A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins

(transcription factors/Kid-1/ZNF2/coiled-coil motif/TIF1/PHD domain)

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Communicated by Alexander Leaf, Harvard Medical School, Charlestown, MA, October 14, 1996 (received for review September 21, 1996)

ABSTRACT The Krüppel-associated box A (KRAB-A) domain is an evolutionarily conserved transcriptional repressor domain present in approximately one-third of zinc finger proteins of the Cys2-His2 type. Using the yeast two-hybrid system, we report the isolation of a cDNA encoding a novel murine protein, KRAB-A interacting protein 1 (KRIP-1) that physically interacts with the KRAB-A region. KRIP-1 is a member of the RBCC subfamily of the RING finger, or Cys2HisCys family of zinc binding proteins whose members are known to play important roles in differentiation, oncogenesis, and signal transduction. The KRIP-1 protein has high homology to TIF1, a putative modulator of ligand-dependent activation function of nuclear receptors. A 3.5-kb mRNA for KRIP-1 is ubiquitously expressed among all adult mouse tissues studied. When a GAL4–KRIP-1 fusion protein is expressed in COS cells with a chloramphenicol acetyltransferase reporter construct with five GAL4 binding sites, there is dose-dependent repression of transcription. Thus, KRIP-1 interacts with the KRAB-A region of Cys2His2 zinc finger proteins and may mediate or modulate KRAB-A transcriptional repressor activity.

Limited numbers of structural motifs have been described for eukaryotic transcription factors. A common motif is the Cys2His2 zinc finger structure that is highly conserved and found in many different species (1). Approximately one-third of all Cys2His2 zinc finger proteins contain evolutionarily conserved regions at their amino terminus, which have been termed Krüppel-associated boxes (KRAB-A and -B), containing a total of about 75 amino acids (2). Similar to regions found in a subset of homeodomain proteins, the paired box and POU domain, the KRAB domain is rich in charged amino acids (2).

Our studies with Kid-1, a KRAB-containing protein with 13 zinc fingers, revealed that its amino terminus, which contains the KRAB-A and -B domains, when fused to GAL4, confers transcriptional repression on promoter constructs with GAL4 binding sites (3). We subsequently established that the KRAB-A regions of Kid-1 and other zinc finger proteins are responsible for the transcriptional repression (4). Similar results were reported by Rauscher and colleagues (5). Site-directed mutagenesis of conserved amino acids in the KRAB-A motif results in decreased repressor activity (4).

Others have reported that the KRAB-A domain, tethered to RNA polymerase II promoters via a GAL4-binding domain, represses transcription in a distance-independent manner (6). The KRAB-A domain, when tethered to the transactivating response element, has also been shown to repress both basal and Tat-mediated human immunodeficiency virus 1 (HIV-1) long terminal repeat-driven gene expression (7). This observation led to the proposal that control of HIV-1 gene expression might be achieved with the KRAB-A domain fused to Tat to generate transdominant negative mutants. Fusion of the KRAB-A domain of human Kox1 to the Tet repressor derived from Tn10 of Escherichia coli results in the production of a tetracycline-controlled hybrid protein that, when bound to the tetO sequences upstream of a cytomegalovirus-driven luciferase reporter construct, represses luciferase expression (8). This repression was found with different promoters and from tetO sequences placed as far as 3 kb from the transcriptional start site. Thiesen and colleagues also found that a 110-kDa protein communoprecipitates with the TetR–KRAB protein, suggesting the presence of an interacting protein. It was the purpose of our studies to identify proteins that interact with the KRAB-A domain to gain insight into potential mechanisms of KRAB-A-mediated repression.

METHODS

Two-Hybrid Screen. A two-hybrid screen was performed to isolate genes encoding proteins that associate with the KRAB-A domain. The two-hybrid screen was carried out in the yeast strain MaV103 using a system devised by Vidal and coworkers (9, 10). The GAL4–GAL80-deleted MaV103 strain is auxotrophic for uracil, leucine, and tryptophan and carries three chromosomally integrated reporter genes whose expression is regulated by different GAL4 responsive promoters: GAL1::HIS3, SPAL10::URA3, GAL1::lacZ.

To construct the KRAB-A bait, the nucleotides encoding the first 54 amino acids of Kid-1 (3), beginning with the initiation codon and containing the KRAB-A domain, were PCR-amplified from pMFH2-GAL4-Kid1A (11). The PCR primers were constructed to incorporate a SalI site in the 5′ end and a BamHI site in the 3′ end. After restriction enzyme digestion, the PCR product was subcloned in-frame with GAL4 into the pPC97 vector to create pPC97/KRAB-A. The pPC97 plasmid has stop codons 3′ from the multiple cloning site in all reading frames and carries the LEU2 yeast selectable marker.

The cDNA library for the yeast two-hybrid screen was constructed in the pPC86 (12) vector modified by adding a 600-bp stuffer into the BglII site to facilitate the recovery of EcoRI- and SpeI-digested clones (a kind gift of Joshua La Baer of the Massachusetts General Hospital Cancer Center). The pPC86 vector carries the TRP1 yeast selectable marker.

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was derived from poly(A) RNA isolated from 12- to 13-day whole murine fetuses and cloned directionally into the EcoRI (5') and SpeI (3') sites of the vector. Fusion proteins are encoded containing the simian virus 40 large tumor antigen nuclear localization sequence and the transactivation domain (AD) of GAL4 at the amino terminus (AD library).

MaV103 yeast cells containing pPC97/KRAB-A were transformed with the AD library by the lithium acetate method (13). Approximately one million transformants were plated onto leucine- and tryptophan-deficient synthetic complete [(SC)-Leu-Trp] medium, replica-plated after 2 days onto (SC)-Leu-Trp-His medium containing 50 mM 3-aminotriazole (3-AT; Sigma) to select for GAL1::His3-dependent His/AT prototrophy, and subsequently replica-plated again onto SC-Leu-Trp-His medium containing 50 mM 3-AT. Twelve positive clones were picked after an additional 3 days of incubation. pPC86 plasmids that conferred resistance to 50 mM 3-AT were cloned were picked after an additional 3 daysof incubation.

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Screening of Murine cDNA Libraries and Sequencing. A cDNA library from embryonic day 13 to 14 kidney constructed in λZAP-II was provided by K. Hession of Biogen. A 500-bp 5’ restriction fragment of the partial length clone obtained by the two-hybrid screen, KRIP-1.2, was used to screen approximately 1.5 × 109 plaques using standard techniques with duplicate filter lifts. Two clones approximately 3 and 3.5 kb long were plaque-purified, subcloned into pBluescript (Stratagene), and sequenced on both strands by the chain-termination method (14). Computer analysis was carried out with the sequence analysis software package of the Genetics Computer Group at the University of Wisconsin, Madison (15).

Northern Blot Analysis. A human multiple tissue Northern blot (CLONTECH) was hybridized at 65°C with the 2582-bp KRIP-1.2 cDNA, labeled with [32P]dCTP by random priming, and washed at 65°C according to the procedure described by CLONTECH.

Transfection Protocols and Chloramphenicol Acetyltransferase (CAT) Assays. COS cells were plated 2 days prior to transfection at a density of 2.5 × 105 cells per 100 mm dish. For transfections, cells were generally exposed to 20 µg of total DNA in 5 ml of DMEM/10% NuSerum (Collaborative Biomedical Products, Bedford, MA)/DEAE-dextran (400 µg/ml)/0.1 mM chloroquine. One microgram of a luciferase-expressing plasmid, pLuSV/T1 (provided by C. S. Shelley, Massachusetts General Hospital) was included for normalization of transfection efficiencies. Three to 4 h after the addition of DNA, medium was removed and cells were shocked for 2 min at room temperature with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS). Cells were then washed once with PBS and new medium was added.

Forty-eight hours after transfection, cells were washed twice with PBS, scraped with a rubber policeman into a microcentrifuge tube, and pelleted. The cell pellet was resuspended in 200 µl of 0.25 M Tris-HCl (pH 7.8) and subsequently broken up by freezing–thawing three times in a dry-ice/ethanol bath and 37°C water bath. The supernatant was assayed for CAT and luciferase activities as described (3). CAT activity is expressed as the ratio [monoaacetylated [14C]chloramphenicol/ (monoaacetylated plus nonacylated) [14C]chloramphenicol] and is normalized to luciferase activity.

Coinmunoprecipitation Studies. To confirm the direct interaction of KRIP-1 with the KRAB-A region of Kid-1 (Kid-1A) or ZNF2 or the entire non-zinc-finger region of Kid-1 (Kid-1N), the 2.6-kb KRIP-1.2 cDNA from pPC86-KRIP-1.2 was subcloned into the expression vector pMT3, which encodes fusion proteins in-frame with the 9-aminocacid hemagglutinin (HA) epitope tag. COS cells were cotransfected with pMT3-KRIP-1.2 and with the expression vector pBXG1 containing an insert encoding GAL4 fusion proteins with Kid-1A, Kid-1N, the KRAB-A region of ZNF2, or the GAL4 binding protein alone. In other studies the vector pMFH was used (11) to encode a GAL4 Kid-1A or Kid-1N fusion protein preceded in-frame by a Flag epitope, from bacteriophage T7. Transfections were done using DEAE-dextran, as described. Forty-eight hours later cells were lysed and cellular proteins were immunoprecipitated for 4 h with anti-HA antibody using protein G-agarose (Boehringer, Mannheim) (16). Proteins were separated on a 10–12% SDS/PAGE gel and electrophoretically transferred to an Immobilon-P membrane (Millipore). Western blot analysis was then performed using a polyclonal antibody to GAL4 or monoclonal antibody to T7 Flag.

Protein Gel and Western Blot Analysis. SDS/PAGE gel and Western blot analysis were performed according to standard protocols (14). An anti-GAL4 antibody (obtained from S. A. Johnston and K. Melcher, University of Texas Southwest Medical Center) was used at a 1:3000 dilution. A commercial monoclonal T7-Tag antibody (IgG2b, k) directed against the Flag epitope was obtained from Novagen and used at 1:3000 dilution. Immune complexes were detected with the Renaissance light detection kit from DuPont.
ized transcriptional repression conferred by KRAB-A in mammalian cells, the GAL4–KRAB-A fusion protein exhibited a strong transcriptional activation function in yeast cells when overexpressed from multicopy plasmids (17, 18) (data not shown). However, when GAL4–KRAB-A was expressed at more moderate levels from the centromeric two-hybrid plasmid, pPC97(12), it exhibited a milder degree of activation of GAL1::HIS3 that could be counteracted by the addition of 50 mM 3-AT in the selective medium lacking histidine. Transformation of yeast cells with the bait pPC97 and the mouse embryonic library in pPC86 with subsequent replica plating of 1.5 × 10^6 transformants to selective medium resulted in the isolation of two yeast clones (containing the inserts KRIP-1.2 and KRIP-1.3). These yeast colonies grew, when glucose was the carbon source, on SC-Leu-His-Trp medium in the presence of 50 mM 3-AT, which selects for yeast cells that have higher levels of HIS3 gene expression, which is required to sustain growth. Each of the two interactor plasmids encoding fusion proteins of the GAL4 activation domain with KRIP-1.2 or KRIP-1.3 (AD^15-2 or AD^15-3 in Fig. 1) were purified and retransformed along with pPC97 encoding the GAL4 DNA binding domain (DB) or the vector containing only the GAL4 DNA binding domain (DB). Growth on SC-Leu-His-Trp medium occurred only when AD^15-2 or AD^15-3 were expressed with DB. Expression of the GAL4 activation domain only with DB or the GAL4 DNA binding domain only with AD^15-2 or AD^15-3 did not permit growth. Similar findings were observed when activity of the URA3 gene was evaluated on SC-Ura-Trp-Leu plates. An additional unique feature of the two-hybrid system used is that the expression of the URA3 gene can be counterselected on medium containing uracil and FOA where cotransformation of the interacting fusion proteins results in prevention of yeast growth (Fig. 1). Finally, the specificity of the interaction between KRIP-1.2 or KRIP-1.3 and KRAB-A
was also shown using the LacZ reporter gene by transferring the colonies to a filter and incubating the filter in 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

The cDNA of clone KRIP-1.2 was 2582 bp long and that of KRIP-1.3 was 2453 bp long. We encountered some difficulty in obtaining a full-length clone likely due to the G+C-rich content of the 5' end of KRIP-1. A cDNA library from embryonic day 13–14 kidney constructed in λZAP-II was screened with a 500-bp 5' restriction fragment of clone KRIP-1.2. Two clones of approximately 3 and 3.3 kb long were plaque-purified and sequenced in pBluescript. Both the 3.0- and 3.3-kb clones were identical to KRIP-1.2 over their 2.6-kb 3' ends and extended 5' beyond KRIP-1.2. The 3.3-kb clone was designated KRIP-1. Its nucleotide and predicted protein sequences are presented in Fig. 2. KRIP-1 encodes an open reading frame of 834 amino acids with a calculated molecular weight of 88.9 kDa. The in vitro-translated protein migrated on SDS-PAGE gels with a molecular mass of approximately 105 kDa (data not shown). The sequence is alanine-rich (10.6% of amino acids), especially at the amino terminus. Upon search of GenBank, the KRIP-1 protein sequence was found to be distinct from but to have very high homology to that of TIF1 (transcriptional intermediary factor 1) (19), a putative modulator of the ligand-dependent activation function of nuclear receptors. Like TIF1, KRIP-1 contains a RING finger domain at its amino terminus, followed by B1 and B2 domains and then a coiled coil domain (20). When KRIP-1.2 and KRIP-1.3 were compared with the KRIP-1 sequence, KRIP-1.3 was found to have a region of nt 695–823 (amino acids 47–89) deleted. This deletion disrupts the RING finger domain. The fact that the KRIP-1.3 peptide interacts with KRAB-A in the interactor screen indicates that the intact RING finger domain is not critical for this interaction. KRIP-1 contains a Cys2-His-Cys3 PHD finger domain (21) near the carboxyl terminus.

Tissue Distribution of KRIP-1. By Northern blot analysis, the KRIP-1 mRNA is approximately 3.5 kb and is expressed in each of the adult mouse tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, and kidney. The highest level of expression was found in testis (Fig. 3).

Direct Interaction Between KRIP-1 and KRAB-A in Mammalian Cells. To establish that the interaction identified by the two-hybrid system can occur in mammalian cells, KRIP-1.2 was coexpressed in COS cells with the KRAB-A regions of Kid-1 or ZNF2 or the non-zinc-finger region of Kid-1, no additional sequence, or the KRAB-A region of Kid-1 respectively. After cell lysis, preparation of soluble (S) and pellet (P) fractions, immunoprecipitation with the anti-HA antibodies, SDS-PAGE resolution of the S, P, and immunoprecipitate fractions (IP), transfer to an Immobilon-P membrane, and reaction with anti-T7 flag antibody, it is apparent that Kid-1N and Kid-1A immunoprecipitate with KRIP-1.2. (c) pMT3-KRIP-1.2 was cotransfected with pBXG1 and KRIP1.2 (Fig. 5).

KRIP-1 Has Transcriptional Repression Activity. The pBXG1/KRIP-1.2 eukaryotic expression construct encoding a GAL4 DNA binding domain–KRIP-1.2 fusion protein was cotransfected with a CAT reporter plasmid containing five GAL4 binding sites (pG5SV-BCAT) into COS cells. When compared with cells cotransfected with the reporter construct and pBXG1/Kid-1N antisense, the CAT activity was lower in cells cotransfected with pBXG1/KRIP1.2 (Fig. 5). When a construct encoding only amino acids 382–834, excluding the RING finger, B1, B2, and coiled coil domain (pBXG1/KRIP-1ΔRBCC), was cotransfected with CAT reporter, the repression was equivalent to that observed with pBXG1/KRIP1.2. A frameshift mutation in pBXG1/KRIP-1ΔRBCC, due to loss of a guanine nucleotide at 1702 that resulted in a stop codon at nt 1727–1729, eliminated the repressor activity of the protein. There was a dosage-dependent repression of CAT activity when the full-length
KRIP-1 was coexpressed as a GAL4 fusion protein with the pG5SV-BCAT reporter (Fig. 6).

DISCUSSION

While there are at least four well-defined types of transcriptional activation domains, serine/threonine-rich, acidic, proline-rich, and glutamine-rich (22), the only well-defined motif other than KRAB-A that has been postulated to mediate transcriptional repressor activity is an alanine-rich domain found in four transcriptional repressors from Drosophila: Krüppel (23), engrailed (24), even-skipped (25), and AEF-1 (23). In other cases where a repressor domain has been delineated, such as Egr-1 (26), SRF (27), and E4BP4 (28), no obvious consensus sequence motifs have been identified. The KRAB-A domain, which is present in approximately one-third of all Cys2-His2 zinc finger proteins, therefore, represents the first widely distributed transcriptional repressor motif. The potential α-helical structure of KRAB domains may mediate protein–protein interactions (2), but this hypothesis had not been previously tested. The fact that the repression afforded by the KRAB-A domain is distance-independent suggests that the mechanism of the repression is not related to steric hindrance and is consistent with a mechanism involving protein–protein interactions.

KRIP-1 belongs to the RBCC subfamily of RING finger proteins (20) that, in addition to the Cys3-His-Cys4 RING finger, contain a second cysteine-rich domain termed the B box, and a coiled coil motif carboxy-terminal to the RING finger. Many members of the RING finger family have been implicated in the control of cell growth, differentiation, and development. The family includes many oncoproteins, such as BRCA1, Mel18, and Bmi1 (20). The RBCC subfamily is particularly notable for the inclusion of a number of known oncoproteins, including Rfp/RET, the acute promyelocytic leukemia protooncogene PML, and T18 (20). Members of the RING finger family have been localized to the nucleus and proposed to interact with DNA. Mutation of cysteines in the RING finger domain of PML disrupts the folding of the protein and prevents PML nuclear body formation (20). Two of the germ-line mutations associated with predisposition to breast cancer occurs in the RING finger domain of the BRCA1 gene (29). Mutations in the B box or leucine coiled coil domain affect the normal interaction of PML with other nuclear proteins, interfering with the formation of nuclear bodies (30). The RBCC motif may be an integrated structural unit (20). The repressor function of KRIP-1 is localized to the carboxyl terminus of the molecule. While we have not identified the repressor domain, it is of interest that the PHD domain is located in this region of KRIP-1.

KRIP-1 is closely related to TIF1, a putative mediator of ligand-dependent activation function of nuclear receptors (19). TIF1, like KRIP-1 (data not shown), is localized in the nucleus of transfected cells. KRIP-1 may modulate or mediate the transcriptional repression of the KRAB-A motif. KRIP-1, like TIF1, may interact with one or more proteins important for the regulation of transcription. KRIP-1 is expressed ubiquitously. It is probable, therefore, that any tissue-specific repression is mediated by the KRAB-A-containing zinc finger protein.

The interaction of KRIP-1 with the KRAB-A domain of zinc finger proteins, if it carried over to other RBCC family members, might help to explain the importance of these proteins in control of proliferation and differentiation. If a KRAB-A-containing zinc finger transcription factor was important to repress proliferation and/or potentiate differentiation, the RBCC protein might mediate these effects. A mutation in the RBCC protein might serve to disrupt the ability of the KRAB-A-containing zinc finger protein to exert its repressor function. Uncontrolled proliferation, loss of differentiation and oncogenesis might be a consequence.
Note. After submitting the sequence of KRIP-1 to GenBank and while this manuscript was being prepared for submission, a study was published by Friedman et al. (31) in which the authors have used a different technique to clone and characterize KAP-1, the likely human homologue of KRIP-1.

We acknowledge Ed Harlow for his support. We thank Dr. Stephen Hsu for his thoughtful input and for reading the manuscript. This work was supported by National Institutes of Health Grants DK 39773, DK 38452, and NS 10828 and a Senior American Cancer Society Fellowship to M.V.