SUPPLEMENTARY MATERIAL

“Repression of B-cell receptor signaling molecules BLNK and BCAP is important for lymphocyte transformation by Rel proteins”

by

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Cells and plasmids

DT40 cells (a gift from J Manley; Columbia Univ. NY) were maintained in RPMI 1640, 10% fetal bovine serum (FBS), 1% chicken serum and 1% antibiotics. Chicken spleen cells (CSC) transformed by Rel proteins, the ALV-transformed immature B-cell line TLT-1 and primary chicken embryo fibroblasts (CEF; Charles River SPAFAS, North Franklin, CT) were maintained in DMEM, 20% FBS, 1% chicken serum and antibiotics. Human 293T cells (ATCC) were maintained in DMEM, 10% FBS and 1% antibiotics. MLBCL-derived Karpas 1106 cells (a gift from A. Karpas; University of Cambridge Clinical School, MRC Centre, UK) were cultured in RPMI 1640, 10% FBS and 1% antibiotics. All cells were cultured at 37°C, 5% CO₂, with the exception of CSC and TLT-1 which were maintained at 40.5°C, 7.5% CO₂. Rel and RelA proteins were stably expressed in DT40 cells by electroporation of bicistronic avian spleen necrosis virus-derived retroviral vectors (pJD214-IRES-puro), created by blunt-end ligation of a NotI/XhoI IRES-puro fragment from pCDNA3.1-Bfl1-IRES-puro into the pJD214 vector (1). mc-Rel, v-Rel, hRelA/v-Rel, cc-Rel, hc-Rel and mRelA cDNAs were subcloned into the Sall site of pJD214-IRES-puro; hRelA was cloned into the ClaI site. Chicken BCAP (chBCAP) and BLNK (chBLNK) were excised from pCDNA3.1-WT BCAP-Flag and pCR2.1BLNK plasmids (gifts from T. Kurosaki, Kansai Medical University, Japan and A.C. Chan, Genentech, USA,
respectively). All were blunt-ligated into the SmaI site of pUC-pJD214-IREs-v-Rel (2). DT40 cells (1x10^7) were electroporated with linearized expression vectors (30µg) at 550V, 25µF on a Gene Pulser (BioRad), followed by selection with puromycin (0.5µg/ml; Sigma) at 24hrs. After limiting dilution in 96-wells, drug-resistant clones were isolated and expanded as established cell lines.

**RT-PCR**

RT-PCR was performed within the linear range of the PCR cycle, as described (2), with primers: GAPDH (cctctctgcaaaatccag, catctgcccatttgatgttg, 23 cycles); BLNK (ttgctgtgaccaagttc, acaccccaaaatgtggtc, 29 cycles); BCAP (gcagccaacccagtccat, gggacaaatccagctggt, 29 cycles) at 94°C 3mins, (94°C 45s, 55°C 30s, 72°C 30s), 72°C 10mins, 4°C.

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

DT40 cells (2.5 x 10^7/ml) stimulated for 10min with anti-chicken IgM (M4, 4µg/ml; a gift from T. Kurosaki, Kansai Medical University, Japan) were lysed in NP-40 buffer (1% NP-40, 150mM NaCl, 20mM Tris [pH 7.5], 1mM EDTA) containing 50mM sodium fluoride, 10µM sodium molybdate and 0.1mM sodium orthovanadate, and protease inhibitor cocktail and analyzed by immunoblotting. Other western blots were performed with extracts prepared in EBC lysis buffer (50mM Tris pH 8.0, 120mM NaCl, 0.5% NP-40, 10% glycerol) with 1X Complete protease inhibitor cocktail (Roche). Total protein concentration was determined by the method of Bradford (3). Proteins were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Schleicher & Schuell) by semi-dry transfer. Membranes were blocked in 5% non-fat milk or BSA in TBST (20mM Tris-HCl pH 7.6, 136mM NaCl, 0.1% Tween-20) and incubated at 4°C overnight with primary antibodies against Rel (Santa Cruz, sc-6955), RelA-N (Rockland, 100-
4165N), actin (Sigma, A2066), phospho-ERK (Cell Signaling, 9101), total ERK (Santa Cruz, sc-94), phospho-Akt (Cell Signaling, 9271), total Akt (Santa Cruz, sc-8312), Rel-RHD (2716; (2), EGFP (Torrey Pines, TP401), chicken BLNK or BCAP (gifts from Dr. T. Kurosaki, Kansai Medical University, Japan). Proteins were detected with HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham Life Sciences). JNK kinase activity was assayed in lysates from untreated or anti-IgM-treated DT40 cell lines (500 µg) prepared in 20mM Tris pH 8.0, 500mM NaCl, 1mM EDTA, 1mM EGTA, 0.25% NP-40 supplemented with protease and phosphatase inhibitors [10mM β-glycerophosphate, 10mM sodium fluoride, 10mM p-nitrophenyl phosphate, 300µM sodium orthovanadate, 1mM benzamidine, 2µM phenylmethanesulphonyl fluoride, 1mM dithiothreitol, 1x protease inhibitor cocktail (Roche)]. Lysates pre-cleared with protein A sepharose were immunoprecipitated in 40mM Tris pH 8.0, 500mM NaCl, 1mM EDTA, 1mM EGTA, 0.1% NP-40 supplemented with protease and phosphatase inhibitors (above) with anti-JNK1 antibody (1 µg; Santa-Cruz Biotechnology sc-474), resuspended in kinase buffer C (20mM HEPES pH 7.7, 2mM MgCl₂) supplemented with protease and phosphatase inhibitors. One-half of the immunoprecipitate was used for in vitro kinase reaction with ³²P-γATP and a GST-c-Jun substrate (2µg; Cell Signaling #9811) followed by transfer to nitrocellulose and autoradiography. The other half of the immunoprecipitate was immunoblotted with anti-JNK1 (Santa-Cruz Biotechnology sc-474).

**Primary lymphoid cell transformation assays**

Primary CSC from three-week old chickens (Charles River SPAFAS, North Franklin, CT; 1.5 x10^7 per 100 µl) partially purified with Ficoll-Paque Plus (Amersham, Biosciences, Sweden) were transformed with retroviral supernatants harvested from CEFs co-transfected with pJD214-IRES-v-Rel derived retroviral vectors (5 µg) co-expressing EGFP, chBLNK or chBCAP-Flag and SW253 Rev-A helper virus DNA (0.1 µg), as described (2). Cells were seeded into soft agar and
transformed colonies were scored after 2 weeks. 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.

**Gel-shift and chromatin immunoprecipitation (ChIP) assays**

Gel retardation assays were performed as described (2) with lysates from transfected 293T cells normalized to equivalent Rel protein levels by immunoblotting with anti-Rel (sc-6955, Santa Cruz). Oligonucleotide probes contained a palindromic κB DNA site (4), the predicted chicken *bcap* (gatetgaattctggatccccacccctctta), *blnk* (gatetgaattctggatccccacccctctta), or human *bcap* (gatetgaattctggatccccacccctctta) or *blnk* (gatetgaattctggatccccacccctctta) NF-κB sites. Supershifts were with an antibody specific for the C-terminus of v-Rel (Ab 1691; (5)).

ChIP assays were according to Upstate Biotechnology’s protocol (Charlottesville, VA) with modifications. Cells (1.5x10^7) suspended in serum free RPMI (10ml) were cross-linked with formaldehyde (1% final) at 37°C for 10mins. After quenching with glycine (0.125M final) for 5mins at 37°C, cells were washed with PBS and nuclei were isolated. Nuclei were lysed in SDS buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8; 300µl) and sonicated 10 times for 10 s each using a sonic dismembrator (Fisher model 300) at setting 30. Lysates (20µl; equivalent to 1x10^6 cells) were diluted to 2ml, precleared for 2hrs using protein-A agarose coated with salmon-sperm DNA (Upstate Biotechnology; 60µl of 50% slurry). Cleared lysates were incubated at 4°C overnight with anti-hc-Rel (1136 or 265, gifts from N Rice, NCI) or PC139 (Oncogene Research Products), Calbiochem normal rabbit IgG (2µg; NI01) or normal rabbit serum (20µl; NS01L). Immunoprecipitated and input (1/50 for DT40) or (1/100 for Karpas 1106) DNA was PCR amplified with primers: ch-*bcap* (tttcaacttcatctgcttt, ccacttcacgttcatctgct, 40 cycles); hu-*bcap* (accaggagaaagctctg, gtttgcagaatgggagtt, 35 cycles); hu-*blnk* (gccagtttgtgattact, 40 cycles).
catgccactgacaacctgat, 35 cycles) or hu-crp2 (6), atgatcccttctgtgtctgcgt, tattgacagcacaaggctcaac, 35 cycles).

**Microarrays and data analyses**

Microarrays were performed with RNA purified with RNeasy MinElute™ (Qiagen). Expression profiles were compared to parental DT40 cells by averaging expression data from three independent cell clones each, hybridized to immune system cDNA microarrays comprised of 3,451 immune cell-specific chicken cDNAs (7). For each array result, spot intensity signals were assessed for signal quality and those identified as poor quality were removed from further analysis. Spot-level ratios were background corrected, log$_2$ transformed, and loess normalized. Sample comparisons were performed using three biological independent experimental replicates that were each compared to a reference. For each comparison, a dye-swapped technical replicate was also performed and the paired results were averaged and used as a single observation. Accordingly, a total of six arrays contributed to three independent observations. Differential expression analysis was performed using CyberT (8), a Bayesian t-statistic methodology that is designed for microarray analyses in studies with low replicate numbers. For our CyberT analysis, we employed the default parameters. Differential gene expression was identified by ranking each gene’s corresponding Bayesian p-values and applying a false discovery rate correction of 5% (9). In addition, we applied a fold-change threshold of ± 1.5 as an additional criterion. Accordingly, relative expression levels for a given gene with a p-value that satisfies the FDR condition and fold-change criteria were identified as differentially expressed. The summary and raw microarray data are available from the GEO database (www.ncbi.nlm.nih.gov/geo/; accession number GSE9544).

**Bioinformatic analyses**
A position specific scoring matrix (10) for searching potential 10-mer NF-κB recognition sites was created using 53 known NF-κB binding sites (11). Background probabilities were calculated using the sequence of all chicken genes in ENSEMBL (12) (WASHUC1 assembly) in addition to 10kb upstream and 10kb downstream sequences. Background probabilities were also used as pseudocounts. For each gene, a 6kb region (within the 3kb upstream and 3kb downstream of the transcription start site) was extracted from ENSEMBL. All 10-mers (both forward and reverse) in this region were scored. Those with scores greater than 6 were considered potential NF-κB binding sites. In case of overlapping sites, the site with the greatest score was reported. All computational work was performed using the Perl programming language.

References for Supplementary Materials


2. Fan Y, Gélinas C. An optimal range of transcription potency is necessary for efficient cell transformation by c-Rel to ensure optimal nuclear localization and gene-specific activation. Oncogene 2007;26:4038-43.


