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Repression of B-Cell Linker (BLNK) and B-Cell Adaptor for Phosphoinositide 3-Kinase (BCAP) Is Important for Lymphocyte Transformation by Rel Proteins

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Abstract

Persistent Rel/nuclear factor- κ B (NF- κ B) activity is a hallmark of many human cancers, and the Rel proteins are implicated in leukemia/lymphomagenesis but the mechanism is not fully understood. Microarray analysis to identify transformation-impacting genes regulated by NF- κ B's oncogenic ν -Rel and c-Rel proteins uncovered that Rel protein expression leads to transcriptional repression of key B-cell receptor (BCR) components and signaling molecules like B-cell linker (BLNK), the B-cell adaptor for phosphoinositide 3-kinase (BCAP) and immunoglobulin λ light chain (Ig λ), and is accompanied by a block in BCR-mediated activation of extracellular signal-regulated kinase, Akt, and c-Jun-NH₂-kinase in response to anti-IgM. The BLNK and BCAP proteins were also down-regulated in lymphoid cells expressing a transformation-competent chimeric RelA/ ν -Rel protein, suggesting a correlation with the capacity of Rel proteins to transform lymphocytes. DNA-binding studies identified functional NF- κ B-binding sites, and chromatin immunoprecipitation (ChIP) data showed binding of Rel to the endogenous *blnk* and *bcap* promoters *in vivo*. Importantly, restoration of either BLNK or BCAP expression strongly inhibited transformation of primary chicken lymphocytes by the potent NF- κ B oncoprotein ν -Rel. These findings are interesting because *blnk* and other BCR components and signaling molecules are down-regulated in primary mediastinal large B-cell lymphomas and Hodgkin's lymphomas, which depend on c-Rel for survival, and are consistent with the tumor suppressor function of BLNK. Overall, our results indicate that down-regulation of BLNK and BCAP is an important contributing factor to the malignant transformation of lymphocytes by Rel and suggest that gene repression may be as important as transcriptional activation for Rel's transforming activity.

Introduction

The vertebrate Rel/nuclear factor- κ B (NF- κ B) transcription factors [c-rel, RelA, RelB, NF- κ B1 (p50/p105), and NF- κ B2 (p52/p100)] play vital roles in immune, inflammatory, and stress responses and are also implicated in oncogenesis. The viral NF- κ B oncoprotein v-Rel and its cellular homologue c-Rel malignantly transform primary chicken lymphocytes *in vitro* and induce leukemia/lymphomas and mammary adenocarcinomas in animal models (1–5).

Consistent with this, Rel/NF- κ B is constitutively activated in many human cancers and is key to tumor survival, pathogenesis, and chemoresistance (6). Activation of Rel/NF- κ B in human tumors often results from constitutive IKK kinase complex activity or from chromosomal amplification, mutation, rearrangement, and/or overexpression of the *c-rel* or *nf- κ b2* genes (7). Nuclear accumulation of the human c-Rel protein is seen in classic Hodgkin's lymphoma (cHL) and primary mediastinal large B-cell lymphoma (MLBCL), and its suppression triggers apoptosis in B cells and sensitized these and other tumor-derived cells to chemotherapy (8–14).

Rel/NF- κ B family members show remarkably different oncogenic potentials in primary chicken lymphocytes, which are readily transformed *in vitro* by v-Rel and c-Rel but not by RelA, RelB, p50/NF- κ B1, p52/NF- κ B2, or the Rel/NF- κ B-activating kinase IKK β (3,4,15). The C-terminal transactivation domains (TAD) of Rel and RelA seem to be important for their different *in vitro* transforming and tumor-inducing capacities, as chimeras composed of the DNA-binding domain of human RelA fused to the TAD of mouse c-Rel, human c-Rel, or v-Rel (hRelA/v-Rel, hRelA/mc-Rel, hRelA/hc-Rel) could transform primary lymphocytes, in contrast to hRelA and chimeras containing the RelA TAD (15). This suggests differential modulation of gene expression important for oncogenesis.

Rel/NF- κ B activates the expression of antiapoptotic genes and promotes tumor cell growth by inducing proinflammatory cytokines and D-type cyclins (16). v-Rel transcriptionally activates expression of c-Jun (AP-1), IRF-4, and IAP1 and promotes alternative splicing of telomerase reverse transcriptase, which play important roles in its ability to transform lymphocytes (17–20). Additionally, recent work uncovered that v-Rel transcriptionally represses SH3BGRL and that this is also important for its transforming activity (21). Here, we show that both v-Rel and c-Rel lead to down-regulation of B-cell receptor (BCR)–signaling molecules B-cell adaptor for phosphoinositide 3-kinase (PI3K; BCAP) and B-cell linker (BLNK; BASH, SLP-65) and that this contributes significantly to v-Rel's transforming activity in lymphocytes. Our results suggest that gene repression may be as important as transcriptional activation for Rel's transforming activity.

Materials and Methods

Cell lines, microarrays and bioinformatic analyses

Rel and RelA proteins were stably expressed at equivalent levels in the chicken DT40 pre-B-cell line (a gift from J. Manley, Columbia University) by electroporation of bicistronic avian spleen necrosis virus-derived retroviral vectors (pJD214-IRES-puro), expressing either chicken, mouse, or human c-Rel (cc-Rel, mc-Rel, hc-Rel), v-Rel, an hRelA/v-Rel chimera, or the human or mouse RelA proteins (hRelA, mRelA), followed by isolation of puromycin-resistant cell lines. Immune cell-specific chicken cDNA microarrays were performed as described (22). Expression profiles were compared with parental DT40 cells by averaging expression data from three independent cell clones expressing each of these proteins using dye-swap experiments, as described in Supplementary Materials. Summary and raw microarray data are available from the GEO database⁷ (accession number GSE9544). Potential NF- κ B-binding sites in the *blnk* and *bcap* regulatory regions were bioinformatically predicted using a position-specific scoring matrix developed as described in the Supplementary Materials.

Anti-IgM stimulation, Western blotting, and *in vitro* kinase assays

DT40 cell lines were stimulated for 10 min with anti-IgM (4 µg/mL; a gift from T. Kurosaki, Kansai Medical University) and analyzed by immunoblotting (23). Other immunoblots were performed as described (15). Antibodies were against Rel, extracellular signal-regulated kinase (ERK), Akt (Santa Cruz), RelA-N (Rockland), actin (Sigma), phosphorylated ERK, phosphorylated Akt (Cell Signaling), Rel-RHD (2716; ref. 24), enhanced green fluorescent protein (EGFP; Torrey Pines), ch-BLNK or BCAP (gifts from T. Kurosaki, Kansai Medical University). c-Jun-NH₂-kinase (JNK) activity was determined in *in vitro* kinase assays with cell lysates immunoprecipitated with anti-JNK1 (Santa Cruz), ³²P-γATP, and a glutathione S-transferase-c-Jun substrate (Cell Signaling), followed by autoradiography and immunoblotting with anti-JNK1, as detailed in the Supplementary Materials.

Reverse transcription-PCR

Reverse transcription-PCR (RT-PCR) was performed within the linear range of the PCR cycle using primers *gapdh* (cctctctggcaagtcacag, catctgccatttgatgttg), *blnk* (ttgctgtgaagcctattca, acacccaaacatgtggat), and *bcap* (gcagccaaccagtagctt, gggacaaatccagccataga).

Gel retardation and chromatin immunoprecipitation assays

Gel retardation assays were performed as described (25) using a palindromic κB DNA site (κB-PD), ch-*bcap* (gatctgaattcgttgggaccccccacctctcctta), ch-*blnk* (gatctgaattcgtcgggaccccccacctctcctta), hu-*bcap* (gatctgaattcgtcgggttctcccacctctcctta), or hu-*blnk* (gatctgaattcgtgggaacttcccacctctcctta) κB sites and 293T cells extracts normalized for equivalent Rel protein levels. An anti-ν-Rel antibody was used for supershifts. Chromatin immunoprecipitation (ChIP) was adapted from Upstate Biotechnology and ref. 21, and used anti-hc-Rel (NR 1136 or NR 265; gifts from N. Rice, National Cancer Institute), PC-139 (Oncogene Research Products), IgG, or normal rabbit serum (NRS; Calbiochem). DNA was PCR-amplified with primers listed in the Supplementary Materials.

Lymphocyte transformation assays

Primary chicken spleen cells (CSC) were transformed with virus harvested from chicken embryo fibroblasts cotransfected with retroviral vectors coexpressing EGFP, ch-BLNK, or ch-BCAP-Flag with ν-Rel. The results of three experiments were calculated as mean ± SD. Animals were used according to the National Cancer Institute Animal Care and Use Committee guidelines under an approved animal study protocol. Additional information is available in the Supplementary Materials.

Results

Microarrays identify a subset of genes down-regulated in DT40 cells expressing Rel proteins

To further clarify the mechanisms that underlie the potent transforming activity of Rel proteins in lymphocytes, we used focused immune system cDNA microarrays (22) to identify genes whose expression is commonly altered in cells expressing NF-κB subunits that transform primary chicken lymphocytes by virtue of a Rel TAD (c-Rel, ν-Rel, and hRelA/ν-Rel). Expression profiles were analyzed in a context independent of their transforming phenotype by stably expressing the human, mouse, chicken, and viral Rel genes, the hRelA/ν-Rel hybrid and human or mouse RelA in the chicken pre-B-cell line DT40 that provided a homogenous lymphocyte background with very low levels of nuclear NF-κB activity (Fig. 1A and data not shown). Three independent cell clones expressing each protein at levels equivalent to those found in ν-Rel-transformed CSCs were analyzed (Fig. 1B and data not shown).

⁷Microarray results are deposited at www.ncbi.nlm.nih.gov/geo/.

As anticipated, several known NF- κ B transcriptional targets like *cyclin d2*, *ikbe*, and *cytokine mip-1 β* were commonly up-regulated in DT40 cell lines expressing the various Rel and RelA proteins (GEO database⁷ accession number GSE9544). Interestingly, a second gene subset was down-regulated in Rel-expressing cells, including important components of the BCR and key players in the BCR-signaling pathway. For example DT40 cells expressing the potently transforming v-Rel showed nearly 25-fold repression of *bcap* (*pik3ap1*) and *ig λ . blnk* (*bash*, *SLP-65*), another important mediator of BCR signaling, was down-regulated by ~9-fold in v-Rel-expressing cells (GEO database⁷ accession number GSE9544).

Consistent with the down-regulation of BCR components and signaling molecules, stimulation of DT40 cells expressing hc-Rel, v-Rel, or hRelA/v-Rel with anti-IgM failed to activate phosphorylated ERK and phosphorylated Akt or induce activation of JNK in contrast to parental DT40 cells and those expressing hRelA (Fig. 1C). Although basal levels of phosphorylated Akt were slightly elevated in v-Rel-expressing cells compared with parental DT40 cells, anti-IgM failed to significantly enhance this response (*lanes 5 and 6*). These results indicate that Rel protein expression in DT40 cells leads to down-regulation of a specific group of genes important for BCR signaling and is correlated with a crippled BCR response.

Suppression of BLNK and BCAP is characteristic of Rel protein expression

Our finding that Ig λ is down-regulated in DT40 cells expressing Rel is consistent with prior studies documenting absence of λ light chain expression in hematopoietic cells transformed by v-Rel (26). However, little is known of the relationship between down-regulation of BLNK and BCAP and Rel-mediated transformation. Both BLNK and BCAP are key adaptor molecules that activate downstream signaling in response to BCR cross-linking (23,27,28). BLNK is a tumor suppressor, and its deficiency is associated with a high incidence of spontaneous pre-B-cell lymphomas (29). On the other hand BCAP participates in the PI3K/Akt pathway that promotes B-cell proliferation and survival and also leads to activation of JNK to promote cell death. Given the potential implications of BLNK and BCAP down-regulation for lymphocyte transformation, we further characterized their expression in Rel-expressing cells.

Consistent with the microarray results, BCAP protein levels were significantly reduced in DT40 cells expressing hc-Rel, v-Rel, or hRelA/v-Rel, compared with parental DT40 cells and those expressing RelA (Fig. 2A). Whereas microarrays suggested repression of *blnk* in cells expressing either Rel or RelA, Western blots of independent cell clones showed that, much like BCAP, BLNK is strongly down-regulated in DT40 cells expressing transformation-competent Rel proteins or the hRelA/v-Rel chimera compared with those expressing hRelA or parental DT40 cells (Fig. 2A, *lanes 2–4* versus *lanes 1 and 5*). BLNK and BCAP proteins were similarly absent in primary CSC transformed either by v-Rel, hRelA/v-Rel, or hc-Rel (Fig. 2B, *lanes 4–6*). In contrast, both BLNK and BCAP were abundantly expressed in the avian leukosis virus (ALV)-transformed immature B-cell line TLT-1 and in parental DT40 cells (*lanes 1 and 2*). Thus, down-regulation of BLNK and BCAP is not common to transformed avian B-cell lines but is rather correlated with expression of transformation-competent Rel proteins. We thus focused further analyses on Rel proteins.

The chicken and human *blnk* and *bcap* regulatory regions contain genuine κ B DNA sites

Bioinformatic analysis of -3 kb to +3 kb surrounding the transcription start site of chicken *blnk* and *bcap* identified a single putative NF- κ B site for ch-*blnk* (CGGGATCCCC at position 263) and two identical tandemly repeated κ B sites upstream of ch-*bcap* (TGGGATCCCC at -336 and -326). Putative NF- γ B sites were similarly predicted in their human counterparts (hu-*blnk* GGGAAGTTCC at -265 and hu-*bcap* CGGGTTCTCC at -2,413). Gel retardation assays with hc-Rel, v-Rel, or RelA/v-Rel proteins expressed in 293T cells confirmed their

interaction with the predicted ch-*blnk* and ch-*bcap* NF- κ B sites, although they differed in efficiency (Fig. 3A and B). A palindromic consensus κ B site served as positive control (κ B-PD). hc-Rel bound more efficiently to the ch-*blnk* κ B site than v-Rel and hRelA/v-Rel. In contrast, the ch-*bcap* κ B site was favored by hc-Rel and v-Rel versus hRelA/v-Rel, suggesting preference for the Rel DNA-binding domain. Despite the seemingly weaker binding of v-Rel and hRelA/v-Rel versus hc-Rel to the ch-*blnk* κ B site, supershifts confirmed their genuine interaction (Fig. 3C).

Because NF- κ B is markedly activated in human MLBCL and cHL due to nuclear accumulation of hc-Rel (8,9,11), we also analyzed the κ B sites predicted in human *blnk* and *bcap* for interaction with hc-Rel in gel retardation assays (Fig. 3D). Like their chicken counterparts, the predicted κ B sites in the hu-*blnk* and hu-*bcap* promoters bound efficiently to hc-Rel *in vitro*. These studies identified genuine NF- κ B-binding sites in the chicken and human *blnk* and *bcap* regulatory regions.

hc-Rel is bound to the *blnk* and *bcap* promoters in chromatin

Next, we used ChIP assays to show direct binding of hc-Rel to the endogenous ch-*bcap* promoter *in vivo* in DT40-hc-Rel cells, but not in parental DT40 cells (Fig. 4A). Anti-hc-Rel specifically immunoprecipitated the ch-*bcap* promoter region in DT40-hc-Rel cells, in contrast to the no antibody, IgG, and NRS controls (lane 5 versus lanes 2–4). The extremely GC-rich nature of the ch-*blnk* regulatory region precluded its analysis by ChIP. Consistent with the binding of Rel proteins to the *bcap* and *blnk* regulatory regions, semiquantitative RT-PCR confirmed repression of *bcap* and *blnk* transcripts in DT40 cells expressing hc-Rel, v-Rel, and hRelA/v-Rel compared with parental DT40 cells (Fig. 4B). This supports the notion that Rel expression is associated with down-regulation of *blnk* and *bcap*.

Interestingly, *blnk* and other BCR signaling components and coreceptors are significantly down-regulated in primary human MLBCL and cHL specimens compared with normal tissue samples (10,30,31). Because nuclear accumulation of hc-Rel is characteristic of these tumors, we used ChIP assays to show that endogenous hc-Rel is bound to the hu-*blnk* and hu-*bcap* promoters in the chromatin of the MLBCL-derived cell line Karpas 1106 (Fig. 4C). Two different anti-hc-Rel antibodies efficiently immunoprecipitated hu-*blnk* and hu-*bcap* compared with the no antibody, IgG, and NRS controls (Fig. 4C, top and middle, lanes 6 and 7 versus lanes 2–4). In contrast, they failed to immunoprecipitate a region from the control human *crp2* gene that lacks an NF- κ B-binding site (bottom, lanes 6 and 7). Anti-hc-Rel antibody PC-139 failed to immunoprecipitate hu-*blnk* and hu-*bcap*, consistent with prior work from our group showing that this antibody does not efficiently pull-down hc-Rel in ChIP.⁸ This further substantiates the specificity of hu-*blnk* and hu-*bcap* ChIP with anti-hc-Rel antibodies NR265 and NR1136. These data show that endogenous hc-Rel in MLBCL-derived tumor cells is bound to the hu-*blnk* and hu-*bcap* promoter regions in the *in vivo* context of chromatin.

Repression of BLNK and BCAP is important for v-Rel-mediated transformation of lymphocytes

Because down-regulation of BLNK and BCAP is seen in DT40 cells expressing Rel proteins, in primary CSC transformed by v-Rel, hRelA/v-rel, or hc-Rel (Fig. 2), and also in human MLBCL and cHL tumor cells that depend on c-Rel for survival (10,30,31), we postulated that their down-regulation might contribute to Rel's function in lymphocyte transformation. We assessed the effect of restoring *blnk* or *bcap* expression on the transformation of primary chicken lymphocytes by v-*rel*, which is the most potent transforming member of the Rel/NF-

⁸L.C. Edelstein and C Gélina, unpublished data.

κ B family. The *blnk* or *bcap* cDNAs were coexpressed along with *v-rel* using a bicistronic retroviral vector, and their efficient expression was confirmed by Western blot (Fig. 5A). Coexpression of either ch-BLNK or ch-BCAP markedly impaired v-Rel-mediated transformation of primary CSC, as it dramatically reduced colony formation in soft agar by 7-fold to 39-fold compared with the EGFP control (Fig. 5B). These results show that suppression of *blnk* and *bcap* expression is important for Rel's transforming activity.

Discussion

Although NF- κ B is best known for its transcriptional activation function, recent work with RelA, RelB, and v-Rel showed that it can down-regulate specific genes and that these can significantly affect its role in apoptosis and oncogenesis (21,32–35). Whereas tumor suppressor alternative reading frame, UV-C, and certain chemotherapeutic drugs can induce RelA to repress transcription of antiapoptotic genes Bcl-xL, XIAP, and/or A20 to sensitize cells to apoptosis, v-Rel down-regulates SH3BGRL that severely impairs its transforming activity although its biological function remains to be determined (21,33,34,36,37). Here, we show that Rel proteins that can transform primary chicken lymphocytes, including a hRelA/v-Rel chimera, down-regulate expression of BCR component Ig λ and BCR signaling adaptors BLNK and BCAP. Their transcriptional down-regulation was most pronounced in cells expressing v-Rel, which is the most potent oncogenic member of the NF- κ B family. We also show that Rel binds to the *blnk* and *bcap* promoters and that expression of BLNK or BCAP strongly antagonizes lymphocyte transformation by v-Rel. These findings underscore that the ability of Rel to induce gene-specific transcriptional repression of these factors is important for its oncogenic activity in lymphocytes.

NF- κ B can use different mechanisms to antagonize gene expression, including direct transcriptional repression as seen for Bcl-xL, X-IAP, TRAF2, c-IAP, FLIP, and SH3BGRL and posttranscriptional repression of IRAK1 and TRAF6 via induction of microRNA (21,33–35,38,39). Although in many cases, the detailed mechanisms remain to be uncovered, down-regulation of Bcl-xL by RelA is promoter-specific and involves association with HDAC1 (33,34). How recruitment of Rel proteins to the *blnk* and *bcap* promoter regions leads to their down-regulation remains to be determined. The histone deacetylase inhibitor trichostatin A (TSA) failed to abrogate down-regulation of *blnk* and *bcap* in DT40 cells expressing Rel (data not shown), but we do not rule out involvement of other TSA-insensitive HDACs. Interaction with other transcription factors might also influence the transcriptional outcome, as shown for the *Drosophila* NF- κ B protein Dorsal that is converted from a transcriptional activator into a repressor via interaction with DSP1 (40). Rel might suppress *blnk* and/or *bcap* expression by recruiting transcriptional repressors and/or by functionally interfering with activators that regulate their expression. Future studies will help to identify the mechanisms involved.

BCR signaling leads to growth arrest and apoptosis in immature B cells, whereas it promotes survival and proliferation of mature B cells via activation of Rel-dependent antiapoptotic and proliferative genes (41–47). Both BLNK and BCAP play important roles in BCR signaling (28,48,49), and coexpression of either of these molecules potently reduced v-Rel's ability to transform lymphocytes. In this regard, why would down-regulation of BLNK and BCAP be important for v-Rel-mediated transformation? The answer might reside in the fact that v-Rel-transformed cells are commonly arrested in the early stages of B-cell differentiation, although it can also transform some more mature B cells, T, myeloid, and dendritic cells (1,50–53), and that BLNK is critically required for B-cell differentiation. Indeed, BLNK is a tumor suppressor and BLNK^{-/-} mice show a high incidence of spontaneous pre-B-cell lymphomas that result from defective B-cell differentiation (29). Reintroduction of BLNK restored BLNK^{-/-} pre-B-cell differentiation and inhibited their capacity to induce lymphoma (54). The long isoform of BLNK was also implicated in promoting BCR-induced apoptosis (55). On the other hand,

BCAP participates in the PI3K/Akt pathway that promotes B-cell proliferation and survival and also leads to activation of JNK upon BCR engagement (27,49). Given the interplay between the NF- κ B and JNK signaling pathways where NF- κ B fails to protect cells when JNK activation is sustained (56), it is tempting to speculate that suppression of BLNK, BCAP, and other BCR components like Ig λ might contribute to v-Rel's ability to block B-cell differentiation and/or cell death to favor transformation of immature B cells. In this context, Rel's ability to activate antiapoptotic and proliferative genes would provide the necessary prosurvival and proliferative activities to malignantly transform lymphocytes in the absence of BCR signaling (14,16,43,44,57–61).

It is interesting to note that suppression of several BCR components and signaling molecules and activation of Rel/NF- κ B are characteristic of human MLBCL and cHL that depend on hc-Rel for survival (10,62). Indeed both tumor types show significantly decreased expression of BLNK, the cell surface immunoglobulin receptor IgM, tyrosine kinase Blk, the Bruton tyrosine kinase-binding protein SAB and PKC β (which act downstream of BCAP), and Akt (which lies downstream of BCAP) are also reduced in MLBCL (10,30,31). Although the mechanisms by which Rel proteins function in lymphocyte transformation might differ across species (63), our findings with v-Rel in chicken lymphocytes raise the possibility that because resistance to apoptosis and defects in cell differentiation are commonly linked with cancer, persistent Rel protein activation might contribute to the tumor phenotype by helping to down-regulate BCR signaling, and at the same time enabling cells to bypass their reliance on BCR signaling for proliferation and survival through Rel's proliferative and antiapoptotic activities. Overall, this study highlights a novel link between Rel protein expression, down-regulation of BLNK and BCAP, and Rel-mediated lymphocyte transformation and suggests that gene repression may be as important as transcriptional activation for the transforming activity of Rel proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of Rel proteins in DT40 cells and their effects on BCR signaling. *A*, Western blot of parental and DT40-derived cell lines stably expressing Rel or RelA proteins. *B*, Western blot showing that Rel protein expression in DT40 cell lines is at levels comparable with those found in v-Rel-transformed CSC. An asterisk marks the position of endogenous chicken c-Rel. *C*, immunoblot showing defective activation of phosphorylated ERK, phosphorylated Akt, and JNK kinase activity in DT40 cells expressing Rel proteins upon BCR cross-linking with anti-IgM compared with parental DT40 cells and those expressing RelA.

Figure 2.

Down-regulation of BLNK and BCAP is characteristic of Rel-expressing cells. *A*, immunoblot showing down-regulation of BLNK and BCAP proteins in DT40 cells expressing different Rel proteins compared with parental DT40 cells and those expressing RelA. Asterisks mark the positions of BLNK and two BCAP variants. *B*, immunoblots revealed absence of detectable BLNK and BCAP expression in primary CSC transformed by either v-Rel, hc-Rel, or hRelA/v-Rel, akin to DT40 cells expressing v-Rel, but not in ALV-transformed TLT-1 or parental DT40 pre-B cells.

Figure 3.

NF-κB-binding sites are present in the *blnk* and *bcap* promoter regions. *A*, immunoblot showing normalized expression of hc-Rel, v-Rel, or hRelA/v-Rel in transfected 293T cells. *B*, gel retardation assay of 293T cell extracts expressing hc-Rel, v-Rel, or hRelA/v-Rel and oligonucleotides containing predicted κB DNA sites from ch-*blnk*, ch-*bcap*, or a palindromic κB site control (κB-PD). Vector-transfected cells (*vector 1*) served as control for hc-Rel and hRelA/v-Rel; more extracts from vector-transfected cells (*vector 2*) served as control for v-Rel. *C*, supershift of v-Rel or hRelA/v-Rel bound to the ch-*blnk* κB site with anti-v-Rel or preimmune serum (*Pre*). *D*, gel retardation assay of 293T cells expressing hc-Rel with oligonucleotides containing the κB sites from human *blnk* or *bcap*.

Figure 4.

hc-Rel is bound to the endogenous *blnk* and *bcap* promoters *in vivo*. A, ChIP of hc-Rel bound to the ch-*bcap* promoter in DT40-hc-Rel cells compared with parental cells. ChIP with anti-hc-Rel, IgG, or NRS was PCR-amplified with primers for the ch-*bcap* promoter. B, RT-PCR showing that Rel protein binding to the ch-*blnk* and *bcap* promoters coincides with reduced *blnk* and *bcap* mRNA levels compared with parental cells. C, ChIP of endogenous hc-Rel bound to the hu-*blnk* and *bcap* promoters in MLBCL-derived Karpas 1106 cells. ChIPs with anti-hc-Rel antibodies PC-139, NR265 or NR1136, IgG, or NRS were PCR-amplified with primers for the hu-*bcap* or *blnk* promoters or *crp2* as control.

Figure 5. BCAP and BLNK strongly antagonize v-Rel-mediated transformation of primary chicken lymphocytes. *A*, immunoblot showing efficient coexpression of BLNK or BCAP and v-Rel. An asterisk marks the position of v-Rel. *B*, coexpression of BLNK or BCAP with v-Rel markedly reduced primary CSC transformation as detected by colony formation in soft agar. Average of three independent assays.