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Mutations in the v-Rel Transactivation Domain Indicate Altered Phosphorylation and Identify a Subset of NF-κB-Regulated Cell Death Inhibitors Important for v-Rel Transforming Activity

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Consistent with the constitutive activation of Rel/NF-κB in human hematopoietic tumors, the v-Rel oncoprotein induces aggressive leukemia/lymphomas in animal models. v-Rel is thus a valuable tool to characterize the role of Rel/NF-κB in cancer and the mechanisms involved. Prior studies by our group identified a serine-rich domain in v-Rel that was required for biological activity. Here, we investigated the molecular basis for the transformation defect of specific serine mutants. We show that the transforming efficiency of these mutants in primary lymphoid cells is correlated with their ability to mediate κB site-dependent transactivation and with specific changes in phosphorylation profiles. Interestingly, coexpression of the death antagonists Bcl-xL and Bcl-2 significantly increased their oncogenicity, whereas other NF-κB-regulated death inhibitors showed little or no effect. The fact that a subset of apoptosis inhibitors could rescue v-Rel transactivation mutants suggests that their reduced transcriptional activity may critically affect expression of defined death antagonists essential for oncogenesis. Consistent with this hypothesis, we observed selection for high endogenous expression of Bcl-2-related death antagonists in cells transformed by weakly transforming v-Rel mutants. These results emphasize the need for Rel/NF-κB to efficiently activate expression of a subset of antiapoptotic genes from the Bcl-2 family to manifest its oncogenic phenotype.

The Rel/NF-κB family of transcription factors plays a key role in regulating immune and inflammatory responses and also participates in the control of cell proliferation and apoptosis, two functions that are critical in cancer. The implication of Rel/NF-κB factors in malignancy was initially suggested by the acute oncogenicity of their viral derivative, the v-Rel oncoprotein. v-Rel induces aggressive and fatal lymphomas in chickens and transgenic mice. This phenotype can be accurately reproduced in vitro through malignant transformation of primary splenic lymphoid cells. Consistent with the oncogenic phenotype of v-Rel, cellular *rel* and *nf-κb* genes are amplified, rearranged, overexpressed, and/or constitutively activated in human lymphoma, leukemia, myeloma, and Hodgkin's disease (reviewed in reference 49). Aberrant *rel*/*nf-κb* genes and/or activity are also observed in solid tumors, including lung, breast, and colon carcinomas. Moreover, *rel*/*nf-κb* genes serve as essential mediators for transformation by oncogenes such as *bcr-abl*, Ha-*ras*, and *RET* (20, 37, 41, 50). These observations highlight the importance of elucidating how Rel/NF-κB functions in oncogenesis.

The inhibition of apoptosis is a common and important feature of malignant cell transformation. Increased cell survival is conducive to neoplasia and also promotes the resistance of tumor cells to chemotherapeutic treatment. In this regard, Rel/NF-κB was implicated in promoting cell survival in response to a wide variety of death-inducing stimuli, including genotoxic agents, although in some situations NF-κB was attributed a proapoptotic activity (1, 4, 26, 59, 67, 75; reviewed in references 3 and 60). The protective activity of Rel/NF-κB is dependent on RNA and protein synthesis and was attributed to its ability to transactivate expression of death-suppressing genes (64, 67). The list of NF-κB target genes that have been reported to date is extensive and includes a number of cell death antagonists (reviewed in references 3 and 46). Among them are the Bcl-2-family members Bcl-xL, Bcl-2, and Bfl-1/A1, the caspase inhibitors c-IAP1, c-IAP2, and XIAP, the zinc finger protein A20, and the tumor necrosis factor (TNF) receptor-associated factors TRAF1 and TRAF2 (7, 10, 11, 15, 16, 25, 31, 33–35, 55, 61, 62, 68, 79, 82). A variant of the immediate-early response gene *iex-1* was also reported to belong to this category, although its role in apoptosis has been the subject of controversy (2, 56, 57, 76; B. Rayet and C. Gélinas, unpublished data).

The mechanism through which Rel/NF-κB factors function in oncogenesis and the cellular genes that they regulate during this process remain open questions. Among the v-Rel target genes that were recently reported to be involved in cell transformation are interferon-responsive factor 4 (IRF-4), the c-Fos and c-Jun components of the AP-1 transcription factor, the chicken inhibitor-of-apoptosis (IAP) homologue, and HMG14, whose product may influence gene transcription in a global fashion (21, 28, 30, 66, 79). The cell cycle progression factor cyclin D1 was reported to be regulated by NF-κB and is probably involved in cell transformation involving Bcl-3 proteins (27, 72). The induction of death-inhibitory genes by v-Rel is likely to be an important and early event in the transformation

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process, as primary chicken lymphoid cells rapidly undergo apoptosis in culture unless a death-inhibitory program is activated by v-Rel (8, 45, 74, 78, 79, 83). v-Rel therefore provides a valuable tool to address the role of NF-κB in oncogenesis and the cellular genes involved in the physiologically relevant context of primary lymphoid cells.

Prior studies from this laboratory, aimed at defining the functional domains of v-Rel necessary for its transforming potential, identified a serine-rich region in its C terminus that was necessary for its transcriptional and biological activities (8). In this analysis, defined Ser-to-Ala mutations were reported to abolish the transforming and antiapoptotic activities of v-Rel, and both activities were restored by most Ser-to-Asp substitutions. However, Ser-to-Asp mutant D10 (S438,439D) displayed wild-type transactivation ability but failed to block apoptosis and to transform cells, suggesting possible changes in the specificity of target gene activation.

Here, we have investigated the molecular basis for the oncogenic defect of this and other v-Rel transactivation mutants by testing whether coexpression of NF-κB-regulated cell death inhibitors would rescue their impaired transforming phenotypes. Surprisingly, our experiments revealed anomalies in this and three other mutants reported in that study (9). We describe the corrected phenotypes of the mutants and show that coexpression of a defined subset of NF-κB-regulated cell death antagonists can efficiently rescue the partially transforming phenotype of v-Rel transactivation mutants, whereas others had little or no effect. We also show that a conservative Arg-to-Lys mutation in the DNA-binding domain of v-Rel severely impaired its DNA-binding activity and specificity and abolished its biological function. In contrast, the S438,439D substitution conferred strong transcriptional and oncogenic phenotypes akin to that of a constitutively active v-Rel protein. This coincided with distinct differences in its phosphopeptide profile, suggesting that phosphorylation of the C-terminal domain of v-Rel may positively regulate its transcriptional activity.

Overall, our findings emphasize the important role of v-Rel-mediated transactivation for its oncogenic potential and suggest that the death program that v-Rel must suppress to transform lymphoid cells is efficiently inhibited by Bcl-xL and Bcl-2.

MATERIALS AND METHODS

Plasmids. Alanine (A6, A7, A10, A6.7, and A6.7.10) and aspartate (D10) point mutants of v-Rel were described previously (8). The R30K, R32K, S438,439D, S438,439T, T6.7.10, and D6.7.10 mutants were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). Mutations were confirmed by DNA sequence analysis with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) or by automated DNA sequencing (Molecular Resource Facility, New Jersey Medical School, Newark, N.J.). Rel proteins were expressed in vitro under the control of the T7 promoter in the pALTER-1 plasmid (Promega Corp., Madison, Wis.). Rel proteins expressed in vivo were driven by the cytomegalovirus (CMV) immediate-early promoter of pJDCMV19SV (19) for transactivation assays or by the spleen necrosis virus long terminal repeat promoter of pJD214 (18) for spleen cell transformation assays.

The pMH105 internal ribosome entry site (IRES) vector was a gift from M. Hannink (University of Missouri). Individual death inhibitors were amplified by PCR from human cDNAs (*bcl-xl*, *bfl-1*, *c-iap1*, *c-iap2*, *xiap*, *traf-1*, and *a20*). Reverse transcription (RT)-PCR was used to amplify *iex-1* from human Jurkat T cells treated with phorbol 12-myristate 13-acetate plus ionomycin. The chicken *bcl-2* cDNA cloned in the pMH105 IRES vector was a gift from T. Gilmore (73). pJD214-IRES-*bcl-xl* was constructed by cloning an IRES sequence from the encephalomyocarditis virus into the unique *Cla*I site of pJD214, followed by insertion of *bcl-xl* cDNA into a unique *Bgl*II site introduced upstream of *Cla*I. pIL-6-κB-Luc was derived from pIL-6-κB-CAT (44) and expressed a luciferase reporter gene under the control of three NF-κB DNA-binding motifs derived from the interleukin-6 (IL-6) promoter (a gift from J. Suh and A. B. Rabson, Center for Advanced Biotechnology and Medicine). The *Renilla* luciferase plasmid pRL-null was used as an internal control (Promega Corp., Madison, Wis.). Plasmid SW253 encodes a replication-competent RevA helper virus (71).

Transient transfection and luciferase assays. Human Tera-2 cells (embryonic carcinoma; HTB-106; American Type Culture Collection, Manassas, Va.) were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, penicillin (100 U per ml), and streptomycin (100 μg per ml; Gibco Life Technology, Grand Island, N.Y.). Cells were maintained at 37°C in a 5% CO_2 incubator. Cells (2×10^5) were seeded in six-well plates in 2 ml of complete medium. On the following day, cells were cotransfected with wild-type or mutant CMV-v-*rel* (1.2 μg), IL-6κBLuc (0.8 μg), and pRL-null DNA (0.015 μg) with Gene Porter (10 μl; Gene Therapy Systems, San Diego, Calif.). Alternatively, cells were transfected with wild-type or mutant CMV-v-*rel* plasmid DNAs (0.75 μg) or an empty CMV vector, an IL-6-κB-luciferase reporter (0.4 μg), and a pRL-null internal luciferase control (0.0125 μg), with the Mirus Trans-IT-LT1 reagent. At 48 h posttransfection, cells were harvested, and dual luciferase assays were performed (Promega Corp., Madison, Wis.).

Transformation of primary chicken spleen cells. Primary spleen cells from 3-week-old chickens (Charles River SPAFAS, North Franklin, Conn.) were transformed by electroporation with spleen necrosis virus-derived retroviral vector DNAs (20 μg) and SW253 helper virus DNA (10 μg), essentially as described (8, 42). The efficiency of spleen cell transformation was significantly improved by carefully screening serum lots used in these assays. Where indicated, cells from individual reactions were divided into four soft agar dishes instead of one to avoid nutrient depletion due to high colony density and further improve transformation efficiencies.

DNA binding assays. Nuclear extracts were prepared as described (58). Briefly, transformed spleen cells from a confluent 10-cm dish (≈2×10^7 cells) or human 293T cells transiently transfected with wild-type or mutant v-*rel* genes were washed several times in Tris-buffered saline and resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail [Boehringer, Mannheim, Germany]). Cells were incubated on ice for 15 min before addition of NP-40 to a final concentration of 0.625%. After centrifugation, nucleus-containing pellets were resuspended in buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail), vortexed vigorously, and rotated for 15 min at 4°C. After centrifugation, supernatants containing nuclear extracts were collected.

Nuclear extracts (3 μg) were incubated with a ^{32}P-labeled double-stranded IL-6-κB oligonucleotide probe (4×10^4 cpm) in 10 mM Tris (pH 7.5)–50 mM NaCl–7.5 mM $MgCl_2$–1 mM EDTA–5% glycerol–5% sucrose–0.1% NP-40–0.5 μg of poly(dI-dC)–5 mM dithiothreitol–10 μg of bovine serum albumin. Where indicated, anti-v-Rel-specific antibody 1967 was added to supershift the complex (1 μl) (77). DNA-protein complexes were analyzed on 5% native polyacrylamide gels. Where indicated, DNA binding reactions were performed with wild-type or mutant v-Rel proteins produced by in vitro translation with a TNT coupled wheat germ extract system (Promega) and pAlter-1 expression vectors driven by the T7 promoter. The DNA-binding efficiencies of the R32K and R30K mutants were compared to that of v-Rel on probes containing a wild-type IL-6-κB site, shown in italics, (GATCTGAATTCGT*GGGATTTTCCC*ACCTCTCCTT) or on G1→A or G1→T IL-6-κB site mutants (GATCTGAATTCGT*A*/*TGGATTTTC CC*ACCTCTCCTT).

Western blotting. Transformed spleen cells were lysed in 50 mM Tris (pH 8.0)–120 mM NaCl–0.5% NP-40–10% glycerol in the presence of protease inhibitor cocktail (Boehringer, Manheim, Germany). Quantitation of total protein concentration was done by the method of Bradford (6). Proteins (30 to 60 μg) were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Secondary antibody detection was performed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, N.J.). Antibodies used in this study were commercially available (Bcl-2 antibody sc-492 and TRAF-1 antibody sc-1831 were from Santa Cruz; Bcl-xL antibody 556361 was from Pharmingen, San Diego, Calif.).

RT-PCR. Total RNA (500 ng) extracted with RNAzol B (Tel-Test, Inc, Friendswood, Tex.) was subjected to RT-PCR with the Titan One-Tube RT-PCR system kit (Boehringer, Manheim, Germany). PCR products were separated on 1% agarose gels.

Northern blotting. Total RNA (25 μg) was separated on 1% formaldehyde–agarose gels and transferred to Hybond-N nylon membranes (Amersham, Piscataway, N.J.). Probes specific for chicken *bcl-xl*, *bfl-1*, or the β-actin gene were synthesized with random primers and Klenow enzyme in the presence of ^{32}P-radiolabeled dCTP and dGTP (NEN Life Science Products, Inc., Boston, Mass.). Blots were exposed to X-Omat film (Kodak, Rochester, N.Y.) and quantified with Image-Quant software (Molecular Dynamics).

Tryptic phosphopeptide analyses. v-Rel-transformed spleen cells (≈4 × 10^7) were washed three times and incubated for 1 h in medium lacking sodium phosphate prior to in vivo labeling with $^{32}P_i$ (1 mCi/ml; Amersham). After 2 h of incubation, dialyzed fetal bovine serum (5%; Gibco) was added, and incubation was continued for another 3 h. Cells were washed three times with phosphate-buffered saline and lysed in EBC buffer (50 mM Tris [pH 8], 120 mM NaCl, 0.5% NP-40, 10% glycerol) containing phosphatase inhibitors (10 mM β-glycerophosphate, 50 mM sodium fluoride, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 10 mM sodium phosphate dibasic) and complete protease inhibitor cocktail (Boehringer, Mannheim, Germany).

Wild-type and mutant v-Rel proteins were immunoprecipitated with anti-v-Rel antibody 1967 and resolved by SDS-PAGE. After transfer, nitrocellulose membranes were exposed for 5 to 12 h to Kodak X-Omat films. Radiolabeled v-Rel protein bands were excised from the membrane and treated with trypsin (10 μg; sequencing grade modified trypsin; Promega Corp., Madison, Wis.) in 50 mM ammonium bicarbonate for 2 h at 37°C, as described (38). An additional 10 μg of trypsin was added, and incubation was continued for 18 h. The resulting peptides were diluted with water (600 μl) and lyophilized. Pellets were resuspended in water (200 μl) and lyophilized again. Samples were resuspended in first-dimension pH 1.9 buffer (10 μl) (5, 65) and spotted on a thin-layer cellulose plate (Merck, Darmstadt, Germany). Electrophoresis in the first dimension was carried out in pH 1.9 buffer at constant 1,000 V (10 to 15 mA) for 1 h. Chromatography in the second dimension was performed in phospho chromatography buffer (5) for 8 h. Thin-layer chromatography plates were exposed to X-Omat films for 3 to 30 days.

RESULTS

A conserved R32K mutation dramatically impairs the DNA-binding activity of v-Rel and abolishes its oncogenicity. Prior studies identified a Ser-to-Asp mutant in the transactivation domain of v-Rel (mutant D10; S438,439D) that displayed wild-type transcriptional activity but failed to malignantly transform primary lymphoid cells. These results suggested that this mutant might be unable to activate the entire collection of genes that v-Rel activates, failing to induce some that are necessary for cell death inhibition and cell transformation (8). We sought to investigate this hypothesis by testing whether coexpression of this mutant with the NF-κB-regulated cell death inhibitor *bcl-xl* would rescue its oncogenic defect.

During the course of these experiments, in-depth analysis of mutant D10 revealed a secondary R32K mutation in the JD214 D10 construct that was used in transformation assays (9). Since this mutation was not present in the D10 mutant that was cloned in other expression vectors previously used to analyze its DNA-binding and transactivation abilities, we investigated the effect of this conservative substitution on the oncogenic activity of v-Rel. As shown in Fig. 1A, the original D10 mutant bearing the additional R32K mutation failed to transform primary splenic lymphoid cells, consistent with the phenotype reported previously (8). However, when isolated from the S438,439D substitution, the R32K mutation alone was sufficient to abolish the transforming ability of v-Rel (Fig. 1A). This suggested that the oncogenic defect reported for mutant D10 most likely derived from the spontaneous R32K mutation rather than from the S438,439D substitution. Analysis of a v-Rel mutant carrying the S438,439D substitution alone confirmed this hypothesis, as this mutant showed strong transforming activity (Fig. 1A). In fact, its oncogenic activity was

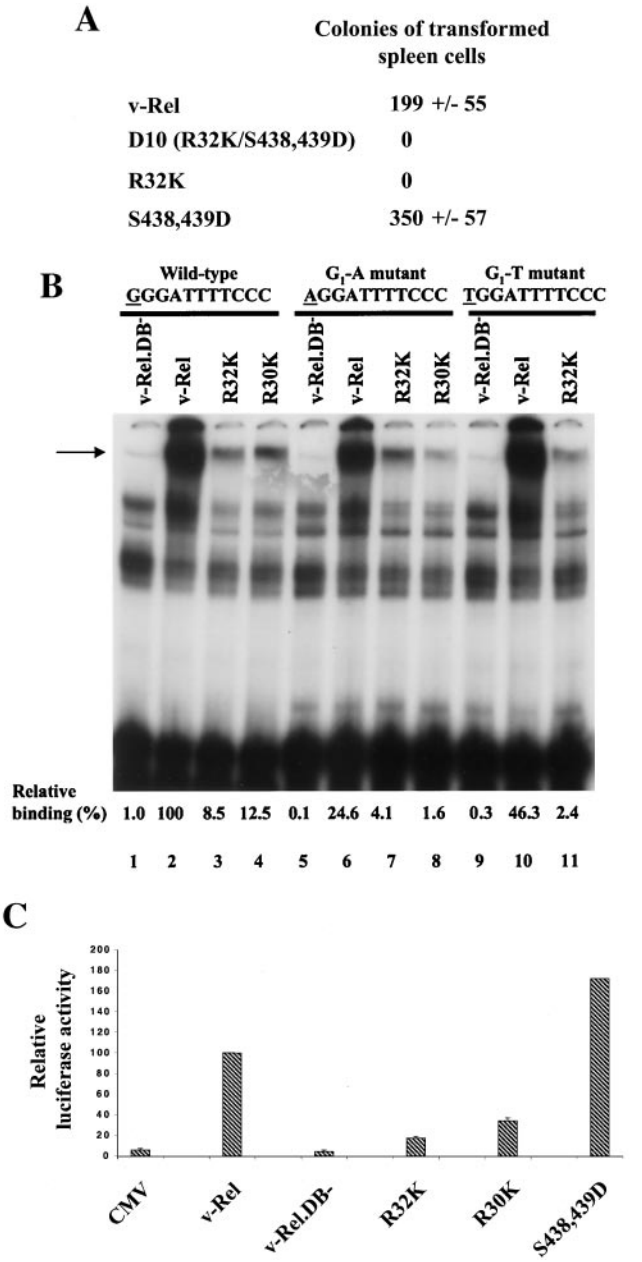


FIG. 1. Biological activity of v-Rel mutants D10 and R32K. (A) Transformation of primary spleen cells by mutant v-Rel proteins. Spleen cells from 3-week-old chickens were cotransfected with the indicated pJD214 retroviral vectors (20 μg) and SW253 helper virus DNA (10 μg) and cultured for 3 days before transfer into soft agar. Transformed colonies were scored 10 to 14 days after transfection. Results represent the average of three independent experiments, each performed in duplicate. (B) Binding efficiency of v-Rel mutant R32K to wild-type or mutant IL-6-κB DNA sites. v-Rel proteins produced by in vitro translation were incubated with wild-type or mutant double-stranded, ^{32}P-labeled IL-6-κB oligonucleotide probes. DNA-protein complexes were analyzed on 5% native polyacrylamide gels. DNA-binding activity was determined with ImageQuant analysis software. (C) Transcriptional activity of wild-type and mutant v-Rel proteins. Tera-2 cells (2 × 10^5) were transfected with Gene Porter with CMV-v-*rel* plasmid DNAs (1.2 μg), IL-6-κB-luciferase reporter (0.8 μg), and the pRL-null internal luciferase control (0.015 μg). At 48 h posttransfection, cells were harvested, and dual luciferase assays were performed. Results show the average of three independent experiments, each performed in duplicate.

significantly greater than that of wild-type v-Rel. Together, these results demonstrated that conservative substitution of a lysine for the arginine at position 32 in v-Rel is sufficient to abolish its biological activity.

Interestingly, the secondary R32K mutation maps within the $R_{27}XXR_{30}XR_{32}XXC$ motif that our laboratory previously implicated in v-Rel DNA binding (32). R32 in v-Rel is predicted to localize at the tip of the DNA recognition loop, according to the X-ray crystal structure of its homologues c-Rel, p50/NF-κB1, p52/NF-κB2, and RelA (12, 14, 17, 23, 29, 43). The corresponding arginine residue in these cellular proteins was shown by site-specific photo-cross-linking and X-ray crystallography to contact the G1 position in the NF-κB DNA recognition site. The two nitrogen groups were found to interact with the O-6 and N-7 positions of the guanine moiety (12, 14, 17, 23, 29, 36, 43). We therefore tested whether the conserved R32K substitution affected the ability of v-Rel to bind to the κB DNA recognition site with a probe derived from the IL-6 promoter (GGGATTTTCCC; Fig. 1B).

In light of the documented participation of the neighboring Arg 57 in p50/NF-κB1, Arg 33 in RelA, Arg 21 in c-Rel, and Arg 52 in p52/NF-κB2 in DNA contact, a corresponding R30K mutant was created in v-Rel as a control. In gel retardation assays, both the R32K and R30K mutant proteins produced by in vitro translation showed substantially reduced DNA-binding activity compared to wild-type v-Rel, although binding was not completely abolished (Fig. 1B, compare lanes 3 and 4 to lane 2). In contrast, a triple mutant, R27,30,32, that our laboratory previously described to be defective for DNA binding, failed to show DNA-protein complex formation (v-Rel.DB$^-$, originally known as mutant 2721 [32]) (Fig. 1B, lane 1). Western blot analysis verified that equal amounts of proteins were used in these assays, and supershift experiments confirmed the presence of wild-type v-Rel or the R32K and R30K mutants in DNA-bound complexes (data not shown).

The dramatically reduced DNA-binding and transforming activities resulting from the R32K mutation were surprising given its conservative nature. Since the corresponding arginine in other Rel/NF-κB family members was suggested to be a determinant of DNA-binding specificity (12, 14, 17, 23, 29, 43), we tested the effect that substitution of adenine for the G1 position in the NF-κB DNA motif would have on the DNA-binding activity of mutant R32K. As shown in Fig. 1B, the binding of mutant R32K was less affected by the G1→A substitution (twofold decrease) than wild-type v-Rel (fourfold decrease) or mutant R30K whose binding was impaired eightfold (compare lanes 6, 7, and 8 to lanes 2, 3, and 4). The latter observation is consistent with the predicted interaction of R30 with the G2 position in the NF-κB site.

Since the binding of R32K was affected to a lesser degree by the G1→A substitution, we next asked whether a G1→T replacement would have a similar effect. Interestingly, the opposite effect was observed. Whereas the binding of wild-type v-Rel was decreased twofold, the R32K mutant was significantly impaired for binding to the T1-substituted probe (fourfold decrease; compare lanes 10 and 11 to lanes 2, 3, 6, and 7). These results are in agreement with a recent study showing more efficient binding of wild-type NF-κB proteins to G1→T than G1→A κB DNA sites (63).

When tested for transactivation, the v-Rel mutants R32K and R30K showed modest activity compared to v-Rel (20 and 30%, respectively), whereas the DNA-binding-negative mutant v-Rel.DB$^-$ had no activity (Fig. 1C). This was correlated with the poor DNA-binding activity of mutants R32K and R30K and their failure to transform primary lymphoid cells. Interestingly, the transcriptional ability of mutant S438,439D was significantly greater than that of v-Rel and was correlated with its markedly higher oncogenic potential (Fig. 1A and C). These data demonstrate that in spite of its conservative nature, the R32K mutation significantly impaired the DNA-binding activity of v-Rel, hence affecting its transcriptional and oncogenic activities. These findings also explain why the previously described oncogenic activity of v-Rel mutant D10 was abolished (8, 9). Moreover, the results of our DNA-binding studies imply a critical role for R30 and R32 in the specificity and affinity of v-Rel for its target recognition motifs and justify their absolute conservation in the DNA recognition loop of all members of the Rel/NF-κB family in vertebrates and *Drosophila melanogaster*.

Substitution of negatively charged aspartic acids for serines 438 and 439 in v-Rel dramatically increases its transcriptional and oncogenic potentials and alters its phosphorylation profile. Another important finding from the experiments described above was that, when separated from the spontaneous R32K mutation, the S438,439D substitution in the transactivation domain of v-Rel significantly enhanced its transforming activity in primary chicken lymphoid cells (Fig. 1A). In these assays, colony formation in soft agar by mutant S438,439D was 175% that of wild-type v-Rel. This sharp increase in oncogenic activity was correlated with its approximately 1.7-fold higher transcriptional activity compared to v-Rel (Fig. 1C). These results indicated that the presence of negatively charged groups at positions 438 and 439 in the transactivation domain of v-Rel enhanced its transcriptional and oncogenic activities. This raised the possibility that negatively charged residues at these positions, such as those conferred by serine phosphorylation, may positively regulate v-Rel activity.

To address this issue, we compared the transforming potential of v-Rel mutants with phosphorylatable threonine substitutions for serines 438 and 439 with that of mutants with alanines or negatively charged aspartic acids (S438,439T; S438,439A; and S438,439D). During the course of these studies, we significantly improved the efficiency of lymphoid cell transformation by carefully screening the serum lots used in these assays. For instance, the number of transformed colonies obtained with v-Rel in Fig. 1A represents a fourfold increase over that obtained in prior studies (8, 32). Moreover, when individual transforming reactions were divided into four soft agar dishes to avoid nutrient depletion and acidification due to high colony density, wild-type v-Rel could yield on average a total of 435 colonies per reaction, which represents a further twofold increase (Fig. 2A).

Under these conditions, mutant S438,439D displayed twice the transforming activity of wild-type v-Rel, consistent with the results described above (Fig. 1A). Importantly, the Ser-to-Ala mutant lacking a negative charge at positions 438 and 439 was much less efficient than mutant S438,439D, showing a transformation efficiency equivalent to that of wild-type v-Rel or mutant S438,439T. Since replacement of serine by aspartic acid can mimic phosphorylation, we used gel retardation and tran-

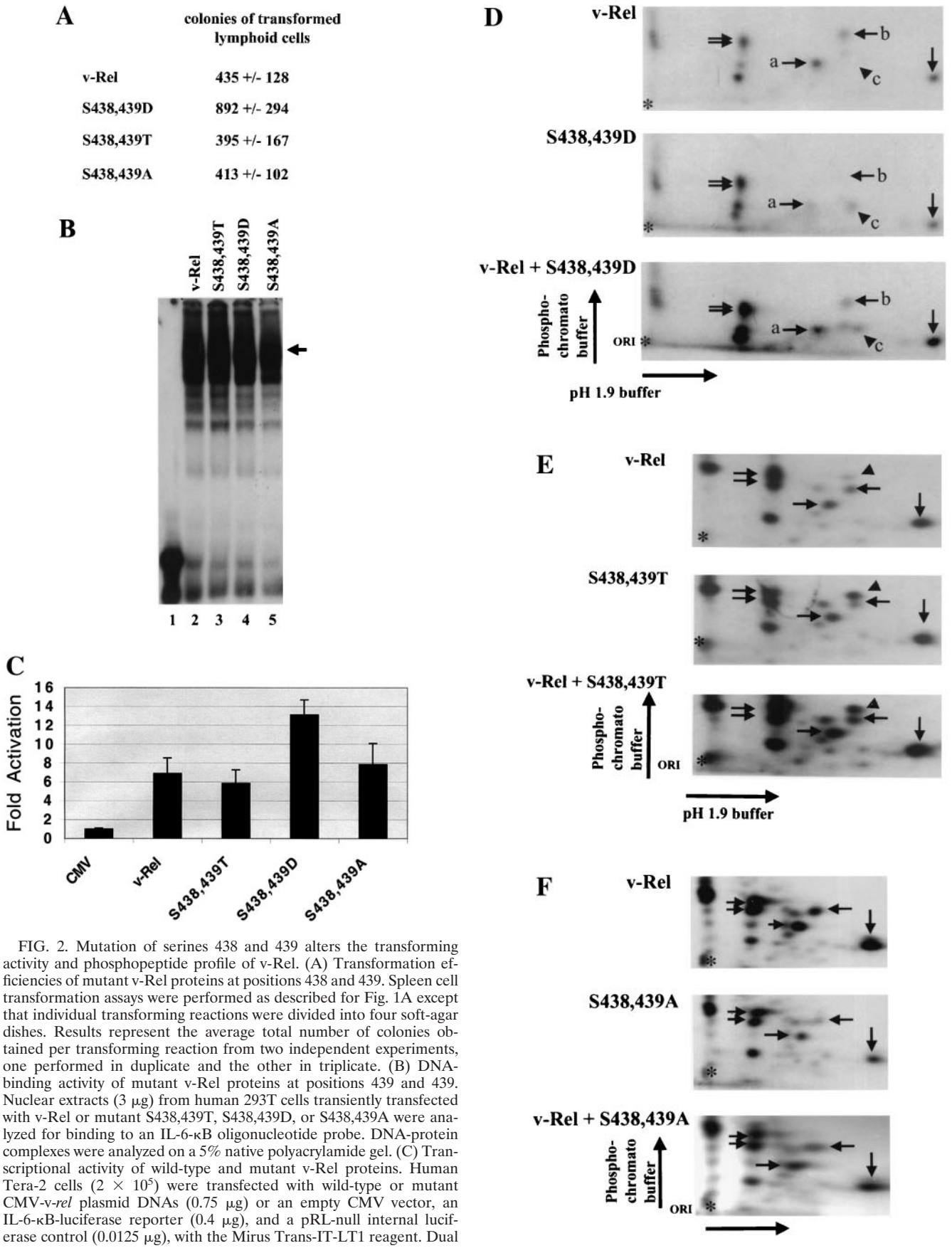


FIG. 2. Mutation of serines 438 and 439 alters the transforming activity and phosphopeptide profile of v-Rel. (A) Transformation efficiencies of mutant v-Rel proteins at positions 438 and 439. Spleen cell transformation assays were performed as described for Fig. 1A except that individual transforming reactions were divided into four soft-agar dishes. Results represent the average total number of colonies obtained per transforming reaction from two independent experiments, one performed in duplicate and the other in triplicate. (B) DNA-binding activity of mutant v-Rel proteins at positions 439 and 439. Nuclear extracts (3 μg) from human 293T cells transiently transfected with v-Rel or mutant S438,439T, S438,439D, or S438,439A were analyzed for binding to an IL-6-κB oligonucleotide probe. DNA-protein complexes were analyzed on a 5% native polyacrylamide gel. (C) Transcriptional activity of wild-type and mutant v-Rel proteins. Human Tera-2 cells (2×10^5) were transfected with wild-type or mutant CMV-v-*rel* plasmid DNAs (0.75 μg) or an empty CMV vector, an IL-6-κB-luciferase reporter (0.4 μg), and a pRL-null internal luciferase control (0.0125 μg), with the Mirus Trans-IT-LT1 reagent. Dual

sient transactivation assays to evaluate the possible effects on v-Rel DNA binding and/or transcriptional activities. As shown in Fig. 2B, mutation of serines 438 and 439 had no effect on the DNA-binding activity of v-Rel. In contrast, mutant S438,439D displayed increased transcriptional activity compared to its serine, alanine, and threonine counterparts (Fig. 2C). In conjunction with the spleen transformation assays shown in Fig. 2A, these results indicate that the presence of negative charges at positions 438 and 439 in the transactivation domain of v-Rel significantly enhanced its transcriptional and oncogenic activities without altering its binding to DNA.

Next, we examined the effect of these substitutions on the overall phosphorylation profile of v-Rel in two-dimensional tryptic phosphopeptide analyses. Chicken spleen cells transformed with v-Rel or mutant S438,439D were labeled in vivo with [^{32}P]orthophosphate, followed by immunoprecipitation with an anti-v-Rel-specific antibody. Radiolabeled v-Rel and S438,439D protein bands were isolated and subjected to digestion with trypsin, and soluble peptides were resolved by two-dimensional separation. A fraction of the soluble tryptic peptides was mixed before resolution on a separate thin-layer cellulose plate to verify the positions of individual phosphopeptides relative to those of v-Rel (Fig. 2D). Consistent with its enhanced activity over the wild type, phosphopeptide analysis of mutant S438,439D showed discrete differences in phosphorylation profile compared to v-Rel that paralleled its distinct transcriptional and biological activities. For example, the disappearance of phosphopeptide spots a and b was accompanied by the appearance of a new phosphopeptide spot in the S438,439D mutant profile compared to that of wild-type v-Rel (arrowhead c). Although the identity of these phosphopeptides is not known at present, the results demonstrate that the presence of a negative charge at positions 438 and 439 modifies the overall phosphopeptide profile of v-Rel.

Next, we compared the phosphorylation profile of v-Rel to those of mutants S438,439T and S438,439A, with threonines or nonphosphorylatable alanines at positions 438 and 439 (Fig. 2E and F). In agreement with its wild-type transforming phenotype, the phosphopeptide profile of mutant S438,439T was similar to that of v-Rel, although the relative intensity of some phosphopeptides varied (e.g., Fig. 2E, arrowhead). Despite differences in the intensity of some phosphopeptides, the profile of mutant S438,439A was also similar to that of v-Rel, consistent with its ability to transform cells as efficiently as the wild type (Fig. 2F). In conjunction with our functional analysis of these mutants, these results suggest that although it is not absolutely needed for function, the presence of negative charges at positions 438 and 439 in the transactivation domain of v-Rel can positively regulate its transcriptional and oncogenic activities. This may result from changes in the overall phosphorylation profile of v-Rel, its structural conformation, or both.

The transforming activity of v-Rel mutants is correlated with their transactivation function. Anomalies that we discovered during the course of our phenotypic analysis of previously described v-Rel transactivation mutants A10 (S438,439A), A6.7 (S398,399,402A), and A6.7.10 (S398,399,402,438,439A) (8, 9) (Fig. 3A) led us to reevaluate the oncogenic activity of these mutants and revisit its correlation with their transcription potential. Their transactivation capacity was tested in transient-transfection assays with an IL-6-κB-luciferase reporter and compared to that of v-Rel and the previously described A6 (S398,399A) and A7 (S402A) mutants (8).

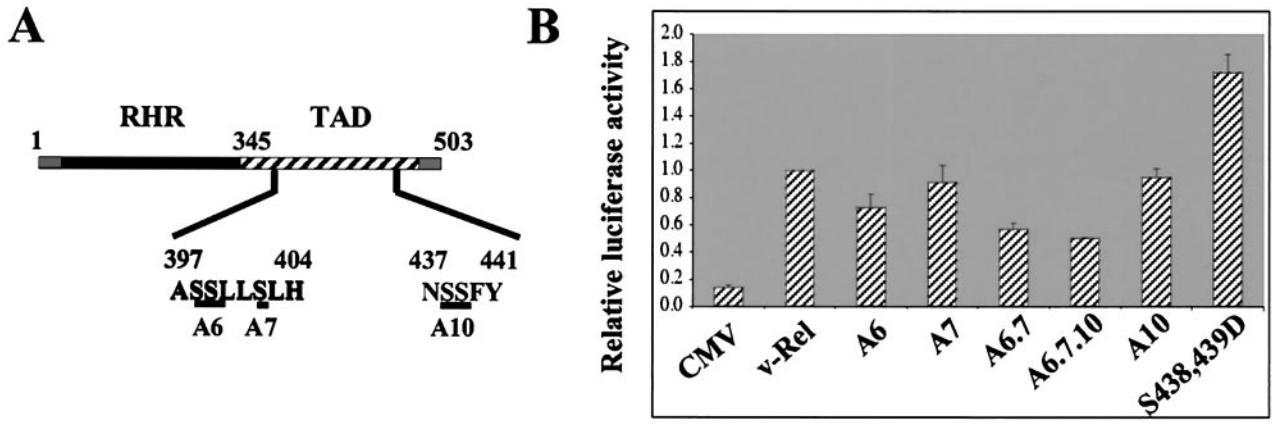
While point mutations A6, A7, and A10 marginally affected the transactivation function of v-Rel, mutants A6.7 and A6.7.10 reproducibly showed 40% and 50% decreases in transactivation ability, respectively (Fig. 3B). Importantly, retrovirus-mediated gene transfer into primary chicken spleen cells revealed an absolute correlation between the transactivation potential of all mutants and their oncogenic activity (Fig. 3C). Indeed, while the Ser-to-Ala A6.7 and A6.7.10 mutants that displayed a 40 to 50% reduction in transcriptional activity could transform cells, they yielded 40 to 60% fewer transformed colonies than their wild-type counterpart. Their significantly impaired biological function did not result from diminished DNA-binding activity, as all mutants showed wild-type DNA-binding potential (Fig. 3D).

Combined, these findings further demonstrate that the transcriptional activity of v-Rel is precisely correlated with its oncogenic potential. The observation that a D6.7.10 mutant with combined aspartic acid substitutions at these positions showed 10 to 20% greater transforming activity than mutant S438,439D further suggests that the presence of a negative charge in the v-Rel transactivation domain is correlated with maximal transforming activity (data not shown). The data also indicate that a certain threshold of transcriptional activity is necessary for efficient cell transformation by v-Rel.

Since mutation of serines 438 and 439 to aspartic acid led to a significant gain of function that was correlated with changes in the overall phosphopeptide profile of v-Rel, we postulated that the partial loss of function of mutant A6.7.10 may also be associated with alterations in its overall phosphorylation profile. In agreement with this hypothesis, two-dimensional tryptic phosphopeptide analysis revealed a number of differences between mutant A6.7.10 and v-Rel that paralleled its reduced transcriptional and oncogenic potentials (Fig. 4). Many phosphopeptides were significantly reduced or eliminated in the A6.7.10 mutant. Phosphoamino acid analysis of a combined T6.7.10 mutant with threonine residues substituted for the serines in question revealed a small increase in the ratio of phosphothreonine to phosphoserine in mutant T6.7.10 (15.3%) compared to v-Rel (9.7%) (data not shown).

While these results may provide some indication that some of the serines targeted by these mutations might be phosphor-

luciferase assays were performed at 48 h posttransfection. The average of two independent experiments, each performed in duplicate, is shown. (D to F) Tryptic phosphopeptide analysis of in vivo ^{32}P-labeled wild-type and mutant v-Rel proteins. Mutants were analyzed individually by two-dimensional separation. To compare the positions of phosphopeptides relative to those of v-Rel, a fraction of the soluble tryptic peptides from wild-type and mutant v-Rel proteins was mixed in a 1:1 ratio before resolution on a separate thin-layer cellulose plate (bottom panels). (D) S438,439D; (E) S438,439T; (F) S438,439A.



C

	Colonies of transformed spleen cells
v-Rel	222 +/- 42
A6	204 +/- 10
A7	237 +/- 96
A6.7	130 +/- 50
A6.7.10	80 +/- 63
A10	312 +/- 51
S438,439D	374 +/- 49

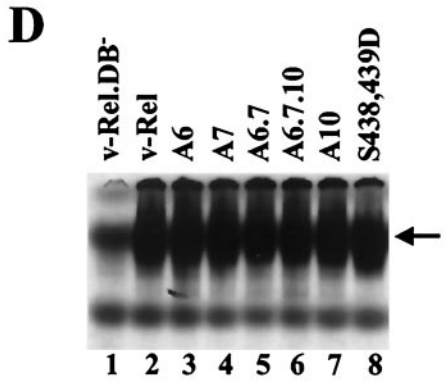


FIG. 3. Effects of Ser-to-Ala mutations on v-Rel function. (A) Schematic representation of serine mutations in the transactivation domain of v-Rel. (B) Transcriptional activity of wild-type and mutant v-Rel proteins. Tera-2 cells (2×10^5) were transfected with Gene Porter with CMV-v-*rel* plasmid DNAs (1.2 μg), IL-6-κB-luciferase reporter (0.8 μg), and pRL-null internal luciferase control (0.015 μg). At 48 h posttransfection, cells were harvested, and dual luciferase assays were performed. Results represent the average of three independent experiments performed in duplicate. (C) Transforming activity of wild-type and mutant v-Rel proteins expressed from the JD214-IRES vector in primary chicken spleen cells. The average of three independent experiments performed in duplicate is shown. (D) In vitro DNA-binding activity of mutant v-Rel proteins. v-Rel proteins were produced by in vitro translation and assayed for binding to a ^{32}P-labeled IL-6-κB oligonucleotide probe. DNA-protein complexes were analyzed on a 5% native polyacrylamide gel.

ylated in v-Rel, the sensitivity of such assays with whole proteins is limited. Consequently, we do not rule out the possibility that the differences in the phosphopeptide profiles that we have observed with v-Rel mutants may result from changes in the phosphorylation of other residues as a consequence of these mutations. While future studies will be needed to precisely map the phosphoacceptor sites in v-Rel, overall our data indicate that phosphorylation may serve to positively modulate its activity.

Cell death inhibitor *bcl-xl* complements the oncogenic defect of partially transforming v-Rel mutants. v-Rel was previously shown to display antiapoptotic activity, and this function was closely correlated with its oncogenic potential (3, 8, 45, 74, 78, 79, 83). In light of these observations, we asked whether the partially transforming phenotype of Ser-to-Ala v-Rel mutants A6.7 and A6.7.10 was caused, at least in part, by diminished antiapoptotic activity. To this end, we investigated whether coexpression of the NF-κB-regulated cell death inhibitor *bcl-xl*, which our laboratory and others previously characterized as a Rel/NF-κB target gene (10), could complement their oncogenic defect. The rationale for this approach was that lymphoid cells expressing a temperature-sensitive mutant of v-Rel could be rescued from undergoing apoptosis by coexpression of the cell death inhibitor *bcl-2* (73).

Spleen necrosis virus-derived retrovirus vector JD214 was used to coexpress v-Rel mutants under the control of the long terminal repeat promoter, along with *bcl-xl* with an internal ribosome entry site (pJD214-v-Rel-IRES-*bcl-xl*). When analyzed in parallel with the mutants alone (Fig. 3C), coexpression of mutant A6.7 or A6.7.10 along with *bcl-xl* restored nearly full oncogenic potential, equivalent to that of v-Rel (compare Fig. 5A and B to Fig. 3C). Western blot analysis confirmed proper expression of *bcl-xl* and v-Rel mutant A6.7.10 (Fig. 5C and data not shown). Importantly, *bcl-xl* failed to complement the transformation-defective phenotype of mutant R32K, which displayed minimal transactivation potential. This suggested once again that a minimum threshold of v-Rel transactivation capacity is needed for oncogenesis and that, while necessary for v-Rel-mediated transformation, expression of a cell death inhibitor is not sufficient to malignantly transform cells. However, since coexpression of the cell death antagonist *bcl-xl* was sufficient to restore wild-type transforming activity on v-Rel

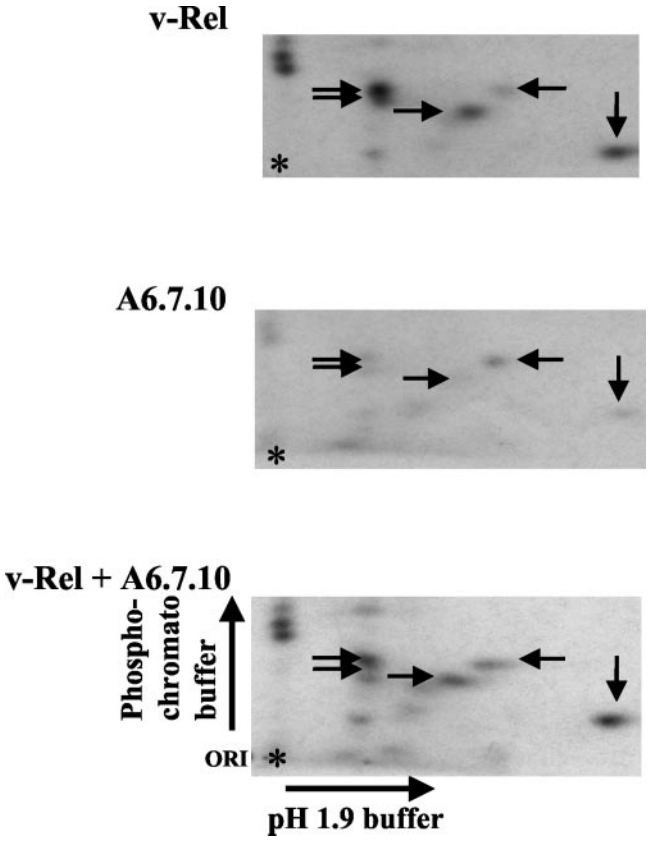


FIG. 4. Mutation of serines 398, 399, 402, 438, and 439 modifies the phosphorylation profile of v-Rel. Tryptic phosphopeptide analysis of in vivo ^{32}P-labeled wild-type v-Rel and mutant A6.7.10. Tryptic peptides from v-Rel and mutant A6.7.10 were analyzed separately (top panels) and in combination (bottom panel).

mutants A6.7 and A6.7.10, these results indicate that their reduced transcriptional activity may critically affect the activation of antiapoptotic genes essential for cell transformation.

A subset of NF-κB-regulated cell death inhibitors rescue the oncogenic defect of v-Rel transactivation mutant A6.7.10. The ability of *bcl-xl* to restore efficient cell transformation by mutant A6.7.10 led us to explore whether any or all of the other NF-κB-regulated death inhibitors that have been described to date could likewise complement its partially transforming phenotype. The MH105-IRES retroviral vector was used for these assays (a gift from M. Hannink). When expressed from this vector, both v-Rel and mutant A6.7.10 showed significantly reduced oncogenic activity in primary lymphoid cells compared to the pJD214 vector (compare Fig. 6A to Fig. 3C). This appeared to result from the significantly lower v-Rel expression levels from this vector (data not shown). Nevertheless, as shown in Fig. 6A, a subset of NF-κB-regulated cell death inhibitors could markedly improve the partially transforming phenotype of mutant A6.7.10, although they differed significantly in efficiency. The antiapoptotic genes *bcl-xl* and *bcl-2* were by far the most efficient in this assay. In contrast, coexpression of v-Rel or mutant A6.7.10 along with *c-iap1*, *c-iap2*, or a control luciferase gene led to a substantial decrease in transformation efficiency.

Expression of the various death inhibitors from the bicistronic virus was confirmed. Expression of the Bcl-2, Bcl-xL, and TRAF-1 proteins was easily detected in cells transformed by their coexpression with v-Rel or A6.7.10 (Fig. 6B, lanes 2, 3, 5, 6, 8, and 9). Since commercially available antibodies against Bfl-1, Iex-1, c-IAP2, Xiap, and A20 proved to be unsuitable to detect expression in immunoblots, cells were analyzed by RT-PCR. In all instances, appropriate expression of the coexpressed gene was confirmed (Fig. 6C). As expected, luciferase activity was detected in extracts from the rare cells transformed by the v-Rel-IRES-luciferase control but not in spleen cells transformed by v-Rel-IRES (Fig. 6D). Combined, these results show that different NF-κB-regulated cell death inhibitors do not exhibit equivalent efficacy in improving v-Rel-mediated transformation. This suggests that the apoptotic pathways inhibited by Bcl-xL and Bcl-2 are likely to be critical targets of the antiapoptotic activity of v-Rel during lymphomagenesis.

The reduced transactivation and oncogenic potentials of mutant A6.7.10, together with the complementation studies described above, led us to analyze some of the few transformed colonies that arose when mutant A6.7.10 was expressed alone for endogenous expression of cell death inhibitors from the *bcl-2* family. Interestingly, the levels of endogenous *bcl-xl* transcript and Bcl-2 protein were consistently elevated in three independent cell clones transformed by A6.7.10 alone compared to v-Rel-transformed cells, despite the fact that the transcriptional activity of mutant A6.7.10 was about 50% lower than that of v-Rel (Fig. 7, compare A6.7.10 IRES to v-Rel). This raised the possibility that cell transformation by A6.7.10 may involve selection for high endogenous expression of *bcl-2* family members *bcl-2* and/or *bcl-xl*. Consistent with this prediction, analysis of a limited sample of cell lines revealed a general tendency for higher *bcl-xl* transcript levels in cells transformed by A6.7.10 IRES alone than in cells transformed by coexpression of A6.7.10 with *bcl-2* in *trans* (Fig. 7).

These results suggest that cell transformation by weakly transforming mutants such as A6.7.10 may need to select for cells with high endogenous antiapoptotic gene expression when no death inhibitor is coexpressed in *trans*. In conjunction with our functional complementation assays, in which *bcl-xl* and *bcl-2* were the most effective in rescuing the partially transforming phenotype of mutant A6.7.10, these results highlight the fundamental importance of v-Rel in antagonizing cell death for efficient manifestation of its oncogenic phenotype. The data also suggest that the threshold of v-Rel-mediated transactivation and the nature of the cell death inhibitors involved are both critical determinants of its transforming potential.

DISCUSSION

In recent years, v-Rel has emerged as a valuable tool with which to study the contribution of the Rel/NF-κB factors to leukemia and lymphomagenesis and to investigate the mechanisms involved. Here, we characterized the properties of v-Rel transactivation mutants. Our studies showed that a threshold of transactivation ability is necessary for efficient lymphoid cell transformation by v-Rel. Indeed, mutant A6.7.10, exhibiting 50% transcriptional activity compared to wild-type v-Rel, was significantly impaired for lymphoid cell transformation. Likewise, mutant A6.7, with a somewhat greater transcriptional

A

	Colonies of transformed cells	
	-IRES	-IRES-*bcl-xL*
v-Rel	222 +/- 42	212 +/- 71
A6	204 +/- 10	244 +/- 32
A7	237 +/- 96	290 +/- 50
A6.7	130 +/- 50	196 +/- 30
A6.7.10	80 +/- 63	183 +/- 51
A10	312 +/- 51	294 +/- 50
S438,439D	374 +/- 49	377 +/- 36
R32K	0	0

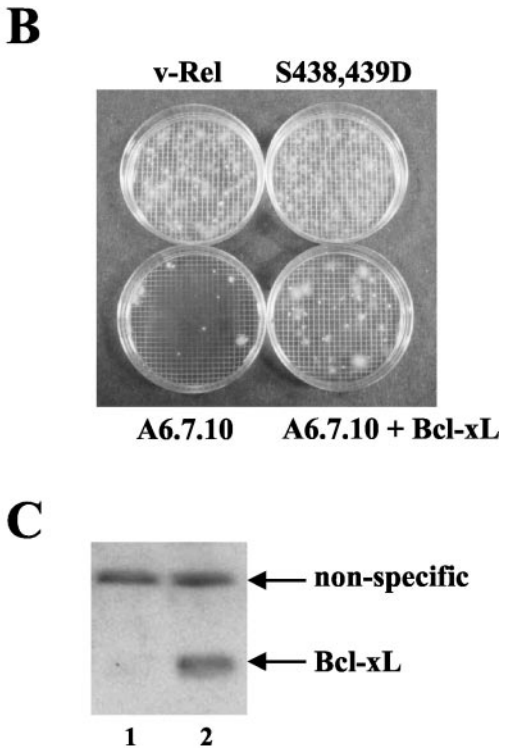


FIG. 5. Cotransformation of primary spleen cells with v-Rel mutants and NF-κB-regulated cell death inhibitor Bcl-xL. (A) Primary chicken spleen cells were electroporated with pJD214-IRES-*bcl-xl*-derived retrovirus constructs for wild-type or mutant v-Rel proteins, along with SW253 helper virus DNA. Colonies of transformed lymphoid cells were scored 2 weeks posttransfection. Results show the average from three independent experiments performed in duplicate. This analysis was conducted in the same series of experiments as that with the JD214-IRES-derived vectors shown in Fig. 3C for comparison. (B) Photograph of a representative transformation assay with JD214-v-Rel-IRES, JD214-S438,439D-IRES, JD214-A6.7.10-IRES, and JD214-A6.7.10-IRES-*bcl-xl* constructs. (C) Western blot analysis of Bcl-xL protein expression. Protein extracts from spleen cells transformed by JD214-A6.7-IRES (lane 1) or JD214-A6.7-IRES-*bcl-xl* (lane 2) were resolved by SDS–15% PAGE, transferred to a nitrocellulose membrane, and incubated with a Bcl-xL-specific polyclonal antibody.

activity than A6.7.10, showed 60% of the wild-type oncogenic potential. Importantly, cell death inhibitors *bcl-xl* and *bcl-2* effectively rescued the partially transforming phenotype of v-Rel transactivation mutants A6.7 and A6.7.10 but not that of mutants lacking significant transcriptional activity, such as those harboring a conservative R32K substitution (D10, R32K).

In contrast to the rescuing activity of *bcl-xl* and *bcl-2*, other NF-κB-regulated cell death inhibitors showed little or no complementing activity in these assays. Somewhat consistent with these results was the observation that the few transformed colonies that arose when mutant A6.7.10 was expressed alone generally displayed higher levels of endogenous *bcl-2* and *bcl-xl* gene expression than those transformed by v-Rel or by mutant A6.7.10 coexpressed with *bcl-2*. Together, our findings indicate that a defined threshold of transcriptional activity is required for v-Rel-mediated transformation and underscore the notion that efficient expression of antiapoptotic genes of the *bcl-2* family is necessary for v-Rel to manifest its oncogenic phenotype.

Conservative mutation in the DNA recognition loop abolishes the transcriptional and biological activities of v-Rel. Investigation into the cause of the transformation-defective phenotype of mutant D10 and of the spontaneous R32K mutation that it harbored revealed an absolute requirement for preserving an arginine at this position for efficient DNA contact, transcriptional activation, and biological activity. This residue is located in the RxxRxRxxC motif that our laboratory previously defined in the Rel homology domain of v-Rel and was shown by X-ray crystallography analysis of various Rel/NF-κB family members to be localized at the tip of the DNA recognition loop (12, 14, 17, 23, 29, 32, 43).

Arg 32 in v-Rel corresponds to Arg 59 in human p50/NF-κB1 and is absolutely conserved among all members of the Rel/NF-κB family, from flies and vertebrates. It is interesting that a histidine residue is found at this position in the Rel-related NF-AT factors, which display weak intrinsic DNA-binding activity (81). Similar to our observations with v-Rel, substitution of an arginine residue for the histidine found in NF-AT vastly improved its DNA-binding activity (81). This further emphasizes the importance of this position in DNA contact.

According to the crystal structures of c-Rel, RelA, p50/NF-κB1, and p52/NF-κB2, R32 in v-Rel is predicted to contact the G1 position in the NF-κB DNA site. Interestingly, the DNA-binding activity of mutant R32K was much less affected by mutation of the G1 moiety in the IL-6-κB DNA motif (*G*GG ATTTTCCC) to an A than wild-type v-Rel or mutant R30K, which is predicted to contact the G2 position. The differential binding of mutant R32K and v-Rel to variants of the NF-κB

A

	Colonies of transformed spleen cells	
	v-Rel	A6.7.10
IRES-	115 +/- 88	8 +/- 11
bcl-xL	179 +/- 71	97 +/- 50
bcl-2	137 +/- 7	60 +/- 46
bfl-1	50 +/- 23	16 +/- 19
a20	73 +/- 63	39 +/- 22
traf-1	75 +/- 48	26 +/- 22
iex-1	93 +/- 55	19 +/- 16
xiap	33 +/- 33	23 +/- 16
c-iap1	40 +/- 9	2 +/- 1
c-iap2	17 +/- 14	2 +/- 2
luciferase	10 +/- 4	1 +/- 1

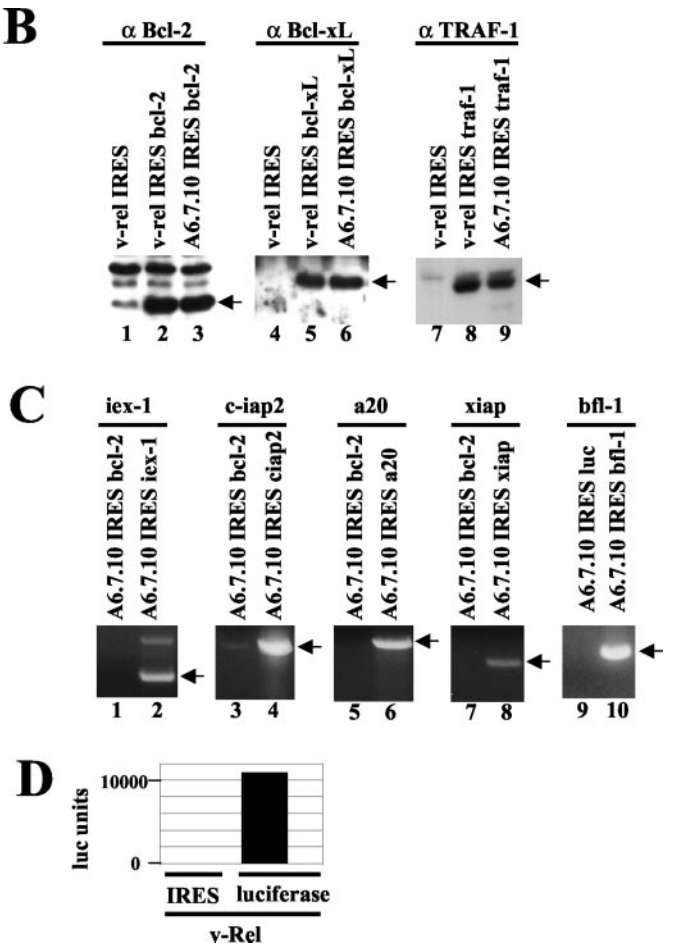


FIG. 6. NF-κB-regulated cell death inhibitors differentially complement the oncogenic defect of mutant A6.7.10. (A) The transforming activity of v-Rel and A6.7.10 proteins coexpressed with NF-κB-regulated cell death antagonists was assayed in primary chicken spleen cells as described for Fig. 5A. A luciferase gene was used as a control. Since introduction of sequences after the IRES in the pMH105 vector decreased expression of v-*rel* genes, results were normalized to v-Rel protein expression levels. The average of four independent experiments is shown. (B) Western blot analysis of cell extracts from transformed spleen cells with antibodies specific for Bcl-2, Bcl-xL, or TRAF-1. (C) RT-PCR analysis of transcripts for *xiap*, *ciap2*, *iex-1*, *bfl-1*, or *a20*. Total RNA (500 ng) from transformed spleen cells was used for reverse transcription. (D) Analysis of luciferase activity in extracts from spleen cells transformed by pMH105 v-Rel-IRES-*luc* (30 μg) was compared to that in control pMH105 v-Rel-IRES cells.

consensus at the G1 position implies that the R32K substitution may affect not only the affinity but also the specificity of v-Rel DNA binding.

Consistent with their significantly decreased DNA-binding potential, the transcriptional activity of mutants R32K and R30K corresponded to 20% and 30% of that of wild-type v-Rel, respectively. This coincided with a complete loss of oncogenicity in primary lymphoid cells, even when the mutants were coexpressed in the presence of cell death inhibitors. Overall, these mutants provide valuable insights into the physical requirements for efficient binding of v-Rel to target sites important for the transcriptional activation of genes involved in transformation. These data also underscore the notion of evolutionary selection for the conservation of an arginine at this position in all members of the Rel/NF-κB family to preserve their biological activity.

Correlation between the phosphorylation profile of v-Rel and its biological activity. Posttranslational modification of the Rel/NF-κB factors has been implicated in modulating their activity. For instance, acetylation of RelA/p65 plays a role in regulating its association with IκBα and consequent nuclear export, DNA-binding, and transcriptional activities, while acetylation of p50 increases its DNA-binding activity (13, 22). The importance of phosphorylation in enhancing the transcriptional activity of Rel/NF-κB factors has also come to light. Phosphorylation of serine 529 in the transactivation domain of RelA is required for TNF-α-mediated activation of NF-κB transcriptional activity and, in conjunction with phosphorylation of S536, in NF-κB-mediated transactivation in response to the AKT pathway (39, 69). While S529 is phosphorylated by casein kinase II, phosphorylation of S536 is controlled by the IκB kinases (54, 70). Moreover, protein kinase A-mediated phosphorylation of RelA facilitates its association with p300 (80). In the C-terminal transactivation domain of c-Rel, S471 was identified as a site important for modulating its transcriptional activity (40).

In this report, we investigated the molecular basis for the oncogenic defect of Ser-to-Ala mutations at positions 398, 399,

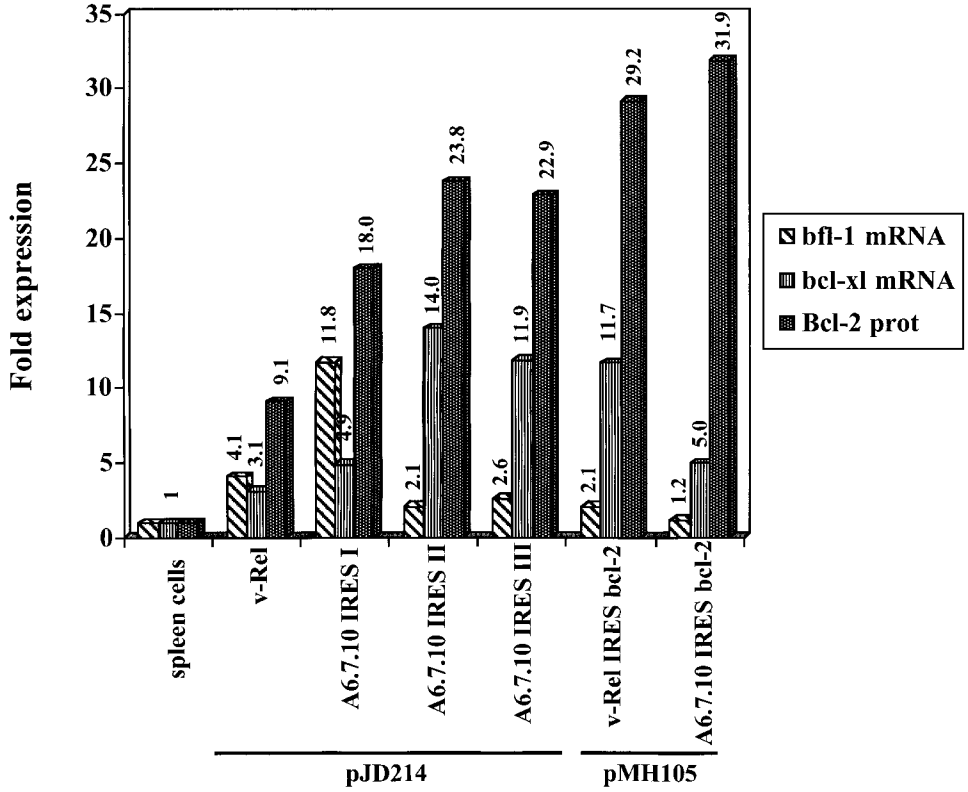


FIG. 7. Selection for high endogenous expression of antiapoptotic genes in spleen cells transformed by a weakly transforming v-Rel mutant. Spleen cells transformed by v-Rel or A6.7.10 expressed alone or together with the cell death inhibitor Bcl-2 were analyzed by Northern blot with radiolabeled chicken *bcl-xl*, *bfl-1*, and actin cDNAs as probes. Bands were quantitated by phosphorimaging. Bcl-2 protein was detected by Western blot analysis with a Bcl-2-specific antibody, and the Bcl-2 bands were quantitated along with that of actin by ImageQuant analysis. The relative ratios of Bcl-xL/actin, Bfl-1/actin, and Bcl-2/actin are plotted.

402, 438, and 439 in the v-Rel transactivation domain. Although individual mutations at these positions had no detrimental effect on v-Rel activity, combined mutations led to a partial yet significant decrease in v-Rel transcriptional and transforming potentials. Indeed, mutant A6.7.10 (S398,399, 402,438,439A) displayed 50% of the transactivation ability of v-Rel. Coincident with its markedly reduced transforming efficiency in primary lymphoid cells, the phosphopeptide profile of mutant A6.7.10 showed significant differences compared to the wild-type protein. This suggests that the mutation of defined serines in the C terminus of v-Rel may influence the overall phosphorylation state of the protein or alter its conformation in a way that alters its tryptic phosphopeptide map. Overall, our results indicate that although phosphorylation of the v-Rel transactivation domain may not be absolutely essential for v-Rel function, it may serve to positively regulate its transcriptional and oncogenic activities. Future studies will be needed to map the in vivo phosphorylation sites in v-Rel and to identify the kinases that can regulate its activity.

S438,439D substitution gives rise to a highly oncogenic gain-of-function mutant. An interesting finding was that Ser-to-Asp mutation of amino acids 438 and 439 in the v-Rel transactivation domain gave rise to a strong gain-of-function mutant which exhibited nearly twofold greater transcriptional and oncogenic activities than wild-type v-Rel (mutant S438,439D). In prior studies, Romero and Humphries reported a v-Rel mutant that showed increased transforming activity toward B cells compared to v-Rel (51). The A40S mutation in question mapped near the DNA-binding domain of v-Rel and was responsible for enhanced DNA-binding activity. The underlying mechanism for the increased biological activity of our S438,439D mutant differs from that of A40S in that S438,439D increased v-Rel transcriptional activity but had no effect on the efficiency of v-Rel DNA binding.

These results are reminiscent of the specific increase observed in p65/RelA transcriptional activity but not DNA binding upon serine phosphorylation in its transactivation domain (39, 69). This raises the intriguing possibility that, by virtue of its increased negative charge or altered conformation, the transactivation domain of mutant S438,439D may be more adept in recruiting specific coactivators and/or components of the transcriptional machinery required for efficient activation of target genes important for oncogenesis. The increased activity of mutant S438,439D may thus derive from the fact that all molecules bear a negative charge at this position compared to wild-type v-Rel, where only a small fraction of the wild-type protein population is phosphorylated in vivo (Rayet and Gélinas, unpublished data). Further studies will help to address these issues.

Selective complementation of partially transforming v-Rel

mutants by *bcl-xl* and *bcl-2*. v-Rel mutants A6.7 and A6.7.10 displayed approximately 50% of the transcriptional and oncogenic activities of wild-type v-Rel. This difference in biological activity may help to define the minimum threshold of v-Rel transcriptional activity needed for efficient manifestation of its oncogenic phenotype. In agreement with this notion, v-Rel mutants R32K and R30K, whose transcriptional activity fell below this threshold (20% and 30% of wild-type activity, respectively), failed to transform cells even when coexpressed with the cell death inhibitor *bcl-xl* (see below). These findings are consistent with a report indicating that a minimum threshold of nuclear v-Rel activity was needed to transform lymphoid cells (53). However, we do not rule out the possibility that the specificity of the v-Rel activation function may also be affected in mutants A6.7 and A6.7.10.

Importantly, the impaired oncogenicity of mutant A6.7.10 was significantly increased by coexpression of either *bcl-xl* or *bcl-2*. This agrees with studies showing that coexpression of *bcl-2* increased the transformation efficiency of temperature-sensitive v-Rel mutants at the permissive temperature (73). The fact that coexpression of a cell death inhibitor alone was sufficient to rescue the oncogenic defect of mutant A6.7.10 suggests that the reduced transcriptional activity of this mutant is likely to critically affect the expression of cell death inhibitors essential for cell transformation, whereas those important for cell proliferation may be affected to a lesser degree or require a lower threshold of expression.

Our observation that several lymphoid cell clones transformed by mutant A6.7.10 expressed alone, in the absence of a cell death inhibitor, showed high endogenous expression of the *bcl-xl* and *bcl-2* genes compared to cells transformed by wild-type v-Rel argues for a model in which cell transformation by weakly transforming v-Rel mutants may select cells in which endogenous expression and/or activation of *bcl-2*-related cell death inhibitors is elevated. In contrast, when coexpressed along with an antiapoptotic gene, selection for rare cells with high expression of these endogenous factors may no longer be necessary, allowing more efficient survival and transformation of primary lymphoid cells by A6.7.10 plus *bcl-xl* or *bcl-2*.

It is possible that A6.7.10 selectively transforms cells that already display elevated expression of an endogenous death inhibitor. Alternatively, A6.7.10 may selectively transform cells in which defined cofactors allow it to more efficiently activate expression of death antagonists. Although it is difficult to discriminate between these two possibilities, our results nevertheless emphasize the requirement for efficient expression of a subset of antiapoptotic genes of the *bcl-2* family in order for v-Rel to manifest its full oncogenic potential. Further support for their mutual implication in oncogenesis stems from the observation that the human c-*rel* gene is often amplified in conjunction with *bcl-2* gene rearrangements in several cases of B-cell diffuse large cell lymphomas (48, 52).

Another important finding was that all NF-κB-regulated cell death inhibitors that have been described are not equally competent in complementing the oncogenic phenotype of mutant A6.7.10. The fact that *bcl-xl* and *bcl-2* were by far the most efficient in these assays suggests that the death pathway(s) that v-Rel must suppress in order to transform cells may be most efficiently inhibited by Bcl-xL and Bcl-2 rather than by other NF-κB-regulated cell death antagonists. Although we do not yet have direct evidence demonstrating that these genes are under the direct transcriptional control of v-Rel in transformed cells, our results suggest that the antiapoptotic pathways activated by v-Rel are likely to act at the level of mitochondria and that the main antiapoptotic targets of v-Rel in cell transformation may include *bcl-xl*, *bcl-2*, and/or survival factors with related activity.

Surprisingly, the prosurvival Bcl-2-related protein Bfl-1 was incapable of efficiently complementing mutant A6.7.10. Its activity may thus not be redundant with that of Bcl-2 or Bcl-xl. Although *bfl-1* has been reported to be associated with several types of cancer (47), either it is not required for v-Rel-mediated transformation, mutant A6.7.10 may not be defective for its activation, or its prosurvival activity may not be sufficient to allow v-Rel to transform cells. The fact that the IAPs failed to complement v-Rel mutants as efficiently as Bcl-2 family members may not be surprising, as c-IAP factors were reported to require coexpression with one another and with TRAF proteins to efficiently protect cells against TNF-α-induced death (68). Overall, the data emphasize the requirement for oncogenic NF-κB factors to act in conjunction with particular cell death inhibitors in order to manifest their full oncogenic phenotype.

It has been proposed that v-Rel leads to abnormal gene expression responsible for cell transformation. Recent data from our laboratory and others showed that cellular Rel proteins are also able to transform primary lymphoid cells (24; Y. Fan, B. Rayet, and C. Gélinas, unpublished data). This suggests that a similar collection of genes involved in tumorigenesis can be activated by both cellular Rel and viral Rel proteins. Altogether, the results described in this report enable us to begin to decipher the cell death inhibitors implicated in lymphomagenesis associated with Rel/NF-κB factors and the role of phosphorylation in regulating v-Rel protein activity. Since Rel/NF-κB is implicated in leukemia and lymphomagenesis, further detailed analysis of its function and regulation is likely to yield important insights into the mechanisms involved.

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