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Rel blocks both anti-Fas- and TNFα-induced apoptosis and an intact Rel transactivation domain is essential for this effect

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Abstract

The v-Rel oncoprotein must be continuously expressed to prevent the apoptosis of transformed lymphoid cells, and also inhibits TNFα-induced cell death. A tetracycline-regulated cell system was used to characterize the functions necessary for the anti-apoptotic activity of Rel proteins. v-Rel mutants defective for DNA binding or transactivation showed no protective effect. Similarly, whereas the transcription-competent c-Rel and RelA proteins inhibited TNFα-induced cytolysis, the transactivation-negative p50/NF-κB1 did not. Importantly, this study is the first to show that c-Rel can also confer significant protection from Fas-mediated cell death. Since the TNFR1- and Fas-signaling pathways involve some intermediates that are common and others that are unique to each pathway, these findings indicate that c-Rel may regulate the expression of genes that function to antagonize either or both death-signaling pathways.

Keywords: Rel; NF-κB; apoptosis; TNFα; Fas

Abbreviations: TNFα, tumor necrosis factor-alpha; TNFR, tumor necrosis factor receptor; TRADD, TNF receptor-associated death-domain protein; TRAF, TNF receptor-associated factor; FADD, Fas-associated death-domain protein; FasL, Fas ligand; CMV, cytomegalovirus; tTA, tetracycline transactivator; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; CHX, cycloheximide; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethylsulfoxide; ECL, enhanced chemiluminescence; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis

Introduction

Apoptosis, also referred to as programmed cell death, is an inducible response to developmental or environmental stimuli that ultimately results in cell suicide. Apoptosis plays a fundamental role in embryogenesis, tumorigenesis, normal tissue turnover, and the immune system (reviewed in Thompson, 1995; White, 1996). Several cytokines have been shown to trigger this process (reviewed in Nagata, 1997). For instance, the production of tumor necrosis factor alpha (TNFα) in immune and inflammatory responses leads to cell proliferation, differentiation, and apoptosis. Some of these events have been shown to involve the activation of the Rel/NF-κB transcription factors (reviewed in Nagata, 1997).

Ligand-mediated activation of TNF receptor I (TNFR1) results in the trimerization of the receptor and the recruitment of TRADD (TNF receptor-associated death domain protein; Banner *et al*, 1993; Hsu *et al*, 1995). The subsequent association of TRAF2 (TNF receptor-associated factor 2) and FADD/MORT1 (Fas-associated death domain protein) initiates two distinct signaling pathways that play critical roles in balancing the cell decision between life and death. While the recruitment of FADD induces apoptosis, that of TRAF2 leads to the activation of NF-κB (Chinnaiyan *et al*, 1996; Hsu *et al*, 1996; reviewed in Nagata, 1997). Increasing experimental evidence indicates that the activation of NF-κB antagonizes cell death (Beg *et al*, 1995; Beg and Baltimore, 1996; Cai *et al*, 1997; Liu *et al*, 1996; Neiman *et al*, 1991; Van Antwerp *et al*, 1996; Wang *et al*, 1996; White *et al*, 1995; Wu *et al*, 1996; Zong *et al*, 1997). Moreover, the expression of a dominant-negative TRAF2 mutant blocks the activation of NF-κB and potentiates the cytotoxic effect of TNFα (Liu *et al*, 1996).

Fas/APO-1 is the principal cell surface receptor involved in the apoptosis of lymphocytes (Nagata and Golstein, 1995). Its ligand FasL belongs to the TNF family and is expressed by activated T-lymphocytes. Activated cytotoxic T-cells expressing Fas undergo apoptosis when they encounter T-cells that express FasL. Antibody-mediated cross-linking of the Fas receptor also induces cell death (Itoh *et al*, 1991; Trauth *et al*, 1989; Yonehara *et al*, 1989). Similar to one branch of the death-signaling pathway triggered by TNFα, the activation of Fas leads to the recruitment of FADD/MORT1 to trigger an apoptotic cascade (Chinnaiyan *et al*, 1995).

The Rel/NF-κB family of transcription factors includes the v-Rel oncoprotein, and its cellular homologs c-Rel, RelA, RelB, p105/NF-κB1, p100/NF-κB2, *Xenopus* X-Rel1, and the *Drosophila* Dorsal, Dif and Relish factors (reviewed in Baldwin, 1996; Verma *et al*, 1995). Cytoplasmic Rel dimers are commonly found associated with one of several IκB inhibitors. Rel protein activation in

response to stimuli results in the phosphorylation and degradation of IκB through the ubiquitin-proteasome pathway (reviewed in Thanos and Maniatis, 1995). The nuclear entry of active NF-κB complexes is followed by their binding to κB DNA sites and the activation of gene expression (reviewed in Baeuerle and Henkel, 1994; Siebenlist *et al*, 1994). Importantly, members of this family have been suggested to participate in the control of programmed cell death. While some studies suggested a positive role for Rel factors in this process, accumulating evidence points to their anti-apoptotic activity (Abbadie *et al*, 1993; Beg *et al*, 1995; Beg and Baltimore, 1996; Cai *et al*, 1997; Grimm *et al*, 1996; Liu *et al*, 1996; Neiman *et al*, 1991; Van Antwerp *et al*, 1996; Wang *et al*, 1996; White *et al*, 1995; Wu *et al*, 1996; Zong *et al*, 1997; reviewed in Sonenshein, 1997).

Previous work indicated that the v-Rel oncoprotein maintains the transformed phenotype of chicken lymphoid cells by preventing their apoptosis (Neiman *et al*, 1991; White *et al*, 1995; Zong *et al*, 1997). Our studies showed that v-Rel can also block TNFα-induced cell death in HeLa cells (Zong *et al*, 1997). The demonstration that v-Rel inhibits programmed cell death in response to different stimuli suggested that its anti-apoptotic activity may be important for its transforming function. Since intact DNA-binding and transactivation domains are required for cell transformation by v-Rel (Diehl and Hannink, 1993; Kumar *et al*, 1992; Sarkar and Gilmore, 1993; Smardova *et al*, 1995; White *et al*, 1996), we directly addressed their role in the anti-apoptotic activity of v-Rel and of other Rel/NF-κB factors. Our studies looking at the ability of Rel proteins to also block Fas-mediated cell death clearly demonstrate that c-Rel inhibits both anti-Fas- and TNFα-induced apoptosis and that its transactivation domain is essential for this effect.

Results

Intact DNA-binding and transactivation domains are required for v-Rel-mediated inhibition of TNFα-induced cell death

Mutant v-Rel proteins defective for DNA binding or transcriptional activation were conditionally expressed using a tetracycline-regulated system and characterized for their ability to protect cells from TNFα-induced apoptosis. In these assays, mutant v-*rel* genes were expressed under the inducible control of a tetracycline transactivator (tTA) protein. The addition of tetracycline to the culture medium prevented the association of the tTA activator with tetracycline operator sites, terminating *rel* gene expression. Stable cell clones were generated in the HeLa-derived HtTA-1 cell line (Gossen and Bujard, 1992). The v-Rel·H mutant is deleted of the C-terminal transactivation domain of v-Rel, and thus encodes the Rel-homology domain alone (Δ331 – 503; Kumar *et al*, 1992). Mutant v-Rel·DB- is defective for DNA binding due to the substitution of alanines for the conserved arginine residues in the RxxRxRxxC v-Rel DNA-binding motif (Figure 1A; Kumar *et al*, 1992).

The inducible expression of mutant v-Rel proteins in HtTA-1-derived cell clones was analyzed in immunoblots

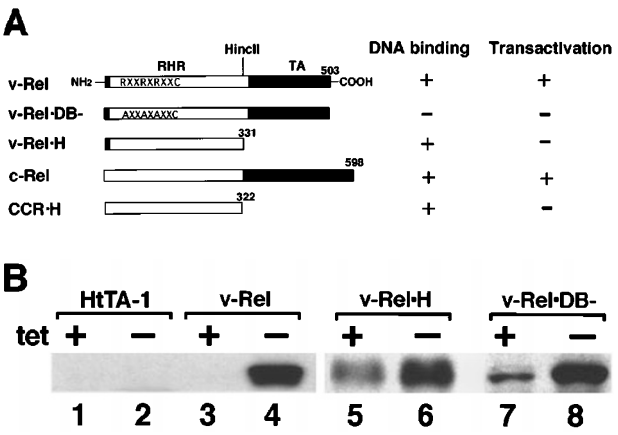

Figure 1 (**A**). Structure, DNA-binding, and transcriptional activities of wild-type and mutant v-Rel and c-Rel proteins. RHR: Rel-homology region; TA: transactivation domain; R: arginine; C: cysteine; A: alanine. (**B**). Immunoblot analysis of wild-type and mutant v-Rel protein expression. Cells from the tetracycline-regulated HtTA-v-Rel, HtTA-v-Rel·H, and HtTA-v-Rel·DB- clones were maintained in the presence of tetracycline (lanes 3, 5, 7). Rel protein expression was induced upon removal of the drug for 48 h (lanes 4, 6, 8). Cells from the parental HtTA-1 clone were cultured in the presence (lane 1) or absence (lane 2) of tetracycline as a control

and compared to that of v-Rel in the tetracycline-regulated HtTA-v-Rel48 cell clone (Zong *et al*, 1997). As shown in Figure 1B, the removal of tetracycline from the culture medium led to the significant accumulation of wild-type and mutant v-Rel proteins, despite some leaky expression observed when the mutant clones were cultured in the presence of tetracycline (compare lanes 4, 6, 8 to lanes 3, 5, 7). As anticipated, no v-Rel expression was detected in the control HtTA-1 parental cells grown in the presence or absence of the drug (lanes 1 and 2).

HtTA-1-derived cell clones were then assayed for their susceptibility to TNFα-induced cell death following the induction of mutant v-Rel protein expression. In agreement with our previous findings, the control HtTA-1 cells underwent massive cell death following treatment with TNFα together with cycloheximide (CHX; Figure 2A, panels a, b; Zong *et al*, 1997). In contrast, wild-type v-Rel conferred significant protection from cytolysis (panels c, d). Importantly, the v-Rel·H mutant deleted of the transactivation domain of v-Rel failed to protect the cells from TNFα-induced apoptosis (panels e, f). This indicated that the Rel-homology domain of v-Rel is not sufficient to inhibit cell death and suggested that sequences mapping to the C-terminus of v-Rel are necessary for its anti-apoptotic activity.

Previous studies from our group and others identified a transactivation domain mapping 3′ to the Rel-homology region of v-Rel and that overlapped with sequences essential for its transforming function (Ishikawa *et al*, 1993; Walker *et al*, 1992; Sarkar and Gilmore, 1993; Chen, Agnès and Gélinas, unpublished observations). Since death signaling involves a series of defined protein-protein interactions, the C-terminus of v-Rel could protect cells from apoptosis because it provides a defined protein interaction domain. Alternatively, it could allow cell survival

because it mediates transcriptional activation. To discriminate between these two possibilities, we investigated whether v-Rel DNA binding was also required to block apoptosis. HtTA-1 cells expressing a v-Rel mutant defective for DNA binding (v-Rel·DB-) were assayed for survival following treatment with TNFα and CHX. Similar to the activation-defective v-Rel·H mutant and in contrast to v-Rel, the v-Rel·DB- mutant failed to protect cells from TNFα-induced cell death (Figure 2A, panels g, h). Quantitation of cell survival by crystal violet staining showed that v-Rel enabled approximately 22% of the cells to escape apoptosis. In contrast, cells expressing the v-Rel·H or v-Rel·DB- mutants did not survive the TNFα treatment (Figure 2B). Together, these experiments demonstrated unequivocally that the anti-apoptotic activity of v-Rel requires both intact DNA-binding and C-terminal domains. The fact that v-Rel·DB- failed to promote cell survival strongly supports that the transcriptional activity of v-Rel is essential to block TNFα-induced cell death.

It is interesting to note that the protection of HtTA-v-Rel48 cells from apoptosis was not absolute. While the removal of tetracycline enabled approximately 22% of the cells to survive the TNFα treatment, many cells still succumbed to cytolysis. An immunofluorescence survey of approximately 200 Hoescht-positive v-Rel48 cells revealed that only a fraction of the cell culture was positive for v-Rel expression when maintained in the absence of tetracycline for 48 h (Figure 3a, b). This survey showed that approximately 30% of v-Rel48 cells were positive for v-Rel expression. This agrees with the survival rate that we observed and further suggests a correlation between v-Rel expression and the ability of cells to escape TNFα-induced apoptosis.

Immunofluorescence analysis also confirmed that the v-Rel·H and v-Rel·DB- proteins that were devoid of protective activity localized to the nucleus, similar to wild-type v-Rel (Figure 3c, and e). The percentage of cells expressing these mutant proteins was also comparable to that of cells

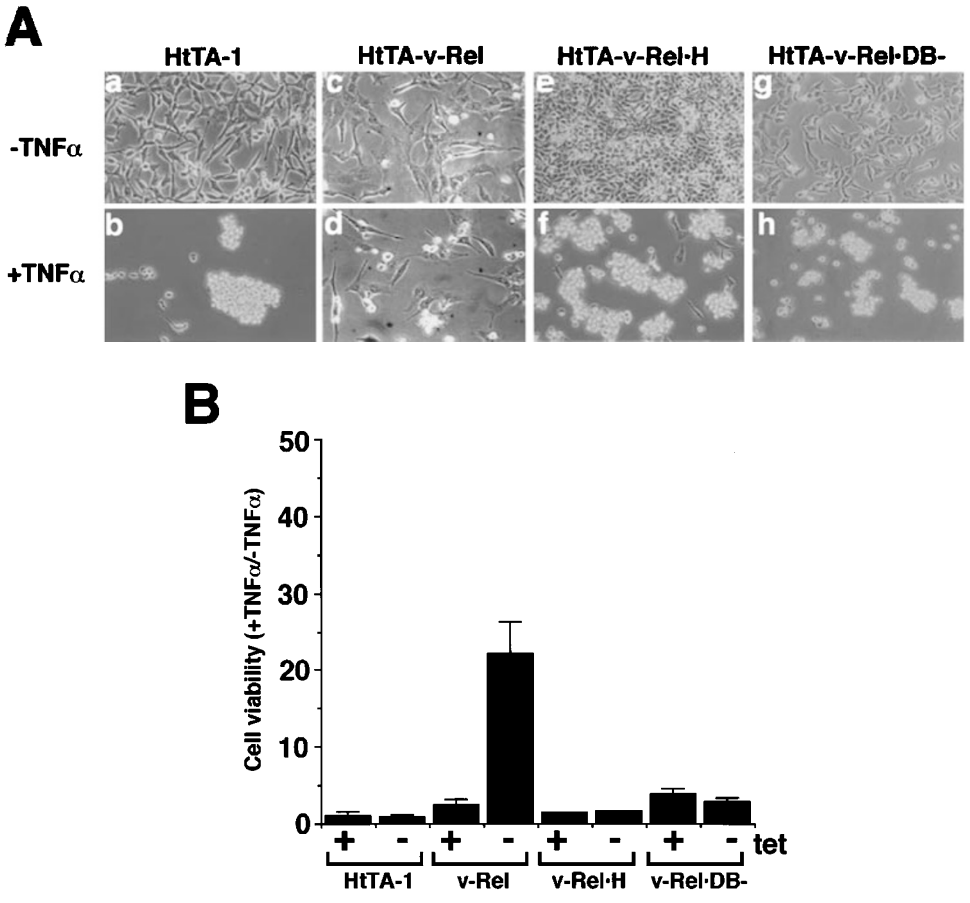

Figure 2 Intact DNA-binding and transcriptional activation domains are required for the anti-apoptotic function of v-Rel. Cells from the parental HtTA-1 clone (panels a, b), and from the HtTA-v-Rel48 (panels c, d), HtTA-v-Rel·H (panels e, f) and HtTA-v-Rel·DB- clones (panels g, h) were induced to express wild-type and mutant v-Rel proteins for 48 h. Apoptosis was induced by addition of TNFα together with CHX for 16 h (panels b, d, f, h). Cells treated with CHX alone were used as a control (panels a, c, e, g). (**A**) Analysis of cell protection from TNFα-induced apoptosis. Cells were photographed at a magnification of 200 $\times$. (**B**) Quantitation of cell survival by crystal violet staining. After incubation with CHX alone or CHX together with TNFα, cell cultures were washed. The attached surviving cells were stained with crystal violet. Relative cell viability represents the optical density ratio of cells treated with TNFα together with CHX over that of cells treated with CHX alone. The average viability observed in three independent experiments is shown. Similar results were obtained by counting whole populations of non-adherent and adherent cells

expressing v-Rel (26% for v-Rel·H; 27% for v-Rel·DB-). It therefore appears that the inability of v-Rel·H and v-Rel·DB- to protect cells from TNFα-induced cell death did not result from a lack of expression, nor from a subcellular localization different from that of wild-type v-Rel.

The transactivation-competent c-Rel and RelA proteins also protect cells from TNFα-induced cell death, while p50/NF-κB1 does not

The chicken c-Rel protein shares extensive sequence homology with v-Rel and strongly activates κB site-dependent transcription *in vitro* and *in vivo* (Capobianco *et al*, 1992; Ishikawa *et al*, 1993; Kamens *et al*, 1990; Walker *et al*, 1992; Xu *et al*, 1993). We therefore tested whether the inducible expression of the transcription-competent c-Rel protein in the HtTA-1-derived CCR43 cell clone would also inhibit TNFα-induced cell death. The removal of tetracycline from CCR43 cells led to the induction of c-Rel expression (Figure 4A, lanes 1, 2) and the concomitant activation of cellular gene expression, as evidenced by the induction of its target MAD-3/IκBα (Figure 4B, lanes 3, 4). c-Rel strongly inhibited apoptosis, with 77% of the cells surviving the TNFα treatment (Figure 4C). In contrast, a C-terminal deletion mutant lacking the c-Rel transactivation domain provided no protection (CCR·H, Figure 4C).

In agreement with our findings with the HtTA-v-Rel48 cell clone, the removal of tetracycline from CCR43 cells did not lead to complete cell death protection. While the removal of the drug enabled 77% of CCR43 cells to survive the TNFα treatment, 23% of the cells still underwent apoptosis. Once again, immunofluorescence assays revealed a good

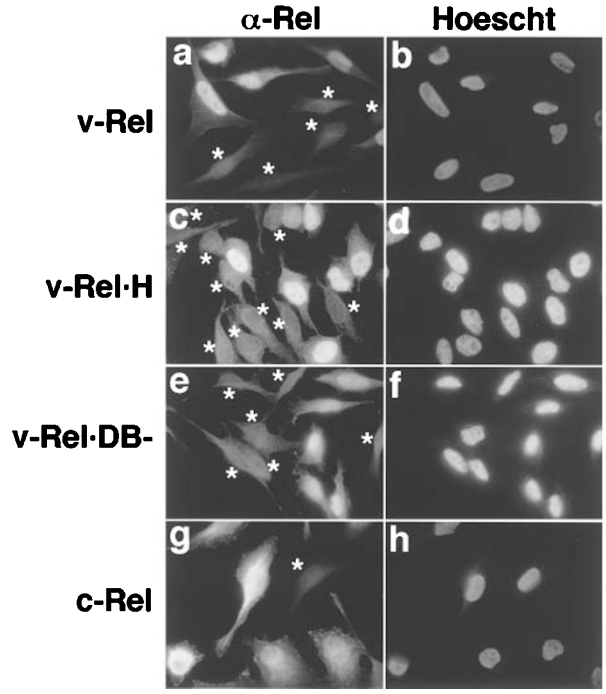

Figure 3 Immunofluorescence analysis of Rel protein expression. HtTA-v-Rel48 (panels **a, b**) and HtTA-v-Rel·H (panels **c, d**), HtTA-v-Rel·DB- (panels **e, f**) and HtTA-CCR43 cells (panels **g, h**) were induced for Rel expression upon the removal of tetracycline for 48 h. Cells were analyzed by immunofluorescence with an anti-Rel antibody. Cells were visualized by staining with fluorescein-conjugated secondary antibody (panels **a, c, e, g**). Nuclei were stained with the Hoescht dye 33258 (panels **b, d, f, h**). Cells that did not show significant Rel protein expression are marked by asterisks (panels **a, c, e, g**). The percentage of cells expressing Rel at the 48 h time point was obtained by surveying an average of 200 Hoescht-positive cells located in 15 different fields

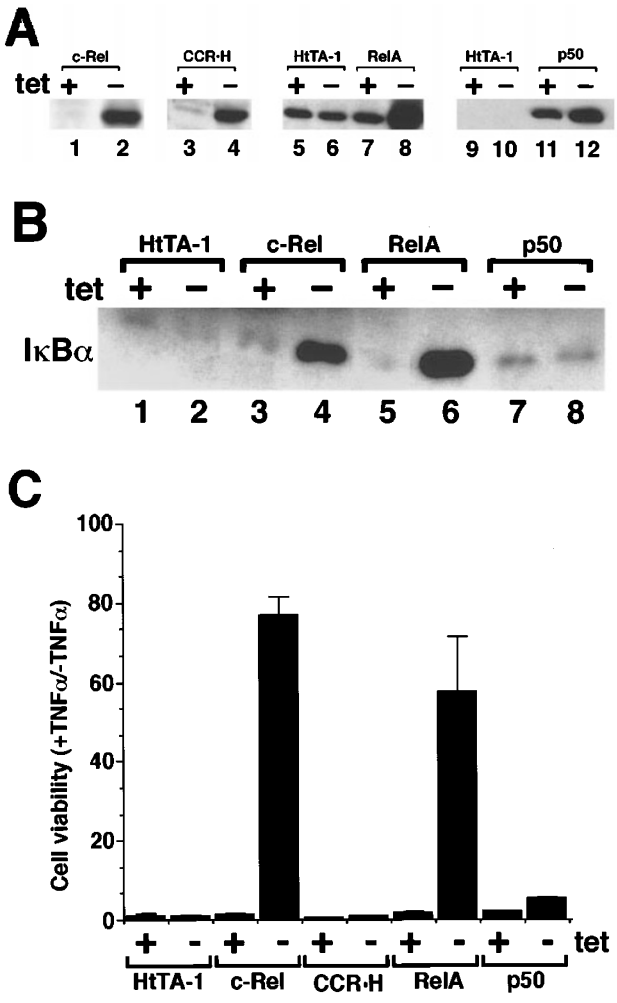

Figure 4 Transcription-competent Rel proteins block TNFα-induced cell death, whereas transactivation-negative proteins do not. (**A**) Immunoblot analysis of c-Rel, CCR·H, RelA and p50 protein expression in tetracycline-regulated HtTA-1-derived cell clones. Cells were maintained in the presence (lanes 1, 3, 5, 7, 9, 11) or absence of tetracycline for 48 h (lanes 2, 4, 6, 8, 10, 12). Extracts were resolved by SDS–PAGE and analyzed by immunoblotting with antibodies specific for c-Rel, RelA or p50. Proteins were visualized by ECL. (**B**) Immunoblot analysis of MAD-3/IκBα expression in HtTA-1-derived cell clones. Cells were maintained in the presence (lanes 1, 3, 5, 7) or absence of tetracycline for 48 h (lanes 2, 4, 6, 8). Immunoblot analysis was performed as described in A, using an antibody specific for MAD-3/IκBα. (**C**) Analysis of cell protection from TNFα-induced apoptosis. The control parental HtTA-1 cell clone and the HtTA-CCR43, HtTA-CCR·H, HtTA-RelA and HtTA-p50 cells were cultured in the presence or absence of tetracycline for 48 h. Cells were treated with CHX alone or CHX together with TNFα. Relative cell viability was determined by crystal violet staining as described for Figure 2B. The average cell viability observed in three independent experiments is shown

correlation between the expression of Rel and cell death protection (Figure 3, panels g, h). In these assays, a survey of approximately 200 Hoechst-positive cells showed that c-Rel was expressed in approximately 74% of HtTA-CCR43 cells maintained in the absence of tetracycline for 48 h, in agreement with the 77% survival rate that we observed (Figure 4C). These results indicate that a minimum threshold of Rel protein expression is necessary to inhibit TNFα-induced cell death.

We further examined the correlation between the transcriptional activity of Rel proteins and cell death protection by investigating the anti-apoptotic activity of two other members of the Rel/NF-κB family: p65/RelA and p50/NF-κB1. While RelA is generally viewed as a strong transactivator, p50 fails to activate gene expression from most κB site-containing promoters (reviewed in Verma *et al*, 1995). RelA and the transactivation-negative p50 protein were expressed under tetracycline-regulated control in HtTA-1 cells. Despite its endogenous expression in parental HtTA-1 cells and in the HtTA-RelA cell clone grown in the presence of tetracycline, significant accumulation of RelA was observed upon the induction of HtTA-RelA cells (Figure 4A, compare lane 8 to lanes 5–7). While some leaky expression of p50 was seen in HtTA-p50 cells cultured in the presence of tetracycline (lane 11), the removal of the drug led to significant accumulation of the protein (lane 12).

Approximately 55% of HtTA-RelA cells survived the TNFα treatment when induced to express RelA, whereas no protection was observed in uninduced HtTA-RelA cells (Figure 4C). We verified that the protective effect of RelA in the induced HtTA-RelA cells correlated with its transcriptional activity by monitoring the endogenous expression of the RelA-regulated MAD-3/IκBα protein. The activation of MAD-3 expression was only observed upon the induction of RelA expression in HtTA-RelA cells (Figure 4B, compare lanes 5 and 6). It therefore appeared that the RelA protein that is endogenously expressed in these cells is not

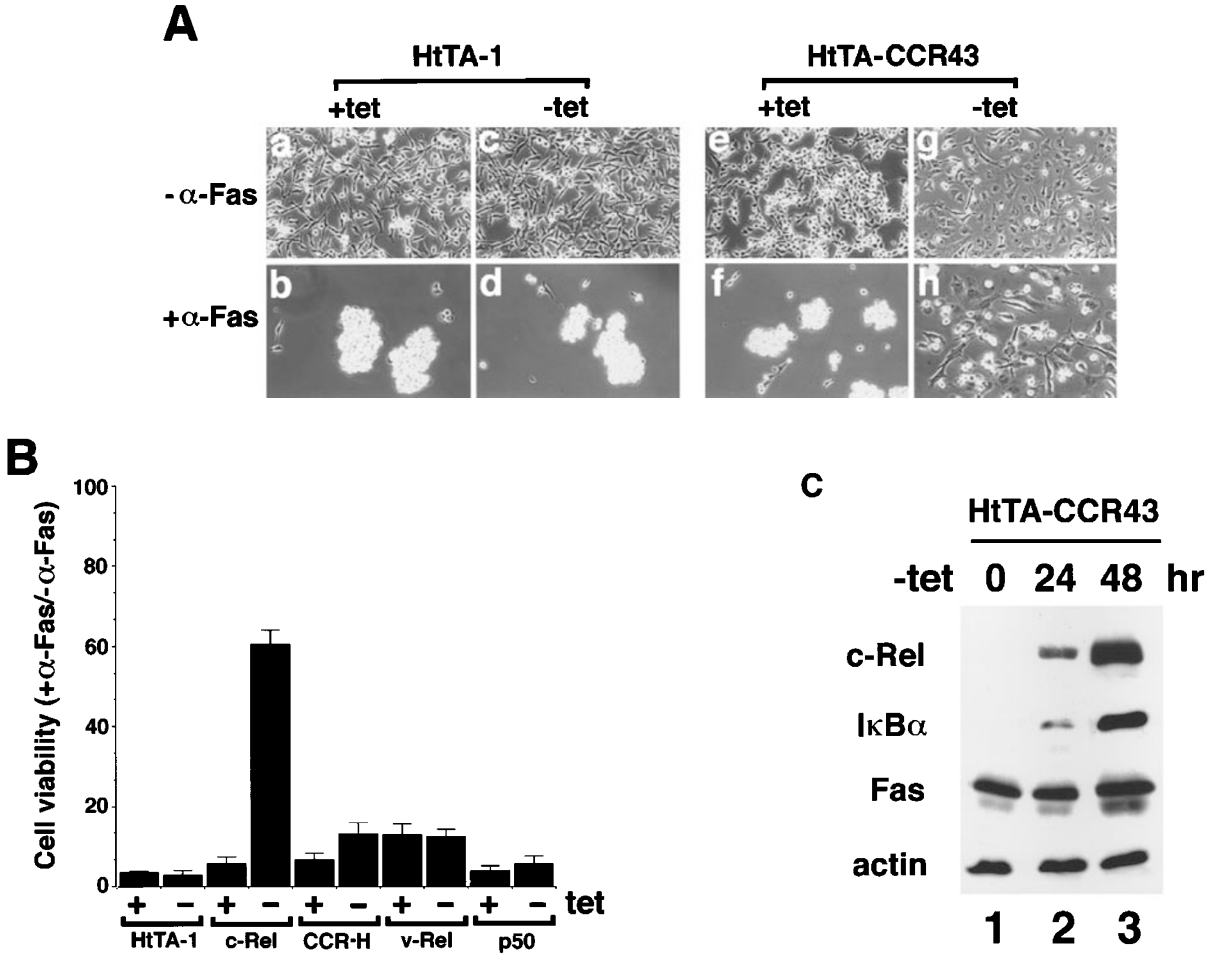

Figure 5 c-Rel blocks Fas-mediated apoptosis. (**A**) HtTA-1 and HtTA-CCR43 cells were cultured in the presence (panels a, b, e, f) or absence of tetracycline (panels c, d, g, h) for 48 h. Cells were treated with CHX alone (panels a, c, e, g) or together with anti-Fas antibodies for 16 h (panels b, d, f, h). The cells were photographed at a magnification of 200 $\times$. (**B**) HtTA-1, HtTA-CCR43, HtTA-CCR·H, HtTA-v-Rel48, and HtTA-p50 cells were treated as described in A. Relative cell viability was determined by crystal violet staining. The average viability observed in three independent experiments is shown. (**C**) c-Rel does not lead to the down-modulation of the Fas/APO-1 receptor. HtTA-CCR43 cells were maintained in the presence of tetracycline or induced for c-Rel expression for 24 or 48 h. Cell extracts were analyzed for the expression of c-Rel, IκBα, Fas/APO-1, or actin by ECL-immunoblot

transcriptionally active. This agreed with the sensitivity of the uninduced HtTA-RelA cells to TNFα-induced cytolysis and with their resistance following the induction of RelA expression (Figure 4C). The fact that the uninduced HtTA-RelA cells have survived more than 30 cell passages also indicated that the endogenous expression of RelA that we observed was not detrimental to the cells.

In contrast to v-Rel, c-Rel and RelA, p50 failed to activate endogenous gene expression and to confer resistance to cell death (Figure 4B, lanes 7 and 8, and Figure 4C). This further emphasized the correlation between the transcriptional activity of Rel-family proteins and their anti-apoptotic effect.

c-Rel also blocks Fas-mediated apoptosis

Similar to TNFα, cells treated either with FasL or with anti-Fas antibodies rapidly undergo apoptosis (Nishimura *et al*, 1995; Ogasawara *et al*, 1995). The TNFR1 and Fas cell death pathways involve some intermediates that are common and others that are unique to each pathway. Therefore, Rel-mediated inhibition of apoptosis triggered through both receptors would indicate the activation of genes that antagonize steps that are common to both pathways. We further characterized the anti-apoptotic activity of Rel proteins by investigating whether c-Rel could also block Fas-mediated cell death.

As anticipated, the control HtTA-1 cells underwent massive cell death following treatment with anti-Fas antibodies together with CHX, regardless of the presence or absence of tetracycline (Figure 5A, panels b, d). In sharp contrast, the expression of c-Rel conferred significant protection from Fas-mediated apoptosis (compare panels f and h). The quantitation of cell survival by crystal violet staining showed that c-Rel enabled nearly 60% of the cells to escape Fas-mediated cell death (Figure 5B). This effect was dependent on the transcriptional activity of c-Rel, since no significant protection was observed with a mutant c-Rel protein deleted of its C-terminal transactivation domain (Figure 5B).

In parallel experiments, v-Rel and p50/NF-κB1 failed to protect against Fas-mediated apoptosis (Figure 5B). This agreed with our observation that c-Rel showed the strongest protective activity in assays of TNFα-induced cell death amongst the Rel proteins that we tested (Figure 4C). Combined, the data demonstrated that c-Rel blocks apoptosis mediated through both the TNFR1 and Fas receptors, and indicated that the activation of cellular gene expression is necessary for this effect.

Since the Fas promoter contains two κB DNA sites (Behrmann *et al*, 1994), an immunoblot analysis was performed to investigate whether c-Rel protected cells from Fas-mediated apoptosis by triggering the down-modulation of the Fas receptor. As shown in Figure 5C, the induction of c-Rel expression in HtTA-CCR43 cells had no detrimental effect on the steady-state levels of the Fas/APO-1 receptor (compare lanes 1–3). In contrast, the expression of the c-Rel-regulated IκBα gene was significantly induced following the removal of tetracycline from CCR43 cells (Figure 5C, lanes 1–3). This demonstrated that c-Rel is transcriptionally active in CCR43 cells and that its protective effect toward Fas-mediated cell death does not involve the downregulation of the Fas/APO-1 receptor.

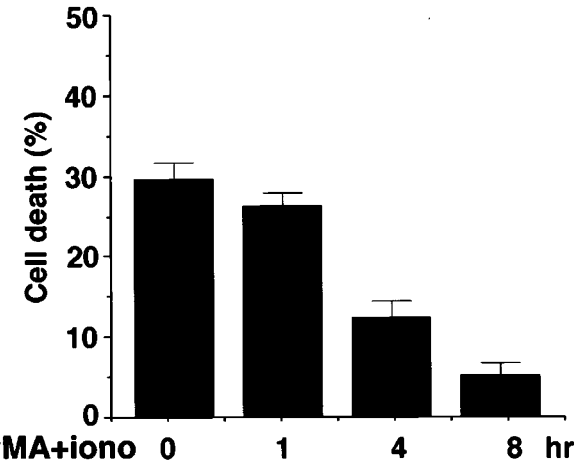

Figure 6 Stimuli that activate endogenous NF-κB activity in Jurkat T-cells promote cell resistance to Fas-mediated apoptosis. Human Jurkat T-cells were stimulated with PMA plus ionomycin for 1, 4 or 8 h, or with DMSO as a control (time 0), and treated with a monoclonal anti-Fas antibody for 16 h. Cell death was quantitated by counting whole cell populations following staining with trypan blue. Relative cell death represents the ratio of trypan blue-staining cells in anti-Fas-treated cells over that in non-treated control cells. The average cell death observed in three independent experiments is shown

To address the ability of Rel to protect cells from Fas-mediated apoptosis under more physiological conditions, we next investigated whether stimuli that induce endogenous Rel/NF-κB activity in T-cells would correspondingly block Fas-mediated cell killing. As shown in Figure 6, the treatment of Jurkat T-cells with PMA plus ionomycin led to significant cell protection from apoptosis in response to anti-Fas antibodies. While HeLa cells are normally resistant to TNFα and Fas-mediated apoptosis in the absence of CHX, these assays in Jurkat cells were performed in the absence of protein synthesis inhibitor. These results are consistent with our experiments showing that ectopically-expressed c-Rel inhibits Fas-mediated cell death.

Discussion

The anti-apoptotic function of Rel proteins requires their transcriptional activity

Accumulating evidence supports a role for the Rel and NF-κB factors in the inhibition of apoptosis in response to various stimuli, ranging from radiation to genotoxic agents (Beg *et al*, 1995; Beg and Baltimore, 1996; Cai *et al*, 1997; Liu *et al*, 1996; Neiman *et al*, 1991; Van Antwerp *et al*, 1996; Wang *et al*, 1996; White *et al*, 1995; Wu *et al*, 1996). Consistent with these findings, our previous studies showed that v-Rel blocks programmed cell death in transformed chicken lymphoid cells and inhibits TNFα-induced apoptosis in HtTA-1 cells (Zong *et al*, 1997). Here we demonstrate that v-Rel mutants defective for DNA binding, or lacking its C-terminal transactivation domain fail to inhibit apoptosis in response to TNFα. While we

do not rule out the possibility that the non-uniform expression of v-Rel in the v-Rel48 cell clone may be due to culture conditions or passage number, immunoblots suggested that it is unlikely to result from a strong induction of IκBα expression (data not shown). Although we observed some clonal variation with respect to the number of cells expressing v-Rel in particular cell clones, our assays showed a good correlation between the percentage of cells expressing v-Rel in independent cell clones and the cell survival rate. These results emphasize our finding that wild-type v-Rel confers protection from TNFα-induced cytolysis. In agreement with these results, the transcription-competent c-Rel and RelA proteins also exhibited protective activity toward TNFα-induced cytolysis, whereas the transactivation-negative p50 protein displayed no anti-apoptotic effect.

Whereas previous studies suggested that Rel-mediated transcription might be important for cell death protection, our experiments provide the first direct evidence that intact Rel DNA-binding and transactivation domains are required for this effect. The demonstration that Rel mutants defective for DNA binding fail to prevent cell death rules out the possibility that protein-protein interactions involving their C-terminal domains are solely responsible for cell death inhibition. Our results rather argue that the anti-apoptotic function of Rel and NF-κB factors requires the activation of cellular gene expression. This model is consistent with studies showing that cell death induced by TNFα is potentiated by inhibitors of RNA or protein synthesis (Itoh *et al*, 1991; Yonehara *et al*, 1989).

In our assays of TNFα-induced cytolysis, c-Rel showed the strongest protective activity amongst all of the Rel proteins tested (Figure 4C). This agrees with its strong transcriptional activity and with studies showing cell protection from TNFα-induced apoptosis in transient transfection assays (Liu *et al*, 1996). Our immunofluorescence experiments also indicated that a minimum threshold of Rel protein expression is necessary to inhibit cell death.

c-Rel also blocks Fas-mediated cell death

Our studies demonstrated that the ectopic expression of c-Rel inhibited Fas-mediated cell death. Our finding that the stimulation of human T-cells with PMA plus ionomycin also led to significant protection from apoptosis in response to anti-Fas antibodies is consistent with this result. This is in contrast with a recent report indicating that the inhibition of NF-κB by a dominant IκBαM protein was not sufficient to sensitize T24 cells to Fas-mediated killing (Van Antwerp *et al*, 1996). Based on the absence of cell killing under these conditions, it was inferred that NF-κB could not rescue cells from Fas-mediated apoptosis. The reason for the discrepancy between our findings and those of Van Antwerp *et al*, (Van Antwerp *et al*, 1996) is unclear, and may derive from differences in cell type or experimental conditions. Since the elimination of NF-κB was not sufficient to sensitize T24 cells to Fas-mediated apoptosis in the absence of CHX, it appears that NF-κB is not what makes T24 cells resistant to Fas-mediated cell death or that NF-κB is not the only factor responsible for their resistance. However, this is not in conflict with our finding that c-Rel can protect cells from Fas-mediated apoptosis.

Death signaling through the TNFR1 and Fas receptors involves some intermediates that are common and others that are unique to each pathway. Our experiments clearly demonstrated that c-Rel can inhibit both TNFα- and Fas-mediated cytolysis. This suggests that c-Rel may regulate the expression of genes that can antagonize death-signaling through steps that are common to the TNFR1 and Fas cell death pathways. These findings will help to focus the search for Rel-regulated genes responsible for the inhibition of cell death.

A striking difference between the TNFR1 and Fas cell death pathways in many cell types is that TNFR1 also induces the activation of endogenous NF-κB factors (reviewed in Nagata, 1997). Given the reported ability of NF-κB factors to block cell death, it is not surprising to find that anti-Fas antibodies induced a stronger apoptotic response than TNFα, with fewer c-Rel-expressing cells surviving the anti-Fas treatment (61%, Figure 5B). It is thus conceivable that the abrogation of death-signaling through Fas may require the action of a stronger apoptosis inhibitor than that through TNFR1, or the accumulation of a higher threshold of inhibitory activity. This agrees with the stronger transcriptional potential of c-Rel in comparison to that of v-Rel or p50 (reviewed in Verma *et al*, 1995), that both failed to enable cells to escape Fas-mediated cell death (Figure 5B). The observation that an independent cell clone expressing v-Rel in the majority of the cell population had a 77% survival rate from TNFα-induced killing but failed to show any resistance to Fas-mediated cell death agrees with this model (data not shown). The ongoing identification of cellular genes whose expression is specifically controlled by c-Rel and the study of their role in the apoptotic process will further address this issue.

Mechanism of cell death inhibition

The signaling events triggered in response to Fas-activation are common to one branch of the TNFR1-signaling pathway (reviewed in Nagata, 1997). Both receptors use FADD/MORT1 as a common signal transducer and share the death mediators downstream of FADD/MORT1. Our finding that c-Rel can block both TNFR1- and Fas-mediated cell death suggests that c-Rel could protect cells by affecting the recruitment of FADD/MORT1 to the activated receptors. Alternatively, c-Rel could affect signaling downstream of FADD/MORT1. Since the DNA-binding and transcriptional activities of Rel are needed to block apoptosis, c-Rel may perhaps affect the expression of factors that interfere with the interaction of death domain effectors common to the TNFR1 and Fas pathways or that act further downstream in the pathways. Our tetracycline-regulated cell systems will help to position Rel within the death-signaling pathway and to identify the cellular genes under Rel control that can block this process. Since the c-*rel* and *relA* genes are amplified or overexpressed in several human tumors (reviewed in Luque and Gélinas, 1997), these findings raise the possibility that the alteration of Rel/NF-κB activity may contribute to malignancy by promoting cell survival.

Materials and Methods

Plasmids

Wild-type and mutant v-*rel*, chicken c-*rel*, human *relA*, and human *nfκb1* genes were conditionally expressed using a tetracycline-regulated system (Gossen and Bujard, 1992). Plasmid pUHD10-3 was used to express v-*rel* and c-*rel* under the control of a minimal Cytomegalovirus (CMV) promoter with heptamerized tetracycline operator sites. v-*rel* mutant v-Rel·DB- is defective for binding to κB DNA sites as a result of alanine substitutions for the arginine residues in the RxxRxRxxC Rel DNA binding motif. This mutant was previously referred to as 2721 (Kumar *et al*, 1992). Mutants v-Rel·H, previously referred to as d*Hin*cII (Kumar *et al*, 1992), and CCR·H (Xu and Gélinas, 1997) contain stop codons inserted at the unique HincII sites of v-*rel* and c-*rel*. This resulted in the truncation of their respective transactivation domains. The mutation in v-Rel·H removed 172 amino acids from the C-terminus of v-Rel; that in CCR·H removed 276 amino acids from the C-terminus of c-Rel. The genes encoding RelA, p50, v-Rel·H and CCR·H were cloned into a pUHD10-3 vector that expressed the hygromycin-B resistance gene from plasmid pHMR272 (Bernard *et al*, 1985; pUHD10-3-hygro).

Cell culture and transfection

HtTA-1 cells, that stably expressed the tTA activator protein, were a gift from Dr. H. Bujard (Gossen and Bujard, 1992). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 × vitamin solution, 1 × non-essential amino acids, and antibiotics (100 units per ml of penicillin, 100 μg per ml streptomycin, and 125 μg per ml of G418 in potency units). Cells were maintained at 37°C in an atmosphere of 5% CO_2. HtTA-1 cells were conditioned to tetracycline-HCl (2 μg per ml, Sigma) for 4 days prior to transfection. The cells were transfected with pUHD10-3-hygro vectors encoding v-Rel·DB-, v-Rel·H, CCR·H, RelA, or p50 using a modified calcium-phosphate procedure (Chen and Okayama, 1987). Cell clones were selected in the presence of hygromycin B (225 units per ml, Calbiochem). Drug-resistant colonies were picked and screened for the inducible expression of RelA, p50, or mutant v-Rel and c-Rel proteins (HtTA-RelA, HtTA-p50, HtTA-v-Rel·DB-, HtTA-v-Rel·H, and HtTA-CCR·H). Tetracycline-regulated cell clones expressing the wild-type v-Rel (HtTA-v-Rel48) or c-Rel proteins (HtTA-CCR43) were described previously (Bash *et al*, 1997; Zong *et al*, 1997). Cell clones were maintained in the presence of tetracycline (2 μg per ml) and refed every other day.

Immunoblotting

Immunoblotting was performed by enhanced chemiluminescence (ECL, Amersham). Cell extracts were prepared in lysis buffer (50 mM Tris HCl pH 7.5, 150 mM sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, 10 μg per ml of leupeptin, 10 μg per ml pepstatin, 20 μg per ml aprotinin, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, and 0.5 mM sodium orthovanadate; Resnitzky *et al*, 1994) and quantitated for total protein concentration by the method of Bradford (Bradford, 1976). Wild-type and mutant v-Rel or c-Rel proteins were detected using rabbit polyclonal antibody Ab#3, that recognizes the conserved Rel-homology domain of v-Rel and c-Rel (Gilmore and Temin, 1986). Expression of RelA (SC-372), IκBα/MAD3 (SC-203), and Fas/APO-1 (SC-715) was detected using antibodies commercially available (Santa Cruz Biotechnology). A polyclonal anti-p50 antibody (Ab#1263) was a gift of Dr. Nancy Rice (ABL-NCI, Frederick, MD, USA).

TNFα and anti-Fas antibody-induced apoptosis

Procedures to induce apoptosis with TNFα or with anti-Fas antibodies were previously described (White *et al*, 1992; Zhang and Winoto, 1996). Briefly, HtTA-1-derived cells containing wild-type or mutant *rel* genes, and the parental HtTA-1 cell line (1×10^5 cells per 35 mm dish) were maintained in the presence of tetracycline (2 μg per ml). Cells were induced for Rel protein expression upon the removal of the drug for 48 h and treated for 16 h with cycloheximide (CHX, 30 μg per ml), alone or together with TNFα (Sigma; 1000 units per ml) or with an anti-Fas antibody (Calbiochem, 1 μg per ml).

For cell death assays in Jurkat T-cells (clone E6.1, ATCC), cells were stimulated with PMA (50 ng per ml) plus ionomycin (1 μM) for 1, 4 or 8 h, or incubated in the presence of 0.5% DMSO as a control (time 0). Cells (1×10^5 per well) were then incubated in 96-well microtiter plates in the presence 100 ng per ml of anti-Fas monoclonal antibody (CH11, PanVera Labs) for 16 h (Armstrong *et al*, 1996).

Crystal violet staining

Cell resistance to apoptosis was quantitated using a modified crystal violet staining assay (Zhang and Winoto, 1996). At 16 h after cell treatment with CHX, alone or together with TNFα or anti-Fas antibodies, the cells were washed twice with PBS, fixed in formaldehyde-saline (10% formaldehyde, 0.9% NaCl), and washed twice with PBS at room temperature. The surviving cells were stained with crystal violet for 5 min at room temperature (10% ethanol, 0.05% NaCl, 0.35% formaldehyde, 0.15% crystal violet dye in PBS). The cells were washed five times with PBS and the dye was eluted with a solution of 1% SDS in PBS. The optical density of the eluate was determined at 595 nm. Relative cell resistance to apoptosis represents the optical density ratio of (TNFα + CHX)- or (anti-Fas + CHX)-treated cells over that of CHX-treated cells.

Immunofluorescence

Cells from the HtTA-v-Rel48 and HtTA-CCR43 clones were seeded onto coverslips and induced for v-Rel or c-Rel expression upon the removal of tetracycline for 48 h. Cells were fixed in 4% paraformaldehyde and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Cells were washed with PBS containing 0.02% Triton X-100 and 10% FBS, followed by incubation with anti-Rel antibody Ab#3 for 1 h at room temperature. Cells were stained with a fluorescein-conjugated goat anti-rabbit secondary antibody (Cappel; 1 : 100 dilution). Nuclei were stained with the intercalating dye Hoechst 33258. Coverslips were mounted in the presence of 2% para-phenylenediamine (Sigma). Cells were photographed at a magnification of 600 ×.

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