Epigenetic Gene Silencing by the SRY Protein Is Mediated by a KRAB-O Protein That Recruits the KAP1 Co-repressor Machinery*[S]

Hongzhuang Peng[‡], Alexey V. Ivanov[§], Hyun J. Oh[¶1], Yun-Fai C. Lau[¶2], and Frank J. Rauscher III[‡3]

From the [‡]Wistar Institute, Philadelphia, Pennsylvania 19104, the [§]Mary Babb Randolph Cancer Center, Department of Biochemistry, West Virginia University School of Medicine, Morgantown, West Virginia 26506, and the [¶]Division of Cell and Development Genetics, Department of Medicine, Veterans Affairs Medical Center, University of California, San Francisco, California 94121

The sex determination transcription factor SRY is a cell fate-determining transcription factor that mediates testis differentiation during embryogenesis. It may function by repressing the ovarian determinant gene, RSPO1, action in the ovarian developmental pathway and activates genes, such as SOX9, important for testis differentiation at the onset of gonadogenesis. Further, altered expression of SRY and related SOX genes contribute to oncogenesis in many human cancers. Little is known of the mechanisms by which SRY regulates its target genes. Recently a KRAB domain protein (KRAB-O) that lacks a zinc finger motif has been demonstrated to interact with SRY and hypothesized to function as an adaptor molecule for SRY by tethering the KAP1-NuRD-SETDB1-HP1 silencing machinery to repress SRY targets. We have critically examined this hypothesis by reconstituting and characterizing SRY-KRAB-O-KAP1 interactions. These recombinant molecules can form a ternary complex by direct and high affinity interactions. The KRAB-O protein can simultaneously bind KAP1 and SRY in a noncompetitive but also noncooperative manner. An extensive mutagenesis analysis suggests that different surfaces on KRAB-O are utilized for these independent interactions. Transcriptional repression by SRY requires binding to KRAB-O, thus bridging to the KAP1 repression machinery. This repression machinery is recruited to SRY target promoters in chromatin templates via SRY. These results suggest that SRY has co-opted the KRAB-O protein to recruit the KAP1 repression machinery to sex determination target genes. Other KRAB domain proteins, which lack a zinc finger DNA-binding motif, may function in similar roles as adaptor proteins for epigenetic gene silencing.

Analysis of the human genome sequence revealed 423 independent KRAB (Krüppel-associated box) domains of KRAB-zinc finger protein (KRAB-ZFP)[4] genes, yielding alternative transcripts that altogether predict at least 742 structurally distinct proteins (1). The KRAB domain is mainly found in the NH_2 termini of Krüppel-related ZFPs, and it is classically divided into an A box and a B box encoded by separate exons (see Fig. 1A and supplemental Fig. S1). By comparing the amino acid sequences of the KRAB domain, KRAB-ZFPs can be classified into several subfamilies: KRAB(AB), KRAB(Ab), and KRAB(AC) (2). Most of the KRAB-ZFPs contain tandem zinc finger modules in their COOH termini. These zinc finger modules can number from 3 to upwards of 40 in a single protein and presumably participate in DNA recognition. KRAB-mediated gene silencing is absolutely dependent on binding to its co-repressor protein, KAP1 (KRAB-associated protein 1) (3–5), which in turn recruits the NURD histone deacetylase complex, SETDB1 histone methyltransferase, small ubiquitin-like modifier protein modification, and the heterochromatin proteins in the HP1 family (6–10). Although much is known of the KRAB silencing mechanism, comparatively little is known about KRAB-ZFP function in higher organisms.

Most KRAB domain proteins harbor zinc finger motifs; however, there are a few exceptions. One of them is KRAB-O (KRAB only), which is an alternatively spliced transcript from *Zfp748* gene (formerly called *Zfp208*, a mouse homologue of the human *ZNF208*); the second is the VHLaK (pVHL-associated KRAB-A), which is an alternatively spliced transcript from *Znf197* gene; and the third is MIF1 (mouse inhibitor of four 1) (11–13). The functional relationship between KRAB domain proteins with or without zinc finger motifs is currently unknown. It has been postulated that KRAB domain proteins without zinc fingers may act as dominant negative regulators for those with zinc fingers, or alternatively these different splice variants might function independently of each other. KRAB-O was identified as an SRY (sex-determining region Y)-interacting protein through the yeast two-hybrid screen (11). KRAB-O and KRAB-ZFP748 contain KRAB-A and KRAB-B boxes, and their transcripts are expressed in embryonic gonads. ZFP748 does not interact with SRY under the same experimental con-

[1] Present address: Dept. of Cell Biology and Anatomy, Rosalind Franklin University of Medicine and Science, North Chicago, IL 60064.

[2] Research Career Scientist of the Department of Veterans Affairs.

[3] To whom correspondence should be addressed: The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104. Tel.: 215-898-0995; Fax: 215-898-3929; E-mail: Rauscher@wistar.org.

[4] The abbreviations used are: ZFP, zinc finger protein; KAP1, KRAB-associated protein 1; RBCC domain, RING finger, B boxes, and Coiled-coil region; GST, glutathione S-transferase; IVT, in vitro translated; HMG, high mobility group; BRG, bridge; h, human; m, mouse; HA, hemagglutinin; ChIP, chromatin immunoprecipitation; WT, wild type; TK, thymidine kinase.

ditions used for KRAB-O interaction analysis. KRAB-O co-localizes with SRY and KAP1 directly and with HP1 indirectly in the nuclei of transfected cultured cells and mouse fetal gonadal cells, suggesting that SRY might use the KRAB-KAP1-mediated repression complexes to regulate the transcription of its target genes. A recent study from the Koopman group (14) shows that more than 100 KRAB genes were found to be expressed in mouse developing gonads, including 19 transcripts encoded by the KRAB-O cluster that were found to be expressed in somatic cells at 11.5 days post-coitum. The knockdown of KRAB genes decreases expression of SOX9 (SRY-related HMG-box 9) in a gonadal cell line stably expressing SRY, suggesting a important role for KRAB-O and other KRAB-ZNF genes in mediating SRY function.

SRY/Sry encodes a putative transcription factor with a HMG DNA-binding domain. The mouse SRY contains an HMG box at its NH_2 terminus, a bridge region (BRG) at its central region, and a glutamine-rich domain at its COOH terminus (see Fig. 2*A*). The human SRY (hSRY) contains additional domains with unknown functions at both the NH_2 terminus and the COOH terminus, but not the Q-domain (see Fig. 2*A*). The HMG box is the only evolutionarily conserved domain among all of the mammalian SRY genes analyzed so far. It binds and bends DNA *in vitro* in a sequence-specific manner (15–17). These properties are essential for SRY as a sex-determining factor because mutations that change these activities are shown to cause gonadal dysgenesis in humans (18). Recent studies have demonstrated that aberrant expression of SRY and other SOX (SRY box-containing) genes is associated with the development and/or progression of certain cancers. SOX17-mediated gene silencing is associated with DNA hypermethylation of a CpG island in the promoter region. The CpG island methylation-dependent silencing of SOX17 occurs in 100% of colorectal cancer cell lines, 86% of colorectal adenoma, 100% of stage I and II colorectal cancer, and other cancers (19). Hence understanding how the SRY and SOX transcription factors regulate their target genes may be important in elucidating the molecular mechanism(s) by which these developmental genes exert their effects on oncogenesis.

SRY has been hypothesized to function as a transcriptional repressor for ovarian differentiation and activator for testicular differentiation during gonadogenesis (11, 20). Although SRY has been demonstrated to repress WNT signaling associated with RSPO1, the ovarian determinant (21–23), and to activate SOX9, a key downstream gene in testis differentiation (24, 25), the exact mechanisms by which SRY and SOX genes exert their transcriptional regulation functions on their target genes are uncertain. Our initial observations are that the BRG of SRY is necessary and sufficient for binding to the KRAB-O protein and that the HMG box can optimize this binding (11).

We postulate that SRY interactions with other co-regulators, particularly KRAB-O, are essential for its functions as either a transactivator or repressor of its target genes. We have performed a systematic analysis for the interaction of SRY/KRAB domains from the KRAB-ZFPs and have characterized these proteins and protein complexes using a biochemical approach. We define the detail of direct interactions between the KRAB-O/KRAB domain and the SRY proteins. The KRAB-O/KRAB domain directly and simultaneously interacts with SRY and KAP1 through specific binding surfaces that support the hypothesis that KRAB-O functions as a bridging molecule between KAP1 and SRY proteins. We also demonstrated that the KRAB-O/KRAB-KAP1-mediated repression complex could down-regulate the hSRY-mediated transcriptional activation of a reporter gene. Therefore, this study provides the biochemical and biological functional evidence to support a working model involving KRAB-O/KRAB-KAP1 interactions for SRY action(s) on sex determination and differentiation.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids—For details of plasmids, see the supplemental "Experimental Procedures."

In Vitro Translation of Proteins and Protein Purification—[^{35}S]Methionine-labeled, *in vitro* translated (IVT) mSRY and hSRY proteins were made using the TNT SP6/T7 coupled reticulocyte lysate system (Promega). The KRAB-O protein was prepared from a GST-KRAB-O fusion protein by thrombin digestion as previous described (2). The His-tagged KAP1-RBCC protein was purified at 4 °C under native conditions as recommended by manufacturer (Qiagen) (26).

Gel Filtration Analysis—Fifty μl of [^{35}S]methionine-labeled, mSRY (IVT) protein, KRAB-O protein, and KRAB-O-mSRY protein complex were analyzed by gel filtration with a Superdex 75 HR 10/30 column (GE Healthcare) equilibrated in phosphate-buffered saline (10 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.0) with 1 mM Tris(2-carboxyethyl)-phosphine. The column was at 4 °C at a flow rate of 0.5 ml/min, and 1-ml fractions were collected. The proteins or protein complex from each fraction was concentrated by deoxycholate-trichloroacetic acid precipitation. The precipitated proteins were resuspended in 100 μl of 0.1 M NaOH. Twenty μl of the resuspended protein sample were resolved on NuPAGE (Invitrogen), and the proteins were visualized by Coomassie Blue stain and autoradiography.

GST Association Assays—The preparation of the GST fusion proteins and the GST association assays were performed essentially as described previously (27, 28). Briefly, 5 μg of freshly prepared GST fusion protein immobilized on glutathione-Sepharose was incubated with 20 μl of IVT SRY proteins or/and with 10 μg of Ni^{2+}-nitrilotriacetic acid-purified recombinant His-tagged KAP1-RBCC protein in 0.5 ml of BB500 buffer for 1 h at room temperature. The protein complexes were washed four times with BB750 buffer, followed by two times with BB500 buffer, and the bound proteins were eluted in 5× Laemmli buffer, resolved by NuPAGE, and visualized with Coomassie Blue stain and autoradiography.

Competition Assay—Ten μg of freshly prepared GST-KRAB fusion protein was first immobilized on glutathione-Sepharose, followed by the binding of IVT SRY and purified KAP1-RBCC proteins under three different binding conditions: 1) Equilibrium: 20 μl of IVT SRY was premixed with 40 μg of purified KAP1-RBCC protein in 0.5 ml of BB500 buffer, and then the mix proteins were added into the GST-KRAB fusion protein and incubated for 1 h at room temperature; 2) GST-KRAB fusion protein was first saturated with KAP1-RBCC protein: 40 μg of purified KAP1-RBCC protein was added to the GST-

KRAB fusion protein and incubated for 1 h at room temperature, and then 20 μl of IVT SRY was added into the reaction and incubated for additional 1 h; and 3) GST-KRAB fusion protein was first saturated with SRY protein: 20 μl of IVT SRY was added to the GST-KRAB fusion protein and incubated for 1 h at room temperature, and the 40 μg of purified KAP1-RBCC protein was added to the reaction and incubated for additional 1 h. The protein complexes were washed and eluted in 5× Laemmli buffer, resolved by NuPAGE, and visualized with Coomassie Blue stain and fluorography.

Transient Transfection—Two cell lines (HEK 293 and U2OS) and three reporter systems (GAL4-TK-Luc, LexA-TK-Luc, and 10xSRY-TK-Luc) were used in transient transfection, respectively. HEK 293 cells or U2OS cells were transiently transfected with one of the reporter plasmids described above, the pcDNA3-β-galactosidase expression plasmids, and the effector plasmids. At 40–72 h post-transfection, the cells were collected and assayed for luciferase activity using the Luciferase Assay System (Promega) and normalized with β-galactosidase activity (Pierce). The cytoplasmic fraction from these cells was prepared in Tween 20 lysis buffer for β-galactosidase and Luciferase assay as described (29). The nuclear pellet was resuspended in Tween 20 lysis buffer containing 500 mM NaCl. The nuclear fraction was used for Western blot analyses with anti-GAL4 (DNA-binding domain) rabbit polyclonal IgG (Santa Cruz), LexA D19 goat polyclonal IgG (Santa Cruz), FLAG-M2 monoclonal IgG (Sigma), and HA monoclonal IgG (Covance) to detect the expressed proteins. For immunoprecipitation and Western blot, the nuclear extracts were immunoprecipitated with HA antibody (agarose immobilized rabbit anti-HA; ICL Lab) in Tween 20 lysis buffer containing 500 mM NaCl and 0.5% Nonidet P-40 overnight at 4 °C. The immunoprecipitated protein complexes were washed with the same buffer three times and eluted in 5× Laemmli buffer and analyzed by Western blot with antibodies as indicated above.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP was performed as described in the supplemental "Experimental Procedures." Immunoprecipitated DNA was purified using Qiaquick PCR purification kit (Qiagen) and analyzed by quantitative PCR on the CPTICON2 (MJ Research Inc.) using SYBR Green technology.

RESULTS

Protein Sequence Analysis for the KRAB Domains from KRAB-O and Other KRAB-ZFPs—The domain structure of the KRAB-O protein is similar to the subclass of KRAB (AB)-ZFPs containing a classical A box and a classical B box except that it has an additional four amino acids at its COOH terminus (Fig. 1*A*). The amino acids in the A and B boxes are highly conserved between KRAB-O and the other KRAB domains (Fig. 1*A*) except for the variance of a few amino acids. Previous studies have mapped the amino acids in the KRAB domain that are important for its transcription repression function (30, 31). Whether these highly conserved amino acids in the context of KRAB-O are critical for its repression function remains to be determined. Previous studies indicate that the A box of a KRAB domain is sufficient for repression function and KAP1-RBCC binding (2, 30). We sought to examine the role of KRAB

domain-containing proteins, not only KRAB-O, but also other KRAB-ZFPs, in the regulation of SRY through the formation of a ternary complex with KAP1.

A Direct Protein-Protein Interaction between the KRAB Domain-containing Proteins and the SRY—Because the amino acid sequence of the BRG region in mSRY and in hSRY is highly conserved, we tested whether KRAB-O/KRAB proteins interact with these proteins with similar affinity. GST association assays were carried out with GST-KRAB-O/KRAB proteins (Fig. 1*A* and supplemental Fig. S1) and IVT SRY proteins (Fig. 2*A*). All of the KRAB domain-containing proteins directly interacted with both mSry and hSRY proteins (Fig. 1*B*). The KRAB-O protein showed a higher affinity for SRY proteins compared with the other KRAB domains. The KOX1-KRAB DV-AA mutant protein, which cannot interact with KAP1, still bound to mSRY and hSRY proteins (Fig. 1*B*), suggesting that SRY molecules may utilize different binding surfaces on KRAB domains than KAP1.

Protein Sequence Analyses of the HMG and BRG Domains of SRY and the RBCC Domain of KAP1—The amino acid sequences of HMG and BRG domains of SRYs among mammalian species are highly conserved. However, there is no significant similarity of the amino acid sequences between the BRG domain of SRY and the RBCC domain of KAP1. Thus, it is suspected that the BRG domain and the RBCC domain recognize different amino acids in a KRAB domain-containing molecule so that the KRAB domain can interact with BRG domain and the RBCC domain simultaneously. To test this hypothesis, the KAP1-RBCC, mSRY, and hSRY proteins were expressed (Fig. 2*A*), and we examined whether both SRY and KAP1-RBCC proteins were able to bind KRAB-O and KRAB simultaneously without interference. The known interacting proteins for KAP1, mSRY, and hSRY are summarized in Fig. 2*A*. These data suggest that two proteins known to associate with KRAB domain-containing proteins, KAP1 and SRY, may form a ternary complex with KRAB family members in a manner that does not require zinc finger motifs.

KRAB-O/KRAB, SRY, and KAP1 Proteins Form a Ternary Stable Complex in Vitro—We previously employed biochemical and biophysical analyses of the KRAB domains and in a complex with the KAP1-RBCC domain (2, 28). The KRAB domain is predominantly monomeric either alone or in a complex with the KAP1-RBCC protein. Gel filtration chromatography was used to test whether the KRAB-O and SRY protein form a stable complex *in vitro*. Under physiological buffer conditions, the IVT mSRY protein alone (calculated molecular mass of the monomer is 25.3 kDa) eluted in a single peak of ≥44 kDa, suggesting that it may be either a dimer or an extended monomer (Fig. 2*B*). The purified KRAB-O protein alone (calculated molecular mass of the monomer is 9.5 kDa) eluted in a single peak of ≤44 kDa, consistent with either a dimeric form of the protein or a monomeric form with asymmetric shape of protein (Fig. 2*B*). We then tested whether KRAB-O and SRY proteins form a stable complex *in vitro*. GST-KRAB-O was immobilized in GST resin and incubated with IVT mSRY. The protein complex was washed intensively, followed by thrombin digestion. The eluted protein complex was loaded on to a Superdex 75 column. Both proteins co-eluted in
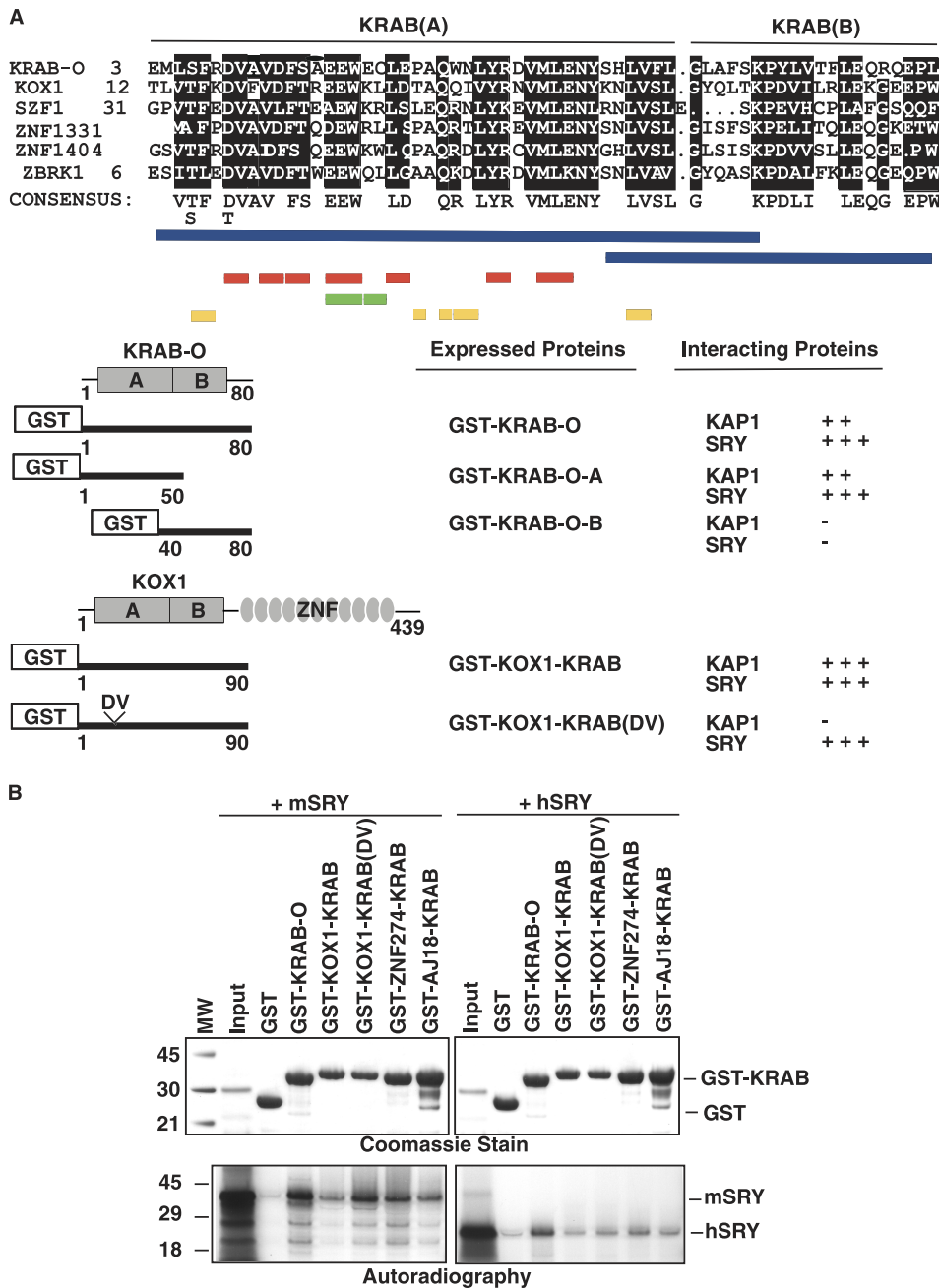
FIGURE 1. **Direct protein-protein interaction between the KRAB domain-containing proteins and the SRY.** *A*, the *top panel* shows the sequence alignment of the KRAB domains from KRAB-O and KRAB-ZFP proteins and the mapping of the binding surfaces of KRAB-O for SRY and KAP1 proteins. The KRAB domain of KRAB-O has an amino sequence that is very similar to other KRAB domains. Each gene encodes the indicated KRAB domain at its NH$_2$ terminus. The *numbers* refer to amino acid positions from various zinc finger proteins. The KRAB domain consensus residues are *highlighted* in *black*. The *periods* represent spaces introduced to obtain maximal alignment. The *blue bar* indicates that the A box is necessary for both KAP1 and SRY binding. The *red bars* represent the mutations in KRAB-O domain abolishing the KAP1 binding. The *green bars* indicate the mutations in KRAB-O domain decreasing the SRY binding. The *yellow bars* indicate the mutations in KRAB-O domain that did not affect KAP1 and SRY binding. The *middle* and the *bottom panels* summarize the interacting proteins for KRAB-O and KOX1 proteins derived from this study by GST association assays. The position of the DV-AA mutation, which disrupts KRAB-mediated repression and association with KAP1, is indicated. The expressed proteins are indicated in the *middle*. The degree of binding of the interacting proteins is indicated by + or − signs at the *right*. The sign − indicates no binding. The sign + + + indicates stronger binding than + +. *B*, direct interaction between the KRAB-O/KRAB domains and SRY proteins. The binding of the GST-KRAB proteins to IVT mSRY and IVT hSRY proteins was detected by GST association assay. The KRAB-O/KRAB proteins directly interacted with both mSRY and hSRY proteins. The *top panel* indicates the GST and GST-KRAB fusion proteins used in each binding reaction mixture. The *bottom panel* shows the associated SRY proteins by the GST-KRAB proteins, and the SRY proteins were detected by autoradiography. *MW*, molecular mass.

the same fractions from the column, suggesting that KRAB-O formed a stable complex with SRY (Fig. 2C). The elution of the KRAB-O protein was shifted one fraction up in complex form, as compared with the pattern when it was eluted alone (Fig. 2, *B* and *C*, compare fraction 10; *B*, *bottom panel* to fraction 9, and *C*, *top panel*). Mouse SRY protein was eluted in the same fraction of the column with or without association with KRAB-O (Fig. 2, *B* and *C*, compare fractions 9 and 10; *B*, *top panel* to fraction 9 and 10, and *C*, *bottom panel*). Taken together, these data suggest that KRAB-O forms a stable complex with mSRY protein, and this complex can be sustained under vigorous chromatography conditions.

To test whether KRAB domain-containing proteins can form a ternary complex with SRY and KAP1, a competition assay was performed with GST-KRAB-O and IVT SRY protein in the presence or absence of KAP1-RBCC protein. KRAB-O, SRY, and KAP1-RBCC proteins formed a ternary protein complex (Fig. 3A). Moreover, the binding of KAP1-RBCC did not interfere with the ability of KRAB-O to bind to SRY, and vice versa, as evidenced by comparing the retention of SRY in the presence (+) and absence (−) of the KAP1-RBCC protein in the competition assay (Fig. 3A). These findings suggest that SRY association with KRAB-O is not dependent on KAP1. To determine whether there is direct interaction between SRY and KAP1, GST association assay was performed with GST-KAP1 and IVT SRY. As expected, the KRAB-O protein directly binds to SRY protein (supplemental Fig. S2). In contrast, no binding of SRY protein to GST or GST-KAP1 was detected (supplemental Fig. S2), suggesting that SRY does not directly interact with KAP1. To determine whether other KRAB domains can form a ternary complex with SRY and KAP1, a competition assay was performed with GST-KOX1-KRAB (WT and DV-AA mutant) proteins and IVT SRY proteins in the pres-
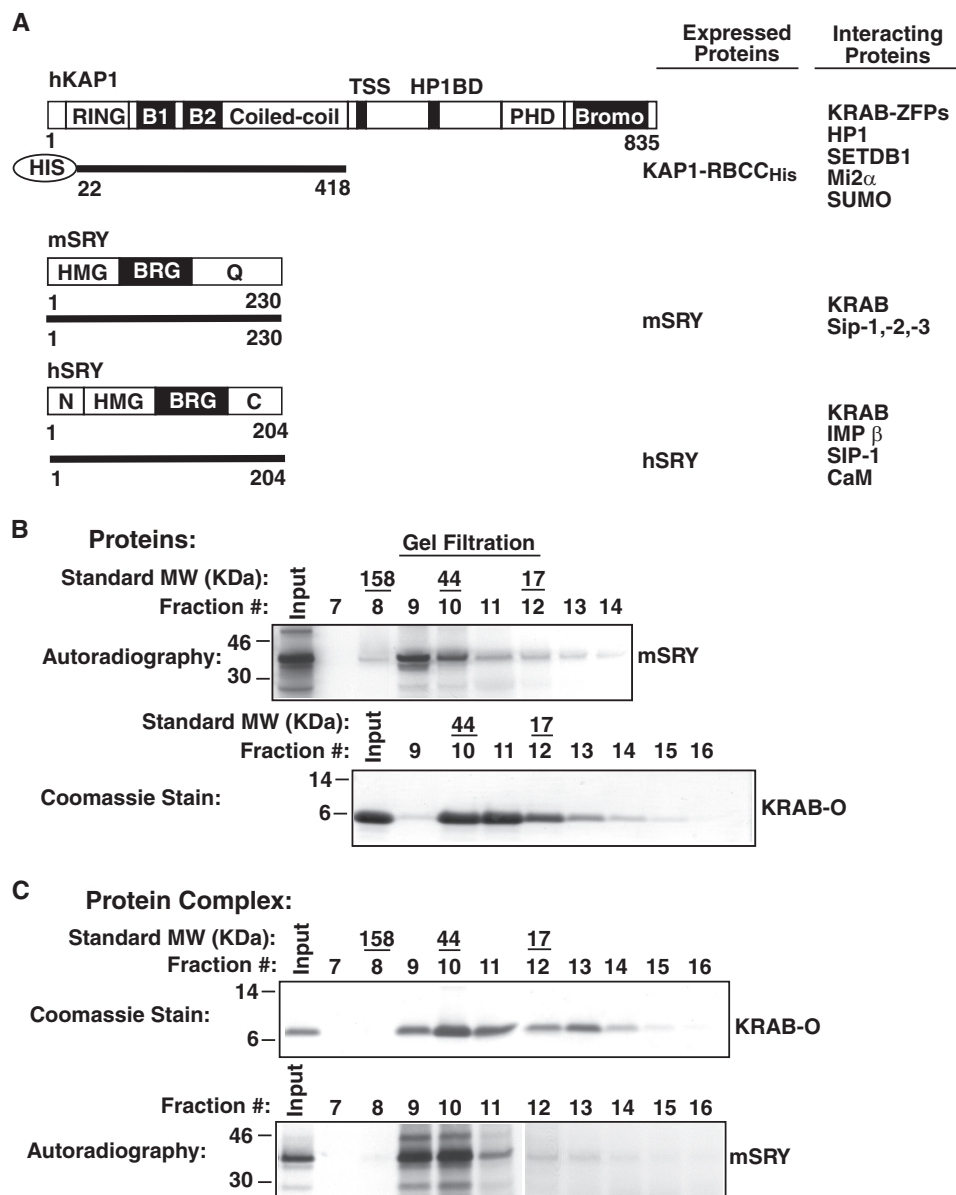
FIGURE 2. **KRAB-O and SRY proteins form a stable complex** *in vitro*. *A*, a representation of hKAP1, mSRY, and hSRY proteins and recombinant derivatives used in this study. The His-tagged KAP1-RBCC (residues 22–418), mSRY (residues 1–230), and hSRY (residues 1–204) proteins were expressed. A summary of the interacting proteins is derived from this study and the literature and is indicated on the *right*. *Sip*, SRY interaction protein; *IMP β*, nuclear import receptor protein importin *β*; *Cam*, calmodulin. *B* and *C*, analyses of KRAB-O protein, mSRY, and a preformed KRAB-O-mSRY complex by gel filtration. The proteins or protein complex were resolved on a Superdex 75 column. The fractions were collected, and the proteins were analyzed by NuPAGE. *B*, the elution profile of individual proteins. The IVT mSRY protein eluted in a single peak of ≥44 kDa (*top panel*). The purified KRAB-O protein eluted in a single peak of ≤44 kDa (*bottom panel*). *C*, the elution profile for a preformed KRAB-O-mSRY complex. The KRAB-O protein shifted one fraction up in the complex compared with when it was alone (fraction 10, *B, bottom panel* to fraction 9; *C, top panel*). The mSRY protein eluted in the same place in protein complex and when it was alone (compared with the *top panel* of *B* and the *bottom panel* of *C*). *MW*, molecular mass.

KRAB-O/KRAB Domains Associate with SRY and KAP1 via Different Binding Surfaces—To examine whether KRAB domains interact with SRY and KAP1 via different binding surfaces, KRAB-O as well as other KRAB domains (WT and mutant) from other ZFPs were used in competition assays, which were performed under three different conditions as described under "Experimental Procedures": 1) equilibrium (Fig. 3*C*), 2) GST-KRAB fusion proteins first saturated with KAP1-RBCC protein (supplemental Fig. S3*A*), and 3) GST-KRAB fusion proteins first saturated with SRY protein (supplemental Fig. S3*B*). The KRAB-O and KRAB domains of other KRAB-ZFPs interacted with SRY and KAP1 proteins simultaneously. The KRAB-O protein showed a higher binding affinity for SRY protein than other KRAB domains under these experimental conditions (Fig. 3*C* and supplemental Fig. S3). These data indicate that KRAB, SRY, and KAP1 formed a ternary complex. Although it did not bind to the KAP1 under all of these experimental conditions, the KOX1-KRAB DV-AA mutant protein still interacted with SRY proteins (Fig. 3*C* and supplemental Fig. S3). These data, when taken together, suggest that KRAB domains associate with SRY and KAP1 via different binding surfaces. Moreover, SRY interacts with the KRAB domains of numerous KRAB-ZFPs in addition to KRAB-O.

To define the molecule binding surfaces on KRAB-O that are specific for KAP1 and SRY, we made deletions and mutations in KRAB-O (Fig. 1*A*). The deletions were made of either the A box or the B box (Fig. 1*A*, *blue*). All of the mutations were

ence or absence of the KAP1-RBCC protein. The DV-AA mutation in the KRAB domain prevents its ability to associate with KAP1. The binding of KAP1-RBCC did not interfere the ability of KOX1-KRAB to bind to SRY (Fig. 3*B*). Moreover, the DV-AA mutant of GST-KOX1-KRAB still exhibited an ability to bind SRY protein, suggesting that the SRY association with KOX1-KRAB is not dependent on KAP1 (Fig. 3*B*). Taken together, these data suggest that KRAB domains can bind KAP1 and SRY simultaneously, and these proteins form a ternary complex *in vitro*.

made by the substitution of the conserved and the nonconserved amino acids to alanines. The effects of these mutations on KAP1 and SRY binding are summarized in Fig. 1*A* (*red and green*, respectively). Mutations that do not affect KAP1 and SRY binding are also noted (Fig. 1*A*, *yellow*). The KRAB-O (deletion and mutant) proteins were expressed as a GST fusion, purified in *Escherichia coli*, and then analyzed for KAP1-RBCC and SRY protein binding (Fig. 4). Previous studies indicated that the A box of KRAB domain is necessary for binding to KAP1 in a manner in which it confers its repression function, whereas the
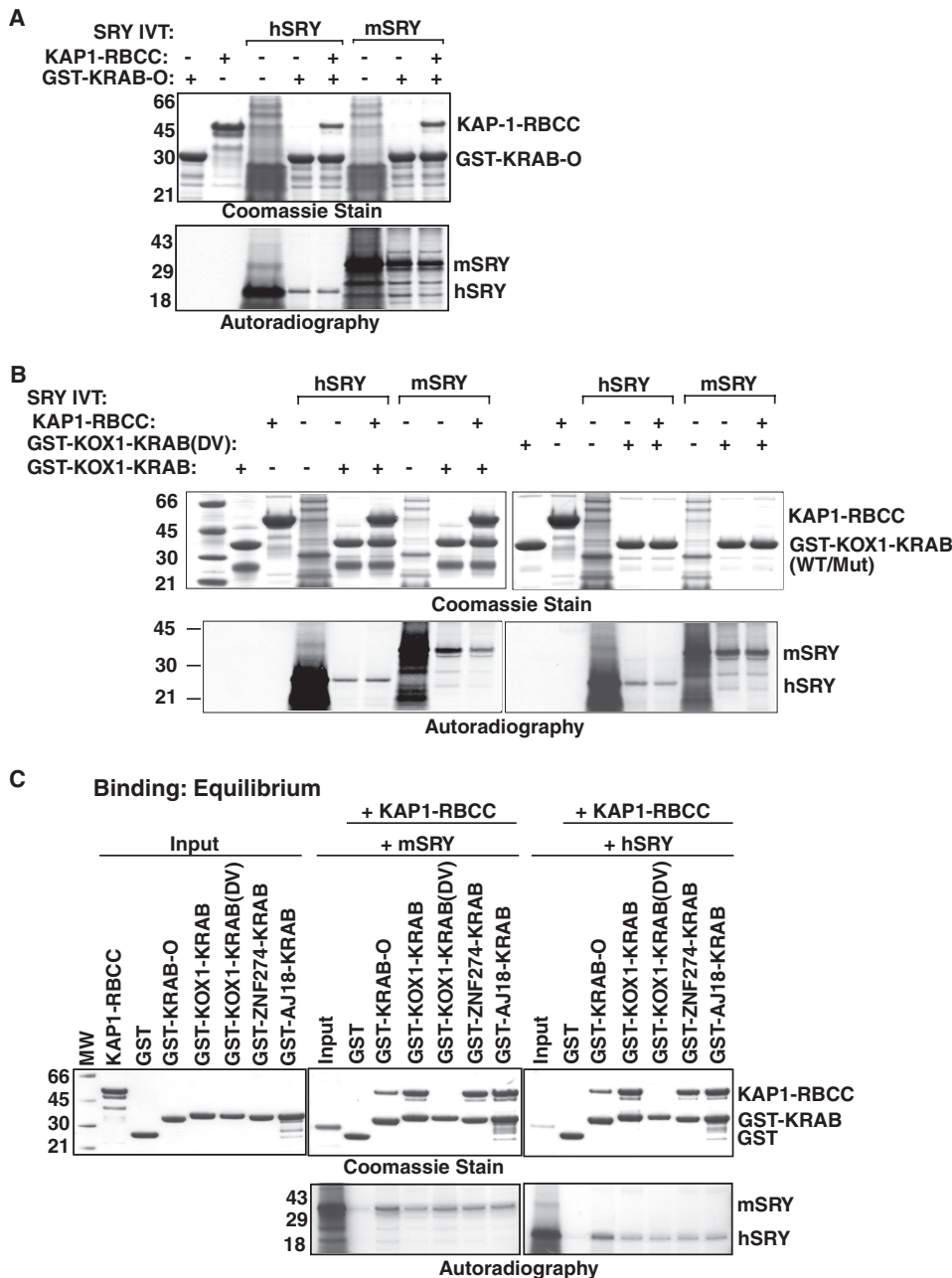
FIGURE 3. *A* and *B*, binding of KAP1 protein to the KRAB-O/KRAB proteins did not disrupt their binding to SRY proteins. Competition assays were performed by mixing either GST-KRAB-O (*A*) or GST-KOX1-KRAB or DV-AA mutant proteins (*B*) and either IVT hSRY or IVT mSRY proteins in the presence or absence of the purified KAP1-RBCC protein. Five μg of GST-KRAB-O/KRAB fusion protein was immobilized on glutathione-Sepharose, followed by adding 20 μl of IVT SRY in the presence or absence of purified KAP1-RBCC protein (40 μg). KRAB-O/KRAB, KAP1, and SRY form a ternary complex, and the SRY association with KRAB-O/KRAB is not dependent on KAP1. The *top panel* indicates the purified proteins in the competition reaction. The *bottom panel* displays the retention of SRY by the GST-KRAB-O protein, and the SRY proteins were detected by autoradiography. *C*, KRAB-O/KRAB domains associate with SRY and KAP1 via different binding surfaces. Competition assays were performed under equilibrium conditions as described under "Experimental Procedures." The *top panel* indicates the purified proteins in the competition reaction. The *bottom panel* displays the retention of SRY by the GST-KRAB-O/KRAB proteins, and the SRY proteins were detected by autoradiography.

the hSRY protein (data not shown). These data suggest that the A box and not the B box interacts with both KAP1 and SRY molecules.

To further map the critical amino acid residues in KRAB-O for the KAP1 and the SRY binding, single and multiple substitutions of the conserved and the nonconserved amino acids to alanines were made in the A box (Fig. 1*A*). For the conserved amino acids in the A box, the substitution of E17A,E18A,W19A was shown to abolish the ability of KRAB-O to interact with KAP1 and decrease SRY binding, suggesting that the amino acids EEW make direct contacts to KAP1 and SRY molecules (Fig. 4). However, substitutions of D9A,V10A; V12A,D13A; F14A,S15A; L22A,E23A; Y30A,R31A; and M34A,L35A,E36A that disrupted the binding of KAP1 did not affect the interaction with mSRY protein, indicating that these amino acids specifically interact with KAP1. A similar result was obtained using hSRY (data not shown). For the nonconserved amino acids in the A box, substitution of E20A,C21A decreased the mSRY binding, whereas it did not affect the KAP1 interaction, suggesting that these amino acids are specific for binding to mSRY (Fig. 4). Similar results for both types of substitutions in KRAB-O were obtained with IVT hSRY protein (data not shown). These data indicate that the KRAB domains associated with SRY and KAP1 via different binding surfaces.

KRAB-O/KRAB Domains Function as a Transcriptional Repression Module—Previous studies demonstrated that the KRAB domains from KRAB-ZFPs could repress transcription when fused to a heterologous DNA-binding domain (30–33). To evaluate the transcriptional regulatory properties of KRAB-O, the KRAB-O domain was fused to a heterologous GAL4 DNA-binding domain. Other KRAB domains of KRAB-ZFPs fused to GAL4 were also generated. The GAL4-KRAB-O and the GAL4-KRAB domain proteins strongly repressed luciferase activity (10–14-fold over vector alone) (Fig. 5*A*, *top panel*). All of the GAL4 fusion proteins exhibited similar levels of expression in mammalian cells (Fig. 5*A*, *bottom panel*), suggesting that the repression activity dem-

B box facilitates this binding and optimizes the repression function (2, 30). The present study reveals that KRAB-O acts like other KRAB-ZFP proteins, the A box is necessary for binding of KAP1, and the B box facilitates this binding (Fig. 4*A*). However, the KRAB-O A box binds mSRY more efficiently than the whole KRAB-O molecule, whereas the KRAB-O B box shows little binding to the mSRY protein. Similar results were obtained for

A

B

Coomassie Stain
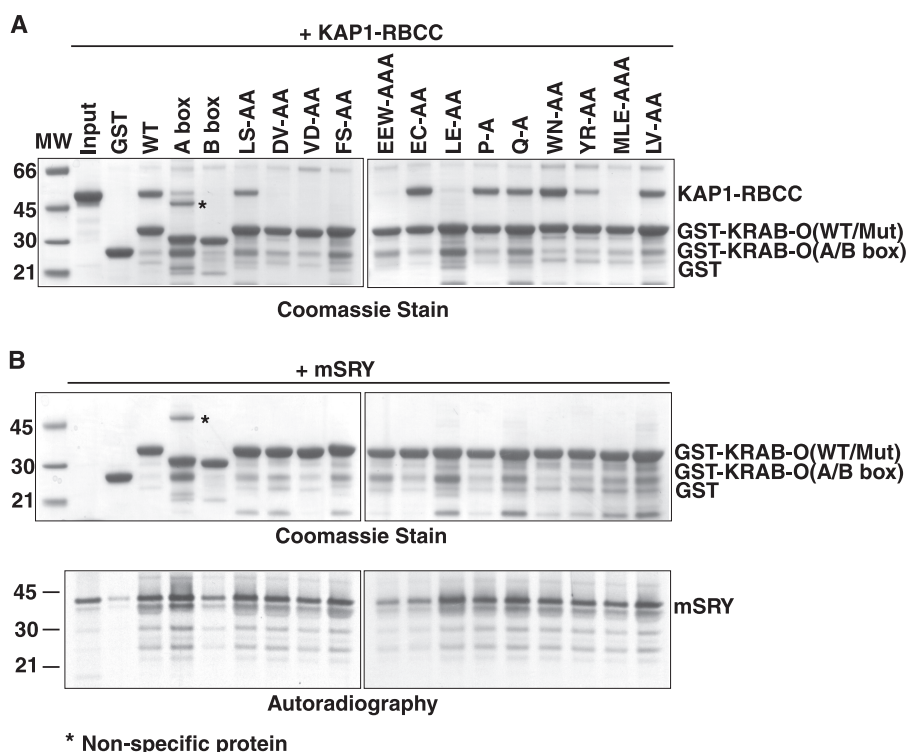
Autoradiography

* **Non-specific protein**

FIGURE 4. **KRAB-O functions as a bridging molecule between the KAP1 and the SRY proteins.** Deletions and mutations of KRAB-O domain were made by mutagenesis. The KRAB-O (deletion and mutant) proteins were expressed as GST fusion, purified in *E. coli*, and analyzed for KAP1-RBCC and SRY protein binding. *A*, the binding of purified KAP1-RBCC protein to WT of GST-KRAB-O but no binding to B box and mutant forms (DV-AA, VD-AA, FS-AA, EEW-AAA, LE-AA, and MLE-AAA) as detected by GST association assay. Forty μg of KAP1-RBCC protein were added to each binding reaction mixture in which 5 μg of GST-KRAB fusion protein was already immobilized on glutathione-Sepharose. *B*, the binding of IVT mSRY to a WT of GST-KRAB-O but less binding to B box and mutant forms (EEW-AAA and EC-AA) as detected by GST association assay. Twenty μl of IVT mSRY were added to each binding reaction mixture in which 5 μg of GST-KRAB fusion protein was already immobilized on glutathione-Sepharose. The *top panel* indicates the GST or GST-KRAB-O proteins in the GST association assay. The *bottom panel* displays the retention of mSRY by the GST-KRAB-O proteins, and the mSRY protein was detected by autoradiography. No binding was detected for GST alone. *MW*, molecular mass.

onstrated by GAL4-KRAB-O is comparable with the other GAL4-KRAB domains. The GAL4-KRAB-O DV-AA mutant protein lost the transcription repression activity (data not shown), which was consistent with the other KRAB DV-AA mutant proteins. Similarly, the GAL4-KRAB-O EEW-AAA mutant protein that abolished KAP1 binding also lost the repression function (Fig. 5*B*). Although the EC-AA mutation decreased SRY binding, it did not affect KAP1 association. As a result, the KRAB-O EC-AA mutant did not diminish repression function (Fig. 5*B*). These data are consistent with the GST association assay (Fig. 4) and suggest that the loss of SRY binding ability does not affect KAP1-mediated repression in this assay system.

We used a cell-based reporter assay in culture cells to investigate the postulated mechanism of KRAB-O/KRAB-KAP1 transcription repression machinery for SRY regulatory action on sex determination and differentiation. Although SRY target promoters were identified using integrated computational and experimental genomics approaches (34) and SOX9 had been demonstrated to be regulated by SRY(24,25), there is currently no definitive repressive target genes of SRY identified in fetal gonads and no cell line that maintains the pre-Sertoli cell properties. The present study utilized two reporter systems, LexA-TK-Luc (a luciferase reporter

gene harboring a minimal promoter with multiple LexA-binding sites) and 10xSRY-TK-Luc (a luciferase reporter gene harboring a minimal promoter with multiple SRY-binding sites) (35), to investigate the SRY-KRAB-O-KAP1 transcription repression pathway. First, SRY was fused to a heterologous LexA DNA-binding domain. The LexA-SRY expression plasmid was co-transfected with KOX1-KRAB (WT or DV-AA mutant) or KRAB-O (WT or mutant), in addition to a LexA-TK-Luc reporter plasmid into U2OS cells. The WT KOX1-KRAB repressed luciferase activity, whereas mutant form DV-AA lost repression activity, indicating that the KAP1 is required for SRY-mediated repression (Fig. 5*C*). We then tested whether KRAB-O with LexA-SRY exerts the repression activity on the 10xSRY-TK-Luc reporter system utilizing SRY-KRAB-O-KAP1 repression complex. We found that the WT KRAB-O repressed luciferase activity as expected (Fig. 5*D*). Three different mutants (DV-AA, EC-AA, and EEW-AAA) of KRAB-O lost their repression activity (Fig. 5*D*). The WT and mutants of KRAB-O with LexA did not repress luciferase activity (data not

shown). The DV-AA mutant failed to bind to KAP1. Although the EC-AA mutant retained its ability to interact with KAP1, it could not tether the KRAB-O-KAP1 complex to SRY. Moreover, the EEW-AAA abolished KAP1 binding and decreased SRY interaction. These data are consistent with the biochemical data (Fig. 4), suggesting that SRY may serve as the tether for KRAB-KAP1-mediated repression in same paradigms. Second, we introduced hSRY, KOX-KRAB (WT and DV-AA mutant), and 10xSRY-TK-Luc into U2OS cells. We demonstrated that hSRY could indeed activate the reporter gene harboring multiple SRY-binding sites at its promoter (Fig. 6) but not the parental reporter without the SRY-binding sites (data not shown). When WT KOX1-KRAB was co-transfected with the hSRY, the SRY transactivation activity was repressed in a dose-dependent manner (Fig. 6). The SRY transactivation activity was not affected when KOX1-KRAB (DV19,20AA) was co-transfected with the hSRY gene (Fig. 6), because this mutant KOX1-KRAB protein failed to bring the KAP1 repression machinery to mediate the SRY reporter gene. These data strongly support the proposed working model of SRY transcription repression via KRAB-O/KAP1 interaction.

SRY Transcription Factor Utilizes the KRAB-O/KRAB-KAP1 Transcription Repression Machinery to Represses Its Target
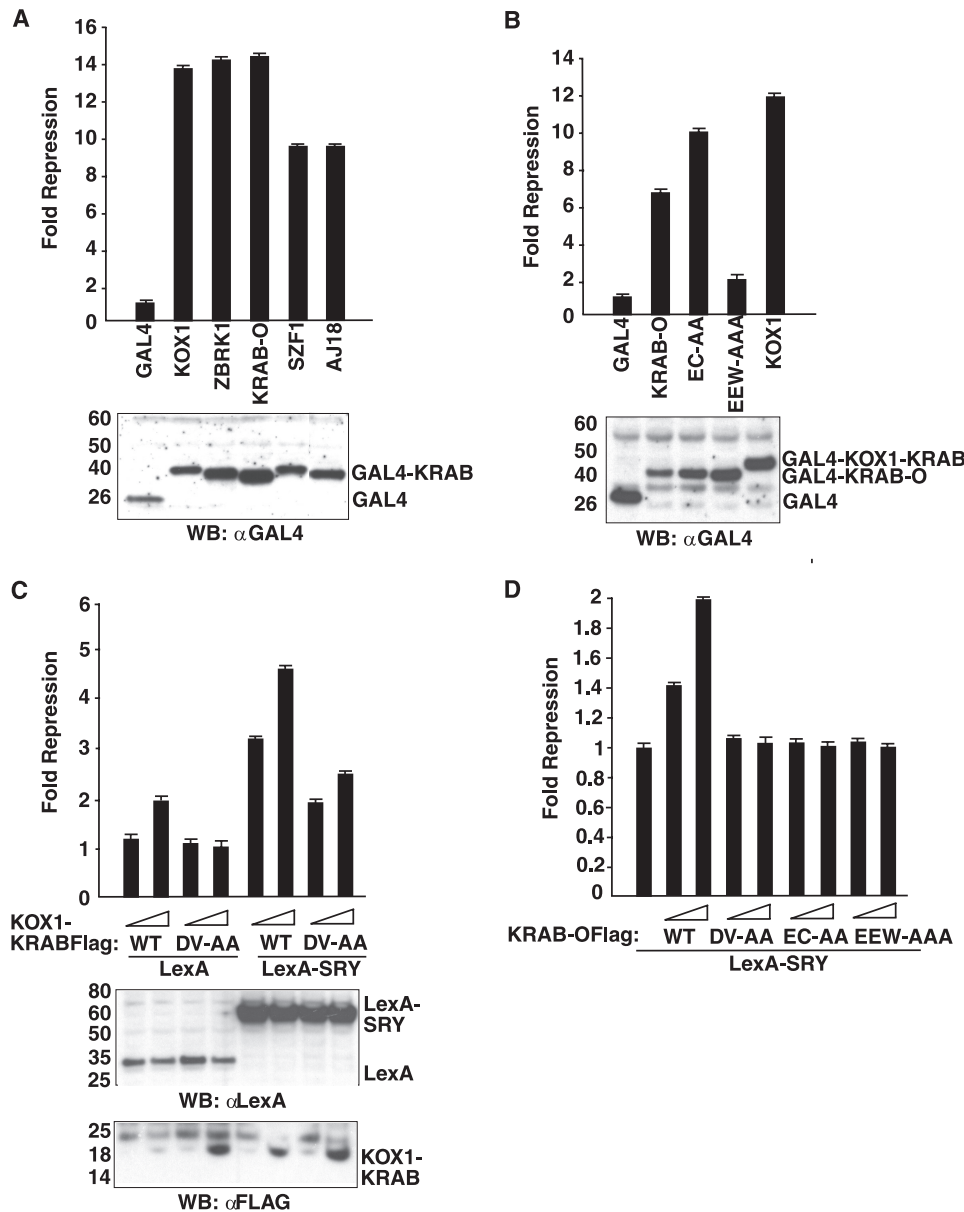
FIGURE 5. **The KRAB-O/KRAB protein expression confers binding site-dependent transcriptional repression of a TK luciferase reporter plasmid.** *A* and *B*, the KRAB-O (WT and mutant) and KRAB domains of KOX1, ZBRK1, SZF1, and AJ18 were expressed with the DNA-binding domain of GAL4, residues 1–147. HEK 293 cells (6-well plate) were transiently transfected with the expression plasmids (0.5 μg) along with a cytomegalovirus-β-D-galactosidase plasmid (0.1 μg) and a TK luciferase reporter plasmid (0.05 μg) containing five GAL4 upstream activating sequence-binding sites. Loss of SRY binding surface did not affect KAP1-mediated repression. *C* and *D*, U2SO cells (6-well plate) were transiently transfected with KOX1-KRAB (WT and DV-AA mutant) and KRAB-O (WT and mutants) (0.1 and 0.3 μg) along with LexA or LexA-SRY (0.5 μg), a cytomegalovirus-β-D-galactosidase plasmid (0.1 μg), and a TK luciferase reporter plasmid (0.05 μg) containing LexA-binding sites. *Fold Repression*, ratio of luciferase activities of empty expression vector relative to that obtained with the indicated expression plasmids. All of the activities were normalized for transfection efficiency based on β-D-galactosidase activity. The stable expression of each protein was determined by Western blot (*WB*) with anti-GAL4 (DNA-binding domain) IgG, anti-LexA IgG, and anti-FLAG IgG. The data are from two independent experiments. Each was performed in duplicate.

Sequence—We had demonstrated that SRY, KRAB-O/KRAB, and KAP1 proteins formed a stable ternary protein complex *in vitro*. We next attempted to determine whether such ternary protein complexes exist *in vivo* using immunoprecipitation and Western blotting strategy. We introduced the hSRY and KRAB-O (WT and mutants) plasmids into U2OS cells. Both WT and mutant KRAB-O proteins were expressed equally in this system (supplemental Fig. S4, *top panel*). However,

WT KRAB-O bound more efficiently to the hSRY than its mutant forms *in vivo* (supplemental Fig. S4, *middle panel*). More importantly, the KAP1 protein was associated with only the WT KRAB-O but not any of the mutant forms of the protein (supplemental Fig. S4, *bottom panel*). These data suggest that SRY, KRAB-O, and KAP1 formed a protein complex *in vivo*. We also observed that the SRY, KOX1-KRAB, and KAP1 could form a protein complex similarly *in vivo* (data not shown). To address the question of whether these protein complexes indeed are recruited to target promoter on the chromatin template, ChIP assay was performed in independent transfections in different cell lines. The hSRY, KRAB-O, and/or KAP1, 10xSRY-TK-Luc reporter genes were transiently transfected into U2OS, and subjected to ChIP analysis with primers specific to the TK promoter. As expected, the hSRY protein bound to the promoter (Fig. 7, *A* and *B*, *top panels*). Consistently, WT and DV-AA mutant KRAB-O proteins were recruited to the TK promoter by SRY (Fig. 7, *A* and *B*, *middle panels*). The enrichment of DV mutant of KRAB-O is equivalent to the WT of KRAB-O. Furthermore, KAP1 was only enriched at the TK promoter in cells transfected with WT KRAB-O but not DV-AA mutant KRAB-O (Fig. 7, *A* and *B*, *bottom panels*). Overall, the trend of the data from Fig. 7 is the same and shows that the level of enrichment of KAP1 is higher in the presence of WT of KRAB-O than in the presence of the DV mutant of KRAB-O. Together, these results suggest that SRY-KRAB-KAP1 protein complexes were formed in the promoter region, and KRAB-KAP1 transcription machinery was recruited to the target promoter through SRY protein.
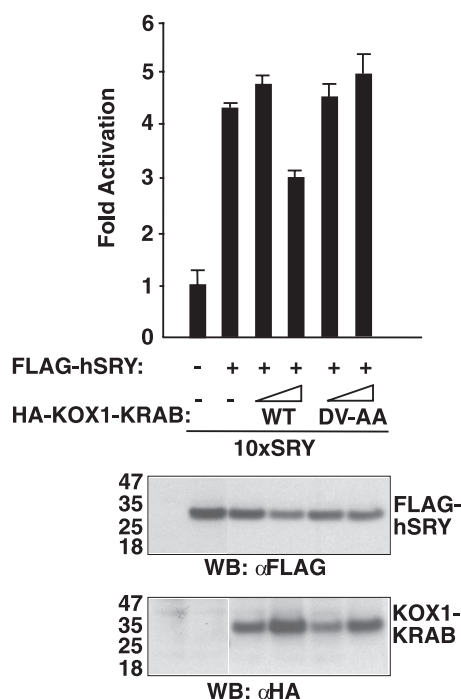
DISCUSSION

In this study, we have characterized the protein-protein interactions for KRAB-O/KRAB, SRY, and KAP1 proteins using various biochemical approaches. We have also evaluated the transcriptional regulation activities of KRAB-O/KRAB and SRY proteins. We have drawn the following conclusions from

FIGURE 6. **KRAB-KAP1 transcription machinery was recruited to the target promoter through SRY protein.** The WT and mutant KRAB-O (0.1 and 0.3 μg), hSRY (0.5 μg), a cytomegalovirus-β-D-galactosidase plasmid (0.1 μg) and 10xSRY-TK-Luc genes (0.05 μg) were transiently transfected into U2OS cells (6-well plate). *Fold Activation*, ratio of luciferase activities of indicated expression plasmids relative to empty expression vector. All of the activities were normalized for transfection efficiency based on β-D-galactosidase activity. The stable expression of each protein was determined by Western blot (*WB*) with anti-FLAG IgG and anti-HA IgG. The data are from two independent experiments. Each was performed in duplicate.

our data. First, the interaction between KRAB-O/KRAB protein and SRY protein is direct, but KRAB-O is not the only KRAB protein capable of interacting with SRY; other KRAB domains from KRAB-ZFPs also interact with SRY. However, it appears that KRAB-O has a higher binding affinity for SRY proteins than other KRAB domains. Second, KRAB-O/KRAB can bind SRY and KAP1 simultaneously, thereby forming a ternary complex *in vitro*. The KRAB-O/KRAB binding to SRY does not interfere with its binding to KAP1 because KRAB-O/KRAB domains associate with SRY and KAP1 via different binding surfaces. Third, the A box of KRAB-O is essential for the KAP1 and SRY protein binding. Although the B box of KRAB-O facilitates the KAP1 binding, it inhibits SRY binding by itself. Fourth, KRAB-O possesses transcription repression activity equivalent to that of other KRAB domains via the KAP1 association. Lastly, SRY, KRAB-O/KRAB, and KAP1 form a ternary protein complex *in vivo* and SRY can recruit such KRAB-O/KRAB-KAP1 repression machinery to repress its target promoter at the chromatin level.

Previous studies using purified recombinant proteins indicate that the KRAB domains from KRAB-ZFPs exist as a monomer either alone or in complex with KAP1 (2, 28). We now demonstrate that KRAB-O also exists as a monomer either alone or in a complex with SRY. This observation is consistent with biochemical and biophysical characterization of the other KRAB domains. On the other hand, only a few biochemical and biophysical characterizations for the SRY protein with its bind-
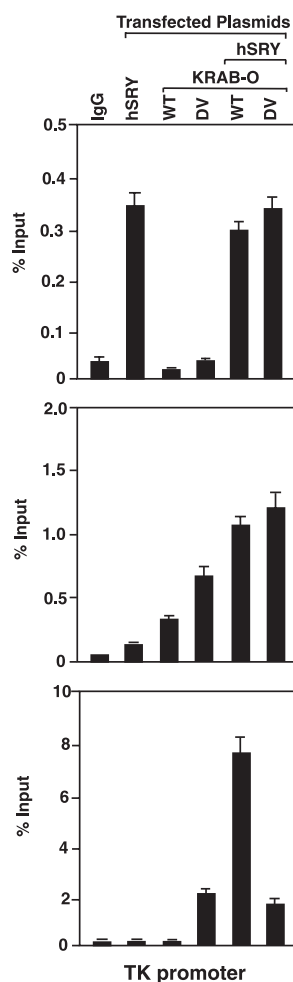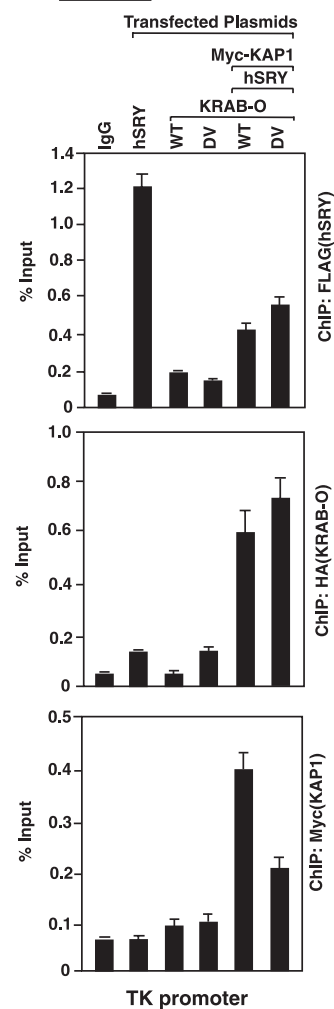

FIGURE 7. **KRAB-KAP1-mediated repression machinery was recruited to the TK promoter through SRY protein.** U2OS cells were transfected with FLAG-hSRY, WT, and DV-AA mutant of HA-KRAB-O and/or Myc-KAP1, together with 10xSRY-TK-Luc plasmid (20 μg DNA was used per 15-cm plate for transfection) and subjected to ChIP analysis. The data are from two independent experiments. Each was performed in duplicate.

ing protein complexes have been reported. We now show that SRY exists as either a dimer or an extended monomer. Moreover, the oligomerization properties of SRY do not change when it forms a complex with the KRAB-O protein. This protein-protein interaction is stable when subjected to *in vitro* biochemical manipulation as detected by a GST association assay and gel filtration. These data provide biochemical evidence for a direct protein-protein interaction between KRAB-O/KRAB and SRY proteins. However, the stoichiometry of KRAB-O/KRAB and SRY molecules and the structure of the protein complex remain to be determined.

KRAB-O has been postulated to function as a bridging molecule between KAP1 and SRY proteins (11). Here we have provided biochemical evidence supporting this hypothesis. In the proposed model, the SRY recruits the KRAB-KAP1 complex as a chromatin modulator. Our studies indicate that the KRAB-O/KRAB domain can simultaneously interact with KAP1 and SRY molecules forming a ternary complex. The binding of KRAB-
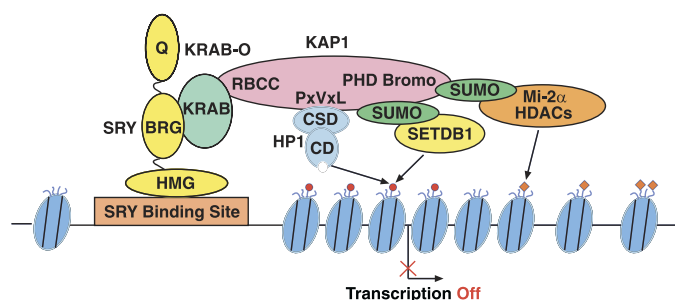
FIGURE 8. **Model for the SRY utilizing the KRAB-O/KAP1-mediated repression complex.** This model is described under "Discussion."

O/KRAB to KAP1 does not interfere its binding to SRY and vice versa. Furthermore, we have mapped the KRAB-O binding surfaces that are specific for KAP1 and SRY molecules. The A box of KRAB-O is necessary for the binding of KAP1, and the B box enhances this binding. This observation is consistent with previous published *in vivo* studies (30, 36). On the other hand, the KRAB-O A box binds a SRY molecule with higher affinity than the whole molecule of KRAB-O, suggesting that the B box somehow interferes with the SRY binding. These data suggest that KRAB-O/KRAB domains may associate with SRY and KAP1 via different binding surfaces. Our mutation studies support this postulation. The conserved amino acids *EEW* (17–19) of KRAB-O (binding surface highlighted in bold italic) directly contact KAP1 and SRY molecules. The mutation of these amino acids abolished or decreased the ability of KRAB-O associated with these proteins *in vitro* (Fig. 1*A*). The conserved amino acids *DVA VDFS* (9–15) are specific for the KAP1 binding, whereas the nonconserved amino acids *EC* (20–21) are specific for the SRY binding. Therefore, the binding surface for the KAP1 is mainly on the amino acid residues *DVA VDF-SAEEWECLE*, whereas the binding surface for the SRY is mainly on the residues *EEWEC* in KRAB-O. These data strongly support the notion that the nonconserved amino acids of the A box are specific for its interactive partner, whereas the conserved amino acids are obligatory for linking the complex to the KAP1 repression machinery.

Consistent with the other KRAB domains, the KRAB domain in KRAB-O functions as a transcriptional repression module. The repression activity of KRAB-O is comparable with the repression activity exhibited by the KRAB domains of other ZFPs. Mutation studies indicated that the loss of the SRY binding surface on KRAB-O did not affect KAP1-mediated repression in GAL4 reporter assay via GAL-KRAB (Fig. 5*B*), although it did affect SRY-mediated repression in a LexA reporter assay via LexA-SRY (Fig. 5*D*). Moreover, the loss of either KAP1 or SRY binding surfaces or both binding surfaces abolished KRAB-O/KAP1-mediated repression in a LexA reporter assay via LexA-SRY, suggesting that SRY serves as the tether for KRAB-O/KAP1-mediated repression (Fig. 5). Furthermore, the KRAB-KAP1-mediated repression attenuated the SRY transactivation activity when it was recruited to the SRY binding sequence promoter via SRY protein (Fig. 6). These results completely support our working model that SRY recruits the KRAB/KRAB-O-KAP1-mediated repression complex to regulate specific target genes (Fig. 8). The data are consistent with the finding that the promoters bound by OCT4 and SRY were also bound by the transcriptional repressor KAP1 by using integrated computational and experimental genomic approaches (34).

The primary goal of this study was to provide biochemical evidence and biological function to support the proposed working model for SRY-mediated gene silencing. In this model, KRAB-O functions as a molecular switch for the action of SRY transcription during sex determination and differentiation. However, equally important are the implications that other yet to be identified KRAB domains lacking zinc finger motifs may function in a similar manner with the other transcription factors. The KRAB domain is currently represented in more than 400 proteins, most of which are C_2H_2 zinc finger proteins and are likely to function as gene-specific repressors. Although we have mainly focused on the SRY-KRAB-O-KAP1 ternary complex, we found that the KRAB domains from other KRAB-ZFPs also interact with SRY proteins. Our findings are complementary to the *in vivo* functional analysis of the SRY-KRAB interaction in mouse sex determination and address the questions raised by their studies (14). Lately it has been reported that OCT4, SRY, and KAP1 bind to a common set of target promoters by using integrated computational and experimental genomics approaches. OCT4 is a key regulator in maintaining the pluripotency and self-renewal of human embryonic stem cells, germ cells, and tumor cells (34). The current working model for SRY utilizing the KRAB-O/KAP1-mediated repression complex is illustrated in Fig. 8. In this model, SRY binds to DNA in a sequence-specific manner through its HMG motif. The DNA-bound SRY protein recruits the KRAB-O/KRAB-KAP1 repression complex via a direct interaction using its BRG domain. Furthermore, the KAP1 co-repressor functions as a molecular scaffold that coordinates activities that regulate chromatin structure, including the HP1 family of heterochromatin protein, NURD histone deacetylase complex, SETDB1 histone methyltransferase, and small ubiquitin-like modifier protein modification, thereby repressing the ovarian differentiating gene, the most important event in the early stages of testis determination and differentiation.

In summary, we have provided the first analysis of direct protein-protein interactions involving KRAB-O/KRAB protein and its physiologically relevant proteins. The KRAB-O/KRAB domain directly interacts with these molecules and functions as a molecular switch for the action of the SRY transcription factor during sex determination and differentiation and/or during oncogenesis when SRY is ectopically expressed in cancer cells. These studies have highlighted the apparently high specificity for recognition inherent in these protein-protein interfaces. Further refinement of these macromolecular interactions could lead to strategies for KRAB-KAP1-mediated repression *in vivo* using synthetic transcription factors.

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