RelB NF-κB Represses Estrogen Receptor α Expression via Induction of the Zinc Finger Protein Blimp1

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Aberrant constitutive expression of NF-κB subunits, reported in more than 90% of breast cancers and multiple other malignancies, plays pivotal roles in tumorigenesis. Higher RelB subunit expression was demonstrated in estrogen receptor alpha (ERα)-negative breast cancers versus ERα-positive ones, due in part to repression of RelB synthesis by ERα signaling. Notably, RelB promoted a more invasive phenotype in ERα-negative cancers via induction of the BCL2 gene. We report here that RelB reciprocally inhibits ERα synthesis in breast cancer cells, which contributes to a more migratory phenotype. Specifically, RelB is shown for the first time to induce expression of the zinc finger repressor protein Blimp1 (B-lymphocyte-induced maturation protein), the critical mediator of B- and T-cell development, which is transcribed from the PRDM1 gene. Blimp1 protein repressed ERα (ESR1) gene transcription. Commensurately higher Blimp1/PRDM1 expression was detected in ERα-negative breast cancer cells and primary breast tumors. Induction of PRDM1 gene expression was mediated by interaction of Bcl-2, localized in the mitochondria, with Ras. Thus, the induction of Blimp1 represents a novel mechanism whereby the RelB NF-κB subunit mediates repression, specifically of ERα, thereby promoting a more migratory phenotype.

NF-κB is a structurally and evolutionarily conserved family of dimeric transcription factors with subunits having an N-terminal region of approximately 300 amino acids that shares homology with the v-Rel oncoprotein (17, 44). The conserved Rel homology domain is responsible for DNA binding, dimerization, nuclear translocation, and interaction with inhibitory proteins of NF-κB (IkBs). Mammals express five NF-κB members, including c-Rel, RelB, RelA (p65), p50, and p52, which can form either homo- or heterodimers. RelB differs from the other members in that it only binds DNA as a heterodimer with either p52 or p50 and interacts only poorly with the inhibitory protein IκB. In most untransformed cells, other than B lymphocytes, NF-κB complexes are sequestered in the cytoplasm bound to specific IκB proteins.

Aberrant activation of NF-κB has been implicated in the pathogenesis of many carcinomas (37). Constitutive activation of c-Rel, RelA, p50, and p52 was first detected in breast cancer (9, 30, 45). RelB appeared to have more limited involvement and functioned predominantly in lymphoid organs and their malignancies (25, 58). For example, Stoffel et al. found p50/RelB complexes in mucosa-associated lymphoid tissue lymphoma (46), and RelB complexes were implicated in Notch1-induced T-cell leukemia (53). More recently, RelB has been implicated in carcinomas of the breast and prostate. RelB was the most frequently detected NF-κB subunit in nuclear preparations from advanced prostate cancer tissue and correlated directly with Gleason score (26), suggesting an association with prostate cancer progression. We observed elevated nuclear RelB levels in mouse mammary tumors driven by ectopic c-Rel expression in transgenic mice or after carcinogen treatment (14, 40). More recently, we demonstrated that RelB synthesis, which is mediated via synergistic transactivation of the RELB promoter by p50/RelA NF-κB and c-Jun/Fra-2 AP-1 complexes, was selectively active in estrogen receptor α (ERα)-negative versus -positive breast cancers and led to the induction of the BCL2 gene (56).

While studying the mechanism of the inverse correlation between RelB and ERα levels in breast cancer, more recently we observed that RelB complexes robustly inhibited ERα (ESR1) gene expression. Since RelB is not by itself inhibitory, we postulated that it controls the expression of an intermediate negative regulatory factor that represses ERα gene transcription. TransFac analysis identified putative binding sites for the B lymphocyte-induced maturation protein (Blimp1), which is expressed from the PRDM1 gene (24) and has been reported to be regulated by NF-κB, although it is not known whether this control is exerted directly or indirectly (22). The Zn finger protein Blimp1 is a repressor of transcription and has been...
shown to function as a master regulator of development of antibody-secreting B lymphocytes and more recently of T-cell homeostasis and function (6, 28), and specification of primordial germ cells (33, 54). Blimp1 was originally identified as a silencer of Ifn-β gene transcription (24). Although the exact mechanism by which repression occurs is not fully understood, Blimp1 possesses DNA-binding activity and can recruit histone deacetylases, histone methyltransferases, and the corepressor Groucho (19, 39, 63). We demonstrate here for the first time that Blimp1 functions as a potent repressor of ERα synthesis in breast cancer cells and is induced by activation of a Bcl-2/Ras pathway by RelB.

MATERIALS AND METHODS

Cell culture and treatment conditions. ERα-positive MCF-7, T47D, ZR-75, and BT474 cell lines and ERα-negative MCF-7 and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in standard culture medium as recommended by the ATCC. The MCF-7 cell line, kindly provided by Philip Leder (Harvard Medical School, Boston MA) was derived from a mammary gland tumor in an ERβ2 double transgenic mouse and cultured as described previously (18). Hs578T cell lines containing human pRelB siRNA or control pRelB sense (si-Control) were established as described previously (56) and grown in the presence of 1 μg/mL of puromycin (Sigma, St. Louis, MO). MCF-7 cell lines containing pBABE and pBABE-Bcl-2 were established during amplification. The pC4bsrR(TO-RelB) construct was generated by subcloning the pRelB siRNA or control pRelB sense (si-Control) were established as described previously (56) and grown in the presence of 1 μg/mL of puromycin. Hs578T or MCF-7 cell lines carrying pSM2c-sh-BCL2 (V2HS_111806; Open Biosystems) or scrambled control were established by exchanging the Bcl-2 carboxy-terminal insertion sequence for an equivalent EGFP-C1 retroviral vector. The Ras C186S mutant and the ERα-positive MCF-7, T47D, ZR-75, and MDA-MB-231 cell lines containing the indicated point mutation, was kindly provided by Tristram G. Parslow (Cambridge, MA) and Pierre Chambon (Strasbourg, France), respectively. The Ras CA (RasV12) vector was as previously described (18). MCF-7 cell lines containing inducible pC4bsrR(TO) retroviral vector. The Ras C186S mutant and the ERα promoter (kindly provided by Ronald Weigel, University of Iowa College of Medicine, Iowa City) using Pfu Hotstart Ultra with the following PCR primers (restriction sites are italicized, and the ERα sequence is underlined): KpnI-prob-3489, 5′-CCGGCCTGGTTACTCCGATGCTCAGACACACGACG-3′; HindIII-prob-1814, 5′-CGCGTCAGGCCTCAAGGCTTCCAGGCTATTGGCGTGG-3′. After amplification, the product was digested with KpnI and HindIII and subcloned into pG3Basiac; sequencing confirmed that no mutations were introduced during amplification. The pC4bsrR(TO-RelB) construct was generated by subcloning the RelB cDNA into the EcoRI restriction site of the doxycycline inducible pC4bsrR(TO-R) retroviral vector. The Ras C186S mutant and the ERα expression vector were kindly provided by Mark R. Philips (NYU School of Medicine, New York, NY) and Pierre Chambon (Strasbourg, France), respectively. The Ras-EGFP-C1 vector was generously provided by Wen-Luan Wendy Hsiao (Hong Kong Baptist University). The Ras CA (RasV12) vector was as previously described (20). The human full-length PRDM1 construct in pcDNA3 was as reported (39). The TBlip vector expressing a dominant-negative (dn)-Blimp1 protein, containing the DNA-binding domain of Blimp1 in the Vpx-puro vector, and the 7-kb human PRDM1 promoter construct in pGL3 was kindly provided by Kathryn Calame (Columbia University, NY) (1, 49), pRelB sense and pRelB siRNA containing control or si-RelB oligonucleotides in pSilRen-RetroQ vector were described previously (41). The human full-length BCL2 constructs in pcDNA3 or pBabe were as reported (42). The BCL2 mutant LI4G, with the indicated point mutation, was kindly provided by Tristram G. Parslow (Emory University School of Medicine, Atlanta, GA). The pRC-CMV-Bcl-B, pRC-CMV-Bcl-acta, and pRC-CMV-Bcl-nt constructs, which were kindly provided by David W. Andrews (McMaster University, Hamilton, Ontario, Canada), were as previously described (64). Briefly, the Bel-acta (mitochondrion-localized Bel-2 mutant) and the Bel-nt (cytoplasm-localized Bel-2 mutant) were established by exchanging the Bel-2 carboxy-terminal insertion sequence for an equivalent sequence from Lacta Acta or by deletion of the Bel-2 carboxy-terminal insertion sequence, respectively. The simian virus 40 β-galactosidase (SV40-β-Gal) reporter vector was as previously reported (57). The estrogen response element (ERE)-TK luciferase construct was as reported elsewhere (18). For transfection into six-well or P100 plates, 3 or 10 μg, respectively, of total DNA was used. For transient transfection, cultures were incubated for 48 h in the presence of DNA and Fugene (Roche Diagnostics Co., Indianapolis, IN) or Geneporter2 (Gene Therapy Systems, Inc., San Diego, CA) transfection reagent. All transient-transfection reporter assays were performed, in triplicate, a minimum of three times. Luciferase assays were performed as described previously (40, 45). Cotransfection of the SV40-β-Gal expression vector was used to normalize for transfection efficiency, as described previously (40). PRDM1 small interfering RNA (siRNA) duplex sequences have been previously described (61). Duplexes (0.8 nM final) were introduced in cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The dsRNA was obtained from Sigma. Antibodies to mouse Blimp1 (NB600-235) and human Blimp1 (ab13700) were purchased from Novus Biologicals and Abcam, respectively. For immunoprecipitation, WCEs were prepared in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, 10 mM Na4O4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 10 μM β-glycerophosphate) and precleared WCEs (50 μg) were incubated overnight at 4°C with antibodies against Bel-2 or Ras and the corresponding normal immunoglobulin G (IgG) in NETN buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, and 1 mM EDTA plus protease and phosphatase inhibitors). Immunoprecipitates were collected using protein A-Sepharose beads, washed four times in NETN buffer, and then subjected to immunoblotting.

EMSA. Nuclear extracts were prepared, and samples (5 μg) were subjected to binding and electrophoretic mobility shift assay (EMSA) as described previously (57). The sequences of the oligonucleotides used were as follows: c-MYC Blimp1 site, 5′-ACAGAAGGGGAGGACTGACGATCG-3′; Puta-1 Blimp1 site, 5′-GATCCGGAAAGGAAAGGGGTCTG-3′; Puta-2 Blimp1 site, 5′-GATCCTTGGAGAAATGTGCAAAC-3′; and Puta-3 Blimp1 site, 5′-GATCCGGCAAGAAGAGCCTGATCGT-3′. The Oct-1 sequence was as reported previously (45). For supershift analysis, 200 ng of goat anti-Blimp1 antibody (Abcam) was incubated overnight with 5 μg of nuclear extract at 4°C; labeled probe was then added, and the mixture was incubated at room temperature for 30 min, as described previously (57).

ChIP assay. Cells were cross-linked with 1% formaldehyde at room temperature for 10 min, and the reaction was stopped by the addition of glycine to a final concentration of 125 mM. After three washes in cold phosphate-buffered saline (PBS), cell pellets were resuspended in chromatin immunoprecipitation (ChIP) lysis buffer (50 mM HEPS [pH 7.5], 150 mM NaCl, and 1% Triton X-100 plus protease inhibitor cocktail) and incubated 30 min on ice. After sonication and centrifugation, the supernatants were incubated with 2 μg of salmon sperm DNA, 150 μg of bovine serum albumin, and 50 μl of protein G-Sepharose for 2 h at 4°C and then centrifuged to preclear. The resulting supernatants were immunoprecipitated overnight at 4°C with 2 μg of Blimp1 antibody (ab13700) plus 50 μl of protein G-Sepharose and 2 μg of salmon sperm DNA. Immunoprecipitates were washed sequentially for 5 min each time at 4°C with ChIP lysis buffer, ChIP lysis high-salt buffer (50 mM HEPS [pH 7.5], 500 mM NaCl, 1% Triton X-100), ChIP wash buffer (10 mM Tris [pH 8], 250 mM LiCl, 0.5% NP-40, 1 mM EDTA) and twice with Triton-EDTA buffer. Immunoprecipitates were then eluted twice with elution buffer (50 mM Tris [pH 8], 1% SDS, 10 mM EDTA) at 65°C for 10 min, and both input and pooled eluates were incubated at 65°C to reverse the cross-linking. DNA was purified with QIAquick PCR purification kit and 1 μl of DNA extraction was used for PCR. (Information on PCR primers and conditions for the ChIP assay is presented in the supplemental material.)
RESULTS

RelB represses synthesis of ERα. The inverse relationship between NF-κB and ERα that typifies many breast cancer cells and tissues has previously been related to the inhibition of NF-κB synthesis or activity by ERα signaling (23, 51, 56). Unexpectedly, we observed the reciprocal repression of ERα activity by RelB in cultures of stable clones of ERα-positive MCF-7 cells that ectopically express elevated RelB levels, as described previously (56). Specifically, MCF-7 clones RELB(1) and RELB(2) showed marked reduction of ERα-driven reporter activity compared to control MCF-7 EV(1) and EV(2) clones, with parental pcDNA3 empty vector (EV) DNA (Fig. 1A). To verify that the observed effects of RelB on ERα activity were not due to clonal selection, transient-transfection analysis was performed using ERα-positive ZR-75 cells with vectors expressing either RelB or binding partners p50 or p52 alone, or in combination. RelB expression resulted in a substantial decrease in ERα-driven reporter activity, which was further decreased upon coexpression of p50 or p52 (Fig. 1B). (Similar data were obtained by using transient transfection of MCF-7 cells [data not shown]). To verify the effects of RelB on endogenous expression of ERα target genes, RNA was isolated from MCF-7/RELB(1) and MCF-7/EV(1) cells. RT-PCR analysis confirmed decreased RNA levels of target genes CATHEPSIN D, Retinoic Acid Receptor α (RARα), p52, and MITA3 (human) in MCF-7/RELB(1) compared to MCF-7/EV(1) cells (Fig. 1C). Similarly, endogenous mRNA levels of CATHEPSIN D and RARα were decreased in ZR-75 and MCF-7 cells upon transient transfection of RelB/p50 or RelB/p52 (Fig. 1D, upper and lower panels). To determine whether RelB decreases ERα levels, transient transfection analysis of vectors expressing RelB in the presence of either p50 or p52 was performed in ZR-75 and MCF-7 cells. Decreased endogenous levels of ERα protein (Fig. 1E) and ERα mRNA (Fig. 1F) were seen with both RelB complexes. (All protein and RT-PCR samples in Fig. 1E and F, respectively, were from the same gels.) ERα has two major promoters, A and B, of which the latter is predominantly used in breast cancer cells. Cotransfection of RelB and p52 expression vectors with the ERα promoter B reporter decreases its activity ~5-fold in MCF-7 and ZR-75 cells (Fig. 1G). Lastly, we monitored the effects of RelB on endogenous ERα levels and ERα promoter B reporter activity in the stable MCF-7/RELB(1) and RELB(2) clones and the control MCF-7/EV(1) and EV(2) clones. RelB expression reduced levels of ERα protein (Fig. 1H) and RNA (Fig. 1I), as well as promoter activity (Fig. 1J). Thus, RelB complexes robustly inhibit ERα expression and activity.

RelB induces expression of the zinc finger repressor protein Blimp1. Since RelB has a transactivation domain and has not been shown to function itself directly as an inhibitor of transcription, we tested the hypothesis that it controls the expression of an intermediate negative regulatory factor that represses ERα. TransFac analysis (at 80% certainty) of promoter B sequences identified three putative binding sites for Blimp1, a master regulator of B and T-cell development. The Zn finger protein Blimp1, which functions as a repressor of key regulatory genes in these cells, is expressed from the gene termed PRDM1 (human) or Blimp1 (mouse) (24). Since expression of Blimp1 has not been reported in epithelial cells, we first tested for endogenous Blimp1 protein in nuclear extracts of human and mouse breast cancer cells. Substantial Blimp1 levels were detectable in ERα-negative Hs578T human breast cancer cells, and more moderate levels were detectable in ERα-negative MDA-MB-231 cells (Fig. 2A). Very low, but detectable Blimp1 levels were seen with ERα positive ZR-75, MCF-7, T47D, and BT474 human breast cancer cell lines (better seen on darker exposures and see Fig. 2B below). The murine NF639 Her-2/neu-driven ERα low breast cancer cell line displayed a moderate level of Blimp1 protein. RT-PCR confirmed the presence of PRDM1 mRNA in human breast cancer cells and demonstrated that the levels of expression were much higher in ERα-negative versus ERα-positive lines (Fig. 2B). To test whether the inverse correlation between Blimp1 and ERα predicted by these findings exists in primary human breast cancers, microarray gene expression datasets publicly available at www.oncomine.org were analyzed. The levels of PRDM1 mRNA were significantly higher in ERα-negative versus ERα-positive breast cancers (Fig. 2C, left panel) in the Van de Vijver_Breast carcinoma microarray data set (reporter number NM_001198) (50) (P = 1.3e-6 [Student t test]), consistent with the analysis of the cell lines. The data from an additional microarray study (Bittner_Breast, reporter number 229964_at, publicly available at www.oncomine.org) confirmed these findings (P = 7.3e-7 [Student t test]) (Fig. 2C, right panel). Thus, PRDM1 gene expression occurs in patient samples and breast cancer cell lines, with higher levels in ERα-negative cells, a finding consistent with the pattern of RelB expression (56).

Previously, we prepared stable Hs578T transfectants expressing either RELB siRNA (Hs578T RELB siRNA) or sense RELB control siRNA (Hs578T control siRNA) (56). Analysis of these cells demonstrated that knockdown of RelB leads to decreased levels of PRDM1 mRNA (Fig. 2D) and Blimp1 protein (Fig. 2E). Conversely, RelB/p52 expression in human
FIG. 1. RelB represses ERα synthesis in breast cancer cells. (A) Stable MCF-7 clones expressing either RELB or EV DNA, isolated as described previously (56) and termed RELB(1), RELB(2), EV(1), and EV(2), were transfected with 0.5 µg of ERE-TK luciferase reporter, 0.5 µg of SV40-β-Gal. Normalized ERE activity, set to 100% in the stable EV cells, was decreased in both RelB-expressing lines (mean ± the SD from three separate experiments). (B) ZR-75 cells were transiently transfected with 0.5 µg of ERE-TK luciferase DNA plus 0.5 µg of the indicated NF-κB subunit expression vectors, 0.5 µg of SV40-β-Gal, and EV pcDNA3 DNA to a 3.0 µg of DNA total. Normalized ERE activity, set to 100% in the EV-transfected cells, was reduced by RelB alone or in combination with p50 or p52 (mean ± the SD from three separate experiments). (C) RNA, isolated from EV(1) and RELB(1) stable MCF-7 clones, was subjected to RT-PCR analysis for expression of ERα target genes CATHEPSIN D (CATHD), RARα, p52, MTA3, and GAPDH, as a loading control. (D) ZR-75 or MCF-7 cells were transiently transfected with 3 µg of each of the vectors expressing RelB and either p50 or p52 or 6.0 µg of EV DNA. Isolated RNA was subjected to RT-PCR analysis for expression of RARα, CATHEPSIN D (CATHD), and GAPDH. (E and F) ZR-75 or MCF-7 cells were transiently transfected with 3.0 µg of vectors expressing RelB and p50 or p52 or EV DNA, WCEs and RNA were isolated, which were subjected to immunoblot analysis for the expression of ERα and β-actin (E) and to RT-PCR analysis for the expression of ERα and GAPDH (F), respectively. (Samples in panels E and F were run on the same gels, and the lanes were brought into contiguous positions.) (G) ZR-75 and MCF-7 cells were transiently transfected with 0.5 µg of ERα proB luciferase reporter plus 0.25 µg of vectors expressing RelB and p52 or 0.5 µg of EV pcDNA3 DNA, 0.5 µg of SV40-β-Gal, and EV DNA to a 3.0 µg of DNA total. Normalized proB activity values are presented as the means ± the SD from three experiments (control EV DNA set to 1). (H and I). Protein and RNA, isolated from RELB(1), RELB(2), EV(1), and EV(2) stable MCF-7 clones, were subjected to immunoblotting for the expression of ERα, RelB, and β-actin (H), and to RT-PCR analysis for the expression of ERα and GAPDH (I), respectively. (J) RELB(1), RELB(2), EV(1), and EV(2) MCF-7 clones were transiently transfected with 0.5 µg of ERα proB luciferase reporter, 0.5 µg of SV40-β-Gal, and EV DNA to a 3.0 µg of DNA total. Normalized proB activity values are presented as the means ± the SD from three experiments (control EV DNA set to 1).
FIG. 2. RelB induces Blimp1 in breast cancer cells. (A) Nuclear extracts were isolated from the indicated human and mouse breast cancer cell lines and subjected to immunoblotting for Blimp1 and β-actin, which confirmed equal loading. (B) RNA, isolated from the indicated human breast cancer lines, was subjected to RT-PCR for PRDM1 for either 28 or 30 cycles (as indicated) and for GAPDH levels. (C) In the left panel, a box plot of data from the Van de Vijver Breast carcinoma microarray data set (reporter number NM_001198) (50) was accessed by using the Oncomine Cancer Profiling Database (www.oncomine.org) and is plotted on a log scale. The data set includes 69 ERα-negative and 226 ERα-positive human primary breast carcinoma samples. A Student t test, performed directly through the Oncomine 3.0 software, showed the difference in PRDM1 expression between the two groups was significant (P = 1.3e-6). In the right panel, a box plot of data from the Bittner study (see Results) showed that the difference in PRDM1 expression between the two groups was significant (P = 7.3e-7). (D) RNA was isolated from stable Hs578T transfectants expressing either RELB siRNA (Hs578T RELB siRNA) or sense RELB (Hs578T control siRNA) (Con) and analyzed for PRDM1 levels. (E) Nuclear proteins were isolated from Hs578T RELB siRNA and Hs578T control (Con) cells and analyzed for Blimp1, lamin B, and RelB levels by immunoblotting. (F) ZR-75, MCF-7, and NF639 cells were transiently transfected with 3 μg each of RelB and p52 expression vectors or 6 μg of EV DNA and RNA subjected to RT-PCR for human PRDM1 or mouse Blimp1 RNA levels. (G) ZR-75, MCF-7, and NF639 cells were transiently transfected with 3 μg each of RelB and p52 expression vectors or 6 μg of EV DNA. Nuclear extracts (ZR-75 and MCF-7 [left panels]) and WCEs (NF639 [right panels]) were analyzed by immunoblotting for Blimp1 and either lamin B or β-actin, respectively. (H and I). Nuclear extracts and RNA, isolated from RELB(1), RELB(2), EV(1), and EV(2) stable MCF-7 clones, were subjected to immunoblotting for the expression of Blimp1 and lamin B (H) and to RT-PCR analysis for the expression of PRDM1 and GAPDH (I), respectively. (J) MCF-7 cells were transiently transfected with 0.5 μg of ERα proB luciferase reporter, 0.5 μg of SV40-β-Gal, and EV DNA to a 3.0 μg of DNA total. Normalized proB activity values are presented as the means ± the SD from three experiments (control EV DNA set to 1).
ZR-75 and MCF-7 cells and in mouse NF639 cells effectively induced PRDM1 or Blimp1 RNA, respectively (Fig. 2F), and Blimp1 protein (Fig. 2G). Furthermore, RelB increased the levels of Blimp1 protein (Fig. 2H) and PRDM1 RNA (Fig. 2I) in the stable MCF-7/RELB(1) and RELB(2) clones, indicating functional RelB complexes can induce Blimp1 expression in ERα-positive or -low breast cancer cells.

Next, we sought to test whether the ERα promoter is indeed repressed by ectopic Blimp1 expression and to localize the functional Blimp1 binding element(s). An ~4-fold reduction in activity of an ERα proB construct (Fig. 2J), which contains all three putative Blimp1 binding sites, was seen with ectopic Blimp1 expression in cotransfection analysis in MCF-7 cells compared to EV DNA. The three Blimp1 sites all contain the core GAAA sequence but differ in surrounding sequences (see Materials and Methods). To identify the functional sites, competition and supershift EMSAs were performed with nuclear extracts of ZR-75 cells ectopically expressing Blimp1 protein or control EV DNA and an oligonucleotide containing the Blimp1 binding site of the c-MYC gene as a probe. Putative site 2, but not sites 1 or 3, competed well for binding to the c-MYC Blimp1 site (Fig. 3A). When used as a probe, the putative site 2 oligonucleotide effectively bound Blimp1 protein (Fig. 3B), as judged by a supershift EMSA (Fig. 3C), confirming that this site is a bona fide Blimp1 element. The position of this confirmed Blimp1 site is indicated on the map in Fig. 3D. ChIP analysis confirmed intracellular binding of Blimp1 following ectopic expression to the ERα promoter B region containing the element in MCF-7 cells (Fig. 3E, upper panels). Furthermore, ChIP analysis in Hs578T cells, which expressed the highest levels of Blimp1, confirmed binding of endogenous Blimp1 to this ERα promoter B region (Fig. 3E, lower panels).

To assess the functional role of the low endogenous levels of Blimp1 in the ERα-positive lines, a dominant-negative form of Blimp1, termed TBlimp or dnBlimp (1), which contains only the DNA-binding domain, was used. Ectopic expression of the dnBlimp caused an increase in ERα protein (Fig. 3F), indicating that endogenous Blimp1 represses ERα in these lines. Consistently, as seen in Fig. 4A below, knockdown of Blimp1 levels using an siRNA strategy in NF639 cells induced ERα protein levels. Conversely, ectopic Blimp1 expression substantially reduced ERα expression in ZR-75 and MCF-7 cells (Fig. 3G), indicating that it can repress the endogenous ERα gene in both lines. Blimp1 expression also led to reduced ERα RNA levels in ZR-75 cells, while inhibition of Blimp1 activity with the dnBlimp led to their induction (Fig. 3H). Of note, expression of the dnBlimp ablated the decrease in ERα expression induced by ectopic RelB in MCF-7 cells (Fig. 3I), further implicating Blimp1 in RelB-induced downregulation of ERα gene expression. Thus, Blimp1 is expressed in breast cancer cells and mediates repression of ERα.

Blimp1 promotes a more migratory phenotype in breast cancer cells via inhibition of ERα. In primordial germ cells, the absence of Blimp1 led to defects in cell migration (33). Given our previous findings showing that ERα-negative breast cancer cells have a more migratory phenotype than ERα-positive ones, we next tested whether Blimp1 regulated migration and reduced expression of epithelial markers, which are required for maintenance of a nonmigratory phenotype. First, an siRNA was used to reduce Blimp1 levels in NF639 cells, which are highly invasive and display robust Blimp1 expression. Knockdown of Blimp1 in NF639 cells decreased migration (Fig. 4A, left panel) and increased expression of E-cadherin and ERα (Fig. 4A, right panel). Conversely, ectopic Blimp1 expression in ERα-positive ZR-75 cells, which repressed ERα levels (Fig. 3G), substantially increased migration (Fig. 4B, left panel) and commensurately decreased levels of E-cadherin and γ-catenin (Fig. 4B, right panel). We next assessed the role of ERα in the ability of Blimp1 to promote a more migratory phenotype. Expression of ERα prevented the increased ability of ZR-75 cells to migrate resulting from Blimp1 expression (3.1-fold versus 1.1-fold) (Fig. 4C, left panel), as well as the decrease in E-cadherin and γ-catenin induced by Blimp1 (Fig. 4C, right panel). Lastly, dnBlimp expression induced the levels of E-cadherin expression in ZR-75 and MCF-7 cells, and these increases were prevented upon knockdown of ERα using siErα RNA (Fig. 4D). Together, these results demonstrate Blimp1 induces a more migratory phenotype and implicate the inhibition of ERα in this control.

Bcl-2 induces Blimp1 via functional interaction with Ras. Previously, we demonstrated BCL2 is a critical RelB target that mediates the migratory phenotype of breast cancer cells (56). For example, knockdown of Bcl-2 in Hs578T cells impaired the ability of the cells to migrate, whereas stable ectopic expression of Bcl-2 in MCF-7 cells (MCF-7/Bcl-2 versus MCF-7/pBABE stable cells) was sufficient to promote a more migratory phenotype via decreasing E-cadherin levels (56). Thus, we tested the hypothesis that Bcl-2 mediates the induction of Blimp1 by RelB. Knockdown of Bcl-2 with the Bcl2 shRNA in NF639 cells led to increased ERα levels (Fig. 5A, left panel), whereas Bcl-2 overexpression decreased the ERα level in ZR-75 and MCF-7 cells (Fig. 5A, right panel), indicating that Bcl-2 can negatively regulate ERα expression. Consistently, knockdown of Bcl-2 levels led to downregulation of Blimp1 protein and Blimp1 RNA levels in NF639 cells (Fig. 5B, upper panels), whereas ectopic Bcl-2 expression robustly induced their levels in these cells (Fig. 5B, lower panels). Similarly, Bcl-2 led to substantial increases in the levels of PRDM1 RNA (Fig. 5C, upper panels) and Blimp1 protein expression (Fig. 5C, lower panels) in both ZR-75 and MCF-7 cells, while ectopic BCL2 shRNA, which inhibited RelB/p52-mediated induction of Bcl-2 (56), prevented the induction of Blimp1 by RelB/p52 in ZR-75 cells, as well as the reduction of ERα levels (data not shown). Lastly, the dnBlimp1 robustly impaired the previously observed ability of Bcl-2 to enhance migration of MCF-7 cells in MCF-7/Bcl-2 cells (Fig. 5D).

Wild-type (WT) Bcl-2, which has been shown to interact with and activate Ras in the mitochondria of cells, leads to the induction of NF-κB and AP-1, whereas a Bcl-2 14G mutant unable to associate with Ras is functionally inactive (7, 15, 35). Notably, Ohkubo et al. identified an AP-1 element upstream of the Blimp1 promoter (34), leading us to hypothesize a role for Bcl-2–Ras interaction in the activation of Blimp1. As a first test, we compared the effects of the WT and the Bcl-2 14G mutant on the PRDM1 promoter reporter in ZR-75 cells. Transfection of a vector expressing WT Bcl-2 resulted in a substantial induction of the PRDM1 promoter activity in ZR-75 cells (mean of 3.5–± 1.7-fold, \( P = 0.05 \)), whereas the Bcl-2 14G mutant led to a reduction in activity of ~5-fold [mean of 0.2–± 0.06-fold, \( P = 0.002 \)]. To assess for association
FIG. 3. Blimp1 represses ERα gene expression in breast cancer cells. (A) Nuclear extracts, prepared from ZR-75 cells transfected with Blimp1 expression vector, were used in competition EMSAs with the Blimp1 element from the c-MYC gene as a probe and a 50-fold molar excess of oligonucleotides containing the c-MYC or putative ERα promoter Blimp1 sites 1, 2, or 3 (P1 to P3). (B) Nuclear extracts, prepared from ZR-75 cells transfected with EV (-) or Blimp1 expression vector (+), were used in EMSAs with the putative ERα gene P1, P2, and P3 Blimp1 sites as a probe. (C) Nuclear extracts, prepared from ZR-75 cells transfected with Blimp1 expression vector, were used in supershift EMSAs in the absence (-) or presence (+) of 200 ng of Blimp1 antibody and the putative ERα gene Blimp1 P2 site as a probe. (D) ERα promoter A and B regions with confirmed Blimp1 site indicated with the element sequence given below, where the core is underlined. (E) ChIP analyses were performed on MCF-7 cells transiently transfected with Blimp1 (top panels) or Hs578T cells (bottom panels) using anti-Blimp1 antibody or the corresponding normal IgG as indicated. The DNA, extracted from the immunoprecipitates or the input, was amplified by PCR using pairs of primers specific to the confirmed Blimp1 site indicated with the element sequence given below, where the core is underlined. (F) ZR-75 and MCF-7 cells were transiently transfected with 5.0 μg of Vxy-puro TBlimp vector expressing the 35-kDa dnBlimp protein or EV DNA, and WCEs were subjected to immunoblotting for ERα, dnBlimp, and β-actin. Densitometry indicated the ERα levels in the ZR-75 and MCF-7 cells were reduced to 28.0 ± 3.4 and 73.9 ± 5.0, respectively, upon ectopic Blimp1 expression compared to EV DNA, set at 100. (H) ZR-75 cells were transiently transfected with 5.0 μg of vector expressing either Blimp1 or dnBlimp protein (left or right panels, respectively) or the corresponding EV DNA, and RNA was subjected to RT-PCR for ERα and GAPDH. (I) MCF-7 cells carrying an inducible C4-R(OR-RelB) RelB construct, plated in the absence or presence of 1 μg of doxycycline/ml, were transiently transfected with 6.0 μg of dnBlimp expression vector. After 48 h, WCEs were analyzed for levels of ERα, RelB, dnBlimp, and β-actin.
FIG. 4. Blimp1 induces a migratory phenotype in breast cancer cells via repression of ERα. (A) NF639 cells were transiently transfected with control siRNA (si-con) or siBlimp1 RNA. (Left panel) Cells were subjected, in triplicate, to a migration assay for 4 h. Cells that migrated to the lower side of the filter were quantified by spectrometric determination of the optical density at 410 nm. The average migration from three independent experiments ± the SD is presented relative to si-con (set at 100%) (*, P = 7.0e−7). (Right panel) WCEs were subjected to immunoblotting for E-cadherin (E-Cadh), ERα, Blimp1, and β-actin. (B) ZR-75 cells were transiently transfected with 6 μg of EV or Blimp1 expression vector. (Left panel) Transfected ZR-75 cells were subjected, in triplicate, to a migration assay for 24 h, as in panel A. *, P = 0.0007. (Right panel) WCEs were analyzed by immunoblotting for Blimp1, E-cadherin, γ-catenin (γ-Caten), and β-actin. (C) ZR-75 cells were transiently transfected with 6 μg of Blimp1 expression vector or EV DNA in the absence or presence of ERα expression vector (1.5 μg) or EV DNA, as indicated. (Left panel) After 24 h, ZR-75 cells were subjected to migration assays for 24 h, as in panel A. The enhanced migration induced by Blimp1 is significantly decreased by ERα., *, P = 0.0001. (Right panel) After 48 h, WCEs were prepared and analyzed by immunoblotting for E-cadherin, γ-catenin, ERα, Blimp1, and β-actin. (D) ZR-75 and MCF-7 cells were transiently transfected with dnBlimp (+) or EV DNA (−). After 24 h, ERα validated Stealth RNAi (+) or Stealth RNAi negative control (−) were introduced in cells using a Lipofectamine RNAiMAX reagent by reverse transfection as previously described (56). After 24 h, WCEs were prepared and analyzed by immunoblotting for E-cadherin, ERα, and β-actin.

of Bcl-2 and Ras in breast cancer cell lines, WCEs were prepared from ZR-75 cells transfected with vectors expressing H-Ras and Bcl-2. Immunoprecipitation of Bcl-2 led to coprecipitation of Ras, whereas control rabbit IgG protein did not (Fig. 6A, left panel). Furthermore, coimmunoprecipitation analysis of endogenous proteins in NF639 cell extracts similarly detected association between endogenous Ras and Bcl-2 (Fig. 6A, right panels). Confocal microscopy confirmed the partial association of transfected Ras-GFP with Bcl-2 in the mitochondria of ZR-75 cells (Fig. 6B and see the projection images in Fig. S1 in the supplemental material).

Interestingly, ectopic Bcl-2 expression led to enhanced membrane and reduced cytosolic localization of endogenous Ras protein in ZR-75 and MCF-7 cells (Fig. 6C). To evaluate the requirement for Ras membrane localization, we compared the effects on ERα levels of expression of the Ras WT versus a Ras C186S mutant, which is unable to localize to the membrane (5, 8). The Ras C186S mutant, which interacts with Bcl-2 (data not shown), was unable to reduce ERα levels or to induce Blimp1 expression in contrast to findings with Ras WT (Fig. 6D). Furthermore, Ras C186S prevented the reduction in ERα levels mediated by Bcl-2 (Fig. 6E) and activation of the PRDM1 promoter (Fig. 6F), suggesting that it functions as a dominant-negative variant. Lastly, we tested the role of mitochondrial localization using two Bcl-2 mutants: Bcl-acta, which localizes to the mitochondria, and Bcl-nt, which predominantly localizes to the cytoplasm (64). Whereas Bcl-acta and Bcl-2 WT cooperated with Ras to induce the PRDM1 promoter, Bcl-nt was unable to induce its activity (Fig. 6G), a finding consistent with an important role for colocalization in the mitochondria.

To test whether Ras signaling is sufficient to induce Blimp1 expression, constitutively active Ras was ectopically expressed in ZR-75 and MCF-7 cells; potent upregulation of Blimp1 was observed (Fig. 7A). We next assessed data publicly available on microarray gene expression profiling of primary human mammary epithelial cells following transformation with various genes. Consistent with the data presented above, a significant induction of PRDM1 mRNA expression was detected upon the expression of activated H-Ras versus GFP (P = 1.0e−7), but not with E2F3, activated β-catenin, c-Myc, or c-Src (2) (Fig. 7B). A significant difference was also observed in PRDM1 mRNA levels between the means of the H-Ras group versus all others combined (P = 1.7e−10). Lastly, we sought to elucidate
FIG. 5. Induction of Blimp1 is mediated via Bcl-2. (A) For the left panels, NF639 cells were transiently transfectected with expression vectors for either control shRNA (contl shRNA) or Bcl2 shRNA, and WCEs were analyzed for E\(_\alpha\), Bcl-2 (which confirmed effective knockdown), and \(\beta\)-actin protein levels. For the right panels, ZR-75 and MCF-7 cells were transiently transfectected with EV or full-length Bcl-2 expression vector. WCEs were analyzed for E\(_\alpha\) and \(\beta\)-actin protein levels and for Bcl-2 (which confirmed the expected changes in levels [data not shown]). (B) NF639 cells were transiently transfectected with expression vectors for either control shRNA (contl shRNA), Bcl2 shRNA (upper panels), or EV or full-length Bcl-2 (bottom panels). For the left panels, WCEs were analyzed for Blimp1 and \(\beta\)-actin. For the right panels, RNA was subjected to RT-PCR analysis determine the levels of Blimp1 and Gapdh. (C) RNA (top panels) and nuclear extracts (bottom panels) were prepared from ZR-75 and MCF-7 cells transfected with control EV DNA or vector expressing Bcl-2 and subjected to RT-PCR for PRDM1 and GAPDH expression (upper panels) and Blimp1 and lamin B levels (lower panels), respectively. (D) Stable mixed populations of MCF-7 cells expressing Bcl-2 or \(\phi\)luc (\(\phi\)) DNA were transiently transfected with EV DNA (\(-\)) or vector expressing dnBlimp (\(+\)). Cells were subjected, in triplicate, to a migration assay for 24 h, as in Fig. 4A. The increase in migration induced by Bcl-2 is significantly reduced by dnBlimp, \(P = 3.8e^{-1}\). Western blot analysis confirmed the expression of the dnBlimp (not shown).

the role of Ras on cell migration induced by Bcl-2 in MCF-7 cells using the dominant-negative function of the Ras C186S mutant. Ras C186S mutant, but not Ras WT, inhibited the Bcl-2-mediated decrease in E-cadherin and ER\(_\alpha\) levels (Fig. 7C) and the ability of MCF-7 cells to migrate (Fig. 7D), a finding consistent with the effects seen above in ZR-75 cells (Fig. 6D to F). Together, these data strongly argue that Bcl-2 association with Ras, likely in the mitochondrial membrane, leads to the induction of the levels of Blimp1 and to a more migratory phenotype (see the scheme in Fig. 7E).

**DISCUSSION**

Our findings identify a novel mechanism whereby the RelB NF-\(\kappa\)B family member acts to repress E\(_\alpha\) gene expression via induction of the zinc finger protein Blimp1. Previously, we demonstrated RelB induces transcription of the BCL2 gene via interaction with C/EBP binding to an upstream CREB site (56) (Fig. 7E). Here, we show that the association of Bcl-2 with Ras, activates this proto-oncogene and leads to expression of the Zn finger repressor Blimp1. In turn, Blimp1 binds to an element upstream of E\(_\alpha\) promoter B, reducing promoter activity and E\(_\alpha\) levels and thereby causing a reduction in the levels of E-cadherin and \(\gamma\)-catenin and a commensurate increase in migratory phenotype in breast cancer cells (see the scheme in Fig. 7E). To our knowledge, this is the first study identifying a crucial role for Blimp1 in repression of E\(_\alpha\) transcription and also in increased migration of cancer cells. Indeed, Blimp1 is a well-known zinc finger transcriptional repressor that is critical for terminal differentiation of B cells into immunoglobulin-secreting plasma cells and functions by attenuating proliferation while promoting the differentiated B-cell gene expression program (10). More recently, Blimp1 has been implicated in T-cell homeostasis (16), where it similarly represses the expression of genes promoting proliferation, e.g., interleukin-2 (IL-2) and Bcl-6 while enhancing IL-10 production. Interestingly, these studies have also indicated that Blimp1 is a critical determinant of primordial germ cells differentiation (33). The absence of Blimp1 led to the formation of primordial germ cells clusters and to defects in primordial germ cell migration, as well as to apoptosis (33). Our findings identify one mechanism for the effects of Blimp1 on migration of breast cancer cells related, in part, to the downregulation of E\(_\alpha\) gene expression. It remains to be determined whether similar regulation of E\(_\alpha\) by Blimp1 occurs in B cells and plays a role in autoimmune disease. Overall, our data identify Blimp1 as a potent negative regulator of E\(_\alpha\) expression and thereby as an activator of the migration of breast cancer cells.

RelB has been reported to activate gene transcription via several different mechanisms. RelB has been shown to induce transcription of the c-Myb oncogene via releasing pausing that occurs during elongation (47). The activation of the BIC, ELC, SDF-1, and Slc promoters by RelB required IKK\(\alpha\) (4). Interestingly, activation of the dapk1, dapk3, c-flip, and birc3 promoters by RelB can be prevented by Daxx, which interacts selectively with RelB but not with RelA or c-Rel (36). In addition to the BCL2 gene (56), RelB induces transcription of several other prosurvival genes, including MNSOD in prostate cancer (60) and MNSOD and SURVIVIN in breast cancer (29). However, RelB was unable to bind to the NF-\(\kappa\)B elements upstream of
FIG. 6. Bcl-2 interaction with Ras induces Blimp1. (A) WCEs (500 μg), prepared from ZR-75 cells transiently transfected with Bcl-2 and Ras expression vectors (left panels) or from untransfected NF639 cells (right panels), were immunoprecipitated with rabbit anti-Bcl-2 or mouse anti-Ras antibodies or the corresponding normal IgG as indicated. Immunoprecipitated proteins were analyzed by immunoblotting for Bcl-2 and Ras. WCEs (5 μg) were used as input. (B) ZR-75 cells, grown on coverslips in six-well dishes, were transfected with 1 μg of Ras-GFP expression plasmid. After staining with MitoTracker, the cells were fixed and subjected to immunohistochemistry for Bcl-2 using an Alexa 647-labeled secondary antibody (purple). Mitochondria are labeled in red with MitoTracker, Ras-GFP is green, and nuclei are labeled with DAPI stain (blue). A composite image was generated from the z-sections and an Orthoganol slice analysis was performed on the composite where XZ is depicted on the horizontal and YZ is depicted on the vertical axis outside the dimension of the composite. Colocalization of Ras and Bcl-2 to the mitochondria is demonstrated by the overlap of signals yielding white spots. Three-dimensional projection images were made from the composites. The Inset shows a 45° rotation of the image of the cell, with arrows indicating areas of colocalization of Ras and Bcl-2 in the mitochondria. (C) ZR-75 and MCF-7 cells were transiently transfected with EV or a vector expressing full-length Bcl-2. After 48 h, cytosol and membrane fractions were prepared as described in Materials and Methods and subjected to immunoblotting for Ras and β-actin. Immunoblotting for VDAC, a mitochondrial ion channel membrane protein, confirmed effective separation of the membrane from the cytoplasmic compartment (data not shown). (D) ZR-75 cells were transiently transfected with EV or Ras WT or Ras C186S mutant protein. After 48 h, WCEs and nuclear extracts were analyzed for levels of ERα and β-actin (upper panels) and Blimp1 and lamin B (lower panels). (E) ZR-75 cells were transiently transfected with EV or vector expressing full-length Bcl-2 in the absence (–) or presence (+) of a vector expressing Ras C186S mutant protein. After 48 h, WCEs were analyzed for ERα protein levels. (F) ZR-75 cells were transiently transfected in triplicate, with 0.5 μg of the 7-kb human PRDM1 promoter-Luc vector, 0.5 μg of Bcl-2 with 0.5 μg of Ras WT (Ras) or 0.5 μg of Ras C186S (C186S) expression vector, 0.5 μg of SV40-β-Gal, and pcDNA3 (EV) to make a total of 3.0 μg of DNA. The luciferase and β-Gal activities were determined, and normalized PRDM1 promoter activity values are presented as the means ± the SD from three separate experiments (control EV DNA set to 1). (G) ZR-75 cells were transiently transfected in triplicate with 0.5 μg of the 7-kb human PRDM1 promoter-Luc vector; 0.5 μg of Ras expression vector with 0.5 μg of either EV DNA, WT Bcl-2, Bcl-acta (mitochondrion-localized Bcl-2 mutant), or Bcl-nt (cytoplasm-localized Bcl-2 mutant) expression vectors (64); 0.5 μg of SV40-β-Gal; and pHc/CMV (EV) to make a total of 3.0 μg of DNA. At 48 h after transfection, luciferase and β-Gal activities were determined, and normalized PRDM1 promoter activity values are presented as the means ± the SD from three separate experiments (control EV DNA set to 1). Using a Mann-Whitney test, we determined a P value of <0.05 between each condition, except for Ras plus WT Bcl-2 and Ras plus Bcl-acta.
the Bcl-xl promoter and led to decreased Bcl-xl gene expression, in contrast to the potent activation seen upon coexpression of either c-Rel or RelA (21). In fibroblasts, RelB has also been reported to suppress expression of several genes, including IL-1α, IL-1β, and tumor necrosis factor alpha, indirectly via modulating IκBα stability (59) and to inactivate p65 via the formation of dimeric complex (27). We report here that RelB can repress expression of the ERα promoter via activation of the zinc finger repressor protein Blimp1. Consistent with this finding, ERα and RelB levels display an inverse pattern of expression in many breast cancer cells and tissues, including inflammatory breast cancers (23, 30, 52, 56).

To our knowledge, this is the first report of a transcription factor that acts as a repressor of ERα promoter activity. Previous work showing repression of ERα promoter transcription in breast cancer cells implicated DNA methyltransferases and histone deacetylases in the regulation (43, 62). To date, the identified transcription factors controlling ERα promoter activity have all been shown to act as positive regulators. deGraffenried et al. (12) and Clark et al. (13) have identified SP1, USF-1, and ERα itself as essential factors required for full ERα gene transcription. The estrogen receptor factor ERF-1 and the estrogen receptor promoter B-associated factor ERBF-1 have been implicated in the positive control of ERα promoter A and B activity, respectively (11, 48). Moreover, our previous study also indicated that the Forkhead box O protein...
3a (FOXO3a) activates ERα promoter B transcription in breast cancer cells (18). Activation of Ras via interaction with Bcl-2 has been previously reported in the context of Ras-mediated apoptosis of T cells (15, 38) and of adipocytes (31), although the findings were somewhat contradictory. Ras–Bcl-2 interaction in T cells blocked Ras-induced apoptosis, whereas in adipocytes it led to the induction of apoptosis via inactivation of Bcl-2 prosurvival function. Bcl-2 has also been shown to interact with Raf, although the resulting effects differed depending upon the cellular conditions. In murine myeloid progenitor cells, Bcl-2 targeted Raf to mitochondrial membranes, allowing this kinase to protect against apoptosis by phosphorylating BAD (55), whereas Taxol-induced activation of Raf was accompanied by the loss of Bcl-2 antiapoptotic function in MCF-7 cells (3). Of note, we observed that a constitutively active Raf could similarly induce Blimpl expression, implicating this Ras-induced pathway in signaling events (results not shown). Interestingly, El-Asiry and coworkers showed that inhibition of Ras or Raf activities led to the reexpression of ERα in MCF-7 cells (32). Our findings that Blimpl mediates repression of ERα can explain these observations. Overall, our study indicates that RelB-mediated induction of Bcl-2 leads to the repression of ERα transcription and thus to a more migratory phenotype of breast cancer cells by activating Ras.

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