Constitutive and Inducible κ B Binding Activities in the Cytosol of v-Rel-Transformed Lymphoid Cells

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Constitutive and inducible κ B binding activities associated with v-Rel and c-Rel in the cytosol of v-Rel-transformed cells have been identified. These activities were resolved by electrophoretic mobility shift assay and chromatographic techniques into a high-molecular-weight protein-DNA complex designated complex I containing v- and c-Rel and lower-molecular-weight complexes II, III and IV which contained only v-Rel and which were stimulated by nucleotides, low pH, and detergent. These experiments suggest that interaction of v-Rel and c-Rel decreases the DNA-binding activity of each.

The oncogene *v-rel* was originally identified as the transforming component of the avian retrovirus reticuloendotheliosis virus-T (25). A mutated homolog of the cellular gene, *c-rel*, *v-rel* induces a rapidly fatal hematopoietic malignancy in young birds. In vitro, *v-rel*-containing retroviruses have been shown to transform both avian fibroblasts and hematopoietic cells (for a review, see reference 8). The mechanism by which v-Rel induces oncogenic transformation, however, remains unclear. The finding that v-Rel and c-Rel are members of the Rel/NF- κ B transcription factor family (15, 21) led to the suggestion that transformation resulted from v-Rel-induced changes in patterns of normal gene expression, perhaps by v-Rel acting as a dominant negative mutant of c-Rel (for a review, see reference 16 and references therein).

The biochemical properties of Rel/NF- κ B proteins have been the subject of extensive analysis. Most family members reside in the cytoplasm in an inactive, but inducible, form by virtue of interaction with a set of inhibitor proteins, I κ B (2). Upon appropriate stimulation, the proteins are translocated to the nucleus and the DNA-binding activity of the Rel/NF- κ B proteins is activated. Our previous analyses have demonstrated that avian c-Rel is also sequestered in the cytoplasm in an inactive state (1). This is presumably the result of its interaction with a set of cellular proteins which include p115 and p124 (the avian homologs of mammalian p100 and p105, respectively), which have been shown to have I κ B-like properties, and pp40, avian I κ B α (9, 11, 28, 32, 34, 35). Only when translocated to the nucleus does avian c-Rel become competent for binding to a consensus κ B site. In sharp contrast, examination of the localization of v-Rel showed that the protein was found in both the cytoplasm and the nucleus of transformed lymphoid cells (17). In addition, both the cytoplasmic and nuclear forms of the protein could bind to a κ B site (19) in electrophoretic mobility shift assays (EMSA). Therefore, unlike c-Rel, whose DNA-binding activities were tightly regulated, v-Rel was found in both compartments and was apparently constitutively active for DNA binding. Interestingly, cytoplasmic v-Rel is part of a protein complex which includes pp40, p115, p124, and p68^{c-rel}, raising questions about the role of pp40, p115, and p124 in the regulation of cytoplasmic v-Rel κ B binding activity (28, 35). In addition, the nature and consequences of the interaction between v-Rel and c-Rel required examination to determine if

v-Rel did indeed behave as a dominant negative mutant of c-Rel.

To understand more fully the biochemical properties of cytoplasmic v-Rel, we have investigated in detail the cytoplasmic κ B binding activities found in v-Rel-transformed lymphoid cells. Cytoplasmic extracts were prepared as described elsewhere (13) from 2.5×10^{10} NPB4 cells, a reticuloendotheliosis virus-T-transformed lymphoid cell line grown in Spinner culture in DMEM (Dulbecco's minimal essential medium) supplemented with 5% newborn calf serum and 2% chick serum (6). These extracts (12 mg) were then assayed by EMSA as described previously by using a consensus κ B site from the immunoglobulin (Ig) enhancer (19; see legend to Fig. 1). As shown in Fig. 1A, lane 6, the constitutive Ig- κ B binding activities in cytosolic extracts from NPB4 cells were resolved into four protein-DNA complexes designated complex I (retained at the origin) to complex IV. Complexes with faster mobilities were not present reproducibly and were not recognized with antibody against v- and c-Rel (Fig. 1C). Therefore, they were considered to be nonspecific. The sequence specificity of these binding activities was evaluated by competitive inhibition with a 200-fold molar excess of wild-type (k) or mutant (m) κ B DNA fragments (Fig. 1C, lanes k and m; see legend for sequence of oligonucleotides). Only wild-type (k) κ B fragments competed for binding effectively, demonstrating that binding was sequence specific.

While these experiments suggested that the cytoplasmic κ B binding activities in v-Rel-transformed cells were constitutively active, work with other Rel/NF- κ B family members showed that the cytoplasmic proteins were usually inactive until induced by agents such as detergent and formamide (3, 14). Therefore, we wanted to determine if any inducible κ B binding activity could be detected in the cytoplasm of v-Rel-transformed cells. To reveal inducible κ B binding activity in NPB4 cytosol, extracts were pretreated with either sodium deoxycholate (DOC) in 8% formamide with or without the nonionic detergent Brij 36T (Fig. 1A) or phosphate buffers which resulted in a final pH range of 6.9 to 5.4 in the binding reaction mixtures (Fig. 1B). These conditions were previously shown to induce the binding activities of NF- κ B (3) and heat shock factor (29) in cell extracts, respectively. Addition of low concentrations of DOC (0.02 or 0.06%) resulted in a fourfold stimulation of the formation of complex II in the absence of Brij 36T (Fig. 1A, lanes 4 and 5). Addition of higher concentrations of DOC (0.6 to 1.8%) inhibited the formation of all four complexes (Fig. 1A, lanes 1 and 2). Conversely, detergent

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FIG. 1. Treatment of NPB4 cytosolic extracts with detergent, low pH, and nucleotide. (A) Stimulation of κ B binding activity in NPB4 cytosolic extracts by various concentrations of DOC-8% formamide in the absence (lanes 1 to 5) or presence (lanes 7 to 11) of 1% Brij 36T. κ B binding activity was measured by EMSA utilizing the oligonucleotides 5'-AGCTTCAACAGAGGGGACTTTCGAGAGGCTCGAG-3' (k5/3, wild-type murine Ig- κ B enhancer motif) and 5'-AGCTTCAACAGAGATCTCTTTCCGAGAGGCTCGAG-3' (Mk5/3, four-point mutant murine Ig- κ B enhancer motif). These oligonucleotides were cloned into the *Hind*III and *Eco*RI sites of pBS/KS+ (Stratagene) to generate pBS-k5/3 and pBS-Mk5/3, respectively. The *Xho*I-*Pst*I fragment containing the wild-type or mutant κ B enhancer motif was excised from the plasmid pBS-k5/3 and end labeled with [α - 32 P]dATP and dGTP. Cytosolic extracts were mixed with 50,000 cpm (0.25 ng) of end-labeled fragment in binding reaction mixtures containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 100 mM KCl, 3 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid], 3 mM ATP, 4% Ficoll, 10% glycerol, and 500 ng of salmon sperm DNA. The binding mixture was resolved on a 4% nondenaturing polyacrylamide gel as described previously (19). Cytosolic extracts (12 mg) were mixed sequentially with 8% formamide and DOC and incubated for 5 min at 25°C. One percent Brij 36T was subsequently added as indicated on the figure. The binding activity of untreated NPB4 cytosol is shown in lane 6. The concentrations of DOC and Brij 36T are shown at the top of each lane. (B) Effect of low pH on the κ B binding activity of cytosolic extracts. NPB4 cytosol (12 mg) was adjusted to the pH indicated above each lane (lanes 1 to 6) with a mixture of 0.2 M Na₂HPO₄ (pH 8.5) and 0.2 M NaH₂PO₄ (pH 2.3) and incubated at 25°C for 10 min. For lanes 7 to 11, NPB4 cytosol was adjusted to the pH indicated above each lane and, after a 10-min incubation, neutralized to pH 7.5 with 0.1 M Tris base. (C) Costimulation of ν - or c -Rel κ B binding activities in NPB4 cytosol by nucleotides and low pH. Stimulation of κ B binding activity

at pH 7.9 or pH 6.0 in the absence (–) or presence (+) of 3 mM ATP was measured by EMSA. The specificity of binding was determined in competition experiments with a 200-fold molar excess of unlabeled DNA fragment containing the wild type and a four-point mutant Ig- κ B enhancer motif (k and m in each set, respectively). The presence of v- or c-Rel in induced (pH 7.9, +ATP) and pH induced (pH 6.0, +ATP) protein-DNA complexes was detected with MAb 46.6 and MAb 6.8 (designated 4 and 6 in each set, respectively). Antibody binding reactions were done by mixing cytosolic extracts with 2.5 μ l (1:5 dilution in phosphate-buffered saline) of either MAb 46.6 or MAb 6.8 ascitic fluids at 4°C for 60 min prior to the DNA-binding reaction. Binding activities in the presence of MAbs to adenovirus E4/6/7 protein or both MAb 46.6 and MAb 6.8 (designated Pi and 6/4 in each set, respectively) are shown.

exchange with ~0.6-fold Brij 36T restored the formation of these complexes to the level of 0.02% DOC stimulation (Fig. 1A, lanes 9 and 10). Higher concentrations of DOC (1.8%) (Fig. 1A, lane 11) remained inhibitory. Since NF- κ B interaction with I κ B is disrupted by such conditions, these results suggest that stimulation of complex II may be due to release of a DNA binding inhibitor, perhaps one of the I κ B proteins, pp40 or p124 (11, 32). The inactivation of κ B binding activity by higher concentrations of DOC presumably results from reversible destabilizing interactions of DOC charge groups with the DNA probe.

The effect of pH on the κ B binding activity was then investigated by adjusting the pH of cytosolic extracts with phosphate buffers at 25°C for 10 min prior to EMSA. Incubation of cytosolic extracts at pHs ranging from 6.9 to 6.0 resulted in a 2.5-fold enhancement in the formation of complexes I through IV, with maximum stimulation occurring at pH 6.4 (Fig. 1B, lanes 2 to 5). Inactivation of binding occurred at pH 5.4 or lower (Fig. 1B, lanes 1 and 11). In addition, novel complexes migrating faster than complex IV were generated at low pH. When equivalent binding reaction mixtures were neutralized to pH 7.5 after a short incubation at the indicated pHs, partial reversal of the low pH stimulation was observed (Fig. 1B, lanes 7 to 10). Similar results were obtained with extracts incubated with citrate buffers (data not shown). These experiments demonstrate that cytosolic extracts of v-Rel-transformed hematopoietic cells contain both constitutive and latent κ B binding activities as judged by the stimulation of binding with anionic detergents and low pH.

The DNA-binding activity of NF- κ B was also reported to be stimulated by nucleotides (24). However, this property has never been examined for v-Rel or c-Rel. Therefore, the modulation of v-Rel κ B binding activity by nucleotides was analyzed with cytoplasmic binding reaction mixtures containing 3 mM ATP. As shown in Fig. 1C (pH 7.9, – and + ATP), the formation of complexes I, II, and IV was stimulated approximately twofold in crude extracts at pH 7.9, as judged by laser densitometry. The influence of ATP was also tested at pH 6.0 to determine if nucleotide stimulated the pH-inducible DNA-binding activity in an additive manner. As can be seen, addition of ATP to binding reaction mixtures adjusted to pH 6.0 resulted in a threefold stimulation of all four complexes as judged by laser densitometry (Fig. 1C, pH 6.0, – and + ATP). Thus, addition of nucleotide to binding reaction mixtures, stimulated at low pH, markedly increased DNA-binding activity associated with complexes I through IV. In each case, the specificity of binding was assayed by competition with a 200-fold molar excess of either wild-type (k) or mutant (m) κ B sites (Fig. 1C, k and m in each set; see legend for sequences of the oligonucleotides used). Finally, the presence of viral and cellular Rel in the protein-DNA complexes was judged by incubation of binding reaction mixtures with either an antibody directed against the adenovirus E4 6/7 proteins as a negative control (designated Pi in Fig. 1C; kindly provided by P. Hearing) or one of two monoclonal antibodies (MAb), MAb 6.8 (designated 6 in Fig. 1C, pH 7.9 and 6.0, +ATP) and MAb 46.6 (designated 4 in Fig. 1C, pH 7.9 and 6.0, +ATP). Both MAb 6.8 and 46.6, which were made by injecting mice with purified

v-Rel, recognize both v-Rel and c-Rel (data not shown). While incubation of NPB4 cytosolic extracts with MAb 6.8 results in a protein-DNA complex with slower mobility and concomitant reduction in complex II (Fig. 1C, Oligo/MAb 6, pH 7.9 and 6.0, +ATP), MAb 46.6 appears to inhibit DNA binding associated with complexes I through IV (panel C, Oligo/MAb 4, pH 7.9 and 6.0, +ATP). These results suggest that v-Rel and/or c-Rel is present in all four protein-DNA complexes, which at pH 6.0 are all stimulated by ATP. Similar results were obtained with 1 mM dCTP, dGTP, dUTP (data not shown).

These results suggest that both constitutive and inducible κ B binding activities are present in v-Rel-transformed cells. To separate these activities and determine the relative contributions of v-Rel and c-Rel to κ B binding, the cytosolic extracts were analyzed by gel filtration chromatography. NPB4 cytosol (650 mg), adjusted to 35% glycerol, was resolved on a Sephacryl S-400 SF column (5.0 by 85 cm; Pharmacia) (see Fig. 3 legend for buffer conditions). Column fractions were subsequently assayed by EMSA with the Ig- κ B enhancer motif to detect v- and/or c-Rel binding activity (19) and by Western blotting (immunoblotting) with SB 66, a polyclonal antibody which recognizes both v-Rel and c-Rel (28, 36).

As can be seen, complex I was separated as a dispersed, high-molecular-mass activity (Fig. 2A, fractions 61 to 91; ≥ 700 kDa) from complexes II through IV (panel A fractions 103 to 124; ~ 450 to 150 kDa). Complexes II through IV, which contained constitutive binding activity, also had inducible v- and/or c-Rel κ B binding activity, since preincubation of cytosolic extracts at pH 6.0 resulted in enhanced DNA-binding activity of these complexes (Fig. 2B, fractions 91 to 124). For subsequent analyses, the peaks of DNA-binding activity were pooled into three fractions, designated A (Fig. 2B, fractions 61 to 70), containing complex I activity; B (panel B, fractions 79 to 91), containing an overlap of complex I through III activity; and C (panel B, fractions 100 to 124), containing complex II through IV activity. The presence of v- and c-Rel in the protein-DNA complexes I through IV was assessed by preincubating extracts with the two MAbs, MAb 6.8 and MAb 46.6. As before, addition of MAb 6.8 retarded the mobility of complex II (Fig. 2A, lane 2) while formation of complexes I through IV was reduced in the presence of MAb 46.6 (panel A, lane 3) at pH 7.9. Inducible κ B binding activity also contained v- and/or c-Rel, as shown by stimulation of cytosolic extracts at pH 6 (Fig. 2B, lane 2) followed by incubation with MAb 46.6 (lane 3).

The elution of v- and c-Rel proteins with these DNA-binding activities was determined by Western blot analysis of column protein fractions with SB 66. The results presented in Fig. 2C indicate that complexes II through IV, which constitute the bulk of the DNA-binding activity, coelute with the minor v-Rel protein peak (fractions 100 to 124, fraction C) and four cross-reactive proteins with M_r s of 36,000, 45,000, 48,000, and 50,000 which presumably represent proteolytic cleavage products of v- or c-Rel. Interestingly, the highest levels of constitutive and inducible κ B binding activities are observed when little c-Rel is detectable in the complex (for example, see fractions 103 to 121 in Fig. 2A and B). Complex I, on the other hand, contains little inducible activity and coelutes with the major v- and c-Rel protein peaks together with two cross-reactive proteins with

FIG. 2. Gel filtration analysis of cytosolic v- and/or c-Rel κ B binding complexes. NPB4 cytosol (650 mg) was resolved on a Sephacryl S-400 SF column (5.0 by 85 cm, flow rate of 4.3 cm/h; see legend to Fig. 3 for conditions). Every third fraction was assayed for binding activity by EMSA (10-ml fraction) with the Ig- κ B enhancer motif (A and B) and by Western blotting (40-ml fraction) with the Rel-specific polyclonal antibody SB 66 (C). Column fractions are shown at the top of each lane. (A) Elution profile of active v- and c-Rel κ B binding complexes. Binding activity of NPB4 cytosol (12 mg) in the absence (lane 1) or presence (lanes 2 and 3) of v- and c-Rel-specific MAb 6.8 (lane 2) and MAb 46.6 (lane 3) is shown. v- and c-Rel κ B binding activities at pH 7.9 are also shown (fractions 61 to 133, lanes 4 to 28). S, sample of the starting extract. (B) Elution profile of inducible v- and c-Rel κ B binding activities. Binding activities of unstimulated NPB4 cytosol, pH 7.9 (lane 1), or pH 6.0 stimulated extract in the

absence (lane 2) or presence (lane 3) of the Rel-specific MAb M46.6 (46) are shown. Binding activities of pH 6.0 stimulated fractions are also shown (fractions 61 to 133, lanes 4 to 28). F, free-labelled probe at the bottom of panels A and B. (C) Western blot analysis of column fractions showing the coelution of v- and c-Rel with κ B binding activities in the A, B, and C fractions. The 36- to 50-kDa proteins may represent cross-reactive proteins or breakdown products of v-Rel. The gel filtration markers (Pharmacia) used were thyroglobulin (T) (86 Å [1 Å = 0.1 nm], 660 kDa), ferritin (F) (60 Å, 450 kDa), catalase (C) (40 Å, 250 kDa), and bovine serum albumin (B), (66 kDa). The voided volume, V_0 , is indicated. Active fractions were pooled as fractions A (76 mg), B (72 mg), and C (244 mg), respectively. Numbers on the left indicate molecular mass in kilodaltons.

M_s s of 48,000 and 50,000, again presumed cleavage products of v-Rel. These observations suggest that the formation of complex I in the A and B fractions may be the result of v- or c-Rel oligomers binding to the κ B site, in accord with the findings of Kochel et al. (22). Alternatively, high-molecular-weight heteromeric v- or c-Rel complexes previously observed in v- or c-Rel immunoprecipitates may make up complex I (10, 22, 23). These experiments demonstrate that v- and c-Rel are present in multiple, constitutive and inducible κ B binding complexes in cytosolic extracts of v-Rel-transformed cells. Interestingly, the major κ B binding activity is found in fractions with low or undetectable levels of c-Rel.

To characterize the constitutive and inducible v-Rel κ B binding activities in complexes I through IV in more detail, the fractionation procedure outlined in Fig. 3 was developed. Specifically, each of the gel filtration A, B, and C fractions was applied directly onto a phosphocellulose P-11 column (1.5 by 5.6 cm, 8 mg of resin ml^{-1} ; Whatman) which was developed with a step gradient of 0.3, 0.5, and 0.85 M KCl. For all three gel filtration fractions, DNA-binding activity was found only in the flowthrough (0.1 M KCl). The fraction A, B, and C P-11 flowthrough was subsequently applied to a Q-Sepharose column (1.5 by 5.6 cm, 5 mg of resin ml^{-1} ; Pharmacia) which was

developed with a linear gradient 0.05 to 0.7 M KCl. Fractions from Q-Sepharose chromatography of the A and B gel filtration peaks were assayed by EMSA and Western blot (Fig. 4A and B). Because of high protein concentrations, the C fraction was analyzed separately (Fig. 3; see below also).

The elution profiles of the κ B binding activity in the A and B fractions from Q-Sepharose chromatography are shown in Fig. 4. The results indicate that chromatography of the A fraction on Q-Sepharose columns resolved complex I into subcomplexes with mobilities similar but not identical to those of complexes II through IV. These subassemblies of complex I eluted at low salt concentrations of 0.15 to 0.2 M KCl (Fig. 4A, peak A-I, fractions 37 to 45). Since identical levels of detectable v-Rel and c-Rel were present in all column fractions as judged by Western blot analysis with the polyclonal antibody SB 66 (Fig. 4A), peak A-II appears to represent inactive v- and c-Rel complexes (panel A, fractions 73 to 83). Interestingly, the newly formed A-I κ B complexes were also recognized by MAb 46.6 and MAb 6.8 by EMSA and were susceptible to low-pH stimulation (data not shown). These results suggest that complex I is stabilized by ionic interactions between multimers of active A-I subcomplexes or with inactive A-II v- and/or c-Rel complexes. These inactive A-II fractions were not inducible since they were unresponsive to pH or detergent treatment (data not shown).

Similar analysis of the gel filtration B fraction indicated that

FIG. 3. Fractionation scheme for constitutive v- and c-Rel binding complexes to the Ig- κ B enhancer motif in NP4 cytosol. The molarities stated in the various fractionation steps refer to concentrations of KCl in elution buffers. Gel filtration chromatography was performed as described in the text at a flow rate of 4.3 ml h^{-1} in buffer TC0.0 (20 mM Tris-HCl [pH 7.9], 10 mM β -mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA, 20% [vol/vol] glycerol) containing 100 mM KCl. All fractionation and column buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.2 mM Na vanadate, 0.2 mM Na molybdate, 30 mM phenyl arsine oxide, 50 mM KF, and 5 mM β -glycerol phosphate. Q-Sepharose chromatography of A and B fractions was carried out as follows. The gel filtration A and B fractions were applied to a phosphocellulose P-11 column, equilibrated with buffer TC0.1 (buffer TC0.0 supplemented with 100 mM KCl), which was subsequently developed with a step gradient of 0.3, 0.5, and 0.85 M KCl in buffer TC0.0. The active flowthrough fractions (0.1 M KCl) were applied to a Q-Sepharose column equilibrated in buffer TQ0.05 (25 mM Tris-HCl [pH 7.5], 10 mM β -mercaptoethanol, 5 mM MgCl_2 , 0.05% Brij 36T, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM KCl), which was developed with a 0.05 to 0.7 M KCl linear gradient in buffer TQ0.0 containing 10% glycerol. The active peak from the A fraction, A-I, eluted at 0.15 to 0.2 M KCl, while the active peak from the B fraction, B-I, eluted at 0.09 to 0.21 M KCl. The gel filtration C fraction was applied directly to a phosphocellulose P-11 column (2.5 by 6.0 cm, 8 mg of resin ml^{-1}) equilibrated in TC0.1. The active flowthrough fraction (0.1 M KCl) was applied to a Q-Sepharose column (2.5 by 4.0 cm, 5 mg of resin ml^{-1}) equilibrated in buffer TQ0.05 which was developed with a 0.05 to 0.7 M KCl linear gradient in buffer TQ0.0 containing 10% glycerol. The active fractions (0.1 to 0.25 M KCl) were applied to an S-Sepharose column (1.5 by 2.5 cm, 5 mg of resin ml^{-1} ; Pharmacia) equilibrated in buffer BS0.05 (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.6], 5 mM MgCl_2 , 10 mM β -mercaptoethanol, 0.05% Brij 36T, 10% glycerol, 1 mM EDTA, 1 mM EGTA 50 mM KCl). The flowthrough fraction was applied to an ss-DNA agarose column (1.5 by 3.8 cm, 3 mg of resin ml^{-1} ; BRL) equilibrated in buffer BD0.1 (50 mM HEPES-KOH [pH 7.9], 10 mM β -mercaptoethanol, 0.05% Brij 36T, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 100 mM KCl). The flowthrough fraction was applied to a Q-Sepharose column (1.5 by 2.5 cm) equilibrated with buffer TQ0.05 and developed with a 0.05 to 0.7 M KCl linear gradient in buffer TQ0.0. The active peak, C-I, was eluted at 0.1 to 0.25 M KCl.

FIG. 4. Coelution of v- and c-Rel with κ B binding activity on Q-Sepharose chromatography. A 50-mg amount of protein from each P-11 or 0.1 fraction of gel filtration peaks A and B and 22.5 mg of protein from ss-DNA agarose fractions of peak C (Fig. 2 and 3) were applied to 10- and 4.5-ml Q-Sepharose columns, respectively, and bound proteins were eluted with a 0.05 to 0.7 M KCl linear gradient. The binding activity in alternate column fractions of A and B fractions was determined by EMSA (A and B). The binding activity of each column fraction of the C fraction is shown in panel C. The binding activity of unfractionated NPB4 cytosolic extract (12 mg) is presented in lane S of panels A, B, and C, and the mobilities of complexes I through IV are indicated on the left of the gels. Proteins in the corresponding column fractions were assayed by Western blotting with polyclonal antibody SB 66 and are shown in panels A, B, and C. The positions of v-Rel and c-Rel are indicated to the right of each Western blot. Column fractions, shown above each lane, are presented in order of increasing salt concentration from left to right. (A) Active and inactive pools of A fraction are designated A-I (fractions 37 to 45; 0.15 to 0.2 M KCl) and A-II (fractions 73 to 83; 0.42 to 0.51 M KCl), respectively. F (second lane) denotes the flowthrough fraction. (B) Active and inactive pools of B fraction are designated B-I (fractions 35 to 47; 0.09 to 0.21 M KCl) and B-II (fractions 71 to 81; 0.49 to 0.55 M KCl), respectively. (C) Active pools of C fraction are designated C-I (fractions 42 to 48; 0.1 to 0.25 M KCl). Numbers on the left indicate molecular mass in kilodaltons.

the majority of the complex II through IV binding activity eluted at low salt concentrations of 0.09 to 0.21 M KCl (designated B-I in Fig. 4B, fractions 35 to 47). While B-I may represent a mixture of complexes II and III and partial assemblies of complex I, the majority of complex I activity eluted in higher salt fractions (Fig. 4B, fractions 55 to 69). Examination of the elution patterns of v- and c-Rel by Western blot with the SB 66 antibody (Fig. 4B) showed that complexes II through IV coeluted with v-Rel (B-I fraction). In contrast to results obtained with gel filtration fraction A, with which c-Rel was found in every Q-Sepharose fraction, the majority of the detectable B fraction c-Rel coeluted with complex I (fractions 55 to 69). These results presumably reflect differences in the stabilities of the Rel protein complexes in the A and B fractions. v-Rel contained within the B-II fractions (Fig. 2B, fractions 71 to 81) appeared to be inactive for DNA binding. Like inactive A-II, B-II was unresponsive to pH or detergent treatment (data not shown). While it is not clear at present why B-II v-Rel is inactive, it is possible that this fraction contains v-Rel in a modification state different from that of the remainder of the cytosolic v-Rel. Our previous results suggested that the DNA-binding form of v-Rel was modified, presumably by phosphorylation, and represented only a small portion of the v-Rel in the infected cell (19).

As were the gel filtration A and B fractions, the C fraction was subjected to phosphocellulose and Q-Sepharose chromatography. Following elution with a 0.05 to 0.7 M KCl linear gradient, however, the active fraction (0.1 to 0.25 M KCl) was applied to an S-Sepharose column (1.5 by 2.5 cm, 5 mg of resin ml^{-1} ; Pharmacia) and a single-stranded (ss)-DNA agarose column (1.5 by 3.8 cm, 3 mg of resin ml^{-1} ; BRL) to reduce nonspecific DNA binding and enrich for v- and/or c-Rel protein complexes II, III, and IV. S-Sepharose chromatography was performed at pH 7.5 rather than at pH 6.5 (the pH optimum for binding) to avoid induction of the latent v- and/or c-Rel κ B binding activities shown in Fig. 2B. The flowthrough from the ss-DNA agarose column was then applied to another Q-Sepharose column (1.5 by 2.5 cm), which was developed with a 0.05 to 0.7 M KCl linear gradient. Resulting fractions were then analyzed by EMSA and Western blot with the SB 66 polyclonal antibody. As shown in Fig. 4C, peak C-I, fractions 42 to 48, rechromatography of the C fraction on Q-Sepharose resulted in elution of a major peak of DNA-binding activity (C-I) which was made up of complexes II, III, and IV. This peak coeluted with the majority of the v-Rel in gel filtration fraction C, low to undetectable levels of c-Rel, and several cross-reactive proteins, which may represent proteolytic cleavage products of v-Rel (Fig. 4C, peak C-I, fractions 42 to 48). This activity was fully inducible (data not shown). Thus, inducible and constitutive binding activities cofractionated in this purification scheme.

The experiments described here were designed to analyze the κ B binding activities present in the cytosol of lymphoid cells transformed by the Rel/NF- κ B family member v-Rel.

While other family members reside in the cytoplasm in an inactive state in most situations (2, 30), v-Rel appeared to be constitutively active (19). In addition, within the cytosol, v-Rel was shown to be in a high-molecular-weight complex with several presumed inhibitor molecules and its normal cellular homolog, c-Rel (10, 11, 28, 32, 35). Our first goal, therefore, was to examine the complexity of the κ B binding activities in NPB4 cytosol and determine if any inducible binding activity was present. Secondly, we wanted to investigate the functional consequences of the interaction of v- and c-Rel with respect to DNA binding since it had been postulated that v-Rel might act as a dominant negative mutant of c-Rel (for a review, see reference 16).

Our results showed that four v- and/or c-Rel-containing protein-DNA complexes could be identified in the cytosol of NPB4 cells by EMSA. These four complexes (I through IV) appeared to be stimulated, albeit differentially, by detergent (which resulted in an increase in complex II), low pH, and nucleotide (which stimulated complexes I through IV). Thus, both inducible and constitutive v- and/or c-Rel κ B binding activities exist in the cytosol of v-Rel-transformed hematopoietic cells. The basis for the differential effect of these agents is unclear but may reflect the presence of more than one class of inhibitor molecules in these complexes. Members of the I κ B family of inhibitors, which includes pp40 and p124 (the avian homolog of the NF- κ B protein p105), are obvious candidates for this activity. These proteins have been shown to selectively inhibit the binding activity of distinct Rel/NF- κ B subunits or heterodimers of these subunits (20, 32). In support of this, the activity of I κ B α and I κ B β was shown to be modulated by DOC-formamide-Nonidet P-40, low pH (2, 37, 38), and phosphorylation (14, 26). However, none of the defined inhibitors of v- or c-Rel binding activity (pp40, p115, or p124) were identified in highly purified cytosolic v-Rel fractions (data not shown). Clearly, further work will be required to precisely determine the composition and biochemical properties of the potential inhibitory proteins associated with v-Rel in the inactive and inducible fractions. Stimulation by nucleotide may point to a stabilizing effect of nucleoside triphosphates on the binding activity of v- or c-Rel. Such an effect was previously demonstrated for several sequence-specific binding proteins such as simian virus 40 T antigen and transcription factors like Ma1T and bovine spleen NF- κ B (12, 24, 33).

While the pattern of inducible and constitutive v- or c-Rel κ B binding activity is complex, it is clearly reminiscent of that observed previously with nuclear extracts from HeLa cells (27) or murine fibroblasts (4) or with the human lymphoid cell lines H9 and Jurkat (31). These patterns of binding activity are, in part, the result of multiple protein complexes associated with the κ B site. Several polypeptides were found complexed with the human immunodeficiency virus type I and simian virus 40 κ B motifs by coupled DNA precipitation assay and high-resolution two-dimensional gel analysis (19, 31). The experiments reported here reinforce the notion that multiple v- and/or

c-Rel protein-protein complexes exist in the cytosol of v-Rel-transformed cells. The functional interaction between all these complexes remains unclear. For example, it will be important to dissect the relationships between active A-I and B-I and inactive A-II and B-II. However, the analyses carried out in this study have allowed a separation of complex I from complexes II, III, and IV, which represent the major protein-DNA species. Initial chromatographic separation showed that complex I (found predominantly in the A fraction) coeluted with the majority of v- and c-Rel in NPB4 cytosol. The minority of v-Rel eluted in fractions which gave rise to the predominant protein-DNA complexes II through IV. When the B fraction, which contained an overlap of complexes I through III, was analyzed in greater detail by Q-Sepharose chromatography, the majority of c-Rel again coeluted with complex I, which exhibited low levels of DNA-binding activity. The fractions containing the minority of v-Rel had the highest levels of κ B binding activity (B-I). With the exception of v-Rel in the Q-Sepharose B-II fractions, these results strongly suggest that v-Rel-containing protein complexes bind DNA more efficiently than v- and c-Rel complexes. They do not rule out the contribution of other proteins to the v-Rel κ B binding complexes II through IV, however. Since complexes II through IV appeared to be approximately 150 to 450 kDa on the basis of gel filtration, in all likelihood multiple proteins contribute to these species. In fact, further purification of the A, B, and C fractions revealed the presence in each of at least 10 proteins, the identities of which are not known (data not shown).

What are the functional consequences of the interactions of v- and c-Rel with respect to transformation? The majority of v-Rel resided in fractions which had the lowest level of DNA-binding activity (A fraction). In contrast, fractions B and C, which had the highest level of DNA-binding activity, contained predominantly v-Rel. c-Rel was not detected at high levels in the active binding fractions except fraction A, which contained complex I. Perhaps the presence of v-Rel down-modulates c-Rel binding to high-affinity κ B motifs. Alternatively, the interactions of v-Rel and c-Rel may significantly alter the binding specificity of c-Rel alone. Finally, these less active or inactive complexes may represent functionally distinct states of v- and c-Rel in which their DNA-binding domains serve as sites of association with each other and, thereby, decrease their ability to bind to DNA. In these models, v-Rel may behave as a dominant negative mutant of c-Rel. However, a portion of v-Rel remains constitutively active and able to influence the transcription of target genes. While early studies suggested that v-Rel could repress transcription, presumably by interfering with c-Rel activity (5), more recent work points to a role for v-Rel in the transcriptional activation of a number of endogenous genes, including HMG14b (7), major histocompatibility complex class I (7), major histocompatibility complex class II and the interleukin 2 receptor (18), and chemokines such as MIP-1 β and an interleukin 8 homolog (30a). Thus, transformation by v-Rel may, in fact, be the result of both v-Rel interference with c-Rel function and transcriptional regulation by constitutively active v-Rel.

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