Two Novel Krüppel-associated Box-containing Zinc-finger Proteins, KRAZ1 and KRAZ2, Repress Transcription through Functional Interaction with the Corepressor KAP-1 (TIF1β/KRIP-1)*

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We have isolated two novel Krüppel-like zinc finger proteins containing the evolutionarily conserved Krüppel-associated box (KRAB), KRAZ1 and KRAZ2, and demonstrated that they repress transcription when heterologously targeted to DNA. Their repression activity appeared to be mediated by the putative corepressor KAP-1 (KRAB-associated protein-1), because KRAZ1/2 bind to KAP-1, but KRAB mutants of KRAZ1/2 that are unable to interact with KAP-1 lack repression activity, and KAP-1 has intrinsic repressor activity and potentiates KRAZ1/2-mediated repression. We dissected the KAP-1 protein into a KRAB-interacting domain and a region necessary for repression. Using a mammalian two-hybrid assay, we further demonstrated that KAP-1 deletions lacking repression activity fused to the VP16 transactivation domain strongly activated transcription when coexpressed with KRAZ1. In contrast, VP16-KAP-1 fusions retaining repression activity resulted in repression. These results provide the first evidence that KAP-1 functionally interacts with KRAB in mammalian cells and seems to exert repressor activity in the DNA-bound KRAB-KAP-1 complex, and they further support the hypothesis that KAP-1 functions as a corepressor for the large class of KRAB-containing zinc finger proteins.

A large number of studies on transcriptional factors has revealed that functional domains of many transcriptional factors are modular. They can be structurally and functionally separated into DNA-binding domains and effector (activation or repression) domains. DNA-binding domains can be classified according to common structural motifs, which are well conserved throughout evolution (1). One of such motifs is the C_{2}H_{2}-type zinc finger repeat that has been estimated to be present in several hundreds of genes, thus being identified as a large class of KRAB-containing zinc finger proteins.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s)AB024004 and AB024005 for KRAZ1 and KRAZ2, respectively.

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1 The abbreviations used are: ZFP, zinc finger protein; KRAB, Krüppel-associated box; PCR, polymerase chain reaction; nt, nucleotide(s); DBD, DNA-binding domain; HA, hemagglutinin; NLS, nuclear localization signal; 6MT, six repeats of the Myc epitope; AD, activation domain; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; TIF, transcriptional intermediary factor.

conserved stretch of seven amino acids (the H/C link) connecting multiple tandem repeats of the zinc finger domain (5). In contrast to the DNA-binding domains, analysis of the structure or targets of effector domains has been hampered by the lack of amino acid sequence homology or structural motifs common among them. Nevertheless, some effector domains can be loosely categorized according to the primary amino acid content. It has been reported that activation domains are often rich in acidic amino acids and/or proline and glutamine (6, 7). Less is known about repression domains; however, some of them are rich in alanine, proline, or charged amino acids (8).

The Krüppel-associated box (KRAB) was first identified as an evolutionarily conserved motif consisting of 75 amino acids that has been assumed to be present in about one-third of the Krüppel-like ZFPs (9). It is found almost exclusively in the N terminus of the Krüppel-like ZFPs that contain zinc finger domains in their C terminus, and is subdivided into KRAB-A and B domain (9). Recently several KRAB domains have been shown to act as potent repressors when heterologously tethered to the promoter (10–13). The KRAB-A domain present in every KRAB domain, but not the B domain, is responsible for such transcriptional repression (10, 11). The KRAB domain is rich in charged amino acids and predicted to fold into two amphipathic α helices, which might thus serve as a protein-protein interaction surface (9). Unlike other alanine- or proline-rich repression domains, the KRAB domain is highly conserved in a subfamily of the Krüppel-like ZFPs sharing a common amino acid sequence and a predicted secondary structure, suggesting that the KRAB domains in many different ZFPs may share a common cellular target(s) (10).

This prediction was recently substantiated by the isolation and characterization of a novel protein that interacts with KRAB, KRAB-associated protein-1 (KAP-1) (14). KAP-1 was also identified as TIF1β or KRIP-1 (15, 16) (hereafter referred to as KAP-1) and found to interact with several different KRAB domains but not with KRAB-A mutants deficient in repression, to enhance KRAB-mediated repression and to repress transcription when directly targeted to DNA (14–16). KAP-1 is a member of the RBCC subfamily of the RING finger proteins that contain the RING finger motif defined as C_{4}HC_{4} finger and followed by one or two B box-type fingers and a putative coiled-coil domain (14–17). Although the functions of these domains are so far unknown, they have been thought to be involved in nucleic acid-protein and/or protein-protein interactions (18, 19). In the C-terminal portion, KAP-1 also contains a C_{4}HC_{4} zinc finger called PHD finger followed by a domain similar to the bromodomain (14–17). The PHD finger is often found in concert with the bromodomain and is also present in a number of proteins implicated in chromatin-mediated transcriptional regulation, thereby suggesting that it might be involved in interactions with chromatin components (20). The bromodomain has also been identified in several proteins that
regulate transcription in the context of large multiprotein complexes and/or through interaction with, or modification of, chromatin (21–23). Thus, it has been proposed to mediate the protein-protein interactions influencing the assembly and/or activity of such complexes, or to be involved in interactions with chromatin (21).

A highly related protein to KAP-1 in the overall structure including the conserved domains is TIF1α, which was originally identified as a putative coactivator of the nuclear receptor (24). However, it was subsequently reported to interact with KRAB and repress transcription when directly recruited to the promoter (15, 17). Therefore, KAP-1 and TIF1α have been postulated to constitute a new family of transcriptional intermediary factors and function as mediators of transcriptional repression for the large class of KRAB-containing ZFPs (17). However, there is no compelling evidence directly indicating that KAP-1 or TIF1α indeed interacts with KRAB in mammalian cells and exerts the repression function in a physiological context of the resultant DNA-bound KRAB–KAP-1/TIF1α complex.

In this report, we describe the isolation and characterization of two novel KRAB-ZFPs, KRAZ1 and KRAZ2, which repress transcription and interact with KAP-1. We further investigated the functional interaction of KRAZ1/2 with KAP-1 using a mammalian two-hybrid assay based on the detailed molecular characterization of the functional domains in KAP-1. Our results present the first evidence that KAP-1 functionally interacts with KRAB in mammalian cells and that KAP-1 seems to function as a corepressor of KRAB-ZFPs in the complex with the DNA-bound KRAB domains.

**EXPERIMENTAL PROCEDURES**

**Isolation of Full-length cDNAs of KRAZ1 and KRAZ2**—Isolation of the cDNAs encoding zinc finger domains using degenerate polymerase chain reaction (PCR) was described previously (25). The EcoRI-BamHI fragments of Mszf42 and Mszf49 (25) were used as hybridization probes to isolate cognate full-length cDNAs from a λgt10 cDNA library that was constructed from C57BL/6 mouse spleen cells stimulated with lipopolysaccharide and interleukin-4 (25). The cDNAs were subcloned into pBluescript KS (+) (Stratagene) and sequenced on both strands using the Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase, FS (Applied Biosystems), and analyzed on a model 373A or 310 automatic DNA sequencer (Applied Biosystems). Sequence homology search was performed using the BLAST program (26).

Screening with the Mszf42 probe resulted in the identification of one cDNA clone carrying a sequence (nt 949–1207) that was highly similar but not identical to Mszf42 (85% identity at nucleotide level) and a region encoding the KRAB-A domain (nt 276–395). This Mszf42-like zinc finger gene was thus referred to as KRAZ1. Two cDNA clones isolated using the Mszf49 probe contained an identical sequence to Mszf49 (nt 1569–1839, numbered in the composite cDNA), but did not extend to the 5′-end of mRNA. To generate the full-length cDNA, the 5′-end of the cDNA was amplified by the 5′-rapid amplification of cDNA ends technique from cDNAs derived from a murine B-lymphoma CH12 F3-2 (27) with specific primers (49AS5; 5′-GGTGGTTGACA-GATTCAGATTCGTTCC-3′ complementary to nt 815–839 or 49AS6; 5′-TTCGGGGTATCTTGATAGAAGACTC-3′ complementary to nt 1219–1243) using the Marathon cDNA amplification kit (CLONTECH) according to the manufacturer’s instructions, and subcloned into pGEM-T vector (Promega). Sequence analysis of seven clones resulted in the identification of an additional 690-nt sequence that precedes the 5′-end of the original cDNA fragments (1571 nt) and contains a region encoding the KRAB-A and B domains (nt 335–514). Mszf49 was therefore renamed KRAZ2 in order to simplify the nomenclature used in this report.

**Plasmids**—Recombiant DNA works were performed according to standard protocols (28). A firefly luciferase reporter plasmid, pGL3-G5SV, was constructed by subcloning the Pou-II-Xhol fragment carrying five GAL4-binding sites excised from pG5BCAT (29) into the MluI (blunted) and Nhel sites upstream of the SV40 promoter of pGL3-Promoter vector (Promega, designated pGL3-5V in this report). Another reporter plasmid, pGL3-G5B, was constructed in two steps. The Xhol-Smal fragment containing five GAL4-binding sites and the E1b TATA promoter excised from pG5BCAT was inserted into the Xhol and EcoRv sites of pBluescript KS (+), and the insert was excised with KpnI and Spe1 and subcloned into the MluI and Nhel sites of the pGL3-Basic vector (Promega).

All of the constructs described below except for VP16-KAP-1 were cloned into the mammalian expression plasmid pEF-BOS bar, which was produced by subcloning the 2.9-kilobase PouII fragment excised from pEF-BOS (30) into the PouII sites of pSV2bar (Kakenseyaku). To construct GAL4 fusions, the KRAB-containing regions of KRAZ1 (amino acids 45–216) and KRAZ2 (7–312) were amplified by PCR from the cDNA clones, and the KRAB-containing region of KOX1 (1–90) (10) and various fragments of the KAP-1 promoter (14) indicated in Fig. 5 were amplified for KAP-1 (440–696) were obtained by PCR from Jurkat cell cDNA using corresponding primers tagged with appropriate restriction sites. For all the KAP-1 fragments, 5′- and 3′-primers were tagged with EcoRI and SalI sites, respectively. The PCR-amplified fragments were digested with relevant restriction enzymes and fused in-frame to the C terminus of the yeast GAL4 DNA-binding domain (GAL4DBD, 1–147) excised from KS (+)–GAL4 (31). Amino acid substitutions in the KRAB domains (DV to AA) were introduced by PCR-mediated site-directed mutagenesis (32). KAP-1 440–696 was constructed by ligating the EcoRI-StuI (30–403) and EcoRI (blunted)-SalI (697–835) fragments prepared from the KAP-1 N421 and 697C constructs, respectively. To express non-DNA-bound forms of the KRAB domains properly in the nucleus, the KRAB regions were tagged N-terminally with the hemagglutinin (HA) tag, alternatively, two HA epitope tags were fused in-frame to the C terminus of KAP-1 (33) and followed by the nuclear localization signal (NLS) of the SV40 large T antigen (34) as follows. The Clal-EcoRI fragment in KS (+)–GAL4 containing GAL4DBD was replaced by a linker (Met-HA CE; 5′-CGATAT-GCCGCACTGATGTACCATACAAGCTCCAGACACGCGG-3′) encoding the initiator methionine (italics) and the HA epitope (underlined), and subsequently the EcoRI-StuI sequence was replaced by a linker (SV40LT-NLS; 5′-AAAGTCGCGGCGAAAGAAGAAAGATGGGTG-AATTTCCTGAGG-3′) carrying the NLS of the SV40 large T antigen (underlined) and a new EcoRI site (italics), resulting in pKS+–HA-NLS. The wild-type or mutated KRAB regions of KRAZ1, KRAZ2, and KOX1 were then fused in-frame to the C terminus of the HA-NLS tag excised from pKS+–HA-NLS. To construct the KAP-1 deletions tagged N-terminally with six repeats of the Myc epitope (6MT) and NLS, the Clal-EcoRI fragment in KS (+)–GAL4 was replaced by the Clal-EcoRI fragment carrying 6MT excised from pC2S+MT mNotchIC (35), and subsequently the EcoRI-StuI sequence was replaced by the SV40LT-NLS linker, resulting in pKS+–6MT-NLS. The KAP-1 deletions were then fused in-frame to the C terminus of the 6MT-NLS tag excised from pKS+–6MT-NLS. Fusion of the KAP-1 deletions with the C-terminal repression domain (AD) (413–490) of herpes simplex virus VP16 were constructed by replacing the EcoRI-StuI sequence in pCMX–VP16-N with the SV40LT-NLS linker and subcloning the KAP-1 deletions into the new EcoRI and SalI sites in-frame to the C terminus of VP16AD-NLS. For generation of GAL4–VP16-fused KAP-1 deletions, GAL4DBD was amplified by PCR from KS (+)–GAL4 with 5′-M13 reverse primer and GAL4–VP16-N with 5′-M13 forward primer and was fused in-frame to the GAL4-VP16 fragment in pCMX–VP16-N with 5′-BglII/II- and 3′-EcoRI-tagged primers. The amplified GAL4DBD and VP16AD regions were digested with Clal/BglII and BglII/EcoRI, respectively, and subcloned between the ClaI and EcoRI sites of KS (+)–GAL4, resulting in pKS+–GAL4–VP16. The KAP-1 deletions were then fused in-frame to the C terminus of GAL4–VP16 excised from pKS+–GAL4–VP16. All the PCR-derived sequences were confirmed by sequencing on both strands. Equivalent expression of the proteins with the expected molecular mass and nuclear localization were confirmed by Western blot analysis and immunohistochemistry.

**Cells, Transfection, and Luciferase Assay**—NIH 3T3 and 293T cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. For the luciferase assay, NIH 3T3 cells plated 24 h prior to transfection at 2 × 10⁵ cells/ well in 24-well plate were transfected with 100 ng of the firefly luciferase reporter plasmid, 4 ng of the sea-pansey luciferase reporter plasmid pRL-SV40 (Promega), and the amounts of expression plasmids indicated in each figure using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc.). Total amounts of expression plasmids were adjusted by adding the empty expression plasmid pEF-BOS bar if necessary. After 48 h of incubation, cell lysates and dual luciferase assays were carried out using the dual-luciferase reporter assay system according to the manufacturer’s instructions (Promega). Firefly and sea-pansey luciferase activities were quantified using a Lumat LB9507 luminometer (Berthold), and firefly luciferase activity was normalized for transfection efficiency as determined by sea-pansey luciferase activity. Each transfection experiment
was performed at least three times in duplicate. The results are presented as the average with standard deviations of duplicates in a representative experiment.

**Immunoprecipitation**—293T cells plated 24 h before transfection at 5 × 10⁶ cells/dish in 60-mm dishes were transfected with 0.4 μg of the expression plasmid for 6MT-NLS-KAP-1 N835 together with 0.4 μg of the plasmids encoding GAL4-KOX1, or their mutants using the CellPhect transfection kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Whole-cell lysates were prepared from 293T cells transfected with the expression plasmids for 6MT-NLS-KAP-1 deletions as described for immunoprecipitation. The cell lysates were incubated with 1–5 μg of GST fusion proteins immobilized to glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The GST fusion proteins were produced by subcloning the KRAB-containing regions of KRAZ1, KRAZ2, and KOX1 identical to those used for GAL4 fusions into pGEX-4T-1 (Amersham Pharmacia Biotech), expressed in *Escherichia coli* BL21 (DE3) and purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

**RESULTS**

**Isolation of Two Novel KRAB-containing ZFPs, KRAZ1 and KRAZ2**—We previously cloned 88 mouse cDNAs encoding 60 different Krüppel-like zinc finger domains using the degenerative PCR method (25) based on the structural characteristics of these factors such as the highly conserved H/C link connecting consecutive finger repeats (5). To further investigate the molecular features and biological functions of the novel zinc finger genes identified, several full-length cDNAs were isolated by screening a cDNA library of mouse spleen cell using cognate zinc finger genes as probes (for details, see under “Experimental Procedures”). Sequence analysis revealed that two of the isolated cDNAs encoded novel Krüppel-like ZFPs containing

![Diagram](image-url)
KRAZ1 and KRAZ2 Repress Transcription When Heterologously Targeted to the Promoter—To address first the possibility that KRAZ1 and KRAZ2 possess transcriptional repressor activity when recruited to the promoter, the KRAB-containing region of KRAZ1 (amino acids 43–216) and KRAZ2 (7–312) were fused to GAL4DBD (Fig. 2A). Expression plasmids for these fusions were transfected into NIH 3T3 cells with a luciferase reporter plasmid carrying five GAL4-binding sites upstream of the SV40 promoter (pGL3-G5SV). This reporter plasmid exhibited a high basal level of transcriptional activity when transfected with the parent expression plasmid or the plasmid encoding GAL4DBD alone (Fig. 2B). Specific repression by GAL4-KRAZ1/2 was detected in a dose-dependent manner (Fig. 2C), as well as by GAL4-KOX1 (Fig. 2B, lane 3), a GAL4 fusion with the KRAB-containing region of KOX1 (1–90) that was described previously to repress transcription (10, 13–15). These GAL4-KRAB fusions did not affect transcription from a reporter plasmid lacking the GAL4-binding site (pGL3-SV) (Fig. 2B, lanes 12–16), and a non-DNA-bound form of KRAB did not significantly repress transcription (lanes 9–11), indicating that repression by KRAB is dependent on site-specific DNA binding and did not result from a nonspecific toxic effect on the transfected cells or titration of the basal transcription machinery. Such specific repression was also observed using another reporter plasmid driven by the thymidine kinase promoter of herpes simplex virus (data not shown), suggesting that KRAB-mediated repression is a universal phenomenon rather than a specific effect on some classes of transcription factors. Furthermore, substitution mutations at the highly conserved amino acids in the KRAB-A domains (DV to AA) disrupted repression activity (Fig. 2C, lanes 6–8), indicating that KRAB-A is responsible for repression.

KRAZ1 and KRAZ2 Share a Common Cellular Cofactor with KOX1 to Repress Transcription—As reported previously (14), repression by KOX1 was dose-dependently abrogated by overexpression of free wild-type KOX1, whereas free KOX1 harboring the DV/AA mutation did not significantly interfere with repression (Fig. 3A), suggesting that a titratable cellular cofactor is required for repression and that the repression ability of KRAB correlates to the interaction with such a cofactor. To determine whether the same cofactor is required for KRAZ1/2-mediated repression, we performed a similar squelching experiment. As shown in Fig. 3B, repression by GAL4-KRAZ1/2 was...
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Fig. 3. KRAZ1/2 share a common cellular cofactor with KOX1. All the expression plasmids were transfected into NIH 3T3 cells with the reporter plasmid pGLO3-G5SV (100 ng). The result is shown as relative luciferase activity. A, squelching of GAL4-KOX1-mediated repression by free KOX1. The GAL4DBD or GAL4-KOX1 expression plasmid (2 ng) was transfected with 250 ng (+) or the indicated amounts of the expression plasmid for HA-KOX1 or HA-KOX1 DV/AA mutant (mut). The diagram indicated at the left illustrates how overexpression of free KOX1 may result in squelching of GAL4-KOX1-mediated repression. PIC, preinitiation complex. B, squelching of GAL4-KRAZ1/2-mediated repression by free KOX1. The GAL4DBD or GAL4-KRAZ1/2 expression plasmid (10 ng) was transfected as in A.

KRAZ1 and KRAZ2 Interact with a Putative Corepressor, KAP-1—KAP-1 was identified as such a cofactor that binds to several KRAB domains including KOX1 but not to KRAB mutants lacking repression activity (14–16). To explore the possibility that KAP-1 represents the cellular cofactor shared by KRAZ1/2 with KOX1, we examined the interaction of KRAZ1/2 with KAP-1 by immunoprecipitation using a Myc-tagged KAP-1 (30–835, designated N835) construct that was expressed in 293T cells together with the GAL4-KRAB fusions. Each GAL4 fusion protein was immunoprecipitated with anti-GAL4DBD antibody, and the precipitated proteins were examined by Western blotting. As shown in Fig. 4, comparable amounts of all GAL4 fusions were precipitated, and KAP-1 was coprecipitated with GAL4-KRAZ1/2 and GAL4-KOX1 (lanes 3, 5, and 7), but not with GAL4DBD alone (lane 2). Furthermore, coprecipitation of KAP-1 with GAL4-KRB mutants (lanes 4, 6, and 8) was strongly abrogated, indicating that KRAB-A is responsible for interaction and the ability of KRAB to interact with KAP-1 correlates to the repression activity. Taken together, these results demonstrate that KAP-1 specifically interacts with KRAB-A of KRAZ1/2 and are consistent with that KAP-1 represents the cellular cofactor required for repression by KRAZ1/2. The interaction between KRAB and KAP-1 seems to be direct, because in vitro-translated KAP-1 also bound to bacterially expressed GST-KRAB fusion proteins (data not shown), although it remains to be excluded that proteins in the extract for in vitro translation might be bridging the interaction.

Mapping of the KRAB-binding Domain in KAP-1—To further characterize the interaction between KRAZ1/2 and KAP-1, we next attempted to delineate the KRAB-binding domain in KAP-1 by GST pull-down assays using a number of Myc-tagged KAP-1 deletions (Fig. 5) expressed in 293T cells and GST-KRAB fusion proteins. As shown in Fig. 6, KAP-1 N835 was able to bind with GST-KRAZ1/2 or GST-KOX1 (lanes 18–20), but not with GST alone (lane 17). In a series of N- or C-terminal deletions of KAP-1, further deletions extending from amino acid 239 to 421 failed to bind to GST-KRAB (Fig. 6). Although expression of KAP-1 697C, 145–421 and 239–421 are consistently low for unknown reason (lanes 33, 36, and 39), 145–421 appeared to bind quite efficiently, and 239–421 alone also could bind (lanes 38 and 41), indicating that KAP-1 239–421, which almost corresponds to the coiled-coil region, is necessary and sufficient for the interaction with KRAB in vitro. Com-
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Intrinsic Repressor Activity of KAP-1 and Mapping of Its Repression Domain—One of the definitive criteria for corepressors is their intrinsic ability to repress transcription when directly targeted to the promoter. We thus constructed KAP-1 N835 directly fused to GAL4DBD and assessed its ability to repress transcription. As shown in Fig. 7A, GAL4-KAP-1 N835 efficiently repressed transcription (lane 7). Similar to KRAB, repression by GAL4-KAP-1 was dependent on site-specific DNA binding, because it did not affect transcription from the reporter lacking the GAL4-binding site, and free KAP-1 did not repress transcription (data not shown).

We next attempted to determine the repressor domain of KAP-1 using GAL4-KAP-1 deletions (Fig. 7A). An internal deletion construct, GAL4-KAP-1 Δ404–696, failed to repress transcription significantly (lane 16), indicating that the deleted region is necessary for repression. Furthermore, the region between the coiled-coil and PHD finger (396–562) seems to be a core domain essential for repression, because further deletions in a series of N- or C-terminal deletions considerably abolished repression activity. However, this region alone was not sufficient for full repression, because GAL4-KAP-1 396–711 repressed transcription less efficiently (7.7-fold repression) than 396C (52.5-fold) or N711 (21.3-fold), which included an N711 repression less efficiently (7.7-fold repression) than 396C (52.5-fold) or N711 (21.3-fold), which included an

overexpression of KAP-1 N835 resulted in an enhancement of GAL4-KRAZ2-mediated repression (lanes 7–11) that was more apparent when repression was partially squelched by free KOX1 (lanes 12–16), whereas overexpression of KAP-1 N835 did not affect transcription in the presence of GAL4DBD alone (lanes 1–6). Moreover, even in the presence of free KOX1, repression was not potentiated by KAP-1 396C, which failed to bind to KRAB (lanes 17–20), suggesting that the functional interaction of KAP-1 with KRAB is required to potentiate KRAB-mediated repression. Repression by GAL4-KRAZ1 was also potentiated by KAP-1 N835 though less efficiently (data not shown). These data suggest that KAP-1 positively regulates KRAZ1/2-mediated repression.

KRAZ1 and KRAZ2 Functionally Interact with KAP-1 in Mammalian Cells—The results described above, however, do not provide direct evidence that KAP-1 interacts with KRAB in mammalian cells and KAP-1 mediates repression in the resultant DNA-bound KRAB-KAP-1 complex. In addition, although KAP-1 was coprecipitated with KRAB expressed in mammalian cells (Fig. 4), even if KAP-1 and KRAB were separately expressed and then mixed with each other in vitro, they were coprecipitated (data not shown). Therefore, interaction proved by such immunoprecipitation does not necessarily indicate actual in vivo interaction.

Thus, we next examined the in vivo functional interaction of KRAB with KAP-1 using a mammalian two-hybrid assay in which GAL4-KRAB was coexpressed with the KAP-1 deletions fused to the VP16 transactivation domain (VP16AD) in NIH 3T3 cells. If GAL4-KRAB can functionally interact with KAP-1 in cells, VP16AD fused to KAP-1 would be recruited to the vicinity of the promoter and expected to activate transcription. As shown in Fig. 5, GAL4-KRAZ1 and GAL4-KOX1 repressed transcription in the presence of VP16AD alone, whereas GAL4DBD alone did not significantly affect transcription in the presence of VP16AD alone or any VP16-KAP1 deletions. Repression by GAL4-KRAZ1 and GAL4-KOX1 was unaffected by VP16-KAP1 N244, 396C, 554C, or 697C, all of which lack the KRAB-binding domain (Figs. 5 and 8A, lanes 3 and 11–13), suggesting that these KAP-1 deletions fail to interact with KRAB also in vivo. In contrast, when GAL4-KRAZ1 or GAL4-KOX1 was coexpressed with VP16-KAP1 N421, N487 or 145–421, which contains the KRAB-binding domain but not repression activity, transcription was strongly activated above the baseline level (Figs. 5 and 8A, lanes 4, 5, and 14) in a dose-dependent manner (for N487, see Fig. 8B). This result clearly indicates that these KAP-1 deletions functionally interact with KRAZ1 and KOX1 in mammalian cells. Although coexpression of GAL4-KRAZ2 with these VP16-KAP-1 deletions did not result in strong activation, repression by GAL4-KRAZ2 was dose-dependently counteracted by VP16-KAP1 N487 (Fig. 8B, lanes 16–20), suggesting that KRAZ2 can also interact with KAP-1 in vivo. KAP-1 145–421 containing the B1/B2 finger and coiled-coil region appeared to interact with KRAB in vivo (Fig. 8A, lane 14) as well as in vitro. Although KAP-1 239–421 further lacking the B1/B2 finger could bind to KRAB in vitro (Fig. 6), VP16-KAP1 239–421 failed to activate transcription (Fig. 8A, lane 15), suggesting that the B1/B2 finger is required for the interaction with KRAB only in vivo but not in vitro.

KAP-1 Exerts Repressor Activity in the DNA-bound KRAB-KAP-1 Complex—More surprisingly, coexpression of VP16-KAP1 N562, N711, N835, 145C, or 239C harboring both KRAB-interacting and repression activity did not result in significant activation of transcription but remained repression especially for N835, 145C, or 239C (Figs. 5 and 8A, lanes 6–10). This result suggests two possibilities. First, they cannot functionally interact with KRAB in vivo, although they can interact...
Schematic representation of the KAP-1 deletion constructs used in this study is shown at the left, with amino acid numbers at both ends. KRAB binding ability of the various KAP-1 deletions was assessed by the GST pull-down assay using GST-KRAZ1 shown in Fig. 6 and is presented as + (bound) and − (not bound). ND, not determined. Intrinsic repressor activity of the KAP-1 deletions was measured by the luciferase assay using GAL4-KAP-1 deletions shown in Fig. 7A and is presented as fold repression relative to GAL4DBD alone. In vivo KRAB binding ability of the KAP-1 deletions was determined by the mammalian two-hybrid assay shown in Fig. 8A (for GAL4-KRAZ1), in which VP16AD alone (Control) or VP16-KAP-1 deletions were coexpressed with GAL4DBD, GAL4-KOX1, or GAL4-KRAZ1 and assayed for their effects on the transcriptional activity of the pGL3-G5SV reporter plasmid. The results are presented as relative luciferase activity (percentage of the activity in the presence of GAL4DBD and VP16AD). The positive results in each assay are boxed. The essential repression domain (396–562) and the KRAB-binding domain (239–421) depicted by these assays are indicated at the bottom.

In conclusion, it is most likely that KAP-1 N562, N711 and N835 can interact with KRAB in vivo and that their repression activity dominates transactivation of VP16AD; therefore, the corresponding VP16-KAP-1 fusions would not enhance transcription but rather cause repression. This prediction is further supported by the careful observation that residual transcriptional enhancement by VP16-KAP-1 N562, N711, or N835 correlates reciprocally to the repressor activity of these KAP-1 deletions reproducibly when coexpressed with either GAL4-KRAZ1 or GAL4-KOX1 (Figs. 5 and 8A, lanes 6–8).

To further directly prove that KAP-1 dominantly suppresses transactivation ability of VP16AD, the KAP-1 deletions were fused to the GAL4DBD-VP16AD chimeric protein (GAL4-VP16). To exclude the influence of other cellular transcription factors, a reporter plasmid, pGL3-G5B, containing the minimal E1b TATA promoter, which shows very low basal transcription in vitro, was used. GAL4-VP16 strongly activated transcription in a dose-dependent manner (Fig. 5D, lanes 5–7), but GAL4DBD alone did not (Fig. 5D, lanes 2–4). Activation by GAL4-VP16 was not affected when fused to KAP-1 N244 com-

FIG. 5. KAP-1 deletion constructs and summary of the characterization of the KRAB-binding and repression domain in KAP-1. Schematic representation of the KAP-1 deletion constructs used in this study is shown at the left, with amino acid numbers at both ends. KRAB binding ability of the various KAP-1 deletions was assessed by the GST pull-down assay using GST-KRAZ1 shown in Fig. 6 and is presented as + (bound) and − (not bound). ND, not determined. Intrinsic repressor activity of the KAP-1 deletions was measured by the luciferase assay using GAL4-KAP-1 deletions shown in Fig. 7A and is presented as fold repression relative to GAL4DBD alone. In vivo KRAB binding ability of the KAP-1 deletions was determined by the mammalian two-hybrid assay shown in Fig. 8A (for GAL4-KRAZ1), in which VP16AD alone (Control) or VP16-KAP-1 deletions were coexpressed with GAL4DBD, GAL4-KOX1, or GAL4-KRAZ1 and assayed for their effects on the transcriptional activity of the pGL3-G5SV reporter plasmid. The results are presented as relative luciferase activity (percentage of the activity in the presence of GAL4DBD and VP16AD). The positive results in each assay are boxed. The essential repression domain (396–562) and the KRAB-binding domain (239–421) depicted by these assays are indicated at the bottom.

FIG. 6. Mapping of the KRAB-binding domain in KAP-1. 293T cells were transfected with the expression plasmids for 6MT-KAP-1 deletions indicated in Fig. 5. Whole-cell lysates were prepared and incubated with GST alone (G), GST-KOX1 (KO), GST-KRAZ1 (K1), or GST-KRAZ2 (K2) immobilized to glutathione-Sepharose 4B, and bound proteins and 3% of the input (I) were subjected to 10% SDS-PAGE and analyzed by Western blotting using anti-Myc antibody. The results shown were obtained with GST-KRAZ1, but identical results were obtained with GST-KOX1 and GST-KRAZ2 (lanes 18 and 20 for KAP-1 N835, data not shown). Positions of the molecular mass markers are indicated on the left side.
pletely lacking repressor activity (Fig. 8D, lanes 8–10) but was severely suppressed by fusion with KAP-1 N835 containing full repression activity (Fig. 8D, lanes 11–13). Similar suppression was observed for GAL4-VP16-KAP-1 N562 and N711 (data not shown). These data clearly indicate that the repressor activity of KAP-1 is dominant to transactivation by VP16AD and further strengthen the notion that VP16-KAP-1 N562, N711, and N835 interact with promoter-bound GAL4-KRAB and dominantly repress transcription in the mammalian two-hybrid assay. Taken together, these results further support the hypothesis that KAP-1 functions as a corepressor for KRAB-ZFPs to exert repressor activity in a physiological context of the DNA-bound KRAB-KAP-1 complex. These findings further support the model that KRAB-ZFPs bind to cis-regulatory sequences by their putative DNA-binding domains, zinc fingers, recruit the corepressors including KAP-1 to the promoter by their KRAB domains, and thereby repress transcription (14–16).

We demonstrated that amino acids 239–421 of KAP-1, which almost correspond to the coiled-coil region, are essential and sufficient for the interaction with KRAB in vitro, but the RING-B1/B2 region is dispensable (Fig. 6). However, involvement of the RING-B1/B2 region in the in vivo interaction with KRAB seems to be complicated but important. The RING finger is essentially dispensable in vivo and also in vitro, but the B1/B2 finger is required only in vivo but not in vitro. These results are in agreement with the report by Moosmann et al. (15) suggesting that the coiled-coil region of KAP-1/TIF1β or TIF1α is essential but not sufficient for the interaction with KRAB in a yeast two-hybrid assay. They clearly indicated that the B1/B2 finger of KAP-1/TIF1β is required for the functional interaction, whereas the RING finger is dispensable (15). Nevertheless, the RING finger is suggested to contribute to the efficient

Fig. 7. KAP-1 repressed transcription when heterologously targeted to the promoter and enhanced KRAB-mediated repression. All of the expression plasmids were transfected into NIH 3T3 cells with the reporter plasmid pGL3-5SV (100 ng). A, mapping of the repression domain in KAP-1. The expression plasmids (250 ng) for the GAL4-KAP-1 deletions used are indicated at the left. The result is shown as fold repression relative to GAL4DBD alone. B, enhancement of KRAB-mediated repression by overexpression of KAP-1. The GAL4DBD or GAL4-KRAZ2 expression plasmid (10 ng) was transfected with the indicated amounts of the expression plasmid for 6MT-KAP-1 N835 or 396C. The HA-KOX1 expression plasmid (25 ng) was added to partially squelch GAL4-KRAZ2-mediated repression. The result is shown as relative luciferase activity.

**DISCUSSION**

It has recently been thought to be a universal molecular mechanism that DNA-binding transcriptional repressors exert their repression activity by recruitment of corepressors in many systems from yeast and Drosophila to mammals (14). Our study, together with the previous reports (14–16), revealed that KAP-1 functions as a universal corepressor for the large class of KRAB-ZFPs, because it fulfills the criteria for corepressors: first, KAP-1 interacts in mammalian cells as well as in vitro with several different KRAB domains including novel ones we isolated, but not with KRAB mutants defective in repression. Second, KAP-1 has intrinsic repressor activity, because KAP-1 efficiently represses transcription when targeted to the promoter. Third, overexpression of KAP-1 potentiates KRAB-mediated repression in a manner dependent on the interaction with KRAB. Fourth, KAP-1 seems to exert the repressor activity in a physiological context of the DNA-bound KRAB-KAP-1 complex. These findings further support the model that KRAB-ZFPs bind to cis-regulatory sequences by their putative DNA-binding domains, zinc fingers, recruit the corepressors including KAP-1 to the promoter by their KRAB domains, and thereby repress transcription (14–16).
FIG. 8. **KRAZ1/2 functionally interact with KAP-1 in mammalian cells and KAP-1 dominantly suppresses transactivation by VP16AD.** The expression plasmids were transfected into NIH 3T3 cells with the reporter plasmid (100 ng) pGL3-G5SV (A-C) or pGL3-G5B (D). The result is shown as relative luciferase activity. **A**, a mammalian two-hybrid assay using GAL4-KRAZ1 and VP16-KAP-1 deletions. The
interaction with KRAB \textit{in vivo} by the observation in our mammalian two-hybrid assay that activation by VP16-KAP1 145–421 is consistently lower than that of VP16-KAP1 N421 (Fig. 8A, compare lanes 4 and 4d). Moreover, the competition experiment revealed that KAP-1 145C lacking the RING finger could partially compete with VP16-KAP1 N487 for binding to KRAZ1, and KAP-1 239C, 145–421, or 239–421 could not (data not shown). It might thus also be possible that a third molecule(s) is required for the stable interaction between KAP-1 and KRAB \textit{in vivo} and that the RING-B1/B2 finger region of KAP-1 is necessary for the interaction with such a third molecule(s).

Amino acids 396–562 of KAP-1, which reside between the coiled-coil and the PHD finger, are considered to be a core domain essential for repression but not sufficient for full repression. Several explanations can be made why the N-terminal portion (the RBCC domain) or the most C-terminal portion (the bromo-like domain) should be required for full repression in addition to the core domain. First, the N- or C-terminal region may contribute to the functional conformation or stability of the KAP-1 protein. Second, KAP-1 might possess two or more independent repression modules that are composed of the N- or C-terminal region together with the core domain. The core domain may thus be shared with both repression modules and would be essential, whereas the N- or C-terminal region would be dispensable as long as the other module remains functional. Third, KAP-1 can function only within a multiprotein complex, and the N or C terminus is required for efficient incorporation of KAP-1 into such a complex through specific protein-protein interaction that is the possible function of these domains, or more simply, there might be a definite threshold of molecular mass necessary for proper formation of such a multiprotein complex.

Several hypotheses have been proposed for the mechanism(s) underlying KRAB/KAP-1-mediated repression (15, 17, 36). One is the reorganization of chromatin into a repressive state by heterochromatin formation, because in the yeast two-hybrid assays, both KAP-1 and TIF1α were found to be associated with mHP1α and mMOD1, which are mouse homologs of \textit{Drosophila} heterochromatin protein 1 that repress transcription by the formation of heterochromatin or a similar structure (17). However, we observed that KAP-1 N562 lacking the putative mHP1α/mMOD1-interacting domain (amino acids 571–579 (17) could repress transcription more effectively (17.8-fold) than KAP-1 554C containing this site (7.7-fold) (Figs. 5 and 7A), in agreement with the finding that a mutation of TIF1α within the HP1-interacting domain that disrupts the interaction in yeast did not affect repression activity in transfected mammalian cells (17). Thus, the formation of repressed chromatin by direct interaction with mHP1α and/or mMOD1 does not seem to be a principle mechanism of KAP-1/TIF1β- and TIF1α-mediated repression, although lack of interaction with mHP1α/mMOD1 in KAP-1 N562 remains to be confirmed.

At present, the most favorable model for KRAB/KAP-1-mediated repression is active repression (8), in which KRAB or KAP-1 interacts in an inhibitory manner with specific proteins of the basal transcription machinery, thereby interfering with the assembly and/or function of them (15, 36). Several lines of evidence support the active repression model rather than the formation of repressive chromatin (15, 36). First, KRAB/KAP-1-mediated repression is quite efficient even in short term transient transfection assays as in this study, as well as in an \textit{in vitro} transcription experiment (36), in which the bulk of the reporter DNA templates would not be fully assembled into chromatin. Second, it was recently demonstrated that KRAB is able to repress transcription by RNA polymerase II and III, but neither by RNA polymerase I nor T7 RNA polymerase in mammalian cells, suggesting that KRAB exerts its repression activity by interfering with some component(s) for RNA polymerase II and III transcription rather than by altering the chromatin structure into a repressive state (37). There are two potential molecular mechanisms proposed for active repression, direct and quenching repression (8). For KRAB/KAP-1-mediated repression, direct repression seems to be more likely than quenching repression, because KRAB has been demonstrated to repress transcription from a number of different polymerase II-dependent promoters (12, 15) and suppress the activating function of various transcriptional activators (36), and moreover, KRAB is able to repress basal promoter activity in addition to activated transcription in an \textit{in vitro} transcription experiment (36).

The active repression mechanism has been considered to involve specific inhibitory protein-protein interactions; however, it is also possible that active repression is accomplished without direct interaction with any components of the transcription machinery, but rather by modification of them. With regard to this possibility, the recent report by Fraser et al. (38) seems to be instructive. They showed that TIF1α possesses intrinsic protein kinase activity responsible for autophosphorylation as well as for phosphorylation of TFIIEα, TAFI128, and TAF1I55. Although functional involvement of such phosphorylation in transcriptional repression and whether KAP-1/TIF1β also has similar activity should be further studied, it is intriguing that KRAB/KAP-1-mediated repression can be regulated through phosphorylation of specific components in the basal transcription complex. It should also be noted that active direct repression and repression by chromatin reorganization are not mutually exclusive (15). The possible existence of multiple repression modules in KAP-1 (this study) and TIF1α (17) might imply repression through both mechanisms.

It was recently proposed that TIF1α and KAP-1/TIF1β constitute a new family of transcriptional intermediary factors (TIFs) that might play a dual role in the control of transcription at the chromatin level (17), because TIF1α was originally identified as a protein enhancing transcription by the nuclear receptor in yeast (24), and KAP-1/TIF1β was recently reported to activate transcription by glucocorticoid receptor and C/EBPβ (39), also because they share common properties not only to repress transcription when targeted to the promoter but also to interact with mHP1α/MOD1 and with the KRAB repressor domains. Therefore, it seems possible that TIFs might function as corepressors or coactivators in a way dependent on the
chromatin status, the promoter context, or the combination of certain types of transcription factors that bring them to the promoter.

It is clearly necessary to elucidate the mechanism(s) and identify the molecular target(s) in KRAB/KAP-1-mediated repression. In addition, it is also important to investigate the biological significance of KRAB/KAP-1-mediated repression. In the mammalian two-hybrid assay, we clearly demonstrated that repression of transcription by GAL4-KRAB can be converted to strong activation when coexpressed with the VP16-fused KAP1 deletions that contain the KRAB-interacting domain but no repression activity, suggesting that these VP16-KAP1 deletions would function as dominant negative mutants with regard to the physiological function of KRAB-ZFPs and/or KAP-1. Therefore, functional investigation using these deletions as a molecular probe in mammalian cells as well as in transgenic animals will facilitate the elucidation of the physiological importance of transcriptional repression by the large class of KRAB-ZFPs and their corepressor KAP-1.

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