Conserved interaction between distinct Krüppel-associated box domains and the transcriptional intermediary factor 1 β

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The Krüppel-associated box (KRAB) domain, originally identified as a 75-aa sequence present in numerous Krüppel-type zinc-finger proteins, is a potent DNA-binding-dependent transcriptional repression domain that is believed to function through interaction with the transcriptional intermediary factor 1 (TIF1) β. On the basis of sequence comparison and phylogenetic analysis, we have recently defined three distinct subfamilies of KRAB domains. In the present study, individual members of each subfamily were tested for transcriptional repression and interaction with TIF1β and two other closely related family members (TIF1α and TIF1γ). All KRAB variants were shown, (*i*) to repress transcription when targeted to DNA through fusion to a heterologous DNA-binding domain in mammalian cells, and (*ii*) to interact specifically with TIF1β, but not with TIF1α or TIF1γ. Taken together, these results implicate TIF1β as a common transcriptional corepressor for the three distinct subfamilies of KRAB zinc-finger proteins and suggest a high degree of conservation in the molecular mechanism underlying their transcriptional repression activity.

The Krüppel-associated box (KRAB) domain is one of the most potent and widely distributed transcriptional repression domains yet identified in mammals (1, 2); it has been estimated that approximately one-third of the 300 to 700 human zinc-finger proteins (ZFPs) of the Krüppel Cys_2His_2-type contain a KRAB domain in their N termini (3). This regulatory domain consists of ≈75 amino acids and is composed of two contiguous modules, the KRAB-A box and the KRAB-B box (3), each encoded by separate exons (4, 5). When fused to a heterologous DNA-binding domain (DBD), the KRAB-A box silences both basal and activated transcription in transfected cells in a dose-dependent manner and over large distances (1, 2, 6, 7). This transcriptional silencing has recently been linked at a molecular level to chromatin remodeling through the demonstration of a physical association between several different KRAB domains and the transcriptional intermediary factor 1 (TIF1) β, a transcriptional corepressor involved in heterochromatin-mediated regulation (8–10). TIF1β, also named KAP-1 (11) or KRIP-1 (12), was demonstrated to interact with numerous KRAB domains but not KRAB mutants deficient in repression, to enhance KRAB-mediated repression, and to silence transcription when directly tethered to DNA (11–13). This silencing activity requires histone deacetylation (9) and may result from the recruitment of a histone deacetylase complex, called N-CoR-1 (14), and/or from an association of TIF1β with members of the heterochromatin protein 1 (HP1) family (9, 10), a class of nonhistone proteins with a well-established epigenetic gene silencing function (for review, see ref. 15).

TIF1β is a member of a family of proteins (8) that also includes TIF1α, a putative nuclear receptor cofactor (8, 16, 17), and TIF1γ, whose function is unknown (18). These proteins are defined by the presence of two conserved amino acid regions: an N-terminal RBCC (RING finger, B boxes, coiled-coil) motif that is involved in KRAB-TIF1β association (19), and a C-terminal region containing a PHD finger and a bromodomain, two characteristic motifs of nuclear proteins known to function at the chromatin level (20–22). Whereas no interaction has been described between TIF1γ and KRAB domains, a yeast two-hybrid interaction was reported between TIF1α and the KRAB domain of the human KOX1 protein (13, 18), suggesting possible cross-talk between KRAB-ZFPs and nuclear receptors (23).

Although more than 10 independent KRAB domain proteins, such as human KOX1 (13), ZNF133 and ZNF140 (ref. 11 and refs. therein), mouse KRAZ1 and KRAZ2 (24), and rat Kid-1 (12), have been shown to interact with TIF1β, almost all of these interaction studies have been performed by using KRAB domains belonging to the AB subfamily. In addition to this subfamily, human and murine genomes contain two other distantly related subfamilies: one carrying the classical KRAB-A box and a highly divergent KRAB-B box, named b, and another containing the classical A box only (25). In the present study, individual members of each KRAB subfamily were tested for transcriptional repression and interaction with members of the TIF1 gene family. Similar to the KRAB(AB) domains, KRAB(Ab) and KRAB(A) variants repress transcription when targeted to DNA through fusion to a heterologous DNA-binding domain in mammalian cells. However, in contrast to the KRAB-B box, the KRAB-b box or the spacer region between the KRAB-A box of the A subfamily and the zinc-finger region does not contribute to the repression activity of the KRAB domain. By using two-hybrid interaction assays, all KRAB domains tested were shown to interact with TIF1β, but not with TIF1α or TIF1γ, suggesting that TIF1β may serve a general role in corepressing transcription by the three distinct subfamilies of KRAB-ZFPs.

Materials and Methods

Plasmids. KRAB cDNA inserts used in this study (see Fig. 1) were all obtained by PCR. MZF22 and MZF13 were previously isolated by screening a cDNA library from the mouse monocytic cell line WEHI-274 (25). HZF4 and 6D were isolated from a human myelomonocytic U-937 cDNA library and a mouse testis

Abbreviations: KRAB, Krüppel-associated box; ZFP, zinc-finger protein; TIF1β, transcriptional intermediary factor 1β; ER, estrogen receptor; ERE, ER element; CAT, chloramphenicol acetyltransferase; AAD, acidic activation domain; OMPdecase, orotidine 5′-monophosphate decarboxylase.

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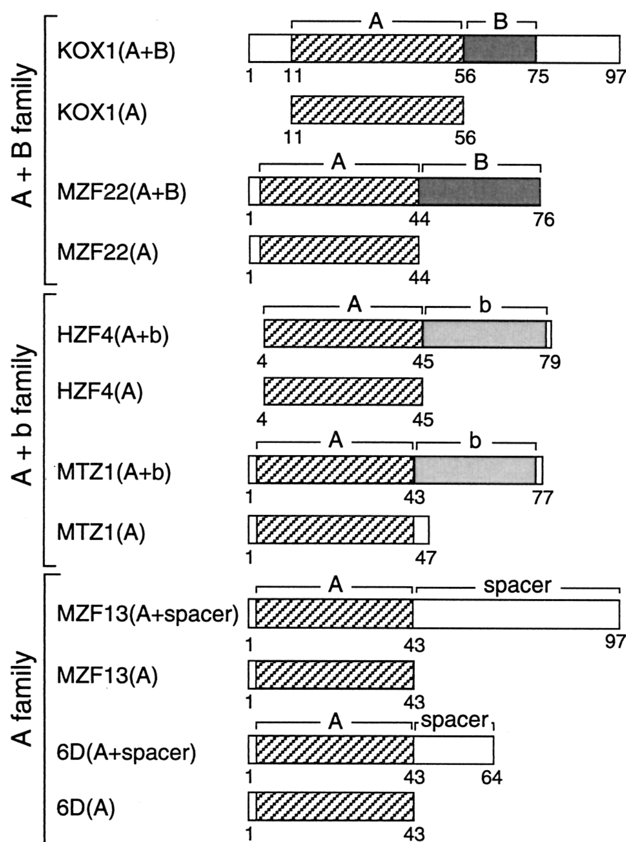

Fig. 1. Schematic representation of the various KRAB domains tested for transcriptional repression and interaction with TIF1 family members. The KRAB A motif is indicated by a striped box. The B motif and the more divergent b motif are depicted by boxes of different shades of gray. Numbers refer to amino acid positions.

cDNA library, respectively (ref. 26; unpublished work). TIF1 cDNAs used in this study correspond to human TIF1γ and mouse TIF1α and TIF1β (European Molecular Biology Data Bank; refs. 8, 16, 18). For transient transfection studies in mammalian cells, the indicated cDNAs were cloned into pG4MpolyII (8). The chimeric protein estrogen receptor (ER)(C)-VP16, which encodes amino acids 176–280 of ERα and amino acids 413–490 of VP16, has been described previously, as well as the reporter gene 17M2-ER element (ERE)-G-chloramphenicol acetyltransferase (CAT) (8). For yeast two-hybrid assays, DBD and acidic activation domain (AAD) fusion proteins were expressed from the yeast multicopy plasmids pBL1 and pASV3, respectively (27). These plasmids express inserts under the control of the phosphoglycerate kinase promoter. pBL1 contains the HIS3 marker and directs the synthesis of epitope (region F of ERα)-tagged ERα DBD fusion proteins. pASV3 contains the LEU2 marker and a cassette expressing a nuclear localized VP16 AAD, preceding a polylinker and stop codons in all reading frames. Details of individual plasmid constructs, which were all verified by sequencing, are available on request.

Transient Transfections. Transient transfections in COS-1 cells as well as CAT and β-galactosidase assays were performed as described previously (8).

Two-Hybrid Interaction Assays. Yeast cells grown in yeast extract/peptone/dextrose or selective medium were transformed by the lithium acetate procedure. Yeast PL3 (Matα *ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::3ERE-URA3*; ref. 27) transformants were grown exponentially for about five generations in selective medium supplemented with uracil. Yeast extracts were prepared and assayed for orotidine 5′-monophosphate decarboxylase (OMPdecase) activity as described previously (27).

Antibodies. mAbs used include: (*i*) anti-GAL4 (1–147), 2GV3 (8); (*ii*) anti-VP16 mAb, 2GV4 (27); and (*iii*) anti-ERα F mAb, F3, raised against the F region of human ERα (27).

Results

Transcriptional Repression Activity of KRAB(AB), KRAB(Ab), and KRAB(A) Domains. Individual members of the three KRAB domain subfamilies, MZF22(AB), HZF4(Ab), MTZ1(Ab), MZF13(A), and 6D(A) (Fig. 1; see also *Materials and Methods*) were analyzed for transcriptional repression when recruited to promoter regions in mammalian cells. GAL4 DBD fusion proteins containing the A box of each KRAB domain, GAL4-MZF22(A), GAL4-HZF4(A), GAL4-MTZ1(A), GAL4-MZF13(A), and GAL4–6D(A) were generated and transfected into COS-1 cells together with a GAL4 reporter containing two GAL4-binding sites (17M2) and an ERE in front of a β-globin (G) promoter-CAT fusion (17M2-ERE-G-CAT; ref. 8). For comparison, assays were also performed with a GAL4 fusion containing the A box derived from the KRAB(AB) domain of the human KOX1 protein [GAL4-KOX1(A); ref. 1]. As shown in Fig. 2 *A–F*, all GAL4-A box fusions led to a dose-dependent repression of the reporter compared with GAL4 alone. A $\approx$10- to 20-fold repression was reproducibly observed with saturating concentrations of GAL4 expression vectors containing the A boxes derived from the KRAB(AB) domains of KOX1 and MZF22 (Fig. 2 *A* and *B*). Under similar conditions, GAL4-A box fusions containing the A boxes from the HZF4 and MTZ1 KRAB(Ab) domains repressed by $\approx$10- to 80-fold (Fig. 2 *C* and *D*, respectively), whereas a 10-fold repression was observed in the presence of the GAL4-A box fusions derived from the MZF13 and 6D KRAB(A) domains (Fig. 2 *E* and *F*, respectively). Thus, similar to the A box of KRAB(AB) domains (1, 2, 6), the A boxes of KRAB(Ab) and KRAB(A) domains have a potent transcriptional repression activity. We also investigated the ability of each GAL4-A box fusion protein to repress VP16-mediated activation of the 17M2-ERE-G-CAT reporter. As shown in Fig. 3, the presence of the ER(C)-VP16 expression plasmid produced a 25-fold stimulation of reporter activity, which was completely abolished by the various GAL4-A box proteins.

The B box has previously been reported to increase the repression activity of the A box (28, 29). Therefore, we tested the transcriptional activity of GAL4-KRAB fusions in which the A boxes of MZF22, MZF4 and MTZ1 were fused to their respective B and b boxes, whereas the A boxes of MZF13 and 6D were fused to their own spacer regions, including the amino acid sequences between the A box and the first zinc-finger motif. Western blot analysis by using an antibody against the GAL4 DBD indicated similar expression levels for all fusion proteins (Fig. 2 legend). As expected, the GAL4-KOX1(A+B) fusion repressed transcription more efficiently than the GAL4 fusion containing the KRAB A box alone (45- vs. 10-fold repression, respectively; Fig. 2*A*). An increase in repression activity was also observed on addition of the MZF22 KRAB-B box to the MZF22 KRAB-A box (Fig. 2*B*). In contrast, addition of the HZF4 and MTZ1 KRAB-b boxes to their respective A box did not significantly change the repression activity of the corresponding fusion proteins [compare GAL4-HZF4(A+b) and GAL4-MTZ1 (A+b) to GAL4-HZF4(A) and GAL4-MTZ1(A), respectively; Fig. 2 *C* and *D*]. Similarly, no effect was observed by adding the spacer regions of MZF13 and 6D to their

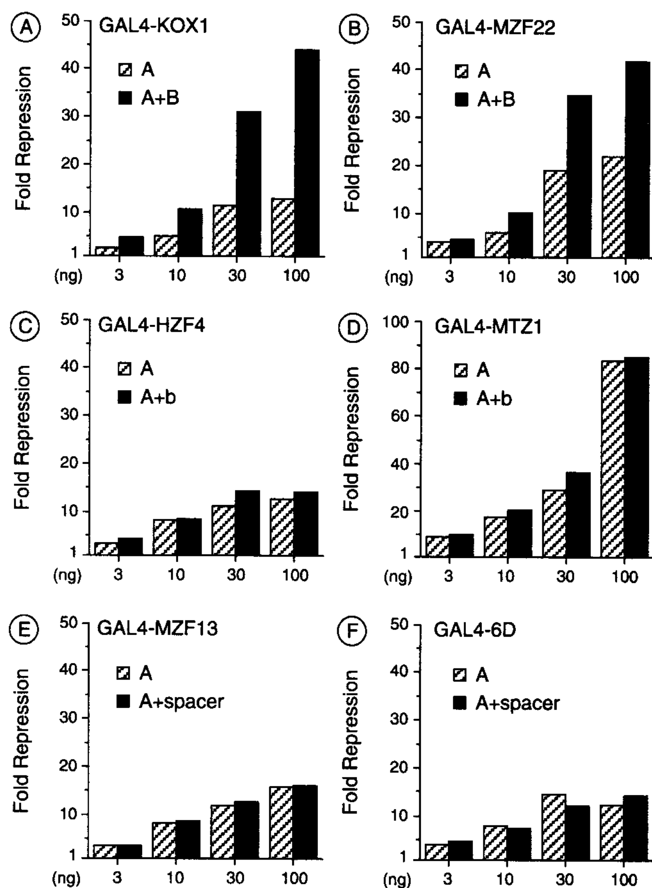

Fig. 2. Transrepressing properties of different KRAB domains. (*A–F*) Increasing amounts of the indicated GAL4 expression plasmids (3, 10, 30, or 100 ng) were transiently transfected into COS-1 cells with 1 μg 17M2-ERE-G-CAT reporter and 1 μg pCH110 (expressing β-galactosidase). The fold repression of each construct was determined by measuring relative CAT activity by using the unfused GAL4 expression vector as a standard. Values (±10%) represent the averages of three independent duplicated transfections after normalization to the internal control β-galactosidase activity of pCH110. Expression of the fusion proteins was confirmed by Western blot by using the antibody 2GV3 against the GAL4 DBD (data not shown).

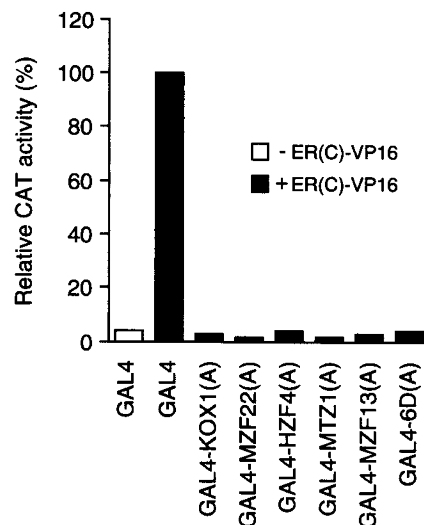

Fig. 3. Repression of VP16 activation by the A boxes of various KRAB(AB), KRAB(Ab) and KRAB(A) domains. 17M2-ERE-G-CAT (1 μg) and pCH110 (1 μg) were cotransfected into COS-1 cells with ER(C)-VP16 (100 ng) together with 250 ng of each GAL4-KRAB(A) fusion protein as indicated. CAT activities are expressed relative to the CAT activity measured in the presence of the unfused GAL4 expression vector (taken as 100%). Values (±10%) represent the averages of three independent duplicated transfections after normalization to β-galactosidase activities.

respective A box [compare GAL4-MZF13(A+spacer) and GAL4–6D(A+spacer) to GAL4-MZF13(A) and GAL4–6D(A), respectively; Fig. 2 *E* and *F*]. Thus, in contrast to the B box, neither the b box nor the spacer region C-terminal to the A box of KRAB(A) domains contribute to the repression activity of the KRAB domain.

Interaction of KRAB(AB), KRAB(Ab), and KRAB(A) Domains with Members of the TIF1 Protein Family. Numerous KRAB domains belonging to the KRAB(AB) subfamily have been shown to interact with the transcriptional corepressor TIF1β in yeast, in mammalian cells as well as *in vitro* (11–13, 24). A yeast two-hybrid interaction was also reported between the KRAB(AB) domain of KOX1 and TIF1α, a TIF1β-related protein (13, 18). The yeast two-hybrid system was therefore used to examine whether KRAB(Ab) and KRAB(A) can interact with TIF1 family members. DBD chimeric proteins consisting of the ERα DBD (residues 176–282; Fig. 4*A*) fused to the A box of each KRAB domain, DBD-KOX1(A), DBD-MZF22(A), DBD-HZF4(A), DBD-MTZ1(A), DBD-MZF13(A), and DBD-6D(A), were coexpressed with the fusion proteins between the AAD of the VP16 protein and any one of the TIF1 family members (AAD-TIF1α, AAD-TIF1β, and AAD-TIF1γ; Fig. 4*A*), in the yeast reporter strain PL3 containing an URA3 reporter gene controlled by three EREs (see Fig. 4*A* and ref. 27). Activation of the reporter was determined by measuring the OMPdecase activity of the URA3 gene product (Fig. 4 *B–G*). When coexpressed with the AAD control, DBD-HZF4(A), but not the other DBD-KRAB(A) fusion proteins, activated the reporter gene above the level of unfused DBD (Fig. 4 *B–G*), indicating that the A box of HZF4 contains an amino acid sequence that can transactivate in yeast but not in mammalian cells (see Fig. 2*C*), at least under the present experimental conditions (cell lines/reporter constructs). No significant increase in OMPdecase activity above the AAD control was detected when the various DBD-KRAB(A) fusion proteins were coexpressed with either AAD-TIF1α or AAD-TIF1γ, whereas under the same conditions, a reporter activation was observed in the presence of AAD-TIF1β (Fig. 4 *B–G*). Thus, KRAB(Ab) and KRAB(A) domains contain A boxes, which, like those of KRAB(AB) domains, are capable of mediating a specific interaction with TIF1β.

DBD-KRAB fusions containing the A box fused to their respective B, b, or spacer region, DBD-KOX1(A+B), DBD-MZF22(A+B), DBD-HZF4(A+b), DBD-MTZ1(A+b), DBD-MZ31(A+spacer), and DBD-6D(A+spacer), were also examined for interaction with the TIF1 family proteins in yeast cells. As observed with DBD-HZF4(A), coexpression of DBD-HZF4(A+b) with unfused AAD transactivated the reporter gene over background levels (Fig. 4*D*). Similar reporter activation was also observed with the DBD fusion including the 6D spacer region [DBD-6D(A+spacer) + AAD; Fig. 4*G*], but not with the other DBD-KRAB chimera tested (Fig. 4 *B*, *C*, *E*, and *F*), indicating that the 6D spacer region may contain an autonomous transactivation function. As previously reported (18), the whole KRAB(AB) domain of KOX1 interacted with both TIF1α and TIF1β, but not TIF1γ [see DBD-KOX1(A+B) + AAD-TIF1α/β/γ; Fig. 4*B*]. Note, however, the modest (≈4-fold) increase in OMPdecase activity in the presence of AAD-TIF1α

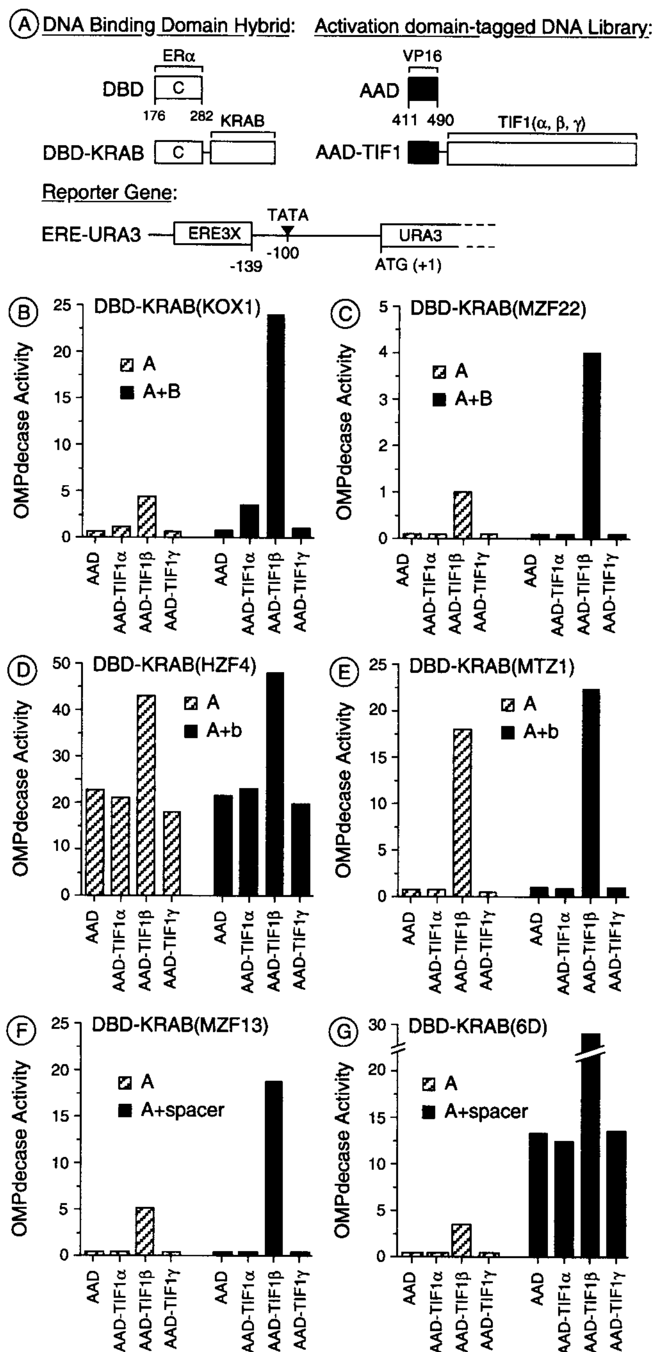

Fig. 4. Yeast two-hybrid analysis of the interaction between different KRAB domains and the TIF1 family proteins. (*A*) Schematic representation of the yeast two-hybrid system used in this study. The DBD of the ERα (amino acids 176–282) and the acidic activation domain (AAD) of VP16 (amino acids 411–490) unfused or fused to the proteins tested for interaction (white boxes) are shown. The URA3 reporter gene, which is regulated by three estrogen response elements (ERE3X) in the yeast reporter strain PL3, is represented below. (*B–G*) Selective interaction of KRAB(AB), KRAB(Ab), and KRAB(A) domains with TIF1β. Plasmids expressing the indicated DBD-KRAB fusions were introduced into PL3 together with either the VP16 AAD (as a control) or the VP16 AAD fused to TIF1α, TIF1β, or TIF1γ. OMPdecase activities determined for each cell-free extract are expressed in nanomolar substrate/minute/milligram protein. The values (±20%) represent the averages of at least three independent experiments. Note that expression of all fusion proteins was confirmed by Western blotting by using antibodies F3 against the F region tag of the ERα DBD and 2GV4 against VP16, respectively (data not shown).

as compared with the 40-fold activation obtained with AAD-TIF1β (Fig. 4*B*). Interestingly, this enhancement was approximately 5-fold higher than that observed by coexpressing AAD-TIF1β with DBD-KOX1(A) (Fig. 4*B*), suggesting that the interaction of TIF1β with KOX1(A+B) may be of higher affinity than that with KOX1(A). As shown in Fig. 4 *C–G*, MZF22(A+B), HZF4(A+b), MTZ1(A + b), MZF13(A+spacer), and 6D(A+spacer) interacted with TIF1β, but not with TIF1α or TIF1γ. As observed with KOX1, DBD-MZF22(A+B) in combination with AAD-TIF1β activated the reporter gene more efficiently than DBD-MZF22(A) tested under the same conditions (Fig. 4*C*)]. Similarly, higher levels of reporter activation were obtained on addition of the spacer region of MZF13 to the MZF13 A box [compare DBD-MZ13(A+spacer) to DBD-MZ13(A); Fig. 4*F*]. In contrast, DBD-MTZ1(A) and DBD-MTZ1(A+b) both activated the reporter gene to similar extents when coexpressed with AAD-TIF1β (Fig. 4*E*). Thus, the B box and the C-flanking region of the A box of the A subfamily, but not the b box, might contribute to the interaction with TIF1β.

Discussion

In addition to the classical KRAB(AB) domains, human and murine genomes contain KRAB(Ab) and KRAB(A) domains (25). Here, we present evidence that the A boxes of these KRAB variants, although distantly related (25), exhibit functional similarity. They all repress transcription when targeted to DNA through fusion to a heterologous DBD in mammalian cells, and they all interact with the transcriptional corepressor TIF1β. The interaction is highly specific, as evidenced by the lack of binding to the related proteins TIF1α and TIF1γ. Thus, although a two-hybrid interaction has previously been reported between the whole KRAB(AB) domain of the human KOX1 protein and TIF1α (13, 18), TIF1β may represent the only member of the TIF1 gene family to be involved in KRAB-mediated repression. Additional support for this specificity of action is the recent finding that mice lacking TIF1β are defective in early postimplantation development (30), which implies that, at least during early embryogenesis, the members of the TIF1 family, although structurally related, exert distinct nonredundant functions. The precise mechanism by which TIF1β may mediate the repression function of KRAB domains has not yet been elucidated. However, several lines of evidence argue for an epigenetic mechanism involving both heterochromatin-binding proteins and histone deacetylases. TIF1β is known to be associated with members of the HP1 family (9, 10), to which it binds directly through a conserved motif (8, 9). HP1 proteins are nonhistone chromosomal proteins associated primarily with pericentromeric heterochromatin, where they are believed to function as regulators of heterochromatin assembly and silencing (reviewed in ref. 15). Moreover, TIF1β was also reported to be an integral component of a histone deacetylase complex (14) and to possess an autonomous silencing function that requires not only HP1 binding but also histone deacetylation (9). Taken together, these results and our present data suggest a conserved mechanism of repression for all KRAB domains, including KRAB(Ab) and KRAB(A) variants, which, through the recruitment of TIF1β and its partners, may induce deacetylation and assembly of heterochromatin-like complexes at specific sites within the genome.

Little is known about the function of the conserved B box. Despite an apparent lack of transcriptional activity, the B box was shown to increase the repression activity of the A box (28, 29). Here, we report two-hybrid interaction data, which, although qualitative, correlate well with the transcriptional repression data. Both KOX1 and MZF22 KRAB(AB) domains exhibited strong repression activity in mammalian cells and similarly interacted with TIF1β more efficiently than their respective A box in yeast cells. Furthermore, our results show

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that, in contrast to the B box, the highly divergent b box does not contribute to the repression activity of the KRAB domain in mammalian cells and does not enhance interaction with TIF1β in yeast. These data indicate that both KRAB(AB) and KRAB(Ab) domains may rely largely on their interaction with TIF1β for their silencing activity. In contrast, no correlation was observed between the transcriptional repression activities of MZF13(A) and MZF13(A+spacer) and their respective TIF1β-binding activities. Both MZF13(A) and MZF13(A+spacer) repressed transcription with similar potency, whereas the interaction of TIF1β with MZF13(A+spacer) appeared more potent than that with MZF13(A). Although how the spacer region of MZF13 may modulate TIF1β interaction in yeast cells remains unclear, our results raise the interesting possibility that this region may exert a cell-specific effect on TIF1β binding. Thus, the recruitment of TIF1β to individual KRAB-ZFPs *in vivo* may depend not only on the A box, but also on the C-flanking region.

The physiological functions of KRAB-ZFPs are at present unknown. However, these potential DNA-binding transcriptional repressors may play an important role in regulating expression of specific genes during cell differentiation and development, as strongly suggested by their temporally and spatially regulated expression patterns (see ref. 25 and refs. therein) and by the finding that TIF1β is essential for early embryogenesis (30). Further molecular and genetic studies should allow us to elucidate the *in vivo* functions of this large family of KRAB-ZFPs and determine which interactions and partners are relevant for these functions.

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