

Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins

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Abstract We report the characterization of three novel members of the KRAB-domain-containing C₂-H₂ zinc finger family (ZNF133, 136 and 140). KRAB (*Krüppel*-associated box) is an evolutionarily conserved protein domain found N-terminally with respect to the zinc finger repeats that encodes the DNA binding domain. ZNF133 and ZNF140 have both the KRAB A- and KRAB B-boxes present at their N-terminus, whereas ZNF136 contains only the KRAB A-box. We have previously demonstrated that the KRAB domains derived from ZNF133 and ZNF140 are potent transcriptional repression domains [Margolin et al. (1994) Proc. Natl. Acad. Sci. USA 91, 4509–4513]. The KRAB domain from ZNF136, containing only subdomain A, is a considerable weaker suppression domain; however, when fused to the heterologous KRAB B subdomain of ZNF10 (KOX1) the two subdomains form a KRAB domain which induces repression as potently as previously reported KRAB domains.

Key words: Zinc finger protein domain; KRAB domain; DNA-protein interaction; GAL4 fusion protein; Transcriptional repression

1. Introduction

Differential regulation of gene expression in eukaryotes is mediated in part by specific binding of transcription factors to DNA sequences. DNA binding proteins can be classified according to the conserved structural motifs they have in common and more than 10 families with distinct DNA binding motifs have been identified [1]. One such motif is the C₂-H₂ (or *Küppel*) type zinc finger repeat first described in *Xenopus* transcription factor IIIA (TFIIIA) [2]. Since then this motif has been found in many proteins with transcriptional regulatory functions e.g., in transcription factor Sp1 and yeast regulatory gene GAL4, in the *Drosophila* *Krüppel* and *hunchback* segmentation genes [3,4], in the Wilms' tumor (WT1) suppressor gene [5] and in Gli-3 which is implicated in Grieg syndrome [6].

Zinc finger proteins (ZFPs) of the C₂-H₂ type consist of tandem arrays of two or more zinc fingers. The basic structural unit is composed of typically 28–30 amino acids with a consensus sequence of CX₂₋₄CX₃FX₅LX₂HX₃₋₄H (X is any amino acid), where the two pairs of cysteines and histidines tetrahedrally coordinate a zinc ion [2]. Each repeated finger unit is

separated by the highly conserved stretch of 7 amino acids TGEKPYX (H/C link) which extends from the last histidine of one finger unit to the first cysteine of the next unit [7] and may play a role in aligning the finger domains within the major groove of the DNA helix [8]. The H/C link consensus sequence has been used to isolate C₂-H₂ type zinc finger genes from *Xenopus* [9], mouse [10], and human [11,12].

It has been estimated that there are 300–700 different ZFP genes in the human genome [11], and similarly high numbers have been found in *Xenopus* [13] and mouse [14]. Many ZFP genes are clustered within the human genome and zinc finger loci have been found to reside predominantly either in telomeric regions or in chromosomal bands known to exhibit chromosome fragility [15–17]. Chromosome 19 carries a disproportionate fraction of the zinc finger genes mapped so far, in particular, a gene complex containing over 40 genes has been identified in chromosomal region 19p12-p13.1 [18]. This cluster consists of KRAB (*Krüppel*-associated box) zinc finger proteins (KRAB-ZFPs) that constitutes a subfamily of up to one third of the C₂-H₂ type ZFPs [19]. The KRAB domain is an evolutionarily conserved domain of about 75 amino acids found N-terminal with respect to the finger domain, and is composed of two contiguous modules: KRAB A- and KRAB B-box [19–22]. The KRAB-ZFPs exhibit a common exon/intron organization with the variant zinc finger repeats present in a single exon and the KRAB-A and -B domain encoded by separate exons [18,21]. The exon/intron structure suggests that isoforms of KRAB-ZFPs with the domain absent or partially deleted could arise by alternative splicing, thus increasing the number of distinct expressed ZFPs.

We have previously characterized a KRAB-ZFP (ZNF141) that maps within the minimal chromosomal region for the Wolf-Hirschhorn syndrome (WHS) at 4p16.3 [23], and have demonstrated that the KRAB domain from ZNF141 is a potent transcriptional repressor [24]. Here we report the isolation and functional characterization of three additional KRAB-ZFPs and show that independent KRAB A and B subdomains can be fused to generate a functional KRAB domain with enhanced repressor activity in comparison to subdomains alone.

2. Materials and methods

2.1. Construction and screening of cDNA library

Total RNA was isolated [25] from a human insulinoma and enriched for mRNA by oligo-(dT) cellulose chromatography [26]. A cDNA plasmid library was constructed from 5 µg mRNA essentially as previously described [27]. After *Bst*XI linker (Invitrogen, San Diego, CA) addition to the double stranded cDNA, a size fractionation was performed on an 0.8% agarose gel to generate two size classes of cDNA: 0.5 to 2 kb and 2 to 10 kb. The cDNA was electroeluted in a dialysis

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Genbank accession numbers: ZNF 133, # U09366; ZNF 136, # U09367; ZNF 140, # U09368.

bag and purified by phenol and chloroform extractions prior to ethanol precipitation.

A modified cDNA cloning vector, pLN86, was constructed from the pVEGT⁺ plasmid [27] by expanding the multiple cloning site with a BstXI recognition site, and the size selected cDNA fractions were ligated into this vector and electrotransformed into ElectroMAX DH10B cells (Life Technologies, Gaithersburg, MD) as described by the supplier. The fraction of the cDNA library with the longer inserts contained 0.2×10^6 clones. Upon plating on LB-agar plates, the colonies were washed off the plates (approximately 10,000 colonies/ml LB-broth, 15% glycerol) and frozen at -80°C for storage.

The high molecular weight library was plated on nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) and hybridized to a radiolabelled degenerate oligonucleotide CA(CT)AC(AG)GG(AGT)-GA(AG)CC(ACT)TA recognizing the HTGKPY linker region of the C₂-H₂ type zinc finger. The hybridization was done at 37°C overnight in 6×SSC, 1% SDS, 10×Denhardt, 10 mM EDTA, 150 μg/ml poly A, 50 μg yeast RNA. The filters were washed 3 times in 6×SSC at 44°C before exposure. One hundred positive colonies were isolated from which 20 were randomly selected. Plasmid DNA was prepared using Qiagen columns (Qiagen Inc., Chatsworth, CA) for further analysis.

2.2. DNA sequencing and analysis

Primer walking DNA sequencing was performed on an A.L.F. sequencer (Pharmacia, Uppsala, Sweden) using fluorescein labelled sequencing primers synthesized on a 394 Applied Biosystem DNA synthesizer (Applied Biosystems, Foster City, CA). Sequence reactions were carried out using the AutoRead Sequence Kit (Pharmacia, Uppsala, Sweden) according to the supplier. Sequences were compiled and analysed using the Sequence Analysis Software Package (version 7) from the Wisconsin Genetic Computer Group.

2.3. Reporter vectors for mammalian cells

The modified luciferase containing plasmids were derived from pGL2-Control (Stratagene), in which the luciferase gene is driven by SV40 enhancer and SV40 promoter elements. In the plasmid 5'-GAL4(+) pGL2-Control, 5 synthetic GAL4 DNA binding sites were cloned in clockwise orientation in the BamHI restriction site at nucleotide position 37.

2.4. Expression vectors

Construction of the GAL4-containing fusion genes were performed using the expression plasmids pM1 and pM2 [28]. This plasmid contains the SV40 early promoter and origin of replication, driving expression of the DNA binding domain of GAL4 (1–147). A polylinker inserted into the gene encoding the carboxy terminus of GAL4 (1–147) allows in-frame cloning and expression of GAL4 fusion proteins. Plasmids containing GAL4-ZNF10, -ZNF133, -ZNF140, -ZNF141 have previously been described [24]. The GAL4 expression plasmid pM1 encoding the KRAB A subdomain of ZNF136, and pM2 encoding the KRAB B subdomain of ZNF10 (KOX1), were constructed via polymerase chain reaction (PCR) amplification of the desired NH₂-terminal KRAB-containing regions using synthetic oligonucleotide primers. The 5' PCR primers contained BglII restriction sites (5' AATACAGATC-TATGGACTCGGTGGCTTTTGGAG for ZNF136; 5' TAAAGATC-TGGTTATCAGCTTACTAAGCC for ZNF10) and the 3' PCR primers contained a BamHI site (5' TCAATGGATCCGGGATTTTCTT-GCTCAGATTCTGA for ZNF136; 5' TCAATGGATCCAAATGC-AGTCTCTGAATCAGG). Standard PCR reactions using Pfu polymerase (Stratagene) were performed using the indicated zinc finger cDNAs as templates. The PCR fragments were isolated from a 6% polyacrylamide gel, cloned and sequenced.

The chimeric KRAB domain between the A subdomain of ZNF136 and the B subdomain of ZNF10 was constructed by making use of PCR based site directed mutagenesis [29]. Two PCR reactions were run, one with the cDNA of ZNF136, the other with the cDNA of ZNF10. The PCR sample of ZNF136 included the 5' primer (5' AATACAGATC-TATGGACTCGGTGGCTTTTGGAG) that contained a BglII site and the 3' primer (5' GATAACCCTATAGAGGCCAGATTC) that is complementary at the 5' end to the 5' primer of the B subdomain. The PCR reaction mix of ZNF10 had a 5' primer (5' TCAATGGATCC-AAATGCAGTCTCTGAATCAGG) complementary at the 5' end to the 3' primer of ZNF136. The 3' primer (5' CTGGCCTCTATAGG-GTTATCAGCTTACTAAG) contained a BamHI site. The complete

hybrid fragment was generated by performing a second PCR using the 5' primer of ZNF136 and the 3' primer of ZNF10. The PCR product was extracted with phenol/chloroform, digested with BamHI and BglII, gel purified and cloned into the BamHI site of pM1 to generate pG-hybrid.

Each fusion protein contained GAL4 (1–147) [30], followed by arginine, asparagine, glycine, serine and the indicated segment of the cDNAs. The methionine encoded at nt 71 in the ZNF10 cDNA [12] was designated as aa 1 for the ZNF10 containing constructs. The segments of the other cDNAs were similarly designated with the first in-frame encoded methionine named aa 1. The cDNAs were fused to GAL4 as follows: ZNF10 nt 71–343 (aa 1–91), nt 232–343 (aa 54–91), nt 71–229 (aa 1–53); ZNF133, nt 449–909 (aa 1–119); ZNF136, nt 72–359 (aa 1–96); ZNF140, nt 273–590 (aa 1–106); ZNF141, nt 157–591 (aa 1–145); ZNF136/ZNF10, nt 72–272/232–343 (aa 1–67/54–91). Sequences of all PCR derived constructs were verified by sequence analysis. The stable expression of chimeric GAL4 fusion protein was confirmed [24].

2.5. Liposome-mediated transfection

5×10^5 – 1×10^6 exponentially growing HeLa cells were plated in a 100 mm culture dish and grown in a CO₂ incubator at 37°C to 80% confluence. The cells were then transfected by using LIPOFECTIN reagent following the manufacturers protocol (Gibco-BRL, Gaithersburg, MD, USA). Each dish of sub-confluent HeLa cells received 5 μg reporter plasmid, 5 μg expression plasmid and 2 μg of the β-D-galactosidase expression plasmid pON260 [31]. 48–72 h after transfection β-D-galactosidase and luciferase activity were determined from the cell extract. The β-D-galactosidase activity was used to normalize the luciferase activity. Each transfection experiment was performed in duplicate three to four times, using at least three different preparations of plasmid DNA. The variation among experiments was 10–20%. There was no overall toxic effects (measured by reduction in cell number or β-D-galactosidase activity) as a result of expressing the GAL4 fusions in transient assays which could account for the repression observed.

2.6. Extraction of cells

Cells (1 – 3×10^6 cells) were collected 24–48 h after transfection and washed twice with PBS and lysed in 200 μl cell extract buffer [32]. Supernatants were obtained by centrifugation and frozen at -20°C . Protein content of the extracts were determined according to Bradford [33].

2.7. Luciferase assay

350 μl luciferase assay buffer [34] was mixed with 10 μl extract and the luciferase activity was determined in a Lumat LB9501 (Berthold, Wildbad, Germany) using intergral mode (10 s at 25°C). D-Luciferin was used at 1 mM.

3. Results

3.1. Isolation and sequencing analysis of KRAB-ZNFs

Zinc finger proteins of the C₂-H₂ type usually have conserved sequences with the TGEKPYX motif separating the tandemly arranged finger domains [11]. A degenerate oligonucleotide probe from this region was used to identify cDNAs with C₂-H₂-type finger domains from a human insulinoma cDNA library. Approximately one hundred positive clones were identified from which twenty were selected randomly for further analysis. Sequence analysis revealed that four cDNA clones belong to a subfamily, since in addition to the finger domain they contain a conserved N-terminal KRAB domain [19]. The characterisation of one of the cDNAs, ZNF141, has previously been reported [23]. The complete nucleotide sequence of the other cDNAs have been deposited in Genbank with the accession numbers, ZNF133: U09366, ZNF136: U09367, and ZNF140: U09368. The predicted amino acid sequences of the zinc finger domains in all three ZNFs conform to the general pattern CX₂CX₁₂HX₃H preceded by a conserved seven amino acid H/C link. ZNF133 contains an open reading frame of 1620

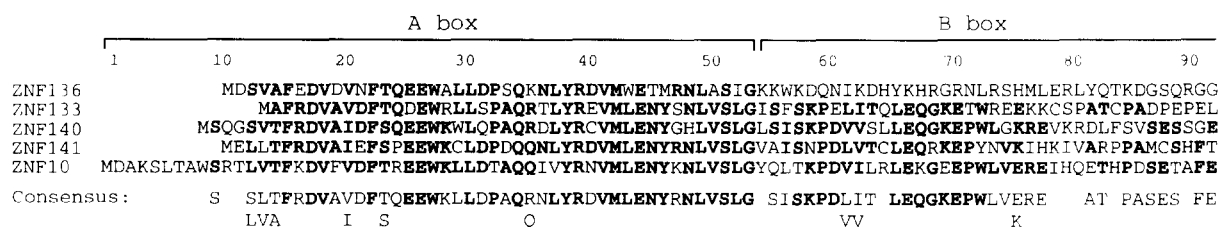


Fig. 1. Comparison of N-terminal KRAB domains from ZNF136, ZNF133, ZNF140, ZNF141 [23], and ZNF10[12]. Highly conserved amino acids are in bold and below is shown the derived consensus sequence from the five KRAB A domains and the four KRAB B domains.

nucleotides that encodes 13 contiguous zinc finger domains. The sequence preceding finger 1 is most likely a degenerate finger, since the amino acid at position 142 is a arginine instead of the predicted cysteine and thus will have destroyed the putative binding capacity. The first 65 amino acids defines the A- and B-box of a KRAB domain with strong homology to those previously characterised (Fig. 1). The predicted molecular weight of the ZNF133 protein is 62.7 kDa. The cDNA contains part of a repetitive ALU element in the 3' untranslated region from nucleotide 2372. A second cDNA, ZNF133a, was isolated which has an identical open reading frame as ZNF133 except at position 662, where the trinucleotide GCA encoding alanine⁷⁴ in the junction between the KRAB B subdomain and the finger domain is deleted. The 5' untranslated region of ZNF133a extends more than 1 kb and is different from ZNF133 (data not shown). The nucleotide sequence of ZNF140 predicts a protein of 457 amino acids with 10 contiguous zinc finger domains and a KRAB domain at the N-terminus (Fig. 1). The predicted molecular weight of ZNF140 is 52.0 kDa. ZNF136 encodes 14 contiguous zinc finger domains followed by one degenerate finger in which the last histidine at position 628 is replaced by a threonine (Fig. 2) and is therefore not expected participate in DNA binding. The KRAB domain in ZNF136 has only the A-box present (Fig. 1) at the N-terminal end. The predicted molecular weight of the protein is 73.4 kDa.

The first 300 base pairs of the cDNA is a repetitive ALU element. Multiple alignment of the three ZNFs peptide sequences reveals a 40-46% identity (50-55% nucleotide identity), whereas the KRAB A peptide domains are 50-70% identical. Northern blot analysis revealed an apparently ubiquitous expression pattern of all three ZNFs (expressed in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas - data not shown).

3.2. The KRAB domain of ZNF136 is a weak repressor

The N-terminal KRAB domains were fused to the GAL4 DNA binding domain and assayed quantitatively for transcriptional repressor activity using the luciferase reporter gene driven by the SV40 promoter in HeLa cells (Fig. 2A). The KRAB domains from ZNF133, ZNF 140 and ZNF10 [12] induce almost complete repression in comparison with GAL4 pG(1-174) alone, as previously described [24]. However, the KRAB domain from ZNF136 encoding the A-box alone only represses the activity to about 30 percent. A similar activity is observed by fusing the A-box from ZNF10 to GAL4 (ZNF10-A).

3.3. The KRAB B subdomain of KOX1 enhances the repressor activity of KRAB A subdomain of ZNF136

In order to assess whether heterologous KRAB A and B

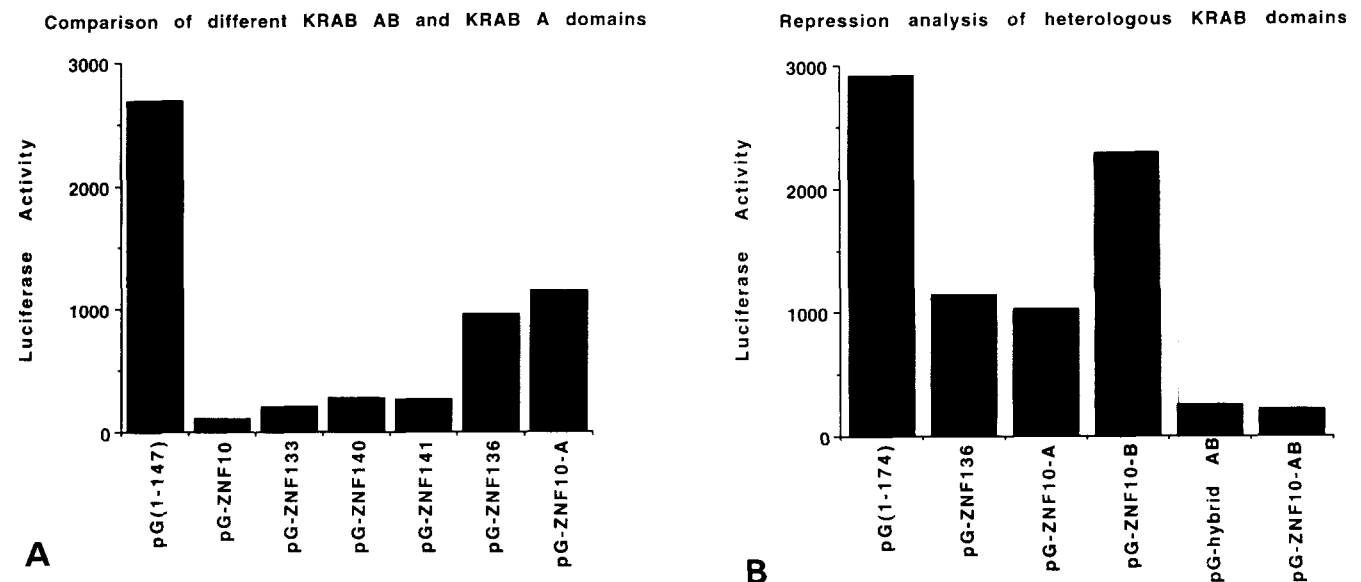


Fig. 2. Transcriptional repression by KRAB domains fused to the GAL4 protein. Transcriptional repressor activity was assayed with the luciferase reporter gene driven by the SV40 early promoter in the HeLa cells. (A) Comparison of different KRAB AB and KRAB A domains. (B) Repression analysis of heterologous KRAB domains.

subdomains are synergistic in their transcriptional repression or are only active in conjunction with their native associated subdomains, the B subdomain from ZNF10 and the KRAB domain of ZNF136 were fused to GAL4 (pG-hybrid). The introduction of a heterologous B subdomain to ZNF136 enhances the repressor activity to similar levels as seen with ZNF133, ZNF140 and the native ZNF10 (Fig. 2B). The ZNF10 KRAB B subdomain alone does not induce significant repression when compared to ZNF136 or ZNF10-A.

4. Discussion

Out of twenty randomly selected ZNF cDNAs we identified four novel ZNFs with KRAB domains in the N-terminal part of the predicted peptide. This finding corroborates the notion that a large fraction of ZNFs in the genome contains the highly conserved KRAB domains [19]. It has previously been shown that a subfamily of KRAB-ZNF genes has a clustered organization in the genome on chromosome segment 19p12 [18]. The KRAB-ZNFs presented here and ZNF141 map at different locations (ZNF133 - 20p11.2; ZNF136 - 19q13.1; ZNF140 - 12q34.33; ZNF141 - 4p16.3) [23,35] indicating a wide distribution of KRAB-ZNF genes in the genome. Whether these KRAB-ZNF genes are part of different gene clusters that are structurally related is not known. Despite a wide distribution in the genome the KRAB-ZNFs conform to the stringent CX₂CX₁₂HX₃HX₇ finger motif with up to 50% conserved amino acid sequence in the zinc finger domain and up to 70% in the KRAB domain.

The KRAB domains from different ZNFs are potent transcriptional repressors when fused to the GAL4 DNA binding domain [24,36,37], and it has been shown that the KRAB A subdomain is sufficient for the induction of transcriptional repression. However, when the repression is measured quantitatively the KRAB A subdomain alone (ZNF136) is a much weaker repressor than the entire KRAB domain (ZNF10, ZNF133, ZNF140 and ZNF141). The question arises whether the KRAB domains only induce complete repression with their native encoded subdomains or whether there is a synergistic effect when heterologous subdomains are fused together. To investigate this, the KRAB B subdomain from ZNF10 was added to the KRAB domain from ZNF136 (which only contains the A subdomain) and fused to GAL4 DNA binding domain. The introduction of a heterologous B subdomain to ZNF136 enhances the repressor activity to similar levels as is seen with ZNF133, ZNF140, ZNF141 and ZNF10 native KRAB domains. The KRAB B subdomain from ZNF10 does not alone induce significant repression demonstrating that the observed activity seen with ZNF136-ZNF10-B is not a simple additive effect from two independent A and B subdomains.

Immunoprecipitation of stably expressed ZNF10 KRAB domains fused to the tetracycline repressor revealed the association of an endogenous protein to the KRAB domain of ZNF10 [34]. This protein designated SMP1 (Silencing Mediating Protein 1) is approximately 110 kDa. It has been emphasized that SMP1 might constitute a putative co-repressor that mediates the repression activity employed by the KRAB domain of any KRAB containing zinc finger protein. In this respect, further investigations are in progress to clarify whether SMP1 binds to the other characterized KRAB domains. It seems likely that the KRAB A versus the KRAB AB domains have varying re-

pressor activities due to altered affinities between SMP1 and the KRAB domains. Alternatively, the repression could be mediated by additional co-repressor proteins that bind predominantly to the KRAB B subdomain.

So far, a KRAB B subdomain has not yet been identified in the genomic region of ZNF136. It has not yet been demonstrated that genomic zinc finger genes encoding both KRAB subdomains can express altered NH₂-terminal modified KRAB domains with different repressor activities. However, our analysis of differential repressor activities of KRAB A versus KRAB AB domains points in this direction.

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