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SLIRP, a Small SRA Binding Protein, Is a Nuclear Receptor Corepressor

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Summary

Steroid receptor RNA activator (SRA), the only known RNA coactivator, augments transactivation by nuclear receptors (NRs). We identified SLIRP (*S*RA stem-*l*oop *i*nteracting *R*NA binding *p*rotein) binding to a functional substructure of SRA, STR7. SLIRP is expressed in normal and tumor tissues, contains an RNA recognition motif (RRM), represses NR transactivation in a SRA- and RRM-dependent manner, augments the effect of Tamoxifen, and modulates association of SRC-1 with SRA. SHARP, a RRM-containing corepressor, also binds STR7, augmenting repression with SLIRP. SLIRP colocalizes with SKIP (Chr14q24.3), another NR coregulator, and reduces SKIP-potentiated NR signaling. SLIRP is recruited to endogenous promoters (pS2 and metallothionein), the latter in a SRA-dependent manner, while NCoR promoter recruitment is dependent on SLIRP. The majority of the endogenous SLIRP resides in the mitochondria. Our data demonstrate that SLIRP modulates NR transactivation, suggest it may regulate mitochondrial function, and provide mechanistic insight into interactions between SRA, SLIRP, SRC-1, and NCoR.

Introduction

Coregulators, functioning as coactivators or corepressors of nuclear receptor (NR) activity, play pivotal roles in mediating hormone action via the regulation of transcriptional efficiency (McKenna and O'Malley, 2002). The discovery of steroid receptor coactivator-1 (SRC-1), a broad-spectrum coactivator (Onate et al., 1995), provided important insight into the mechanisms underlying transcriptional activation by NRs. Since then, a large family of coregulators has been discovered, each of which is selectively recruited by specific NRs in a ligand- and tissue-specific manner to cognate response elements in the genome (Robyr et al., 2000; McKenna and O'Malley, 2002).

For the estrogen receptor (ER), a NR that plays a key role in the proliferation of breast cancer cells (Herynk and Fuqua, 2004), a large number of coregulators have been identified. These include SRC-1 (Onate et al., 1995), SHARP (Shi et al., 2001), and SRA (Lanz et al., 1999). Remarkably, SRA (steroid receptor RNA activator) is the only known coregulator that has the capacity to coactivate as an RNA and for this reason stands alone in its functional characteristics.

SRA plays an important role in mediating 17β-estradiol (E2) action (Lanz et al., 1999, 2003). Its expression is aberrant in many human breast tumors, suggesting a potential role in tumorigenesis (Murphy et al., 2000). Despite evidence that an alternative splice variant of SRA exists as a protein (Chooniedass-Kothari et al., 2004), it has been conclusively shown that SRA can function as an RNA transcript to coactivate NR transcription (Lanz et al., 1999, 2002, 2003). While it is currently hypothesized that SRA acts as an RNA scaffold for other coregulators at the transcription initiation site, the precise mechanism by which SRA augments ER activity remains unclear.

Recent findings have identified protein interactors of SRA and provided insight into the putative mechanisms underlying SRA's transcriptional coactivation ability. Specifically, SHARP (SMRT/HDAC1 associated repressor protein) is a NR corepressor that interacts with SRA in vitro and contains three RNA recognition motifs (RRMs) (Shi et al., 2001). These RRMs are required by SHARP to repress SRA-augmented E2-induced transactivation (Shi et al., 2001). Another ER coregulator that binds SRA in vitro and copurifies with SRA from cell extracts is p72 (Wantanabe et al., 2001). Thus, although SRA-protein interactions impact significantly on NR activity and signaling, the specifics of interactions remain unclear, and the identity of SRA binding proteins whose function is dependent upon targeting specific SRA substructures is unknown.

We sought to identify SRA binding proteins using a specific stem-loop structure of SRA (stem-loop structure 7, STR7) that was identified both as important for its coactivator function (Lanz et al., 2002) and also as a target for proteins from breast cancer cell extracts. Here we describe the isolation and characterization of SLIRP (SRA stem-loop interacting RNA binding protein), a widely expressed small SRA binding protein, that is a repressor of NR signaling. SLIRP functions in an additive manner with SHARP to further repress ER activity and augments the estrogen antagonistic effects of 4-hydroxytamoxifen (Tam) and ICI 182780 (ICI).

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Although SLIRP is localized predominantly to the mitochondria, it is actively recruited to hormone-responsive promoters where it modifies NR cofactor recruitment and transactivation. Interestingly, SLIRP lies adjacent to SKIP (Ski-interacting protein), another NR coregulator (MacDonald et al., 2004), in the genome and antagonises SKIP's coactivation of the ER. Taken together, these data suggest a key role for SLIRP as a corepressor, modulating several NR pathways.

Results

SRA STR7 Is a Target for Proteins in Human Breast Cancer Cells

SRA is a complex RNA molecule predicted to contain multiple stable stem-loop structures (Figure 1A) (Lanz et al., 2002). STR7, an 89 nucleotide (nt) sequence, is the largest and one of the most stable stem-loop SRA structures that functions in a cooperative manner with other stem loops to augment ER transactivation (Lanz et al., 2002). Based on these observations, we first investigated if SRA STR7 was a target for RNA binding proteins in RNA electrophoretic mobility shift assay (REMSA) studies. While weak RNA-protein complexes (RPCs) formed with cytoplasmic extracts from MCF-7, MDA-MB-468, and HeLa cell lines, two strong RPCs were evident in the nuclear extracts of each (Figure 1B). Addition of excess unlabeled STR7 effectively abrogated RPC formation (Figure 1C, lane 3). However, addition of ~100-fold excess of either unlabeled vector transcript (pBlue) or yeast tRNA competitor RNA did not diminish the formation of RPCs in HeLa cell nuclear extracts (Figure 1C, lanes 4–7). Taken together, these data indicate a highly specific interaction between SRA STR7 and nuclear proteins from human cancer cells.

UV crosslink (UVXL) studies were performed to further characterize RPC formation with the STR7 riboprobe. Multiple STR7-protein complexes were identified from each of the nuclear cell extracts (Figure 1D). Although many bands were common, there were some significant differences between cell types. For example, two RPCs, of ~39 and 40 kDa, were more prominent in extracts from MDA-MB-468 cells than in MCF-7 and HeLa extracts (Figure 1D). These data show that an array of nuclear proteins bind SRA STR7 in vitro.

Cloning of SLIRP, a SRA Binding Protein

To isolate SRA binding proteins, we used SRA STR7 as bait in a yeast three-hybrid screen (SenGupta et al., 1996) of a primary human breast cancer cDNA library (Byrne et al., 1998). Mfold secondary (2º) structure analysis indicated that the STR7 stem-loop structure was preserved in the hybrid RNA transcribed from the pIIIA/MS2-2 bait construct. From the screen, we isolated a cDNA clone that contained an open reading frame with a 3′ untranslated region (UTR) and polyadenylated (poly A) tail (Figure 2A). The cDNA sequence predicted a protein of 109 amino acids (aa), with a M_r of 12.7 kDa. Database analysis revealed that this clone, which we termed SLIRP, was identical to human sequences Hypothetical Protein DC50 (GenBank accession number [GAN], AF271779) and Chr 14 Open Reading Frame 156 (C14orf156) (GAN, BC017895).

The SLIRP mRNA sequence (GAN, AY860853) predicts a protein composed almost entirely of an RRM (Figure 2A) containing RNP1 and RNP2 submotifs (Burd and Dreyfuss, 1994). The RRM domain in SLIRP shares substantial aa homology with SHARP (Figure 2B), a SRA corepressor (Shi et al., 2001), and nucleolin, a canonical RRM-containing protein (Bouvet et al., 1997). Significantly, homology between SLIRP and SHARP, particularly within key interacting residues of their RRMs (Figure 2C), suggests these molecules may bind the same RNA targets, i.e., SRA STR7.

The aa sequence of SLIRP is highly conserved across human, rat, and mouse species (Figure 2D). Curiously, rat and mouse SLIRP homologs are surrounded by the same genes as human SLIRP. This striking sequence conservation suggests an important function for SLIRP in multiple species. Of interest, human SLIRP is positioned within 1750 nt of SKIP, on Chr 14q24.3 with no intervening genes. SKIP is a vitamin D receptor (VDR) corepressor implicated in oncogenesis (Barry et al., 2003; MacDonald et al., 2004). SKIP is expressed in breast cancer tissue and regulates ER transactivation (Barry et al., 2003). Colocalization of these genes suggested that SLIRP and SKIP may participate in the same NR pathways and could be coordinately expressed.

SLIRP Is Expressed Widely in Human Tissues and Cancer Cells

In normal human tissue, SLIRP mRNA is ubiquitously expressed, but in varying amounts, with the highest levels in heart, liver, skeletal muscle, and testis (Figure 3A). SLIRP was readily detected in a variety of cell lines, including SK-BR-3, MCF-7, HMEC, MDA-MB-468, LNCaP, and COS-7 (Figure 3B) and increased in HeLa, Calu-6, and HepG2 cells. Notably, we found that SLIRP expression across multiple tissues (Figure 3A) and cell lines (Figure 3B) was similar to that of SRA (refer to Lanz et al. [1999], Figure 1B).

We generated polyclonal SLIRP antisera (ab) and demonstrated SLIRP protein (M_r ~12.7 kDa) expression in multiple human cell lines, including those derived from breast, prostate, and lung carcinomas (Figure 3C). The ab was highly specific for human SLIRP, with virtually no cross reactivity with a number of other species (see Figure S1 in the Supplemental Data available with this article online). Expression of SLIRP protein varied across different breast cancer cell lines, and in some cells, discordant levels of SLIRP mRNA and protein were observed (e.g., HeLa cells). While expression of SLIRP and SKIP was similar, there was little evidence that their expression at the mRNA or protein level was coordinately regulated.

Immunohistochemistry (IHC) with SLIRP ab on human primary breast cancer tissue showed SLIRP staining in normal ductal tissue, but little in the surrounding stroma (Figures 3Da and 3Db). Intense SLIRP staining was noted in carcinoma tissue (Figure 3Dd) compared to control (Figure 3Dc). Staining was evident throughout the cell, but predominantly with punctate, cytoplasmic distribution (Figures 3Db and 3Dd).

Characterization of SLIRP's Interaction with SRA

To confirm SLIRP's interaction with SRA in vivo, we performed immunoprecipitation-RT-PCR (IP-RT-PCR)

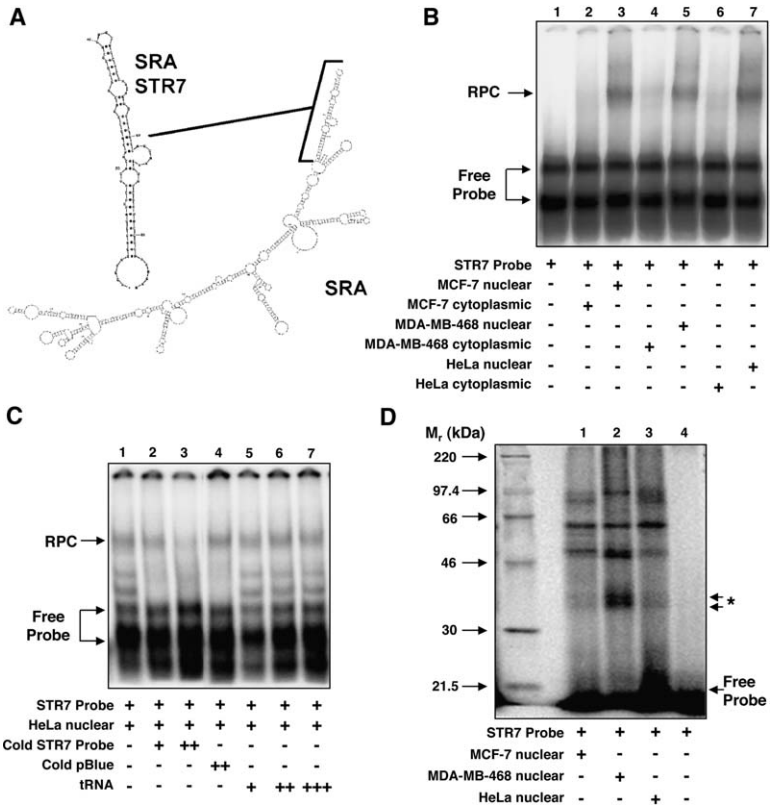

Figure 1. SRA STR7 Is Bound by Human Breast Cancer Cell Proteins

(A) Mfold secondary structure plot of SRA (Zucker, 2003). ΔE value for the full-length SRA structure was −243.1 kJ mol^{-1}. STR7 (labeled) is one of the most stable substructures of SRA.

(B) Comparison of nuclear and cytoplasmic MCF-7, HeLa, and MDA-MB-468 extract binding to SRA STR7 via REMSA.

(C) REMSA with SRA STR7 showing reduced complex formation with unlabeled "cold" competitor STR7, but not excess cold pBlue vector or tRNA. RPC, RNA-protein complex. +–+++, increasing cold competitor RNA.

(D) UV crosslink assay with nuclear extracts from cell lines in (B). Cell extract (30 μg) was incubated with labeled STR7, UV irradiated, RNase A digested, resolved by SDS-PAGE, and detected by PhosphorImager after transfer to PVDF membrane. [^{14}C] molecular weight markers were used as size standards. Arrows with asterisk highlight 39 and 40 kDa RPCs in MDA-MB-468 cells.

assays with SLIRP ab. Using HeLa (Figure 4A), MCF-7, and MDA-MB-468 cells (data not shown), SRA coimmunopurified with SLIRP (Figure 4A, lane 5), but not β actin (Figure 4A, lane 6). Thus, SLIRP closely interacts with SRA mRNA in several different cancer cell lines. As SRA also copurifies with SRC-1 (Lanz et al., 1999), we examined the effects of reducing intracellular SLIRP levels with siRNA and found a corresponding increase in SRC-1 associated with SRA (Figures 4B and 4C). This suggests that competition may exist between SRC-1 and SLIRP for association with SRA in vivo, which could directly impact on their coregulator effects.

Using recombinant SLIRP proteins (Figure 4D), both GST-SLIRP (Figure 4E, lane 2) and cleaved SLIRP (data not shown) bound STR7 avidly, while GST alone did not (Figure 4G, lane 3). Addition of increasing amounts of unlabeled (cold) STR7 probe efficiently competed out the complex (Figure 4E, lanes 3 and 4). In contrast, neither addition of excess unlabeled pBlue (Figure 4E, lanes 5 and 6) nor high amounts of tRNA significantly affected SLIRP-STR7 complex formation (data not shown). Taken together, these results indicate that SLIRP binds STR7 in vitro with a high degree of specificity.

We found binding of GST-SLIRP to the SRA SDM7 probe (a SRA STR7 mutant containing several stem-loop point mutations and having reduced transactivation activity compared to wild-type) (Lanz et al., 2002) was consistently reduced compared to the STR7 probe (up to 2.9-fold) (Figure 4F, lane 6). SLIRP binding to the SDM7 probe could also be overcome with excess unlabeled SRA STR7 (lanes 7 and 8).

Given the homology between SLIRP and SHARP within their RRM domains, we next examined if SHARP could also bind SRA STR7. A GST-SHARP fusion protein (Shi et al., 2001) containing SHARP's three RRM domains (GST-SHARP-RRM) bound STR7 avidly (Figure 4G, lane 1), while GST-SHARP-RD (containing only its repression domain) did not (Figure 4G, lane 2). These data indicate that the SHARP RRMs may compete with SLIRP for binding to SRA STR7.

SLIRP Represses SRA-Mediated Nuclear Receptor Coactivation

As SLIRP was identified from a human breast cancer library and SRA is an activator of ER signaling (Lanz et al., 1999), we were particularly interested in assessing SLIRP's potential role as a modulator of E2 action. In transfection assays in HeLa cells using an E2-responsive reporter, we found that SRA coactivated reporter activity approximately 3- to 4-fold (Figure 5A), as previously reported. When cotransfected with SRA, SLIRP repressed SRA-augmented coactivation by up to 3-fold in a dose-dependent manner (Figure 5A). These data define SLIRP as an ER corepressor. Addition of SLIRP to cells cotransfected with SRA and treated with Tam or ICI further enhanced the E2-antagonistic activities of these compounds (Figure 5A).

We next investigated whether SLIRP repressed other NRs. Cotransfection of SLIRP with NR reporters and SRA into HeLa cells resulted in strong repression of glucocorticoid (GR), androgen (AR), thyroid (TR), and VDR-mediated transactivation (Figure 5B), indicating that SLIRP can modulate several different NR signaling pathways. To determine if SLIRP could act on orphan NRs, we used a peroxisome proliferator-activated receptor δ (PPARδ) agonist GW501516 (Oliver et al., 2001) and a

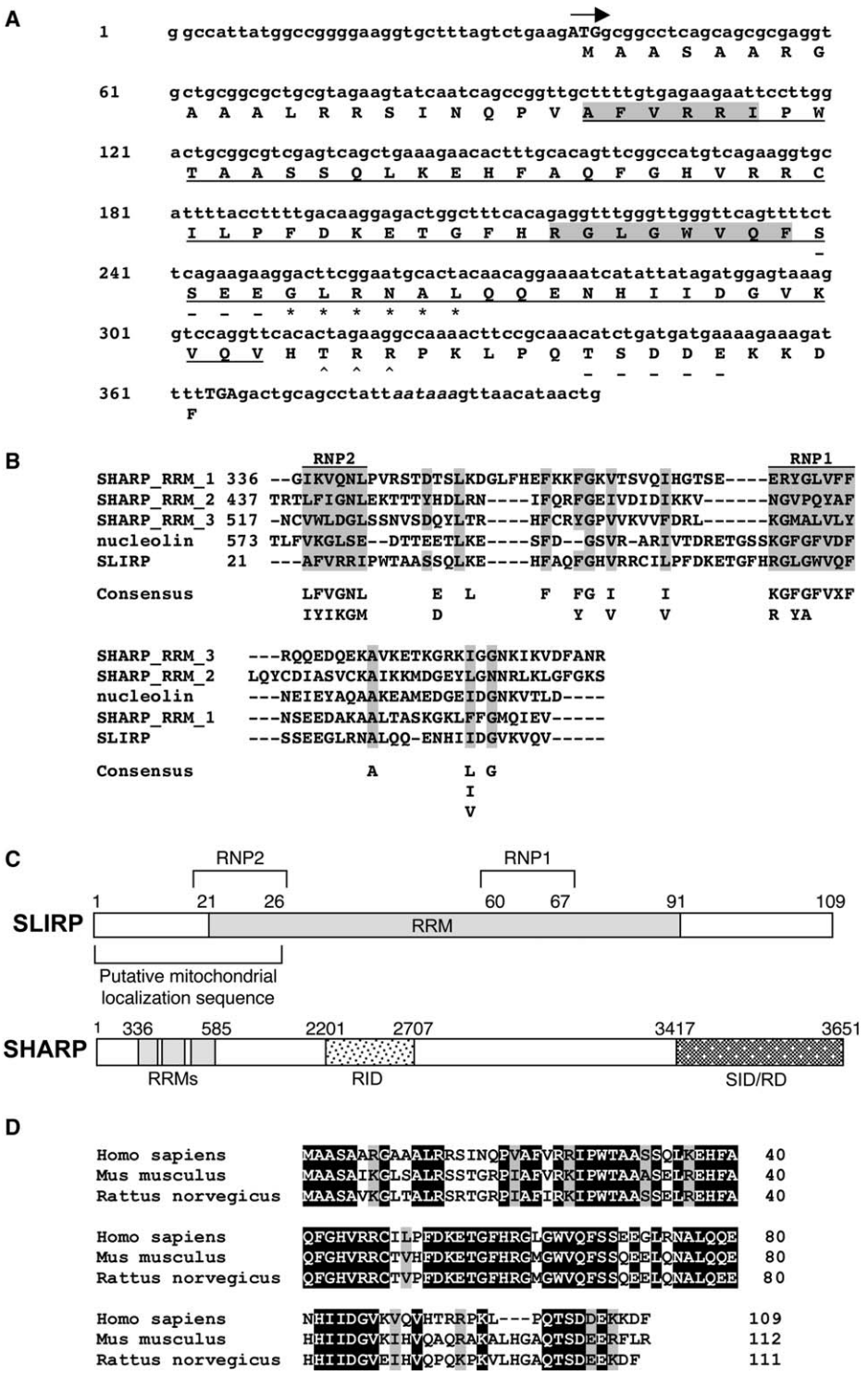

Figure 2. SLIRP Is an RRM-Containing SRA Binding Protein

(A) Nucleotide and amino acid (aa) sequence of SLIRP. The entire mRNA is shown. Arrow denotes sequence isolated via yeast three-hybrid screen, start and stop codons are in capitals, italics denote poly A signal. SLIRP contains a highly conserved RRM (underlined) with consensus RNP2 and RNP1 submotifs (highlighted). *, ∧, and − denote putative N-myristoylation, protein kinase C phosphorylation, and casein kinase II phosphorylation sites, respectively.

(B) Sequence alignment comparing SLIRP, SHARP and nucleolin RRMs. Black boxes indicate aas conserved with consensus RRM sequence described by Burd and Dreyfuss (1994).

(C) SLIRP and SHARP functional domains. RRM, RNA recognition motif. RID, receptor interaction domain. SID/RD, repression domain. Numbers denote aa sequence position.

(D) Alignment of human, mouse, and rat SLIRP aa sequences illustrates high degree of homology between species. Black, aa identity; gray, similarity; white, no homology.

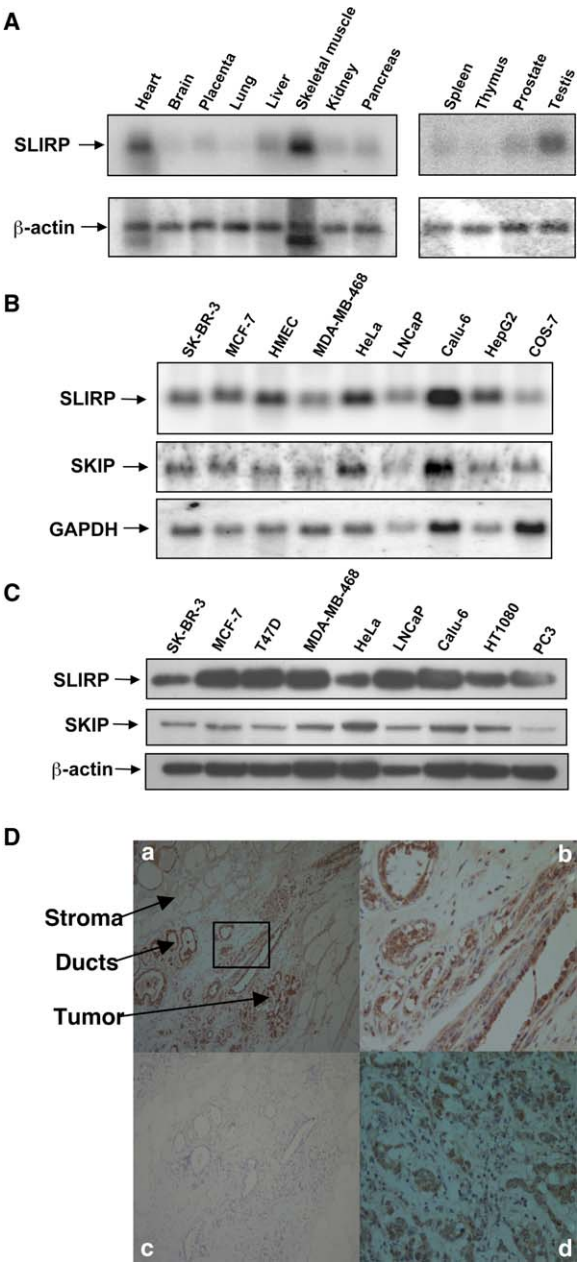

Figure 3. SLIRP Is Widely Distributed in Normal Human Tissues and Cancer Cell Lines

(A) Northern analysis of SLIRP compared with β-actin in normal human tissues.

(B) Northern analysis of SLIRP in cancer cell lines. mRNA from human breast (SK-BR-3, MCF-7, MDA-MB-468), prostate (LNCaP), lung (Calu-6), cervical (HeLa), and liver (HepG2) cell lines and normal mammary (HMEC), and monkey kidney (COS-7) cells probed with SLIRP, SKIP, and GAPDH probes.

(C) Immunoblot of protein lysates from breast (SK-BR-3, MCF-7, MDA-MB-468, T47D), cervical (HeLa), prostate (LNCaP, PC3), lung (Calu-6), and fibrosarcoma (HT1080) cells using SLIRP, SKIP, or β-actin abs.

(D) SLIRP in primary human breast cancer tissue. Sections (20 × a,c; 40 × b,d) from a human breast ductal cancer were probed with SLIRP ab (Da, Db, and Dd) and compared with sections from the same tumor with no ab (Dc). Arrows denote stroma, ducts, and tumor tissue. Box in (Da) denotes region magnified in (Db) (40×).

PPAR-Luc reporter (PPARE) (Dressel et al., 2003). SRA augmented the activation by the agonist ~2-fold, which was repressed (up to 2.9-fold) by SLIRP (Figure 5B). These data suggest that SLIRP has broad corepressor activity within the NR superfamily.

To complement our SLIRP overexpression studies, we investigated the effects of SLIRP siRNA on dexamethasone (Dex)-responsive reporter activity. In cells with reduced endogenous SLIRP expression, we found a 10-fold increase in GRE-luc activity, further confirming that SLIRP acts as a NR corepressor (Figure 5C).

SLIRP Modulates SHARP- and SKIP-Mediated Coregulation of NR Activity

The high aa sequence homology between SHARP and SLIRP and their avid binding to SRA STR7 in vitro suggested that a functional interaction may exist between these molecules in vivo. When cotransfected with SRA, SHARP repressed SRA-mediated coactivation of the E2-responsive reporter (Figure 5D, lane 4) as previously reported (Shi et al., 2001). When SLIRP was cotransfected with SHARP and SRA, an additional 2-fold repression of SRA-augmented coactivation was observed (Figure 5D, lane 5). Thus, SHARP and SLIRP appear to act in an additive fashion to enhance repression of the E2-responsive reporter.

We investigated the effects of SKIP on SLIRP repression in further transfections (Figure 5D, lanes 6–12). In the presence of transfected SKIP alone, reporter activity was increased ~2-fold, consistent with SKIP functioning as a coactivator of ER transactivation. In the presence of cotransfected SRA, an additive effect was observed with a total increase in activity of ~6-fold. When SLIRP was added, reporter activity was reduced by more than 5-fold. Thus, in the presence of SRA, SLIRP is a potent repressor of SKIP-mediated coactivation. When we reduced endogenous SLIRP expression using siRNA, SLIRP repression was abrogated. Reduction of SKIP expression with siRNA reversed SKIP's coactivation effect. When expression of SLIRP and SKIP were both reduced, an intermediate reporter activity resulted. Taken together, these data validate the functional role of each of these coregulators on ER transactivation and suggest that a competitive interaction exists between SLIRP and SKIP in NR signaling.

SLIRP Function Requires an Intact RRM Domain

To investigate the structural and functional significance of the RRM domain within SLIRP, we assessed the properties of proteins with mutations to this motif (Figure 5E). Based on binding predictions from other RRM-containing proteins, arginine 24 and 25 were mutated to alanines (R24,25A) in the RNP2 submotif, and within the RNP1 domain, leucine 62 was mutated to alanine (L62A). A double mutant (DM) containing both the R24,25A and L62A substitutions was also prepared. In REMSA studies, each of the mutations markedly reduced binding to the SRA STR7 probe (Figure 5F, lanes 4–9 and Figure S2). In transfections, each mutant partially relieved the SLIRP-mediated repression (Figure 5G, lanes 4–6), indicating the requirement of an intact RRM domain for SLIRP to function as a repressor of E2-induced SRA coactivation.

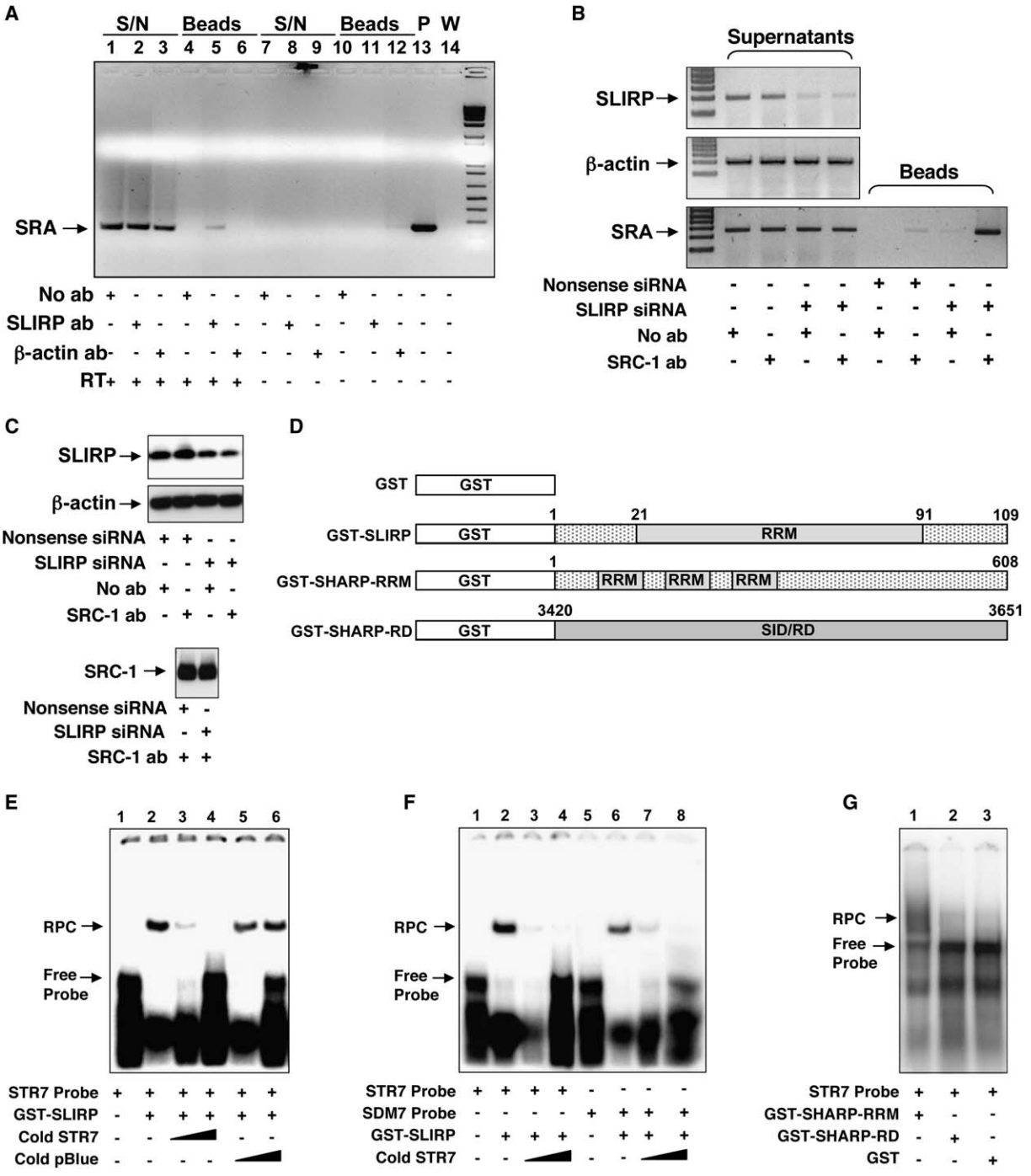

Figure 4. SLIRP Associates with SRA In Vivo, Regulates SRC-1-SRA Association, and Binds SRA STR7 In Vitro

(A) SLIRP associates with SRA in vivo. SRA was detected by RT-PCR in supernatant samples or following immunoprecipitation with beads plus SLIRP but not β-actin ab. No product generated from RT− samples. P, SRA expression plasmid; W, no template. Arrow denotes 260 bp SRA-specific PCR product.

(B and C) SLIRP knockdown augments SRC-1 association with SRA. (B) Lysates of MCF-7 cells treated with SLIRP siRNA were incubated with no ab or SRC-1 ab and SRA detected as above. SLIRP was significantly knocked down without affecting β-actin or SRA. Substantially more SRA copurified with SRC-1 in SLIRP siRNA-treated cells than nonsense controls. (C) Immunoblot confirming SLIRP protein knockdown without affecting SRC-1.

(D) Schematic of plasmids used in REMSA studies were the following: GST alone, GST-SLIRP (wild-type), GST-SHARP-RRM (SHARP aas 1–608), and GST-SHARP-RD (SHARP aas 3420–3651).

(E) Binding of recombinant GST-SLIRP to SRA STR7. REMSA demonstrating specific binding of STR7 by GST-SLIRP (lane 2) is reduced with unlabeled "cold" STR7 (lanes 3 and 4, up to 100-fold excess), but not excess cold pBlue (lanes 5 and 6).

(F) REMSA demonstrating that GST-SLIRP binds SRA STR7 more avidly than SDM7 mutant probe. Binding to both probes reduced following addition of up to 100-fold excess "cold" STR7 competitor (lanes 3 and 4 and 7 and 8).

(G) STR7 is bound by GST-SHARP-RRM (lane 1), but not with either GST-SHARP-RD (lane 2) or GST alone (lane 3).

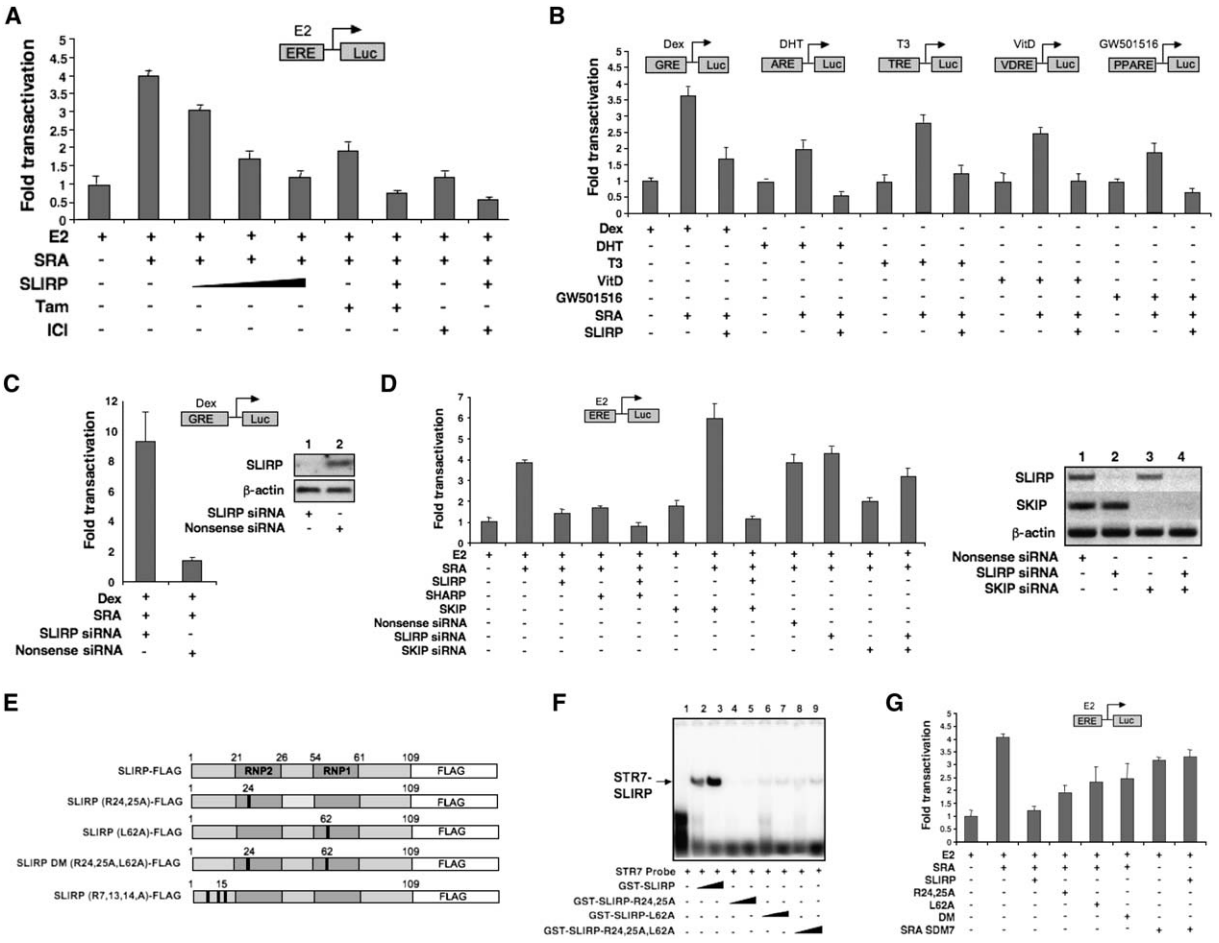

Figure 5. SLIRP Is a SRA-Dependent Repressor of NR Transactivation that Interacts with SHARP and SKIP and Requires an Intact RRM for Coregulation

(A) SLIRP represses ER transactivation, which is augmented by Tam and ICI. HeLa cells were cotransfected with ERE-luciferase (Luc), expression vectors for ERα ± SRA, and increasing amounts of SLIRP. After 24 hr, cells were treated for 8 hr with E2 prior to assessment of Luc activity (normalized to protein). Tam or ICI were added where indicated at the same time as E2. All results are representative of triplicate experiments; error bars represent standard deviation.

(B) SLIRP represses signaling of multiple NR pathways. HeLa cells were cotransfected with either a GRE-Luc, ARE-Luc, TRE-Luc, VitD-Luc, or PPARE-Luc reporter plus corresponding AR, TR, VDR, PPARδ, SRA, and SLIRP expression vectors and incubated with ligand (Dex, DHT, T3, VitD, GW501516) for 8 hr, and Luc activity was determined as above.

(C) Targeted reduction of SLIRP expression potentiates GR transcription. HeLa cells were cotransfected with GRE-Luc and siRNA directed against either SLIRP or a nonsense target. After 48 hr, cells were treated with Dex (8 hr) prior to assessment of Luc activity. Immunoblot confirmed reduced endogenous SLIRP expression in SLIRP siRNA-treated cells (lane 1) compared with nonsense (lane 2) relative to β-actin.

(D) SLIRP augments SHARP's repression and antagonizes SKIP's coactivation of ER. HeLa cells were cotransfected with ERE-Luc and expression vectors for ERα alone, and/or empty, SHARP, SLIRP, or SKIP vectors, ±siRNA (nonsense, SLIRP, or SKIP). RT-PCR confirmed reduced SLIRP and SKIP expression in siRNA-treated cells (right panel).

(E) Plasmids for expression of wild-type and mutant SLIRP with carboxy-terminal FLAG epitope.

(F) Mutation of the RRM domain abrogates binding of SLIRP to SRA. REMSA using labeled SRA STR7 probe and increasing amounts of GST-SLIRP fusion proteins (wild-type, mutants R24,25A, L62A, or double mutant R24,25A,L62A).

(G) SLIRP mutants have reduced ability to repress ER activity. HeLa cells were transfected with ERE-Luc and either wild-type (SLIRP-FLAG) or mutated SLIRP-FLAG (R24,25A, L62A, DM) expression vectors together with SRA or SRA SDM7 (stem-loop mutant) and reporter activity assessed as in (A), above.

To examine the functional specificity of the SLIRP-STR7 interaction in vivo, we utilized the SRA-SDM7 mutant (Lanz et al., 2002), in which the stem-loop structure is mutated but preserved (Figure 5G). This mutation decreased SRA-mediated coactivation to ~70% of wild-type levels. Furthermore, when we cotransfected SLIRP with SRA-SDM7, SLIRP was unable to function as a repressor. These data suggested that a direct interaction between SLIRP and STR7 is critical for SLIRP's repressive activity.

SLIRP Is Recruited to Endogenous NR Target Promoters

To determine if SLIRP is recruited to E2- and Dex-responsive promoters, we performed chromatin immunoprecipitation (ChIP) assays. We found that SLIRP was recruited to the E2-responsive pS2 promoter within 60 min of ligand treatment (Figure 6A), but this association had returned to undetectable levels by 120 min. ERα binding increased in response to ligand returning to basal levels within 120 min. In contrast, HuD, another

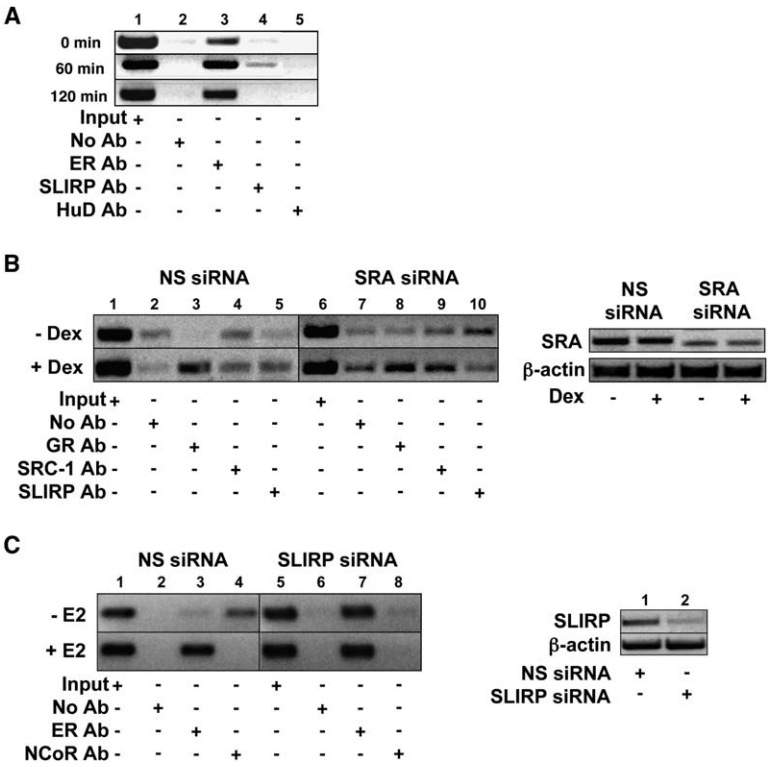

Figure 6. SLIRP Is Recruited to Endogenous Promoters and Modulates NCoR Recruitment

(A) ChIP assay demonstrating recruitment of SLIRP and ER, but not HuD, to the pS2 promoter of MCF-7 cells in response to E2. Sheared, genomic, MCF-7 DNA used as input control.

(B) Recruitment of SLIRP to the metallothionein promoter is regulated by SRA. HeLa cells treated with SRA siRNA or nonsense siRNA (NS siRNA) for 3 days were incubated with Dex, and then ChIP assays were performed with either GR, SRC-1, or SLIRP ab (left). RT-PCR shows knockdown of SRA without affecting β-actin (right).

(C) SLIRP regulates NCoR association with the pS2 promoter. MCF-7 cells were treated with either SLIRP siRNA or NS siRNA (3 days) followed by E2 for 45 min before ChIP assay using ER or NCoR ab as above (left). RT-PCR demonstrating SLIRP knockdown is shown (right).

well-characterized RRM-containing RNA binding protein (Chung et al., 1996), was not recruited to the DNA. These data confirmed that SLIRP can closely associate with the response element of an E2-regulated gene.

To investigate the mechanism by which SLIRP might mediate its effect at the transcriptional level, we performed ChIP assays in HeLa cells treated with SRA siRNA. Interestingly, in cells with reduced SRA expression, ~50% less SLIRP was recruited to the Dex-responsive metallothionein promoter (Figure 6B, lane 10). This suggests that the presence of SRA is critical for recruiting SLIRP to the promoter and consequently mediating SLIRP's repressive effects.

To investigate interactions of SLIRP with other corepressors, we performed ChIP studies in cells treated with SLIRP siRNA. In the absence of E2, NCoR is recruited to the pS2 promoter together with a small amount of ER (Figure 6C). However, in cells with reduced SLIRP, NCoR could no longer be detected on the promoter, and ER recruitment was significantly higher. This suggests a key role for SLIRP in facilitating recruitment of NCoR to the promoter.

SLIRP Is Predominantly Mitochondrial

Based on our transfection and ChIP data, we envisaged that SLIRP would be a predominantly nuclear protein. However, imaging studies using the SLIRP ab revealed endogenous SLIRP to have a filamentous cytoplasmic distribution confined predominantly to the mitochondria (Figure 7A, top panel). A similar pattern was observed using ab to HSP-60, a mitochondrial-specific protein (Figure 7A, second row) (Gupta and Knowlton, 2005). In cells transfected with FLAG-tagged SLIRP, we found that SLIRP colocalized with another mitochondrial-specific protein cytochrome c oxidase (Figure 7A, third row). Sequence analysis of SLIRP revealed an N-terminal 26 aa domain highly predictive of an amphipathic α-helical mitochondrial targeting sequence conserved between the mouse, rat, and human genomes (see Figure 2C). This mitochondrial signal sequence is evident in the 3D predicted structure of SLIRP as an independent helix linked to the RRM (Figure 7C). To evaluate the importance of the N-terminal signal sequence, we compared the intracellular localization of SLIRP-FLAG versus FLAG-SLIRP constructs. Interestingly, SLIRP-FLAG localized to the mitochondria, whereas FLAG-SLIRP was pancellular (Figure 7A, bottom two rows), consistent with the notion that the N-terminal mitochondrial signal sequence is critical for targeting SLIRP to the mitochondria.

To further investigate the mitochondrial location of endogenous SLIRP, we examined primary human breast tissue with SLIRP and HSP-60 abs. A punctate cytoplasmic staining pattern, characteristic of mitochondria, was observed with both SLIRP and HSP-60 abs (Figure 7B). Taken together, these data confirm that SLIRP resides predominantly in the mitochondria and that interference with the N-terminal signal sequence can substantially alter the intracellular distribution of the protein.

To evaluate the functional importance of the mitochondrial signal sequence, we generated a triple mutant of the first three arginines in SLIRP (R7,13,14,A) (see Figure 5F). When cotransfected into HeLa cells with SRA, this SLIRP mutant had reduced ability to function as a repressor (Figure 7D). These data suggested that the mitochondrial signal sequence is required for maintaining corepressor function, raising the possibility that SLIRP has bifunctional capacity as a NR corepressor in the nucleus and mitochondria.

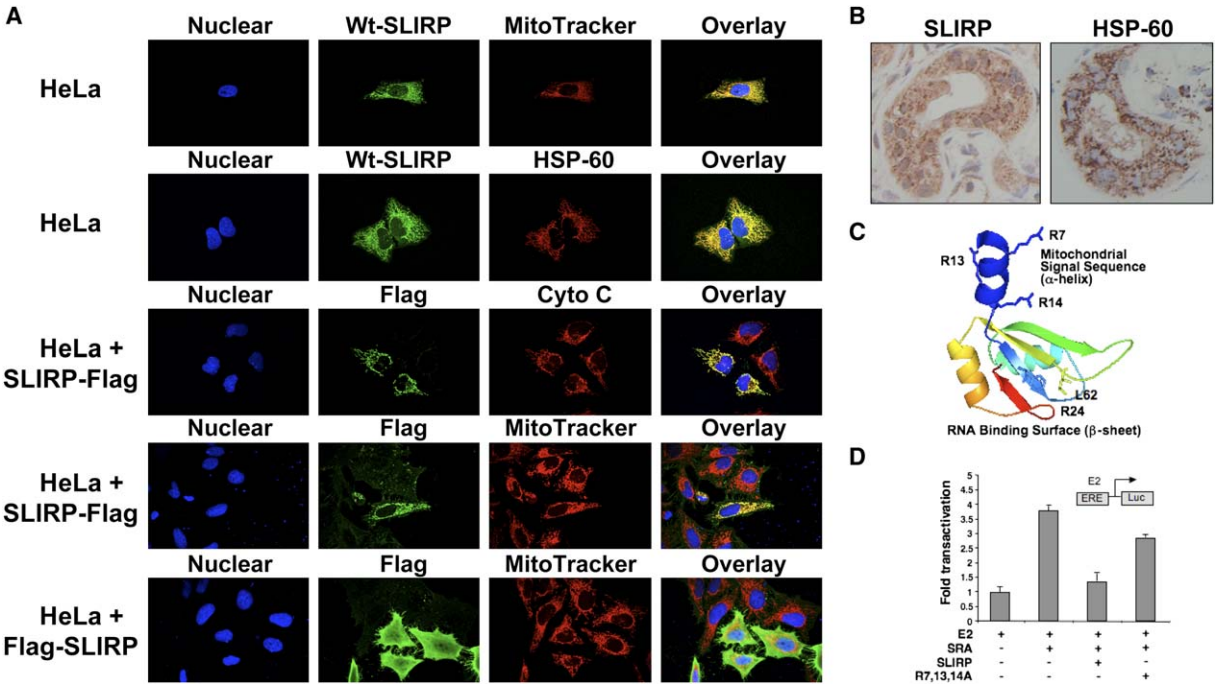

Figure 7. SLIRP Localizes Predominantly to the Mitochondria

(A) Simultaneous mitochondrial (red, Mitotracker), nuclear (blue, Hoescht 33258), and endogenous SLIRP protein staining (green, rabbit polyclonal sera, Alexa Fluor 488 secondary ab) of HeLa cells. Overlaying of confocal images reveals colocalization of SLIRP and the mitochondria (yellow) (top row). Endogenous SLIRP also colocalizes with mitochondria-specific HSP-60 (second row). Transfected SLIRP-FLAG colocalized with cytochrome c (middle row) and Mitotracker stain (fourth row). Transfected FLAG-SLIRP was pancellular and did not colocalize with the Mitotracker stain (bottom row).

(B) SLIRP and HSP-60 stain similarly in human breast cancer tissue. IHC of primary human breast cancer tissue using either SLIRP or HSP-60 abs. Ducts stained readily with both abs in a punctate cytoplasmic pattern, consistent with a mitochondrial location for HSP-60 and SLIRP.

(C) Three-dimensional modeling of the SLIRP protein predicts a mitochondrial localization signal in the amino terminal 26 aas. Residues subjected to point mutation are indicated.

(D) Mutations in the SLIRP mitochondrial sequence relieve its repressive activity. HeLa cells were transfected as in Figure 5A with ERE-luc, ERα, wild-type SRA, and either wild-type SLIRP or the R7,13,14A mutant. Results are representative of triplicate experiments; error bars represent standard deviation.

Discussion

SRA coregulates NR pathways and has been implicated in tumorigenesis. However, the mechanisms by which SRA mediates its effects remain to be elucidated. We identified SLIRP as a protein that binds to SRA in vitro and in vivo and is a potent repressor of E2, glucocorticoid, androgen, thyroid hormone (T3), and VitD action. In addition, SLIRP represses orphan NR activity, as shown by its effects on PPARδ-mediated transactivation. SLIRP, which is composed almost entirely of an RRM region, is widely expressed in normal human tissues while elevated in skeletal muscle, heart, liver, and testis. Furthermore, SLIRP is also widely expressed at the mRNA and protein level in multiple cancer cell lines, and IHC studies confirm its presence in primary human breast tumors.

Here we show that SLIRP interacts specifically with STR7 in vitro and with endogenous SRA in vivo. Shi et al. (2001) showed that the coregulator activity of SHARP requires its RRM. We observed similar findings with SLIRP in that discrete single and double aa substitutions of the RRM domain significantly reduced its SRA binding and corepression activities. We also demonstrate that SHARP (via its RRM domain) interacts with STR7, raising the possibility that SHARP and SLIRP may compete for binding to SRA, consequently affecting ER-regulated gene expression.

The SRA STR7 stem loop is the longest and one of the most stable identified by 2º structure predictions and accounts for a substantial proportion of SRA's overall coactivator activity (Lanz et al., 2002). Our SRA mutation data show that STR7 is required for SLIRP to act as a corepressor, further strengthening the case for a direct interaction between STR7 and SLIRP in vivo. Reduction of endogenous SLIRP expression increases SRA's coactivation ability, suggesting not only that this interaction is functionally relevant, but also that SLIRP could play an important tumor-suppressor role in SRA-activated NR pathways. The additive repressive effect of SHARP and SLIRP, both of which bind to SRA STR7, suggests these proteins could function to significantly downregulate ER signaling in breast cancer cells.

Complex Interactions between SLIRP and Other NR Coregulators at Hormone-Responsive Promoters

Our studies provide new insight into the mechanism of interaction between SRA, SLIRP, and SRC-1. In particular, we found that recruitment of SLIRP to an endogenous Dex-responsive promoter is regulated by the amount of SRA in the cell. Furthermore, we have shown

that SRC-1 and SLIRP appear to compete for association with SRA. Specifically, when SLIRP levels are reduced, SRC-1 association with SRA increases. This is consistent with the opposing function of these two coregulators: the association of SRC-1 with SRA results in coactivation, while association of SLIRP with SRA results in corepression.

Our ChIP data in cells with reduced SLIRP expression provide additional mechanistic insights. The results suggest that SLIRP is essential for mediating NCoR's association with the promoter in the absence of E2. Most interestingly, in SLIRP siRNA-treated cells, not only is binding of NCoR abrogated, but the ER is strongly recruited, suggesting that removal of SLIRP from the cell alters the promoter state from one of repression to activation.

We were intrigued to observe that SKIP and SLIRP colocalize to human Chr 14q24.3 and that this genomic distribution is conserved across species. It raised the possibility that they may be coordinately regulated, as is the case for Grb7 and HER2 that lie adjacent to each other on human Chr 17 (Daly, 1998). However, our expression data did not support this idea. Furthermore, our transfection and siRNA studies suggested that they have opposing and possibly competitive effects on estrogen signaling rather than working in concert. Given the interaction between SKIP and NCoR in VDR transactivation (Leong et al., 2004), our studies with SKIP and NCoR suggest a complex role for SLIRP in modulating VDR signaling. Although loss of heterozygosity (LOH) has been described at Chr 14q31.2 in breast tumors (Martin et al., 2001; O'Connell et al., 1999), LOH in the genomic area of SLIRP/SKIP in breast cancer has not been described.

SLIRP Is a Predominantly Mitochondrial NR Corepressor

The increased expression of SLIRP in high-energy demand and mitochondria-rich tissues such as skeletal muscle, heart, and liver is consistent with its predominantly mitochondrial location. Multiple imaging studies suggest that more than 90% of SLIRP is located in the mitochondria, raising the possibility that it may function both in the nucleus and mitochondria to regulate NR activity. Whether this is via interactions with SRA or other mitochondrial RNA targets is unknown. In addition, SLIRP's capacity to represses PPARδ-mediated signaling suggests a potential role in regulating lipid homeostasis in energy-rich tissues.

The role of NRs in the mitochondrion, affecting cell survival and energy homeostasis, has recently come under close scrutiny. Studies in breast cancer tissues suggest that mitochondrial ER plays a role in tumor cell survival (Pedram et al., 2006). Additionally, T3 can induce transcription in the absence of nuclear factors acting via mitochondrial TR (Scheller and Sekeris, 2003). GR interacts with NFκB subunits (Tao et al., 2001) present in the mitochondria (Cogswell et al., 2003), and putative GREs exist in some key components of the mitochondrial genome-encoded oxidative phosphorylation pathway (Psarra et al., 2006). Taken together, these data provide a foundation for NR and coregulator action in the mitochondria and a rationale for SLIRP's presence there.

The discovery of SRA, the first RNA coactivator, led to a paradigm shift in our understanding of NR coregulation and hormone action. With the identification of SLIRP, a new SRA binding protein, we provide the most detailed characterization of a direct SRA-protein interaction to date. The expression of SLIRP in a variety of cancers, its functional corepression of multiple NR signaling pathways, and its capacity to regulate NCoR promoter recruitment and SRC-1 association with SRA as well as interactions with SHARP and SKIP suggest that SLIRP may play an important role in regulating a broad range of NR activities and therefore potentially tumorigenesis. Moreover, its expression in energy-rich tissues, mitochondrial location, and repression of PPARδ-mediated signaling suggest that SLIRP may participate in controlling lipid and energy metabolism, with roles in both the nucleus and mitochondria. Further studies to elucidate the role of SLIRP in each of these cellular locations should contribute substantially to the biology underlying hormone-dependent tumor growth and the control of body metabolism.

Experimental Procedures

Cell Culture

MCF-7, MDA-MB-468, SK-BR-3, T47D, LNCaP, PC-3, HepG2, Calu-6, HT1080, COS-7, HeLa cell lines obtained from the American Type Culture Collection, HMEC from Dr. Roger Reddel. Cells were grown as per supplier's recommendations or as previously described (Giles et al., 2003) and used within 20 passages of original stock for all experiments.

Yeast Three-Hybrid Screen of Human Breast Cancer Library

Screening and plasmid isolation protocols were as described (Sen-Gupta et al., 1996). Analysis of SRA and STR7 structures was performed using Mfold (Zucker, 2003). pIIIA/MS2-2 and *S. cerevisiae* L40coat were gifts of Dr. Marvin Wickens. Dr. Jennifer Byrne donated the human breast cancer cDNA library (Byrne et al., 1998). Colonies underwent stringency tests as described (Park et al., 1999).

SLIRP Plasmid Constructs, Sequences, and Vectors

SLIRP coding domain was amplified from the yeast three hybrid clone with SLIRP (sense) 5′ cgc gga tcc gcg gcc tca gca gca 3′, SLIRP (reverse) 5′ gcg cgg atc cta ggc tgc agt ctca 3′ primers and subcloned into BamHI cut pCMV-FLAG 7.1 for transfection assays and pGEX-6P2. STR7 oligonucleotides (sense) 5′ agg agg cag gta tgt gat gac atc agc cga cgc ctg gca ctg ctg cag gaa cag tgg gct gga gga aag ttg tca ata cct gta aag aa 3′ and (reverse) 5′ ttc ttt aca ggt att gac aac ttt cct cca gcc cac tgt tcc tgc agc agt gcc agg cgt cgg ctg atg tca tca cat acc tgc ttc ct 3′ were cloned into EcoRV-digested pBluescript II KS+ (Stratagene) to generate labeled riboprobes. To generate SRA SDM7, underlined residues above were mutated to tcc, ctc, and ctc as outlined (Lanz et al., 2002). pBluescript vectors were linearized and riboprobes generated as described (Thomson et al., 1999). SLIRP mutants (R24,25A and L62A) were prepared by PCR-based mutagenesis and subcloned into BamHI-cut pCMV-FLAG 7.1 and pGEX-6P2 as above. Sequence alignments of SLIRP and SHARP and SLIRP interspecies comparisons were performed using algorithms described by Stothard (2000).

REMSA and UVXL

REMSA and UVXL were performed as described (Thomson et al., 1999). In competition REMSA, up to 100-fold excess unlabeled pBlue, STR7, or tRNA was used. Large-scale recombinant GST protein expression was performed as described (Giles et al., 2003). Recombinant proteins were digested with PreScission Protease (Sigma).

Transfection and Luciferase Assays

HeLa cells were transfected in RPMI containing 5% stripped serum using FuGENE6 (Roche) with equal molar ratios of control empty and or cDNA expression plasmids plus the following: 50 ng/well ERα, TRβ, AR, PPARδ; 0.6 μg/well ERE-Luc, GRE-Luc, TRE-Luc,

ARE-Luc, VDRE-Luc, PPARE-Luc; 0.0625 μg/well pSCT or 0.08 μg/well pSCT-SRA, pSCT-SRA-SDM7a; 0.049 μg/well pCMV-FLAG-7.1 or 0.05-0.5 μg/well FLAG-SLIRP, FLAG-SLIRP-R24,25A, FLAG-SLIRP-L62A, FLAG-SLIRP-DM; 0.3 μg/well pCMX or 0.5 μg/well pCMX-SHARP; 100 ng/well pCGN or pCGN-SKIP. After transfection, cells were cultured for 24 hr prior to addition of E2, Dex, DHT (10 nM), T3 (1 nM), VitD (100 nM), GW501516 (500 nM, Alexis Biochemicals), Tam (1 μM), ICI (1 μM, Tocris), or equal volume of absolute ethanol or DMSO. After 8 hr, lysates assayed for luciferase activity relative to protein levels using Luciferase (Promega) and Protein Assays (Bio-Rad) on a FluorStar Optima (BMG).

RNA Interference

Cells were transfected with siRNA complexes directed against SRA, SLIRP, SKIP, or control (nonsense) (Dharmacon RNA Technologies, final concentration 20–200 nM) using Lipofectamine 2000 (Invitrogen). To assess SLIRP knockdown on GRE-luc activity, reporter plus pSCT or pSCT-SRA and siRNA conjugates were added simultaneously. After 48 hr, cells were induced with Dex (10 nM) and lysates processed as described above. For SLIRP and SKIP siRNA studies, cells were transfected with siRNA for 72 hr, ERα and ERE-Luc co-transfected at 48 hr, and E2 (10 nM) added 8 hr prior to harvest. SKIP (sense) 5′-cat tca act ctg gag cta aac aga-3′ and (reverse) 5′-tcc gat cag caa tgt aga ggg ct-3′; SLIRP (sense) 5′-gcg ctg cgt aga agt atc aa-3′ and SLIRP (reverse) 5′-tcg att ccg aag tcc ttc tt-3′; and β-actin (sense) 5′-gcc aac aca gtg ctg tct gg-3′ and β-actin (reverse) 5′-tac tcc tgc ttg ctg atc ca-3′ primers were used to quantitate targets by RT-PCR.

Northern Analysis

Human tissue blots obtained from BD Biosciences (7759-1, 7760-1). Total RNA isolated using TRIzol LS (GIBCO-BRL), Poly A RNA using Poly ATtract (Promega). Samples (2 μg) were electrophoresed and transferred to Hybond-N membrane (Amersham). Human SLIRP, SKIP, or β-actin cDNAs were random primer labeled ([^{32}P]dCTP) and hybridized with membranes for 2–5 hr, washed to 0.1 × SSC/0.1% SDS at 65ºC, and visualized by PhosphoImager.

Immunoblotting

Cell lysates were resolved by SDS-PAGE and transferred onto PVDF Membrane (Roche). SLIRP was detected with rabbit polyclonal antisera raised against GST-SLIRP protein (1:250). SRC-1 (SC6096), SKIP, ERα (SC7207), and β-actin abs (Abcam ab 6276) were used with HRP conjugated anti-rabbit or anti-mouse secondary abs and ECL Plus (Amersham).

Immunohistochemistry

Full-face sections of primary human breast cancer tissue were immunostained using standard protocols. SLIRP polyclonal ab or HSP-60 ab (SC13115) was used at 1:2500, with biotinylated goat anti-rabbit and anti-mouse ab (Chemicon IHC Select) followed by streptavidin-horseradish peroxidase as secondary and tertiary reagents. Sections were vizualised with diaminobenzidine (DAKO) followed by a light counterstain with haematoxylin.

Immunoprecipitation RT-PCR Assay

Method was as described (Giles et al., 2003). Using MCF-7, MDA-MB-468, or HeLa cells with either SLIRP ab, SRC-1 ab, β-actin ab, or no ab, coimmunopurifying SRA detected using the following primers: SRAEIV(sense), 5′-tga tga cat cag ccg acg cct-3′ and SRAEIV(reverse) 5′-gct gca gat ttc tct tca ttg-3′. SLIRP and β-actin mRNAs were detected using primers as above.

ChIP Assay

Assay was as described (Dowhan et al., 2005). MCF-7 and HeLa cells were treated with 100 nM E2 for 0-120 min or Dex 100 nM for 15 min prior to fixation. Soluble chromatin incubated with 4 μg ERα, SLIRP polyclonal, HuD (SC5979), SRC-1, NCoR, or GR (a 50:50 combination of SC1002 and SC1004) abs. Recovered DNA fragments were amplified with either pS2 (sense) 5′-ggc cat ctc tca cta tga atc act tct gc-3′ and (reverse) 5′-ggc agg ctc tgt ttg ctt aaa gag cg-3′ or metallothionein (MTA2) (sense) 5′-act cgt ccc ggc tct ttc ta-3′ and (reverse) 5′-agg agc agt tgg gat cca t-3′primers.

Imaging Studies

HeLa cells cultured on glass cover slips were incubated with Mito-Tracker (Molecular Probes), SLIRP, HSP-60, or cytochrome c (SC7159) ab and Alexa Fluor 488 goat anti-rabbit (Molecular Probes) secondary ab added. Cover slips were bathed in 100 ng/ml Hoechst 33258/PBS, washed, and mounted in Vectashield (Vector Laboratories). For FLAG imaging, cells were transfected 24 hr prior to staining as above and visualized using a BioRad MRC1000 confocal microscope.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/22/5/657/DC1/.

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Accession Numbers

The GenBank accession number for the SLIRP sequence reported in this paper is AY860853.