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RelB/p52 NF-κB Complexes Rescue an Early Delay in Mammary Gland Development in Transgenic Mice with Targeted Superrepressor IκB-α Expression and Promote Carcinogenesis of the Mammary Gland

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Classical NF-κB (p65/p50) transcription factors display dynamic induction in the mammary gland during pregnancy. To further elucidate the role of NF-κB factors in breast development, we generated a transgenic mouse expressing the IκB-α S32/36A superrepressor (SR) protein under control of the mouse mammary tumor virus (MMTV) long terminal repeat promoter. A transient delay in mammary ductal branching was observed in MMTV-SR-IκB-α mice early during pregnancy at day 5.5 (d5.5) and d7.5; however, development recovered by mid- to late pregnancy (d14.5). Recovery correlated with induction of nuclear cyclin D1 and RelB/p52 NF-κB complexes. RelB/p52 complexes induced *cyclin D1* and c-*myc* promoter activities and failed in electrophoretic mobility shift assay to interact with IκB-α–glutathione *S*-transferase, indicating that their weak interaction with IκB-α can account for the observed recovery of mammary gland development. Activation of IKKα and NF-κB-inducing kinase was detected by d5.5, implicating the alternative NF-κB signaling pathway in RelB/p52 induction. Constitutively active IKKα induced p52, RelB, and cyclin D1 in untransformed mammary epithelial cells. Moreover, mouse mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene treatment displayed increased RelB/p52 activity. Inhibition of RelB in breast cancer cells repressed cyclin D1 and c-Myc levels and growth in soft agar. These results implicate RelB/p52 complexes in mammary gland development and carcinogenesis.

NF-κB/Rel is a structurally and evolutionary conserved family of transcription factors distinguished by the presence of an N-terminal 300-amino-acid region, termed the Rel homology domain. The Rel homology domain is responsible for DNA binding, dimerization, nuclear translocation, and binding of Rel factors to the IκB inhibitory proteins (reviewed in reference 25). Mammals express five NF-κB members, of which RelB, c-Rel, and p65 (RelA) are synthesized as mature products and contain a C-terminal transactivation domain. In contrast, p50 and p52 are synthesized as longer precursors that have C-terminal ankyrin repeats and can serve as inhibitory molecules. The p105 and p100 precursor proteins require proteolytic processing to produce the mature p50 and p52 subunits, respectively, which lack a transactivation domain (46). In most untransformed cells other than B lymphocytes, NF-κB complexes are sequestered in the cytoplasm bound to specific inhibitory proteins, termed IκBs (2), which have been found to display specificity of interaction (reviewed in references 54 and 59). For example, IκB-α most strongly interacts with p65 and c-Rel and only weakly with RelB, p50, and p52. Activation of NF-κB can occur via multiple pathways. Stimuli such as tumor necrosis factor (TNF) and interleukin-1 lead to induction of p65/p50 complexes with IκB-α via the canonical pathway involving activation of the IKK complex, consisting of the kinases IKKα and IKKβ (33). In particular, activation of the IKKβ kinase leads to phosphorylation of IκB-α on S32/36 and its rapid proteasome-mediated degradation (7, 12), allowing for translocation of the free NF-κB to the nucleus (reviewed in reference 59). Thus, an IκB-α protein with the S32/36A mutation, which is resistant to phosphorylation and subsequent degradation, is termed superrepressor (SR)-IκB-α. A recently reported alternative pathway involves induction of RelB/p52 complexes upon stimulation by such TNF family receptors as lymphotoxin β and CD40 (15, 34, 62). This signaling leads to activation of the NF-κB-inducing kinase (NIK) and subsequent IKKα-mediated phosphorylation of the p100 component of RelB/p100 cytoplasmic complexes (44, 61), resulting in its processing to p52. Studies by Bravo and coworkers (18) indicate that the resulting RelB/p52 complexes do not interact well with IκB-α and, thus, are largely free to migrate to the nucleus.

NF-κB factors have been implicated in the pathogenesis of breast cancer and in development of the normal mammary gland. We and others have demonstrated aberrant constitutive activation of NF-κB factors in human and rodent breast cancers (14, 29, 35, 41, 51). High levels of nuclear NF-κB were found in the majority of primary human and rodent breast tumor tissue samples, breast cancer cell lines, and carcinogen-transformed mammary epithelial cells (14, 29). Inhibition of

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the constitutive NF-κB activity in human breast cancer cell lines via ectopic expression of IκB-α induced apoptosis (51) or reduced transformed phenotype (42). Furthermore, inhibition of NF-κB has been found to promote sensitivity to chemotherapy (16, 40, 55). Conversely, ectopic expression of c-Rel resulted in resistance to transforming growth factor β1-mediated inhibition of proliferation (50). Accelerated degradation of the IκB-α inhibitory protein was observed in carcinogen-transformed cells (28), and several kinases involved in the induction of NF-κB have been implicated in human breast cancer, including IKKα, IKKβ, and protein kinase CK2 (42). Using a mouse mammary tumor virus (MMTV) c-*rel* mouse model, we demonstrated a causal role of ectopic c-Rel in breast cancer; 31.6% of transgenic mice developed one or more mammary tumors at an average age of 19.9 months (41). The tumors overexpressed several NF-κB-regulated genes that control cell proliferation, transformation, and survival, i.e., *cyclin D1*, c-*myc*, and *Bcl-x(L)* (1, 11, 20, 26, 30). Furthermore, the untransformed mammary glands had poor regression of the ductal tree after lactation, consistent with previous studies demonstrating a prosurvival role for NF-κB (40, 48, 60) and its involvement in mammary gland development in mice (4, 9, 13, 53). In particular, activation of p65/p50 classical NF-κB complexes was observed by the earliest time point of pregnancy examined, day 10.5 (d10.5) (5, 13). An overall increase in lateral ductal branching was observed upon transplantation of mammary tissue from neonatal IκB-α-deficient versus normal mice into cleared wild-type mouse mammary fat pads (4). Cao et al. (10) have shown that mice null for the IKKα kinase display defective mammary gland development, due to decreased induction of cyclin D1, and have further shown that IKKα is an essential link in signaling via RANK ligand. Here we have further examined the role of NF-κB in development of the mammary gland in mice. Using an MMTV long terminal repeat (LTR) promoter-driven S32/36A SR-IκB-α mouse, we report that an early delay in mammary gland development associated with decreased cyclin D1 levels at d5.5 to d7.5 was surprisingly followed by recovery by d14.5. Analysis of the mechanism of recovery indicates that NF-κB plays a bimodal role in mammary gland development, with activation of IKKα during pregnancy leading to a delayed induction of RelB/p52 via the alternative pathway. Activation of these NF-κB complexes was observed in mouse mammary tumors induced by treatment with the polycyclic aromatic hydrocarbon 7,12-dimethylbenz (*a*)anthracene (DMBA) and in human breast cancer cell lines. Inhibition of RelB decreased cyclin D1 and c-Myc expression and repressed transformed phenotype. Overall, these findings implicate the RelB/p52 alternative pathway in both carcinogenesis and normal development of the breast.

MATERIALS AND METHODS

Isolation of MMTV-SR-IκB-α transgenic mice. The 1.3-kb HindIII/NotI fragment from the human pRc/β-actin-IκB-α S32/36A vector, containing the human Ser32,36Ala SR-IκB-α coding region, was blunt end ligated into the MMTV-LTR plasmid, which directs expression chiefly to the mammary epithelium, with ras 5′ untranslated sequences provided upstream of the cDNA and a simian virus 40 (SV40) intron and polyadenylation signal downstream (41, 47), yielding the pMMTV-SR-IκB-α plasmid. The direction of the insert was confirmed by restriction mapping and DNA sequencing by the Molecular Biology Core at Boston University School of Medicine. Plasmid sequences were removed by restriction digestion at the SalI and SpeI sites, leaving the MMTV-LTR sequence, ras 5′ untranslated sequences, SR-IκB-α cDNA, and the SV40 intron and poly(A) addition signal sequence. The excised transgene construct was gel purified and microinjected into pronuclei of fertilized one-cell zygotes from FVB/N mice. These zygotes were reimplanted into pseudopregnant foster mothers, and the offspring were screened for the presence of the transgene by Southern blotting, as we have described previously (41). Carriers were bred to establish three independent transgenic lines. Mice were housed in a two-way barrier at the Boston University School of Medicine Transgenic mouse facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

Preparation of mammary gland whole mounts. The number 4 mammary fat pad was dissected en bloc, spread on a glass microscope slide, and then fixed in Tellyesniczky's fixative (70% ethanol–formaldehyde–glacial acetic acid [20:1:1]) for at least 24 h. The tissue was then rinsed for 1 h in running tap water and immersed in acetone overnight. The acetone was replaced and left overnight again, and this was repeated for a third time. On the fourth day, the slides were treated with the following solutions, each for a duration of 2 h: 100% ethanol, 95% ethanol, 70% ethanol, water, and carmine dye solution, and then rinsed overnight with running tap water. The slides were treated with a large excess of the following reagents each for 2 h: 50% ethanol, 70% ethanol, 95% ethanol, and 100% ethanol. Following overnight incubation in methylsalicylate, they were then stored in fresh methylsalicylate. The slides were photographed with Kodak Select Elite chrome slide film using a Nikon AF camera and Leika MZ6 microscope.

RNA analyses. Frozen breast tissue from mammary glands 4 and 5, with the lymph node removed, was pulverized in liquid nitrogen with a mortar and pestle, and total RNA was extracted with the Ultraspec-II RNA isolation system (Biotecx Laboratories Inc.). To remove contaminating DNA, RNA samples were digested for 30 min at 37°C with RQ1 RNase-free DNase (Promega Corporation), according to the manufacturer's directions. For reverse transcriptase PCR (RT-PCR), 5-μg RNA samples were reverse transcribed with SUPERSCRIPT RNase H^- RT in the presence of 200 ng random primers (all reagents from Invitrogen Life Technologies). For PCR, Immolase DNA polymerase (Bioline Inc.) was used to amplify a 287-bp fragment of the transgene with primers specific to human IκB-α (5′-CTTATATCCACACTGCACACTGC-3′ and 5′-TTTCACCCCACATCACTGAA-3′) at an annealing temperature of 50°C. As a control for RNA quality, a 425-bp fragment of β-actin mRNA was amplified by 25 PCR cycles using the primers 5′-CACTGGCATCGTGATGGACT-3′ and 5′-CGGATGTCCAGGTCACACTT-3′ at an annealing temperature of 50°C.

EMSA. Nuclear proteins were extracted from frozen tissue powders of mammary glands 1 to 3 as we have described recently (41). The sequence of the URE NF-κB-containing oligonucleotide from the c-*myc* gene is as follows: 5′-GATCCAAGTCCGGGTTTTCCCCAACC-3′ (20). The core element is underlined. The NF-1 oligonucleotide has the following sequence: 5′-GATCCTTTTGGATTGAAGCCAATATGATAA-3′ (31). Nuclear extract samples (5 μg) were subjected to electrophoretic mobility shift assay (EMSA), as described elsewhere (51). For antibody supershift analysis, the binding reaction was performed in the absence of the probe, the appropriate antibody was added, and the mixture was incubated for 16 h at 4°C. The probe was then added, the reaction mixture was incubated an additional 30 min at 25°C, and the complexes were resolved by gel electrophoresis, as above. Antibodies used included the following: anti-RelA (C-20), sc-372; anti-c-Rel (N), sc-70; anti-p50 (NLS), sc-114; anti-RelB (C-19), sc-226 (all from Santa Cruz Biotechnology). In addition, rabbit polyserum 1495 specific for p52 was kindly provided by N. Rice and M. Ernst (National Cancer Institute, Frederick, Md.). For IκB-α blocking experiments, IκB-α–GST fusion protein was added to the binding reaction mixture after the 30-min incubation and incubated for an additional 1.5 h at 4°C, as described elsewhere (51).

Immunoblot analysis. Whole-cell extracts were prepared from mammary glands 1 to 3 in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 1% sodium sarcosyl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, 1 mM dithiothreitol [DTT]) or in PD buffer (40 mM Tris [pH 8.0], 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.1% NP-40). Nuclear extracts were prepared as described above. Samples (40 μg) were separated by electrophoresis in 8% polyacrylamide–SDS gels, transferred to a 0.45-μm-pore-size polyvinylidene difluoride membrane (Millipore), and subjected to immunoblotting, as described previously (51). Antibodies used were against human and mouse IκB-α (C-15; sc-203), anti-p52 (K-27; sc-298), anti-NIK (H248; sc-7211), anti-p-NIK (Thr-559-R; sc-12597-R) (all from Santa Cruz Biotechnology), and anti-cyclin D1 monoclonal Ab-3 (Oncogene). Antibodies specific for other NF-κB subunits were as described above. Data were quantified by

densitometry using a Molecular Dynamics densitometer or Scion Image software.

Cell lines and transfection conditions. NMuMG, which is an untransformed, immortalized mouse mammary epithelial cell line, was cultured as described previously (49). MCF-10F is a human mammary epithelial cell line established from a patient with fibrocystic disease, which does not display malignant characteristics (8). The D3-1 mammary epithelial cell line was derived using DMBA-mediated transformation of MCF-10F cells and cultured as described previously (8). The Hs578T tumor cell line, which was derived from a carcinosarcoma and is epithelial in origin, was grown as described previously (51). The RelB, p52, and Bcl-3 expression vectors have been described elsewhere (41). The pRC-β-actin-IKKαSS/EE vector in which serines 176 and 180 of IKKα were mutated to glutamine, rendering it constitutively active, and empty vector DNA were kindly provided by F. Mercurio (Celgene Signal Research Division, San Diego, CA) (33). The pSIREN RelB-sense and pSIREN siRelB expression vectors (43) were a kind gift of Finn-Eirik Johansen (Rikshospitalet University Hospital, Oslo, Norway.) The *cyclin D1* promoter construct −1745 bp WT-Luc was a kind gift of R. G. Pestell (Georgetown University, Washington, D.C.) (26). The p1.6 Bgl Myc-CAT WT vector contains the murine c-*myc* promoter (−1141 to +513) with the WT NF-κB URE and IRE binding sites, respectively (21). For luciferase reporter assays, NMuMG cells were transfected with 2.5 μg DNA in six-well plates using Fugene reagent (Roche Diagnostics Corporation), with the SV40 promoter β-galactosidase reporter vector (pSV40-β-gal), which was used to normalize transfection efficiency, as previously described (1). For stable transfectants, MCF-10F cells were transfected in p100 dishes with 10 μg of either pRc-β-actin promoter empty vector parental or pRc-β-actin-IKKαSS/EE vectors along with 400 μg/ml G418 (Sigma) for 4 days and then grown in the presence of 200 μg/ml G418. Hs578T or D3-1 cells were transfected in p60 dishes with 10 μg of either pSIREN RelB-sense or pSIREN siRelB and allowed to grow for 48 h before selection for 1 week with 4 μg/ml puromycin (Sigma). Cells were subsequently maintained in 2 μg/ml puromycin.

IKKα kinase assay. Whole-cell extracts were prepared in PD buffer, as above, and a kinase assay was performed as described elsewhere (42). Briefly, 150 μg of extract in a 500-μl volume were precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech AB) for 1 h at 4°C. The IKK complexes were then isolated by immunoprecipitation using 1 μg of antibody against IKKα (M-280) sc-7182 (Santa Cruz Biotechnology Inc.) and subjected to a kinase assay at 30°C for 45 min in kinase buffer C (20 mM HEPES [pH 7.7], 2 mM $MgCl_2$, 10 μM ATP, 3 μCi of [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) containing 200 ng of WT IκB-α–GST fusion protein (wt-IκB-α-GST) as substrate (33). Alternatively, the S32,36A mutant IκB-α-GST protein (S32,36A-IκB-α-GST) was used to assess kinase specificity. The kinase reaction was stopped by the addition of 2× SDS-polyacrylamide gel electrophoresis sample buffer, and proteins were subjected to SDS-polyacrylamide gel electrophoresis analysis and visualized by autoradiography.

Generation of DMBA-induced murine mammary tumors. Female FVB/N mice at 5 weeks of age were administered 1 mg DMBA in 0.2 ml sesame oil by intragastric gavage weekly for 6 weeks, for a total dose of 6 mg/mouse. Mice were bred continuously following treatment with DMBA and palpated weekly for tumor formation. Upon necropsy, tumors were harvested and immediately sectioned. Sections of tumors were snap-frozen for preparation of nuclear and cytoplasmic extracts. Normal mammary glands from female WT FVB/N mice, matched in age and reproductive history, were similarly processed.

Soft agar assay. D3-1 cells were plated, in triplicate, at 4.0×10^3/ml in top plugs consisting of complete medium and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, ME). Plates were subsequently incubated for 2 weeks in a humidified incubator at 37°C. Cells were stained with 2 ml of crystal violet solution and washed extensively with water, and colonies were counted using a microscope at 40× magnification. Four random fields were counted from each of the triplicate samples, and average values are presented ± the standard deviation.

RESULTS

Generation and characterization of MMTV-SR-IκB-α transgenic mice. To clarify the role of NF-κB in mammary development, we generated a mouse model in which human SR-IκB-α was expressed under control of the hormone-responsive MMTV-LTR promoter. Three founders were isolated (lines 14, 15, and 36), as judged by Southern blot analysis (data not shown), and bred to homozygosity. We first sought to verify SR-IκB-α protein expression in the various founder lines. At day 18.5 of the first pregnancy, cytoplasmic extracts were prepared from mammary glands of transgenic lines 14, 15, and 36 and from a WT FVB/N mouse as a control. Samples were subjected to immunoblot analysis for IκB-α, using an antibody that recognizes both human (38-kDa) and murine (37-kDa) IκB-α proteins (Fig. 1A). The relative positions of human and murine IκB-α were determined using an extract from Hs578T cells as a control for human IκB-α (data not shown). All of the transgenic lines displayed expression of the 38-kDa SR-IκB-α protein; however, the ratio of human to mouse IκB-α appeared slightly higher in the mammary glands of lines 15 and 36 compared to line 14. To confirm human IκB-α RNA expression in the transgenic animals, MMTV-SR-IκB-α line 15 mice were bred to induce transgene expression, and total RNA was isolated from the mammary gland at d18.5 of pregnancy. RNA samples were analyzed by RT-PCR, using primers specific for the human IκB-α transgene (Fig. 1B, upper panel). As positive and negative controls, respectively, RNA isolated from the human breast cancer D3-1 cell line, or from mammary glands of WT mice at d18.5 of pregnancy, was similarly analyzed. Expression of the SR-IκB-α transgene was observed in the mammary gland of line 15, but not the WT mouse, as expected. Analysis of β-actin mRNA by RT-PCR confirmed the integrity of the RNA (Fig. 1B, middle panel), and PCR in the absence of RT verified that the samples were free of genomic DNA contamination (Fig. 1B, lower panel). Overall, these results confirm that the three transgenic lines express the human IκB-α transgene.

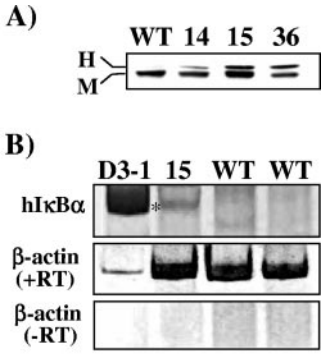

FIG. 1. MMTV-LTR-driven SR-IκB-α transgene expression in FVB/N mice. (A) Expression of SR-IκB-α protein in founder lines. Mammary glands were harvested from WT FVB/N mice or from the indicated MMTV-IκB-α founder line mice at day 18.5 of the first pregnancy. Whole-cell extracts were prepared from these glands and subjected to immunoblot analysis for IκB-α, using an antibody that preferentially recognizes human IκB-α. The positions of human and murine IκBs are indicated by "H" and "M," respectively. (B) Transgenic SR-IκB-α expression. Total RNA was prepared from mammary glands of WT FVB/N or line 15 SR-IκB-α mice at day 18.5 of pregnancy or from the human D3-1 breast cancer cell line and subjected to RT-PCR using primers specific for human IκB-α in order to verify transgene expression or for β-actin in the presence or absence of RT to verify transcript integrity and lack of DNA contamination. *, position of human IκB-α cDNA band.

MMTV-SR-IκB-α mice display a transient early delay in mammary gland development. To assess the effects of SR-

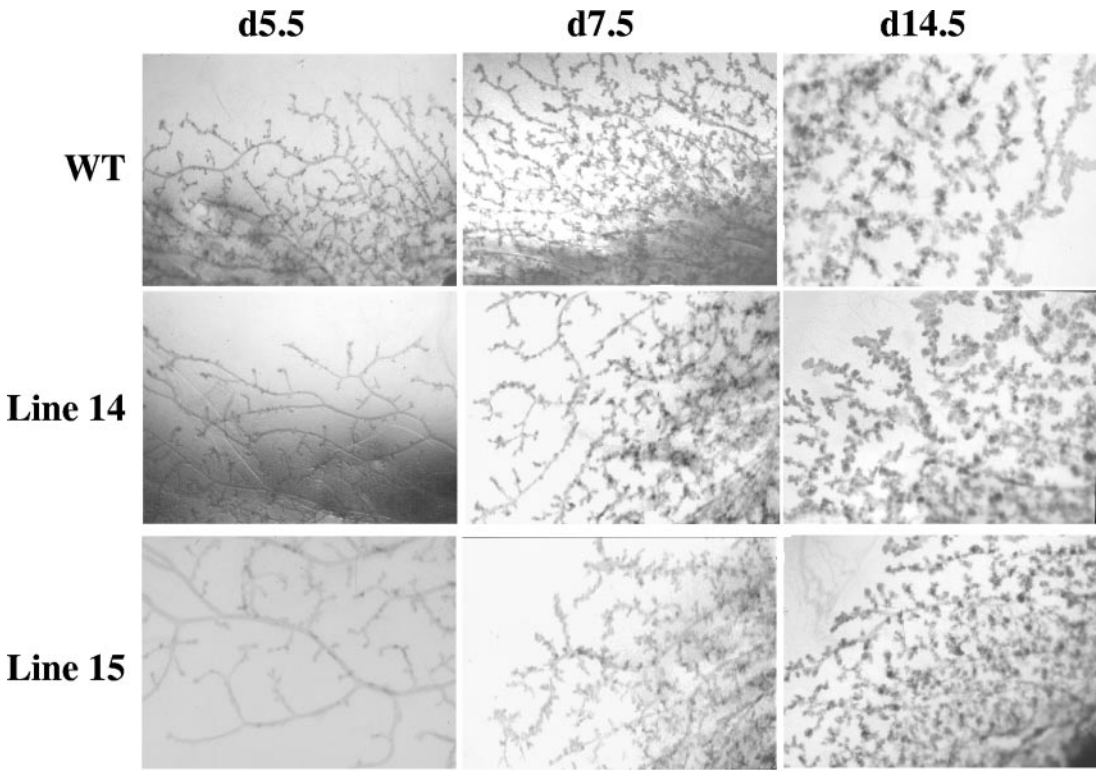

FIG. 2. Mammary glands of MMTV-SR-IκB-α transgenic mice display an early developmental delay. The fourth mammary gland of WT FVB/N or line 14 and 15 MMTV-SR-IκB-α mice was removed at the indicated day of pregnancy and subjected to whole mount analysis.

IκB-α expression on mammary gland development, whole mount analysis was performed. Whole mounts were prepared from the fourth mammary gland at days 5.5, 7.5, and 14.5 of pregnancy of WT and lines 14 and 15 MMTV-SR-IκB-α mice (Fig. 2). In the WT mice, ductal branching appeared to begin at d5.5 and was more extensive by d7.5. By 14.5 days of pregnancy, extensive alveolar formation was noted in the WT mice. In both lines of the MMTV-SR-IκB-α mice, development of the mammary gland appeared substantially delayed at early pregnancy at days 5.5 and 7.5 compared to WT mice, with a decrease in ductal branching and alveolar development (Fig. 2). Surprisingly, however, gland development appeared to recover by mid- to late pregnancy at d14.5, and extensive alveolar branching was seen even in the presence of SR-IκB-α expression. Moreover, the transgenic mice were observed to nurse their pups normally.

Cyclin D1 expression is transiently delayed in the MMTV-SR-IκB-α mouse mammary gland. The nuclear expression of cyclin D1, which has been shown to play a critical role in mammary gland development (22), was assessed. Nuclear extracts were prepared and subjected to immunoblot analysis (Fig. 3). Activation of cyclin D1 in the mammary gland was seen between days 5.5 and 7.5 of pregnancy in WT mice (Fig. 3A). In contrast, MMTV-SR-IκB-α line 14 mice displayed almost no induction of cyclin D1 at d7.5 but by d14.5 of pregnancy exhibited levels of cyclin D1 essentially comparable to the WT mouse (Fig. 3B), although some variability was noted in β-actin loading control levels. Similar data were obtained with line 15 (data not shown). To determine whether a delayed induction of nuclear levels of p65 was responsible for the recovery of cyclin D1 expression in the transgenic animals, immunoblot analysis was performed. In nuclear extracts from mammary glands of WT mice, an induction of p65 levels occurred between days 5.5 and 7.5 and the levels remained elevated out to d14.5, essentially paralleling the changes in cyclin D1 (Fig. 3A), consistent with previous reports on the role of p65 in the mammary gland (5, 13). In contrast, MMTV-SR-IκB-α mice demonstrated almost no increase in the nuclear level of p65 throughout the course of pregnancy (Fig. 3B), as expected with ectopic IκB-α expression in the glands. These data are representative of three independent experiments. Thus, the increase in expression of cyclin D1 in the mammary glands of transgenic mice occurs with a transient delay, recovering by d14.5 of pregnancy, confirming the important role of this cyclin in mammary gland formation (22); however, induction of p65-containing NF-κB complexes cannot account for the observed rescue, consistent with the ectopic expression of the SR-IκB-α.

Mammary glands of MMTV-SR-IκB-α mice display a delayed increase in NF-κB binding during pregnancy. To assess the effects of MMTV-SR-IκB-α expression on overall NF-κB binding, nuclear extracts were prepared from virgin WT FVB/N and MMTV-SR-IκB-α mice and at days 5.5, 7.5, and 14.5 of pregnancy. Samples were subjected to EMSA using the NF-κB element upstream of the c-*myc* promoter, which has been shown to bind all members of the NF-κB family (30). In the extracts of virgin WT FVB/N mouse mammary glands, low levels of NF-κB binding activity were detected (Fig. 4A, upper

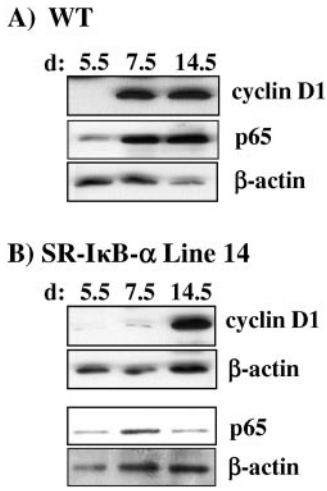

FIG. 3. Induction of cyclin D1 and NF-κB p65 is delayed in the mammary gland during pregnancy in MMTV-SR-IκB-α mice. Nuclear extracts were prepared from mammary glands 1 to 3 of WT FVB/N mice (A) and SR-IκB-α line 14 mice (B) at the indicated days of the first pregnancy and subjected to immunoblot analysis for cyclin D1 and p65. The expression of β-actin was used to normalize for loading.

panel): band 1, p65/p50, and band 2, p50 homodimers, as reported previously (5). Although some variability was seen between animals, the level of NF-κB binding increased through day 14.5 of pregnancy with extracts from mammary glands of WT animals. Interestingly, by day 7.5 of pregnancy, another NF-κB complex, band 1b, became apparent, which migrated slightly faster than the p65/p50 complexes. Nuclear extracts from mammary glands of MMTV-SR-IκB-α mice at days 5.5 and 7.5 of pregnancy displayed relatively lower levels of formation of band 1a (p65/p50), consistent with the reduced levels of nuclear p65 protein, as described above (Fig. 3). Importantly, by d14.5, extracts from the transgenic animals displayed NF-κB binding essentially comparable to WT animals, with a predominant band 1b (Fig. 4A, upper panel). NF-1 binding confirmed extract integrity and essentially equal protein loading (Fig. 4A, lower panel).

Supershift EMSA was performed to test for the presence of the p52, p50, p65, c-Rel, and RelB NF-κB subunits in nuclear extracts from WT FVB/N and MMTV-SR-IκB-α mice at d14.5 of pregnancy. Addition of antibodies against either RelB or p52 removed band 1b (Fig. 4B), while antibodies against p50 or p65 removed band 1a. Based on this analysis, the complexes have the following compositions: band 1a, p65/p50; band 1b, RelB/p52; band 2, p50/p50. These have been renamed band 1 (p65/p50), band 2 (RelB/p52), and band 3 (p50/p50), respectively.

To confirm the induction of RelB/p52 nuclear levels, we next performed immunoblotting for these subunits as a function of time of pregnancy. Nuclear extracts from WT FVB/N and MMTV-SR-IκB-α line 15 mice at days 5.5, 7.5, and 14.5 of pregnancy were subjected to immunoblot analysis for p52 and RelB. A low level of nuclear p52 and RelB was observed at day 5.5, which increased by day 7.5 and remained elevated out to day 14.5 in both the WT (Fig. 5A) and transgenic (Fig. 5B) animals. Overall, the induction of nuclear RelB/p52 occurred with a time course that correlates with recovery of mammary gland development in the transgenic MMTV-SR-IκB-α mice.

RelB/p52 complexes activate the *cyclin D1* promoter in mammary epithelial cells. While previous studies had shown that p65/p50 and other NF-κB complexes can activate the *cyclin D1* promoter (26, 41), RelB/p52 complexes had not been evaluated. To test directly whether RelB/p52 complexes were capable of promoting the induction of cyclin D1 during pregnancy, transfection analysis was performed. Untransformed mouse mammary epithelial NMuMG cells were cotransfected with the *cyclin D1* promoter reporter construct, SV40 β-Gal DNA to normalize for transfection efficiency, and vectors expressing p52 and RelB, or p65 and p50, or the empty vector DNA. Expression of p65/p50 effectively activated the *cyclin D1* promoter compared to the empty vector DNA (Fig. 6A), in agreement with our previous findings (41). The RelB/p52 complexes robustly activated the *cyclin D1* promoter at a level equivalent to or better than p65/p50 (Fig. 6A). Thus, induction of RelB/p52 complexes can activate *cyclin D1* promoter activity.

RelB/p52 binding activity in mammary gland extracts is resistant to inhibition by IκB-α. As discussed above, Bravo and coworkers (18) have shown that RelB/p52 complexes only weakly associate with IκB-α in comparison to p65/p50 or p50/RelB. To assess directly the ability of IκB-α to inhibit RelB/p52 complex binding in the mammary gland nuclear extracts, EMSA was performed in the presence of 0, 0.25, or 0.5 μg IκB-α–GST fusion protein. Addition of IκB-α–GST to nuclear extracts from WT animals caused a dose-dependent decrease in binding of band 1 p65/p50 complexes and more minor removal of band 3, leaving a more pronounced apparent RelB/p52 complex (band 2) (Fig. 6B). To confirm the presence of RelB in this complex, supershift EMSA was performed with either RelB or p65 antibody in the presence of 0.25 μg IκB-α–GST fusion protein. The RelB antibody completely removed band 2, whereas the p65 antibody removed only the complex in band 1 remaining in the presence of a low level of IκB-α–GST protein. The effects of addition of 0, 0.25, or 0.5 μg IκB-α–GST fusion protein were also assessed on NF-κB binding of extracts from the transgenic animals. Complex formation with nuclear extracts of mammary glands from the MMTV-SR-IκB-α line 15 mice showed less abrogation of binding with increasing doses of IκB-α–GST, consistent with the endogenous effects of IκB-α in the mouse mammary gland. Addition of an antibody to RelB in the presence of 0.5 μg IκB-α–GST protein resulted in the disappearance of band 2, whereas the anti-p65 antibody had almost no effect. Thus, our findings are consistent with the previous work indicating RelB/p52 complexes are resistant to inhibition by SR-IκB-α (18). Taken together, the data suggest that the induction of RelB/p52 complexes during mid- and late pregnancy plays a functional role in the activation of cyclin D1 (and potentially other NF-κB target genes) and therefore mediates recovery of mammary epithelial cell proliferation.

IKKα and NIK activities are induced early during pregnancy. Mice null for IKKα display decreased lobuloalveolar development of the mammary gland (9). To investigate IKKα expression and kinase activity during mammary gland development, samples of whole-cell extracts prepared from mammary glands of virgin mice and from animals at days 5.5, 7.5,

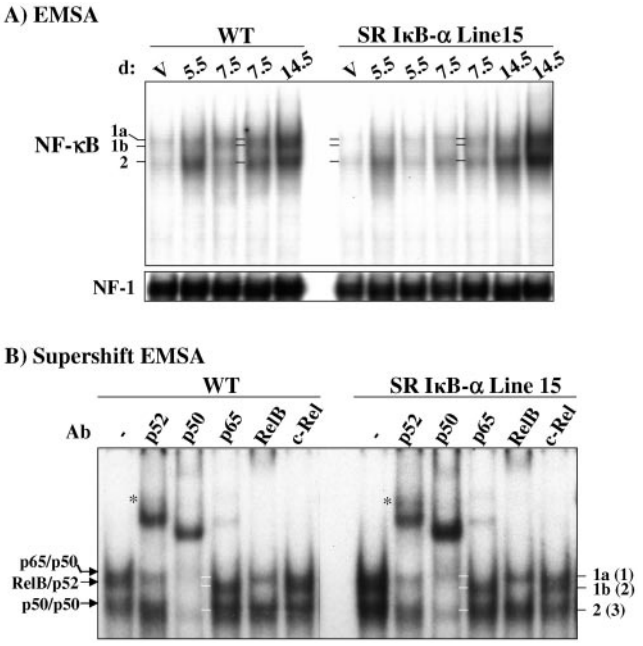

FIG. 4. WT and transgenic mouse mammary glands display a delayed induction of RelB/p52 NF-κB binding activity during pregnancy. (A) Nuclear extracts were prepared from WT FVB/N and MMTV-SR-IκB-α mammary glands 1 to 3 at the indicated day of the first pregnancy, and samples (5 μg) were analyzed by EMSA for NF-κB binding using the c-*myc* URE NF-κB binding element as probe. NF-1 binding was used to verify equal loading. Lines indicate positions of NF-κB complexes, numbered bands 1a, 1b, and 2. (B) Identification of active NF-κB subunits. Nuclear extracts from mammary glands of WT FVB/N and MMTV-SR-IκB-α line 15 mice at day 14.5 of pregnancy were subjected to supershift analysis using antibodies specific to p52, p50, p65, RelB, and c-Rel. The positions of identified p65/p50, RelB/p52, and p50 homodimer complexes are indicated. *, position of a nonspecific band seen with NF-κB probe and p52 antibody alone. Complexes 1a, 1b, and 2 were renumbered bands 1, 2, and 3, respectively, as indicated.

and 14.5 of first pregnancy were subjected to immunoblot analysis for IKKα (Fig. 7A). IKKα protein levels increased over the course of pregnancy. To test for activation of the IKKα kinase, samples containing 150 μg protein were immunoprecipitated using an IKKα antibody and subjected to a kinase assay using as substrate either WT-IκB-α–GST or SR-IκB-α–GST, which cannot be phosphorylated by IKKα (Fig. 7B). IKKα activity was extremely low in virgin animals and increased only slightly by day 5.5. By d7.5 a substantial increase in IKKα activity was noted, which remained elevated out to d14.5, consistent with the induction of RelB/p52 nuclear levels.

The induction of the IKKα in the alternative pathway is frequently mediated via NIK (34). As an initial test of the involvement of NIK, we monitored the total level of NIK protein as a function of pregnancy. Whole-cell extracts were subjected to immunoblot analysis for NIK (Fig. 7C, upper panel). Virgin mice expressed a low level of NIK, which increased slightly by d5.5 of pregnancy. The levels of the active, phosphorylated NIK protein (32) were next assessed. Phosphorylated NIK appeared between days 5.5 and 7.5 of pregnancy (Fig. 7C, middle panel). This phosphorylated form persisted through to d14.5 of pregnancy, at which time a more rapidly migrating species was induced as well. These findings are consistent with the mammary phenotype seen in alymphoplasia (*aly*/*aly*) mice, which fail to express active NIK and display abnormal mammary gland lobuloalveolar development (36). Thus, both NIK and IKKα are activated during pregnancy, and the time course of their activation is consistent with the induction of RelB/p52 levels in the nuclei of the mammary gland shown above.

Constitutive IKKα induces RelB/p52 and cyclin D1 levels in untransformed mammary epithelial cells. To directly assess the ability of IKKα expression to alter RelB/p52 and cyclin D1 levels in breast epithelial cells, the untransformed MCF-10F human epithelial cell line was used. MCF-10F cells were transfected with an HA-tagged constitutively active variant of IKKα (IKKαEE), and the effects on p52, RelB, and cyclin D1 levels were tested. Transfection with an empty vector DNA was used as control. Ectopic constitutively active IKKα expression led to an increase in the levels of p52 and RelB, which appeared selective, as levels of p50 were unaffected (Fig. 8). Immunoblotting for HA confirmed expression of the tagged IKKα protein. Consistent with the increase in RelB/p52, ectopic IKKα resulted in an increase in levels of cyclin D1. These findings strongly implicate activation of IKKα and resulting induction of RelB/p52-containing complexes in control of cyclin D1 and thereby mammary gland development.

Carcinogen-induced mammary tumors display activated RelB/p52 complexes. Recently, we showed that tumors arising in MMTV–c-*rel* mice frequently display aberrant activation of RelB/p52 subunits (41). To address the potential role of the alternative pathway in mammary carcinogenesis, the expression of these subunits in mammary tumors induced by the administration of the polycyclic aromatic hydrocarbon DMBA was examined. Female FVB/N mice were given six weekly doses of 1 mg DMBA, bred continuously, and palpated weekly for tumor formation. Of 20 mice treated with DMBA, 17 developed mammary tumors: 9 squamous cell carcinomas (SCC), 2 adenosquamous carcinomas, 1 papillary adenocarcinoma, 1 mixed spindle-adenosquamous, 1 spindle cell tumor, and 1 microacinar carcinoma (15a). To assess NF-κB activity in tumors of a homogenous histological phenotype, nuclear extracts were prepared from five mammary SCC, the most abundant type identified among the DMBA-induced tumors, and analyzed by immunoblotting for p65, c-Rel, RelB, p50, and p52 (Fig. 9). Age-matched normal mammary gland from untreated WT FVB/N mice that had been continuously bred was used for comparison. As a control for detection of the various subunits, a whole-cell extract from pooled normal FVB/N spleens was used. RelB and p52, as well as c-Rel, were highly overexpressed in three of five squamous cell carcinomas analyzed compared to the normal mammary gland (Fig. 9). SCC2 had a particularly high level of RelB, even compared to the other tumors. Levels of p65 in the tumors appeared to be only moderately upregulated compared to the normal mammary gland. Surprisingly, p50 levels were higher in the normal mammary gland than the tumors; however, the migration pattern of the protein suggested it represented a clipped form. (This shorter p50 peptide has been observed previously in extracts from normal mouse mammary glands and untransformed cell lines [39].) Extracts from untreated mouse mammary glands contained low levels of RelB and p52 (better seen on a darker

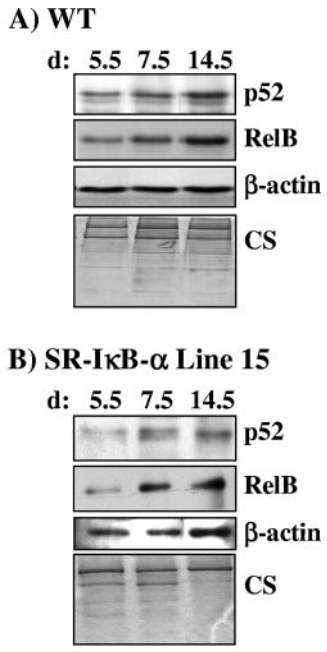

FIG. 5. NF-κB p52 and RelB are induced similarly during pregnancy in both WT FVB/N and MMTV-SR-IκB-α transgenic mice. Nuclear extracts were made from mammary glands of WT (A) and transgenic line 15 (B) mice at the indicated days of pregnancy and subjected to immunoblot analysis using antibodies against p52 and RelB. Expression of β-actin and Coomassie blue stain (CS) was used to control for equal loading. The p52 band sometimes runs as a doublet in both WT and IκB extracts.

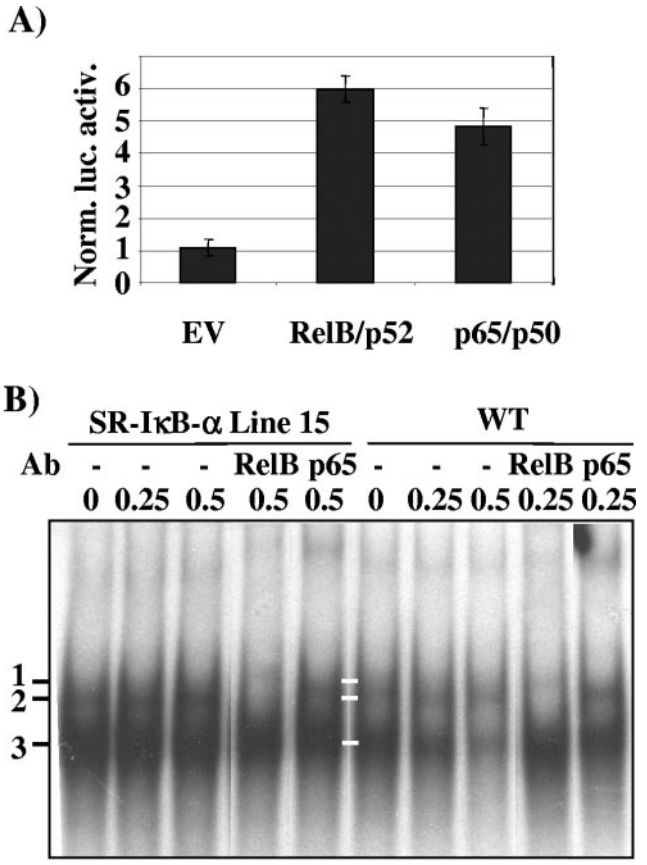

FIG. 6. RelB/p52 NF-κB complexes induce *cyclin D1* promoter activity and interact poorly with IκB-α. (A) NMuMG untransformed murine mammary gland cells were transiently transfected, in triplicate, with 1 μg of −1745 bp WT *cyclin D1* luciferase gene reporter construct and 0.5 μg of pSV40-β-Gal in the presence of 0.5 μg each of RelB and p52 or p65 and p50 plasmid expression vectors or 1.0 μg of the empty vector. After 48 h, cultures were harvested, normalized for β-Gal activity, and assayed for luciferase activity. Values for luciferase activity, relative to stimulation with empty vector DNA (EV), are presented after normalization to β-Gal activity (norm. luc. activ.). The error bars represent the standard deviations. (B) Nuclear extracts were prepared from MMTV-SR-IκB-α line15 or WT mammary glands at day 14.5 of first pregnancy, and samples (5 μg) were subjected to EMSA for NF-κB binding in the presence of 0, 0.25, or 0.5 μg IκB-α–GST protein, as indicated. Alternatively, samples were incubated overnight at 4°C using an antibody against either p65 or RelB proteins prior to the binding reaction and addition of IκB-α–GST. The positions of bands 1, 2, and 3 are indicated.

exposure). Essentially equal loading was confirmed by Coomassie staining.

To test whether the RelB/p52 subunits were actively binding, three squamous cell carcinomas (SCC1 to SCC3) were selected for supershift analysis. Nuclear extracts from these three tumors and a normal mammary gland from an untreated WT FVB/N animal were compared (Fig. 10). The NF-κB binding activity in mammary gland from a previously pregnant WT animal consisted of three complexes, as expected (Fig. 10). Band 1 was confirmed to contain primarily p50 and p65, as judged by the reduction in its formation upon addition of an antibody against either subunit. Band 2 shifted with antibody against p52 and lightened with antibody against RelB, consistent with RelB/p52 heterodimer. Band 3 shifted completely with an antibody against p50 and therefore likely contains p50 homodimers.

Various tumors yielded different patterns of binding, compared to each other and to normal mammary gland (Fig. 10). Extracts from SCC1 exhibited a binding pattern similar to that seen with the normal mammary gland extracts. However, the relative level of RelB appeared to be higher in the SCC1 tumor, as judged by the lack of a visible RelB supershifted band with the WT gland extract even after a 2-week-long exposure (data not shown). SCC2 had the highest nuclear levels of c-Rel and RelB, as judged by the immunoblot in Fig. 9, and this was reflected in the NF-κB binding activity (Fig. 10). In this sample, the predominant upper band detected corresponded to band 2 and appeared to contain RelB and p50 or p52. An additional middle band was seen, termed band 4, which could be identified as consisting of c-Rel and p52. The SSC2 sample may also contain p52 homodimers or p50/p52 heterodimers. Interestingly, SCC3, which by immunoblot assay seemed to display the least induction of nuclear NF-κB of the three tumors analyzed, had substantial NF-κB binding activity in the form of RelB/p52 or p50, c-Rel/p52 or p50, and p50 homodimers (Fig. 10). Significantly, all three tumors exhibited increased RelB/p52 binding activity compared to the normal gland, which is likely to play an important role in mammary tumorigenesis.

Repression of RelB reduces tumorigenicity in transformed cells. To directly assess the effects of RelB in tumorigenesis, in

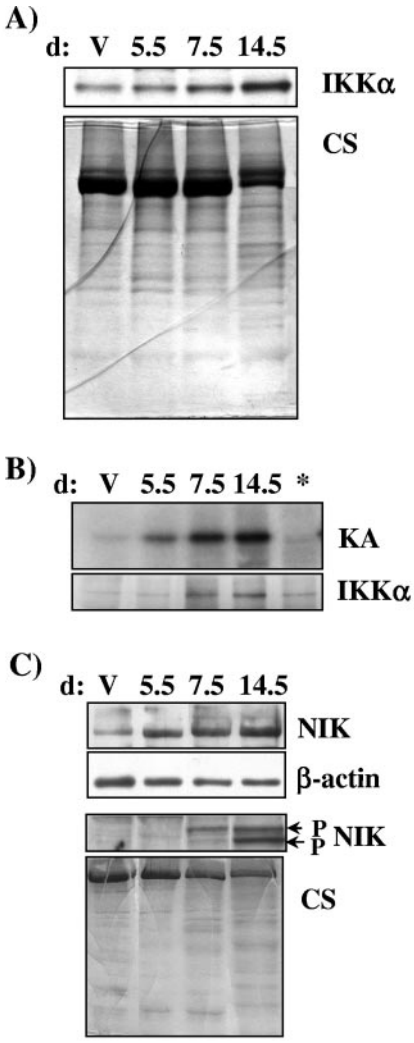

FIG. 7. Induction of the NF-κB alternative pathway during pregnancy. (A) IKKα levels are induced during pregnancy. Whole-cell extracts (40 μg) from mammary glands of WT FVB/N mice at the indicated day of pregnancy were subjected to immunoblot analysis for IKKα. Coomassie blue stain (CS) was used to verify equal loading. (B) IKKα kinase activity is induced during pregnancy. Whole-cell extracts were prepared from mammary glands of WT FVB/N mice at the indicated day of pregnancy, and samples (150 μg) were assayed for IKKα kinase activity using WT IκB-α–GST as substrate. Alternatively, mutant S32/36A IκB-α–GST was used with pooled extract samples to control for nonspecific kinase activity (*). Subsequently, the membrane was analyzed by immunoblot analysis for IKKα as control. (C) NIK is induced during pregnancy. Mammary glands of WT FVB/N mice were harvested at the indicated day of pregnancy, and whole-cell extracts were isolated and subjected to immunoblot analysis for NIK and phospho-NIK. β-Actin or CS was used to verify equal loading. Arrowheads indicate phosphorylated NIK.

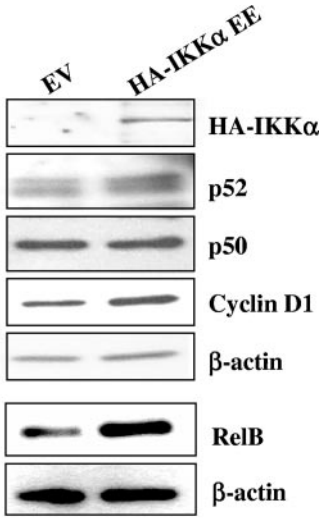

FIG. 8. IKKα induces cyclin D1, p52, and RelB expression. MCF-10F untransformed human mammary epithelial cells were stably transfected with an HA-tagged constitutively active mutant of IKKα termed IKKα-EE or with empty vector DNA (EV). Whole-cell extracts were prepared after 48 h of serum starvation and analyzed by immunoblotting for levels of HA (as a measure of ectopic IKKα expression), p52, p50, RelB, cyclin D1, and β-actin, which confirmed equal loading.

vitro analyses were performed. Unfortunately, we were unable to establish cell lines from the DMBA-induced murine mammary tumors. Therefore, experiments were performed in Hs578T human breast cancer cells and D3-1 DMBA-transformed mammary epithelial cells, which were both found to express substantial levels of RelB (see below). In order to determine the involvement of RelB in tumor cell proliferation, RelB was repressed in Hs578T breast cancer cells, using stable transfection with pSIREN siRelB expression vector or, as a control, the pSIREN RelB-sense vector (43). Expression of siRelB strongly repressed nuclear RelB compared to the control (Fig. 11A). RelB repression resulted in inhibition of nuclear levels of cyclin D1. Moreover, cells expressing siRelB also displayed slower proliferation than RelB sense control cells (data not shown). Thus, RelB is necessary for maintenance of normal cyclin D1 expression and proliferation of Hs578T breast cancer cells.

A drop in c-Myc levels was also noted with the siRelB expression vector in Hs578T cells (Fig. 11A). As c-Myc is induced during late pregnancy (52), it might represent an additional RelB/p52 target gene in the mammary gland. Thus, we next sought to assess the role of RelB in c-*myc* induction in mammary epithelial cells using transfection analysis. Untransformed mouse mammary epithelial NMuMG cells were cotransfected with the p1.6 Bgl *myc*-CAT WT promoter reporter construct, the pSV-40 β-Gal expression vector to normalize for transfection efficiency, and vectors expressing p52 and RelB, or p65 and p50, or the empty vector DNA. Expression of p65/p50 effectively activated the c-*myc* promoter compared to the empty vector DNA (Fig. 11B). The RelB/p52 complexes robustly activated the c-*myc* promoter at a level equivalent to or better than p65/p50 (Fig. 11B). No induction was seen with c-*myc* promoter constructs with mutated NF-κB elements, as expected (data not shown). Thus, induction of RelB/p52 complexes can activate c-*myc* promoter activity.

To further characterize the involvement of RelB in DMBA-mediated cellular transformation, D3-1 DMBA-transformed mammary epithelial cells were stably transfected with either the siRelB expression vector or its control vector expressing the sense sequence. After 1 week in selection, nuclear RelB expression was found to be substantially decreased (Fig. 12A). Growth in soft agar, a hallmark of transformation, was next

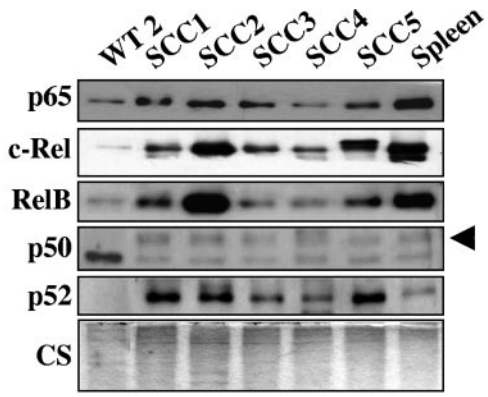

FIG. 9. Carcinogen-induced murine mammary squamous cell carcinomas exhibit high nuclear levels of p52 and RelB. WT FVB/N normal mammary gland or DMBA-induced squamous cell carcinomas (SSC1 to SSC5) were harvested. Nuclear extracts were prepared and subjected to immunoblot analysis for p65, c-Rel, RelB, p50, and p52. Coomassie blue staining was used to control for loading. A whole-cell extract from pooled FVB/N spleens was used as a control for expression of the different NF-κB subunits. The black arrowhead indicates the position of a 50-kDa protein band, as judged by the 50-kDa band in the Precision Plus protein standards (Bio-Rad) run alongside the sample extracts.

assessed. Soft agar assays were incubated for 2 weeks. D3-1 cells expressing the sense RelB insert formed large colonies, similar to the parental D3-1 cells; however, cells expressing siRelB formed very few colonies, and the colonies were smaller than those formed by cells expressing the sense RelB RNA (data not shown). Quantitation of total colony numbers revealed that siRelB expression reduced colonies numbers by 75 to 85% (Fig. 12B). Moreover, siRelB-expressing cells grew more slowly than control cells (data not shown). Thus, RelB is important for control of genes that regulate cell proliferation, as well as oncogenic transformation.

DISCUSSION

Here we have demonstrated, for the first time, that the alternative IKKα signaling pathway activates RelB/p52 complexes in the mammary gland by day 7.5 of pregnancy, and these complexes, which are resistant to the expression of the SR-IκB-α transgene, promote mid- to late-stage development of the mammary gland. Interestingly, RelB/p52 complexes are also induced by DMBA, and these likely play a role in the observed mammary carcinogenesis. Expression of the SR-IκB-α transgene resulted in a reduction of ductal branching in early pregnancy, which recovered during mid- to late pregnancy. The initial delay is consistent with previous reports of the importance of p65/p50 in regulating early breast development (4). The later recovery of ductal branching and, in particular, alveolar development can be attributed to the activation of NIK and IKKα between days 5.5 and 7.5 of pregnancy, which promotes the nuclear localization of RelB/p52 and thereby activation of the *cyclin D1* promoter. Importantly, the poor ability of IκB-α to interact with and prevent nuclear localization and binding of the released RelB/p52 complexes appears responsible in large measure for the observed rescue. Thus, NF-κB activation during mammary development follows a bimodal pattern. In virgin mice and at the early stages of pregnancy, p65/p50 and p50 homodimers are the major active complexes, while by mid- to late-stage pregnancy RelB/p52 activity is strongly induced as well, and it is this induction which promotes the completion of mammary gland development. DMBA-induced tumors in female Sprague-Dawley rats were typified by activation of NF-κB (51). Here we showed that murine tumors induced by DMBA frequently display a significant nuclear RelB/p52 induction. Similarly, the mammary tumors isolated from MMTV–c-*rel* transgenic mice that contained high RelB also expressed high nuclear p52, suggesting the alternative pathway was also active in these tumors (41). Moreover, repression of RelB in human breast cancer cells

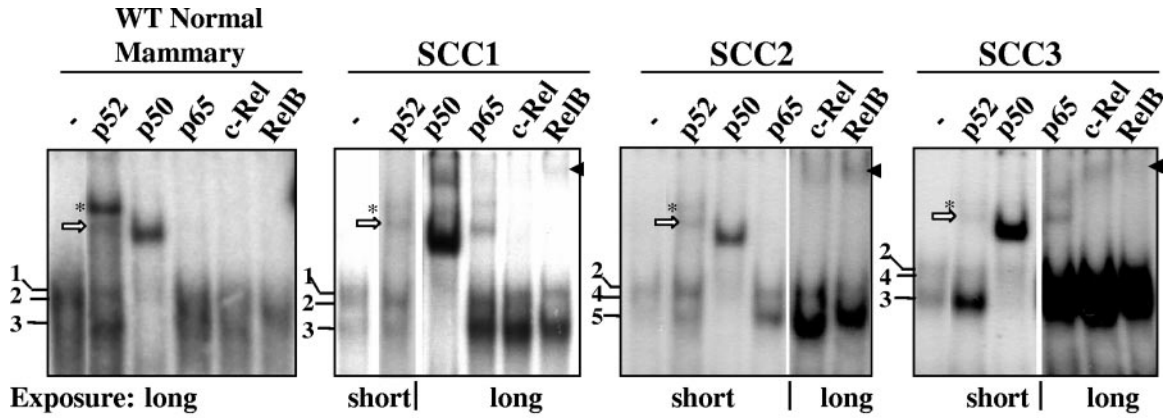

FIG. 10. DMBA-induced mammary squamous cell carcinomas exhibit increased RelB/p52 binding activity. Nuclear extracts from WT FVB/N normal mammary gland or from DMBA-induced mammary squamous cell carcinomas (SCC1, SSC2, and SSC3) were analyzed on different gels by supershift analysis for NF-κB binding activity, using supershifting antibodies against p52, p50, p65, c-Rel, and RelB. Supershift analyses were aligned at the top of the gel; however, complexes ran to slightly different positions in the separate gels and did not align perfectly with the corresponding bands on the other gels. Numbered lines indicate positions of identified subunit compositions as follows: 1, 65/p50 or p65/p52; 2, RelB/p52 and maybe some RelB/p50; 3, p50 homodimer; 4, c-Rel/p50 or c-Rel/p52; 5, p50/p52 or p52/p52. Supershifts were exposed for various time periods in order to more clearly discern supershifting bands. Short exposures were 4 days for SCC1 and overnight for SCC2 and SCC3. Long exposures were 1 week for WT, SCC2, and SCC3 and 2 weeks for SCC1. White arrow, position of a supershifted p52 band; black arrowhead, position of a supershifted RelB band; *, position of a nonspecific band seen with probe and antibody alone.

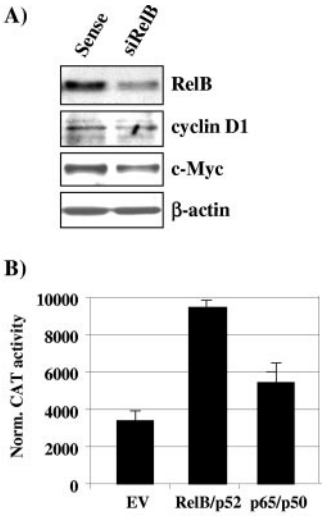

FIG. 11. RelB complexes are required for maintenance of cyclin D1 and c-*myc* expression in breast cancer cells. (A) Hs578T cells were stably transfected with the pSIREN RelB-sense (sense) and pSIREN siRelB (siRelB) expression vectors. Nuclear extracts were prepared, and immunoblot analysis was performed for RelB, cyclin D1, c-Myc, and β-actin, which confirmed equal loading. (B) NMuMG cells were transiently transfected with 1 μg of the p1.6 Bgl-*myc*-CAT reporter construct with WT NF-κB sites and 0.5 μg of pSV40-β-Gal in the presence of 0.5 μg each of RelB and p52 or p65 and p50 plasmid expression vectors or 1.0 μg of the empty vector. After 48 h, cultures were harvested, normalized for β-Gal activity, and assayed for luciferase or chloramphenicol acetyltransferase (CAT) activity as appropriate. Values for CAT activity are presented after normalization to β-Gal activity. The error bars represent the standard deviations.

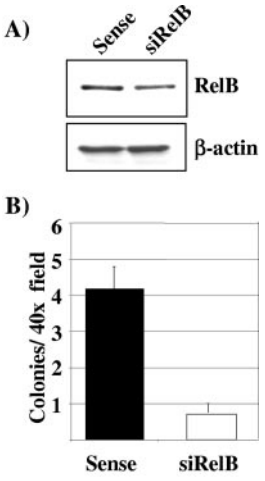

FIG. 12. Inhibition of RelB reduces the anchorage-independent phenotype of DMBA-transformed breast cancer cells. D3-1 cells were stably transfected with the pSIREN RelB-sense (sense) and pSIREN siRelB (siRelB) expression vectors. (A) After 1 week of selection, nuclear extracts were prepared, and RelB expression was assessed by immunoblotting. Analysis of β-actin expression confirmed equal loading. (B) Soft agar assays were performed in triplicate, and colony number was quantitated in four random fields from each plate. Error bars represent the standard deviations.

resulted in inhibition of cyclin D1 and c-Myc and a reduced ability to grow in soft agar. Overall, these findings suggest a major role for activation of RelB/p52 in carcinogenesis as well as in late-stage development of the mammary gland.

While the specific roles of the differential expression of NF-κB complexes during mammary development remain unclear, there are several intriguing possibilities. Importantly, it is known that the NF-κB binding elements of different genes exhibit selective preference for various NF-κB complexes. For example, while the c-*myc* URE is capable of binding all members of the NF-κB family (30), it is not transactivated by Bcl-3/p52 complexes (data not shown), in contrast to the *cyclin D1* promoter (58). Furthermore, an NF-κB element in the *BLC* (B-lymphocyte chemoattractant) promoter preferentially binds RelB/p52 (3). In contrast, the two NF-κB elements in the *Bcl-x(L)* promoter are unable to bind RelB/p50, and the gene is completely unresponsive to RelB/p50-mediated transactivation (27). These findings are consistent with differential roles for the various NF-κB complexes. Thus, we propose that that the early induction of p65/p50 during mammary development serves primarily to induce epithelial cell proliferation and ductal branching, while the late induction of RelB/p52 preferentially activates a set of target genes involved in the promotion of differentiation of mammary epithelial cells into milk-producing alveoli, although some targets appear responsive to all members of the family, e.g., c-*myc* and *cyclin D1*. Interestingly, the alternative NF-κB pathway was first shown to be critical in B-cell maturation and formation of secondary lymphoid organs (44). It has since been implicated in osteoclast differentiation (37), supporting the theory that RelB/p52 functions as a major regulator of differentiation and cellular maturation. Interestingly, neither the RelB nor the p52 knockout mouse has been reported to display aberrant mammary gland development (24, 57); however, the expected phenotype would be quite subtle and require specific examination.

While our studies have found that induction of RelB/p52 complexes appears to correlate with recovery of mammary gland development in the MMTV-SR-IκB-α transgenic mice, it is possible that other factors may also be involved. It has been shown that Bcl-3/p52 complexes potently activate the *cyclin D1* promoter in mammary epithelial cells (41, 58) and may therefore be involved in epithelial proliferation during pregnancy. However, as discussed above, only RelB/p52 was able to induce the c-*myc* promoter, while Bcl-3/p52 complexes could not (data not shown), implying that RelB/p52 complexes may be more potent mediators of proliferation than Bcl-3/p52 complexes. Moreover, the importance of RelB in mediating proliferation was underscored by the finding that specific repression of RelB via siRNA inhibited both cyclin D1 and c-Myc expression and slowed proliferation of mammary tumor cells. These data strongly indicate that the RelB/p52 complexes induced during pregnancy likely play a functional role in mediating mammary epithelial cell proliferation during development of the normal mammary gland and in tumorigenesis.

Cao et al. (9) proposed that the upstream signaling event leading to activation of IKKα in the mammary gland was signaling via the receptor activator of NF-κB (RANK)/RANK ligand (RANKL). RANK is a member of the TNF receptor family and can lead to NF-κB activation. RANKL- or RANK-deficient mice display a failure of lobuloalveolar mammary development, resulting in mammary glands in which the maximal extent of alveolar development is comparable to WT

glands at day 10.5 of pregnancy (23). Significantly, both IKK$\alpha^{AA/AA}$ kinase-inactive knock-in mice and cyclin D1-deficient mice display a similar mammary phenotype as RANKL-deficient mice (9, 22, 45), and transgenic expression of cyclin D1 in the IKK$^{AA/AA}$ knock-in mice rescues mammary development (9). Moreover, the timing of the failure of lobuloalveolar development in these mice correlates well with what we have found to be the onset of significant RelB/p52 binding activity. Interestingly, Aly mice, in which the kinase function of NIK is inactivated, exhibited a defect in mammary development (36). However, the alternate pathway via IKKα can be activated by other kinases, including Akt (38), and these signaling pathways may converge during pregnancy to induce nuclear RelB/p52 complexes.

Interestingly, RelB expression does not appear to be grossly affected by the inhibition of p65/p50 in the MMTV-SR-IκB-α transgenic mice, despite the role of classical NF-κB in transactivation of the *relB* promoter (6). One factor that may be influencing RelB expression in the mammary gland is the vitamin D receptor (VDR). The VDR is dynamically regulated during pregnancy, and its ablation results in accelerated lobuloalveolar development of the mammary gland, as well as a delay in postlactational mammary regression (63). The VDR has been shown to repress the *relB* promoter (19); hence, relief of repression by VDR during pregnancy may be one mechanism whereby RelB is induced. Another candidate for transcriptional regulation of RelB during mammary development is the AP-1 family of transcription factors. Recent work in our lab has shown a role for c-Jun and Fra-2 in transactivation of the *relB* promoter (56). Interestingly, tumors in the MMTV-c-*rel* transgenic mice, with high nuclear p52 and RelB (41), expressed high levels of AP-1 family members (X. Wang and G. E. Sonenshein, unpublished observations), leading us to speculate on the role of AP-1 in mammary gland tumorigenesis. Significantly, AP-1 activity is regulated by JNK, which is, in turn, strongly activated as a result of RANKL signaling (17), providing yet another possible mechanism whereby RANKL/RANK signaling may be exerting its effects in the mammary gland. Overall, our studies strongly implicate signaling through IKKα and the alternative RelB/p52 pathway as an important regulator of mammary lobuloalveolar cell proliferation during late pregnancy and in carcinogenesis in vivo, suggesting that these NF-κB family members may represent potential therapeutic targets that are resistant to repression by IκB-α.

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