wild type Bright (Fig. 2), did not bind to the MAR probe despite the presence of the ARID domain. These results imply that the MAR DNA binding activity of Bright requires the self-association domain as well as the ARID domain.

REKLESβ-mediated Self-association Is Necessary but Not Sufficient for DNA Binding—Next we examined the MAR DNA-binding properties of REKLESβ point mutants produced in vitro by transcription/translation in reticulocyte lysates (Fig. 4A). Even though point mutations G532A (Fig. 4, lane 14–16), G532P (lane 17–19), or Y535A (lane 11–13) did not impair the self-association of Bright (Fig. 2 and data not shown), suggesting that these residues play a concerted role in Bright-Bright interaction and/or contribute to a different REKLESβ-mediated function(s).
ing, indicating that post-translational phosphorylation of this highly conserved (Fig. 1A) tyrosine does not play a role. Two additional self-association-competent point mutants, G537A and L539A, also failed to bind to the IgH V₁1 promoter-associated MARs (data not shown).

DNA binding activity of some of these point-mutated proteins could be rescued following their extraction from COS-7 cells after transient transfection (Fig. 4, C and D). Although GFP-fused wild type Bright and the Y535F mutant maintained DNA binding activity as predicted from the in vitro assays, indistinguishable binding complexes were now observed for G532A (Fig. 4D, lanes 10 and 11) and L539A (data not shown). Moreover, G532P now could form a specific DNA-binding complex (Fig. 4D, lanes 8 and 9), but it migrated more slowly, as observed for the REKLES spacer mutant (d500–521 in lane 10 of Fig. 3). The restoration of DNA binding activity of these mutants might result from structural refolding of mutant proteins by cellular factors, such as chaperones, or by heteromeric interactions with proteins not expressed in reticulocyte lysates. However, the DNA binding of two other mutants, Y535A and G537A, did not appear to be rescued, despite their equivalent (to wild type) levels of expression in COS-7 cells (data not shown). These data indicate that REKLESβ-dependent Bright self-association is necessary but not sufficient for Bright to bind DNA.

**FIGURE 3.** The REKLESβ subdomain is required for the DNA binding activity of Bright/ARID3a. GFP, GFP-Bright, and mutants summarized in Fig. 1B were prepared by in vitro transcription/translation. EMSA was performed using a 32P-labeled, IgH V₁1/S107 promoter-associated MAR (Tx125) as probe. Supershift was performed with anti-Bright anti-serum; supershifted complexes are observed in the wells. WT, wild type Bright. Ab, antibody.

**FIGURE 4.** REKLESβ-mediated self-association is necessary but not sufficient for DNA binding. GFP-Bright mutants were prepared either as in vitro transcription/translation lysates (A and B) or as nuclear extracts of transiently transfected COS-7 (C and D). Approximately equivalent expression and input was confirmed by Western blotting (A and C). EMSA and supershift were performed as in Fig. 3. WT, wild type Bright; p, pre-immune serum. Expression in COS-7 is capable of rescuing DNA binding of some (G532P and G532A) but not all (Y535A) substitution mutants. Signal in the well at the top of the gel on treatment of antibody (Ab) may reflect binding of probe by an antibody-induced aggregation of the protein. Artificial aggregation of the ARID DNA binding domain may be sufficient for probe binding even in the absence of REKLES-dependent protein multimerization.

**FIGURE 4D—**One explanation for the cellular rescue observed in Fig. 4D is that some self-association-competent mutants fail to multimerize to the correct stoichiometry required for DNA binding. To test this, we analyzed the relative mobility of such Bright mutants, produced in vitro (Fig. 5A) or following cell transfection (Fig. 5B) by native gel electrophoresis. Regardless of how they were derived, DNA-binding-competent wild type Bright (Fig. 4, lanes 1) and Y535F (lanes 3) formed slowly migrating complexes, consistent with their ability to properly multimerize. Based on the mobility of protein standards (Fig. 5) and gel filtration chromatography (data not shown), we estimated the sizes of these complexes as tetrameric or greater. Likewise, irrespective of derivation, REKLESβ-deficient mutant (d521–541) migrated as a monomer (compare lanes 2 of Fig. 5, A and B). However, mobility of mutants (G532P and G532A), whose DNA-binding deficiencies were rescued when expressed in cells (Fig. 4D), shifted from monomeric to tetrameric (compare lanes 4) or greater (compare lanes 5). Therefore, the DNA-binding competency of these Bright mutants is correlated with their ability to form tetramers or perhaps larger multimers.
Even though a significant fraction of Y535A (lanes 4) and G537A (data not shown) formed apparent tetramers when expressed in COS-7 cells, they were still unable to bind DNA (Fig. 4D). This suggests that these mutations create a different and more severe defect in REKLESβ that cannot be suppressed by factors supplied by COS-7. Subcellular fractionation of COS-7 transfectants indicated that the multimerization of Bright and its various REKLES/H9252 mutants is indistinguishable between the cytoplasm (C) and nucleus (N).

The data of Fig. 5 along with previous results (1) prompted the question as to whether REKLES mediates multimerization of Bright when bound to DNA. We simultaneously produced in vitro various combinations of wild type Bright and a DNA binding-competent truncation (residues 238–601) and then examined the resulting complexes by EMSA. As expected for a tetramer (Fig. 6A and B), full or partial rescue of multimerization of Bright mutants from COS-7 cells indicates indistinguishable multimerization between the cytoplasm (C) and nucleus (N).

The deletion of the spacer region between REKLESα and REKLESβ (Fig. 1B, mutant d500–521) resulted in more slowly migrating DNA-binding complexes (Fig. 3, lane 10). Employing the same mixing strategy as above, we observed that the d500–521-DNA complexes are composed of greater than four Bright molecules (Fig. 6C). Based on mobility and size, we estimated...
Multifunctional Domain in Bright/Dril1/ARID3a

A

| M12.4 | BCL1 |
|-------|------|
| W C N np ch mn | W C N np ch mn |

B

| α-GFP | α-α-Tubulin | α-Histone H1 | α-Lamin B |
|-------|-------------|--------------|-----------|
| GFP   |             |              |           |
| Bright|             |              |           |
| 1-541 |             |              |           |
| 1-493 |             |              |           |
| d453-500 |        |              |           |
| d500-521 |        |              |           |
| d521-541 |        |              |           |
| Y535F |             |              |           |
| Y535A |             |              |           |
| G532P |             |              |           |
| G532A |             |              |           |
| G537A |             |              |           |
| L539A |             |              |           |

FIGURE 7. The REKLESβ subdomain is required for nuclear matrix association. A, Bright localizes within the nuclear matrix of mature B cell lines, M12.4 and BCL1. Proteins were extracted either as whole cell lysates (W) or as cytoplasmic (C) or nuclear (N) fractions. Nuclear protein was further fractionated into soluble nuclear (np), chromatin-associated (ch), or nuclear matrix (nm)–associated in equal volumes of buffer, and either −15% (whole cell lysates, cytoplasmic, and nuclear) or −40% (nuclear protein, chromatin, and nuclear matrix) of each fraction was analyzed by SDS-PAGE/anti-Bright immunoblotting. B, NMTS of Bright resides in REKLESβ. Wild type or the indicated GFP-Bright mutants were expressed in COS-7 cells, and fractionations were performed as described under “Experimental Procedures.” Bright localization was determined by SDS-PAGE/anti-GFP Western blotting. Purity of fractions was assessed by blotting with anti-α-tubulin (soluble, s), anti-histone H1 (ch), and anti-lamin B (nm).

the complex as pentameric or larger. Multimerization of d500–521 to a size greater than wild type also occurred in the absence of DNA (data not shown). These results indicate that Bright complexes larger than tetrameric can specifically bind to the IgH MARs and that proper spacing between REKLESβ and REKLESα is required for tetramerization.

REKLESβ Is Required for the Nuclear Matrix Association of Bright—IgH-associated MARs bound by Bright are components of the nuclear matrix (21). To determine whether Bright is also matrix-associated, mature B cells (Fig. 7A, M12.4 and BCL1) were biochemically fractionated (19) and Western-blotted with anti-Bright antibody (Fig. 7A). As expected, the nuclear component of Bright partitioned primarily (in M12.4) or partially (in BCL1) into the nuclear matrix. Similar results were observed for pre-B (7023) and terminally differentiated (J558) B cell lines (data not shown).

To determine the nuclear matrix targeting sequences (NMTS), we carried out the same biochemical fractionations on COS-7 cells transiently transfected with GFP-Bright wild type or mutants of Fig. 1B. As shown in Fig. 7B, controls for soluble (α-tubulin), chromatin (histone H1), and nuclear matrix (lamin B) fractions revealed no cross-contamination. GFP-only was detected primarily in the soluble fraction, whereas Bright (residues 1–601), as in B cells (Fig. 7B), was detected in the soluble and nuclear matrix fractions. We next tested deletion mutants that contained an intact nuclear localization signal (NLS) (residues 461–466, see Ref. 5). Mutant 1–541 associated with the nuclear matrix, but further deletion of 48 residues (mutant 1–493) lost matrix association and accumulated quantitatively in the soluble cytoplasmic/nuclear fraction. Therefore, the REKLESβ-spanning 493–541 region contains the NMTS. Internal deletions within the REKLESβ domain confirmed that the NMTS resides within REKLESβ (residues 521–541) and that the REKLES spacer (residues 500–521) is not required. However, REKLESβ is not sufficient for nuclear matrix targeting, as it could not direct Bright to the matrix in the absence of the NLS (mutant 453–500). Fractionation of individual REKLESβ point mutants of Fig. 1B revealed no single mutation capable of eliminating nuclear matrix association (Fig. 7B). Therefore, residues within the REKLESβ domain critical for MAR binding and/or tetramerization stoichiometry are critical for nuclear matrix targeting.

REKLESβ Domain Is the Interface for Both Homo- and Heteromerization of ARID3—As with Bright, the ARID3 paralogue, Bdp/ARID3b, is expressed in B cells (22). Sequence alignment confirmed the high similarity of the REKLESβ domain between the two proteins (Fig. 1A). Because of this similarity and because REKLESβ seems to be sufficient for Bright self-association, we reasoned that Bright and Bdp might interact through the same domain. Using the same COS-7 transient transfection approach described above, we observed that myc-Bright specifically interacted with GFP-Bdp, and conversely, myc-Bdp interacted with GFP-Bright (Fig. 8, lanes 3 and 6). To determine whether REKLESβ participates in this interaction, we co-expressed myc-Bdp and GFP-Bright mutant proteins (Fig. 8). myc-Bdp did not co-immunoprecipitate with either the N-terminal domain or the ARID-domain (Fig. 8, lanes 7 and 8) but did co-precipitate with the REKLES-containing C-terminal domain (lanes 9 and 10). Further N-terminal deletion (residues 500–601) maintained the Bdp interaction (Fig. 8, lane 10), indicating that the N-terminal REKLESα region was dispensable. However, deletion of the C-terminal REKLESβ-containing portion of the domain (mutant 1–493) eliminated Bdp interaction (data not shown). Examination of two internal deletions within this C-terminal region indicated that loss of residues 500–521 had no effect, whereas loss of 521–541 eliminated Bright-Bdp interaction. Thus the REKLESβ domain is the interface for both homo- and heteromerization of ARID3.

Bdp-ARID3b Interaction Traps Bright in the Nucleus by Suppression of Its REKLESβ-mediated NES Activity—Bright accumulates in a heterogeneous pattern within unsynchronized cell populations, exhibiting a range of localization from strictly cytoplasmic to strictly nuclear (5). Whether expressed endogenously in B cell lines (M12.4 and BCL1; Fig. 9A) or ectopically as a GFP fusion in COS-7 cells (Fig. 9B), Bright localized to the nucleus and to the cytoplasm. However, Bdp localized strictly to the nucleus in B cells (Fig. 9A). In COS-7 cells examined 24 h
Although Bdp was able to exert a dominant effect on Bright localization, no such change in the cellular localization of Bdp was observed by increasing the levels of transfected Bright significantly above those of Bdp (data not shown).

Bdp co-expression resulted in nuclear mislocalization of the cytoplasmic-only NLS mutant (K466A) of Bright (Fig. 9D). Because Bright K466A retains a functional NES, its nuclear accumulation indicates that Bdp provides nuclear retention activity to the heteromeric complex in addition to imparting nuclear localization activity to Bright, i.e. Bdp-Bright complexes are retained in the nucleus by virtue of suppression of Bright NES activity.

**DISCUSSION**

Bright/ARID3a is a transcription factor whose DNA binding is mediated by the ARID domain (1). We previously demonstrated that Bright shuttles between the cytoplasm and nucleus and mapped its NLS within REKLESα and its NES within REKLESβ (5). Here we showed that REKLESβ can function as a distinct domain necessary and sufficient for interactions of Bright with itself or with its parologue, Bdp/ARID3b. Nixon et al. (23) reported that Bright can exist as dimers in Chinese hamster ovary cells and B cell lines. We also observed Bright homodimers in vitro (data not shown), but the majority of Bright self-associates into tetramers and higher order multimers when produced either in vitro or in transfected COS-7 cells.

Bright appears to multimerize identically within the nucleus and in the cytoplasm. This indicates that its nucleocytoplasmic shuttling is not dependent on alternative self-assembly stoichiometry, even though several of the same REKLES residues (e.g. Tyr-535 and Gly-532) are indispensable for both functions (5).

Previous studies of ARID1 (e.g. Swi/SNF) and ARID5 (e.g. Mrf-2) subfamily members demonstrated that in these proteins the ARID domain alone is sufficient for DNA binding (13, 24, 25). That ARID3a/Bright requires the REKLES domain for DNA binding was suggested previously (1). This finding was confirmed and extended to include a requirement for REKLES-dependent multimerization. Bright tetramers form regardless of whether specific DNA-binding sites are present. Thus, DNA binding is not required to induce multimerization of Bright. We found that the stoichiometry of the Bright homeric complex, whether free or assembled upon DNA, is dependent upon the length of the spacer region between REKLESα and REKLESβ (Fig. 1A). However, the presence of the ARID domain and the multimerization of Bright are not sufficient for DNA binding. Single amino acid substitution mutations within REKLESβ (Y535A and G537A) did not detectably alter multimerization while abolishing (or severely reducing) DNA binding. Additional regulatory mechanisms for DNA binding as well as for self-association through the REKLESβ domain remain to be identified.

Because Bright binds to MARs upstream of the S107 V141 promoter and MARs flanking the intronic enhancer, we speculated that Bright may regulate IgH gene expression by recruiting the IgH loci to the nuclear matrix (9). We showed here that a large fraction of Bright accumulates within the nuclear matrix fraction in mature B cell lines or when ectopically expressed in non-B cells. Structure-function and secondary structure analyses of other MAR-binding/nuclear matrix-residing proteins,
including SATB1 and AML, have provided no clear consensus for NMTS function (26–29). In Bright, the nuclear matrix targeting sequence was identified as the REKLES subdomain, although it has no detectable sequence or structural homology with these other previously identified NMTSs. Point mutations within this domain did not affect nuclear matrix targeting, even although they eliminated nuclear export and/or DNA binding. Control of the relative subnuclear abundance of Bright among chromatin, soluble, and nuclear matrix compartments might be exerted through the REKLESβ-associated NMTS, but this hypothesis has been difficult to test in lieu of mutations that distinguish NMTS from other REKLESβ-mediated multifunctionality.

Unlike Bright/ARID3a, which actively shuttles between the nucleus and cytoplasm, we found its paralogue Bdp/ARID3b to localize exclusively to the nucleus. We showed that Bdp enhances the nuclear localization of Bright by interaction through its REKLESβ. However, Bright was incapable of re-localizing Bdp, suggesting a dominant effect for Bdp on the cellular localization of both proteins. The increased nuclear localization of Bright-Bdp heteromers appears to result from an increased nuclear retention activity. The previous observation that nuclear Bright abundance is maintained despite reduction of its cytoplasmic levels during growth factor starvation (5) is consistent with such a mechanism. REKLESβ-mediated interactions with Bdp and/or the nuclear matrix should facilitate nuclear retention and presumably enhance accessibility for MAR DNA binding. Bdp is expressed ubiquitously (22) and thus could be key in regulating Bright function by regulating its cellular localization in B cells. In addition, Bright interactions with several nonparalogous proteins, including Sp100, LYSp100, and Ubc9, have been mapped roughly to the REKLES region (9, 11).

Whether these interactions are also mediated through the REKLESβ domain or whether, as with Bdp, they alter other functions of REKLESβ remains to be determined.
Although the ARID family members share highly similar DNA binding domains (7), only ARID3a/Bright has been reported to undergo nucleocytoplasmic shuttling (5). Our observations suggest that, via the REKLES domain, the cellular localization of ARID3 is integrated with its ability to bind DNA. A similar argument has been made for STAT1 (30). However, unlike STAT1, the position of the NES of Bright is in REKLESβ and not in the DNA binding domain. The importance of the conserved REKLESβ tyrosine (Tyr-535) for both DNA binding and NES activities draws a parallel with STAT1. Dephosphorylation of a conserved tyrosine within the DNA binding domain of STAT1 correlates with nuclear export, leading to the suggestion that its NES is masked when tyrosine-phosphorylated STAT1 is bound to DNA (31). In contrast, ARID3 Tyr-535 is not phosphorylated, and conservative (Y535F) but not conservative (Y535A) substitutions maintain NES activity. Although the crystal structure of tyrosine-phosphorylated STAT1 bound to DNA supports the masking hypothesis (3), the ARID3a/Dri structural analysis (13) was limited to the DNA binding domain. Thus, it remains to be determined whether physical interaction of REKLES and ARID domains occur. We speculate that they do, as it best explains why at least two self-association-competent, multimerization-competent REKLESβ substitutions (Y535A and G537A) abolished DNA binding regardless of whether the proteins were synthesized in vitro or in cells. Physical ARID-REKLES interaction may also account for our previous observation that NES-deficient Bright (G532A) failed to trans-activate a reporter gene under the control of the S107 V_{141}-associated MARs, even though the mutant can form tetrameric protein-DNA complexes and interact with the nuclear matrix (5).

Our results identify REKLES as a multifunctional domain. Coupling the overlapping NLS, NES, nuclear matrix retention, and (potentially) transactivation elements of REKLES with ARID domain-mediated DNA binding provides an elegant mechanism for functional control. Critical issues that remain to be resolved include the structural details of a putative ARID-REKLES interaction, whether nuclear export and DNA binding of Bright compete for REKLESβ-required functions, and the mechanism of Tyr-535 modulation of many of the REKLESβ functions that together determine the fate and activity of nuclear Bright.

Acknowledgments—We thank J. C. Colbourne and the Daphnia Genome Database for access to unpublished Daphnia genomic sequence used in Fig. 1; Maya Ghosh for excellent technical assistance; and the members of our laboratory for critical review of the manuscript.

REFERENCES

1. Herrscher, R. F., Kaplan, M. H., Lelsz, D. L., Das, C., Scheuermann, R., and Tucker, P. W. (1995) Genes Dev. 9, 3067–3082
2. Kaplan, M. H., Zong, R. T., Herrscher, R. F., Scheuermann, R. H., and Tucker, P. W. (2001) J. Biol. Chem. 276, 21325–21330
3. Goepel, P., Montalbano, A., Ayers, N., Kompfner, E., Dickinson, L., Webb, C. F., and Feeney, A. J. (2002) J. Immunol. 169, 2477–2487
4. Webb, C. F., Smith, E. A., Medina, K. L., Buchanann, K. L., Smithson, G., and Dou, S. (1998) J. Immunol. 160, 4747–4754
5. Kim, D., and Tucker, P. W. (2006) Mol. Cell. Biol. 26, 2187–2201
6. Peper, D. S., Shvarts, A., Brummelkamp, T., Douma, S., Koh, E. Y., Daley, G. Q., and Bernards, R. (2002) Nat. Cell Biol. 4, 148–153
7. Wilsker, D., Probst, L., Wain, H. M., Maltais, L., Tucker, P. W., and Moran, E. (2005) Genomics 86, 242–251
8. Kortschak, R. D., Tucker, P. W., and Saint, R. (2000) Trends Biochem. Sci. 25, 294–299
9. Webb, C. F., Zong, R. T., Lin, D., Wang, Z., Kaplan, M., Paulin, Y., Smith, E., Probst, L., Bryant, I., Goldstein, A., Scheuermann, R., and Tucker, P. (1999) Cold Spring Harbor Symp. Quant. Biol. 64, 109–118
10. Patsialou, A., Wilsker, D., and Moran, E. (2005) Nucleic Acids Res. 33, 66–80
11. Iwahara, J., Ishihara, M., Daughdriill, G. W., Ford, J., and Clubb, R. T. (2002) EMBO J. 21, 1197–1209
12. Zhu, L., Hu, J., Lin, D., Whiston, R., Itakura, K., and Chen, Y. (2001) Biochemistry 40, 9142–9150
13. Wilsker, D., Patsialou, A., Zuminbrun, S. D., Kim, S., Chen, Y., Dallas, D. B., and Moran, E. (2004) Nucleic Acids Res. 32, 1345–1353
14. Shandala, T., Kortschak, R. D., and Saint, R. (2002) Int. J. Dev. Biol. 46, 423–430
15. Zong, R. T., Das, C., and Tucker, P. W. (2000) EMBO J. 19, 4123–4133
16. Kim, J. H., Kang, J. S., and Chan, C. S. (1999) J. Cell Biol. 145, 1381–1394
17. Webb, C. F., Das, C., Eneff, K. L., and Tucker, P. W. (1991) Mol. Cell. Biol. 11, 5206–5211
18. Johnson, D. R., Levant, S., and Bale, A. E. (1995) BioTechniques 19, 192–195
19. Reyes, J. C., Muchardt, C., and Yaniv, M. (1997) J. Cell Biol. 137, 263–274
20. Webb, C. F., Das, C., Eaton, S., Calame, K., and Tucker, P. W. (1991) Mol. Cell. Biol. 11, 5197–5205
21. Cockerill, P. N., Yuen, M. H., and Garrard, W. T. (1987) J. Biol. Chem. 262, 5394–5397
22. Numata, S., Claudio, P. P., Dean, C., Giordano, A., and Croce, C. M. (1999) Cancer Res. 59, 3741–3747
23. Nixon, J. C., Rajaiya, J., and Webb, C. F. (2004) J. Biol. Chem. 279, 52465–52472
24. Whiston, R. H., Huang, T., and Itakura, K. (1999) Biochem. Biophys. Res. Commun. 258, 326–331
25. Kim, S., Zhang, Z., Upchurch, S., Isern, N., and Chen, Y. (2004) J. Biol. Chem. 279, 16670–16676
26. Alvarez, J. D., Yasui, D. H., Niida, H., Joh, T., Loh, D. Y., and Kohwi-Shigematsu, T. (2000) Genes Dev. 14, 521–535
27. Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P., and Kohwi-Shigematsu, T. (2002) Nature 419, 641–645
28. Cai, S., Han, H. J., and Kohwi-Shigematsu, T. (2003) Nat. Genet. 34, 42–51
29. Seo, J., Lozano, M. M., and Dudley, J. P. (2005) J. Biol. Chem. 280, 24600–24609
30. Reich, N. C., and Liu, L. (2006) Nat. Rev. Immunol. 6, 602–612
31. ten Hoeve, J., de Jesus Ibarra-Sanchez, M., Fu, Y., Zhu, W., Tremblay, M., David, M., and Shuai, K. (2002) Mol. Cell. Biol. 22, 5662–5668