Zik1, a Transcriptional Repressor That Interacts with the Heterogeneous Nuclear Ribonucleoprotein Particle K Protein*

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Oleg N. Denisenko§§, Bruce O'Neill‡, Jerzy Ostrowski‡, Isabelle Van Seuningen‡, and Karol Bomztyk¶

From the ‡Department of Medicine, University of Washington, Seattle, Washington 98195 and the ¶Institute of Protein Research, Pushchino, Moscow Region 142292, Russia

The heterogeneous nuclear ribonucleoprotein particle (hnRNP) K protein is comprised of multiple modular domains that serve to engage a diverse group of molecular partners including DNA, RNA, the product of the proto-oncogene vav, and tyrosine and serine/threonine kinases. To identify additional K protein molecular partners and to further understand its function, we used a fragment of K protein as a bait in the yeast two-hybrid screen. The deduced primary structure of one of the positive clones revealed a novel zinc finger protein, hereby denoted as Zik1. In addition to the nine contiguous zinc fingers in the C terminus, Zik1 contains a KRAB-A domain thought to be involved in transcriptional repression. Zik1 and K protein bound in vitro and co-immunoprecipitated from cell extracts indicating that in vitro their interaction is direct. Expression of Gal4 DNA-binding domain-Zik1 fusion protein repressed a gene promoter bearing Gal4-binding elements, indicating that from cognate DNA elements Zik1 is a transcriptional repressor. The known diverse nature of K protein molecular interactions and now the identification of a K protein partner that is a transcriptional repressor lends support to the notion that K protein is a remarkably versatile molecule that may be acting as a docking platform to facilitate communication among molecules involved in signal transduction and gene expression.

K protein was first discovered as a component of the heterogeneous nuclear ribonucleoprotein particle (hnRNP) from which it derives its name (1, 2). As a component of hnRNP, K protein binds poly(C) RNA, but not other RNA homopolymers (1), and might be involved in mRNA processing. K protein binds RNA via the highly conserved KH (K homology) domains (3) that are present in a variety of other RNA-binding proteins (4, 5). In vitro, K protein also recognizes the c-myc promoter CT

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†To whom correspondence should be addressed: Dept. of Medicine, 356521, University of Washington, Seattle, WA 98195. Tel.: 206-685-8581; Fax: 206-685-8661; E-mail: karolb@u.washington.edu.

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MATERIALS AND METHODS

Cell Lines—The rat glomerular epithelial cells, the murine pre-B lymphocytes 70Z/3, the human epidermoid KB, and the monkey kidney COS cell lines were grown as described before (20).

Reagents—The bacterial expression vector pGEX-KT was provided by Dr. J. Dixon (University of Michigan). The mammalian expression vector pM2 was kindly provided by Dr. I. Sadowski (University of British Columbia, Vancouver, Canada). Glutathione agarose beads were obtained from Sigma.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted essentially as described previously (21). Cells or animal tissues was washed with PBS. 2.0 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) was added to each plate or to 100 mg of animal tissue to lyse the cells. The final RNA was dissolved in 25 μl of water and used for Northern blot analysis. RNA was analyzed essentially as described previously (22). After first denaturing in formaldehyde and formamide at 65°C for 15 min, the RNA sample was cooled to 4°C on ice. 10 μg of total RNA per lane was resolved by electrophoresis for 4 h at 70 V using 1.4% agarose gel containing 2.2 M formaldehyde. RNA was transferred overnight in 20 × SSC to Hybond N+ membrane (Amersham, Arlington Heights, IL), and dried at 80°C for 45 min. The membranes were prehybridized for 2 h at 42°C in hybridization buffer (50% formamide, 5 × Denhardt’s solution, 5 × SSC, 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 0.1 mg/ml yeast tRNA). After prehybridization, 32P-labeled cDNA probe (2 × 106 cpm/ml) was added and hybrid-
Zinc Finger Protein

Cloning of a Novel Protein That Interacts with hnRNP K—The N-terminal two-thirds of K protein (amino acids 1–318) (14) was used as a bait in a two-hybrid screen to isolate K protein molecular partners. A partial cDNA of 366 base pairs in length was isolated and sequenced, and this cDNA represented a fragment of a novel gene. This partial cDNA was used to screen a Agt10 cDNA library (30) derived from the murine pre-B cell line, 22D6, and 3 putative clones were isolated and subcloned into a pBluescript vector. The longest clone was used for sequencing of both strands.

The complete nucleic acid and deduced amino acid sequences of the 3042-long cDNA clone are shown in Fig. 1. The ATG located at position +245 is an initiation codon which starts the longest open reading frame. This clone encodes a protein of 463 amino acids, with a calculated molecular weight of 52,778 and a pl of 8.5. The cloned cDNA contains a short 5′ (244-base pair) and a long 3′ (1406-base pair) untranslated region (Fig. 1.). Comparison of the deduced primary structure of the protein with sequences in the data base revealed that the cDNA encodes a protein with the N-terminal segment (amino acids 14–47) similar to Ki61, a zinc finger protein enriched in the rat kidney (31, 32) (Fig. 2C). Notably, this region contains the Kruppel-associated type A box (KRAB-A). This highly conserved motif is present in approximately one-third of all zinc finger proteins and has been shown to mediate transcriptional repression (32). The C terminus of the cloned protein (amino acids 215–463) consists of nine contiguous repeats that are similar or identical to the C3H2 zinc finger consensus sequence (YxCxxCxxxFXxxxxLxxHxxHTGKP) (Fig. 2B) (33). The cDNA fragment isolated in the two-hybrid screen represented the 59–180-amino acid stretch of the full-length protein. Therefore, this 122-amino acid-long stretch contains the K protein-binding domain (Figs. 1 and 2A). We have designated this novel protein Zik1, for zinc-finger protein interacting with K protein.

Tissue Distribution of Zik1 Transcripts—The full-length cDNA was used as a probe to define the tissue distribution of Zik1 transcripts. RNA was isolated from a variety of mouse tissues and cells grown in culture, separated by size on an agarose-formaldehyde gel, and blotted onto a nylon membrane. Fig. 3 illustrates the autoradiogram of the Northern blot. In the tissues tested, a 3.9-kilobase transcript was most abundant in ovaries (lane 2) and liver (lane 4), and, although present, the message levels in brain (lane 1) and muscle (lane 3) were very low. RNA was also present in the (lane 3) purov caracinoma KB (34) (lane 5), the murine pre-B lymphocyte 70Z/3 cell line (35), and the rat primary glomerular epithelial line (20) (lane 7). Comparison among the tissues and cell line tested...
demonstrates that the level of expression is by far highest in the glomerular epithelial cells. Antibodies to GST-Zik1 fusion protein in Western blot detected a band corresponding to Zik1 in glomerular epithelial but not in COS cells (data not shown). It is interesting to note that Kid1, the zinc finger protein that shares most sequence similarities to Zik1, is also expressed preferentially in the kidney (31).

**Interaction of Zik1 with K Protein Is Direct**—To determine whether or not the interaction of K protein with Zik1 is direct, we tested the ability of 35S-labeled Zik1 in vitro translated to bind to glutathione beads bearing the GST-K fusion protein (14). After incubation with Zik1, beads were washed, boiled in the presence of SDS, and proteins were resolved by SDS-PAGE. The Coomassie Blue-stained and autoradiographed gel is illustrated in Fig. 4. In agreement with the yeast two-hybrid screen, these results revealed that Zik1 binds to the beads bearing the full-length K protein fused to GST (lane 1). Zik1 also binds to several K protein deletion fragments, the GST-K13 (amino acids 1–337) (lane 2) and GST-K3 (amino acids 171–337) (lane 5), but not to the GST-K8 (amino acids 1–108) (lane 4) or the GST-K12 (amino acids 1–209) (lane 3) deletion mutants. These results suggest that the binding of Zik1 to K protein is direct and specific and allow us to map the Zik1-binding site to the 209–337-amino acid domain of K protein.

**Interaction of Zik1 with K Protein Is Regulated by Nucleic Acids**—K protein binds to RNA and to single- and double-stranded DNA in a sequence-selective manner (6, 9, 15). Because the function of K protein might be regulated by its binding to RNA and DNA, we next tested the effect of cognate nucleic acid motifs on the interaction of Zik1 with K protein. Results of this series of experiments are illustrated in Fig. 5. The binding assay was performed as described above (Fig. 4) in the presence or absence of RNA or DNA. These results showed that poly(C)RNA, which binds K protein, blocked Zik1 binding to K protein, while poly(A), which does not bind K protein (1), did not affect binding of Zik1 to glutathione beads bearing K protein (compare lanes 3 and 4 with lane 2). Similarly, a double-stranded synthetic oligonucleotide containing the \( \kappa B \) motif and the single-stranded synthetic oligonucleotide containing the antisense \( \kappa B \) motif, both of which bind K protein (9), blocked Zik1 binding (compare lanes 7 and 9 with lane 6), while the single-stranded sense \( \kappa B \), which does not bind K protein, did not alter Zik1 binding (compare lane 8 with lane 6). These results demonstrate that cognate RNA and DNA sequences both block the interaction of Zik1 with K protein. This experiment, in conjunction with the observation that K protein phosphorylation is regulated by nucleic acid binding (9, 14, 15), indicates that K protein function is highly regulated by cognate nucleic acids.
RNA and DNA motifs.

Co-immunoprecipitation of Zik1 and K Protein from Cell Extracts—To determine whether Zik1 and K protein interact in vivo in mammalian cells, we used monoclonal antibody directed against Gal4 DNA-binding domain to determine whether K protein co-immunoprecipitated with either Gal4 or Gal4-Zik1 fusion proteins when they were co-expressed in COS cells. Western blot analysis using an antibody directed against K protein (Fig. 6) showed that the anti-Gal4 antibody precipitated K protein (lane 2) when it was co-expressed with Gal4-Zik1 fusion protein, but not when it was co-expressed with Gal4 protein alone (lane 1). This experiment provides further evidence that Zik1 and K protein can interact in vivo.

Zik1 Contains Transcriptional Repressor Activity—Zik1 contains the KRAB-A domain and shares sequence similarity with the transcriptional repressor Kid1, suggesting that Zik1 might also affect transcription. To determine the transcriptional activity of Zik1, either the full-length Zik1 (amino acids 1–463) or its KRAB-A domain (amino acids 1–54) were fused in-frame to the Gal4 DNA-binding domain (amino acids 1–147) in the mammalian expression vector pM2 (36). These constructs were transiently co-expressed with the reporter luciferase gene plasmids in either rat glomerular epithelial or COS cell lines. The results of luciferase activity assay are shown in Fig. 7. The
full-length Gal4-Zik1 and more so its KRAB-A domain substantially repressed transcription of the luciferase gene driven by a promoter containing 5 Gal4 DNA elements (shaded bars), but did not diminish activity of the same promoter lacking the Gal4-binding motifs (clear bars). These results demonstrate that, like Kid1 (31), Zik1 can act as a transcriptional repressor. The observation that K protein can engage both a transcriptional repressor and the TFIID TATA-binding protein may explain why expression of K protein can both activate and repress gene promoters (8, 11).

Identification of a transcriptional repressor that directly interacts with K protein further expands the repertoire of K protein molecular partners: sequence-specific RNA and DNA, Vav, both tyrosine and serine/threonine kinases, TATA-binding protein, and now Zik1. What are the possible implications of this versatility? Not only is K protein very interactive, but it is also abundant, and is present both in the nucleus and the cytoplasm (9, 10, 15). The diversity of K protein molecular interactions, K protein abundance, and wide intracellular distribution would fit a model in which K protein acts as a universal docking platform. The yeast two-hybrid screen revealed that K protein can interact with itself at two different sites that K protein can interact with itself at two different sites. When transcribed, K protein co-immunoprecipitates with Zik1 from COS cell extracts. K protein was transiently co-expressed in COS cells with either Gal4 DNA-binding domain (Gal4) or Gal4 DNA-binding domain-Zik1 fusion protein (Gal4-Zik1) using mammalian expression vectors. 48 h following transfection, cells were harvested and extracts were prepared for immunoprecipitation. 50 µg of cell extracts were incubated with 1.0 µg of anti-Gal4 monoclonal antibody. The immune complexes were recovered with protein A beads and centrifugation. After several washes, proteins were eluted from the beads by boiling in loading buffer, and proteins were resolved by SDS-PAGE and were electro-transferred to polyvinylidene difluoride membrane for immunoblotting with anti-K protein antibody (14). K protein is marked by the arrow.

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**Fig. 5.** Effects of nucleic acids on the interaction of K protein with Zik1. Binding of 35S-labeled Zik1 (load) to glutathione beads bearing full-length GST-K protein was done as in Fig. 4, in the presence or absence of (a) 10 µg/ml poly(C) RNA (poly C), (b) poly(A) RNA (poly A), or (c) double-stranded synthetic oligonucleotide containing the B motif (ds-B), or (d) single-stranded synthetic oligonucleotides containing sense (ss-sB) or antisense (ss-aB) B motif (clear box). Zik1 co-immunoprecipitates with K protein when (a) no RNA, (b) poly(C) RNA, (c) poly(A) RNA, or (d) either DNA or RNA is present in the interaction buffer. Luciferase activity was measured using a luminometer (37) and was normalized for protein concentration. The data shown are representative of one out of five experiments. (data not shown), suggesting that it has the potential to form oligomers. If so, this would provide the K protein with a great capacity to permit simultaneous docking of a number of proteins and thus provide an environment for multilateral protein cross-talk. As exemplified by the K protein-Zik1 (Fig. 5) and K protein-K protein kinase (9, 14, 15) interactions, a cross-talk among the docked proteins could be regulated by sequence-specific nucleic acids. The potential of K protein to simultaneously engage and allow cross-talk between signaling molecules such as Src or Vav, with transcriptional repressors such as Zik1, opens up an exciting avenue to investigate.

**Fig. 6.** K protein co-immunoprecipitates with Zik1 from COS cell extracts. K protein was transiently co-expressed in COS cells with either Gal4 DNA-binding domain (Gal4) or Gal4 DNA-binding domain-Zik1 fusion protein (Gal4-Zik1) using mammalian expression vectors. 48 h following transfection, cells were harvested and extracts were prepared for immunoprecipitation. 50 µg of cell extracts were incubated with 1.0 µg of anti-Gal4 monoclonal antibody. The immune complexes were recovered with protein A beads and centrifugation. After several washes, proteins were eluted from the beads by boiling in loading buffer, and proteins were resolved by SDS-PAGE and were electro-transferred to polyvinylidene difluoride membrane for immunoblotting with anti-K protein antibody (14). K protein is marked by the arrow.

**Fig. 7.** Effect of Zik1 on transcription of luciferase reporter gene. A, plasmids used in transient cell transfections. Reporter plasmids, luciferase reporter gene pG5bL3 enh plasmid containing SV40 enhancer and minimal promoter either with (shaded box) or without (clear box) 5 Gal4-binding elements. Expression plasmids, mammalian vector, pM2, was used for expression of either Gal4 DNA-binding domain alone (Gal4) or a fusion of Gal4 DNA-binding domain with either the full-length Zik1 (amino acids 1–463) (Zik1) or the N-terminal 54 amino acids of Zik1 (A). B, effect of expression of Gal4 DNA-binding domain and Gal4 DNA-binding domain-Zik1 fusion constructs on expression of luciferase reporter gene in COS (COS) and rat glomerular epithelial (GEC) cells. 10 µg of expression plasmid with 5 µg of luciferase reporter plasmid were used for cotransfections in both cell lines. 48 h following transfection, cells were pelleted and were lysed in reporter lysis buffer. Luciferase activity was measured using a luminometer (37) and was normalized for protein concentration. The data shown are representative of one out of five experiments. (data not shown), suggesting that it has the potential to form oligomers. If so, this would provide the K protein with a great capacity to permit simultaneous docking of a number of proteins and thus provide an environment for multilateral protein cross-talk. As exemplified by the K protein-Zik1 (Fig. 5) and K protein-K protein kinase (9, 14, 15) interactions, a cross-talk among the docked proteins could be regulated by sequence-specific nucleic acids. The potential of K protein to simultaneously engage and allow cross-talk between signaling molecules such as Src or Vav, with transcriptional repressors such as Zik1, opens up an exciting avenue to investigate.

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