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Constitutive RelB Activation in v-Src-Transformed Fibroblasts: Requirement for IκB Degradation

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Abstract RelB, an NF-κB/Rel-related transacting factor, was initially identified as an immediate-early gene product in fibroblasts and subsequently shown to exhibit constitutive DNA binding activity in lymphoid cells. The data presented in this report show that RelB is also constitutively active, as monitored by electrophoretic mobility shift assay, in the v-Src-transformed fibroblast cell line, SR1. By contrast, nontransformed parental (3Y1) cells displayed inducible NF-κB activity; RelB activity was also observed, although to a lesser extent, in two additional v-Src-transformed fibroblast lines. RelB activation in SR1 cells did not require an increase in RelB expression or result from a decrease in the levels of IκBα or p105, proteins previously shown to bind to and inhibit the activity of the Rel proteins. Numerous studies have shown that stimulus-dependent Rel activation requires degradation of IκBα, p105 or other member of the IκB family, and that this process is precluded by agents that inhibit proteasome activity. We show that treatment of SR1 cells with proteasome inhibitors abolishes RelB activity and thus suggest that RelB in these cells is associated with IκB and that v-Src transformation activates RelB by accelerating IκB proteolysis. Additional data show that serum and tumor necrosis factor-α (TNF-α) increase RelB protein levels in 3Y1 cells and that this process is blocked by proteasome inhibitors. J. Cell. Biochem. 73:237–247, 1999.

Key words: IκB; NF-κB; Rel, κB

The Rel proteins are ubiquitous stimulus-dependent transcriptional regulators that interact with related decameric DNA motifs termed κB sites [Siebenlist et al., 1994]. Rel target genes are numerous and diverse and encode proteins that participate in inflammatory, proliferative, oncogenic, and apoptotic responses [Siebenlist et al., 1994; Baeuerle and Henkel, 1994; Beg and Baltimore, 1996; Wang et al., 1996; Van Antwerp et al., 1996; Liu et al., 1996]. Members of the Rel family include c-Rel, v-Rel, RelB, p65, p50, and p52; p65 and p50 comprise the prototypic Rel complex termed NF-κB. Each Rel protein contains a 300-amino acid "Rel homology domain" that mediates DNA binding, nuclear translocation, and dimerization. The Rel proteins contact κB sites as dimers and, at least in vitro, all combinations are possible with the exception of those involving RelB, which are limited to RelB/p50 and RelB/p52 [Dobrzanski et al., 1994]. c-Rel, p65, and RelB contain potent *trans*activation domains in their C-termini [Bull et al., 1990; Schmitz et al., 1991; Dobrzanski et al., 1993], whereas p50 and p52, which are synthesized as ankyrin-containing precursors (p105 and p100, respectively), lack this domain and, as homodimers, may repress transcription [Schmitz et al., 1991; Franzoso et al., 1992; Kang et al., 1992].

In the absence of stimulus, the Rel proteins are present in the cytosol complexed to an inhibitory protein termed IκB [Baeuerle and Baltimore, 1988; Beg and Baldwin, 1993]. When bound to IκB, the Rel proteins cannot translocate to the nucleus or interact with DNA (i.e., are inactive). Several forms of IκB have been identified: (1) the prototypic and ubiquitous IκBα and IκBβ [Zabel and Baeuerle, 1990; Haskill et al., 1991; Thompson et al., 1995], (2) the recently discovered IκBε [Whiteside et al., 1997; Li and Nabel, 1997; Simeonidis et al., 1997], (3) the oncoprotein BCL-3 [Ohno et al.,

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1990], (4) the unprocessed forms of p50 and p52—p105 and p100, respectively [Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990], and (5) the C-terminal halves of p105 and p100—IκBγ and the putative IκBδ, respectively [Inoue et al., 1992a; Liou et al., 1992]. Each IκB contains multiple ankyrin repeats, which are required for IκB/Rel interaction [Inoue et al., 1992b; Hatada et al., 1992], and each preferentially recognizes different Rel complexes. IκBα and IκBβ, for example, interact primarily with p65- and c-Rel-containing complexes [Baeuerle and Baltimore, 1989; Beg et al., 1992; Beg and Baldwin, 1993; Thompson et al., 1995; Dobrzanski et al., 1994], whereas BCL-3 specifically recognizes p50 and p52 homodimers [Nolan et al., 1993]. Additional studies indicate that different IκBs modulate different types of κB responses; IκBα, for example, is involved in the rapid and transient activation of Rel proteins, whereas IκBβ mediates delayed but persistent Rel activation [Thompson et al., 1995; Johnson et al., 1996].

Studies initially done on IκBα [Beg et al., 1993; Henkel et al., 1993], and subsequently on other IκBs [Thompson et al., 1995; Whiteside et al., 1997], demonstrate that Rel activation requires IκB degradation. In response to stimulus, IκB is phosphorylated at specific serine residues and is consequently ubiquitinated and targeted to proteasomes [Brown et al., 1995; Chen et al., 1995, 1996; Traenckner et al., 1995; McKinsey et al., 1996; Whiteside et al., 1997]. Agents that induce these events, and that thus allow Rel complexes to enter the nucleus, bind κB sites and initiate transcription (i.e., that activate Rel complexes), include cytokines, growth factors, viruses, and stress-inducing factors, such as ultraviolet (UV) light, oxidants, and ionizing radiation [Siebenlist et al., 1994]. Two IκB kinases, designated IKKα and IKKβ, have been identified recently; in response to cytokines (e.g., tumor necrosis factor-α [TNF-α]), these kinases phosphorylate IκBα and IκBβ at sites that trigger their degradation [Didonato et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Mercurio et al., 1997]. Whether other κB-activating agents act via these kinases, or whether additional IκB kinases, perhaps with different substrate specificities, exist is unknown. As noted above, p105 and p100 are IκBs; in response to stimulus, the C-terminal halves of these molecules are selectively degraded, and consequently, active Rel/p50 and Rel/p52 complexes are released [Fan and Maniatis, 1991; Rice et al., 1992; Mercurio et al., 1993; Palombella et al., 1994]. In a limited number of systems (e.g., B cells, neurons, and thymocytes [Sen and Baltimore, 1986; Kaltschmidt et al., 1994; Sen et al., 1995], the Rel proteins escape IκB inhibition and become constitutively active.

NF-κB is the predominant inducible κB DNA binding activity in most cell types, whereas RelB (with p50 or p52) represents the major constitutive κB activity of terminally differentiated B cells, splenic B cells, and thymocytes [Lernbacher et al., 1993; Liou et al., 1994; Weih et al., 1994; Dobrzanski et al., 1994]. Consistent with this latter observation, gene knockout studies have shown that RelB is essential for hematopoietic function [Weih et al., 1995]. Although participation of RelB in non lymphoid function is suggested by the finding that RelB is an immediate-early gene product in serum-stimulated fibroblasts [Ryseck et al., 1992], it is noted that RelB is not appreciably active in fibroblasts, even in the presence of serum or other stimulus [Lernbacher et al., 1993; Olashaw, 1996]. RelB activation may therefore be a lymphoid-specific phenomenon or, in nonlymphoid cells, may occur only in limited circumstances. We have identified a v-Src-transformed fibroblast cell line (termed SR1) in which RelB is highly active in the absence of exogenously added stimulus, and thus demonstrate that constitutive RelB activity is not restricted to lymphoid cells. Our studies address the mechanism leading to RelB activation in SR1 cells, and suggest that v-Src transformation activates RelB by promoting IκB degradation.

MATERIALS AND METHODS

Cell Culture

NIH-3T3 and Balb/c-3T3 mouse fibroblasts, 3Y1 rat fibroblasts, and their transformed counterparts have been described previously [Yu et al., 1993]. Cells were grown in Dulbecco-Vogt modified Eagle's medium supplemented with 10% calf serum, 4 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin in humidified 5% CO_2/95% air.

Electrophoretic Mobility Shift Assay (EMSA)

Cells in 100-mm plates were rinsed with, and scraped into, phosphate-buffered saline (PBS) and lysed in buffer A [Dignam et al., 1983]

containing 1% Nonidet P-40 (NP-40). Nuclear pellets were incubated in a small volume of buffer C [Dignam et al., 1983] with 0.45 M NaCl, and the extracted material was diluted to give concentrations of the buffer ingredients as specified for buffer D [Dignam et al., 1983]. Nuclear extracts were normalized for protein and incubated at 4°C with antibody for 1–4 h or glutathione-S-transferase (GST)-IκBα [Kowalik et al., 1993] for 15 min in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1.8 μg/ml salmon sperm DNA, 0.2% NP-40, and 20 μg/ml bovine serum albumin (BSA). Double-stranded radiolabeled oligonucleotide (1 ng/reaction) containing the κB sequence of the mouse κ light chain gene [Lenardo et al., 1989] was added, and the mixture was incubated for 20 min at room temperature. This κB sequence recognizes NF-κB and RelB with comparable affinity [Ryseck et al., 1992]. Samples were electrophoresed on 6% polyacrylamide gels containing 25 mM Tris, 22 mM borate, and 0.25 mM EDTA. After electrophoresis, the gels were dried and exposed to film overnight. In some experiments, nuclear extracts were prepared essentially as described by Sartor et al. [1997]; similar results were obtained using this method and the method outlined above.

Immunoblotting

Cells were lysed in buffer A [Dignam et al., 1983], and nuclear material was removed by centrifugation. Normalized cytosolic extracts (100 μg) were resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred from the gel to nitrocellulose. The nitrocellulose was blocked in PBS supplemented with 5% instant milk and 1% Tween-20, and incubated with antibody for 1 h at room temperature. Proteins recognized by the antibody were detected by enhanced chemiluminescence using a horseradish peroxidase (HRP)-coupled secondary antibody as specified by the manufacturer (Amersham).

Materials

Antibodies were purchased from Santa Cruz Biotechnology. N-tosyl-L-phenylalanine (TPCK) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were obtained from Sigma Chemical Co. (St. Louis, MO), and calpain inhibitor 1 was from Calbiochem (San Diego, CA). Human recombinant TNF-α was purchased from Promega (Madison, WI).

RESULTS

Constitutive RelB DNA Binding Activity in SR1 Cells

κB DNA binding activity was assessed in parental (3Y1) and v-Src-transformed (SR1) rat fibroblasts by EMSA. Confluent cultures were prestarved for two days in medium containing 0.1% serum; at this time, most cells are in G_1 [Yu et al., 1993]. Cells then received TNF-α (20 ng/ml) for 30 min or no addition. Nuclear extracts were prepared, normalized for protein, and incubated with antibody to p65, RelB, p50, or p52, and subsequently with a radiolabeled double-stranded oligonucleotide probe containing the κB site of the κ light chain gene. As shown in Figure 1, κB binding activity in untreated 3Y1 cells was not detectable (lanes 1 and 11). Addition of TNF-α to these cells stimulated the formation of a complex (lanes 2 and 12) that was supershifted by antibody to p65 (lane 3) and p50 (lane 13) but was essentially unaffected by antibody to RelB (lane 4) or p52 (lane 14), and thus is NF-κB (referred to in text as p65). Untreated SR1 cells (lanes 5 and 15), on the other hand, expressed an activity that was blocked by RelB antibody (lane 7), supershifted by p50 antibody (lane 16), and unaffected by p65 (lane 6) or p52 (lane 17) antibody. Thus, in SR1 cells, a complex consisting of RelB and p50 (referred to in text as RelB) is active in the absence of exogenous stimulus. Addition of TNF-α to SR1 cells did not appreciably activate RelB (cf. lanes 6 and 9), and a small increase in p65 activity was observed (lane 10). 3Y1 cells, therefore, exhibit predominantly TNF-α-inducible p65 activity, whereas SR1 cells display constitutive RelB activity.

κB DNA binding activity was also determined in two additional v-Src-transformed cell lines, Balb/Src and NIH/Src, and their nontransformed counterparts, Balb/c-3T3 and NIH-3T3, respectively. Balb/c-3T3 and NIH-3T3 cells displayed an activity that was stimulated by TNF-α (Fig. 2A, cf. lanes 1 and 2, and 8 and 9), supershifted by p65 antibody (lanes 3 and 10), and essentially unaffected by RelB antibody (lanes 4 and 11). Thus, like 3Y1 cells, these cell lines exhibit TNF-α-inducible p65 activity. Similar to SR1 cells, κB DNA binding activity was apparent in Balb/Src and NIH/Src cells in the absence of stimulus (cf. lanes 1 and 5, and 8 and

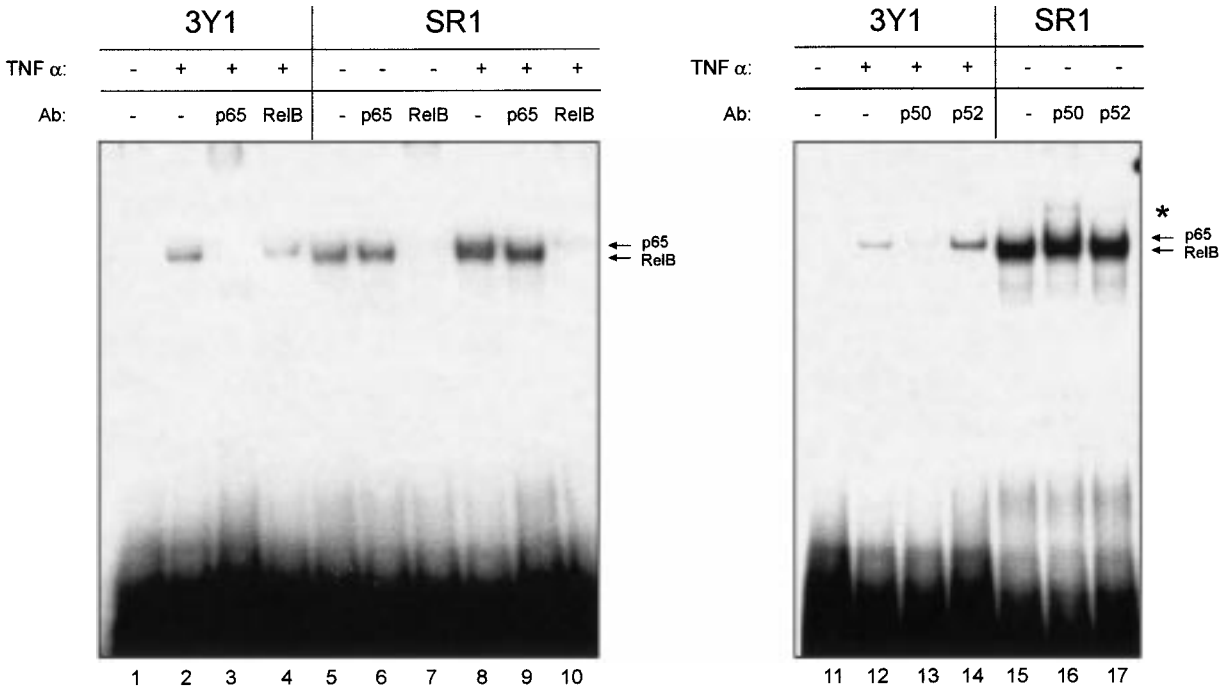

Fig. 1. Constitutive RelB/p50 DNA binding activity in SR1 cells. Confluent 3Y1 and SR1 cultures were prestarved for 48 h in medium containing 0.1% serum. Cells then received either no addition or 20 ng/ml tumor necrosis factor-α (TNF-α) for 30 min. Nuclear extracts were prepared, and equal amounts of protein were incubated antibody (Ab) to p65, RelB **(A)**, p50 or p52 **(B)**, or 2 h at 4°C, and subsequently, with a radiolabeled oligonucleotide probe containing a κB site. Reaction mixtures were electrophoresed on nondenaturing polyacrylamide gels. Specificity of the antibodies was verified by peptide blocking experiments and by monitoring their effects on Rel proteins overexpressed in COS cells. *, p50 supershift. A p50 supershift is apparent in lane 13 in autoradiograms exposed for longer periods of time (data not shown). The inability of p50 antibody to completely supershift this complex presumably reflects the low affinity of p50 antibodies for p50 heterodimers [Kieran et al., 1990; Lernbacher et al., 1993]. The difference in intensity of the bandshift in lane 2 versus lane 4 was not routinely seen.

12). In contrast to SR1 cells, however, the constitutive activity in these transformed mouse cell lines consisted of both RelB- and p65-containing complexes: RelB antibodies abolished the lower portion of this activity (lanes 7 and 14); whereas p65 antibodies supershifted/blocked the upper portion (lanes 6 and 13); thus, the faster migrating complex seen in lanes 6 and 13 represents RelB, and the slower migrating complex observed in lanes 7 and 14 represents p65. In Balb/Src cells, p65 (lane 7) and RelB (lane 6) were activated to comparable extents, whereas in NIH/Src cells, p65 was the major constitutive activity (cf. lanes 13 and 14). TNF-α significantly increased p65 activity in Balb/Src (Fig. 2B, cf. lanes 3 and 6) and NIH/Src (data not shown) cells but had no effect on RelB activity in either of these lines (cf. lanes 2 and 5, and data not shown). It is unclear why different complexes are activated in different cell lines. However, because SR1 cells express high levels of RelB activity in the absence of p65 activity, they were chosen for further studies addressing the mechanism of RelB activation. Activation of NF-κB in T-cell hybridomas expressing v-Src has also been reported [Eicher et al., 1994].

RelB DNA Binding Activity in SR1 Cells Does Not Require an Increase in the Amount of RelB Protein

In lymphoid cells, high RelB activity is associated with high RelB expression [Lernbacher et al., 1994]. To determine whether a similar correlation applies to fibroblasts, levels of RelB protein and activity were compared in 3Y1 cells and SR1 cells in different culture conditions. Cultures were either sparse or dense and in medium containing 10% or, for 48 h, 0.1% serum. Cytosolic extracts were prepared, normalized for protein and immunoblotted with antibody to RelB (Fig. 3A) or, for comparative purposes, p65 (Fig. 3B); nuclear extracts were analyzed by EMSA (Fig. 3C). Irrespective of cell density, in the presence of 10% serum, RelB protein levels were similar in SR1 cells, which exhibited RelB activity, and 3Y1 cells, which

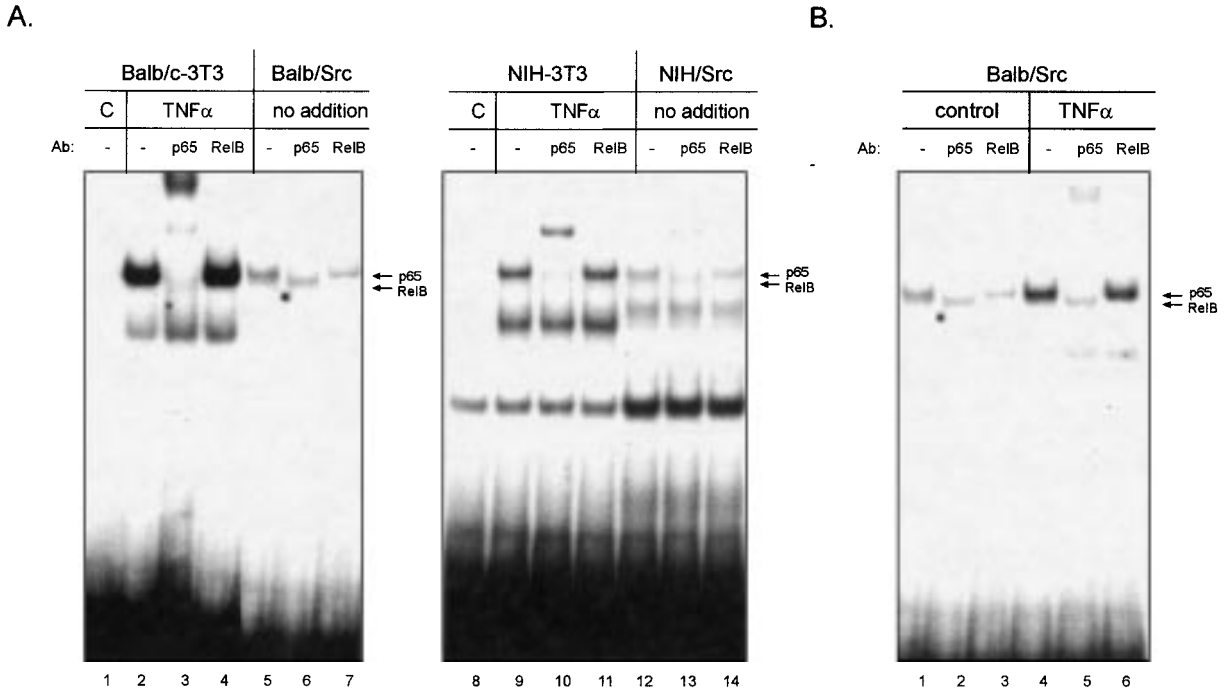

Fig. 2. Constitutive κB DNA binding in Balb/Src and NIH/Src cells. **A:** Confluent Balb/c-3T3, NIH-3T3, Balb/Src, and NIH/Src cells were prestarved for 48 h in medium containing 0.1% serum. Parental cells received no addition (C) or 20 ng/ml TNF-α for 30 min. **B:** Prestarved Balb/Src cells were treated with or without 20 ng/ml TNF-α for 30 min. A,B: Nuclear extracts were prepared, incubated with antibody (Ab) to p65 or RelB, and analyzed for κB DNA binding activity by electrophoretic mobility shift assay (EMSA).

did not (Fig. 3A,C, cf. lanes 1 and 3, and 5 and 7). RelB activity in SR1 cells therefore does not require increased RelB expression. It is noted that we [Olashaw, 1996] and others [Baldwin et al., 1991; Duckett et al., 1995] have shown previously that although 10% serum stimulates κB activity when added to quiescent cultures, growing cells in medium containing 10% serum have little or no detectable κB activity; this presumably reflects depletion of the stimulating factor from the serum. Interestingly, the data presented in Figure 3 also show that serum starvation elevated the amount of RelB protein in SR1 cells but not in 3Y1 cells (Fig. 3A, lanes 4 and 8); the reason for this is unknown. Increases in RelB protein were accompanied by small increases in RelB activity (Fig. 3C, lanes 4 and 8), suggesting that enhancement of RelB expression, while not required, may govern the extent of RelB activation in SR1 cells. Unlike RelB, levels of p65 were unaffected by serum concentration and were comparable in 3Y1 and SR1 cells (Fig. 3B). This finding is in agreement with previous studies showing that p65 is constitutively expressed [Ueberla et al., 1993].

Increased Levels of IκBα and p105 in SR1 Cells

To address the possibility that decreases in the amount of IκB mediate RelB activation in SR1 cells, the levels of two IκBs, IκBα, and p105 were examined in 3Y1 cells and in SR1 cells in different culture conditions. These IκBs were chosen, as (1) IκBα has been shown previously to interact with RelB, albeit with low affinity [Dobrzanski et al., 1994]; and (2) p105 is the precursor of p50, the RelB binding partner in these cells (Fig. 1). Thus, IκBα and p105 are potential modulators of RelB activity in 3Y1 cells, and loss of these inhibitors due to decreased synthesis (IκBα) or increased processing (p105) could deregulate RelB activity in SR1 cells. As in the experiment described in Figure 3, cells were either dense or sparse and in medium containing either 10% or 0.1% serum. Immunoblot analysis of normalized cytosolic extracts showed that, regardless of culture condition, the relative amounts of IκBα and p105 were higher—not lower—in SR1 as compared to 3Y1 cells (Fig. 4A,B). This finding is similar to previous studies demonstrating increased levels of IκBα mRNA in mature B cells

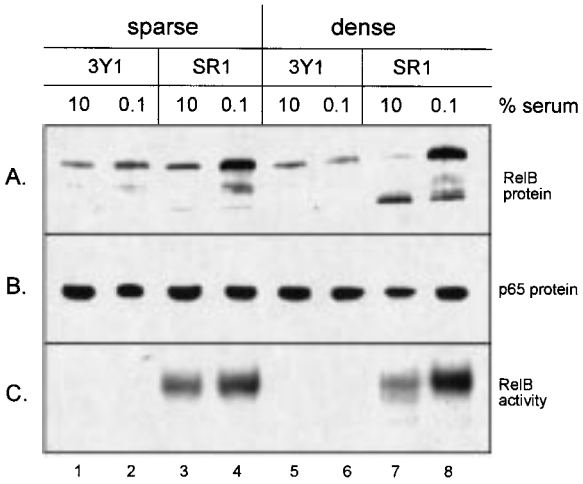

Fig. 3. Comparison of RelB protein levels in 3Y1 and SR1 cells. Sparse and dense 3Y1 and SR1 cultures in medium containing 10% serum were either starved in medium containing 0.1% serum for 48 h or left untouched. Cytosolic extracts were normalized for protein and immunoblotted with antibody to RelB **(A)** or p65 **(B)** as described in Materials and Methods. κB DNA binding activity was determined in nuclear extracts by electrophoretic mobility shift assay (EMSA). **C:** It is noted that only a small percentage of total RelB (<10%; N. Olashaw, unpublished data) is active (i.e., nuclear) in SR1 cells; thus, measurement of cytosolic RelB approximates the amount of total RelB. A: The top band is RelB, and the lower two bands are RelB degradation products.

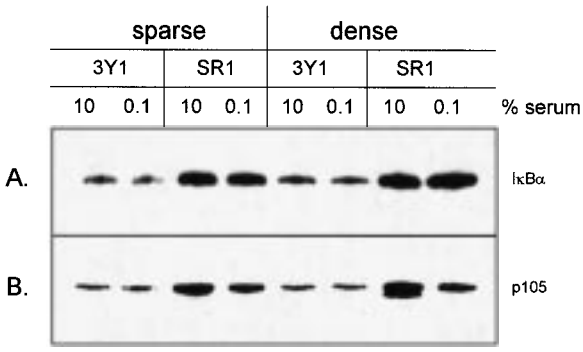

Fig. 4. Comparison of IκBα and p105 protein levels in 3Y1 and SR1 cells. Sparse and dense 3Y1 and SR1 cultures in medium containing 10% serum were either starved in medium containing 0.1% serum for 48 h or left untouched. Cytosolic extracts were normalized for protein and immunoblotted with antibody to IκBα **(A)** or p105 **(B)**, as described in Materials and Methods.

(which exhibit constitutive c-Rel activity), as compared with pre-B cells [Miyamoto et al., 1994] and, given the presence of κB sites in the IκBα and p105 promoters [Cogswell et al., 1993; LeBail et al., 1993], not unexpected. The enhanced expression of IκBα and p105 in SR1 cells suggests that RelB in SR1 cells is functional, i.e., that it stimulates transcription from κB sites. IκBα expression was also increased in COS cells transiently transfected with RelB [Dobrzanski et al., 1993], although not in thymus extracts of transgenic mice that overexpressed RelB [Weih et al., 1996].

RelB DNA Binding Activity in SR1 Cells Requires IκB Degradation

Although levels of IκBα and p105 are increased in SR1 cells, is is possible that v-Src transformation abrogates the ability of RelB to interact with these or other IκBs or, conversely, that the IκB responsible for regulating RelB activity in SR1 cells no longer functions in this respect. To determine whether RelB activity is subject to, or independent of, IκB control, SR1 cells were treated for various times with calpain inhibitor 1 (see Fig. 6A–C) or TPCK (see Fig. 6D). Numerous studies have shown that these agents, by virtue of their capacity to inhibit proteasome activity, block IκB degradation and, consequently, κB activation [Henkel et al., 1993; Mellits et al., 1993; Finco et al., 1994; Palombella et al., 1994; Alkalay et al., 1995; Didonato et al., 1995; Lin et al., 1995; Thompson et al., 1995; Whiteside et al., 1997]; inhibition of IκBα phosphorylation by TPCK has also been observed [Finco et al., 1994; Alkalay et al., 1995]. As shown in Figure 5A, exposure of SR1 cells to calpain inhibitor 1 for 8 h virtually abolished RelB DNA binding activity. The loss of RelB activity was not due to a loss of RelB protein; as presented in Figure 5B, levels of RelB protein remained constant throughout the experimental period. Previous studies have shown that the transcription factor STAT3, which modulates transcription via interaction with the Sis-inducible-element (SIE), is constitutively active in v-Src-transformed fibroblasts [Yu et al., 1995; Cao et al., 1996]. In contrast to RelB binding activity, SIE binding activity was unaffected by calpain inhibitor 1, thus attesting to the specificity of the response (Fig. 5C). TPCK also ablated RelB activity in SR1 cells, as well as TNF-α-stimulated p65 activity in 3Y1 cells (Fig. 5D). These findings demonstrate that RelB activity in SR1 cells is susceptible to inhibition by IκB, and suggest that v-Src transformation activates RelB by accelerating IκB proteolysis.

RelB Expression in 3Y1 Cells Correlates With NF-κB Activation

Previous studies have shown that RelB mRNA expression is induced by serum in quies-

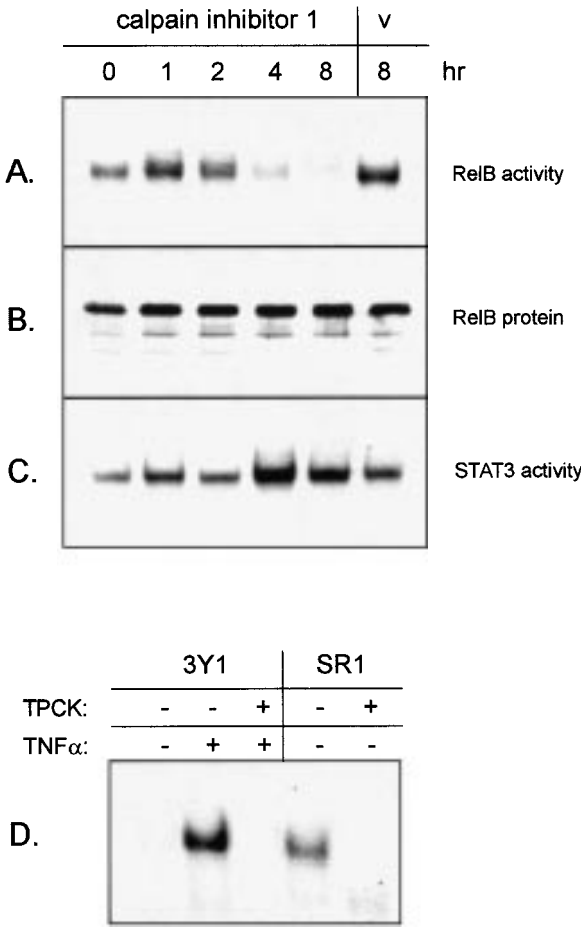

Fig. 5. Inhibition of RelB DNA binding activity in SR1 cells by proteosome inhibitors. SR1 cells were exposed to 200 mM calpain inhibitor 1 for the indicated times or to ethanol (v) as a vehicle control for 8 h (A–C). The DNA binding activities of RelB **(A)** and STAT3 **(C)** were determined by EMSA using a κB probe and an SIE probe, respectively. RelB protein levels **(B)** were determined in cytosolic extracts by immunoblotting. **D:** 3Y1 cells were treated with 28 μM TPCK for 3 h, and subsequently with 20 ng/ml TNF-α for 45 min. SR1 cells received TPCK for the indicated times. κB DNA binding activity was determined by electrophoretic mobility shift assay (EMSA).

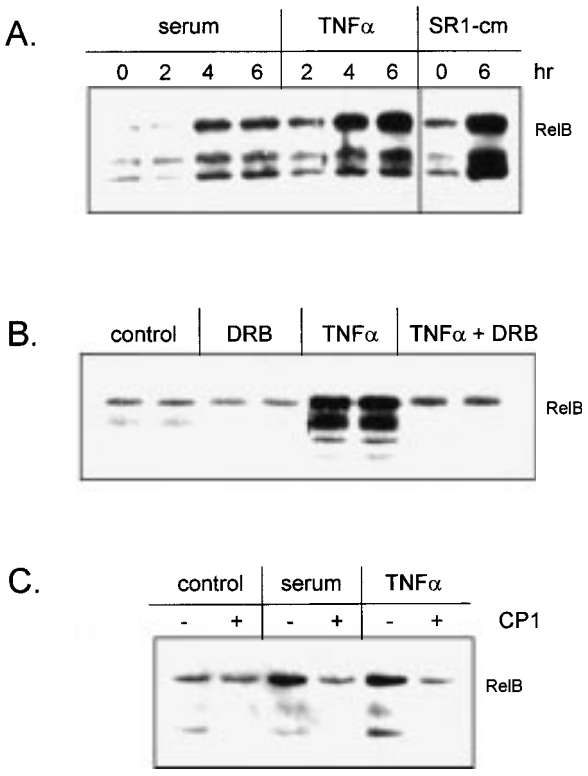

Fig. 6. Effects of DRB and calpain inihibitor 1 on inducible RelB expression in 3Y1 cells by DRB and calpain inhibitor 1. All procedures were done on density-arrested serum-starved 3Y1 cells. **A:** 3Y1 cells received 10% serum, 20 ng/ml TNF-α or SR1-conditioned medium (SR1-cm) for the indicated times. **B:** 3Y1 cells were either untreated (control), or treated with 20 ng/ml TNF-α, 100 μM DRB or both for 6 h. **C:** Density-arrested 3Y1 cells were pretreated with 200 μM calpain inhibitor 1 (Cp1) for 4 h and subsequently with 10% serum or 20 ng/ml TNF-α for 6 h. Normalized cytosolic extracts were immunoblotted with RelB antibody. SR1 conditioned medium was prepared by incubating confluent SR1 cultures overnight in medium containing 0.1% serum. Conditioned medium was centrifuged or filtered to remove floating cells.

cent serum-starved NIH-3T3 cells in a protein synthesis-independent manner [Ryseck et al., 1992]. As shown in Figure 6A, serum (10%) also increased levels of RelB protein when added to quiescent serum-starved 3Y1 cells, as did TNF-α and SR1-conditioned medium. DRB, an inhibitor of mRNA synthesis, blocked the TNF-α-induced increase in RelB protein levels (Fig. 6B), indicating that RelB expression in 3Y1 cells is modulated at the level of transcription or by mRNA stability. In NIH-3T3 cells, the pattern of RelB expression in response to serum and cycloheximide mimicked that of p105, a known κB gene product [Ryseck et al., 1992; Cogswell et al., 1993], and our data show that RelB protein levels are increased by agents—serum [Baldwin et al., 1991; Olashaw et al., 1992], TNF-α (Figs. 1 and 2) and SR1-conditioned medium (data not shown)—that activate NF-κB in fibroblasts. To determine whether RelB expression required κB activity, as suggested by these observations, we incubated 3Y1 cells with serum or TNF-α in the presence or absence of calpain inhibitor 1. As shown in Figure 6C, this inhibitor ablated the increase in RelB protein levels induced by these agents. Although effects of calpain inhibitor 1 on proteins other than IκB cannot be excluded, this

finding is consistent with the premise that RelB expression is κB dependent.

DISCUSSION

Our data demonstrate constitutive RelB DNA binding activity in the v-Src-transformed fibroblast cell line, SR1 (Fig. 1). RelB activity was also apparent, albeit at lower levels, in two additional v-Src-transformed fibroblast lines, Balb/Src and NIH/Src (Fig. 2). This is the first demonstration of constitutive RelB activity in nonlymphoid cells, and our studies address the mechanism of activation of RelB in SR1 cells. RelB is expressed at higher levels (aproximately 20-fold) in lymphoid, as compared with nonlymphoid organs [Weih et al., 1996; Lernbacher et al., 1994], and high RelB expression is thought to account, in part, for RelB activation in lymphocyte populations [Lernbacher et al., 1994]. Although increases in RelB expression may contribute to RelB activity, our data show that RelB activation in SR1 cells does not require an increase in the amount of RelB protein. In medium containing 10% serum, SR1 cells, which displayed high levels of RelB activity, had similar amounts of RelB protein as did parental 3Y1 cells, which had no detectable RelB activity (Fig. 3). We have also found that TNF-α and SR1-conditioned medium increase RelB protein levels but do not activate RelB in 3Y1 cells (Figs. 1 and 6; data not shown). Thus, up-regulation of RelB expression per se is not sufficient for RelB activation.

Constitutive RelB activity in lymphoid cells is also thought to result from inefficient interaction of RelB with IκBα [Dobrzanski et al., 1994; Weih et al., 1995; Ferreira et al., 1998]. RelB/p50 and, in particular, RelB/p52 complexes, both of which are present in lymphocytes, have a lower affinity for IκBα than does NF-κB [Dobrzanski et al., 1994] and, as proposed by Lernbacher et al. [1994], post-translational modifications of RelB may further abrogate its capacity to bind this inhibitor. In accord with these findings, Ferreira et al. [1998] found that overexpression of a nondegradable form of IκBα inhibited NF-κB but not RelB/p50 activity in thymocytes. Additional studies on lymphocytes indicate that the capacity of RelB to associate with IκBγ, BCL-3, and IκBβ is also limited [Dobrzanski et al., 1994; Kistler et al., 1998]. Thus, constitutive RelB activity in lymphoid cells presumably reflects the lack of IκB-mediated RelB retention in the cytosol. It is noted that IκBδ has been shown to bind RelB when these proteins are overexpressed in COS cells [Dobrzanski et al., 1995]; whether IκBδ exists in vivo, however, is unclear. Our studies show that RelB activity in SR1 cells is abolished by agents (calpain inhibitor 1, TPCK) that inhibit IκB degradation (Fig. 5) and thus indicate that RelB in SR1 cells is associated with and susceptible to inhibition by IκB. Constitutive RelB activity in fibroblasts, therefore, apparently requires continued degradation of IκB, a process perhaps initiated by v-Src. In support of v-Src involvement, previous studies have demonstrated increased κB-mediated transcription in cells transiently transfected with this oncoprotein [Qureshi et al., 1992; Eicher et al., 1994].

We do not know which IκB interacts with and modulates the activity of RelB in SR1 cells. Although RelB binds to, and is inactivated by, IκBα in vitro (data not shown), RelB activity in vivo most likely is not controlled by IκBα. Our data show that levels of IκBα are higher in SR1, as compared with 3Y1 cells (Fig. 4) and thus are inconsistent with accelerated turnover of this inhibitor in SR1 cells. In addition, the loss of RelB activity observed in cells treated with calpain inhibitor 1 was not accompanied by an increase in the amount of IκBα (Fig. 5, data not shown). Increases in the amount of IκBα may, however, account for the lack of TNF-α-inducible p65 activity in SR1 cells (Fig. 1). It is also unlikely that RelB associates with IκBα in 3Y1 cells. TNF-α induces IκBα degradation but does not appreciably activate RelB in these cells (Fig. 1 and data not shown), despite that fact the levels of RelB are not limiting (Fig. 3) and in fact are up-regulated by TNF-α (Fig. 6). Our data also do not support an involvement of p105 or p100 in RelB activation in SR1 cells. Levels of p105, like those of IκBα, were higher in SR1 versus 3Y1 cells (Fig. 4), and RelB/p52 complexes, which would result from processing of RelB/p100 complexes, were not detected in SR1 cells. With regard to the remaining IκBs, it is noted that IκBβ does not associate with RelB either in vitro or in pre-B cells [Kistler et al., 1998] and that neither BCL-3 nor IκBγ affected RelB transcriptional activity when overexpressed in Jurkat cells [Dobrzanski et al., 1994]. Whether IκBε interacts with RelB is unknown; however, data showing increased IκBε expression in TNF-α-treated fibroblasts [Simeonidis et al., 1997] are consistent with a role of this

inhibitor in modulating RelB and NF-κB activity in this cell type.

Although NF-κB is capable of inducing the expression of IκBα and p105 [Cogswell et al., 1993; LeBail et al., 1993; Cheng et al., 1994, the role of RelB in these processes is unclear. Increased amounts of IκBα were observed in COS cells transiently transfected with RelB [Dobrzanski et al., 1994], whereas neither IκBα nor p105 were up-regulated in thymus extracts of transgenic mice that overexpressed RelB [Weih et al., 1996]. In addition, Cheng et al. [1994] reported that RelB, in contrast to p65, was unable to induce transcription of a reporter gene via κB sites in the IκBα promoter when co-transfected with p50 into endothelial cells. On the other hand, Ferreira et al. [1998] found that IκBα was efficiently expressed in thymocytes in the absence of p65 activity, and suggest that RelB stimulates IκBα expression from sites in the IκBα promoter that are distinct from prototypical κB elements.

Previous studies have shown that the addition of serum to quiescent, serum-starved NIH-3T3 fibroblasts induces the expression of RelB [Ryseck et al., 1992]. As shown here, serum, as well as TNF-α and SR1-conditioned medium, also increased RelB protein levels in quiescent, serum-starved 3Y1 cells (Fig. 6). RelB expression in response to serum and TNF-α was ablated by calpain inhibitor 1 (Fig. 6) and, thus, presumably is κB dependent. Despite its increased expression, RelB in 3Y1 cells was not appreciably activated, at least not during a 6-h exposure to the inducing agent (data not shown). Whether RelB is activated at a later point in the cell cycle, or perhaps in response to a different agent, is unknown. Also, as RelB is constitutively active in SR1 cells, it is surprising that RelB protein levels were similar in SR1 cells and 3Y1 cells in medium containing 10% serum (Fig. 3). This finding suggests that κB activation may be necessary but insufficient for RelB expression.

As constitutive RelB activity in fibroblasts has not been observed previously, SR1 cells provide a unique opportunity to evaluate the role of RelB in fibroblasts and in transformation. Involvement of the Rel proteins in transformation is supported by numerous studies. For example, NF-κB DNA binding activity is increased in metastatic rat mammary cells [Nakshatri et al., 1997], and elevated κB transcriptional activity has been observed in cells transformed by activated Ras and Bcr-Abl [Finco et al., 1997; Reuther et al., 1998]. Our data showing constitutive RelB, and also NF-κB, activity in v-Src-transformed fibroblasts further support a role of κB gene products in oncogenesis.

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