Interaction of Two Multifunctional Proteins

HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN K AND Y-BOX-BINDING PROTEIN*

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The heterogeneous nuclear ribonucleoprotein (hnRNP) K, a component of the hnRNP particles, appears to be involved in several steps of regulation of gene expression. To gain insight into mechanisms of K protein action, we performed two-hybrid screens using full-length hnRNP K as a bait. Several novel protein partners were identified, including Y-box-binding protein (YB-1), splicing factors 9G8 and SRp20, DNA-methyltransferase, hnRNP L, and hnRNP U. In vitro binding studies and co-immunoprecipitation from cellular extracts provided evidence for direct interaction between hnRNP K and YB-1. Two distinct domains in YB-1 were responsible for binding to K protein. Each protein was able to transcribe transcription from a polyadenylidine-rich promoter; however, this effect was reduced when K and YB-1 proteins were coexpressed suggesting a functional interaction between these two proteins.

Activation of gene expression in eukaryotes encompasses multiple processes including chromatin reorganization, followed by the assembly of transcription factors onto promoter elements, processing of pre-mRNA transcripts, and export of the mRNA into the cytoplasm and translation. Although each of these steps has been intensively explored, little is known of how these processes are linked and what factors are involved. RNA-binding proteins, components of the hnRNP1 particles, may play such a role (1). The formation of these particles is closely coupled to transcription. There are over 20 proteins that bind to pre-mRNA, which are collectively known as the heterogeneous nuclear ribonucleoproteins. These proteins are thought to promote formation of pre-mRNA secondary structure and can influence splice site selection. Some hnRNP proteins shuttle between the nucleus and cytoplasm and potentially can influence mRNA export and translation. RNA-binding proteins not only associate with nascent pre-mRNA but also have been found to contact the basal transcriptional machinery and can directly bind to DNA regulatory elements within the promoter itself (reviewed in Ref. 2). Moreover, recent data suggest that components of hnRNPs may serve as a structural and/or functional link between RNA metabolism and nuclear architecture (3).

The hnRNP K protein, a component of the hnRNP particle, exhibits many of the functions described above. It has a wide tissue distribution and is detected in the nucleus and cytoplasm (4). K protein contains both a classical bipartite-basic nuclear localization signal and the novel K nuclear shuttling domain that allow it to shuttle between nucleus and cytoplasm by utilization of one of the two pathways for nuclear entry (5).

K protein has a strong affinity for polyuridylic-acid-rich RNA and for single-stranded DNA. K protein is also known to interact with many proteins involved in several cellular processes. Because hnRNP K interacts with many protein kinases such as Src, protein kinases C, and the proto-oncogene vav (6–8), it has been proposed that hnRNP K is involved in signal transduction and may act as a "docking platform" mediating cross-talk between these molecules (9). K protein may facilitate the ability of other factors to stimulate or repress transcription by recruiting gene-specific or general components of the transcription machinery to promoters, such as TBP, SP1, and the transcriptional repressor, Zik1 (10–12).

In addition to its involvement in signal transduction and transcription, K protein may participate in the regulation of other nucleic acid-dependent processes. For example, binding of K protein to Eed (13), a putative silencer of homeotic genes, may reflect participation of hnRNP K in chromatin reorganization. K protein-mediated silencing of the lipoygenase mRNA through the binding to the CU-rich 3′-untranslated region (14) represents an example of hnRNP K involvement in translation.

Taken together, these studies suggest that K protein is involved in multiple processes that comprise gene expression and may serve as a link between signal transduction pathways and nucleic acid-dependent processes (7, 15). To gain more insight into the mechanisms of K protein action, we performed two-hybrid screens using full-length hnRNP K as a bait. We identified several novel protein partners, including YB-1 transcription factor, splicing factors 9G8 and SRp20, DNA-methyltransferase, hnRNP L, and hnRNP U. In this work we describe the interaction between K protein and YB-1, another multifunctional protein.

MATERIALS AND METHODS

Plasmids—For two-hybrid analyses, cDNA encoding full-length K protein was inserted into multiple cloning sites of plasmid pGBT9, which expresses the Gal4 DNA binding domain (CLONTECH). In frame fusion was verified by sequencing using the DyeDeoxy terminator cycle sequencing kit (PE Applied Biosystems) and by in vitro transcription/translation analysis. Two cDNA libraries were used in the two-hybrid screen. The mouse cDNA library was generated by random-primed cDNA synthesis from a 9–10-day-old mouse embryo (Dr. S. Hollenberg) and size-selected for inserts in the range of 350–700 nucleotides. This library was inserted into the transcription activation domain vector, splicing factors 9G8 and SRp20, DNA-methyltransferase, hnRNP L, and hnRNP U. In this work we describe the interaction between K protein and YB-1, another multifunctional protein.

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‡ The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; TBP, TATA-binding protein; GST, glutathione S-transferase; CSD, cold shock domain; PAGE, polyacrylamide gel electrophoresis; YB, Y-box.
cDNA was subcloned into the NotI site of pBluescript II SK (Stratagene), and the resulting plasmid was denoted pSK-YB-1. To create the YB-1 C mutant, pSK-YB-1 was digested with SalI and religated. The NotI/SalI fragment was isolated and inserted into the NotI/SalI sites of pET28a.

The 0.7-kilobase SalI fragment from pSK-YB-1 containing the N terminus of EFI/YB-1 was subcloned into the SalI site of the pSK vector. This mutant was named YB-1 NI. The NII, cold shock domain (CSD), IIII, C, and CIII fragments were created by polymerase chain reaction and subcloned into pET28a vector (Fig. 5). All constructs were verified by sequencing.

Flag-YB-1 was generated by insertion of YB-1 cDNA into the EcoRI/Xhol sites of pI8-Flag mammalian expression vector.

PSGS-K and pSG 5 was a gift from Dr. A. Ostareck-Lederer. pSG 5-YB-1 was created by inserting the NotI fragment of EFI/YB-1 into pSG5.

The plasmids pgt-TBP, p536CT3wt, and p536CT3mut were a gift from Dr. D. Levens. CT3wt-c-fos and CT3mut-c-fos promoter elements were cut out from p536CT3wt and p536CT3mut, respectively, and subcloned into the pGL3-basic vector (Promega). All plasmids were purified using the QiAfilter Plasmid Maxi Kit (Qiagen).

**Yeast Strains, Transformations, and Growth Conditions—**Transformation of yeast cells was carried out by the method of Klebe et al. (16). Yeast transformants were selected and cultivated on SD synthetic dextrose medium (2% dextrose, 0.67% yeast nitrogen base without amino acids) supplemented with essential amino acids and nucleotides.

The yeast strain HFC7 (MATa, ura3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, gal1–542, gal80–538, lys2::Gal-HIS3, ura3::(GAL 17-mers), CYC1-LacZ) was used to perform primary screening. Histidinotroph prototroph clones were assayed for β-galactosidase activity by a filter assay. Positive clones were then transformed into the yeast strain SFPY326 (MATa, ura3–52, his3–200, ade2–101, lys2–801, trp1–901, leu2–3, 112, can1, gal1–542, gal80–538, ura3::Gal1-LacZ) and tested for β-galactosidase activity.

β-galactosidase expression was detected either by qualitative determination using a filter lift assay (17) with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate or quantitatively by β-D-galactopyranoside hydrolysis measured photometrically at 420 nm upon permeabilization of cells (17). The enzymatic activity was normalized to cell culture density expressed as arbitrary units.

**Synthesis and Purification of GST Fusion Proteins—**GST-K and GST-TBP constructs were described previously (6, 7).

In vitro transcription and translation was performed using the TNT T7 Quick coupled transcription/translation system as per the manufacturer’s protocol (Promega, Madison, WI).

**In Vitro Binding Studies—**2.5 μl of the 35S-labeled products were added to a suspension of 20 μl of glutathione or 10 μl of either poly(A), (C), (G), (U), or (I) beads in 100 μl of binding buffer (12). After mixing for 30 min at 4 °C, the beads were washed three times with 400 μl of binding buffer and then boiled with 30 μl of SDS loading buffer; proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

**Transient Transfections—**HeLa cells were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum in 60- or 100-mm dishes and were transfected using SuperFect Transfection Reagent as per the manufacturer’s protocol (Qiagen). Either pRL-null plasmid (Promega) encoding Renilla luciferase or pSVgal plasmid (Promega) encoding β-galactosidase was co-transfected to normalize transfection efficiency. The same protocol was used to transfect NIH3T3 cells. Luciferase activity assay was performed according standard protocols (Promega). β-Galactosidase activity was measured using a Galacto-light plus chemiluminescent reporter assay system (Tropix).

Cell extracts were prepared by a modified version of the method of Dignam et al. (18) as described previously (7). Protein concentration was measured using the DC method (Pierce).

**Immunoprecipitation and Western Blotting—**Transfected HeLa cells were grown for 48 h, and cell extracts were prepared as described above. Rabbit polyclonal anti-K protein antibody 54 was added to 500 μl of cell extract, and the mixture was incubated overnight at 4 °C with low speed rotation. 20 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnolog) were added to the sample and incubated for 1 h at room temperature. The beads were washed four times with 1 ml of ELB buffer, and the proteins were eluted by boiling in 30 μl 1x loading buffer, separated by SDS-PAGE, and transferred to Immobilon-P membrane (Millipore). The blots were probed with monoclonal M2 anti-Flag antibodies (Sigma). The chemiluminescence detection was performed with ECL Western blotting detection reagents according to standard protocol (Amersham Pharmacia Biotech).

**RESULTS**

Identification of New Protein Partners of K Protein Using the Yeast Two-hybrid System—Although recent data indicate that hnRNP K protein is involved in many cellular processes, the mechanism of its action is largely unexplored. Thus the identification of new protein partners of K protein provides important information about its cellular function. We performed a two-hybrid screen with full-length K as a bait using two cDNA libraries. The Gal4BD-K fusion protein did not have transactivation activity by itself when expressed in Saccharomyces cerevisiae. Yeast strain HF7C expressing Gal4BD-K protein was transformed with 9–10-day-old mouse embryo or Jurkat cell cDNA library; transformants were selected as described under “Materials and Methods." Putative positive clones were transferred into SFPY326 yeast strain and tested for the expression of β-galactosidase. False positives were excluded by their ability to activate transcription of His and lacZ reporter genes in the absence of the bait. Sequencing of twenty-one true positive clones from the mouse cDNA library identified several new protein partners of hnRNP K (Table 1). Screening of the Jurkat cDNA library yielded two true positive clones encoding hnRNP L, a component of hnRNP, and the pre-mRNA splicing factor SRp 20. The interaction with splicing factors and hnRNP L suggests that K protein participates in the processing of pre-mRNA. Interaction with scaffold factors and DNA-methyltransferase may reflect the role of K protein in chromatin organization. Seven clones represented YB-1 cDNA with different lengths of insert and different strength of interaction with the bait (Fig. 1). We were particularly interested in YB-1 protein because it shares many functional similarities with hnRNP K and appears to be involved in many cellular processes.

**K Protein Interacts with YB-1 in Vitro—**To confirm the two-hybrid screen we examined whether YB-1 could interact with K protein in vitro. In these experiments we used the rat homologue of YB-1, EFI1X. In vitro translated 35S-labeled YB-1 was incubated with agarose beads bearing GST-K protein, as described previously (7). After incubation, beads were washed three times with an excess of binding buffer and one time with the same buffer containing 1% Nonidet P-40. Samples were boiled in SDS loading buffer, and proteins were resolved by SDS-PAGE. In agreement with the two-hybrid screen, a strong interaction between YB-1 and GST-K was observed in vitro (Fig. 2A, lane 2). In contrast to the mammalian K protein, the yeast homolog of hnRNP K, PBP2p (20), did not interact with YB-1 (Fig. 2A, lane 1). Of note, the YB-1 family proteins are not represented in the yeast genome (confirmed by a search of the completed yeast genome sequence). For some RNA-binding proteins it is known that RNA mediates protein-protein interaction. For example, the primary steps of Sam68 dimerization require cellular RNA (21). Because both K and YB-1 are known to be RNA-binding proteins, we next tested whether the interaction between these proteins can be mediated by RNA. We
performed the binding experiments in the presence of RNase and found no change in the strength of binding of YB-1 to GST-K (data not shown). This indicates that YB-1 interacts with hnRNP K directly; however, the possibility that RNA regulates this interaction cannot be excluded. To test this possibility, the binding of \(^{35}\text{S}\)-labeled YB-1 to GST-K was performed in the presence of several homopolymeric RNAs, poly(A), (C), (G), (I), and (U) (Fig. 2 B, lanes 1–5). These experiments were carried out in two different ways. In one set of experiments, \(^{35}\text{S}\)-labeled YB-1 and GST-K beads were incubated in the presence of polynucleotides; in another set, GST-K beads were preincubated with polynucleotides for 30 min before \(^{35}\text{S}\)-labeled YB-1 was added to the reaction mixture. Identical results were obtained in both experiments. As shown in Fig. 2B (lane 1), poly(U) decreased the interaction of YB-1 with K protein, whereas the other polynucleotides did not alter this binding. When incubated under the same binding conditions, but without K protein, YB-1 was able to bind poly(U) and poly(G) beads (data not shown).

Fig. 3. Mapping of K protein domain responsible for binding to YB-1. GST-K mutants (A) were incubated with \textit{in vitro} translated \(^{35}\text{S}\)-labeled YB-1. Beads were washed three times with binding buffer, and bound proteins were eluted with SDS buffer as described under “Materials and Methods” and then resolved by SDS-10\% PAGE. 30\% of the input used in each reaction (load) was run in lane 9. The \textit{arrow} marks the position of the \textit{in vitro} translated \(^{35}\text{S}\)-labeled YB-1. The gels were stained (Coomassie Blue) and autoradiographed (B).
and 288–321 abolished binding to 35S-labeled YB-1 (data not shown). Thus KI (amino acids 240–337) is the minimal region responsible for hnRNP K-YB-1 interaction. To map the interactive region in YB-1 that binds K protein, we created a series of deletion mutants (Fig. 5C). The in vitro binding experiments were performed as described above. Surprisingly, we observed equally strong binding of both N-terminal and C-terminal parts of YB-1, indicating the presence of multiple interactive regions (Fig. 5, A and B). The binding of NII polypeptide (amino acids 58–150), which includes the conservative RNA binding CSD followed by 26 amino acids, was as strong as that of the full-length YB-1 (Fig. 5A, lanes 7). As shown in Fig. 5A, the NIII polypeptide, containing 26 amino acids immediately downstream of the CSD, maintained a strong interaction with GST-K (Fig. 5A, lane 9). No interaction was found with the “cold shock” domain (Fig. 5A, lane 8).

Fig. 5B illustrates mapping of the second interactive region localized in the C terminus of YB-1. The CI polypeptide did not interact with GST-K protein (lane 11); however, the binding of the CII polypeptide containing the amino acids 271–320 was strong (lane 12). Thus the second interactive region responsible for binding to GST-K is located in the last 50 amino acids of the C terminus of YB-1 (amino acids 271–320). These results are in agreement with our two-hybrid system data. As shown in Fig. 1, the highest β-galactosidase activity was observed with clones 1 and 2, which contained residues located just downstream of the cold shock domain. Clone 5 had the part of the C-terminal interactive region resulting in an increase of β-galactosidase activity in comparison to clones 3 and 4, which are missing this C-terminal region. The middle part of YB-1 has been shown to be responsible for its multimerization and for binding to other protein partners of YB-1 (22, 23). The presence of two distinct interactive regions for K protein in YB-1 is a novel observation.

Next, we examined whether TATA-binding protein, a known partner of hnRNP K, can interact with YB-1. As in the case of GST-K, the same NII (amino acids 124–150) (Fig. 5A, lane 6) and CII (amino acids 271–320) polypeptides (Fig. 5B, lane 6) were responsible for the binding of YB-1 to TBP.

**Co-immunoprecipitation of K Protein and YB-1 from Cell Extracts**—Because the hnRNP K is highly expressed in HeLa cells, we tested whether endogenous K protein can interact with overexpressed Flag-tagged YB-1. HeLa cells were transfected with Flag- YB-1 construct. Cellular extracts were prepared and examined for the presence of K protein-YB-1 complex by co-immunoprecipitation with either pre-immune or anti-K serum followed by Western blotting with anti-Flag antibodies (as described under “Materials and Methods”). In cells transfected with Flag-YB-1 only immune anti-K serum precipitated Flag-YB-1 (Fig. 5, lane 4). Although expression levels of both hnRNP K (4) and Flag- YB-1 are high (lanes 7 and 8), the magnitude of their detectable interaction appears to be lower.
The Role of K Protein-YB-1 Interaction in Gene Expression—

One of the functional similarities between hnRNP K and YB-1 is their high affinity binding to single-stranded polypurine-pyrimidine-rich sequences. K protein and YB-1 have been shown to interact with a C-rich DNA sequence, termed the CT element upstream of the c-myc gene (24, 25). Furthermore, overexpressed hnRNP K and TBP synergistically activated transcription of a CT element-dependent reporter gene in vivo (10). Recent studies revealed that the chicken homolog of YB-1 protein, chk-YB-1b, binds efficiently to the well conserved polypurine tract found in α1(1) collagen gene promoter from rat, mouse, human, and chicken (26). Thus the promoters containing CT-rich elements could be an appropriate system to understand the significance of hnRNP K-YB-1 interaction (Fig. 7).

Because hnRNP K and YB-1 exhibit transcriptional activities from the sequence CCCTCCCCA, known as the CT element of the human c-myc gene (10, 24, 25), we tested how the coexpression of K and YB-1 proteins affects the activity of a luciferase reporter gene. HeLa cells were transfected with hnRNP K and Flag-tagged YB-1 expression constructs and with a plasmid encoding the luciferase reporter gene driven by a synthetic promoter containing three repeats of the CT element of either wild-type CT3 or a mutated version, CT3mut, upstream of the minimal c-fos promoter described in Ref. 10. As shown in Fig. 7A, hnRNP K alone did not have an effect on the luciferase expression level. Flag-YB-1 slightly activated transcription from the CT3 wild-type promoter element but did not affect transcription from the promoter-containing mutated CT3 element. When both proteins were coexpressed in HeLa cells, the activation effect of YB-1 from the wild-type CT3 element was reduced. Similar results were obtained in NIH3T3 cells (Fig. 7B). YB-1 activated transcription from the wild-type promoter but not from the mutant. Interestingly, in this cell line hnRNP K also activated the CT element in a sequence-specific manner. The sequence-specific trans-activation effect of both K protein and YB-1 was reduced when both proteins were coexpressed. These transfection experiments show that hnRNP K and YB-1 are functionally linked. It should be noted, however, that both proteins had only a modest effect on the expression of the reporter gene. As mentioned above, K and YB-1 proteins are abundant, and the high level of the endogenous proteins may have blunted the reporter gene expression.

Discussion

In this paper we describe the interaction between K protein and YB-1. There are many indications that hnRNP K and YB-1 are functionally similar proteins. As described in the Introduction, K protein is involved in many steps of transcriptional regulation, interacting with general and sequence-specific transcription factors, and binding sequence-specific polypurine-pyrimidine-rich DNA motifs within promoter regions. It has been proposed that K protein acts as a scaffold or architectural transcription factor that promotes assembly of sequence-specific transcription factors and basal transcription machinery on the promoter elements (10). YB-1 protein possesses similar functional properties. It has been shown to modulate transcription through binding single-stranded polypurine-pyrimidine-rich DNA
sequences and by interacting with transcription factors (10–12). Like K, protein YB-1, is known to be involved in repressing and activating transcription (11, 27). YB-1 is thought to activate transcription of human polyomavirus JC by recruiting another trans-activator (p65 or RelA) to the viral promoter (28). YB-1 can act on a single gene, the matrix metalloproteinase 2 gene, in a positive or negative manner that is dependent upon the cellular context (29). The reported transcriptional activity of YB-1 protein is strongly dependent on the intracellular environment. For example, the synergistic activity of YB-1 and Pur α proteins on the JCV enhancer-promoter region has been observed in glial cells but was not detected in other cell lines (23). The transactivation properties of YB-1 on myosin light chain 2v gene promoter have been shown in cardiac myocytes, where the expression of YB-1 resulted in a 3.4 higher reporter gene activity in comparison to the control, but no activation was detected in COS-1 cells (30). A moderate level of transcription activation of matrix metalloproteinase-2 promoter by YB-1 was observed in mesangial cells but not in glomerular epithelial cells (29). Taken together, these data and our transfection experiments (Fig. 7) clearly show that transcriptional activity of hnRNP K and YB-1 is cell type-dependent. Both proteins are likely acting as scaffolds or docking platforms for multiprotein complexes that include transcription factors. Acting as docking platforms, they may respond to changes in extracellular environment at sites of nucleic acid-dependent processes (7, 15).

Both proteins have multiple interactive domains. K protein contains two different types of nucleic acid binding domains, the KH domains and the RGG cluster, and the KI region responsible for protein-protein interaction. The same is true for YB-1. Safak et al. (23) have shown that YB-1 protein interacts through the region that includes the entire cold shock RNA binding domain with the transcription factor Pur α. Our data indicate that the N-terminal interactive domain (NIID) in YB-1 responsible for K protein binding overlaps with the Pur α binding region but does not include the cold shock domain. Moreover, the CII peptide, which has a strong affinity for hnRNP K and TBP (Fig. 5B, lanes 6 and 12), does not bind Pur α (23).

These data indicate that K and YB-1 proteins may simultaneously interact with more than one of their partners to form multiple protein or protein-nucleic acid complexes. It is further plausible that the interaction of YB-1-hnRNP K with one of their partners (nucleic acid and proteins) may serve to regulate the interaction with another partner. For example, as shown in Fig. 2B (lane 1) the interaction with poly(U) abolished the binding of K to YB-1.

When both YB-1 and hnRNP K proteins are coexpressed their transcriptional activity is reduced. HnRNP K and YB-1 interact in vitro with the general transcription factor TATA-binding protein (Figs. 4 and 5). It has been suggested that TBP-mediated activation of transcription can be regulated by a variety of gene-specific transcription factors (31). As shown previously, cotransfection of K protein and TBP resulted in activation of transcription from the CT-rich promoter element (10). Our preliminary findings indicate that coexpression of YB-1 and TBP has the same effect on the CT-rich promoter element. In vitro data show that YB-1 binds K protein and TBP through the same domains (Fig. 5), suggesting that the two factors may compete for YB-1. We propose that gene-specific activation of transcription, particularly from the CT element containing promoters, may be achieved by increasing concentration of either hnRNP K TBP or YB-1 TBP complexes explaining the increased reporter gene expression when either K or YB-1 was transfected. Coexpression of K and YB-1 proteins may favor formation of K-YB-1 complexes, which might be transcriptionally inactive, accounting for the decreased reporter gene expression. A similar effect has been shown for Sp1 and C/EBP β transcription factors where the interaction with K protein abolished their trans-activation ability (11, 32).

In summary, we show interaction between two functionally similar factors hnRNP K and YB-1. Because both proteins are involved in many steps of gene expression such as transcription, translation, and nuclear transport it is plausible that these processes are regulated by the interaction of hnRNP K and YB-1. Future studies will be designed to address this issue.

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