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TGFβ1 Inhibits NF-κB/Rel Activity Inducing Apoptosis of B Cells: Transcriptional Activation of IκBα

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Summary

TGFβ1 treatment of B cell lymphomas decreases c-*myc* gene expression and induces apoptosis. Since we have demonstrated NF-κB/Rel factors play a key role in transcriptional control of c-*myc*, we explored the effects of TGFβ1 on WEHI 231 immature B cells. A reduction in NF-κB/Rel activity followed TGFβ1 treatment. In WEHI 231 and CH33 cells, we observed an increase in IκBα, a specific NF-κB/Rel inhibitor, due to transcriptional induction. Engagement of surface CD40 or ectopic c-Rel led to maintenance of NF-κB/Rel and c-Myc expression and protection of WEHI 231 cells from TGFβ1-mediated apoptosis. Ectopic c-Myc expression overrode apoptosis induced by TGFβ1. Thus, downmodulation of NF-κB/Rel reduces c-Myc expression, which leads to apoptosis in these immature B cell models of clonal deletion. The inhibition of NF-κB/Rel activity represents a novel TGFβ signaling mechanism.

Introduction

The transforming growth factor-β1 (TGFβ1) exerts its growth inhibitory properties on several cell types, including B lymphoid cells (Massaguè, 1990; Roberts and Sporn, 1990; Stavnezer, 1996). Treatment of murine immature B cell lymphomas, such as WEHI 231 and CH31, with TGFβ1 leads to a block at the G1/S transition, followed by apoptosis (Warner et al., 1992; Fischer et al., 1994). More recently, it has been demonstrated that TGFβ1 treatment causes apoptosis in normal human B cells and in Epstein–Barr virus (EBV)-negative lymphoma B cell lines (Chaouchi et al., 1995; Lomo et al., 1995). It has been proposed that TGFβ1 exerts its growth inhibitory effects through the down-regulation of the activity of genes involved in cellular proliferation such as cyclin-dependent kinases (Howe et al., 1991; Ewen et al., 1993; Hannon and Beach, 1994), the retinoblastoma susceptibility product (pRb) (Laiho et al., 1990; Pietenpol et al., 1990) and the c-*myc* proto-oncogene (Coffey et al., 1988; Pietenpol et al., 1990). For example, TGFβ1-induced apoptosis of WEHI 231 or CH31 cells is preceded by a decline in c-*myc* expression (Warner et al., 1992; Fischer et al., 1994).

The c-*myc* gene has been strongly implicated in the regulation of cellular death. Overexpression or inappropiate expression of the c-*myc* gene promoted apoptosis in myeloid and fibroblast cells upon removal of factors required for cell proliferation (Askew et al., 1991; Evan et al., 1992). Furthermore, addition of antisense oligonucleotides against c-*myc* to immature T cells and some T cell hybridomas prevented T cell receptor–mediated apoptosis (Shi et al., 1992). In contrast, the evidence in immature B cell models of tolerance, such as WEHI 231 and CH31 and CH33 cells (Boyd and Schrader, 1981; Kim et al., 1979; Monroe and Seyfert, 1988; Ralph, 1979), has correlated the drop in c-Myc expression with induction of apoptosis. An early transient increase followed by a dramatic decline in levels of c-*myc* RNA and protein precedes anti-immunoglobulin receptor–mediated induction of apoptosis of WEHI 231 and CH 31 B cells (Ales-Martinez et al., 1988; Benhamou et al., 1990; Hasbold and Klaus, 1990; Levine et al., 1986; Maheswaran et al., 1991; McCormack et al., 1984). In WEHI 231 and CH 33 cells stably transfected to express an immunoglobulin D (IgD) surface molecule, both anti-IgM and anti-IgD treatments led to transient increases of c-*myc* expression, but only anti-IgM caused a decline in c-*myc* RNA levels below baseline followed by apoptosis, whereas anti-IgD treatment maintained c-*myc* expression at or above control levels and no induction of apoptosis was noted (Tisch et al., 1988; Ales-Martinez et al., 1988). Similarly, mutants of WEHI 231 that failed to induce apoptosis in response to anti-immunoglobulin displayed sustained c-*myc* expression (Hibner et al., 1994). Furthermore, addition of c-*myc* antisense oligonucleotides prevented anti-immunoglobulin receptor and TGFβ1-mediated apoptosis in the murine WEHI 231 and CH31 cell lymphomas (Fischer et al., 1994); however, the treatment with c-*myc* antisense oligonucleotide, but not the mismatched control, led to the stabilization of c-Myc protein and to protection against apoptosis.

Previously, we identified two κB elements within the murine c-*myc* gene, termed the upstream and internal regulatory elements, URE and IRE, respectively (Duyao et al., 1990; Kessler et al., 1992b), which regulate c-*myc* expression (Duyao et al., 1992; Kessler et al., 1992a; La Rosa et al., 1994). Classical NF-κB is a heterodimeric transcription factor formed of two subunits, NFKB1 (p50) and RelA (p65) (Ghosh et al., 1990; Kieran et al., 1990; Ruben et al., 1991; Nolan et al., 1991). Other members of the NF-κB/Rel family expressed in mammals include the proto-oncogene c-Rel, NFKB2 (p52), and RelB (Gilmore, 1991; Neri et al., 1991; Schmid et al., 1991; Ryseck et al., 1992; Bours et al., 1992). The Rel family of factors is involved in the regulation of a diverse group of genes that play important roles in immune, acute phase, and inflammatory responses, cell proliferation, and differentiation (Grilli et al., 1993; Baeuerle and Henkel, 1994). Furthermore, evidence indicates these factors mediate signals in immunoglobulin gene rearrangement (Kentner et al., 1993). Rel factors are ubiquitously expressed in most non-B cells as inducible dimeric complexes (Baeuerle, 1991), sequestered in the cytoplasm by a family of inhibitor proteins, termed IκBs, that includes IκBα and IκBβ (Baeuerle and Baltimore, 1988; Davis et al., 1991; Haskill et al., 1991; Thompson et al., 1995). A wide range of stimuli promotes nuclear translocation of

Rel factors by a mechanism that involves phosphorylation and ubiquitination of these IκB proteins (reviewed by Grilli et al., 1993; Verma et al., 1995).

Exponentially growing WEHI 231 cells express predominantly p50/c-Rel complexes and minor amounts of classical NF-κB (p50/RelA) (Rice and Ernst, 1993; Miyamoto et al., 1994; Liou et al., 1994; Lee et al., 1995). Recently, we demonstrated that both basal and anti-immunoglobulin–modulated c-*myc* gene expression in WEHI 231 cells are controlled in large part by NF-κB/Rel binding (Lee et al., 1995). Anti-immunoglobulin treatment led to a significant early transient induction of binding and activity of NF-κB/Rel species followed by a loss of binding below basal levels at later timepoints; these changes paralleled the increases and decreases in c-*myc* gene transcription (Lee et al., 1995). Here, we investigated the possible role of NF-κB/Rel factors in mediating the effects of TGFβ1 in WEHI 231 and CH33 cells. We found that TGFβ1 treatment of WEHI 231 cells decreased NF-κB/Rel binding activity. This decrease could be related, in part, to elevated levels of IκBα mRNA and protein resulting from increased transcription of the IκBα gene in both lines. Ablated apoptosis of WEHI 231 cells was observed with TGFβ1 upon costimulation with a soluble form of the CD40 ligand (CD40L), which led to maintained NF-κB/Rel and c-Myc expression, or upon ectopic expression of c-Rel or c-Myc. These results indicate inhibition of NF-κB/Rel downmodulates c-Myc expression inducing death of these B cells, and demonstrate a novel signaling pathway for TGFβ1 through NF-κB/Rel that has potential implications for multiple processes within the immune system in addition to apoptosis, including activation of cell proliferation, differentiation, and gene rearrangement.

Results

TGFβ1 Down-Regulates NF-κB/Rel Activity

To determine whether TGFβ1 treatment can alter NF-κB/Rel activity, WEHI 231 cells were cultured in the absence or presence of 2 ng/ml TGFβ1 for 3 and 6 hr and nuclear extracts prepared. Electrophoretic mobility shift analysis (EMSA) was performed with fragments containing the two κB sites within the c-*myc* gene, termed the URE and IRE (Duyao et al., 1990; Kessler et al., 1992b). As expected, nuclear extracts from untreated WEHI 231 displayed several constitutive specific binding complexes with the URE (Figure 1, left) and IRE (Figure 1, right) (Lee et al., 1995; Schauer et al., 1996). Previous EMSA supershift studies have identified the five fastest migrating complexes with the URE as follows: band 1, p50 homodimer; band 2, p50/c-Rel; band 3, p50/Rel A; band 4, heterodimer containing p50 and an as yet unidentified partner; band 5, c-Rel homodimer; band 6, unidentified (Lee et al., 1995; data not shown). For the fragment containing the IRE, the three fastest migrating complexes are identical to those with the URE and the fourth as a homodimer of c-Rel (Schauer et al., 1996). A slight decrease in binding was detectable by 3 hr of TGFβ1 treatment, and by 6 hr overall NF-κB/Rel binding to both the URE and IRE was significantly reduced. This is more dramatically seen by 9 hr of TGFβ1 treatment, in a separate experiment, illustrated below (see Figure 5); furthermore, only much more minor alterations observed in the binding activity of AP-1 complexes confirmed the specificity of these changes (data not shown; see Figure 5). Thus, TGFβ1 selectively reduces NF-κB/Rel binding.

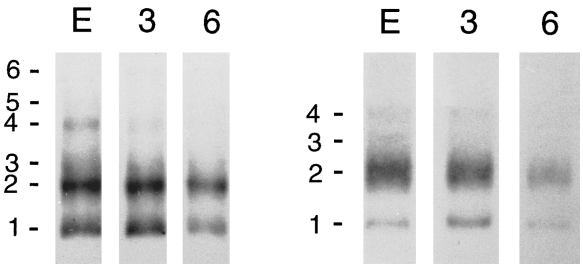

Figure 1. TGFβ1 Treatment Inhibits NF-κB/Rel Binding Activity in WEHI 231 Cells

Equal amounts of nuclear extracts (5 μg protein), prepared from WEHI 231 cells in exponential growth (E) and following treatment with 2 ng/ml TGFβ1 cells for 3 or 6 hr, were used in EMSA with radiolabeled DNA fragments containing (left) the κB element upstream of the murine c-*myc* promoter (URE), or (right) the internal regulatory region (IRE) within exon 1 of the murine c-*myc* gene. The six URE complexes are as follow: band 1, p50 homodimer; band 2, p50/c-Rel; band 3, p50/RelA; band 4, p50 and an unidentified subunit; band 5, c-Rel homodimer, band 6, unidentified (Lee et al., 1995). The four IRE nucleoprotein complexes are as follows: band 1, p50 homodimer; band 2, p50/c-Rel; band 3, p50/RelA; band 4, c-Rel homodimer (Schauer et al., 1996).

TGFβ1 Induces IκBα Synthesis

The observed inhibition of NF-κB activity prompted us to explore the possibility of induction upon TGFβ1 treatment of IκBα, an inhibitor protein of NF-κB/Rel capable of sequestering these dimeric species in the cytosolic compartment (Baeuerle, 1991; Verma et al., 1995). Cytoplasmic extracts were isolated from WEHI 231 cells treated with TGFβ1 for 0, 3, 6, and 9 hr, and subjected to immunoblot analysis. TGFβ1 treatment led to induction of IκBα protein levels after 3 hr, and levels continued to increase throughout the time course (Figure 2A). The effect of TGFβ1 was selective for the IκBα product, in that IκBβ protein remained constant throughout the 9 hr period (data not shown).

IκBα protein in WEHI 231 cells is normally labile with a half-life of approximately 30 min (Miyamoto et al., 1994). To test for stabilization of IκBα protein, WEHI 231 cells were treated with the protein synthesis inhibitor cycloheximide (CHX) in the presence or absence of TGFβ1. Cytoplasmic extracts were monitored by immunoblotting for IκBα expression (Figure 2B). IκBα displayed the normal rapid rate of decay following TGFβ1 treatment. In addition, a similar decay of the IκBα protein was observed when the cells were incubated for 6 hr with TGFβ1 prior to CHX treatment (data not shown). Thus, TGFβ1 does not stabilize IκBα protein.

TGFβ1 Induces IκBα mRNA Synthesis

To determine whether the increased protein expression was due to increased steady-state RNA levels, the effect of TGFβ1 treatment on IκBα mRNA was measured. After 3 hr of TGFβ1 treatment, an approximately 2.5-fold induction in IκBα mRNA level was detected, and the level

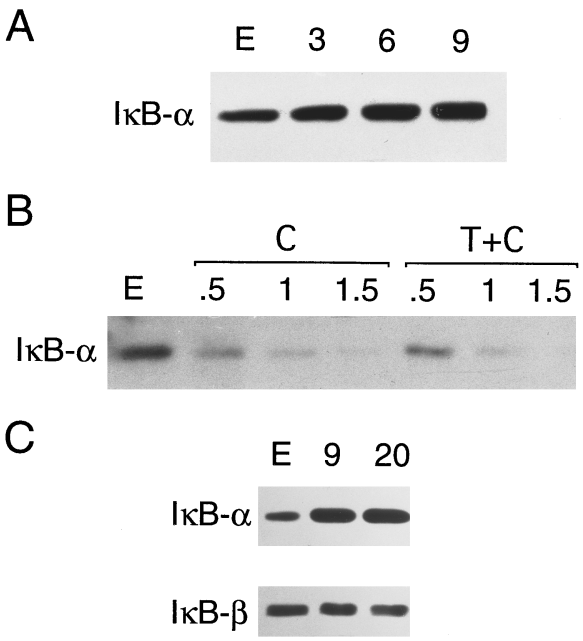

Figure 2. TGFβ1 Treatment Increases IκBα Expression in WEHI 231 and CH33 Cells

(A) TGFβ1 increases IκBα protein levels in the cytoplasm of WEHI 231 cells. Cytoplasmic protein was isolated from cells in exponential growth (E) or following TGFβ1 treatment for the indicated timepoints (hr) and subjected to immunoblotting using a polyclonal antibody against IκBα. Based on molecular mass markers, the IκBα band has a molecular mass of 38 kDa.

(B) TGFβ1 treatment does not lead to IκBα protein stabilization in WEHI 231 cells.

(C) Cells in exponential growth (E) were treated with 10 μg/ml CHX in the absence or presence of TGFβ1 (T). Cytoplasmic extracts, isolated after 0.5, 1, or 1.5 hr of treatment, were analyzed by immunoblotting for IκBα.

(C) TGFβ1 increases IκBα protein levels in the cytoplasm of CH33 cells. Exponentially growing CH33 cells (E) were incubated in the presence of TGFβ1 for 9 and 20 hr, and cytoplasmic extracts analyzed by immunoblotting for IκBα and IκBβ proteins, as above. The band corresponding to IκBβ protein has a molecular mass of 49 kDa.

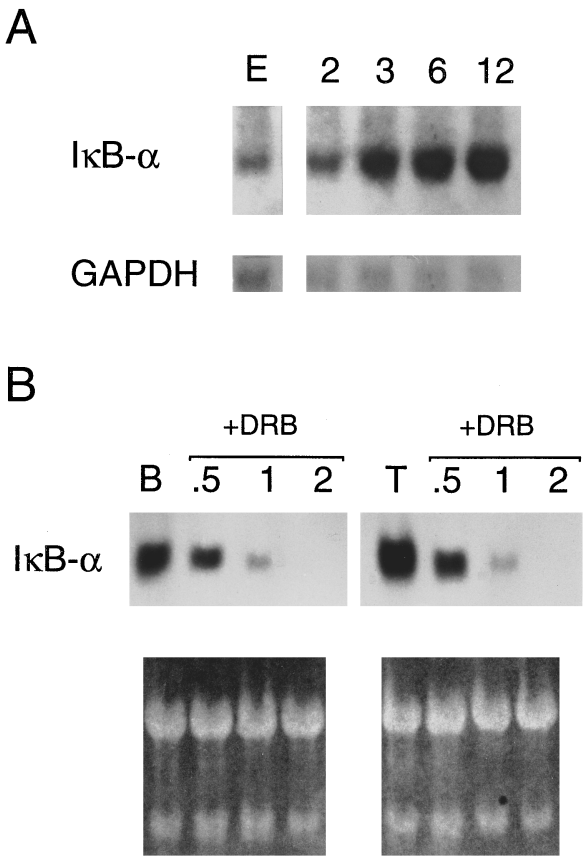

Figure 3. TGFβ1 Induces the IκBα mRNA Levels but Does Not Alter the Stability of the Transcript in WEHI 231 Cells

(A) TGFβ1 increases steady-state levels of IκBα RNA. WEHI 231 cells in exponential growth (E) were exposed to TGFβ1 for 2, 3, 6, or 12 hr. Total RNA was isolated and subjected to Northern blot analysis for expression of IκBα and GAPDH, as control for equal loading.

(B) TGFβ1 treatment does not alter the stability of IκBα mRNA. Following treatment of WEHI 231 cell cultures for 9 hr with TGFβ1 (T) or carrier BSA (B), as control, 25 μg/ml DRB was added and total RNA isolated after 0, 0.5, 1, and 2 hr. Northern blot analysis was performed for mRNA expression of IκBα. Ethidium bromide staining, used to confirm RNA integrity and equal loading of the samples, is shown in the bottom panel.

remained elevated for up to 12 hr (Figure 3A). Equal loading of RNA samples was demonstrated by rehybridization of the same filter for the housekeeping gene glyceraldehyde-3-P-dehydrogenase (GAPDH). We next determined whether an increase in the half-life of the IκBα mRNA could account for the increase in transcript level upon TGFβ1 exposure, using 5, 6-dichlorobenzimidazole riboside (DRB), a selective inhibitor of RNA polymerase II. Control and WEHI 231 cells that had been treated with TGFβ1 for 9 hr were incubated with 25 μg/ml DRB and total RNA was isolated at various timepoints (Figure 3B). A $t_{1/2}$ of approximately 30 min was observed in control cells (Figure 3B). The IκBα mRNA levels decayed in response to DRB treatment with the same kinetics in the TGFβ1-treated cultures. An induction of the steady-state levels of IκBα mRNA levels was seen in response to TGFβ1 treatment (Figure 3B), as expected. Thus, TGFβ1 does not stabilize the normally labile IκBα mRNA from decay.

To test whether TGFβ1 increased the rate of IκBα gene transcription, nuclear run-on analysis was performed. Nuclei were isolated from exponentially growing WEHI 231 cells or following treatment with TGFβ1 for 2.5 hr. A significant increase in the rate of IκBα gene transcription was detected at the 2.5 hr timepoint (Figure 4, left), consistent with the observed induction of mRNA levels at 3 hr. Densitometric scanning of this and a duplicate experiment gave an average fold increase of 3.1 ± 0.36 when normalized to β-actin. In contrast, no effect on pMT2T, the parental vector for the IκBα clone or pUC19 plasmid DNA was detectable, which suggests equal RNA loading (Figure 4, left). Similarly, the rate of transcription of β-actin and Max, the c-Myc binding partner, was unaffected by TGFβ1 treatment, consistent with the lack of change in their mRNA levels over the time course (data not shown). Thus, an increase of IκBα gene transcription following TGFβ1 treatment appears responsible for the increase of IκBα steady-state mRNA and protein levels in the WEHI 231 cell line.

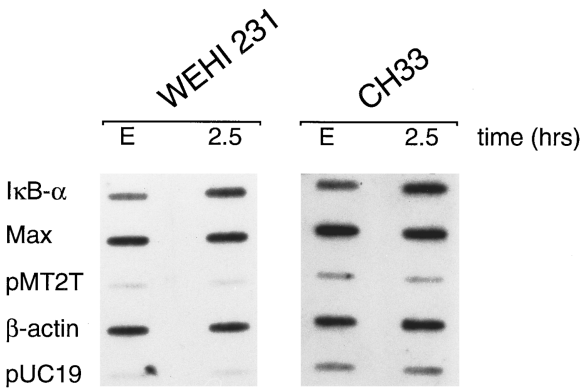

Figure 4. TGFβ1 Treatment of WEHI 231 and CH33 Cells Increases the Rate of IκBα Gene Transcription

Nuclei were isolated from (left) WEHI 231 or (right) CH33 cells in exponential growth or following treatment with TGFβ1 for 2.5 hr and subjected to run-on analysis. The radiolabeled RNA products were analyzed with double-stranded DNA corresponding to the human IκBα, rat β-actin, and murine Max genes. pMT2T and pUC19 DNA was used as a measure of nonspecific hybridization.

TGFβ1 Induces IκBα Gene Expression in the CH33 Cell Line

To test whether TGFβ1-mediated induction of IκBα gene was unique to the WEHI 231 cell line or more general to other immature B cell lymphomas, the CH33 cell line was similarly analyzed. Following 20 hr of TGFβ1 treatment, CH33 cells display extensive DNA laddering (Fischer et al., 1994; data not shown). Cytoplasmic extracts were isolated from CH33 cells treated with 2 ng/ml TGFβ1 for 9 and 20 hr and analyzed by immunoblotting for IκBα protein. Treatment for 9 hr with TGFβ1 led to an increase in IκBα protein levels that persisted throughout the time course (see Figure 2C). TGFβ1 treatment did not affect IκBβ expression (see Figure 2C). We next sought to evaluate whether TGFβ1 increased the rate of IκBα gene transcription in CH33 cells (Figure 4, right). A 2.1-fold increase in the rate of IκBα gene transcription, normalized to β-actin, was measured in nuclear run-on analysis at the 2.5 hr timepoint, similar to the results with WEHI 231 cells. As seen above, TGFβ1 treatment had no detectable effect on β-actin or Max gene expression or on hybridization to plasmid DNA, used as controls for equal loading (Figure 4, right). Thus, TGFβ1 selectively induces IκBα gene expression in murine B cell lymphomas.

CD40 Ligand Prevents TGFβ1-Driven Apoptosis and Induces NF-κB/Rel Levels

The observation that engagement of surface CD40, a member of the tumor necrosis factor receptor (TNFR) superfamily (Banchereau et al., 1994), was sufficient to neutralize the anti-immunoglobulin–mediated apoptosis of WEHI 231 cells (Tsubata et al., 1993) prompted us to ask whether CD40L treatment could similarly override TGFβ1-driven apoptosis. WEHI 231 cells were treated for 20 hr with TGFβ1 alone or together with CD40L, and apoptosis measured by DNA fragmentation analysis. As seen in Figure 5A, the extensive DNA laddering induced by TGFβ1 treatment was prevented upon costimulation with CD40L.

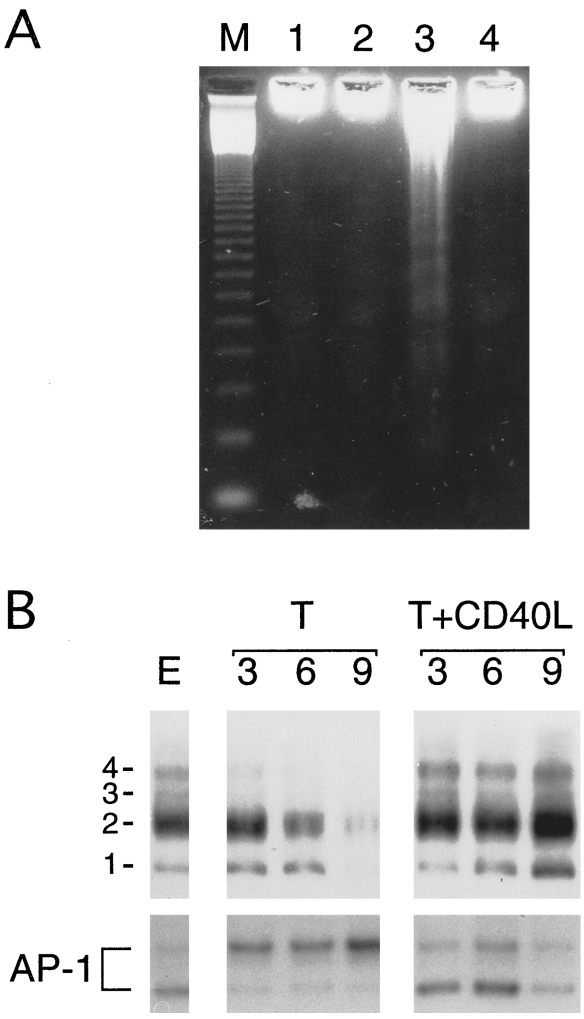

Figure 5. CD40L Prevents TGFβ1-Driven Apoptosis and Inhibition of NF-κB/Rel Binding in WEHI 231 Cells

(A) CD40L prevents TGFβ1-driven apoptosis. WEHI 231 cells in exponential growth (lane 1) were treated with BSA (lane 2) or TGFβ1 alone (lane 3) or in combination of CD40L and CD8α (CD40L) (lane 4), and analyzed for apoptosis after 20 hr using DNA fragmentation assay. M, 123 marker DNA.

(B) CD40L prevents TGFβ1-mediated down-regulation of NF-κB/Rel binding activity. Nuclear extracts were isolated from untreated WEHI 231 cells (E) or following treatment with TGFβ1 alone (T) or in combination with CD40L (T + CD40L) for 3, 6, and 9 hr. Samples (5 μg) were subjected to EMSA using the IRE κB element, described in the legend to Figure 1. The specificity of the changes in binding activity was confirmed using EMSA with a radiolabeled AP-1 oligonucleotide.

Recently, we have observed that CD40L treatment of WEHI 231 cells caused an increase in NF-κB/Rel binding and prevented the decline following anti-immunoglobulin treatment (Schauer et al. 1996). Costimulation with CD40L similarly reversed the decrease in nuclear extract binding to the IRE mediated by TGFβ1 (Figure 5B). In addition, CD40L appeared to abrogate the changes in AP-1 binding induced by TGFβ1. As expected, the TGFβ1-mediated drop in κB binding could be related to a decrease in nuclear levels of RelA and c-Rel protein (Figure 6A). In contrast, costimulation with TGFβ1 and CD40L up-regulated their expression. We next examined the effects of costimulation with CD40L on the

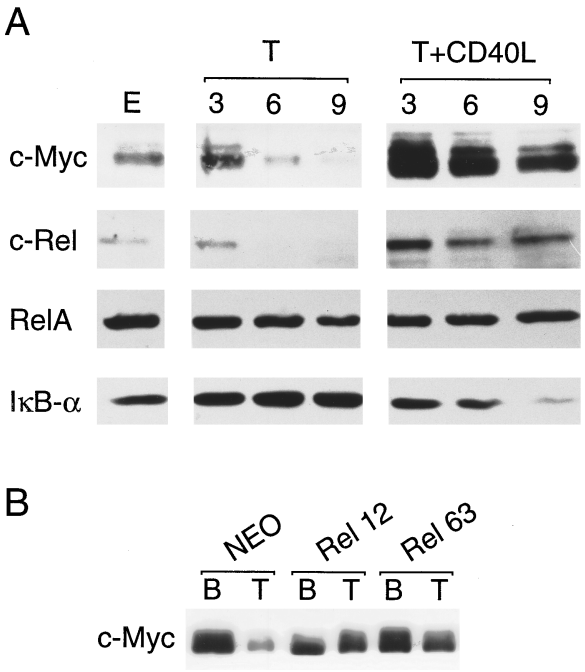

Figure 6. Maintenance of Nuclear NF-κB/Rel Activity Resulting from CD40L Treatment or Ectopic Expression of c-Rel Ablates TGFβ1-Driven Drop in c-Myc Expression

(A) CD40L treatment overrides TGFβ1 downmodulation of NF-κB/Rel and c-Myc expression in WEHI 231 cells. Nuclear and cytoplasmic protein was isolated from WEHI 231 cells in exponential growth (E) or following treatment with either TGFβ1 alone (T) or in combination with CD40L (T + CD40L) for 3, 6, or 9 hr. Samples (40 μg) were subjected to immunoblot analysis, using antibody preparations against c-Myc, c-Rel, RelA, and IκBα. The doublet corresponding to c-Myc has a molecular mass of 67/69 kDa.

(B) Ectopic expression of c-Rel ablates the decrease in nuclear c-Myc protein levels. WEHI 231 stable transfectant lines, prepared with either pSV2*neo* vector (NEO) alone or with the murine c-Rel expression vector (Rel 12, Rel 63), were incubated for 12 hr in the presence of BSA (B) or TGFβ1 (T). Samples of nuclear proteins (20 μg) were subjected to immunoblotting using an affinity-purified c-Myc antibody.

induction of IκBα protein levels upon TGFβ1 treatment (Figure 6A). TGFβ1 treatment alone resulted in an increase above baseline of cytoplasmic levels of IκBα, as seen above. This increase was reversed by CD40L treatment in combination with TGFβ1. In fact, a dramatic decrease of IκBα protein levels well below baseline values was observed, suggesting signals regulating expression of the inhibitor protein induced by CD40L overrode those of TGFβ1 (Figure 6A). Thus, an increase in overall nuclear NF-κB/Rel binding was induced by CD40L, which could be correlated, at least in part, with a decrease in IκBα expression.

CD40L or Ectopic Expression of c-Rel Maintains c-Myc Expression

To assess whether the changes in NF-κB/Rel activity results in altered c-*myc* gene expression, RNA was isolated and analyzed by reverse transcription polymerase chain reaction. The reduction below baseline of c-*myc* mRNA levels after 20 hr of TGFβ1 treatment was reversed by costimulation with CD40L, resulting in an overall induction of c-*myc* expression (data not shown). To confirm the RNA was functional, the effects on c-Myc protein expression were monitored. Nuclear protein extracts of WEHI 231 cells, treated with TGFβ1 for the indicated times in the absence or presence of CD40L, were subjected to immunoblot analysis. As expected, within 6 hr of TGFβ1 treatment alone, a decline in c-Myc nuclear protein levels was observed (Figure 6A), consistent with kinetics of the drop in c-*myc* mRNA levels (Warner et al., 1992; data not shown). The combined treatment of TGFβ1 and CD40L led to an increase in c-Myc protein levels that were maintained above basal levels for up to 9 hr (Figure 6A). Thus, CD40L costimulation leads to the maintenance of elevated levels of c-*myc* expression.

Since sustained c-*myc* expression correlated directly with Rel-related factor activity, we tested for the ability of ectopic expression of c-Rel to modulate c-Myc protein levels. Transfected WEHI 231 cells were selected with G418 following electroporation with murine c-*rel* expression vector (La Rosa et al., 1994) and pSV2*neo*, as described previously (Wu et al., 1996), or pSV2*neo* alone, as control. Two stable WEHI 231 c-Rel transfectant clones, Rel 12 and Rel 63, and one control pSV2*neo* transfectant clone (NEO 5) were characterized by immunoblotting for their levels of nuclear c-Rel protein following TGFβ1 treatment. Rel 12 and Rel 63 retained c-Rel expression for up to 12 hr, whereas the NEO 5 control cells showed marked down-regulation of c-Rel levels (data not shown). When these same nuclear lysates were analyzed for c-Myc expression, Rel 12 and Rel 63 were found to maintain c-Myc protein levels following TGFβ1 treatment, whereas the expected decline was noted in NEO 5 cells (Figure 6B). These clones were next tested for their sensitivity to TGFβ1-mediated apoptosis. Compared with the NEO 5 cells, both the Rel 12 and Rel 63 clones displayed more extensive survival and proliferation even after 48 hr of TGFβ1 treatment, as judged by cell numbers (data not shown) and trypan blue exclusion. The values for the percentage of trypan blue–positive (dead) cells, determined in triplicate cultures, were 12.8 ± 1.6% for Rel 12, 10.5 ± 2.0% for Rel 63, and 35.9 ± 1.4% for NEO 5. Lastly, to confirm that the cells retained TGFβ1 responsiveness, the induction in IκBα protein expression was confirmed following 12 hr of TGFβ1 treatment (data not shown). Thus, c-Rel expression leads to elevated c-Myc expression and rescue from apoptosis following TGFβ1 treatment.

Ectopic Expression of c-Myc Overrides TGFβ1-Driven Apoptosis of WEHI 231 Cells

To test directly the role of CD40L-mediated maintenance of c-Myc on apoptosis of WEHI 231 cells induced by TGFβ1, the effects of ectopic expression of c-*myc* were monitored. Stable transfectants were prepared with the human c-*myc* expression vector pM21 (Stone et al., 1987), and assayed by immunoblotting for c-Myc expression. The pM21 clones displayed normal levels of c-Myc protein when compared with the pSV2*neo* stable clones (Figure 7A; data not shown). Following TGFβ1 treatment of the pM21 clone P17, an elevated level of c-Myc protein was retained for up to 20 hr. These findings are in marked contrast with the down-regulation of

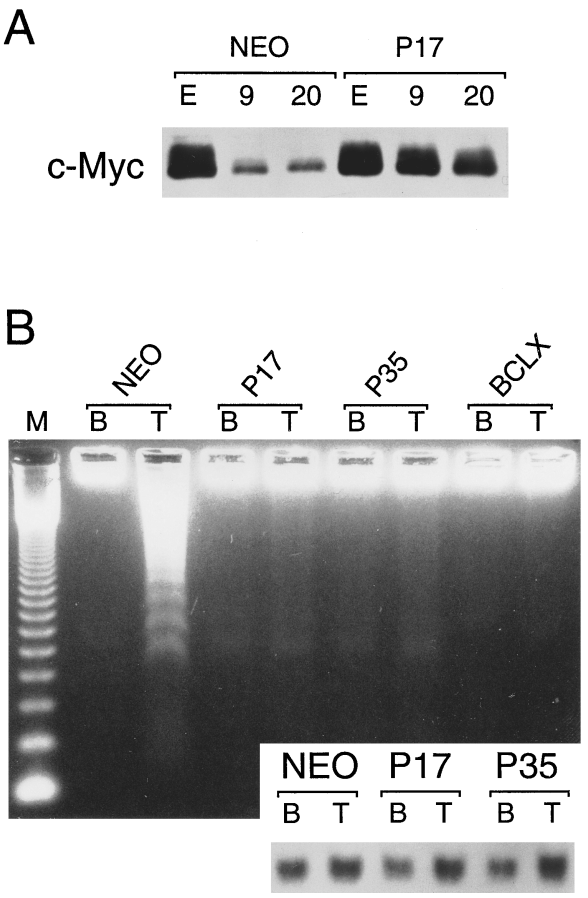

Figure 7. Ectopic Expression of c-Myc Prevents Apoptosis of WEHI 231 Cells Induced by TGFβ1

(A) Stable transfectants retain high levels of c-Myc expression following TGFβ1 treatment. Stable transfectant lines of WEHI 231 cells, prepared with either pSV2*neo* alone (NEO) or with the pM21 c-*myc* expression vector (P17), were incubated in the absence (E) or presence of TGFβ1 for 9 and 20 hr. Nuclear proteins (40 μg) were subjected to immunoblotting for c-Myc, as above.

(B) Ectopic expression of c-Myc prevents apoptosis upon TGFβ1 exposure. Two stable c-Myc WEHI 231 clones (P17 and P35) or control Neo cells were treated with TGFβ1 for 20 hr, and subjected to the DNA fragmentation assay of apoptosis. Alternatively, a stable WEHI 231 line expressing Bcl-x_L (JMBCLX) was similarly analyzed. M, 123 marker DNA. Inset, TGFβ1 treatment leads to induction of IκBα RNA levels in the c-Myc lines. RNA was isolated from the P17 and P35 c-Myc and Neo control clones, treated for 9 hr with TGFβ1 (T) or BSA (B), as control, and samples (20 μg) were analyzed for IκBα levels.

c-Myc observed in the control NEO 5 cells (Figure 7A) and in the parental WEHI 231 cells (see Figure 6A). We next tested two of the c-Myc clones, P17 and P35, for their sensitivity to apoptosis following TGFβ1 treatment. A significant protection from apoptosis, as judged by the absence of DNA fragmentation, was observed. The NEO 5 cells remained sensitive to TGFβ1-driven apoptosis (Figure 7B). In addition, the pM21 clones displayed extensive survival and were proliferating even after 48 hr of TGFβ1 treatment (data not shown). The P17 and P35, as well as control NEO 5 cells, retained TGFβ1 responsiveness, as judged by the increase in IκBα gene expression following incubation for 9 hr with TGFβ1 compared with carrier bovine serum albumin (BSA) (Figure 7B, inset). These results indicate that the maintenance of c-Myc levels, mediated by ectopic expression or CD40L, is sufficient to override the effects of TGFβ1 treatment on viability of WEHI 231 cells.

Bcl-x_L but not Bcl-2 has been found to inhibit anti-immunoglobulin–mediated apoptosis of WEHI 231 cells (Gottschalk et al., 1994). A constitutive Bcl-x_L expressing WEHI 231 cell line (JMBCLX) displayed significant resistance to apoptosis induced by TGFβ1 treatment (Figure 7B). Interestingly, expression of Bcl-x_L did not counteract the growth arrest induced by TGFβ1 exposure (data not shown). Thus, expression of Bcl-x_L overrides the signal promoting apoptosis induced by TGFβ1; its action appears to be mediated further downstream within the apoptosis cascade.

Discussion

In this study, we have shown a novel signaling pathway for TGFβ1 via a decrease in NF-κB/Rel activity. An induction of IκBα protein levels and gene transcription was mediated by TGFβ1 treatment. The reduction in NF-κB/Rel binding correlated with a drop in c-Myc levels. Costimulation with CD40L neutralized the TGFβ1-mediated effects and resulted in the induction and maintenance of NF-κB/Rel activity and c-Myc expression. Similarly, ectopic expression of c-Rel led to sustained levels of c-Myc following TGFβ1 treatment. Both treatment with CD40L and ectopic c-Rel expression, as well as ectopic expression of c-Myc, led to protection from TGFβ1-driven apoptosis. These results indicate a common pathway for induction of physiologic cell death of immature B cells by TGFβ1 and anti-immunoglobulin: a drop in NF-κB/Rel activity that results in a drop in c-*myc* expression. In agreement with these findings, we have found that forced down-regulation of c-Myc/Max activity via overexpression of Mad led to the induction of apoptosis of WEHI 231 cells (M. W. et al., submitted). Thus, the drop of c-Myc expression, rather than its overexpression, appears responsible for the signaling that triggers the initiation of apoptosis of these immature B cells. Two major regulatory mechanisms have been identified for deletion of autoreactive B cells: physical deletion and functional inactivation of self-reactive B cells, termed clonal deletion and anergy, respectively (reviewed by Nossal, 1994). It has been postulated that clonal deletion plays a major role during normal B cell development (Nossal, 1994). Our findings with the WEHI 231 and CH 33 lines, widely used as models for clonal deletion, suggest that Rel-related factors and c-*myc* play major roles in mediating the signals leading to B cell death, as well as rescue via CD40L.

TGFβ1 Signals Through NF-κB/Rel

TGFβ1 acts as a pleiotropic immunosuppressant factor through inhibition of B and T cell function, including inhibition of secretion of lymphokines and activation of cell proliferation (Massaguè, 1990; Stavnezer, 1996). Further, TGFβ1-deficient mice presented severe dysfunction in immune and inflammatory systems such that massive inflammatory cell infiltration in several organs

and abnormal production of interferon γ, TNFα, and MIP1α were noted (Shull et al., 1992). Among its immunosuppressant activities, TGFβ1 has been implicated in the induction of apoptosis in several B cells (Rotello et al., 1991; Oberhammer et al., 1992). In particular TGFβ1 induced apoptosis of murine and human B cell lymphomas and of normal human B cells (Warner et al., 1992; Chaouchi et al., 1995; Lomo et al., 1995). Our finding that TGFβ1 stimulation leads to a downmodulation of NF-κB/Rel activity suggests that investigation of the possible role of this family of factors in many of the observed immunosuppressant effects of TGFβ1 is now needed. Furthermore, we have observed that phorbol ester–mediated activation of NF-κB/Rel in WEHI 231 cells is blocked by TGFβ (M. A. and G. E. S., unpublished data), raising the possibility of a much wider role for this novel signaling pathway in modulation of cytokine stimulation of multiple cell types. Lastly, these results support an pRb-independent mechanism for the TGFβ1-mediated drop in the rate of c-*myc* gene transcription, since the decline in c-*myc* expression precedes by 7–9 hr the appearance of active hypophosphorylated pRb (Fischer et al., 1994).

The decline in Rel factor binding following TGFβ1 treatment correlated with an increase in IκBα expression. However, given the complexity of the regulation of NF-κB/Rel activity, the possible involvement of other mechanisms leading to the decreased binding of these factors cannot be ruled out. The induction of IκBα by TGFβ1 was completely abrogated by costimulation with the protein synthesis inhibitor CHX (Figure 2B; data not shown), suggesting that IκBα up-regulation by TGFβ1 requires de novo protein synthesis. These findings suggest that activation of IκBα is not a result of a direct effect of TGFβ1. Of potential relevance is the recent finding that TGFβ receptor type I can interact, in vitro, with the immunophilin protein FKBP12 (Wang et al., 1994); FKBP12 is capable of modulating the calcineurin-dependent pathway and therefore NF-κB/Rel expression (Schreiber, 1991). Two groups have recently demonstrated that glucocorticoids (GC) can induce IκBα synthesis and thus interfere with NF-κB/Rel activity (Auphan et al., 1995; Scheinman et al., 1995a, 1995b), suggesting that GC immunosuppressant activity is, in part, attributable to Rel downmodulation. In this case, however, a direct effect of GC on IκBα gene transcription was indicated. Dexamethasone treatment of HeLa cells increased IκBα gene transcription within 15 min, and cotreatment with CHX resulted in the superinduction of IκBα expression. IκBβ expression was found to be unchanged in response to GC treatment of HeLa cells, similar to the effects of TGFβ1 in B cell lymphomas.

NF-κB/Rel and Apoptosis

Recently, we demonstrated that anti-immunoglobulin receptor–mediated apoptosis of WEHI 231 cells is accompanied by down-regulation of NF-κB/Rel activity (Lee et al., 1995). More recently, we have found that treatment with various inhibitors of NF-κB/Rel species such as N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and pyrrolidinethiocarbamate (PDTC) promotes apoptosis in normal murine splenic B lymphocytes and WEHI 231 cells (Wu et al., 1996). Lastly, microinjection of an IκBα–GST protein, and not control GST protein, induced apoptosis of WEHI 231 cells. Taken together, our data strongly suggest that apoptosis of B cells is promoted by down-regulation of NF-κB/Rel; furthermore, they lead to the prediction that a similar mechanism may apply with other agents, e.g., dexamethasone, that similarly reduce Rel factor activity.

NF-κB/Rel species have been implicated in the regulation of B lymphocyte apoptosis by other groups. Transformation of bursal lymphocytes by v-Rel protected these cells from apoptosis induced by follicular dispersion or by exposure to γ radiation (Neiman et al., 1991). Similarly, Gilmore and coworkers demonstrated that chicken splenic lymphocytes, transformed by a temperature-sensitive mutant of v-Rel, undergo apoptosis once they are shifted to the nonpermissive temperature, suggesting that v-Rel expression protected from apoptosis (White et al., 1995). Recently, RelA-deficient mice displayed embryonic lethality within 14 days of gestation, which was accompanied by massive liver degeneration due to apoptosis of hepatocytes (Beg et al., 1995). In this latter case, however, it was not established whether the absence of RelA expression was directly involved in the cellular death. Interestingly, TGFβ1 is also capable of inducing apoptosis in liver cells (Oberhammer et al., 1992).

CD40-Mediated Down-Regulation of IκBα

Surface engagement of CD40, a member of the TNFR superfamily (Smith et al., 1994), overrides the anti-immunoglobulin and TGFβ1-mediated growth arrest of WEHI 231 cells (Santos-Argumedo et al., 1994). Moreover, CD40L costimulation protected mature B and WEHI 231 cells from anti-immunoglobulin–mediated apoptosis (Tsubata et al., 1993). CD40L similarly rescues WEHI 231 from cellular death induced by TGFβ1. This effect correlates with downmodulation of cytosolic expression of IκBα gene. The mechanism by which engagement of CD40 leads to IκBα down-regulation is currently under investigation. Recently, a new class of proteins with a common functional domain, termed TNF receptor–associated factors (TRAF), capable of interacting with members of the TNFR superfamily, has been identified (Rothe et al., 1994). In particular, the cytoplasmic domain of CD40 has been found to be associated with two members of the TRAF family, TRAF2 (Rothe et al., 1995), and CRAF or TRAF 3 (Cheng et al., 1995), responsible for the downstream signaling that results in NF-κB/Rel activation. The results presented here suggest the hypothesis that TRAF species are involved in the signaling by CD40 that results in the down-regulation of IκBα gene expression.

Experimental Procedures

Cell Culture, Treatment, and Transfection Conditions

WEHI 231 and CH33 cells were maintained in Dulbecco's modified Eagles medium (DMEM), supplemented as described previously (Lee et al., 1995). For treatment, cells were incubated for the indicated periods of time with 2 ng/ml TGFβ1 (R and D Systems, Minneapolis, Minnesota or Austral Biological, San Ramon, California) dissolved in 0.1% carrier BSA or 2 ng/ml BSA as control. CD40L, which was prepared as a soluble fusion protein as described previously

(Francis et al., 1995), and anti-CD8 reagent were provided by T. Rothstein (Boston University Medical School, Boston, Massachusetts). Supernatants containing CD40L and anti-CD8 were used at optimal concentrations (1:8 and 1:40, respectively) determined on the basis of proliferative assays (Francis et al., 1995). The WEHI 231 c-Myc and c-Rel stable transfectants were prepared using 40 μg pM21 human c-*myc* (Stone et al., 1987) or 38 μg murine c-*rel* expression vector (La Rosa et al., 1994), respectively, and 2 μg pSV2*neo* DNA. Cells were transfected by electroporation as described (Lee et al., 1995). After 24 hr, 1.2 mg/ml G418 (GIBCO Laboratories, Gaithersburg, Maryland) was added to the medium and selective growth conditions maintained for approximately 2 weeks. Clones were isolated by limiting dilution. For analysis of DNA laddering, approximately 10^6 cells were used according to the procedure of Smith et al. (1989).

RNA Isolation and Analysis

Total cellular RNA was isolated by the guanidinium method and samples (20 μg) subjected to Northern blot analysis, as described elsewhere (Lee et al., 1995). Probes used include the mouse c-*myc* cDNA clone pM-c-*myc*54 (Stanton et al., 1983), the murine Max cDNA clone pTAwtMax (Arsura et al., 1995), the human IκBα clone pMT2T-IκBα (Brown et al., 1995), and a GAPDH clone (Dugaiczyk et al., 1983). Quantitation by scanning densitometry was performed using a Molecular Dynamics 300A computing densitometer.

Run-On Analysis

Nuclei isolation and transcription analysis was performed as described by Greenberg and Ziff (1984). Double-stranded DNA was used for the following probes: human IκBα, pMT2T-IκBα (Brown et al., 1995); murine Max pMT2T-Max (Arsura et al., 1995); human MAD, pUHD10-3-Mad (provided by R. dePinho, Albert Einstein Medical School, New York); rat β-actin (Bond and Farmer, 1983).

EMSA

The 221 bp fragment of the murine c-*myc* gene, spanning BglII to AccI sites, including base pairs −1139 to −921 relative to the P1 promoter, which contains κB URE, was labeled and used in EMSA, as previously described (Duyao et al., 1990). The 93 bp fragment containing the IRE from the murine c-*myc* gene, which spans the region from +418 to +505 bp relative to the P1 promoter (Kessler et al., 1992b) was amplified via polymerase chain reaction and EMSA performed as described previously (Schauer et al., 1996). The binding reaction for AP-1 was performed with a double-stranded oligonucleotide with the following sequence: 5′-GATCGCCATGTGACTC ATTAC-3′ (Wu et al., 1996).

Western Blots

For isolation of cytoplasmic proteins, washed cells were resuspended in cold 10 mM Tris (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, and 10 μg/ml leupeptin. After incubation on ice for 10 min, cells were lysed by addition of Triton X-100 to 0.5%, and nuclei removed by centrifugation. For detection of nuclear proteins, washed cells were resuspended in cold 10 mM Tris (pH 7.6), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 and incubated 5 min on ice. The nuclei were resuspended in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% Na lauryl sarcosine, 1 mM EDTA, 10 mg/ml leupeptin, 0.2 mM PMSF, and 0.2 M DTT and DNA was sheared and removed, as described previously (Arsura et al., 1995). Samples of nuclear and cytoplasmic protein extracts (40 μg) were subjected to electrophoresis on a 10% polyacrylamide–SDS gel, transferred to PVDF membrane (Millipore, Bedford, Massachusetts) and Western blotting performed, as described (Arsura et al., 1995). To detect IκB proteins, antibodies specific for IκBα (provided by U. Siebenlist, National Institutes of Health, Bethesda, Maryland), or IκBβ (SC 371, Santa Cruz Biotechnology, Santa Cruz, California) were employed. An affinity-purified murine c-Myc antibody (provided by S. Hann, Vanderbilt University, Nashville, Tennessee), v-Rel (8541) antibody (provided by Nancy Rice, National Cancer Institute, Frederick, Maryland) or Rel A (SC109) antibody (Santa Cruz Biotechnology) was used.

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