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Regulation by phosphorylation of the zinc finger protein KRC that binds the κB motif and V(D)J recombination signal sequences

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ABSTRACT

The DNA binding protein KRC (for κB binding and recognition component of the V(D)J recombination signal sequence) belongs to a family of large zinc finger proteins that bind to the κB motif and contains two widely separated DNA binding structures. In addition to the κB motif, KRC fusion proteins bind to the signal sequences of V(D)J recombination to form highly ordered complexes. Here, we report that KRC may be regulated by post-translational modifications. Specific protein kinases present in the nucleus of pre-B cells phosphorylated a KRC fusion protein at tyrosine and serine residues. Such protein modifications increased DNA binding, thereby providing a mechanism by which KRC responds to signal transduction pathways. KRC is a substrate of epidermal growth factor receptor kinase and P^{34}cdc2 kinase *in vitro*. Our results suggest that activation of the KRC family of transcription factors may provide a mechanism by which oncogenic tyrosine kinases regulate genes with κB-controlled gene regulatory elements.

INTRODUCTION

The DNA binding protein KRC [for κB binding and putative recognition component of the V(D)J recombination signal sequence (RSS)] is a member of a growing family of zinc finger proteins that bind to the κB or related motifs (1–3). Based on the sequence similarities, these proteins are divided into three classes (1) represented by KRC and the two MHC class I gene enhancer binding proteins, MBP1 (4–7) and MBP2 (8–9). These are among the largest DNA binding proteins identified (mol. wt >250 kDa; 7) and are characterized by the presence of four to eight highly conserved C_2H_2 zinc fingers. Similar zinc fingers are also found in proteins of lower eukaryotes, e.g. schnurri in *Drosophila* (10–12) and sem-4 in *Caenorhabditis elegans* (13). The organization of the zinc fingers in this family of proteins is distinct in that four are arranged in two widely separated pairs. In addition, each pair constitutes an integral part of a DNA binding structure named the ZAS domain (3). A ZAS domain contains a pair of zinc fingers, a glutamic acid/aspartic acid-rich acidic sequence and a serine/threonine-rich sequence. The cellular functions of the ZAS proteins are under investigation. They have been shown to be positive transcription regulators of target genes. The ZAS domains interact with κB-like *cis*-acting regulatory elements present in the promoter or enhancer regions of genes mainly involved in immune responses. For example, human MBP1, also known as PRDII-BF1, has been shown to activate transcription of the human immunodeficiency virus enhancer (14). Its mouse counterpart, αA-crystallin binding protein 1 (αA-CRYBP1), also acts as a transcription activator for the αA-crystallin gene during the development of the eye lens (15). In addition to transcriptional regulation, the ZAS proteins may be involved in DNA recombination and in embryonic development. The products of the *KRC* gene, in addition to the κB motif, also bind to the consensus sequences of V(D)J recombination (1,2,16). The *schnurri* gene in *Drosophila*, which affects dorsal ectodermal patterning, may be involved in the decapentaplegic, a TGF-β ligand, signaling pathway (10,11). The *sem-4* gene in *C.elegans* controls neuronal and mesodermal cell development (13). Although these ZAS proteins are important mediators of cellular processes, little is known about the regulation of their functions.

To study the biochemistry and to elucidate the function of KRC, we produced KRC fusion proteins in *Escherichia coli*. A fusion protein, Mbp/Rc_{490}, was produced containing 490 amino acids of KRC including the carboxyl ZAS2 domain and five copies of Ser/Thr-Pro-X-Arg/Lys (S/T-P-X-R/K) fused to the C-terminus of maltose binding protein (Mbp) (1,2). The S/T-P-X-R/K motif is predominantly found in proteins with gene regulatory functions (17), may serve as phosphorylation sites for P^{34}cdc2 (18) and has been shown to bind DNA at the minor groove (19). The formation of highly ordered complexes between Mbp/Rc_{490} and DNA suggests that KRC might be essential in assembling the transcription or recombination complexes (2). Here, we demonstrate that the DNA binding of Mbp/Rc_{490} is under post-translational control and that KRC may be a substrate for protein kinases involved in growth and in the cell cycle.

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MATERIALS AND METHODS

Recombinant proteins, nuclear extracts and KRC antiserum

Mbp and Mbp/Rc$_{490}$ were produced in *E.coli* TB1 strain and purified on amylose resins (2). The p50 subunit of NF-κB (Promega, catalog no. E3770), epidermal growth factor receptor kinase/epidermal growth factor (EGF-RK; CalBiochem, catalog no. 324858-Q) and P^{34}cdc2/cyclin B (P^{34}cdc2; New England BioLabs, catalog no. 6020S) were purchased. The mouse pre-B cell line 22D6 (a gift from Dr David Baltimore, Massachusetts Institute of Technology, Cambridge, MA) was cultured in RPMI medium 1640 (Life Technologies) supplemented with 10% fetal calf serum and 55 µM β-mercaptoethanol. Nuclear extracts were prepared according to the 'mini-extracts' method (20). For protein inactivation, nuclear extracts were either heated at 65°C for 15 min or incubated with 1 µg trypsin (Boehringer Mannheim) at 37°C for 1 h. Polyclonal antiserum was raised against Mbp/Rc$_{490}$ in rabbits using standard procedures (21).

Electrophoretic mobility shift assay (EMSA)

EMSA using a DNA fragment containing the κB motif labeled with [^{32}P]dCTP by the Klenow fragment of DNA polymerase was performed as previously described (2). For 'supershift' assays, after a 15 min incubation, polyclonal KRC antiserum (dilutions of 1:1 to 1:256) was added to the DNA–protein binding reactions and incubation was continued for another 10 min before gel loading.

Protein phosphorylation

Mbp or Mbp/Rc$_{490}$ (~2 µg) was incubated with 200 µM ATP (Sigma) in the presence of [γ-^{32}P]ATP (~5 µCi, 6000 Ci/mmol; Amersham) and 22D6 nuclear extracts (~50 ng) at 37°C in modified buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 10 mM DTT, 20% glycerol and 100 µM $ZnCl_2$). Phosphorylation of Mbp/Rc$_{490}$ by EGF-RK (0.5 U) or P^{34}cdc2 (1 U) was performed according to the manufacturers' instructions.

SDS–PAGE, southwestern blot analysis and phosphoamino acid analysis

Protein samples were resolved by 4–20% gradient SDS–PAGE. Total proteins were visualized by staining gels with Coomassie brilliant blue and [^{32}P]phosphoproteins were visualized by exposing dried gels to X-ray films or with a PhosphorImager (Molecular Dynamics). The relative radioactivity was determined using the software ImageQuant. Southwestern blot analysis using a DNA fragment containing the κB motif labeled with [^{32}P]dCTP by the Klenow fragment of DNA polymerase was performed as previously described (1). Phosphoamino acids were determined by thin layer chromatography according to published procedures (22,23).

RESULTS

Incubation with nuclear extracts increases the DNA binding of KRC fusion proteins

The conditions for detecting the association of DNA binding proteins with nuclear factors by EMSA was established using the p50 subunit of NF-κB expressed in bacteria and a κB DNA labeled with [^{32}P]dCTP by the Klenow fragment of DNA polymerase. NF-κB are DNA binding proteins that, like KRC, bind to the κB motif (24). The protein and DNA mixtures were supplemented with or without nuclear extracts prepared from the pre-B cell line 22D6. After a 15 min incubation, DNA–protein complexes and free DNA were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography. p50 formed a slow migrating DNA–protein complex with the κB DNA (Fig. 1A, lane 1). The addition of the nuclear extracts (~2 µg) resulted in the formation of a more slowly migrating species (Fig. 1A, lane 2). Thus, the association of p50 with protein factors can be demonstrated by the supershift assay. Likely, p50 forms heteromers with another member of the NF-κB family, p65, which is present in the nucleus of pre-B cells (24,25).

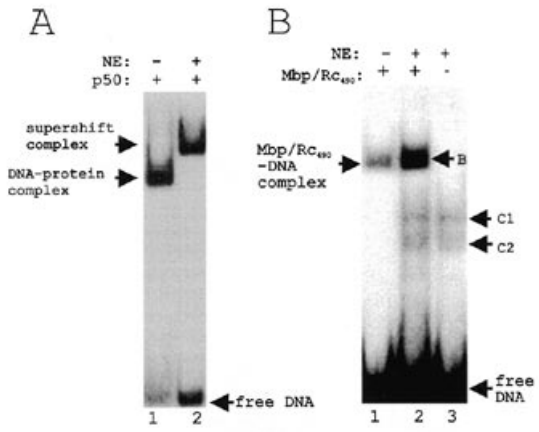

Figure 1. Nuclear extracts increase the DNA binding of KRC fusion proteins. (**A**) Autoradiogram of EMSA of the p50 subunit of NF-κB, with or without 22D6 nuclear extracts (2 µg) and ^{32}P-labeled κB DNA fragment. (**B**) Autoradiogram of EMSA of Mbp/Rc$_{490}$ (0.5 µg), nuclear extracts of 22D6 cells (0.5 µg) or both and ^{32}P-labeled κB DNA fragment.

To determine if the association of KRC with nuclear factors could be detected with the above procedures, supershift assays were performed using a KRC fusion protein, Mbp/Rc$_{490}$. Mbp/Rc490 contains 490 amino acids of KRC including the ZAS2 DNA binding domain fused to the C-terminus of Mbp (2). The KRC fusion protein (0.5 µg) bound to the κB motif to form a slow migrating DNA–protein complex (Fig. 1B, lane 1). The addition of 22D6 nuclear extract to the binding reaction did not supershift the Mbp/Rc$_{490}$–DNA complex (designated complex B in Fig. 1B, lane 2). However, the amount of complex B seen in the presence of nuclear extract and Mbp/Rc$_{490}$ was ~20-fold more than the complex seen in the presence of Mbp/Rc$_{490}$ alone. The result suggests that Mbp/Rc$_{490}$ may not associate with nuclear factors under the experimental conditions employed or that such complexes may have a similar gel mobility to Mbp/Rc$_{490}$. Alternatively, nuclear factors may have modified Mbp/Rc$_{490}$ and increased its DNA binding affinity. Two faster migrating complexes, labeled C1 and C2, were observed in these experiments (Fig. 1B, lanes 2 and 3). They were probably DNA–protein complexes of NF-κB. NF-κB is present in the nuclear extracts of pre-B cells (24,25). These complexes were not observed in Figure 1A because p50, a member of the NF-κB family, was used in that experiment.

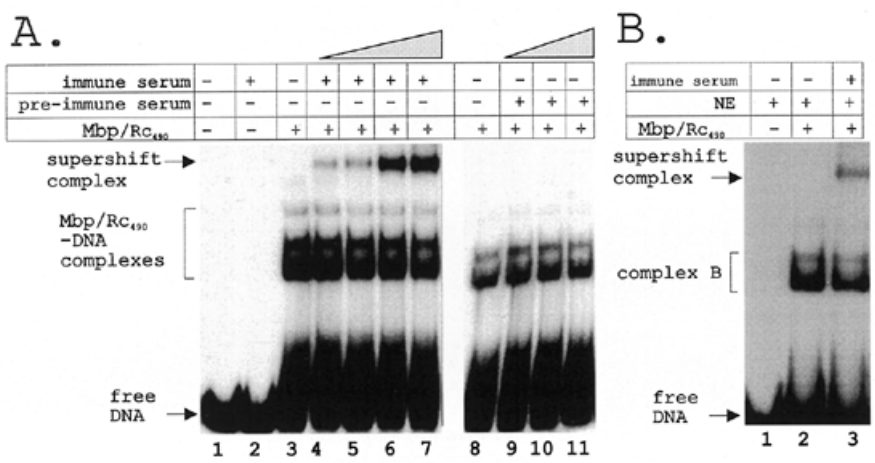

Figure 2. KRC antiserum forms supershift complexes with KRC fusion proteins in EMSA. Autoradiograms of (**A**) Mbp/Rc$_{490}$ (0.5 µg) and (**B**) Mbp/Rc$_{490}$ (0.2 µg) and nuclear extracts (0.1 µg) incubated with a ^{32}P-labeled κB DNA fragment with or without KRC antiserum. The dilutions of the KRC immune serum from right to left in (A) are 1, 8, 64 and 256 and preimmune serum are 1, 2 and 4. The KRC antiserum was diluted 2-fold in (B).

The ability of Mbp/Rc$_{490}$ to form 'supershift' complexes (large complexes of DNA, DNA binding protein and antibodies) in EMSA was demonstrated using rabbit polyclonal antiserum to Mbp/Rc$_{490}$. The addition of the KRC antiserum to the mixture of Mbp/Rc$_{490}$ and κB DNA resulted in the formation of a more slowly migrating species in a dosage-dependent manner (Fig. 2A, lanes 1–7). The KRC antiserum itself did not form distinct complexes with the κB DNA (Fig. 2A, lane 2) and the preimmune serum was inactive in the supershift assay (Fig. 2A, lanes 8–11). Furthermore, the addition of the KRC antiserum also supershifted complex B formed when nuclear extracts were present in the binding mixture (Fig. 2B). The data suggest that factors are present in pre-B cell nuclear extracts that can increase the DNA binding of Mbp/Rc$_{490}$. The factor is likely a protein(s) because heat treatment (65°C for 15 min) or incubation with trypsin (1 µg, 37°C for 1 h) abrogated the ability of the nuclear extracts to increase the DNA binding of Mbp/Rc$_{490}$ (data not shown).

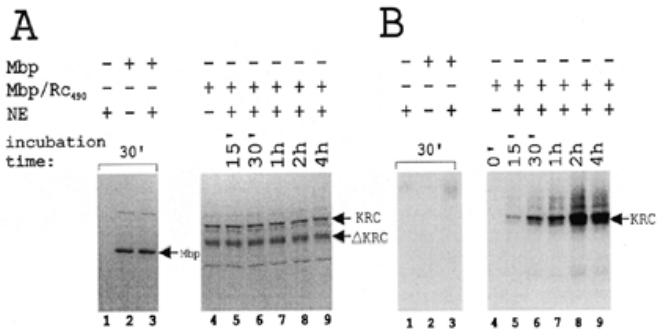

Figure 3. Phosphorylation of Mbp/Rc$_{490}$ by nuclear extracts. Mbp or Mbp/Rc$_{490}$ (2 µg each) were incubated with [γ-^{32}P]ATP with or without nuclear extracts (NE, 50 ng) and subjected to SDS–PAGE. (**A**) Coomassie blue staining of the protein gel. (**B**) An autoradiogram of the dried gel showing that Mbp/Rc$_{490}$ but not Mbp incorporated ^{32}P.

Phosphorylated KRC has a higher DNA binding affinity

Because the most prevalent form of protein modification is phosphorylation (26,27), we examined the phosphorylation of Mbp/Rc$_{490}$ by the nuclear extracts. The incorporation of ^{32}P into the fusion protein was used as an indicator of protein phosphorylation. Mbp/Rc$_{490}$ (~2 µg) was incubated with nuclear extracts in the presence of [γ-^{32}P]ATP at the time intervals shown in Figure 3. After the incubation, the samples were analyzed by SDS–PAGE. Proteins were first visualized by staining with Coomassie brilliant blue (Fig. 3A). The vector-encoded Mbp is ~50 kDa (lanes 2 and 3) and Mbp/Rc$_{490}$ is ~100 kDa (lanes 4–9). In addition, notable amounts of partially degraded Mbp/Rc$_{490}$ products of ~70 kDa (ΔKRC) were also observed in these samples. Initial experiments were performed to establish the conditions for efficient phosphorylation of Mbp/Rc$_{490}$ and for minimal background phosphorylation. In these experiments, the amount of nuclear extracts used was 50 ng, which was beyond the detection limit of Coomassie brilliant blue. Subsequently, the gel was dried and exposed to X-ray film to visualize ^{32}P-labeled proteins. A prominent phosphorylated species corresponding to the ~100 kDa Mbp/Rc$_{490}$ was observed (Fig. 3B). The amount of ^{32}P incorporation increased with the time of incubation and maximal phosphorylation was observed at 2 h (Fig. 3B). As controls, Mbp alone, Mbp/Rc$_{490}$ alone and Mbp with nuclear extract did not result in the incorporation of ^{32}P into these proteins (Fig. 3B, lanes 2–4). Furthermore, no ^{32}P incorporation was observed for ΔKRC, although its amount was comparable with the 100 kDa Mbp/Rc$_{490}$. This observation suggests that the missing portion of the 70 kDa degradation product is essential for protein phosphorylation, by harboring the phosphorylation sites or by configuring the protein so as to allow phosphorylation. We conclude that Mbp/Rc$_{490}$ can be phosphorylated by the nuclear extracts and that the phosphorylation sites are likely limited to the KRC portion of the fusion protein.

The DNA binding ability of Mbp/Rc$_{490}$ with respect to its state of protein phosphorylation was examined by southwestern analysis. Proteins that are not linked covalently will be dissociated

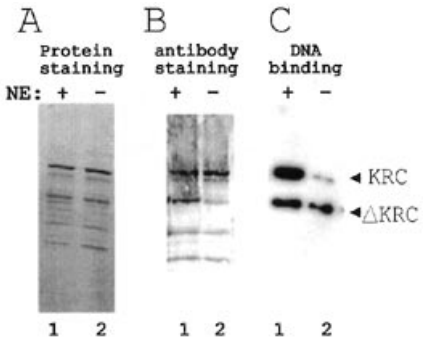

Figure 4. DNA binding of KRC fusion proteins is dependent on the state of phosphorylation. Mbp/Rc$_{490}$ (2 µg) was incubated with ATP (0.2 mM) with (lane 1) or without pre-B cell 22D6 nuclear extracts (NE, 50 ng) (lane 2) at 37°C for 30 min and then subjected to SDS–PAGE. (**A**) Coomassie blue staining of the protein gel. (**B**) Immunoblot analysis of Mbp/Rc$_{490}$ with KRC antiserum. (**C**) Southwestern blot analysis of proteins transferred onto nitrocellulose membranes and hybridized to ^{32}P-labeled κB DNA.

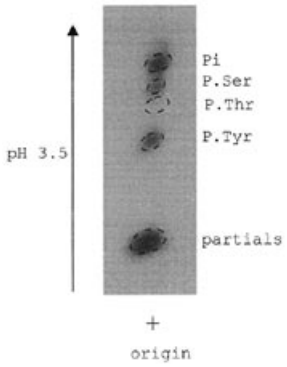

Figure 5. Phosphoamino acid analysis. Mbp/Rc$_{490}$ was incubated with [γ-^{32}P]ATP and pre-B cell 22D6 nuclear extracts for 30 min and subjected to SDS–PAGE. After autoradiography, ^{32}P-labeled Mbp/Rc$_{490}$ was purified from dried gels and hydrolyzed with hydrochloric acid. The phosphoamino acid composition was determined by thin layer chromatography. Encircled areas represent the location of phosphoserine (P.Ser), phosphothreonine (P.Thr) and phosphotyrosine (P.Tyr) based on ninhydrin staining. The buffer pH, direction of electrophoresis, products of partial acid hydrolysis (Pi) and origin of the sample spotted onto thin layer chromatographic plates are shown.

during SDS–PAGE. Therefore, this experiment would rule out the possibility that increased DNA binding merely results from protein association or stabilization. Mbp/Rc$_{490}$ was incubated with or without nuclear extract before SDS–PAGE. Staining of proteins in the gels by Coomassie brilliant blue or on nitrocellulose membrane filters with KRC antiserum showed that the amounts of Mbp/Rc$_{490}$ in the samples were comparable (Fig. 4A and B, lanes 1 and 2). The 100 kDa Mbp/Rc$_{490}$, ΔKRC and two smaller degradation products were observed. Duplicated protein samples were transferred onto membrane filters and the filters hybridized to a ^{32}P-labeled κB DNA. Two κB DNA binding species of ~100 and ~70 kDa were observed in each sample (Fig. 4C, lanes 1 and 2). While the DNA binding affinity of the smaller 70 kDa ΔKRC species was comparable between the two samples, the DNA binding affinity of the larger 100 kDa species was enhanced ~10-fold by incubation with the nuclear extract. Thus, these data, in conjunction with the data of Figure 3 showing phosphorylation by nuclear extracts of the 100 kDa species but not the 70 kDa species, suggest that protein phosphorylation increases the DNA binding of Mbp/Rc$_{490}$.

KRC was phosphorylated at serine and tyrosine residues

As an initial effort to identify the KRC kinase(s), the amino acid residues in Mbp/Rc$_{490}$ which were phosphorylated by the nuclear extracts were determined. After incubating Mbp/Rc$_{490}$ with [γ-^{32}P]ATP and nuclear extract, samples were subjected to SDS–PAGE. [^{32}P]Mbp/Rc$_{490}$ was excised from dried gels and hydrolyzed to amino acids with hydrochloric acid. Phosphoamino acids were determined after electrophoresis in the presence of phosphoamino acid standards followed by autoradiography. Radioactivity was observed for tyrosine and serine, but not for threonine (Fig. 5). Thus, the data suggest that the KRC kinase has dual enzymatic specificity or that more than one kinase is involved.

KRC may be a substrate for EGF-RK and P^{34}cdc2

Computer-assisted sequence analysis identifies potential kinase recognition sites in KRC. Multiple phosphorylation sites including targets for cAMP- and cGMP-dependent protein kinase, protein kinase C and casein kinase II are present. Of particular interest are the recognition motifs for EGF-RK and P^{34}cdc2. EGF-RK is a tyrosine kinase with a target consensus sequence E/D-Y-I/L/V. A potential EGR-RK target sequence, E-Y-I, is present at amino acids 1708–1710, 10 residues upstream of zinc finger 4 (Fig. 6A). P^{34}cdc2 is a cyclin-dependent kinase and is activated by association with cyclin B. The target sequence for P^{34}cdc2 is S/T-P-X-R/K (18). The S/T-P-X-R/K motif is frequently present in gene regulatory proteins and can form a β-turn I structure that can bind DNA at the minor groove (17,19). Mbp/Rc$_{490}$ has five copies of the S/T-P-X-R/K motif. To examine if EGF-RK and P^{34}cdc2 may phosphorylate KRC, we incubated these kinases with Mbp/Rc$_{490}$ before EMSA. The result showed that both EGF-RK and P^{34}cdc2 could alter the DNA binding of Mbp/Rc$_{490}$, but with antagonistic effects (Fig. 6). EGF-RK increased the DNA binding affinity of Mbp/Rc$_{490}$ by ~9-fold while P^{34}cdc2 decreased the DNA binding by half.

DISCUSSION

KRC cDNA was isolated from a mouse thymocyte expression cDNA library by probing with a DNA ligand containing four copies of the RSS (1). Subsequently, KRC fusion proteins produced in *E.coli* were shown to have dual DNA binding specificities to the RSS and to the κB motif (1,2,16). The DNA binding specificities together with the lymphoid expression conform to the notion that KRC may be involved in V(D)J recombination (1). The ability of a KRC fusion protein, Mbp/Rc$_{490}$, to form highly ordered multimeric DNA–protein complexes with short DNA ligands further suggests that KRC could play a role in bringing distal variable region gene segments of the immune receptors together for V(D)J recombination (2). DNA binding and protein multimerization are likely to be intrinsic characteristics of KRC because the KRC portion of the fusion protein retains these properties after the Mbp domain was removed by enzymatic digestion of Mbp/Rc$_{490}$ with Factor Xa (2). One advantage of utilizing fusion proteins in these experiments is that they can be produced easily and economically in bacteria.

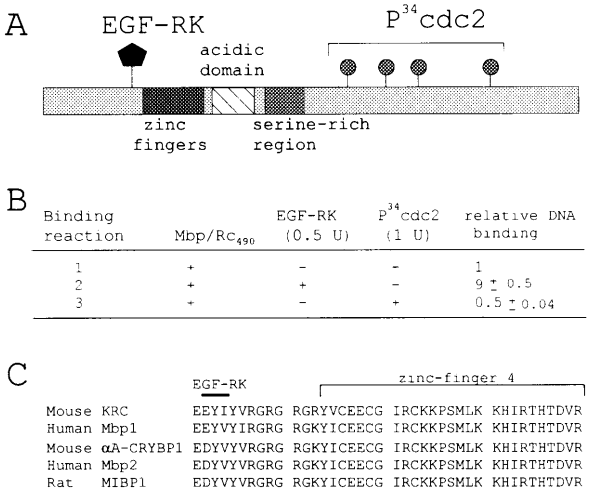

Figure 6. Structural domains and motifs present in the KRC portion of Mbp/Rc$_{490}$ and the effect of EGF-RK and P^{34}cdc2 on DNA binding of Mbp/Rc$_{490}$. (**A**) A diagram showing the structural domains and motifs present in the KRC portion of Mbp/Rc$_{490}$. (**B**) The effect of pretreating Mbp/Rc$_{490}$ with EGF-RK or P^{34}cdc2. Mbp/Rc$_{490}$ (1 or 2 µg) was preincubated with EGF-RK (0.5 U) or P^{34}cdc2 (1 U) at 37°C for 30 min. One tenth of the protein samples was subjected to EMSA using a ^{32}P-labeled κB DNA fragment. The relative radioactivity was determined by exposing the dried gels with a PhosphorImager and the software ImageQuant (Molecular Dynamics). For comparison, the relative DNA binding of untreated Mbp/Rc$_{490}$ was tentatively assigned as 1. The data were compiled from four experiments. (**C**) Conservation of the EGF-RK recognition site, E/D-Y-I/L/V, in KRC-related proteins upstream of zinc finger 4.

Additionally, the vector-encoded sequences can often be used to purify the fusion proteins by affinity chromatography. It is generally assumed that DNA binding proteins produced in bacteria have similar properties as in eukaryotes, although protein modification such as phosphorylation that is known to modulate DNA binding (26,27) is uncommon in bacteria.

KRC fusion proteins bind RSS and κB DNA motifs (1,2,16). Regulation of KRC's DNA binding ability occurs via RNA processing and by post-translational modification. Previously, we have shown that multiple differentially spliced transcripts of KRC were produced in thymus and brain (3,16). Alternative RNA splicing is a common feature of the *KRC* family of genes: alternatively spliced transcripts were also observed in *MBP1* (28), *αA-CRYBP1* (15), and *schnurri* (11). The signature of the KRC family of proteins is the presence of a pair of ZAS DNA binding domains. These domains, ZAS1 and ZAS2, are widely separated and have distinct DNA binding properties (9,14,16,28). Alternative splicing modulates the DNA binding ability of these proteins by selectively deleting one or both of the ZAS-encoding domains (16,28). In the case of *KRC*, alternative splicing involves a gigantic 5487 bp exon, a 459 bp region within this very large exon or a 176 bp exon (16). These DNA regions encode either the ZAS1 or the ZAS2 domain, suggesting that these alternatively spliced transcripts direct the production of proteins with varying DNA binding capability. The proteins resulting from these processed transcripts would have distinct N-termini and the number of ZAS domains varies from two to none (16).

Not only is KRC's DNA binding ability regulated by differential RNA splicing events encoding the ZAS domains, in this study, we found that it is also regulated by post-translational modifications. The DNA binding affinity of KRC may be modulated by phosphorylation. Here, we show that the DNA binding affinity of Mbp/Rc$_{490}$ was increased upon phosphorylation by nuclear extracts. The protein kinase(s) present in the nuclear extracts of pre-B cells that phosphorylates Mbp/Rc$_{490}$ has yet to be identified. The KRC kinase that phosphorylated tyrosine or serine residues and enhanced DNA binding by Mbp/Rc$_{490}$ is present mainly in the nucleus. Cytoplasmic extracts had minimal effects on DNA binding of the fusion protein (data not shown).

We examined the effect of EGF-RK and P^{34}cdc2 on DNA binding of Mpb/Rc$_{490}$ to determine if they are able to phosphorylate KRC. These candidate kinases were chosen because an overt structural and functional correlation might be established between their phosphorylation of distinct residues or motifs in KRC and DNA binding (Fig. 6). The delineation of these sites will direct future experiments to identify specific tyrosine or serine residues whose phosphorylation is important for DNA binding by site-directed mutagenesis. However, the fact that the nuclear extracts were isolated from unstimulated cells suggests that the activity present in the nuclear extracts that phosphorylates Mbp/Rc$_{490}$ and increases its DNA binding affinity is most likely distinct from EGF-RK.

The enhancement of DNA binding of Mbp/Rc$_{490}$ by EGF-RK may explain how oncogenic tyrosine kinases lead to cell transformation. The notion that the KRC family of proteins could be involved in oncogenic transformation stems from the fact that several PRDII-controlled promoters were activated in v-*src*-transformed cells (29) and that the transcription of *MBP1*, a *KRC*-related gene that binds specifically to PRDII, was induced by the virus (7,30). PRDII was originally identified as a positive regulatory domain in the promoter of the β-interferon gene (7) and was subsequently found in the promoters of other genes, including the long terminal repeat of human immunodeficiency virus-1 (14). Therefore, it was suggested that oncogenic tyrosine kinases may play a role in the induction of viruses with PRDII-controlled promoters (29). However, the fact that the induction of *MBP1* mRNA lags behind the induction of β-interferon seems to make *MBP1* as the limiting transcription regulator of β-interferon less likely (7). Here, we show that EGF-RK increases DNA binding of Mbp/Rc$_{490}$. The conservation of the EGF-RK recognition site in the KRC family of transcription factors (Fig. 6C) leads to the speculation that oncogenic tyrosine kinases may activate the KRC family of proteins by phosphorylation prior to induction of transcription.

Somatic V(D)J recombination is essential for the generation of the diversity of immune receptors (31). Because V(D)J recombination involves double-strand DNA cleavage (32) that is potentially deleterious during DNA replication or cell division, this process should be prohibited during the S and M phases of the cell cycle. One mechanism by which V(D)J recombination may be linked to the cell cycle clock is through periodic phosphorylation of the recombination-activating gene *RAG2* (33,34). RAG2 is targeted for rapid degradation near the G$_1$-S phase boundary upon phosphorylation at Thr$_{490}$ by one or more cyclin-dependent kinases (34). P^{34}cdc2 is a cyclin-dependent kinase that causes a subtle but significant decrease in DNA binding by Mbp/Rc$_{490}$. The recognition site for P^{34}cdc2 is S/T-P-X-R/K and this motif has also been shown to bind DNA at the minor groove. Multiple copies of similar repeats are found in other KRC-related proteins (3) as well as in other proteins mainly with a gene regulatory function (17). The phosphorylation of S-P-X-R/K has been

shown to modulate histone–DNA interactions (35). In the H1 and H2B histones of sea urchin spermatozoa, these P^{34}cdc2 recognition motifs appear to be sites of phosphorylation by a kinase that is active in a developmental stage-specific manner in spermatids and upon fertilization in the zygote (36,37). This observation has suggested a role of these sites in the packaging of DNA through phosphorylation-dependent DNA–protein interactions (37). While the implications of DNA binding by KRC are not yet definitive, one may speculate that KRC regulates transcription and/or accessibility of target DNA by specific binding to RSS and to the κB motifs. Although these observations are suggestive, additional studies are required to determine whether EGF-RK and P^{34}cdc2 play a role in the phosphorylation of endogenous KRC. The major conclusion of this study is that phosphorylation may be a key mechanism regulating the DNA binding ability of the KRC family of proteins.

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REFERENCES

1 Wu,L.-C., Mak,C.-H., Dear,N., Boehm,T., Foroni,L. and Rabbitts,T.H. (1993) *Nucleic Acids Res*., **22**, 5067–5073.
2 Mak,C.H, Standtmann,J. and Wu,L.-C. (1994) *Nucleic Acids Res*., **22**, 383–390.
3 Wu,L.-C., Liu,Y., Strandtmann,J., Mak,C.-H., Lee,B., Li,Z. and Yu,C.Y. (1996) *Genomics*, **35**, 415–424.
4 Singh,H., LeBowitz,J.H., Baldwin,A.S. and Sharp,P. (1988) *Cell*, **52**, 415–423.
5 Maekawa,T., Sakura,H., Sudo,T. and Ishii,S. (1989) *J. Biol. Chem*., **264**, 14591–14593.
6 Baldwin,A.S., LeClair,K.P., Hairinder,S. and Sharp,P.A. (1990) *Mol. Cell. Biol*., **10**, 1406–1414.
7 Fan,C.-M. and Maniatis,T. (1990) *Genes Dev*., **4**, 29–42.
8 Rustgi,A., van't Veer,L.J. and Bernards,R. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8707–8710.
9 van't Veer,L.J., Lutz,P.M., Isselbacher,K.J. and Bernards,R. (1990) *Proc. Natl Acad. Sci. USA*, **89**, 8971–8975.
10 Grieder,N.C., Nellen,D., Burke,R., Basler,K. and Affolter,M. (1995) *Cell*, **81**, 791–800.
11 Arora,K., Dai,H., Kazuko,S.G., Jamal,J., O'Connor,M.B., Letsou,A. and Warrior,R. (1995) *Cell*, **81**, 781–790.
12 Matunis,E., Tran,J., Gonczy,P., Caldwell,K. and DiNardo,S. (1997) *Development*, **124**, 4383–4391.
13 Basson,M. and Horvitz,H.R. (1996) *Genes Dev*., **10**, 1953–1965.
14 Seeler,J.-S., Muchardt,C., Suessle,A. and Gaynor,R.B. (1994) *J. Virol*., **68**, 1002–1009.
15 Brady,J.P., Kantorow,M., Sax,C.M., Donovan,D.M. and Piatigorsky,J. (1995) *J. Biol. Chem*., **270**, 1221–1229.
16 Mak,C.-H., Li,Z., Allen,C.E., Liu,Y. and Wu,L.-C. (1998) *Immunogenetics*, **48**, 32–39.
17 Suzuki,M. (1989) *J. Mol. Biol*., **207**, 61–84.
18 Pearson,R.B. and Kemp,B.E. (1991) *Methods Enzymol*., **200**, 62–81.
19 Churchill,E.M.A. and Suzuki,M. (1989) *EMBO J*., **8**, 4189–4195.
20 Schreiber,E., Matthias,P., Müller,M.M. and Schaffner,W. (1989) *Nucleic Acids Res*., **17**, 6419.
21 Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22 Boyle,W.J., van de Geer,P. and Hunter,T. (1991) *Methods Enzymol*., **201**, 110–149.
23 Cooper,J.A., Bartholomew,M.S. and Hunter,T. (1983) *Methods Enzymol*., **99**, 387–402.
24 Ghosh,S., Gifford,A.M., Riviere,L.R., Tempst,P., Nolan,G.P. and Baltimore,D. (1990) *Cell*, **62**, 1019–1029.
25 Fujita,T., Nolan,G.P., Ghosh,S. and Baltimore,D. (1992) *Genes Dev*., **6**, 775–787.
26 Jackson,S.P. and Tjian,R. (1988) *Cell*, **55**, 25–33.
27 Hunter,T. and Karin,M. (1992) *Cell*, **70**, 375–387.
28 Muchardt,C., Seeler,J.-S., Nirula,A., Shurland,D.-E. and Gaynor,R.B. (1992) *J. Virol*., **66**, 244–250.
29 Dehbi,M., Beaulieu,S., Cohen,E.A. and Bedard,P.A. (1994) *Oncogene*, **9**, 2399–2403.
30 Fan,C.M. and Maniatis,T. (1989) *EMBO J*., **8**, 101–110.
31 Tonegawa,S. (1983) *Nature*, **302**, 575–581.
32 Roth,D.B., Nakajima,P.B., Menetski,J.P., Bosma,M.J. and Gellert,M. (1992) *Cell*, **69**, 41–53.
33 Lin,W.-C. and Desiderio,S. (1993) *Science*, **260**, 953–959.
34 Lin,W.-C. and Desiderio,S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2733–2737.
35 Hills,C.S., Rimmer,J.M., Green,B.N., Finch,J.T. and Thomas,J.O. (1991) *EMBO J*., **10**, 1939–1948.
36 Hill,C.S., Packman,L.C. and Thomas,J.O. (1990) *EMBO J*., **9**, 805–813.
37 Hill,C.S. and Thomas,J.O. (1990) *Eur. J. Biochem*., **187**, 145–153.