KAP-1, a novel corepressor for the highly conserved KRAB repression domain

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The KRAB repression domain is one of the most widely distributed transcriptional effector domains yet identified, but its mechanism of repression is unknown. We have cloned a corepressor, KAP-1, which associates with the KRAB domain but not with KRAB mutants that have lost repression activity. KAP-1 can enhance KRAB-mediated repression and is a repressor when directly tethered to DNA. KAP-1 contains a RING finger, B boxes, and a PHD finger; the RING-B1-B2 structure is required for KRAB binding and corepression. We propose that KAP-1 may be a universal corepressor for the large family of KRAB domain-containing transcription factors.

[Key Words: Corepressor; RING finger; KRAB domain; zinc-finger]

Received April 23, 1996; revised version accepted July 9, 1996.

Transcriptional regulation of gene expression is mediated primarily by DNA sequence-specific transcription factors, which are generally composed of a DNA-binding domain and one or more separable effector domains that may activate or repress transcriptional initiation (for review, see Tjian and Maniatis 1994; Zawcl and Reinberg 1995). Activation domains may function by directly interacting with components of the basal transcriptional machinery, thereby nucleating, stabilizing, and/or facilitating the assembly and initiation of the RNA pol II transcription complex. Alternatively, activation domains may function through a novel class of intermediary molecules variously termed adaptors or coactivators (for review, see Guarente 1995). These intermediary molecules must be tethered to the DNA template via protein–protein interactions with the effector domain of the DNA-bound transcription factor, thus serving to bridge the activation domain to the ultimate downstream target.

In contrast to our knowledge of activators, less is known regarding the mechanisms utilized by repression domains. Not surprisingly, repression domains may contact components of the basal transcriptional machinery [Fondell et al. 1993, 1996; Baniahmad et al. 1995; Sauer et al. 1995; Um et al. 1995] or molecules with properties suggestive of corepressor function. Emerging models of repressor–corepressor interaction include hairy–groucho in Drosophila [Paroush et al. 1994; Fisher et al. 1996], SSN6/TUP1–MCM1/IRAS2 in yeast [Kelley et al. 1992; Cooper et al. 1994; Tzamaras and Stuhl 1994], MAD/MAX–mSin3 in mammalian cells [Ayer et al. 1995; Schreiber-Agus et al. 1995], and N-CoR or SMRT interaction with the nuclear hormone receptor family [Chen and Evans 1995; Horlein et al. 1995]. A common theme among these examples is that mutations in the DNA-bound repression domain that abolish interaction with the corepressor molecule also eliminate the repression function.

The KRAB [Krüppel-associated box] is a repression domain [Fig. 1] that is encoded by many transcription factors. It was originally identified in humans as a conserved amino acid sequence motif at the amino termini of proteins that contain multiple TFIIIA/Krfippel-class Cys2–His2 [C2H2] zinc fingers in their carboxyl termini [Bellefroid et al. 1991]. The KRAB domain has now been identified in frog, rodent, and human zinc-finger proteins [Altaba et al. 1987; Thiessen et al. 1991; Constantinou-Deltas et al. 1992; Witzgall et al. 1994; Vissing et al. 1995]. Between 300 and 700 human genes encode C2H2 zinc-finger proteins [Klug and Schwabe 1995], and one-third of these contain KRAB domains [Bellefroid et al. 1991]. To date ~65 unique KRAB domain sequences have been isolated. The KRAB domain homology [Fig. 1] consists of ~75 amino acids, is divided into A and B boxes [on the basis of common intron–exon boundaries], and is predicted to fold into two charged amphipathic helices [Bellefroid et al. 1991].

The KRAB domain is a potent, DNA binding-dependent repression domain [Margolin et al. 1994; Witzgall et al. 1994; Pengue et al. 1995; Vissing et al. 1995]. The minimal KRAB repression module is ~45 amino acids, and substitutions for conserved residues within this domain abolish repression. To date, 16 KRAB domains have been demonstrated to be potent repressors, suggesting that this is a common property of independently en-
Friedman et al.

Figure 1. Comparison of the KRAB domains from various zinc-finger proteins. The amino acid sequences of 16 KRAB domains (retrieved from sequence data bases and referenced in the text) are aligned. Each gene encodes the indicated KRAB domain at its amino terminus, the superscript numeral at the left of each sequence indicates the position of the amino acid with respect to the initiator methionine. The zinc fingers encoded by each gene are not shown. The KRAB NK10 text) are aligned. Each gene encodes the indicated frequent residue in that position. The substitu-

tions DV--* AA and MLE--~ KKK in the KOX1 KRAB domain dramatically reduce repression activity (Margolin et al. 1994). A dot indicates a gap introduced to facilitate alignment.

coded KRAB domains. Thus, the KRAB-zinc-finger protein (KRAB-ZFP) family represents a large, mechanistically unexplored class of transcriptional repressor molecules.

KRAB-ZFPs are likely to play important regulatory roles during development. Expression of the KRAB-ZFP KID1 is temporally and spatially restricted to the developing mouse kidney (Witzgall et al. 1993). A cluster of >40 KRAB-ZFP-encoding genes has been identified on human chromosome 19p12-p13.1, and several members of this cluster are differentially expressed during T-cell ontogeny and activation (Bellefroid et al. 1993). Expression of the KRAB-ZFPs HPF4, HTF10, and HTF34 is down-regulated during myeloid differentiation (Bellefroid et al. 1991). Finally, a number of KRAB-ZFPs are candidate genes for human diseases on the basis of chromosomal location (Tommerup et al. 1993; Crew et al. 1995).

The mechanism of repression by the KRAB domain is not known. In this study, we have identified KRAB-associated protein-1 (KAP-1), a novel protein that binds to the KRAB domain and functions as a transcriptional corepressor. KAP-1 is thus a potential mediator of repression for the large class of KRAB domain-containing transcription factors.

Results

Evidence for a titratable cellular factor required for KRAB domain-mediated repression

The KRAB domain of KOX1 (Fig. 1) is a potent repressor of transcription when fused to the GAL4 DNA-binding domain, and repression by GAL4–KRAB requires DNA binding (Margolin et al. 1994). To determine whether the KRAB repression domain and the WT1 repression domain function via similar titratable cofactors, we attempted to squelch GAL4–WT1-mediated repression with the KRAB-encoding plasmid (Fig. 2B). Transcriptional repression mediated by GAL4–WT1 was unaffected by increasing concentrations of KRAB [lanes 12,13], suggesting that these two repression domains do not share a common target detectable by this squelching assay.

Purification of a 100-kD KRAB domain-binding protein

We sought to identify cellular factors that bind to KRAB domains using affinity chromatography. We constructed glutathione-S-transferase fusion genes (Fig. 3) encoding the wild-type (GST–KRAB) and a mutant KRAB domain (GST–KRAB, DV → AA) that lacks repression activity.
These proteins were soluble and highly expressed in E. coli (Fig. 3A). Sepharose beads bound to either wild-type or mutant proteins were incubated with a nuclear extract from [35S]methionine-labeled cells (Fig. 3B). The beads were washed with stepwise increases in salt concentrations, and the proteins that remained bound after each wash were analyzed by SDS-PAGE (Fig. 3B). A 100-kD protein (p100) was retained by the wild-type GST–KRAB protein but not the mutant GST–KRAB(DV → AA) protein. A 100-kD protein was also retained by the GST–KRAB in a single-step purification procedure after extensive washing in buffer containing 0.5 M NaCl and 0.1% Triton X-100 (Fig. 3C). GST-fusion proteins encoding KRAB domains from four other proteins, ZNF133, ZNF140, KRK-1, and EEK-1 (Fig. 3C), bound to p100, suggesting that this may be a general property of KRAB domains. p100 failed to bind to two KRAB-domain substitution mutations (DV → AA, Fig. 3B, and MLE → KKK; data not shown) that greatly diminish repression activity (Margolin et al. 1994).

A KRAB-binding protein of identical mobility was also detected in extracts from Rh30, COS1, and RD cells, suggesting that p100 is conserved in mouse, monkey, and human (data not shown). Moreover, when a 32P-labeled recombinant KRAB protein was used as a probe in a Far Western blot assay, a 100-kD protein was detected that was not observed when an identical blot was probed with 32P-labeled KRAB (DV → AA) protein (data not shown). These results suggest that the KRAB–p100 interaction is direct. The correlation between loss of repression and loss of binding to p100 observed with the KRAB mutants indicates that p100 may play a critical role in KRAB-mediated repression.

Cloning of KAP-1

A preparative-scale purification of KRAB-binding proteins from calf-thymus extract yielded a 100-kD protein that bound to wild-type GST–KRAB but not mutant GST–KRAB(DV → AA). The purified protein was subjected to microsequence analysis, and five peptide sequences were obtained. The amino acid sequence of one of the peptides was used to design degenerate oligonucleotide primers that were used in coupled reverse transcription–polymerase chain reaction (RT–PCR). A 70 bp PCR product was found to encode the original peptide sequence and was used as a probe to isolate a 3.1-kb cDNA clone from a human testis cDNA library.

The nucleotide sequence of this clone revealed a 300-bp untranslated region (UTR) followed by a 2.5-kb open reading frame, a 300-bp 3′-UTR, and a poly(A +) tail. The predicted initiator methionine codon is within a favorable sequence context for translation initiation (Kozak 1992) and is immediately preceded by an in-frame stop codon. The longest open reading frame encodes a polypeptide of 835 amino acids (Fig. 4A), with a calculated mass of 89 kD and a pI of 7.6. In addition, all five peptide sequences obtained in the original purification are encoded by the predicted open-reading frame. A BLAST search (Altschul et al. 1990) with the protein and nucleotide sequences indicated that we had isolated a novel gene which we named KAP-1 ([KRAB-associated protein]).

Analysis of the predicted amino acid sequence of KAP-1 revealed several regions similar to previously identified protein motifs (Figs. 4A,B). At the amino terminus a region rich in alanine is followed by a RING finger (which conforms to the consensus C3HC4) and B1 and B2 boxes, each of which are regions of conserved cysteine and histidine spacing often found immediately carboxy-terminal to the RING finger (for review, see Freemont 1993). A region of predicted coiled-coil structure (Lupas et al. 1991) is then followed by another cysteine/histidine-rich structure recently identified as a PHD finger (Aasland et al. 1995). The extreme carboxyl terminus displays significant similarity to the bromo-domain (Haynes et al. 1992). The overall architecture of KAP-1 is similar to the TIF1 protein (LeDouarin et al.
The KAP-1 cDNA encodes a 100-kD KRAB-binding protein

To verify that the KAP-1 cDNA encodes the 100-kD KRAB-binding protein, we performed coupled in vitro transcription and translation (IVT). A 100-kD protein (Fig. 5A, lane 6) was produced that was immunoprecipitated (lane 5) by antiserum raised against recombinant KAP-1 (amino acids 423–589). The KAP-1 antiserum immunoprecipitated a protein of identical mobility (lane 2) from 35S-labeled COS1 nuclear extracts. Transfection of an expression vector containing the KAP-1 cDNA into COS1 cells resulted in an approximately fivefold increase in the amount of this protein (lane 3). We conclude that the KAP-1 cDNA encodes a full-length protein.

To confirm that KAP-1 is identical to the protein we originally purified, we performed a serial GST–KRAB purification/immunoprecipitation experiment using nuclear extract from KAP-1-transfected COS1 cells (Fig. 5B). To allow us to distinguish endogenous KAP-1 from the product of the transfected KAP-1 cDNA, we placed a seven-residue epitope tag (which is recognized by antimyc monoclonal antibodies) at the carboxyl terminus of KAP-1. Following transfection, the p100 KRAB-binding protein was purified from 35S-labeled COS1 nuclear extracts by use of a GST–KRAB resin (Fig. 5B, lane 4). The protein was then eluted from the resin with glutathione and immunoprecipitated with either anti-KAP-1 serum or myc-tag monoclonal antibody. The 100-kD protein purified on the GST–KRAB resin comigrated with immunoprecipitated KAP-1 (lanes 2 and 3) and was precipitated by the KAP-1 and myc-tag antisera (lanes 6,7), but not by preimmune serum (lane 5). Identical results were obtained with endogenous KAP-1 protein from COS1 cells in a sequential GST–KRAB Western blot assay (data not shown). These results strongly suggest that the KAP-1 cDNA encodes the p100 KRAB domain-binding protein first identified in nuclear extracts.

KAP-1 forms a ternary complex with a DNA-bound KRAB domain in vitro

If KAP-1 plays a role in KRAB-mediated repression, it should be able to form a stable complex with a DNA-bound KRAB domain. To detect such ternary complexes, we used the electrophoretic mobility shift assay (EMSA). In these experiments the KRAB domain was fused to the DNA-binding domain of the human PAX3 protein. The PAX3–KRAB protein was produced by IVT (Fig. 6A) and DNA binding of the PAX3–KRAB protein was detected via EMSA with a 32P-labeled c5 PAX3 recognition sequence (Goulding et al. 1991). When the PAX3–KRAB protein was preincubated with increasing amounts of COS1 nuclear extract, a new complex with reduced mobility was observed (Fig. 6A, lanes 3-6). Incubation of wild-type PAX3 IVT with nuclear extract failed to generate the new complex (data not shown). Moreover, the PAX3–KRAB[DV → AA] protein failed to generate this slowly migrating complex (lanes 8–12) following incubation with nuclear extract. Furthermore, purified, recombinant wild-type KRAB protein but not mutant KRAB[DV → AA] protein was an effective competitor of complex formation (Fig. 6B).

These data suggest that the new gel shift complex is composed of DNA-bound PAX3–KRAB protein complexed to a KRAB-domain-binding protein present in the nuclear extract. We identified this factor as KAP-1 using an extract from COS1 cells transfected with myc-tagged KAP-1 (Fig. 7A); the lower mobility complex was supershifted and/or disrupted by antisera specific for PAX3, KRAB, KAP-1, and the myc epitope (lanes 4–7), but not by preimmune serum (lane 3). Together, these results strongly suggest that we have reconstituted the DNA-bound KRAB–KAP-1 complex in vitro.

To isolate KRAB–KAP-1 complexes from a cellular milieu, we transfected COS1 cells with the PAX3–KRAB
Amino-acid sequence of KAP-1. (A) The predicted amino acid sequence of the protein encoded by the open reading frame. Conserved cysteine and histidine residues of the RING, B1, B2, and PHD fingers are circled. The five peptides indicate the segment of KAP-1 expressed in Escherichia coli and utilized for antibody production. (B) Alignment of the KAP-1 RING finger, PHD finger, and bromodomain-like regions with the corresponding sequences of related proteins. Residues in aligned proteins sequenced are underlined. The relative locations of the RING, B1, B2, coiled-coil, and bromodomains are indicated. The brackets are taken from Eckner et al. (1994). Dashes indicate gaps inserted for alignment. Accession numbers (NCBI sequence ID): TIF1, 998813; amino acids are indicated. The middle line summarizes the percent identity between KAP-1 and TIF1 by region.

**Figure 4.** Amino-acid sequence of KAP-1. [A] The predicted amino acid sequence of the protein encoded by the KAP-1 open reading frame. Conserved cysteine and histidine residues of the RING, B1, B2, and PHD fingers are circled. The five peptides indicate the segment of KAP-1 expressed in Escherichia coli and utilized for antibody production. [B] Alignment of the KAP-1 RING finger, PHD finger, and bromodomain-like regions with the corresponding sequences of related proteins. Residues in aligned proteins sequenced are underlined. The relative locations of the RING, B1, B2, coiled-coil, and bromodomains are indicated. The brackets are taken from Eckner et al. (1994). Dashes indicate gaps inserted for alignment. Accession numbers (NCBI sequence ID): TIF1, 998813; PML, 239750; RAD18, 131780; MI-2, 761718; RBP2, 138858; p300, 627657; TAF250, 115942. [C] Comparison of KAP-1 and TIF1. The regions of homology to previously identified motifs are denoted by shaded boxes; regions characterized only by a richness in particular amino acids are indicated. The middle line summarizes the percent identity between KAP-1 and TIF1 by region.

expression plasmid. Nuclear extracts from transfected cells were then tested by EMSA with the 32P-labeled c5 DNA binding site (Fig. 7B). The predominant gel shift complex comigrated with the complex reconstituted in vitro, and it was supershifted or disrupted by the PAX3, KRAB, and KAP-1 antisera (Fig. 7B). These results suggest that a stable KRAB–KAP-1 complex is extractable from transfected cells; it remains possible, however, that association occurs after extraction from the nucleus.

The RING-B1-B2 region of KAP-1 is required for binding to the KRAB domain

To identify the region of KAP-1 that is required for the interaction with the KRAB domain, we constructed an amino-terminal deletion of KAP-1 [KAP-1(239-835)], which lacks the RING-B1–B2 domains [Fig. 8A], but includes the complete predicted coiled-coil domain and the remainder of the protein. As shown in Figure 8C, this KAP-1(239-835) protein was properly expressed in transfected COS1 cells as detected by immunoprecipitation with anti-KAP sera from the nuclear extract (lane 1), but it failed to bind to GST–KRAB resin (lane 2). Note that full-length endogenous KAP-1 is properly bound [lane 2]. This suggests that the amino terminus of KAP-1 is the site of KRAB binding.

**KAP-1 has the functional properties of a corepressor**

One of the defining properties of coactivator/repressor molecules is their ability to influence transcription only when brought to specific target genes by a DNA-bound
transcription factor. This model provided us with two predictions that were tested with regard to KAP-1. First, we expected an increase in the nuclear concentration of KAP-1 to increase the rate of formation of the DNA-bound KRAB–KAP-1 complex and, if that step is rate-limiting, to thereby increase the efficiency of KRAB-mediated repression. This prediction was confirmed by the experiment depicted in Figure 8B, in which transfection of increasing amounts of KAP-1 expression vector enhanced repression by PAX3–KRAB up to fivefold, but had a minimal effect in the absence of cotransfected PAX3–KRAB. It is noteworthy that the KAP-1[239-835] protein, which fails to interact with the KRAB domain, had a minimal effect in the absence of cotransfected KAP-1 amino acids 293-835 to the GAL4-1-147 DNA-binding domain. The amino-terminal RING-B1-B2 structures were not included, thereby ensuring that any effect on transcription was not attributable to interactions with endogenous KRAB-containing proteins. The GAL4–KAP-1, 293-835 fusion protein significantly repressed transcription of the 5xGAL4-TKCAT reporter (Fig. 9A).

In summary, these data suggest that KAP-1 contains an intrinsic DNA binding-dependent repression function and supports the model that KAP-1 is a corepressor that mediates repression by the KRAB domain.

**Discussion**

We have utilized the KRAB repressor domain to identify and clone a gene encoding a novel nuclear protein (KAP-1) that displays the hallmarks of a corepressor. We offer a number of lines of evidence that suggest that KAP-1 plays a key role in mediating KRAB domain repression: (1) KAP-1 binds to multiple KRAB repression domains and the KRAB–KAP-1 interaction can be reconstituted in vitro; (2) mutations in the KRAB domain that abolish repression concomitantly abolish the interaction with KAP-1; (3) overexpression of KAP-1 enhances KRAB-mediated repression in a manner dependent on the presence of the domain in KAP-1 that binds the KRAB domain; and (4) KAP-1 itself is a repressor when fused to a DNA-binding domain. Together, these findings are consistent with a simple model in which a DNA-bound KRAB domain recruits the KAP-1 corepressor to the promoter via direct protein–protein interactions (Fig. 9B). Repression based on recruitment of a corepressor may be similar to hairy-groucho interactions in Drosophila (Paroush et al. 1994), MATa2/MCM1–SSN6/TUP1 interactions in yeast (Kelche et al. 1992; Cooper et al. 1994; Tzamarias and Struhl 1994), p53-E1b (Yew et al. 1994), thyroid hormone receptors–N-CoR, or SMRT interactions (Chen and Evans 1995; Horlein et al. 1995) in mammalian cells. It will be interesting to determine whether the mechanisms utilized by these corepressor molecules converge at a common step in the transcriptional initiation process.

KAP-1 encodes at least four different classes of cysteine/histidine-rich motifs: The RING finger, B1 box, B2 box, and PHD finger. These structural motifs support its role as a corepressor and also suggest potential mechanisms of repression. The amino-terminal RING finger motif is identified by the signature C3HC4 spacing of cysteine and histidine residues (Freemont 1993), and our data support its proposed function as a protein–protein interface (Barlow et al. 1994; Borden et al. 1995). RING-finger-containing proteins have been strongly implicated in cell growth regulation and transcription, these genes include the tumor suppressor BRCA-1 (Miki et al. 1994), the proto-oncogene PML, which is fused to the retinoic-acid receptor-α (RARα) in acute promyelocytic leukemia (Kakizuka et al. 1991), and the Drosophila protein msl-2 (Kelley et al. 1995; Zhou et al. 1995). msl-2 is particularly suggestive of a role for the RING finger in the regulation of chromatin structure; it is localized to male X chromosome chromatin and is required for the hypertranscription that is responsible for Drosophila dosage compensation.
The PHD finger is a cysteine/histidine-rich structure that is distinguished from the RING finger and LIM domain by containing a consensus of C_{4}H_{3} that spans 50–80 residues [Aasland et al. 1995]. Many PHD-finger-containing proteins have been implicated in chromatin-mediated transcriptional modulation. These include products of the *Drosophila* genes *trithorax* and *polycomblike* and the human gene HRX, which is fused to AF10/AF17 in the t(11:17) translocation in acute leukemia [Chaplin et al. 1995]. Likewise, although the bromodomain of KAP-1 is imperfect, it is interesting to note that bromodomains are found in the adaptor proteins p300, CBP, and GCN5, as well as in the SWI2/SNF2 component of the yeast SWI/SNF transcriptional activation complex of proteins [Laurent et al. 1991; Yoshimoto and Yamashita 1991; Georgakopoulos and Thireos 1992; Chrivia et al. 1993; Eckner et al. 1994]. Thus, the overall organization of KAP-1 supports its classification as a corepressor and leads to the speculation that KAP-1 represses transcription by a chromatin-mediated mechanism.

The protein most homologous to KAP-1 is TIF1, a putative coactivator for nuclear hormone receptor-mediated transcriptional activation [LeDouarin et al. 1995]. TIF1 was cloned in a yeast two-hybrid screen for proteins that are able to enhance transactivation by the AF-2 region of the retinoid-X receptor-γ (RXRγ), and it was found to interact with several members of the nuclear hormone receptor family. Although KAP-1 and TIF1 share a remarkably similar organization of motifs, they are clearly not encoded by the same gene: The overall amino acid sequence homology is only 31%. The loop region between C5 and C6 of the TIF1 RING is much longer and highly divergent compared with the analogous region in KAP-1. In addition, the regions between the coiled-coil domain and the PHD fingers of KAP-1 and TIF1 are only weakly similar. Finally, the amino-acid sequence of the TIF1 bromodomain conforms much more stringently to the consensus that defines the domain than does that of KAP-1. Whether the similarity between KAP-1 and TIF1 is actually reflected in significant cross-talk between the hormone receptor and KRAB-ZFP families of transcription factors is an intriguing question and remains to be tested.

In conclusion, we have isolated a corepressor, KAP-1, for the KRAB repression domain. This discovery provides a new and potentially unifying paradigm for the enormous number of KRAB-domain-containing zinc-finger proteins in the human genome. A future determination of the downstream targets of KAP-1 may yield im-

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**Figure 6.** Detection of DNA-bound KRAB–KAP-1 complexes formed in vitro. (A) The binding of PAX3–KRAB to COS1 nuclear proteins was detected by EMSA with in vitro translated PAX3–KRAB [lanes 2–6], PAX3–KRAB(DV → AA) [lanes 7–12], a 32P-labeled DNA probe containing the e5 PAX3 binding site, and a COS1 nuclear extract, as described in Materials and methods. The specific e5-binding complex formed in the absence of COS1 nuclear extract is indicated by a small arrow; the more slowly migrating complex formed upon addition of the nuclear extract is indicated by a large arrow. (B) Competition of higher-order complex formation by purified GST–KRAB (0.6, 1.25, 2.5, 5, 10, and 20 μg; lanes 2–7) or GST–KRAB(DV → AA) (0.6, 1.25, 2.5, 5, 10, and 20 μg; lanes 8–13). The EMSA was performed as in A, but with a constant amount of COS1 nuclear extract. GST-fusion proteins were expressed in bacteria, purified on glutathione–Sepharose (Pharmacia), and eluted with glutathione as described in Materials and methods.
Figure 7. Identification of components of the KRAB–KAP-1 complex. (A) Use of specific antisera to identify components of the e5-binding complex. The EMSA was performed as in Fig. 6A, with the addition of the indicated antisera to the binding reactions. (B) Detection of PAX3–KRAB–KAP-1 complexes in nuclear extracts. The EMSA was performed using the [32P]-labeled e5 probe and nuclear extract from COS1 cells transfected with an expression plasmid encoding PAX3–KRAB. Antisera were added to the binding reactions as indicated. The arrow indicates the specific e5 binding complex containing PAX3–KRAB–KAP-1.

Materials and methods

Expression and reporter vectors

The plasmids encoding the GAL4[1-147] fusions to KOXI[1-90], KOXI[1-90; DV → AA], and to WT1[1-298] have been described [Madden et al. 1993; Margolin et al. 1994]. For the squelching experiments, KOXI[1-161] was subcloned into the pCB6* expression vector. The GAL4-KAP-1 plasmid was generated from a partial KAP clone isolated from a KZAP NK cell library (Stratagene) that includes KAP-1 nucleotides 718-2679 (amino acid residues 293-837) flanked by 5xGAL4-TKCAT and TKCAT reporter plasmids have been described [Madden et al. 1993; Morris et al. 1991]. Each transfection experiment was performed at least three times in duplicate. The numbers derived are the averages of duplicates in a single experiment and variability was within 10%.

Generation of GST-fusion proteins and purification of KAP-1

The cDNAs encoding KOXI[1-90] and the DV → AA mutant were subcloned into the pGEX-2TK vector [Pharmacia]. The ZNF133 and ZNF140 GST-fusion plasmids were generated with cDNAs kindly provided by H.-J. Theisen. Regions of each cDNA including the KRAB domain (ZNF133 residues 1–198 and 191, 193). Each transfection experiment was performed at least three times in duplicate. The numbers derived are the averages of duplicates in a single experiment and variability was within 10%.

Cell culture, transfections, and CAT assays

NIH-3T3 mouse cells, human Rh30, and COS1 monkey kidney cells were grown and transfected as described (Margolin et al. 1994). Expression of all constructs was confirmed by immunoprecipitation with the appropriate antisera [Madden et al. 1991, 1993; Morris et al. 1991]. The CMV vector expressing β-galactosidase, pON260, was used in all transfections, and β-galactosidase activity was used to normalize transfection efficiency in cell extracts for the CAT assay. Cell harvesting, CAT assays, and quantitation were performed as described [Madden et al. 1991, 1993]. Each transfection experiment was performed at least three times in duplicate. The numbers derived are the averages of duplicates in a single experiment and variability was within 10%.

Figure 8. Enhancement of KRAB-mediated repression by exogenous KAP-1. (A,B) Cells were transfected with the indicated amounts (in micrograms) of expression plasmid encoding PAX3–KRAB, and with 5 μg [lanes 5,8], 10 μg [lanes 6,9], or 15 μg [lanes 7,10] of expression plasmid encoding KAP-1 or KAP-1[239-835], along with 2.5 μg of the reporter plasmid. The CAT assay and quantification were performed as described in Materials and methods. The amount of CAT activity in the absence of PAX3–KRAB and KAP-1 was assigned a level of 100% activity; other activities are reported as a percentage of this value. The degree of repression by 3 μg of PAX3–KRAB expression plasmid in the presence of 0, 5, 10, and 15 μg of KAP-1 plasmid is graphically represented in the right panel. (C) Deletion of the RING-B1-B2 eliminates both binding to the KRAB domain and enhancement of KRAB-mediated repression. An expression plasmid encoding KAP-1[239-835] was transfected into COS1 cells, and transfected cell extracts were used in an anti-KAP-1 immunoprecipitation [lane 1] or purification on GST–KAP bound to glutathione–Sepharose [lane 2]. Full-length endogenous KAP-1 and exogenous KAP-1[239-835] are indicated by arrows.
Figure 9. Repression by a GAL4-KAP-1 fusion protein. (A) Increasing amounts of GAL4-KAP-1(293-835) expression plasmid (0.01, 0.05, 0.25, and 6.25 μg; lanes 2–6 and 8–12) were transfected into NIH-3T3 cells with either the 5xGAL4-TKCAT reporter (lanes 1–6) or the TKCAT reporter (lanes 7–12). The amount of CAT activity for each reporter in the absence of GAL4-KAP-1(293-835) was assigned a level of 100% activity; other activities are reported as a percentage of this value. (B) A model of KRAB-ZFP-KAP-1 interactions which may mediate repression. We suggest that a KRAB-ZFP protein bound to its domains then mediates repression. It is speculated that the intrinsic repression domain of KAP-1 nominally comprised of the carboxy-terminal segment containing the PHD and bromodoms then mediates repression. It is speculated that the mechanisms of KAP-1-mediated repression involve chromatin structure alteration [signified by the black box] or direct interactions with components of the basal transcription machinary.

ZNF140 residues 1–137) were cloned into the vectors pGEX-4T-3 and pGEX-2TK, respectively. The GST–KKR-1 fusion plasmid was generated by the subcloning of a region including the KRAB domain (residues 30–290) into the pGEX-4T-3 vector. All GST-fusion proteins were expressed in bacteria and purified according to the manufacturer's instructions (Pharmacia). We routinely obtained >98% purity at concentrations of 1–2 μg of GST–KRAB protein bound per ml of 50:50 Sepharose slurry. For analytical purification of KAP-1 from NIH-3T3 cells, [35S]methionine labeling and nuclear extract preparation were performed as described [Fredericks et al. 1995]. Labeled nuclear extract [50 μl] was precleared for 1 hr at 4°C with 20 μl of GST–Sepharose [5 μg protein/μl resin] in a total volume of 1 ml of NEB (10 mM HEPES, pH 7.5, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 2 μg/ml leupeptin, 2 μM pepstatin A, and 0.04 U/ml aprotinin), and homogenized in a Waring blender and Dounce homogenizer. KCl and glycerol were added to 500 mM and 20%, respectively, and the extract was centrifuged at 146,000g for 1 hr. Proteins in the supernatant were fractionated by 33% ammonium sulfate precipitation. The resulting precipitate was resuspended in 20 ml of NEB and dialyzed exhaustively in the same buffer. Approximately 10 ml of this calf thymus extract was loaded onto a 1-ml bed volume GST-fusion protein–glutathione–Sepharose column, the column was washed with >5 bed volumes of NEB + 1% Triton-X100, and eluted with three bed volumes of elution buffer (20 mM glutathione, 100 mM Tris-HCl, pH 8.0, and 120 mM NaCl). The column fractions were analyzed by SDS-PAGE and Coomassie blue staining. Selected fractions from several purifications were pooled and concentrated in Centricon-50 concentrators according to the manufacturer's instructions [Amicon]. A total of ~50 μg of KAP-1 was obtained from ~100 gm of frozen calf thymus, the result of three independent purification procedures. Protein blotting, in situ trypsin digestion, reverse phase HPLC separation of peptides, mass spectrometry analysis of fractions, and peptide microsequencing were performed by the Wistar Protein Microsequencing Facility as described in Best et al. (1995).

Cloning of KAP-1

The sequence of the peptide QGSGSSQPMVEQEGYFGSGD-DPYSAAEPH was used to design a degenerate primer pair (including BamHI and EcoRI sites to facilitate cloning) for use in RT–PCR. The template for RT–PCR was poly(A+) bovine kidney RNA (Clontech). The RT–PCR was performed as follows: 1 μg of poly(A+) RNA was reverse-transcribed with an oligo(dt) primer and SuperScript II enzyme (Gibco-BRL) according to the manufacturer's instructions. A fraction of the reverse-transcription reaction was then amplified by PCR with the degenerate primer pair under the following conditions: 37°C, 15 min, followed by five cycles of 94°C, 30 sec; 48°C, 30 sec; 70°C, 1.5 min. A 70-bp product was gel-purified, digested with BamHI and EcoRI, and cloned into the pGEM-7zl +1 plasmid (Promega). The DNA sequence of the clone was found to encode the original peptide in a single reading frame. A human expressed sequence tag sequence that displayed 98% nucleotide sequence identity to the 70-bp product was used as a hybridization probe to isolate a 3.1-kb CDNA clone from a human testis cDNA library (Clontech). The KAP-1 CDNA was subcloned into the pcDNA3 vector [Invitrogen] for in vitro transfection and expression in mammalian cells. The modified 7 residue myc-tag amino acid sequence, EQKLISE, was fused to the carboxyl terminus by incorporating it into an oligonucleotide primer followed by PCR. The KAP-1[239-835] deletion was constructed by PCR of the entire region using a 5′ primer which included a Kozak consensus sequence (Kozak 1987) and an initiator methionine codon. The resulting product was cloned into the pCDNA3 mammalian expression vector [Invitrogen]. All PCR products were sequenced on both strands to guard against errors.

Sequence analysis

The KAP-1 cDNA was sequenced on both strands by a combination of manual dye-exchange sequencing (Ausubel et al. 1993) and automated sequencing on an Applied Biosystems cycle sequencing apparatus. DNA and protein sequence homology searches
Antisera, immunoprecipitations, and immunohistochemistry

Nucleotides 1270–1755 of the KAP-1 cDNA were amplified by PCR and subcloned into the pQE30 hexahistidine-fusion bacterial expression vector (Qiagen), expressed in bacteria, and purified with nickel-chelate chromatography (Qiagen). Then hexahistidine-tagged KAP-1 (1–354) was used to generate anti-KAP-1 sera in C3H mice. Soluble protein was mixed with Freund's adjuvant and injected subcutaneously. The bleeds from five mice were pooled and tested in immunoprecipitations of labeled, in vitro transcribed/translated KAP-1. The same single bleed pool was used in all experiments. The anti-KOX1 serum was generated in rabbits against hexahistidine-KOX1 (1–161) and anti-PAX3 serum was generated as described (Fredericks et al. 1995).

For immunoprecipitations, COS1 cells were labeled with [35S]-methionine and harvested in ELB buffer plus protease inhibitors (250 mM NaCl, 50 mM HEPES, pH 7.5, 0.1% NP-40, 1 mM EDTA, 0.1 mM PMSF, 2 μg/ml leupeptin, and 11 μg/ml aprotinin). Immunoprecipitations were performed in 1 ml of ELB plus protease inhibitors. One microliter of preimmune or anti-KAP-1 sera (or 1 μg of myc-tag monoclonal antibody) was added and incubated for 2 hr at 4°C, followed by the addition of 100 μl of a 10% slurry of Protein A-Sepharose (Pharmacia) and rotation at 4°C for 30 min. The pellets were washed five times with 1 ml of ELB, boiled in 2X SDS gel loading buffer, and analyzed by SDS-PAGE and fluorography. For the sequential GST association/immunoprecipitations, proteins bound to GST–KRAB were eluted in batch in five bed volumes of elution buffer (20 mM glutathione, 100 mM Tris-HCl, pH 8.0, and 120 mM NaCl) at room temperature for 30 min. Eluted proteins were then immunoprecipitated in 1 ml ELB as described above.

Electrophoretic mobility shift assay

EMSA was performed essentially as described (Fredericks et al. 1995). The PAX3–KRAB fusion protein was transcribed/translated in vitro and used in all assays unless otherwise noted. Binding reactions were assembled in 20 μl of binding buffer [final concentration 20 mM HEPES (pH 7.6), 50 mM NaCl, 0.2 μg poly[d(I-C)], 0.5 mM DTT, 5 mM MgCl2, 10% glycerol]. In vitro translated PAX3-KRAB was incubated in the above buffer with nuclear extracts/antisera/competitor proteins for 20 min at room temperature, and then 1 μl of c5 probe (107 cpm/μl) was added and the reaction was incubated for 10 min at 30°C. Amounts of antibodies used were as follows: 1 μl of a 1:10 dilution of anti-KAP-1 sera, 1 μl anti-PAX3 sera, 1 μl of a 1:5 dilution of anti-KOX1 sera, or 0.1 μg myc-tag monoclonal antibody. In experiments involving addition of competitor proteins, GST–KRAB and GST–KRAB(DV → AA) were purified on glutathione–Sepharose and eluted as described above. For assays of PAX3–KRAB complexes formed in vivo, PAX3–KRAB and KAP-1 expression plasmids were transfected into COS1 cells, and nuclear extracts were prepared as described above. DNA–protein complexes were resolved on 1.5-mm 4% native polyacrylamide gels by electrophoresis at 400 V for 1 hr in 45 mM Tris-borate (pH 8.3)/45 mM boric acid per 1 mM EDTA buffer. EMSA gels were dried and visualized by autoradiography.

Acknowledgments

We thank Drs. Amitabha Basu and Chin Howe for assistance with thymus extract preparation; Dr. Gerd Maul for immunofluorescence analyses; Dr. Laura Benjamin for assistance with RT–PCR assays, David Reim and the Wistar Institute Microchemistry Core Facility for peptide sequencing, Drs. Hildegund C.J. Ertl and Zhi Quan Xiang for assistance in production of polyclonal mouse sera, and Dr. Cory Abate (Center for Advance Biotechnology and Medicine) for the gift of anti-myc-tag monoclonal antibody. We also thank Drs. Shelley Berger, Mitch Lazar, Henrik Vissing (Novo Nordisk, Denmark), George Pendergast, Paul Lieberman, Giovanni Rovera, and Peter Traber for helpful discussion and Dawn Gillespie for preparation of the manuscript. J.R.F. is supported by the Medical Science Training Program, University of Pennsylvania School of Medicine. W.J.F. is supported by the Wistar Institute Cancer Training Grant CA09171. D.E.J. is supported by a Susan G. Komen Breast Cancer Foundation Fellowship. E.G.N. is supported by DK49210 and DK45191. D.W.S. is supported by CA66671 and CA25874. F.J.R. is supported by National Institutes of Health grants CA52009, Core grant CA10815, DK49210, GM54220, DAMD17-96-1-6141, ACS NP-954, and the Irving A. Hansen Memorial Foundation, the Mary A. Rumsey Memorial Foundation, and the Pew Scholars Program in the Biomedical Sciences.

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*Genes Dev.* 1996, 10:
Access the most recent version at doi:10.1101/gad.10.16.2067