Constitutive Nuclear Factor-κB–RelA Activation Is Required for Proliferation and Survival of Hodgkin's Disease Tumor Cells

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Abstract

The pathogenesis and etiology of Hodgkin's disease, a common human malignant lymphoma, is still unresolved. As a unique characteristic, we have identified constitutive activation of the transcription factor nuclear factor (NF)-κB p50-RelA in Hodgkin/Reed-Sternberg (H/RS) cells, which discriminates these neoplastic cells from most cell types studied to date. In contrast to other lymphoid and nonlymphoid cell lines tested, proliferation of H/RS cells depended on activated NF-κB. Furthermore, constitutive NF-κB p50-RelA prevented Hodgkin's lymphoma cells from undergoing apoptosis under stress conditions. Consistent with this dual function, Hodgkin's lymphoma cells depleted of constitutive nuclear NF-κB revealed strongly impaired tumor growth in severe combined immunodeficient mice. Our findings identify NF-κB as an important component for understanding the pathogenesis of Hodgkin's disease and for developing new therapeutic strategies against it. (*J. Clin. Invest.* 1997. 100:2961–2969.) Key words: cell cycle • apoptosis • IκBα • lymphoma • Reed-Sternberg cells

Introduction

Hodgkin's disease (HD)[1] is identified histologically by the presence of multinucleated Reed-Sternberg (RS) and mononucleated Hodgkin (H) cells surrounded by a background of lymphocytes, plasma cells, eosinophils, histiocytes, and stromal cells in the affected lymph nodes. The pathogenesis of HD and the cellular origin and clonality of its malignant components, RS and H cells, are still unclear, due to the scarcity of these cells in the malignant tissue (for a review, see references 1–3). H/RS cells have been investigated by histochemical characterization or single cell PCR in lymph node specimens, or after establishment of cell lines from neoplastic cells obtained from pericardial effusions. In general, the established cell lines display similar cytological features and heterogeneous surface marker expression as the H/RS cells in biopsy specimens (2). When injected into severe combined immunodeficient (SCID) mice, similar disseminated growth and organ infiltration are observed as in human HD (4). Surface marker and genotype analysis have suggested that H/RS cells carry B lymphoid, T lymphoid, monocytic or in many cases even shared characteristics of these cell lineages. Several attempts have been made to identify common molecular markers or other strictly shared features for H/RS cells. Although molecules like CD30 or restin (5, 6) are often found associated with H/RS cells, their expression is neither confined to nor always associated with H/RS cells. EBV is likewise found often, but not regularly, associated with HD. It is still a matter of debate whether H/RS cells are in fact clonal. Clonality of H/RS cells has been indicated by V_H gene rearrangements determined by PCR of micromanipulated cells from tissue sections (7). These single cell studies further indicated that the subgroup of H/RS cells tested should be derived from germinal center B cells, given the high frequency of somatic mutations in their rearranged V_H genes (7). In contrast, a similar study claimed that H/RS cells are in fact polyclonal, but contamination with non-H/RS cells in single cell manipulation cannot be excluded (7, 8).

Despite the apparent heterogeneity of H/RS cells, many of the clinical and histopathological features of HD correlate well with the deregulated, high expression of various cytokines, growth factors, and cell-surface receptors typical for H/RS cells (2). The strong cytokine production of H/RS cells has incited a search for deregulated transcription factors, and we have identified as a potential common marker constitutively activated nuclear factor (NF)-κB p50-RelA (p50–p65) both in primary H/RS cells derived from a pericardial effusion and in all different cell lines tested (9).

Transcription factor NF-κB is a mediator of inducible gene expression in response to inflammatory cytokines and pathogens, and is known for its crucial roles in the immune system (for a review, see references 10–12). In most cell types, with the exception of certain neurons (13), nuclear p50-RelA is observed only transiently in response to diverse inducers. RelA does not contribute to the constitutive NF-κB activity known in mature B cells, which consists mainly of c-Rel or RelB along with p50 or p52 (14–16). Several observations have suggested a role for NF-κB and IκB gene products in cell proliferation, transformation, and tumor development (12, 17). Members of the mammalian NF-κB/Rel and IκB gene families have been identified as potential protooncogenes, as in the case of p100/lyt-10 or Bcl-3. Although to date there have been no direct

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1. *Abbreviations used in this paper:* ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility–shift assay; HD, Hodgkin's disease; H/RS, Hodgkin/Reed-Sternberg; LMP, latent membrane protein; NF, nuclear factor; SCID, severe combined immunodeficient.

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demonstrations, indirect evidence suggests that NF-κB/Rel proteins could be directly involved in cell growth control. NF-κB controls the expression of a number of growth-promoting cytokines. In fact, a nuclear NF-κB–like DNA-binding activity is induced during the G_0 to G_1 transition after serum stimulation (18). Interestingly, the NF-κB transactivation potential appears to be linked to signaling that controls cell-cycle progression. Cyclin-dependent kinases regulate RelA through interactions with the coactivator p300 (19). Although a direct role of NF-κB to promote cell growth has not been established, both antiapoptotic and proapoptotic functions have been described. NF-κB protects fibroblasts against TNF-α–induced apoptosis, as has been shown with cells from RelA-deficient mice (20). Furthermore, Jurkat or fibroblastic cells underwent enhanced apoptosis after treatment with TNF-α, ionizing radiation, or daunorubicin when nuclear translocation of NF-κB was blocked (21–23). Likewise, downregulation of the c-Rel activity in a B lymphoma cell line resulted in spontaneous apoptosis (24). These data suggest that some of the NF-κB/Rel target genes have antiapoptotic functions. However, in neurons, glutamate-induced cell death requires the induction of NF-κB (25), and in human breast carcinoma cells, TNF-α–induced apoptosis is independent of NF-κB (26), indicating cell type differences in apoptotic functions of NF-κB.

We show here that activated NF-κB RelA is a characteristic property of H/RS cells in lymph node biopsy samples from patients suffering from Hodgkin's disease. With a dominant negative inhibitor of NF-κB stably introduced into H/RS cell lines, we demonstrate that constitutive NF-κB–RelA activation is essential for both proliferation and survival of HD tumor cells. These data demonstrate for the first time a direct requirement of NF-κB for cell growth, and a significant and common deregulation of this transcription factor in a human neoplastic disease. We postulate that Hodgkin's lymphoma is a malignancy of deregulated NF-κB RelA which protects these transformed cells against apoptotic elimination.

Methods

Immunostaining of biopsy samples. Biopsy samples were stained using the TSA-Indirect kit (DuPont-NEN, Boston, MA). The anti-RelA anti–nuclear localization signal mAb was obtained from Boehringer Mannheim (Mannheim, Germany). In brief, slides were blocked and incubated in H_2O_2 for 30 min at room temperature, followed by incubation with the primary antibody for 60 min. After standard washing and blocking steps, slides were incubated with biotinylated secondary antibody for 30 min and washed again. After incubation with streptavidin-conjugated horseradish peroxidase for 30 min and washing, slides were incubated with biotinyl tyramide for 10 min. Slides were washed, incubated with streptavidin-conjugated horseradish peroxidase, and washed again. Counterstaining was performed with hematoxylin to stain nuclei blue.

Transfections and DNA-binding assays. HD-MyZ, L540, HeLa, and MOLT-4 cells were grown as described (9). Cells were transfected with IκBαΔN encoding amino acids 71–317 in pcDNA3 (27) or with the empty expression vector by electroporation. Stable clones were selected with G418 (GIBCO BRL, Gaithersburg, MD). Cytosolic and nuclear extracts, Western blotting, and electrophoretic mobility–shift assay (EMSA) were performed as described (28). EMSA was performed with the H2K site as probe and H2K and AP-1 sites as competitors. Anti-IκBα and anti-RelA antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For reporter gene assays, cells were transfected with 5 μg each of HIV–chloramphenicol acetyl transferase (CAT) (29) and pCMVβ (Clontech, Palo Alto, CA) per 2×10^5 cells in 9-mm culture dishes by the lipofection technique (QIAGEN Inc., Chatsworth, CA). 24 h after transfection, cells were extracted by a freeze–thaw procedure, and CAT activity was determined with CAT-ELISA (Boehringer Mannheim). The mean values were corrected for transfection efficiency.

Cell-cycle progression. Cells at 50% confluence were synchronized by incubation with 3 μM aphidicolin for 24 h. After addition of fresh medium and trypsin treatment, 5×10^5 cells were fixed for 1 h in ethanol at −20°C, then precipitated (5 min of 2,000 rpm at 4°C), washed with PBS, and resuspended in 0.5 ml PBS (40 U/ml RNase A, 0.1 mg/ml propidium iodide). After incubation for 30 min at 37°C, DNA contents were determined by FACS® (Becton Dickinson, Mountain View, CA) using the Cellquest program.

Determination of apoptotic cells. After induction of apoptosis by serum withdrawal, cells were washed twice with ice-cold PBS and resuspended in 200 μl of PBS, 0.1% NaN_3, 10% human IgG (Venimmun; Behring, Marburg, Germany). Cells were stained for 30 min with FITC-labeled annexin V. Apoptotic cells were determined on a FACSort® (30).

Xenotransplantation of HD cells. HD-MyZ cells (10^5–10^7), either mock-transfected or stably expressing IκBαΔN, were injected subcutaneously into C.B.-17 SCID/SCID mice. The mice were obtained from our own breeding colony and kept in isolators under stringent conditions. Microbiological controls were performed regularly by the addition of sterile sentinel animals to the colony. Tumors were measured in two dimensions, length (a) and width (b), by means of calipers in millimeters. Tumor volume (V) was calculated according to $V = ab^2/2$, where a is the longer of the two measurements.

Results

Abundant activated NF-κB RelA in RS cells from patients suffering from HD. In a previous study, we have shown that seven different HD cell lines as well as primary tumor cells from a pericardial effusion shared high constitutive nuclear NF-κB p50-RelA activity (9). This activity was specific for HD cells and was not observed in anaplastic large cell lymphoma (reference 9, and data not shown).

The presence of constitutive nuclear p50-RelA in all Hodgkin cell lines tested suggests that this activity could, in fact, be a marker for HD cells; no such common marker has yet been described. To address this possibility and to verify that deregulated RelA is also detectable in RS cells in lymph node sections of patients suffering from this disease, biopsy samples from 10 patients were tested with an anti-RelA antibody. This antibody recognizes the nuclear localization signal epitope and thus preferentially free RelA, but not RelA bound to IκBα (31).

When lymph node sections of HD patients were analyzed, the majority of the RS cells in all of the samples tested were positive for free RelA (Fig. 1). Interestingly, not only nuclei but the entire cells were strongly stained, indicating the high amount of activated NF-κB in these cells. Similarly, high levels of constitutive NF-κB DNA binding-activity were detected in both cytoplasmic and nuclear fractions of cultured HD cells (data not shown). In only a few cases did we observe staining of small mononuclear cells in the histological sections, whereas $>$ 90% of mononucleated Hodgkin cells or multinucleated cells with RS morphology were positive in all patients analyzed. Several sections from non-Hodgkin's lymphoma, including anaplastic large cell lymphoma, did not reveal activated RelA in the malignant cells (data not shown). The presence of high amounts of activated RelA in almost all H/RS cells in the biopsy samples is thus in agreement with the data

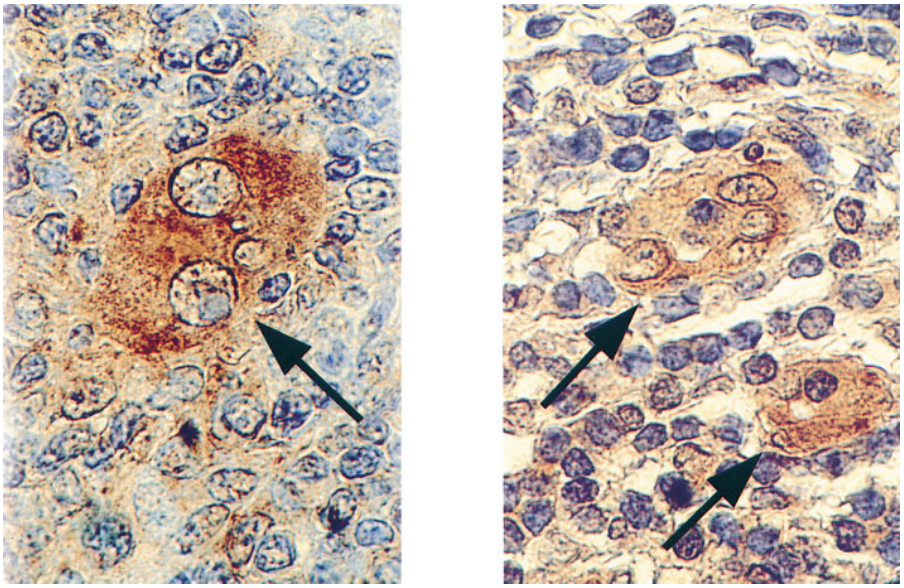

Figure 1. Abundant NF-κB–RelA activity in RS cells of HD patients. Immunostaining of biopsy samples from patients suffering nodular sclerosis–type HD with an anti-RelA antibody. Two representative samples are shown from a total of 10 patients. *Arrows*, Stained RS cells. More than 90% of the RS cells were positive for RelA in each case. Specificity of staining was confirmed by peptide competition (not shown). No comparable NF-κB–RelA staining was observed in lymph node samples from patients suffering from anaplastic large cell lymphoma or other non-Hodgkin's lymphoma (not shown).

obtained with H/RS cell lines, and strongly suggests that activated RelA is a common property of the malignant cells associated with HD.

Generation of HD cell lines with artificially blocked nuclear NF-κB activity. To study the functional biological consequences of constitutive p50-RelA activity in H/RS cells, HD-MyZ cells were stably transfected with IκBαΔN, a dominant-negative IκBα mutant that lacks the 70 amino-terminal amino acids (Fig. 2). IκBαΔN is able to bind to NF-κB and to undergo basal turnover, but it cannot release NF-κB upon cellular stimulation since it lacks serine and tyrosine residues required for signal-dependent activation (27, 32–36). Several clones were selected and analyzed for IκBαΔN protein expression (Fig. 2 *A*). Interestingly, the IκBαΔN expression levels inversely correlated with the expression of endogenous IκBα (Fig. 2 *A*; compare lanes *1* and *2*, *left panel*, with lanes *1–4*, *right panel*), in accordance with previous observations that NF-κB p50-RelA stimulates transcription of the IκBα gene (37). Correlating with its expression level, IκBαΔN caused a strong downregulation of the constitutive nuclear p50-RelA DNA-binding activity (Fig. 2 *B*, *left panel*, lanes *1–4*). Specificity and subunit composition of nuclear NF-κB p50-RelA in mock-transfected HD-MyZ cells were confirmed by antibody supershifting and DNA competition experiments (Fig. 2 *B*, *right panel*, lanes *1–5*). The binding activities of other transcription factors (such as AP-1) remained unaffected by IκBαΔN (data not shown). Depending on the IκBαΔN expression level, not only constitutive p50-RelA but also transient induction of NF-κB in these cells by TNF-α was strongly impaired or blocked almost completely (Fig. 2 *C*, lane *1* versus lanes *2–4*). As expected, this induction was partially inhibited in the presence of the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) (lane *4*). The signal-unresponsiveness of IκBαΔN was also reflected by its inertness towards degradation compared with endogenous IκBα (Fig. 2 *C*, lanes *5–8*). Endogenous IκBα but not IκBαΔN was degraded rapidly after short stimulation (lane *5* versus lane *6*) and resynthesized after prolonged stimulation (lane *7*). To assess whether the constitutive NF-κB activity is functional, HIV-1–CAT reporter constructs were transiently transfected into mock-transfected HD-MyZ cells, and revealed an NF-κB binding site–dependent activation by constitutive p50-RelA (Fig. 2 *D*). In contrast, IκBαΔN-expressing clones did not show any NF-κB–dependent reporter gene activation (Fig. 2 *D*). Thus, constitutive NF-κB activity in H/RS cells is functional and is specifically and efficiently blocked by IκBαΔN.

Similarly, L540 Hodgkin cells were stably transfected with IκBαΔN (Fig. 3). L540 cells displayed a much higher level of constitutive NF-κB activity than HD-MyZ cells (9). The expression of IκBαΔN (Fig. 3 *A*) led to a strong reduction in the constitutive NF-κB activity (Fig. 3 *B*, lanes *1* and *2* versus lanes *3* and *4*).

Constitutive nuclear NF-κB RelA is required for the proliferation of HD tumor cells. One important role of aberrant, constitutive nuclear p50-RelA in Hodgkin's lymphoma tumor cells may be to lock the cells into an activated state, mimicking continuous growth-promoting signaling. Therefore, we investigated whether blocking of endogenous NF-κB would affect cell growth in vitro. A comparison of the cell number of HD-MyZ–IκBαΔN clones and –mock-transfected clones at several time points after the start of the cell culture revealed that the loss of constitutive NF-κB strongly decreased cell growth (Fig. 4 *A*). Cell clones expressing lower amounts of IκBαΔN displayed an intermediate reduction in the proliferation rate (not shown), reflecting a dose–response relationship between nuclear NF-κB levels and cell growth. No differences in spontaneous cell death could be observed, indicating that reduced cell growth under normal cell culture conditions is due to reduced cell-cycle progression, rather than to augmented apoptosis rates. This was confirmed with a cell-cycle analysis of synchronized cells. Cells were synchronized with aphidicolin, which blocks cell-cycle progression in G_1. Upon aphidicolin release, mock cells proceeded rapidly into S-phase within 3 h and subsequently showed normal cell-cycle progression (Fig. 4 *B*). In contrast, almost complete suppression of cell-cycle progression in G_1 could be observed in IκBαΔN

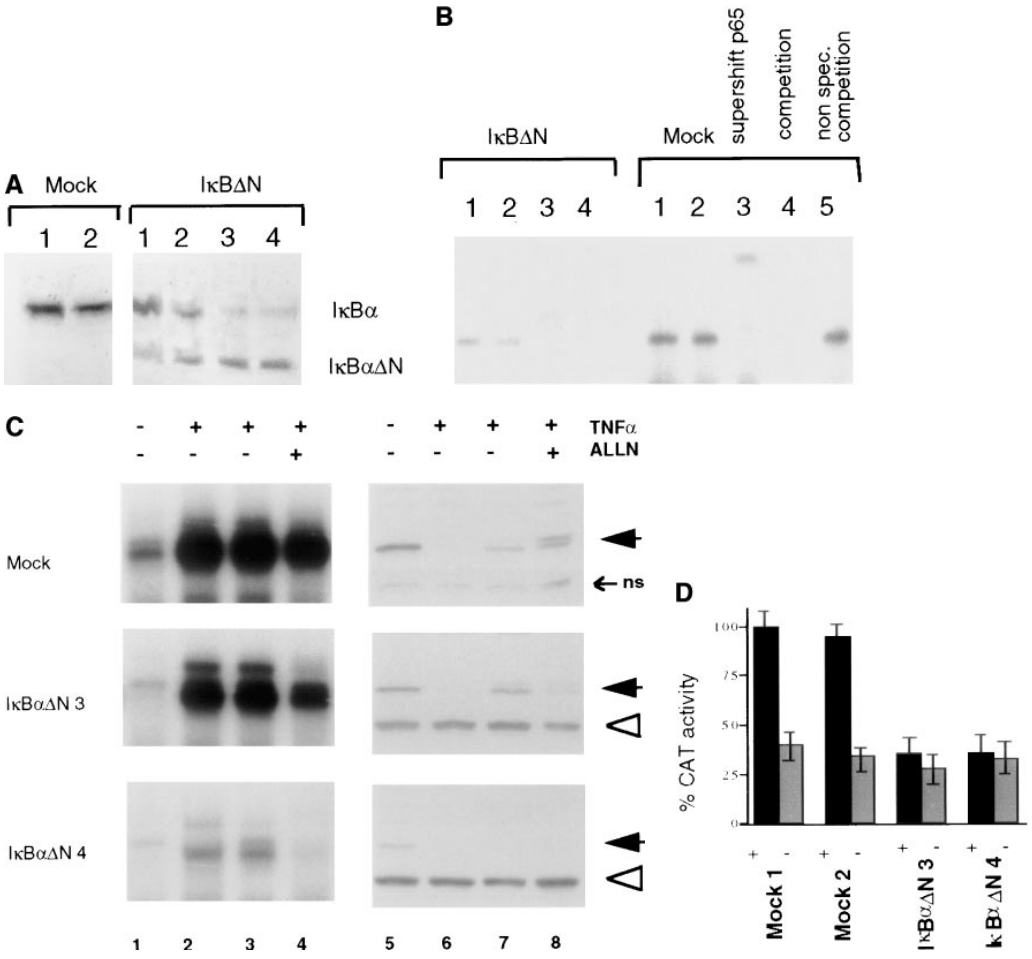

Figure 2. Overexpression of IκBαΔN downregulates constitutive NF-κB/Rel activity in HD-MyZ cells. (*A*) Western blot analysis of cytosolic endogenous IκBα and transfected IκBαΔN in mock-transfected controls (*left*) or stably transfected HD-MyZ clones (*IκBΔN*, *right*), IκBΔN1–IκBΔN4 (lanes *1–4*). (*B*) EMSA analysis of constitutive nuclear NF-κB DNA-binding activity in IκBαΔN-expressing cells (*left*, lanes *1–4*, clones IκBΔN1-IκBΔN4, respectively) or mock-transfected cells (*right*, lanes *1–5*). The constitutive p50-RelA activity (*right*, lanes *1* and *2*) was supershifted with an anti-RelA antiserum (lane *3*) and specifically inhibited with a binding site for NF-κB but not for AP-1 (lanes *4* and *5*). (*C*) IκBαΔN blocks transient induction of NF-κB by TNF-α in a dose-dependent manner. Mock-transfected cells (*top panels*) or cells stably expressing IκBαΔN (clone IκBΔN3, *middle panels*; clone IκBΔN4, *bottom panels*) were treated with TNF-α for 20 (lanes *2* and *6*) or 40 min (lanes *3* and *7*) or for 20 min with TNF-α after preincubation with the proteasome inhibitor ALLN (lanes *4* and *8*). Cellular extracts were analyzed for induced NF-κB DNA-binding activity by EMSA (lanes *1–4*; only DNA complexes are shown) and in Western blots for endogenous (*black arrowheads*) or ectopic (*white arrowheads*) IκBα expression (lanes *5–8*). *ns*, Nonspecific band. (*D*) Downregulation of NF-κB–dependent reporter gene activation by IκBαΔN. IκBαΔN–HD-MyZ clones 3 and 4 or two mock transfectants were transiently cotransfected with HIV–CAT reporter constructs with (+) or without (−) NF-κB–responsive elements and with a β-galactosidase reporter.

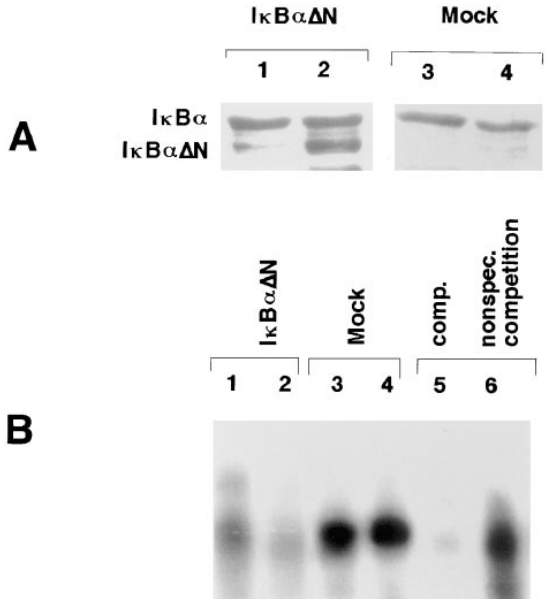

Figure 3. L540 cells were stably transfected with IκBαΔN. (*A*) Western blot of mock- (lanes *3* and *4*) or IκBαΔN-transfected L540 cells (lanes *1* and *2*). The position of wild-type and mutant IκBα is indicated. (*B*) EMSA with extracts of IκBαΔN-transfected (lanes *1* and *2*) or mock-transfected L540 cells (lanes *3* and *4*). Extracts from a mock-transfected clone were analyzed by competition with specific (NF-κB) or nonspecific binding site oligonucleotides (lanes *5* and *6*). Free DNA is not shown.

clones within the observed time period. Interestingly, IκBαΔN clones (1 and 2), in which NF-κB activation was blocked only partially (Fig. 2), were less affected in cell-cycle progression (not shown). Thus, blocking of endogenous constitutive NF-κB in H/RS cells leads to reduced cell growth in vitro due to impaired cell-cycle progression.

In the same fashion, L540 Hodgkin cells revealed reduced proliferation rates when overexpressing IκBαΔN compared with mock transfectants (Fig. 4 *C*). The growth reduction was less pronounced than that observed for HD-MyZ cells, consistent with the higher NF-κB activity that had to be downregu-

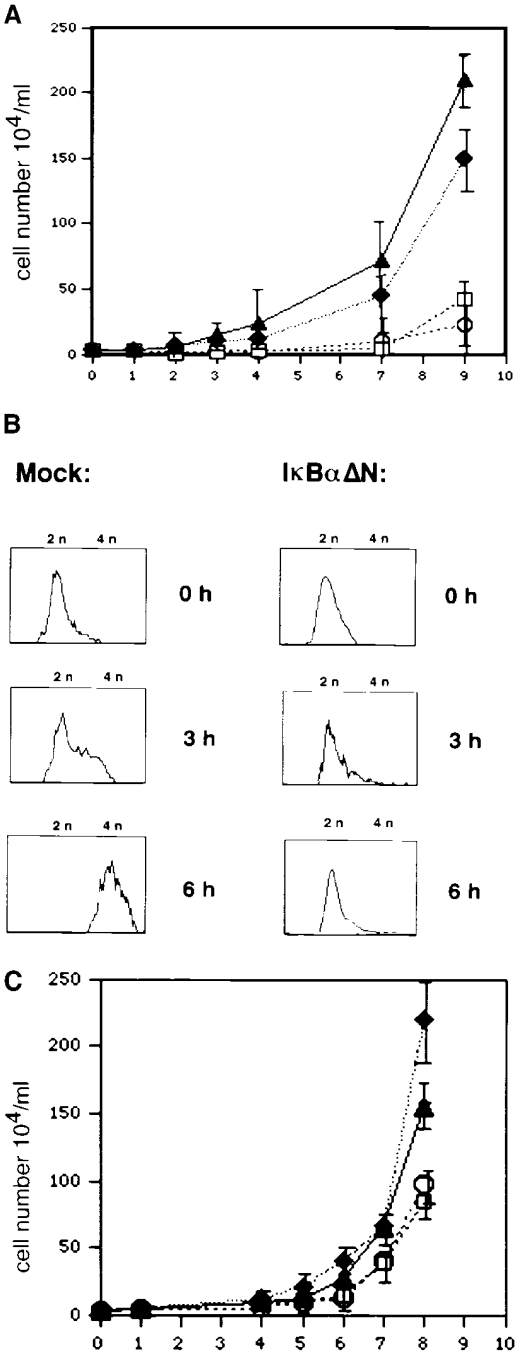

Figure 4. Blocking of endogenous NF-κB in Hodgkin cells suppresses proliferation by interfering with cell-cycle progression. (*A*) Proliferation rates of IκBαΔN-expressing (clone 3, *squares*; clone 4, *circles*) or mock-transfected (*filled symbols*) HD-MyZ cells. The cell density at various days after seeding is indicated. (*B*) FACS® analysis of progression into S-phase of IκBαΔN-transfected (*right*) or mock-transfected (*left*) HD-MyZ cells at 0, 3, or 6 h after release from aphidicolin-induced cell-cycle arrest. (*C*) Proliferation of L540 clones expressing IκBαΔN (clone 1, *squares*; clone 2, *circles*) compared with mock transfectants (*solid symbols*), as cell numbers determined at various days after start of the cell culture. The experiments shown in *A* and *C* were performed in triplicate.

lated in L540 cells and the higher residual constitutive activity (Fig. 3 *B*, and data not shown).

To assess whether the role of constitutive RelA for cellular growth is specific for H/RS cells, other cell types were stably transfected with IκBαΔN and analyzed for proliferation rates (Fig. 5). Neither MOLT-4 T cells (Fig. 5 *A*) nor HeLa cells (Fig. 5 *B*) showed any effect on cellular proliferation when expressing IκBαΔN compared with mock-transfected cells (Fig. 5 *A* and *B*, *bottom panels*). For each cell line, IκBαΔN expression efficiently reduced transient NF-κB inducibility by TNF-α (Fig. 5, *middle panels*). The expression of transfected IκBαΔN in these cells was in a similar order compared with endogenous IκBα (Fig. 5, *top panels*).

We also observed that significantly less stable IκBαΔN transfectants could be isolated from HD-MyZ or L540 Hodgkin's cells compared with MOLT-4 or HeLa cells, consistent with a severe growth disadvantage of Hodgkin's tumor cells with strongly reduced constitutive NF-κB (data not shown). Thus, our results indicate that constitutive RelA plays a crucial role in supporting proliferation specifically of HD tumor cells.

Constitutively activated NF-κB RelA protects Hodgkin's tumor cells against stress-induced apoptosis. To investigate a potential role for constitutive NF-κB p50-RelA in programmed cell death of Hodgkin cells under environmental stress conditions, we induced apoptosis in cultured HD-MyZ cells by serum starvation. IκBαΔN-expressing HD-MyZ cells underwent apoptosis almost completely, whereas mock clones or untransfected cells (not shown) were resistant to apoptosis within a time frame of 5 d (Fig. 6 *A*, *top* and *bottom*). In the same fashion, L540 cells expressing IκBαΔN but not mock transfectants were strongly driven into apoptosis after growth factor withdrawal (Fig. 6 *B*). Thus, constitutive p50-RelA appears to serve as a survival factor, and prevents cell death when Hodgkin's cells are put under stress conditions. In contrast to Hodgkin's cells, MOLT-4 T cells, devoid of constitutive NF-κB, showed increasing numbers of apoptotic cells when deprived of growth factors, and IκBαΔN expression did not affect this process (Fig. 6 *C*).

An antiapoptotic function of RelA has also been suggested previously to explain embryonal liver degeneration in RelA-deficient mice (38), and it has been proposed that c-Rel protects B lymphocytes against spontaneous apoptosis (24). Furthermore, a protective function of transiently induced NF-κB has also been reported for TNF-α–mediated cell death in embryonic fibroblasts and in a fibrosarcoma cell line (20, 21, 23). In contrast, in other cell types, NF-κB was either required or completely dispensable for apoptosis (25, 26).

NF-κB activity is required for growth of Hodgkin cell tumors in mice. A recently established SCID mouse model for HD (4) allows in vivo study of the effects of artificial downregulation of NF-κB. To determine the role of NF-κB in tumor growth, IκBαΔN-expressing HD-MyZ cells were transplanted into SCID mice. In contrast to mock-transfected or untransfected cells, which developed rapidly growing tumors, IκBαΔN-expressing cells showed strongly diminished tumor growth (Fig. 7). The small tumors observed at a prolonged time after transplantation of IκBαΔN-expressing cells (Fig. 7) had a different, soft consistency, with liquid-filled vacuoles (not shown). Thus, constitutive nuclear NF-κB supports Hodgkin's tumor growth in vivo, most probably by enhancing cellular proliferation and by conferring protection against apoptosis.

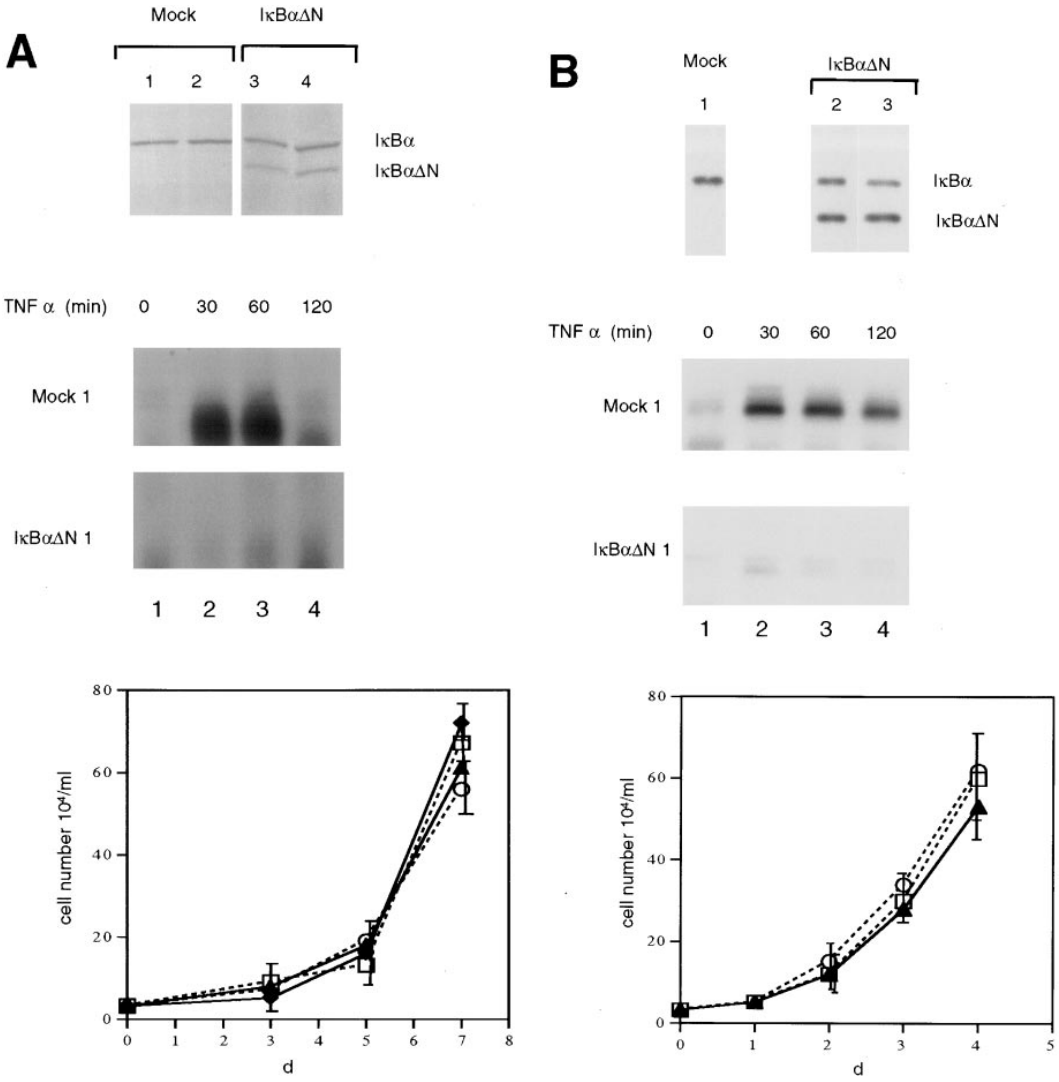

Figure 5. Expression of IκBαΔN in MOLT-4 or HeLa cells does not affect proliferation rates. (*A*) MOLT-4 cells were stably transfected with IκBαΔN. Western blot analysis (*top*) shows levels of wild-type and mutant IκBα in mock transfectants and stably expressing clones. EMSA (*middle*) of NF-κB induction by TNF-α in a mock-transfected or IκBαΔN-expressing clone. Cells were stimulated with TNF-α for the indicated times. Free DNA is not shown. Proliferation curves (*bottom*) of MOLT-4 cells, stably expressing IκBαΔN (clone 1, *squares;* clone 2, *circles*), and of mock-transfectants (*solid symbols*). (*B*) HeLa cells were transfected with IκBαΔN. Analysis of cell clones is as described for MOLT-4 cells in *A*.

Discussion

Constitutively activated RelA as a common marker and survival factor of RS cells. Several members of the NF-κB/Rel/IκB families of transcription regulators have been identified in aberrant forms in hematopoietic neoplasms, although neither a causative role for malignant growth nor a particularly high incidence has ever been established. In this study, we have elaborated a critical function of NF-κB in HD, one of the least understood human malignant lymphomas. We demonstrated previously that constitutive activation of NF-κB p50-RelA (p50–p65) is a characteristic property of H/RS cells, which discriminates these neoplastic cells from most other cell types studied to date. Activated RelA was detected in all H/RS cell lines tested, which were derived from different HD subtypes, as well as in primary H/RS cells obtained from a pericardial effusion (9). We showed here by in situ immunolabeling of lymph node sections from patients suffering from HD of the nodular sclerosing type, the most common HD subtype, that high-level RelA activity was detected in the RS cells, which represent the diagnostic hallmark for this disease. Strikingly, > 90% of the RS cells in the inspected histological sections were positive for activated RelA. In a double staining experiment, we also found expression of CD30 on the RS cells positive for activated RelA (data not shown). Sections from non-Hodgkin's lymphoma did not reveal activated RelA in the malignant cells (data not shown). Since constitutively activated RelA is not found frequently in other cell lines and cell types, its presence in HD-derived cell lines, primary neoplastic, and RS cells in lymph node sections must be regarded as highly significant. In all other cell types analyzed to date, RelA is only activated rapidly and transiently in response to a variety of signals. Constitutive NF-κB–RelA activation in the absence of obvious exogenous stimuli or viral infection has only been detected to date in cultured neurons isolated from the hippocampus or cerebral cortex (13). In contrast to H/RS cells, the constitutive nuclear NF-κB/Rel activity characteristic of B lymphocytes consists of c-Rel and RelB (15, 16).

We have addressed the functional significance of NF-κB for HD tumor cells by blocking both constitutive and inducible nuclear NF-κB activities in Hodgkin L540 and HD-MyZ cells using a signal-unresponsive IκBα mutant (IκBαΔN). These well-characterized cell lines are derived from nodular-sclerosing type HD with myelomonocytic or T cell–like features, re-

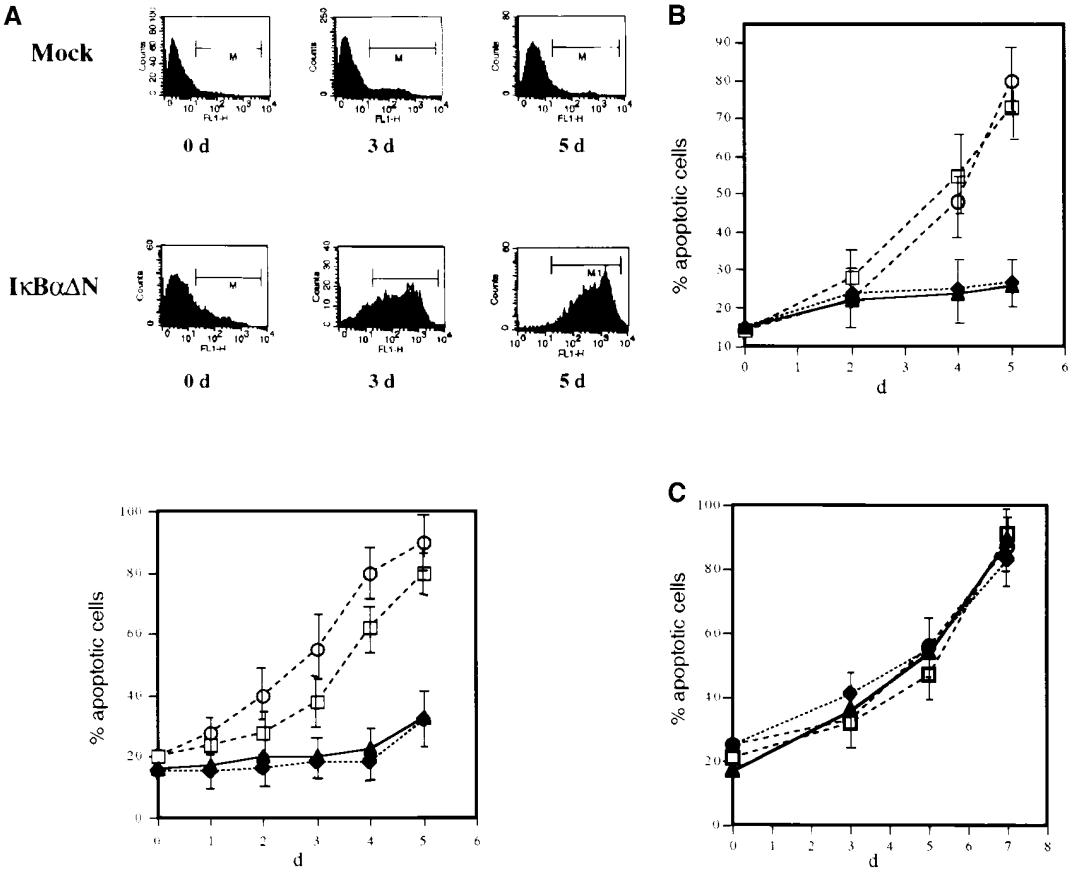

Figure 6. Inhibition of nuclear NF-κB renders Hodgkin cells sensitive to serum starvation–induced apoptosis. (*A*) Apoptosis of mock- or IκBαΔN-transfected HD-MyZ cells was induced by serum (growth factor) depletion. *Top*, Representative FACS® analysis of mock transfectants or IκBαΔN clone 4 cells at 3 or 5 d after serum withdrawal, respectively. *Bottom*, Fraction of apoptotic cells in mock-transfected (*solid symbols*) or IκBαΔN-transfected HD-MyZ clones (clone 3, *squares*; clone 4, *circles*) determined at various days after serum withdrawal. (*B*) Fraction of apoptotic cells measured after serum withdrawal in IκBαΔN-transfected (clone 1, *squares*; clone 2, *circles*) and mock-transfected (*solid symbols*) L540 cells. (*C*) Fraction of apoptotic cells measured after serum withdrawal in IκBαΔN-transfected (clone 1, *squares*; clone 2, *circles*) and mock-transfected (*solid symbols*) MOLT-4 cells. The experiments in *A* (*bottom*), *B*, and *C* were performed in triplicate.

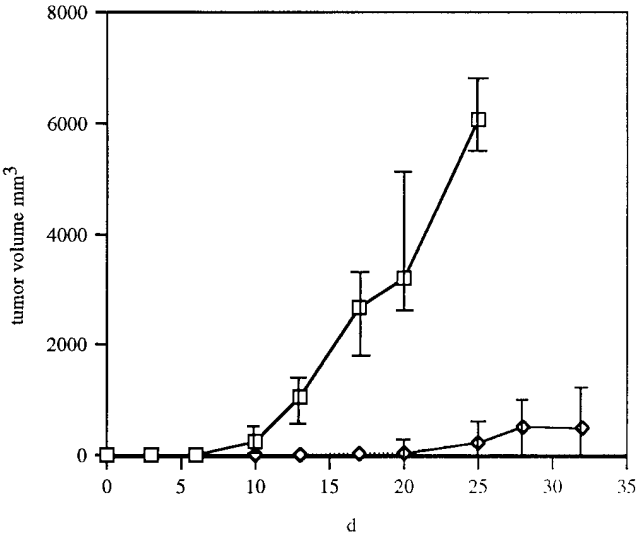

Figure 7. Impaired tumor growth of Hodgkin cells devoid of constitutive NF-κB activity. Tumor volumes were measured for xenotransplanted IκBαΔN-transfected (clone 4, *diamonds*) or mock-transfected (*squares*) HD-MyZ cells at different days after subcutaneous injection (10^6 cells). The mean values of four independent experiments are shown.

spectively (9, 39). Downregulation of nuclear NF-κB suppressed cell growth of these Hodgkin's tumor cells due to impaired cell-cycle progression. In contrast, growth of other lymphoid or nonlymphoid cell lines tested (HeLa, MOLT-4) was not affected by IκBαΔN expression. Furthermore, the lack of constitutive NF-κB strongly enhanced the sensitivity of the modified Hodgkin's cells to undergo apoptosis after growth factor withdrawal. This dual function of NF-κB in vitro was consistent with its role in tumor growth in vivo: HD-MyZ cells expressing IκBαΔN revealed strongly impaired tumor growth after xenotransplantation into SCID mice.

Since the observed effects were obtained by stable IκBαΔN expression, a formal possibility would be that an NF-κB–unrelated property was selected when these cell lines were established. We rule this out because both proapoptotic and antiproliferative effects correlated in a dose–response—like fashion with the expression of IκBαΔN, and only affected cell lines with constitutive RelA activity.

Possible mechanisms of constitutive RelA activation in HD tumor cells. An interesting question is how the constitutive RelA activity in H/RS cells is acquired. The EBV-encoded latent membrane protein (LMP)1 is a known activator of NF-κB (40), but the absence of EBV in most of the cell lines tested excludes the possibility that LMP1 expression accounts for the activation of RelA. However, we cannot rule out that infection

with a hitherto unknown virus leads to RelA activation. A further candidate is CD30, like LMP1 a receptor belonging to the TNF-receptor superfamily and frequently expressed on HD cells. Stimulation of CD30 can induce nuclear translocation of p50-RelA, involving the signal-transducing molecules TNF receptor–associated factor 1 and 2 (41, 42). As with EBV, CD30 is not expressed by all H/RS cells. Furthermore, CD30 is not confined to H/RS cells and is also expressed by anaplastic large cell lymphomas, which do not contain constitutively activated RelA (9).

We noted low steady state IκBα protein amounts in H/RS cells, which indicated high turnover of IκBα (9). High nuclear RelA levels are expected to cause a strong transcriptional upregulation of the IκBα gene, leading to a sequestering of excess free RelA. This has been demonstrated impressively with overexpressed RelA in transgenic animals that is masked efficiently by upregulated IκBα and kept in the cytoplasm (43). Therefore, continuous elevated degradation of IκBα should account for the constitutive RelA activity in HD cells. To this end, we can speculate about two possibilities. H/RS cells could stimulate NF-κB activation by an autocrine mechanism involving the expression of NF-κB–regulated receptors and cytokines. Alternatively, H/RS cells may have acquired defects in components that regulate NF-κB release from IκBα, resulting in constitutive NF-κB activation. These components may be any of the kinases, phosphatases, or other signal-transducers normally involved in NF-κB–activation pathways.

Constitutive RelA and cellular proliferation. We showed for the first time that activated NF-κB directly promotes proliferation. A role for RelA in signal transduction pathways controlling T and B lymphocyte proliferation has also been deducted from data obtained with RelA-deficient lymphocytes (44). Other studies with knockout mice have shown that not only RelA, but also RelB, c-Rel, and p105 at some point play a role in B cell proliferation (45, and references therein).

Since IκBαΔN expression specifically inhibited growth of HD but not HeLa or MOLT-4 cells, devoid of constitutive RelA activity, a direct functional coupling of NF-κB with general cell-cycle control proteins seems unlikely. As an alternate possibility, NF-κB could directly regulate genes whose products promote cell cycle in a cell type–specific fashion. The IκBαΔN-unaffected growth of MOLT-4 or HeLa cells also excludes the possibility that the antiproliferative effects could be due to the requirement of low-level nuclear NF-κB/Rel activities for some essential cellular functions. A functional interaction between NF-κB RelA with p300 and the cyclin-dependent kinase 2–cyclin E complex has been demonstrated recently, although its significance for cell growth has yet to be established (19).

A possible level of interference with tumor growth in the animal model is the dependency of cellular adhesion molecule expression on NF-κB (46). It has been maintained previously that in transformed cell lines, antisense oligonucleotides or RNA to RelA inhibited both in vitro growth and the ability of cells to adhere to an extracellular matrix and ablated tumor growth in mice (47, 48). We did not observe any consistent effect reflecting impaired adherence of IκBαΔN-expressing HD cells (not shown). As a further possible mechanism, an autocrine action of growth factors could lock NF-κB p50-RelA into a permanently activated state. In fact, HD cells typically express high levels of various cytokines and their receptors, several of which are either known inducers of NF-κB or are regulated by NF-κB at the transcriptional level.

An antiapoptotic function of RelA in H/RS cells. The HD cells were protected by constitutive NF-κB against growth factor withdrawal–induced apoptosis, similar to the protective effect of transiently induced NF-κB in fibrosarcoma, fibroblast, or HeLa cell lines against TNF-α, daunorubicin, or radiation-induced cell killing (20–23). Recent evidence that the inhibition of NF-κB RelA did not affect TNF-induced apoptosis in MCF7 cells (26) clearly demonstrates cell type differences in the involvement of NF-κB in apoptosis. In several B cell lymphoma and normal splenic B cells, blocking of the constitutive c-Rel activity resulted in spontaneous apoptosis without the requirement of further apoptotic signals (24). This is in contrast to the situation found in Hodgkin's cells, which in the absence of activated RelA undergo apoptosis only when put under stress conditions. Since IκBαΔN binds efficiently to both RelA and c-Rel, possible differences in these factors for antiapoptotic or cell growth functions have to be considered. Whereas both HD lines used in this study contain constitutive nuclear RelA, L540 but not HD-MyZ also contains nuclear c-Rel (9). Therefore, the common target for the equivalent effects observed in the two cell lines upon IκBαΔN expression should be RelA, although a similar function of c-Rel cannot be excluded at this stage. In fact, it is intriguing that in addition to constitutive RelA, several HD lines contain activated c-Rel (9), indicating a resemblance to B cells.

The putative genes that may be the target for the antiapoptotic action of NF-κB in HD cells and other cell types have yet to be identified. Candidate genes with known antiapoptotic functions are A20, encoding a zinc finger protein, and mitochondrial manganous superoxide dismutase (49, 50). The expression of both is induced by NF-κB (26, 51).

A recent single cell analysis restricted to CD30-positive cells suggested that H/RS cells are derived from B lineage cells and, because of their high degree of Ig variable gene somatic mutations, possibly stem from germinal centers (7). Although these studies cannot exclude a more complex lineage origin, constitutive NF-κB would explain nicely the apoptosis resistance required to survive in the germinal center, if H/RS cells were B cell–derived clones with crippling mutations. Interestingly, all H/RS cells tested were positive not only for constitutive RelA, but also for Oct-2 (9), a hallmark for B cells.

Given that p50-RelA deregulation is a characteristic common feature for RS cells in lymph nodes and a number of HD-derived cell lines, it may be useful as a diagnostic tool and specific target for therapeutic intervention. RelA-based immunohistochemistry may serve to detect residual diseased cells after chemotherapy, in particular for advanced stages of the disease. Studies are under way to screen systematically the various types of lymphoma for the contribution of persistently activated NF-kB.

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