Biochemical Analysis of the Kruppel-associated Box (KRAB) Transcriptional Repression Domain

SPECTRAL, KINETIC, AND STOICHIOMETRIC PROPERTIES OF THE KRAB-KAP-1 COMPLEX*

The Kruppel-associated box (KRAB) domain is a 75-amino acid transcriptional repressor module commonly found in eukaryotic zinc finger proteins. KRAB-mediated gene silencing requires binding to the RING-B box-coiled-coil domain of the corepressor KAP-1. Little is known about the biochemical properties of the KRAB domain or the KRAB-KAP-1 complex. Using purified components, a combination of biochemical and biophysical analyses has revealed that the KRAB domain from the KOX1 protein is predominantly a monomer and that the KAP-1 protein is predominantly a trimer in solution. The analyses of electrophoretic mobility shift assays, GST association assays, and plasmon resonance interaction data have indicated that the KRAB binding to KAP-1 is direct, highly specific, and high affinity. The optical biosensor data for the complex was fitted to a model of a one-binding step interaction with fast association and slow dissociation rates, with a calculated $K_d$ of 142 nM. The fitted $R_{\text{max}}$ indicated three molecules of KAP-1 binding to one molecule of the KRAB domain, a stoichiometry that is consistent with quantitative SDS-polyacrylamide gel electrophoresis analysis of the complex. These structural and dynamic parameters of the KRAB/KAP-1 interaction have implications for identifying downstream effectors of KAP-1 silencing and the de novo design of new repression domains.

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 either activate or repress transcriptional initiation (1–3). Much progress has been made in understanding how activation and repression domains of a DNA-bound protein transmit signals that modulate transcription via the basal transcriptional machinery. Both activation and repression domains may function by directly interacting with components of the basal transcriptional machinery to modulate transcription, or these domains function through cofactors that regulate transcription via a network of protein/protein interactions to regulate downstream targets (for review, see Refs. 2 and 4–10). The paradigm is now well established for coactivators and corepressors to function as bridging molecules between transcription factors and either the basal transcription apparatus or chromatin components, resulting in the regulation of target genes (11).

Modular transferable repression domains have emerged as a set of highly conserved structural motifs in families of transcription factors. These conserved repression domains include the BTB/POZ, WRPW, SNAG, SCAN, and Kruppel-associated box (KRAB)1 (12–16). We have focused on the KRAB domain as a model system for the analysis of repression modules (17–19) (Fig. 1). The KRAB domain was originally identified as a conserved motif at the NH$_2$ terminus of zinc finger proteins (ZFPs) (13) and was shown to be a potent, DNA binding-dependent transcriptional repression module (18, 20, 21). KRAB-ZFPs have been primarily described in higher vertebrate species, where their functions are largely unknown. Among the estimated 500–700 human Kruppel-type Cys$_2$-His$_2$ ZFPs (22), one-third contain KRAB domains (13). The KRAB domain homology consists of approximately 75 amino acid residues and is predicted to fold into two amphipathic helices that are involved in protein/protein interactions (13, 23). The minimal repression module is approximately 45 amino acid residues, and substitutions for conserved residues abolish repression (18). More than 10 independently encoded KRAB domains have been demonstrated to be potent repressors of transcription, suggesting that this activity is a common property of this domain. Thus, the KRAB-ZFP family represents a large class of transcriptional repressor molecules.

KRAB-ZFPs appear to play important regulatory roles during cell differentiation and development. The KRAB-ZFPs ZNF43 and ZNF91 exhibit expression that is mainly restricted to lymphoid cells, suggesting roles as transcriptional regulators specific for lymphoid cell differentiation (24, 25). Other KRAB-ZFPs, such as HPF4, HTF10, and HTF34, are down-regulated during myeloid differentiation (13). SZF1, a KRAB-ZFP specific to CD34 stem cells, is down-regulated in differentiated hematopoietic derived cell lines (26). A number of KRAB-ZFPs are candidate genes for human diseases based on their chromosomal locations (27, 28). There are more than 40 KRAB-ZFPs supported in part by National Institutes of Health Grants CA 52009, CA 10815, DK 49210, and GM 54220; American Cancer Society Grant NP-854; the Irving A. Hansen Memorial Foundation; the Mary A. Rumsey Memorial Foundation; and the Pew Scholars Program in theBiomedical Sciences. To whom correspondence should be addressed: The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104. Tel.: 215-898-0955; Fax: 215-898-3929; E-mail: rauscher@wistar.upenn.edu.

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1 The abbreviations used are: KRAB, Kruppel-associated box; RBCC, RING finger, B boxes, and coiled-coil region; NTA, nitroliotriacetic acid; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; bv, baculovirus; ZFP, zinc finger protein; C$_{\text{E}}$, polyoxyethylene 5-octyl ether; GST, glutathione S-transferase; HP1BD, HP1 binding domain.
encoding genes that have been identified on human chromosome 19p13 and more than 10 KRAB-ZFPs genes clustered on chromosome 19q13 (29, 30), many of which exhibit hematopoietic specific expression (31). Some of these KRAB-ZFPs are selectively expressed in certain leukemia cell lines representing different lineages (31).

We hypothesized that KRAB domain repression may be mediated by a common cellular co-factor. We identified and cloned a protein, KAP-1, that binds to the KRAB repression domain using affinity chromatography (17). This protein was subsequently identified by other investigators in yeast two-hybrid screens and designated TIF1β and KRIP-1 (32, 33). KAP-1 is a member of an emerging superfamily of transcriptional co-regulators, including TIF1α and TIF1γ (17, 34, 35). The TIF1 family encodes the signature RBCC motif that designates the RING-B box-coiled-coil tripartite structure. This motif probably functions as a cooperative protein/protein interaction motif (19, 36, 37). The definitive element of the tripartite motif is the RING finger, which is found almost exclusively in the N-terminal position in RBCC proteins and is likely to contribute specificity and/or multimerization properties to the tripartite motif. Mutational analyses have confirmed the requirement for the RING finger for proper biological function (for reviews, see Refs. 36–39). The second signature motif of the RBCC domain is the B-box (40). Two B-box motifs are often found immediately COOH-terminal to the RING finger in the RBCC domain. The third RBCC signature motif is a coiled-coil domain that displays helical amphiphilic character and is probably required for homo- or heterotypic interaction. Many biological functions of RBCC proteins have been shown to be dependent on multimerization via this coiled-coil region (36).

KAP-1 appears to function as a universal corepressor for KRAB domain proteins (17). The RBCC domain of KAP-1 is both necessary and sufficient for the KRAB domain binding (19). Oligomerization of the KAP-1-RBCC is required for binding KRAB domain, and all three components of the tripartite motif appear to cooperate in KRAB recognition. The central region of KAP-1 contains the HP1 binding domain (HP1BD), which directly binds to mammalian homologues of the heterochromatin protein, HP1 (41). A stable quaternary complex can be formed between DNA, a KRAB-ZFP, KAP-1, and HP1. The COOH terminus of KAP-1 includes a plant homeo-domain finger and bromodomain, and this region is able to repress transcription when tethered to DNA using a helical amphipathic DNA-binding domain.2 Thus, KAP-1 is composed of an independent KRAB-recognition domain and at least two independent repression domains.

It has been firmly established that KAP-1 is required for KRAB domain-mediated transcriptional repression. The evidence includes the following. 1) KAP-1 binds to multiple KRAB repression domains both in vitro and in vivo; 2) KRAB domain mutations that abolish repression decrease or eliminate KAP-1 binding; 3) overexpression of KAP-1 enhances KRAB-mediated repression; 4) the KRAB domain does not repress in cells that lack KAP-1. These results support a model in which KRAB-ZFPs repression transcription by recruiting the KAP-1 corepressor via the RBCC domain. To understand the interaction between the KRAB domain and KAP-1 in more detail, we have purified the KRAB domain and employed a comprehensive set of biochemical and biophysical approaches to analyze the complex.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids—The plasmids pQE30 GAL4-KRAB (1–90), pQE30 KOXI-KRAB, GST-KRAB, GST-KRAB(DV), pQE30 KAP-1-RBCC, and pVL1392 KAP-1-RBCC have been described previously (17–19). The pQE30 KOXI-(1–161) plasmid was generated via polymerase chain reaction using KOXI cDNA as a template. A 5’ oligonucleotide, which incorporated a BamHI site 5’ to the initiation methionine, and a 3’ oligonucleotide, which incorporated a stop codon preceding a HindIII site after amino acid 161 of KOXI, were used to amplify the desired sequence. The polymerase chain reaction product was digested and cloned into the corresponding sites of the pQE30 vector (Qiagen). The protein thus contains the NH2-terminal amino acid residues MRGSHHHHHHG, followed by residues 1–161 of the KOXI protein. The pQE30 KAP-1-(22–618) plasmid was generated by subcloning an XmaI fragment encoding residues 22–618 of human KAP-1 from a plasmid that contained the full-length human KAP-1 cDNA (17) into the corresponding sites of pQE30 (Qiagen). The protein contains the NH2-terminal amino acid residues MRGSHHHHHHGSCHELGT, followed by residues 22–618 of the KAP-1 protein and then the COOH-terminal sequence GRPAKLN encoded by the vector. DNA sequencing of both strands confirmed all of the plasmids generated by polymerase chain reaction.

Protein Purification—The purification of KOXI-KRAB protein was performed at room temperature under denaturing conditions followed by renaturation on the column (42) as described previously (19). The KAP-1-RBCC protein expressed from bacterial and baculovirus vectors was purified using nondenaturing conditions as described previously (19). To reconstitute the complex, the KAP-1-RBCC and the KOXI-(1–161) proteins were first purified under denaturing conditions and then eluted from the Ni2+-NTA-agarose with 300 mM imidazole and pH 4.5. The eluted KOXI-(1–161) and KAP-1-RBCC proteins were then mixed at a 1:1 molar ratio in a volume of 20 ml of buffer containing 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0, 10% glycerol, 20 mM ZnSO4, and 0.5 mM diethiothreitol. These proteins were then renatured during dialysis by a stepwise 1:1 serial dilution of urea from 4 to 0 M in P300 buffer (10 mM Na2HPO4, 1.4 mM KH2PO4, 2.7 mM KCl, 450 mM NaCl, pH 7.0, 1 mM phenylmethylsulfonyl fluoride) plus 10% glycerol, 20 mM ZnSO4, and 0.5 mM diethiothreitol using five changes of the buffer over a 2-day period. After dialysis, the insoluble protein was removed by centrifugation, and the soluble fraction was concentrated in an Amicon concentrator.

Gel Filtration Analysis—The soluble KOXI-KRAB protein derived from on-column renaturation was chromatographed on a Superdex 200 HR 10/30 column equilibrated in P300 buffer plus 10% glycerol and 10 mM polyoxyethylene 5-octyl ether (C12E8) (Sigma). The KAP-1-(22–618) protein and the KOXI-(1–161)-KAP-1-RBCC complex were then purified using a Superose 6 HR 10/30 column equilibrated with the P300 buffer plus 10% glycerol. The columns were run at 4 °C at a flow rate of 0.5 or 0.5–1 ml fractions were collected. Aliquots of each fraction were analyzed for protein content by SDS-PAGE and Comassie Blue staining.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed essentially as described previously (19, 43). The purified recombinant GAL4-KRAB was incubated with purified Escherichia coli- or baculovirus-expressed KAP-1-RBCC protein for 15 min at 30 °C. In the complex formation assays, the KRAB protein was added to the reaction simultaneously with the GAL4-KRAB and KAP-1-RBCC proteins, or the KRAB protein was pre-incubated with the KAP-1-RBCC protein for 15 min at 30 °C. One µl of 32P-labeled GAL4 probe (105 cpm/µl) was then added, and the reaction was incubated for an additional 15 min at 30 °C. The DNA-protein complexes were resolved on native polyacrylamide gels by electrophoresis in 45 mM Tris borate, pH 8.3, 1 mM EDTA buffer at 4 °C. The EMSA gels were dried and visualized by autoradiography. The GAL4 probe was the double-stranded synthetic oligonucleotide 5’-GAT CCC GGA GGA CAG TAC TCC GT-3’, which was labeled with [32P]ATP as described (43).

Circular Dichroism—The CD spectra (190–240 nm) were measured on a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co.) at 25 °C. The CD spectra were recorded using a 100-µl cell containing a 0.2-mm path length. The sample was at a concentration of 1 mg/ml in P300 buffer plus 10% glycerol. Spectra were analyzed using the SOFTSPEC software supplied by the manufacturer.

Analytical Ultracentrifugation—Prior to analytical ultracentrifugation, the proteins were purified by gel filtration. The KOXI-KRAB protein in P300 buffer plus 10% glycerol was incubated with 20 mM C6E6 for 1 to 4 °C by rocking. The sample was then loaded onto a 10% sucrose cushion before gel filtration. The KOXI-KRAB protein was then subjected to gel filtration in the same buffer containing 10 mM C6E6. The KAP-1-(22–618) protein was purified by gel filtration in P300 buffer plus 10% glycerol without detergent. Sedimentation equilibrium experiments were performed in an Optima XL-a ultracentrifuge, using either the absorbance (280 nm) optics (for KOXI-KRAB) or the interference optics

2 D. Schultz, unpublished data.
For each experiment, three cells were assembled with 12-mm double-sector centerpieces and quartz or sapphire windows, respectively. The cells were loaded with 110 ml of reference buffer (P300 plus 10% glycerol) or 110 ml of sample at three different protein concentrations. The experiments were performed at 4 °C and using various speeds between 16,000 and 43,400 rpm. The absorbance or fringe displacement data were collected every 6 h until equilibrium was reached, as determined by comparing successive scans using the MATCH program, and the data were edited using the REEDIT program. Analysis of sedimentation equilibrium data was performed using the NONLIN program (44).

The partial specific volume of the protein was calculated according to Laue et al. (45). Three data sets from different loading concentrations were fitted simultaneously. Examination of the residuals and minimization of the variance determined the goodness of fit.

**Analysis of Stoichiometry—** The KOX1-(1–161)/KAP-1-RBCC complex, which was formed by co-renaturation as described above, was purified by gel filtration, and the peak fraction of the complex was concentrated by deoxycholate-trichloroacetic acid precipitation (46). The precipitated proteins were resuspended in 30 ml of 0.1 M NaOH and resolved on 10% SDS-PAGE with known amounts of KOX1-(1–161) and KAP-1-RBCC proteins. The proteins were visualized with Coomassie Blue stain and quantitated by densitometry on a MultiImage Light Cabinet instrument (Alpha Innotech). The data were analyzed with the program AlphaImager 2000 version 4.03.

**GST Association Assays—** The preparation of the GST fusion proteins and the GST association assays were performed essentially as described previously (41). Briefly, 5 μg of freshly prepared GST fusion protein immobilized on glutathione-Sepharose was incubated with 10 μg of Ni²⁺-NTA-purified recombinant His₆-tagged protein in 100 ml of BB500 buffer (20 mM Tris, pH 7.9, 500 mM NaCl, 0.2 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride), and the bound proteins were eluted in 5× Laemmlı buffer, resolved by SDS-PAGE, and visualized with Coomassie Blue stain.

**Kinetic Analysis—** Kinetic studies of the direct protein/protein interaction between the KRAB domain and KAP-1 were done using optical biosensors-BIAx and BIA3000 Instruments (Biacore, Inc. Uppsala, Sweden). All experiments were done at 25 °C. The Biacore TM sensor surface CM5 was activated by a 7-min incubation with amine coupling reagent as recommended by the manufacturer. The amine coupling reagents were N-hydroxysuccinimide and N-ethyl-N′-(dimethylaminopropyl)carbodiimide (Biacore, Inc.). One hundred μl of anti-GST polyclonal antibody (Biacore, Inc.) at a concentration of 10–50 μg/ml in 10 mM sodium acetate, pH 5.0, was then injected at a flow rate of 5 μl/min, resulting in 4000 response units being captured. The remaining coupling sites were blocked by injection of 35 μl of ethanolamine-HCl.
a heterogeneous oligomeric analyte using a Langmuir 1:1 integrated equation; 2) a trivalent interaction fitted to three different models: 1) a one-step binding interaction, 2) a two-step binding interaction, and 3) a heterogeneous oligomeric analyte interaction (48, 49).

RESULTS

KRAB Domain Sequence Analysis and Purification of Recombinant Proteins—A search of the nonredundant nucleotide and protein sequence data bases has revealed that the KRAB domain is present in more than 130 independent proteins (data not shown). Given this abundant distribution, it is surprising that no detailed biochemical or structural analysis of this domain has yet been undertaken. We began our studies by aligning and analyzing the most well characterized members of the KRAB domain family (Fig. 1A). One of the founding members of this family is KOX1, which encodes an NH2-terminal KRAB domain and 11 COOH-terminal C2H2 class zinc finger motifs (Fig. 1B). Each of the other proteins encodes COOH-terminal zinc fingers (not shown) and the indicated KRAB domain sequences in the NH2 terminus of the protein. The region of KRAB homology in each protein generally extends for about 75 contiguous residues and is often divided into A and B boxes. This division is based on the fact that separate exons often encode A and B boxes in the genomic structure of many KRAB-ZFPs and the observation that alternative splicing can generate protein isoforms lacking either the A or B box (13). The KRAB domain shows remarkable homology among the members. The consensus sequence contains almost completely conserved blocks of sequence including the residues DV, EEW, LD, VMLENY, and KP (Fig. 1A). Amino acid substitutions within any of these conserved residues have been shown to abolish the repression activity (18). A structural prediction program (Chou-Fasman) suggests that the KOX1-KRAB domain possesses a helical content of approximately 35% with two major helices. These helices have an amphipathic nature as defined by helical wheel analysis. These predictions suggest that the KRAB domain provides an interface for protein/protein interactions and that it may bind to the KAP-1 corepressor via a helix/helix interaction. To further investigate the KRAB/KAP-1 interaction, we initiated biochemical and biophysical analyses of the KRAB domain.

The KRAB domain that we have utilized in these studies corresponds to amino acids 1–90 and 1–161 of the KOX1 zinc finger protein (18) (Fig. 1, A and B). We selected the KRAB domain from KOX1 for the following reasons. 1) The KOX1-KRAB domain was originally utilized to isolate the KAP-1 corepressor, and mutations in this domain that concomitantly abolish repression and KAP-1 binding are well characterized (17). 2) The KOX1-KRAB domain is highly expressed in E. coli autonomously or as a GAL4 fusion and is well behaved in protein reconstitution assays (19). 3) The GAL4-KOX1-KRAB(1–90) fusion (hereafter designated GAL4-KRAB) is a potent, KAP-1-dependent, and DNA binding-dependent transcriptional repressor in vivo (18) (Fig. 1B). We expressed and purified both KOX1-(1–90) (designated KOX1-KRAB) and KOX1-(1–161) domains from E. coli using Ni2+-NTA chromatography under denaturing conditions, followed by a renaturation protocol that yielded soluble, highly active proteins (Fig. 2A). The SDS-PAGE analysis revealed that purified KOX1-KRAB protein migrates with an apparent molecular mass of 15.5 kDa, which is slightly larger than its predicted molecular mass of 13.9 kDa (Fig. 2A). The KOX1-(1–161) protein migrates with an apparent molecular mass of 23 kDa, which is slightly larger than its calculated molecular mass of 20.5 kDa (Fig. 2A). The slightly aberrant migration may be due to the highly charged nature of the KRAB domain.

The KAP-1-RBCC protein produced in E. coli was purified on Ni2+-NTA under native conditions (19) (Fig. 1C). SDS-PAGE analysis (Fig. 2A) revealed that the KAP-1-RBCC protein migrated with an apparent molecular mass of 46 kDa, a value
close to its predicted molecular mass of 45.9 kDa. The KAP-1-RBCC was also expressed in Sf9 insect cells using a baculovirus expression vector (hereafter designated bv.KAP-1-RBCC). The bv.KAP-1-RBCC was purified to near homogeneity under native conditions using Ni²⁺-NTA chromatography and migrated near to its predicted monomeric molecular mass of 47.7 kDa on SDS-PAGE (Fig. 2A), suggesting that bv.KAP-1-RBCC is not subjected to extensive post-translational modification. We also expressed a larger version of the KAP-1, KAP-1-(22–618), which includes the RBCC domain and the HP1 binding domain (HP1BD) (19, 41). The KAP-1-(22–618) protein produced in E. coli was not soluble and therefore was purified under denaturing conditions, followed by step dialysis renaturation. The KAP-1-(22–618) protein migrated with an apparent molecular mass of 68 kDa in SDS-PAGE, a value close to its predicted molecular mass of 66 kDa (Fig. 2B).

The GST, GST-KRAB, and GST-KRAB(DV- AA) fusion proteins were purified to homogeneity and were analyzed for the ability to bind the purified E. coli-expressed KAP-1-(22–618) and the KAP-1-RBCC proteins. Significant binding of the KAP-1-(22–618) and the KAP-1-RBCC proteins was observed for the GST-KRAB protein but was negative for the control GST protein and GST-KRAB(DV) mutant protein (Fig. 2B), supporting our previous results demonstrating that the interaction between the KRAB domain and the RBCC domain of KAP-1 is direct and specific. Moreover, these purified proteins are biochemically well behaved, which makes them useful reagents for quantifying the affinity of this interaction in the optical biosensor assays.

**Oligomerization Properties of the Purified KOX1-KRAB and KAP-1-RBCC Proteins**—To investigate the mechanisms behind the KRAB/KAP-1 interaction, we employed biochemical and biophysical analyses of the individual components and the KOX1-KRAB-KAP-1-RBCC complex. Gel filtration chromatography was used to estimate the hydrodynamic size (Stokes radius) of the proteins. Our initial studies showed that under physiological buffer conditions, the KOX1-KRAB protein eluted in a broad peak at approximately 670 kDa, suggesting that it was a soluble self-aggregate upon purification from E. coli (data not shown). We attempted to generate nonaggregated KRAB protein using the detergent C₈E₅. The C₈E₅ is a non-denaturing detergent (50) that forms micelles that are neutrally buoyant, allowing a reversible association to occur between monomer and oligomer states of the protein in analytical ultracentrifugation. When the KOX1-KRAB protein preparation was incubated with C₈E₅, two molecular weight species (designated KOX1-KRAB_HMW and KOX1-KRAB_LMW) were separated by gel filtration in the presence of this detergent (Fig. 3). The molecular mass of the KOX1-KRAB_HMW was estimated at 670 kDa, and the KOX1-KRAB_LMW was estimated at 32.7 kDa. Thus, even in the presence of detergents, the KRAB domain has a significant tendency for aggregation as evidenced by the presence of the KOX1-KRAB_HMW fraction under these conditions. The KRAB_LMW material may either be a dimer or an extended monomer.

Previous studies have indicated that the RBCC domain (residues 22–418) of KAP-1 is the minimal KRAB binding region and that it exists as an apparent trimer in the absence or presence of the KRAB domain (19). However, we were unable to determine the mass of the KRAB-KAP-1-RBCC complex by analytical ultracentrifugation due to low protein recoveries and aggregation during the sedimentation equilibrium run. Instead, we used nondenaturing PAGE to estimate the native size of the KOX1-KRAB protein, the KAP-1-RBCC protein, and a preformed KOX1-KRAB-KAP-1-RBCC complex (data not shown). The estimated molecular size for the individual components and the complex is slightly higher than expected. However, it is consistent with the observations made for the individual components and the complex in gel filtration studies (Fig. 3) (19).

**Secondary Structure of the Purified Recombinant Proteins**—The CD spectra of the KOX1-KRAB domain show that the protein has a high degree of secondary structure (Fig. 4). Analysis of the spectra using SOFTSPEC software shows an excellent agreement with a protein containing 32.4% helix, 27.5% β-sheet, 9.2% turn, and 31% random coil for the purified KOX1-KRAB protein (Fig. 4A) and 25.3% helix, 39.8% β-sheet, 10.2% turn, and 24.7% random coil for the purified KOX1-KRAB_LMW protein (Fig. 4B). These experimental values are consistent with secondary structure predictions, suggesting that about
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35% of the KOX1-KRAB domain is helical (Fig. 1A). The CD spectra of the KAP-1-RBCC protein and the KOX1-KRAB-KAP-1-RBCC complex purified from gel filtration also show that the proteins have a high degree of secondary structure (data not shown). These data indicate that the proteins were all folded under these experimental conditions.

Ultracentrifugation Studies—To obtain an estimate of the molecular mass of the KOX1-KRABLMW and the KAP-1-(22–618) proteins, we performed equilibrium sedimentation experiments using analytical ultracentrifugation (Fig. 5). Analysis of the KOX1-KRABLMW protein was performed at 4 °C and 43,400 rpm, and the concentration of protein versus radius data was fitted with various models of self-association using nonlinear regression (Fig. 5A). The data were best described by a model describing a monomer-trimer (n = 2.9) equilibrium. Some nonreversibility was consistently observed with the highest concentration cell, which showed a slightly weaker affinity compared with the two lower concentration cells. The basis for this discrepancy may be a small degree of irreversible aggregation or other nonideal behavior. Hence, the Kd of 230 ns determined for the two lower protein concentrations should most accurately describe this association. This observation, that the predominant oligomeric state of the KAP-1-(22–618) is a trimer, is consistent with the previous findings for KAP-1-RBCC (19). The fact that this KAP-1-(22–618) trimer (monomer mass of 66 kDa) migrated with an apparent molecular size of 600 kDa compared with an apparent size of 158 kDa by gel filtration for the KAP-1-RBCC trimer (monomer mass of 45.9 kDa) indicates that the KAP-1-RBCC domain is globular, while the larger KAP-1-(22–618) oligomer is highly asymmetric.

Direct Interaction between the KOX1-KRAB Domain and KAP-1-RBCC Domain—We have previously shown that the E. coli-expressed KAP-1-RBCC protein was able to form a complex with the DNA-bound KRAB domain in EMSAs (19). We used this assay to compare the ability of E. coli and baculovirus-expressed KAP-1-RBCC to directly bind the KRAB domain (Fig. 6A). Binding of the GAL4-KRAB protein to a canonical 32P-labeled synthetic oligonucleotide containing the GAL4 recognition sequence yielded the expected mobility shift (Fig. 6A, lane 2). When increasing amounts of purified E. coli KAP-1-RBCC protein were incubated with the GAL4-KRAB protein and DNA, a new mobility shift was observed (Fig. 6A, lanes 3–7). This supershift contains the ternary complex of DNA-GAL4-KRAB-KAP-1-RBCC. The studies described above were exclusively performed with bacterial expressed proteins that lack any eukaryotic post-translational modifications and the potential to interact with endogenous cellular partner proteins. To determine if our observations extend to eukaryotic cell-expressed proteins, we expressed the KAP-1-RBCC in Sf9 insect cells using a baculovirus expression vector. The KAP-1-RBCC was highly expressed as a soluble protein in Sf9 cells (Fig. 2). We then characterized the bv.KAP-1-RBCC protein in the GAL4-KRAB EMSA assay. When increasing amounts of purified bv.KAP-1-RBCC protein were added to a constant amount of the GAL4-KRAB protein, a complex with reduced mobility was observed that exactly co-migrates with the complex formed with E. coli-produced KAP-1-RBCC (Fig. 6B). Moreover, the concentrations of either baculovirus or E. coli-produced proteins required to supershift a given amount the GAL4-KRAB were similar (Fig. 6, compare A and B). These results indicate that both E. coli- and baculovirus-expressed KAP-1-RBCC are equally active in KRAB domain binding.

To compare the activity of the KOX1-KRABLMW and KOX1-KRABLMW proteins purified from the gel filtration for binding to the KAP-1-RBCC protein, EMSA assays were performed. We first formed the GAL4-KRAB-KAP-1-RBCC complex using con-
stant levels of each protein. The resulting supershift is shown in Fig. 7A, lane 2. When increasing amounts of the KOX1-KRABLMW were added to the binding assay simultaneously with the GAL4-KRAB, very little loss of the supershift was observed (Fig. 7A, lanes 3–6). This suggests that the aggregated form of the KRAB domain binds very poorly to the KAP-1-RBCC domain. However, under the same conditions, the KOX1-KRABLMW is a very efficient competitor of the GAL4-KRAB/KAP-1-RBCC interaction (Fig. 7A, lanes 8–11). When the KAP-1-RBCC and KOX1-KRABLMW protein were preincubated, the latter protein was still a poor competitor for the added GAL4-KRAB protein (Fig. 7B, lanes 3–6). However, the KOX1-KRABLMW was still a very effective competitor when it was preincubated with the KAP-1-RBCC protein. These results strongly suggest that the most active form of the KRAB domain is the apparent monomeric form.

We next determined the stoichiometry of the KRAB-KAP-1 complex. The KOX1-(1–161) protein and the KAP-1-RBCC protein were purified under denaturing conditions and renatured together at a 1:1 molar ratio. The complex was purified by gel filtration and analyzed by SDS-PAGE. The molar ratio of the KOX1-(1–161) protein to KAP-1-RBCC protein in the complex was estimated to be 1:3.08 (Fig. 8), suggesting that one KRAB domain monomer binds KAP-1-RBCC trimer. This result is consistent with the observations for the KOX1-KRAB, KAP-1-RBCC, and KRAB-KAP-1-RBCC complex in gel filtration (Fig. 3) (19) and analytical ultracentrifugation (Fig. 5) (19).

Kinetic Studies of the Interaction between KRAB and KAP-1 Repressor—In order to determine the kinetic parameters of the KRAB/KAP-1 interaction, we used real time optical biosensor technology. The proteins used in the kinetic studies were expressed and purified to homogeneity (Fig. 9A). Fig. 9B shows the overlay plot of increased binding of the KAP-1-(22–618) protein as a function of concentration at low surface density of the GST-KRAB protein (100 response units). The GST tag of the recombinant KRAB protein was used to orient the protein, and it is assumed that the KRAB surface displays freely accessible binding epitopes to the KAP-1-(22–618) protein. The contact time was kept below 5 min to minimize sample dispersion. The kinetics of binding of the KAP-1-(22–618) to the GST-KRAB was fitted to three models: 1) a simple one-step interaction equilibrium in which one molecule of GST-KRAB interacts with three molecules of KAP-1-(22–618); 2) a model describing one molecule of GST-KRAB binding to monomer, dimer, and trimer of KAP-1-(22–618); and 3) a model describing the trimeric binding of KAP-1-(22–618) in which species with KRAB binding one, two, and three molecules of KAP-1-(22–618) are in equilibrium. The collected data best fit the model that describes concentration versus radius data for three loading concentrations of the KOX1-KRAB (circles). The three data sets were fitted globally with a model describing monomers and small amounts of aggregates, using the nonlinear regression program NONLIN (44). Each data set yielded different association constants, indicating the species were not in reversible equilibrium. The solid lines represent the calculated fits. Panels a–c show the residuals of the fitted curves to the data points at the three protein concentrations, from the highest to the lowest concentration.

Fig. 5. Analytical ultracentrifugation analysis of KOX1-KRAB and KAP-1-(22–618). A, sedimentation equilibrium analysis of KOX1-KRAB was performed at 43,400 rpm and 4 °C. Panel d shows the concentrations versus radius data for three different initial loading concentrations at equilibrium. The three data sets were fitted globally with a model describing a monomer-trimer (n = 2.9) equilibrium, using the nonlinear regression program NONLIN (44). The highest concentration showed a 4-fold weaker binding affinity compared with the two lower concentrations, suggesting some nonreversibility at higher protein concentrations. Solid lines represent the calculated fits. Panels a–c show the residuals of the fitted curves to the data points at the three protein concentrations, from the highest to the lowest concentration.
scribed a simple one-step interaction equilibrium, in which one molecule of GST-KRAB binds three molecules of KAP-1-(22–618). The KAP-1-(22–618) protein was found to bind to the KRAB protein in a dose-dependent manner (Fig. 9B). The solid lines represent the best global fits to a simple 1:1 Langmuir binding model. Using this model, a $x^2$ value was calculated to be 0.936, indicating a good fit. The fitted $R_{\text{max}}$ (maximum response) was consistently 3-fold higher after subtracting the nonspecific contribution of the GST-KRAB(DV) control surface to the interaction. This indicates that 3 mol of KAP-1-(22–618) bound per mol of GST-KRAB captured on the target. The kinetic parameters were calculated by fitting the data globally to a model for a single step with variable $R_{\text{max}}$. The interaction parameters for a 1:3 stoichiometry of GST-KRAB to KAP-1-(22–618) are shown in Table I. The on and off rates for the KRAB/KAP-1 interaction determined from the sensor analysis were $1.7 \times 10^3 \text{ M}^{-1} \text{ S}^{-1}$ and $2.42 \times 10^{-2} \text{ S}^{-1}$, respectively, yielding a $K_d$ of 142 nM.

**DISCUSSION**

In this study, we have systematically evaluated the oligomeric states and hydrodynamic properties of the KRAB and KAP-1 recombinant proteins as well as the binding affinity and stoichiometry of KRAB/KAP-1 association. We conclude the following from our data. 1) The KRAB domain exists as a monomer in the absence or presence of the RBCC domain of KAP-1 protein. 2) The interaction between a KRAB and the KAP-1 protein is direct; it apparently does not require post-translational modifications and is stable to in vitro biochemical manipulation. 3) A stable ternary complex can be formed be-

**FIG. 6.** Binding of purified *E. coli* or baculovirus-expressed KAP-1-RBCC to a wild-type KOX1-KRAB domain as detected by EMSA. EMSA was performed as described under "Experimental Procedures." Each reaction contained a constant amount of purified GAL4-KRAB (100 ng), a $^3$P-labeled DNA probe containing the canonical GAL4 binding site, and increasing amounts of purified *E. coli*-expressed KAP-1-RBCC (A) or baculovirus-expressed KAP-1-RBCC (B). The arrow indicates the DNA-GAL4-KRAB binary complex. The bracket represents the slower migrating DNA-GAL4-KRAB-KAP-1-RBCC complex. FP, free probe.

**FIG. 7.** Purified KOX1-KRAB actively competes purified KAP-1-RBCC binding to GAL4-KRAB. A competition of the GAL4-KRAB-KAP-1-RBCC complex formation by the purified KOX1-KRABLMW and KOX1-KRABHMW. A constant amount of GAL4-KRAB (100 ng) and KAP-1-RBCC (1 μg) was incubated with increasing amounts of either KOX1-KRABHMW (250, 500, 1000, and 2000 ng) (lanes 4–7) or KOX1-KRABLMW (63, 125, 250, and 500 ng) (lanes 8–11) prior to the addition of DNA probe. B, a constant amount of purified KAP-1-RBCC (1 μg) was incubated with increasing amounts of KOX1-KRABLMW (250, 500, 1000, and 2000 ng) (lanes 4–7) or KOX1-KRABHMW (63, 125, 250, and 500 ng) (lanes 8–11) for 15 min at 30 °C. A constant amount of GAL4-KRAB (100 ng) was then added, and the reaction was incubated for an additional 15 min at 30 °C. The GAL4 probe was then added and incubated with the complex for 15 min at 30 °C. The arrow indicates the DNA-GAL4-KRAB binary complex. The bracket represents the slower migrating DNA-GAL4-KRAB-KAP-1-RBCC complex. FP, free probe.
between DNA, a DNA-bound KRAB domain, and the KAP-1-RBCC protein; therefore, the KRAB/KAP-1 interaction does not inhibit DNA binding in the system we used. 4) The stoichiometric analysis shows that one molecule of KRAB domain directly interacts with three molecules of KAP-1-RBCC. 5) The real time kinetic analysis of the protein-protein interaction...
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using the optical biosensor indicated that the formation of this complex is a single-step event with fast association and slow dissociation rates, indicating that this interaction occurs with high affinity.

Previous studies using purified recombinant proteins indicated that the RBCC domain of KAP-1 is necessary and sufficient for the interaction with the KOX1-KRAB domain, and this interaction is direct and specific (19). We now demonstrate that this interaction does not require post-translational modifications. The purified bv-KAP-1-RBCC migrated as a single polypeptide band consistent with its predicted monomeric molecular mass as detected by SDS-PAGE when expressed in eukaryotic cells. The bv-KAP-1-RBCC protein behaved identically with the E. coli-expressed protein in all of the biochemical binding assays (19) and was equally active in KRB domain interaction as detected by EMSA assay. Moreover, this interaction is stable in vitro biochemical manipulation as detected by gel filtration, sucrose gradient sedimentation (19), GST association assay, and optical biosensor assay. Furthermore, binding of a preformed KAP-1-H1P1 complex was observed with the GST-KRAB protein, suggesting that a ternary KRAB-KAP-1-H1P1 complex can be readily formed in vitro. These data suggest that KAP-1 may serve as a bridging molecule between the KRAB-ZFP and the downstream effectors-H1P1 proteins via separate protein-protein interaction domains, as defined by the KAP-1-RBCC and the KAP-1-H1P1BD, respectively. This evidence supports the current working model for KRAB-ZFP-KAP-1-mediated transcriptional repression (see below).

Although it has been demonstrated that the KRAB domain is a potent, DNA-binding-dependent transcriptional repression module (18, 20, 21, 51), little is known about its structure and the protein-protein interface of the KRAB-KAP-1 complex. We have demonstrated that KRAB binding induces and/or stabilizes the oligomeric species (trimer) of the KAP-1-RBCC protein (19). We now present the first demonstration that the KRAB domain functions as a monomer to bind to the homotrimer of the RBCC domain of corepressor KAP-1. Gel filtration indicates that the KOX1-KRAB domain is an apparent monomer in solution, although a significant amount of KOX1-KRAB protein aggregates. Analytical ultracentrifugation experiments showed that the KRAB domain is the predominant monomer. We confirmed these studies using low molecular weight (monomer) and high molecular weight species (aggregates) of the KOX1-KRAB protein in an EMSA competition assay. This study clearly shows that the monomeric species of the KRAB is an effective competitor of the GAL4-KRAB-KAP-1-RBCC complex, suggesting that the monomeric KRAB protein has high binding affinity with the KAP-1-RBCC protein. Together, these observations substantiate the relationship between the structure and function of the protein.

It has been demonstrated that the KAP-1-RBCC domain must properly homo-oligomerize in order to bind the KRAB domain (19). However, the oligomeric state of the KRAB domain and the stoichiometry of KRAB-KAP-1 complex have not been determined. We attempted to determine the stoichiometry of the KRAB-KAP-1-RBCC complex from the analytical ultracentrifugation experiment. Unfortunately, we were unable to obtain useful data starting with a preformed KOX1-KRAB-KAP-1-RBCC complex, because the protein concentrations of the complex that could be obtained were too low and the sample partially aggregated under the conditions required for the ultracentrifugation experiments. Therefore, alternative approaches were applied to determine the stoichiometry of the protein/protein interaction complex. First, non-denaturing PAGE analysis of a preformed KOX1-KRAB-KAP-1-RBCC complex shows that the molecular mass of the complex is about the sum of the predicted monomeric molecular mass of the KRAB and trimeric molecular mass of the KAP-1-RBCC. Second, SDS-PAGE analysis of a preformed KOX1-KRAB-KAP-1-RBCC complex indicates a 1:3 stoichiometry for the KRAB protein and KAP-1-RBCC protein. Third, protein-protein interaction analysis using optical biosensors demonstrated binding with a 1:3 stoichiometry of the KRAB protein and KAP-1-RBCC protein. The data are best described by a one-step interaction model in which one molecule of GST-KRAB binds to three molecules of KAP-1(22-618) to form a complex. This is consistent with the results obtained from analytical ultracentrifugation and gel filtration analysis of the same materials employed in the optical biosensor experiments. The binding of a KRAB domain to a trimer of KAP-1(22-618) protein is a single-step event with fast association and slow dissociation rates, indicating a high binding affinity of the complex. It will be interesting to determine the dynamic parameters of the multimeric complexes involving KRAB-ZFP, KAP-1, and H1P1 protein-protein interaction using an optical biosensor.

Taken together, all data presented here further support the current working model for the KRAB-ZFP-KAP-1-mediated transcriptional repression (19). A KRAB-ZFP binds target gene DNA sequence-specifically through its array of C$_2$H$_2$ zinc fingers. The DNA-bound KRAB domain recruits the KAP-1 corepressor via direct interaction with the RBCC domain. The KAP-1 apparently self-assembles into a homotrimer, which is the active form of the protein that binds the KRAB domain. The HIP1BD, plant homeo-domain finger, and bromodomain of KAP-1 comprise the surfaces that mediate gene silencing via interaction with the downstream targets. One of the direct targets of KAP-1 was defined by us and others as the heterochromatin protein HP1 (34, 41). The HP1 proteins are a small family of non-histone chromosomal proteins, some of which are tightly associated with silenced heterochromatin. We have shown that KAP-1 directly binds to recombinant HP1 protein via KAP-1-H1P1BD and that a stable quaternary complex can be formed between DNA, KRAB-ZFP, KAP-1, and HP1. HP1 and KAP-1 extensively colocalize to heterochromatotic regions in interphase nuclei. KAP-1 binds to HP1, resulting in recruitment of the KRAB-ZFP-bound target gene to heterochromatin and subsequent silencing of gene expression. According to this model, it is speculated that the KAP-1 serves to nucleate HP1-mediated repression on a template and that the oligomerization of KAP-1 as it binds the KRAB domain has a role in the nucleation of heterochromatin.

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Biochemical Analysis of the Kruppel-associated Box (KRAB) Transcriptional Repression Domain: SPECTRAL, KINETIC, AND STOICHIOMETRIC PROPERTIES OF THE KRAB-KAP-1 COMPLEX
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