The Proto-oncoprotein SYT Interacts with SYT-interacting Protein/Co-activator Activator (SIP/CoAA), a Human Nuclear Receptor Co-activator with Similarity to EWS and TLS/FUS Family of Proteins*

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The proto-oncoprotein SYT is involved in the unique translocation t(X;18) found in synovial sarcoma SYT-SSX fusions. SYT has a conserved N-terminal domain (SNH domain) that interacts with the human paralog of *Drosophila* Brahma (hBRM) and Brahma-related gene 1 (BRG1) chromatin remodeling proteins and a C-terminal transactivating sequence rich in glutamine, proline, glycine, and tyrosine (QPGY domain). Here we reported the isolation of the ribonucleoprotein SYT-interacting protein/co-activator activator (SIP/CoAA), which specifically binds the QPGY domain of SYT and also the SYT-SSX2 translocation fusion. SIP/CoAA is a general nuclear co-activator and an RNA splicing modulator that contains two RNA recognition motifs and multiple hexapeptide repeats. We showed that the region consisting of the hexapeptide motif (YQ domain) is similar to the hexapeptide repeat domain found in EWS and in TLS/FUS family proteins. The YQ domain also resembles the QPGY region of SYT itself and like all these other domains acts as a transcriptional activator in reporter assays. Most interestingly, the last 84 amino acids adjacent to YQ down-modulate by 25-fold the YQ transactivation of the reporter gene, and both domains are important for SIP/CoAA binding to SYT. In addition, SYT acts together with SIP/CoAA in stimulating estrogen and glucocorticoid receptor-dependent transcriptional activation. Activation is hormone-dependent and requires functional hBRM and/or BRG1. The stimulation is strongly reduced if the N-terminal region of hBRM/BRG1 (amino acids 1–211) is deleted. This region encompasses the SNF11 binding domain (amino acids 156–211), which interacts specifically with SYT *in vivo* and *in vitro*.

Chromosomal translocations that result in oncogenic transformation frequently involve the fusion of two genes that express novel chimeric proteins (1, 2). In many cases these proteins are aberrant transcription factors. In synovial sarcoma, the unique chromosomal translocation t(X;18)(p11.2;q11.2) results in the fusion of the proto-oncogene *SYT* on chromosome 18 to either of three closely related genes *SSX1, SSX2,* and *SSX4* on chromosome X. This chromosomal translocation is found in more than 90% of synovial sarcomas cases (3). The resulting chimeric genes express SYT-SSX1, SYT-SSX2, or SYT-SSX4 fusion proteins in which the 8 C-terminal amino acids of SYT, at the main translocation point, are replaced by the last 78 amino acids of the C-terminal end of the SSX proteins (4–7).

The proto-oncoprotein SYT is ubiquitously expressed in cells during embryogenesis (8) and in the adult (4). Although the protein is localized in the cell nucleus, it has no recognizable nucleic acid-binding motifs, and its biological function is still unclear. SYT has two recognized functional domains as follows: (i) a conserved N-terminal SNH[5] domain (amino acids 15–73), and (ii) a region rich in glutamine (Q), proline (P), glycine (G), and tyrosine (Y), called the QPGY domain, which encompasses the C-terminal half of the protein (amino acids 187–387) (9).

The SNH domain binds a conserved region known as the SNF11 binding domain (SNF11 BD) situated at the N terminus of the two highly homologous human proteins hBRM and BRG1 (amino acids 158–214 for hBRM; amino acids 156–211 for BRG1) (10). In the yeast homolog Snf2, this domain binds the SNF11 protein (11) giving the homonymous name of SNF11 BD to the two human counterpart domains, although no human SNF11 paralog is known. hBRM and BRG1 are the ATPase subunits of the ATP-dependent chromatin remodeling complexes found in human cells, and the two proteins are homologs to the yeast subunits Snf2 and Sth1 and to the *Drosophila* protein Brahma (12–15). In human cells, there are two major SWI/SNF complexes named BAF (or SWI/SNF-A) and PBAF (or SWI/SNF-B) that possess similar functions but differ in some protein subunit composition (16–19). BAF contains either one of the two ATPases hBRM or BRG1 whereas PBAF contains only BRG1. Another signature that confirms distinctness is the fact that the BAF250 protein (also called p270/p250) is present in the BAF complex and not in PBAF, and polybromo (or BAF180) is only found in the PBAF complex. There is also good evidence that endogenous SYT is associated with native human SWI/SNF complexes. On the other hand, the C-terminal region of the fusion,

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[5] The abbreviations used are: SNH, SYT N-terminal homology domain; hBRM, human paralog of *Drosophila* Brahma; BRG1, Brahma-related gene 1; ERE, estrogen response element; ER, estrogen receptor; E2, 17β-estradiol; GRE, glucocorticoid-response element; GR, glucocorticoid receptor; SIP/CoAA, SYT-interacting protein/co-activator activator; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TRBP, thyroid hormone receptor-binding protein; PBS, phosphate-buffered saline; BD, binding domain; RRMs, RNA recognition motifs; GST, glutathione *S*-transferase; RGG, arginine-glycine-glycine; CBP, cAMP-response element-binding protein-binding protein.

encoding the SSX1 C terminus (amino acids 310–387), binds to core histones and oligonucleosomes (20). The AF10 protein, which is a putative transcription factor deregulated in t(10;11)-positive acute leukemias, was also found to interact with the SNH domain of SYT (21). Finally, a role independent from transcriptional activation has also been reported for SYT. The SYT protein can interact with the p300 histone acetyltransferase, but not CBP, for the regulation of cell adhesion possibly by activating the $\beta1$ integrin/fibronection receptor. When cells undergo G_1 arrest because of contact inhibition, p300 binds to the N-terminal region of SYT in the cell nucleus. The interaction is lost if the first 43 amino acids of SYT (which contains part of the SNH domain) are deleted (22).

The QPGY domain of SYT resembles the N-terminal activation region of the EWS and FUS/TLS family of proteins (9) and is even more closely related to the N-terminal region of BAF250 (20), which (as described above) is part of the BAF complex and confers specificity to this complex (19). The SYT QPGY domain appears to be composed of degenerate repeats based on the amino acids glutamine, proline, glycine, and tyrosine, but it is not possible to discern a consensus repeat sequence (9) as recognized in EWS protein (23). Most interestingly, the transactivating QPGY region can also interact with itself, but there are no other proteins that are known to bind to this domain (10).

In this work we have focused our attention on the QPGY domain of SYT. Transfection experiments that we have carried out previously, with SYT fused to the DNA binding domain of GAL4, demonstrate that the QPGY repeat region stimulates the expression of reporter constructs (9, 10, 24). Thus, although it has no obvious DNA binding domain, SYT may be a transcriptional co-activator that binds to unknown sequence-specific DNA-binding proteins. We performed a yeast two-hybrid screen to identify new proteins that directly interact with the QPGY domain. Here we describe a novel interaction between the QPGY domain of SYT and the general co-activator ribonucleoprotein SYT-interacting protein/co-activator activator (SIP/CoAA) (25–27). SIP/CoAA also binds to SYT-SSX fusion proteins and may be important in the formation of synovial sarcoma.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Cloning of Full-length cDNA of SIP/CoAA—The yeast two-hybrid system was used to clone the SIP/CoAA cDNA from a library made from the synovial sarcoma cell line SS255. A DNA fragment corresponding to amino acids 159–314 was subcloned into the plasmid pYTH9 and stably integrated into the yeast cell line Y190. Expression of the bait was detected by Western blot analysis using a hemagglutinin antibody (Insite Biotech). 250,000 colony-forming units were screened using the lithium acetate transfection method. The transfected yeast was then plated onto trp⁻-leu⁻-his⁻ selection agar containing 25 mM 3-aminotriazole. Positive clones were assayed for β-galactosidase activity in a filter assay as described in the Clontech protocol. The insert of one of the positive clones (clone 60) was cloned into pBluescript KS+ (Stratagene) and sequenced using fluorescent dye terminator Taq-FS ready reaction kit (Applied Biosystems). The sequence of the 5′-end of SIP/CoAA was obtained using genomic DNA. cDNA was radioactively labeled and used to probe the PAC library RPCI5 (28). 40 positive PACs were obtained and fluorescently fingerprinted (29). Tiling sets were obtained using FPC software (30), and central PAC 845F19 was identified as containing the full-length SIP/CoAA and localized to chromosome 11q13.3. The PAC 845F19 clone was used as template in a PCR with a 5′-primer (TTCG-GACGTTTTGCCTGTCG) derived from an EST sequence (accession number 610833) together with the reverse primer CTGCTTCCT-

TCTCCATGTGA. The PCR product was subsequently combined with the C-terminal half of SIP/CoAA into pBIND vector (Promega) to obtain a full-length cDNA clone of SIP/CoAA in a mammalian expression vector (GenBank™ accession number AF080561). Sequence alignment was carried out using Clustal analysis (www.ebi.ac.uk/clustalw/). Motif characterization was done using Fuzzpro (www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/fuzzpro.html), Multiple EM for Motif Elicitation (MEME), and Motif Alignment and Search Tool (MAST) (meme.sdsc.edu/meme/website/intro.html).

Plasmid Description and Construction—Mammalian two-hybrid vectors pBIND (GAL4) and pACT (VP16) were used in the CheckMate mammalian two-hybrid assays (Promega). Full-length VP16-SYT and VP16-SYT-SSX2 were described previously (10), and GAL4-SIP/CoAA was obtained as described above. Each deletion clone was generated by PCR using *Pfu* polymerase (Stratagene). The vector pGEX-4T-1 (Amersham Biosciences) was used to clone GST-SIP/CoAA fusion for *in vitro* interactions assays. Clones expressing FLAG-tagged SIP/CoAA, SYT, SYT-SSX2, hBRM, and BRG1 were prepared using the pCMV-Tag2 vector (Stratagene). Constructs pcDNA5/FRT/TO-BRG1 FL and pcDNA5/FRT/TO-BRG1ΔSNF11 expressing full-length BRG1 and BRG1Δ1–211 were prepared using the pcDNA5/FRT/TO expression vector (Invitrogen). These clones were derived, by restriction enzymes, from the full-length GAL4-SIP/CoAA, VP16-SYT, VP16-SYT-SSX2, pcDNA4/HisMax-hBRM, and pcDNA4/HisMax-BRG1 vectors. pcDNA4/HisMax-hBRM and pcDNA4/HisMax-BRG1 vectors were provided kindly by Dr. S. Mittnacht. The pTAT3-*luc* expressor vector, containing three tandem GREs (pGRE-*luc*), the rat pRSV-6RGR (pGR), the pGL3–2XERE-PS2 (pERE-*luc*), and pSG5-mERα (pERα), were described previously (31, 32) and were kindly donated by Dr. M. D. Vivanco and Prof. M. G. Parker.

Antibodies and Immunoprecipitations—Polyclonal anti-SYT 903 rabbit antibody was described previously (10). Polyclonal anti-SIP/CoAA 907 rabbit antibody was raised and affinity-purified as described for anti-SYT 903 (10) by using the synthetic peptide C+RSPTKSSLDYRRLPD from the C-terminal region of SIP/CoAA (Eurogentec). Anti-SYT 903 antibody was cross-linked using Seize X Protein A immunoprecipitation kit (Perbio) as indicated in the text. Commercially available anti-VP16 activation domain 2GV-4 mouse monoclonal (Euromedex) and anti-FLAG M2-agarose-conjugated mouse monoclonal antibodies (Sigma) were used for immunoprecipitations. Protein G beads (Sigma) were used to immunoprecipitate mouse monoclonal antibodies.

Untransfected nuclear and cytoplasmic cell lysates of HeLa cells (350 mM NaCl and 100 mM NaCl, respectively) were prepared as described previously (33). Briefly, 8×10^5 cells were scraped off by using cold phosphate-buffered saline (PBS), pelleted, and resuspended in 400 μl of cold 100 mM NaCl buffer (100 mM NaCl, 10 mM HEPES-KOH, pH 7.9, at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche Applied Science)). The cells were allowed to swell on ice for 10 min and then vortexed gently for 10 s. Samples were centrifuged, and the supernatant obtained (cytoplasmic fraction) was used immediately for co-immunoprecipitation or Western blot analysis. The pellet was then resuspended in 100 μl of cold 350 mM NaCl buffer (350 mM NaCl, 20 mM HEPES-KOH, pH 7.9, at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche Applied Science)) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation, and the extraction process was repeated a second time. The two 350 mM supernatants (nuclear fractions) were combined and diluted 1:2 with 100 mM NaCl buffer to 400 μl

and used immediately for co-immunoprecipitation or Western blot analysis.

Whole cell lysates of transfected COS7 cells were obtained using M-PER mammalian protein extraction reagent (Perbio) according to the manufacturer's instructions. For the immunoprecipitations, cell lysates were prepared in the presence of protease inhibitors (Roche Applied Science) and phosphatase inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 10 mM glycerol phosphate) and incubated with anti-FLAG M2-agarose-conjugated mouse monoclonal antibody for 4 h at 4 °C or with anti-VP16 activation domain 2GV-4 mouse monoclonal antibody for 1 h at 4 °C and for a further 3 h after 50 μl of 50% (v/v) protein G-Sepharose (Sigma; preequilibrated in PBS buffer) was added. Protein G beads were collected by centrifugation, and the pellets were washed five times in cold PBS buffer.

Beads were eluted using ImmunoPure IgG elution buffer, pH 2.8 (Perbio), incubated at room temperature for 5 min or 2× LDS sample buffer (Invitrogen) incubated at room temperature for 10 min. The eluted proteins were passed through a Spin-X centrifuge tube filter containing a cellulose acetate membrane of 0.22-μm pore diameter (Perbio) to avoid any contamination with residual antibody conjugate beads. Samples were analyzed by protein electrophoresis.

GST Pull-down Assay—^{35}S-Labeled proteins were generated using the TNT T7 quick-coupled transcription/translation system (Promega). Pull downs with *in vitro* ^{35}S-radiolabeled luciferase and SYT full-length were performed as described previously (10).

Immunopurification and in Vitro Interaction of FLAG-SIP/CoAA with VP16-SYT and VP16-SYT-SSX2 from Transfected Cell Lysates— Immunopurification and *in vitro* interaction of FLAG-SIP/CoAA with VP16-SYT and VP16-SYT-SSX2 was performed as described previously (10). Briefly, FLAG-SIP/CoAA, VP16-SYT, and VP16-SYT-SSX2 were immunopurified from cell lysates from separately transfected COS7 cells using anti-VP16 activation domain clone 2GV-4 mouse monoclonal antibody (Euromedex) with protein G-Sepharose (Sigma), and anti-FLAG M2-agarose-conjugated mouse monoclonal antibody (Sigma). FLAG-SIP/CoAA was then eluted and added to the purified VP16-SYT or VP16-SYT-SSX2 proteins bound to the beads via anti-VP16 mouse monoclonal antibody. Beads were collected by centrifugation after 4 h of incubation and washed in cold PBS buffer. Bound proteins were eluted and analyzed by Western blot.

Immunoblot Analysis—Proteins from whole cell lysates and immunoprecipitations were analyzed by NuPAGE 4–12% BisTris SDS-PAGE (Invitrogen) and transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore). Blots were incubated with the indicated primary antibody and then with the species-specific horseradish peroxidase-conjugated secondary antibody (Bio-Rad), and bands were detected by chemiluminescence (ECL detection reagents, Amersham Biosciences).

Transient Transfections and Two-hybrid Assays—The CheckMate mammalian two-hybrid dual reporter *firefly*/*Renilla* luciferase assay (Promega) was used to detect protein-protein interactions. For each transfection different combinations of plasmid DNA of the mammalian vectors pBIND and pACT (Promega) expressing full-length or deletion constructs of SYT and SIP/CoAA were transfected together with the luciferase reporter vector pG5-*luc* according to the protocol as described previously (10). Duplicate transfections were carried out in COS7 cells (monkey kidney fibroblast) using 6-well plates and Superfect reagent (Qiagen). The experiments were repeated at least three times independently and the results presented as the average ± S.D. GAL4 and VP16 parental vectors (provided by Promega) were used for nega-

tive controls. *Firefly* luciferase values were obtained using a microtiter plate luminometer (Dynex). Results were normalized for transfection efficiency by the use of co-expressed *Renilla* luciferase gene located on the pBIND vector, and for levels of protein expression by Western blot analysis to control for variations in protein expression level of transfected constructs.

The SW13 cell line was normally cultured in Dulbecco's modified Eagle's medium with Glutamax I (Invitrogen), supplemented with fetal calf serum (Invitrogen) and streptomycin sulfate (0.1 g/liter)/benzylpenicillin sodium (0.06 g/liter). 24 h before transfection SW13 cells were plated in 6-well plates and grown in phenol-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% charcoal-stripped fetal calf serum (GlobePharm), streptomycin sulfate (0.1 g/liter)/benzylpenicillin sodium (0.06 g/liter), and Glutamax I (1×) (Invitrogen). Cells were transfected with pERE-*luc* (150 ng) and pERα (150 ng) or pGRE-*luc* (150 ng) and pGR (150 ng) together with 400 ng of the indicated combinations of the following constructs: FLAG-hBRM FL, FLAG-SIP/CoAA FL, and FLAG-SYT FL or pcDNA5/FRT/TO-BRG1 FL and pcDNA5/FRT/TO-BRG1ΔSNF11 expressing full-length BRG1 and BRG1Δ1–211. FLAG-tagged or pcDNA5/FRT/TO empty vectors were added so that the same amount of DNA was used in each transfection. Transfections were carried out using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. 24 h after transfection, the medium was aspirated and replaced with phenol-free Dulbecco's modified Eagle's medium (Invitrogen) containing 10% charcoal-stripped fetal calf serum, streptomycin sulfate (0.1 g/liter)/benzylpenicillin sodium (0.06 g/liter), and Glutamax I supplemented either with 10 nM E2 (Sigma) or 100 nM dexamethasone (Sigma), and 0.1% ethanol was used as a vehicle control. 24 h post-hormone treatment, cells were washed in cold PBS and lysed in 500 μl/well of Passive Lysis Buffer (Promega) according to the manufacturer's specifications. Luciferase values were obtained by testing 20 μl of the cell lysates using a microtiter plate luminometer (Dynex). Results were normalized for transfection efficiency by the use of co-expressed pRL-SV40-*luc* vector (150 ng) that constitutively expresses the *Renilla* luciferase. Levels of protein expression were also determined by Western blot analysis to control for variations in the protein expression level of transfected constructs. The experiments were repeated at least three times independently and each time in duplicate. The results shown represent the average ± S.D.

RESULTS

SIP/CoAA, a Novel SYT-binding Protein—In an attempt to isolate new proteins that interact with the transactivating QPGY domain of SYT, we screened a cDNA library made from the synovial sarcoma cell line SS255 using the yeast two-hybrid technique. The bait consisted of the C-terminal QPGY activation domain (amino acids 160–387) fused to a GAL4 DNA binding domain. A screen of 2.5×10^5 transformants yielded three clones that grew on trp$^-$-leu$^-$-his$^-$ selection medium and that were positive in the β-galactosidase assay. Sequence determination of these clones revealed that the inserts were identical, with the coding sequence of the inserts in-frame with the GAL4 DNA binding domain. Specificity of interaction was shown when the DNA from these clones was isolated and retransformed into yeast expressing C-terminal SSX2 and C-terminal PRCC (34) as negative controls. No growth in trp$^-$-leu$^-$-his$^-$ plates was observed in this study.

The protein isolated from the screening was originally novel and named SIP (SYT interacting protein, GenBank^{TM} accession number AF080561). SIP is now known as a new nuclear receptor co-activator, called CoAA (co-activator activator), which has a role at the interface of

FIGURE 1. **Schematic representations of the primary structures of SYT, SIP/CoAA, EWS, and TLS/FUS proteins.** *Vertical striped box* indicates the SYT N-terminal homology domain (SNH); *black boxes* indicate the RRM; *gray boxes* indicate the repeat regions (QPGY or YQ domains); *hatched boxes* indicate the multiple repeats of arginine-glycine-glycine tripeptide (RGG).
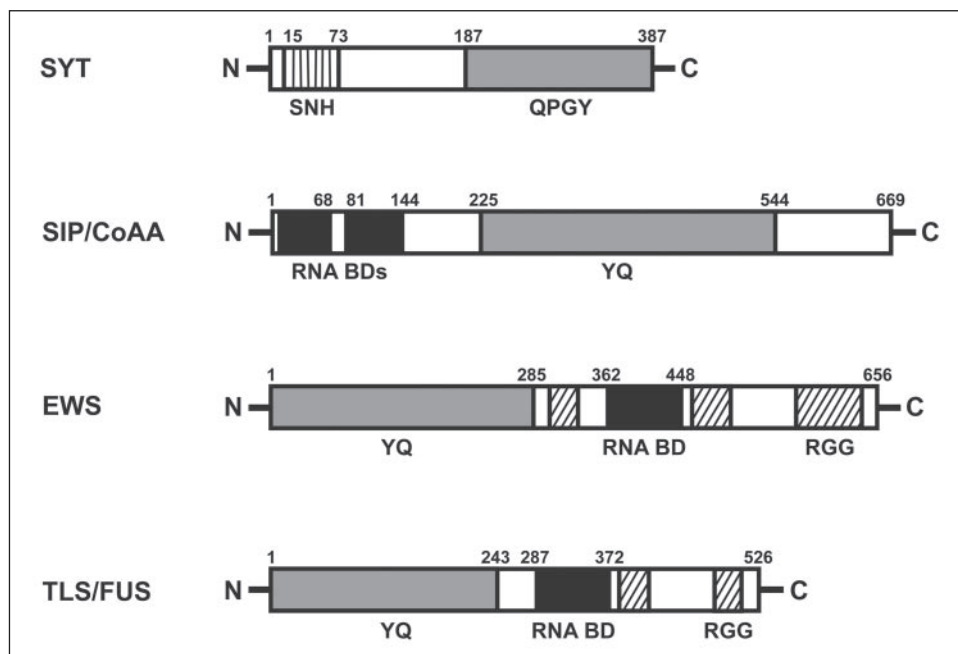
transcriptional co-activation and RNA splicing (25–27). In this work we use the name SIP/CoAA to identify this protein.

Sequence Analysis of SIP/CoAA—SIP/CoAA encodes a protein of 699 amino acids in length that belongs to a family of RNA-binding proteins characterized by the presence of two RNA recognition motifs (RRMs) at its N-terminal end (25). Outside the RNA-binding motifs, Iwasaki *et al.* (25) reported the presence of a domain (amino acids 307–584) that interacts with the thyroid hormone receptor-binding protein (TRBP) and of a consensus motif *XYXXQ*, throughout the same domain, present in >20 copies. The motif was characterized with a tyrosine at the second residue and a glutamine most commonly in position 5, where *X* represents a small amino acid that can include Gly, Ala, Ser, and Pro. Here we analyzed the consensus repeat region in more detail, and we show the homology with similar repeats identified in the EWS (23) and in TLS/FUS (35, 36) family of proteins. *EWS* and *TLS/FUS* genes were first recognized to be involved in different chromosomal translocations in many types of sarcomas and leukemias (1, 37, 38). The encoded proteins are known to be RNA-binding proteins with a single RRM motif and multiple repeats of arginine-glycine-glycine (RGG) tripeptide involved in RNA binding (39). The RGG tripeptides are absent in the SIP/CoAA sequence. SYT, SIP/CoAA, EWS, and FUS/TLS domains are compared in Fig. 1. Our detailed analysis of the repetitive motif of SIP/CoAA shows that it contains a hexapeptide repeated 27 times between amino acid 225 and 544 (Fig. 2, *a* and *b*). The spacing between the repeats differs, ranging from 1 to 14 amino acids, with the most recurrent distance of 6 amino acids occurring 12 times. A comparison of the 27 consensus repeats is shown in Fig. 2*b*. The tyrosine at position 2 and the glutamine at position 5 are invariant. The percentage occurrence of amino acids in the other positions is shown in Fig. 2*c*. When the repetitive sequence has been compared with EWS and TLS/FUS repeats, the tyrosine at position 2 is always conserved with the glutamine occurring at position 4 (in EWS and TLS/FUS) or 5 (in SIP/CoAA), and it resembles the same sequence pattern (Fig. 2*d*). Most interestingly, there is a similar amino acid occurrence in the QPGY domain of SYT itself, although in this case it is difficult to identify a specific recurrent peptide motif with the tyrosine and glutamine residues (9). The homology between SYT and SIP/CoAA does not extend outside the peptide repeat

domain. We named the SIP/CoAA repeat region the YQ domain, which refers to the most conserved amino acids of the hexapeptide motif.

SIP/CoAA Interacts Specifically with SYT in Vitro and in Vivo—*In vitro* binding studies were carried out to confirm that the interaction between SYT and SIP/CoAA is specific and direct. GST-SIP/CoAA fusion protein (amino acids 93–669) was purified and immobilized on glutathione-Sephadex beads and incubated with *in vitro*-translated ^{35}S-labeled full-length SYT or luciferase, the latter as a negative control. Radiolabeled SYT associates with GST-SIP/CoAA (Fig. 3*a*, *lane 5*) but not with GST protein alone (*lane 6*). Controls for specificity mixing GST-SIP/CoAA or GST alone with radiolabeled luciferase were also negative (Fig. 3*a*, *lanes 3* and *4*, respectively). Fig. 3*a*, *lanes 1* and *2*, shows the ^{35}S-labeled luciferase and SYT inputs that correspond to 15% of the input material used in the pull-down assay. Approximately 30% of the full-length radiolabeled SYT bound to the SIP/CoAA protein (amino acids 93–669). Radiolabeled SYT has a multiband pattern, which may be explained due to early terminations during *in vitro* transcription/translation.

To confirm the interaction of the endogenous proteins *in vivo*, immunoprecipitation assays were performed using HeLa cell lysates. Anti-SYT 903 antibody cross-linked to protein A beads was used to immunoprecipitate SYT. Western blots were then analyzed as indicated in Fig. 3*b*. Low pH elution buffer and 2× LDS sample buffer were used sequentially to elute SYT and SIP/CoAA proteins from the beads and minimize cross-reaction with the same species-specific second antibody in Western blot analysis. Fig. 3*b*, *top panel,* shows Western blots for co-immunoprecipitated endogenous SIP/CoAA, and Fig. 3*b bottom panel* shows the immunoprecipitated endogenous SYT. Using protein A-agarose conjugate beads with cross-linked antibody, we repeatedly detected cross-reaction in Western blots with the Ig heavy chain (55 kDa) of the anti-SYT 903 antibody used for immunoprecipitation (see Fig. 3*b*, *lanes 6* and *9*) and to some extent with protein A beads (*lane 3*). Both bands overlap the 54-kDa band of SYT.

Immunoprecipitations were performed with cell lysates prepared in low salt buffer (100 mM NaCl) and followed by high salt extraction (350 mM NaCl) buffer. Low salt extraction lysate (Fig. 3*b*, *input lane 7*), which isolates the cytoplasmic fraction, also contains some SYT protein that
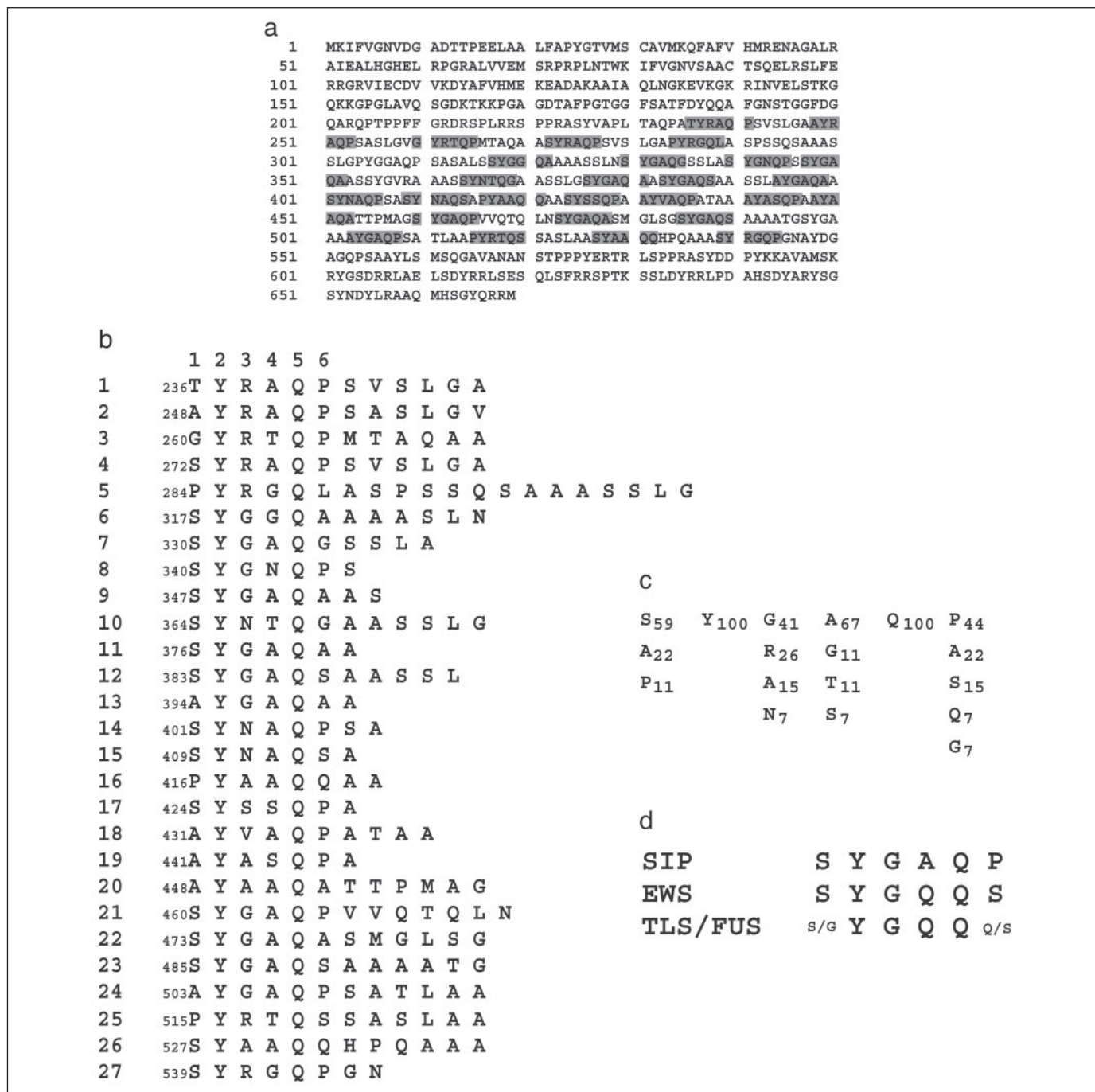
FIGURE 2. **Analysis of the SIP/CoAA repeat domain.** *a,* the SIP/CoAA amino acid sequence. The central domain of SIP/CoAA contains a sequence of six amino acids that are repeated 27 times between amino acids 225 and 544. The *shaded areas* represent the amino acid hexapeptide repeats. *b,* the repeat domain of SIP/CoAA organized to highlight the presence of the repeat motif. In this region tyrosines (*Y*) and glutamines (*Q*) always occur in position 2 and 5, respectively, and characterize the motif that is repeated 27 times. The repeats are spaced by a different number of amino acids ranging between 1 and 14. *c,* the most frequent residues with their frequency of occurrence at a given position in the motif in percent. *d,* the consensus sequence of the SIP/CoAA repeat compared with the consensus repeat found in EWS and TLS/FUS.

probably has low affinity to chromatin and requires lower ionic strength to be extracted from the chromatin matrix. Confocal microscopy analysis showed SIP/CoAA protein to be located in the nucleus and the cytoplasm (data not shown). The high salt extraction lysate (Fig. 3*b*, *inputs, lanes 1* and *4*) isolates the nuclear fraction. *Inputs* in *lanes 1, 4* and *7* of Fig. 3*b* represent 2.5% of the total lysate used in each immunoprecipitation, and 10% of the co-immunoprecipitated proteins was loaded in *lanes 3, 6,* and *9*. Cross-linked anti-SYT 903 antibody specifically co-precipitates SIP/CoAA protein in Fig. 3*b, lane 6.* We have estimated that in this experiment ~6–8% of SIP/CoAA was associated

with SYT in the nucleus. No co-precipitated bands were detected with the same nuclear lysate and protein A beads without cross-linked anti-SYT 903 antibody (beads only in Fig. 3*b*, *lanes 1–3*). Cross-linked beads with anti-SYT 903 did not co-precipitate SIP/CoAA from the cytoplasmic cell lysate (Fig. 3*b*, *lanes 7–9*) indicating that binding in the nuclear fraction (*lanes 4–6*) is physiological and functional. Binding may occur when these proteins are tightly bound in transcription complexes on the chromatin matrix. During the preparation of the cytoplasmic cell lysate two additional fainter bands are observed, respectively, below and above the expected endogenous SYT and SIP/CoAA protein bands (Fig. 3*b*,

FIGURE 3. **Direct interaction of SYT with SIP/CoAA** *in vitro* and *in vivo*. *a,* bacterially produced GST-SIP/CoAA (amino acids 93–669) and GST protein alone were purified with glutathione-Sepharose beads and incubated with *in vitro* translated [^{35}S]methionine-labeled full-length SYT (^{35}S SYT FL) or luciferase (^{35}S Luc) proteins, the latter used as a negative control. Bound proteins were resolved by SDS-PAGE and visualized with a PhosphorImager. Specific binding is observed between GST-SIP/CoAA and SYT (*lane 5*). *Lane 1,* input of *in vitro* translated ^{35}S-labeled luciferase; *lane 2,* input of *in vitro* translated ^{35}S-labeled SYT; *lane 3,* GST-SIP/CoAA incubated with ^{35}S-labeled luciferase; *lane 4,* GST protein alone incubated with ^{35}S-labeled luciferase; *lane 5,* GST-SIP/CoAA incubated with ^{35}S-labeled SYT; *lane 6,* GST protein alone incubated with ^{35}S-labeled SYT. *b,* HeLa cell lysates used for immunoprecipitation (*IP*) were prepared using low salt extraction buffer (100 mM NaCl, *lanes 7–9*) followed by high salt extraction buffer (350 mM NaCl, *lanes 1–6*). Western blots were probed with rabbit anti-SYT 903 and rabbit anti-SIP/CoAA 907 antibodies as indicated in the figure. *Lanes 1* and *4,* high salt extraction inputs. *Lanes 2* and *3,* immunoprecipitation using beads only in high salt lysate. The elution steps were performed using pH 2.8 elution buffer (*lane 2*) and 2× LDS sample buffer at room temperature (*RT*) (*lane 3*). *Lanes 5* and *6,* co-immunoprecipitation of endogenous SIP/CoAA using cross-linked anti-SYT 903 antibody in high salt lysate. The elution steps were performed using pH 2.8 elution buffer (*lane 5*) and 2× LDS sample buffer at room temperature (*lane 6*). *Lane 7,* low salt extraction input. *Lanes 8* and *9,* immunoprecipitation using cross-linked anti-SYT 903 antibody in low salt lysate. The elution steps were performed using pH 2.8 elution buffer (*lane 8*) and 2× LDS sample buffer at room temperature (*lane 9*).
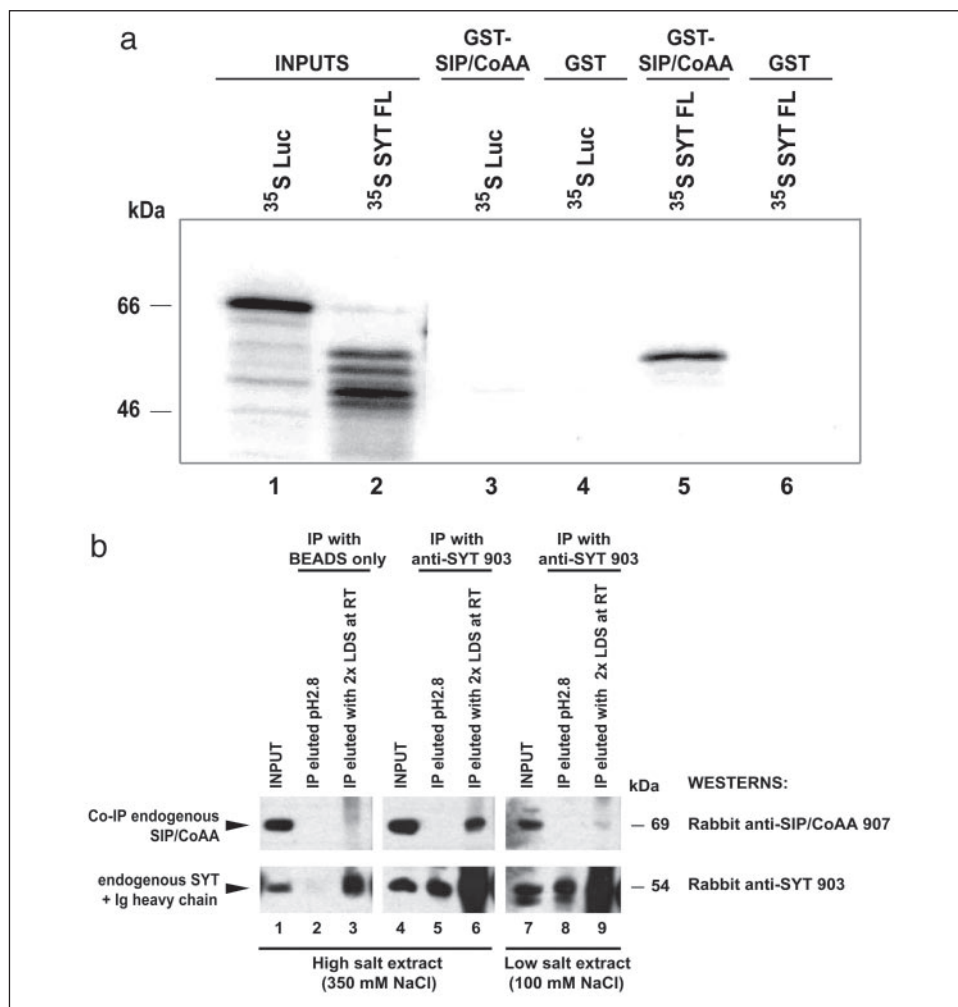
lane 7). The second high salt extraction on the same sample (nuclear fraction) extracts a single band (Fig. 3*b, lanes 1* and *4*). To determine whether the doublets observed were due to a different phosphorylation status of the endogenous proteins, low salt extraction lysates were incubated in the presence or absence of λ protein phosphatase (data not shown). Doublets were not affected by λ protein phosphatase treatment because there was no band shift from the top to the lower band, as would be expected if the top band was more highly phosphorylated. Evidence that SYT protein does not appear to be phosphorylated was also discussed previously (10, 40). These results indicate that the SIP/CoAA protein interacts specifically with the SYT protein *in vitro* and *in vivo* and that the interaction is specific and occurs in the cell nucleus.

The YQ Repeat Region of SIP/CoAA Acts as a Transcriptional Activating Domain, whereas the C-terminal End Cis-represses YQ Transactivation—The repeat motif at the N terminus of EWS and at the C terminus of SYT (the QPGY domain) has been found previously to activate transcription when fused to the GAL4 DNA binding domain in transient transfection experiments, and the activation is enhanced when the regions flanking the repeat motifs were removed (9, 10, 24, 41). Here we investigated whether the YQ domain in SIP/CoAA also has comparable transcriptional activating properties because of its similarity to EWS and SYT repeats. The full-length SIP/CoAA and the two deletion constructs C and B were cloned into the mammalian expression vector pBIND so that they were fused downstream of the GAL4 DNA binding domain. These constructs (named GAL4-SIP/CoAA FL, GAL4-SIP/CoAA C, and GAL4-SIP/CoAA B, respectively) or the

pBIND parental vector alone (GAL4 only) were transfected into COS7 cells together with pG5-*luc* reporter vector. The pG5-*luc* vector contains five upstream activating sequence GAL4-binding sites upstream of a TATA box minimal adenovirus promoter linked to the *firefly* luciferase gene. The results showed that no activation of the *firefly* reporter gene was detected when GAL4 is fused to the SIP/CoAA full-length construct (Fig. 4, *GAL4-SIP/CoAA FL, transfection 1*) but an increase of the luciferase value was detected in construct C when the two RNA binding domains are removed (*GAL4-SIP/CoAA C, transfection 2*). More notably, when the last 84 amino acids were also removed, and only the YQ domain was left in-frame with the GAL4-binding site, the construct strongly activated luciferase expression (683-fold) (Fig. 4, *GAL4-SIP/CoAA B, transfection 3*). Thus, proteins that carry these similar repeat patterns, although not identical, are able to activate transcription when used in reporter assays. Most interestingly, the transactivating ability is fully revealed only when the repeat region is expressed with the surrounding regions of the protein removed and in particular for SIP/CoAA, with the deletion of the last 85 amino acids (amino acids 585–669). To investigate the hypothesis of a repressing domain located at the C-end of SIP/CoAA, we constructed a plasmid in which DNA encoding the SIP/CoAA C-terminal 85 amino acids 585–669 was fused downstream of the VP16 region in the GAL4-VP16 vector. We called the construct GAL4-VP16-SIP/CoAA A. GAL4-VP16 contains the GAL4 DNA binding domain fused immediately upstream of the herpes simplex virus VP16 activation domain, which is known to be a strong activator. Expression constructs GAL4-VP16 and GAL4-VP16-SIP/CoAA
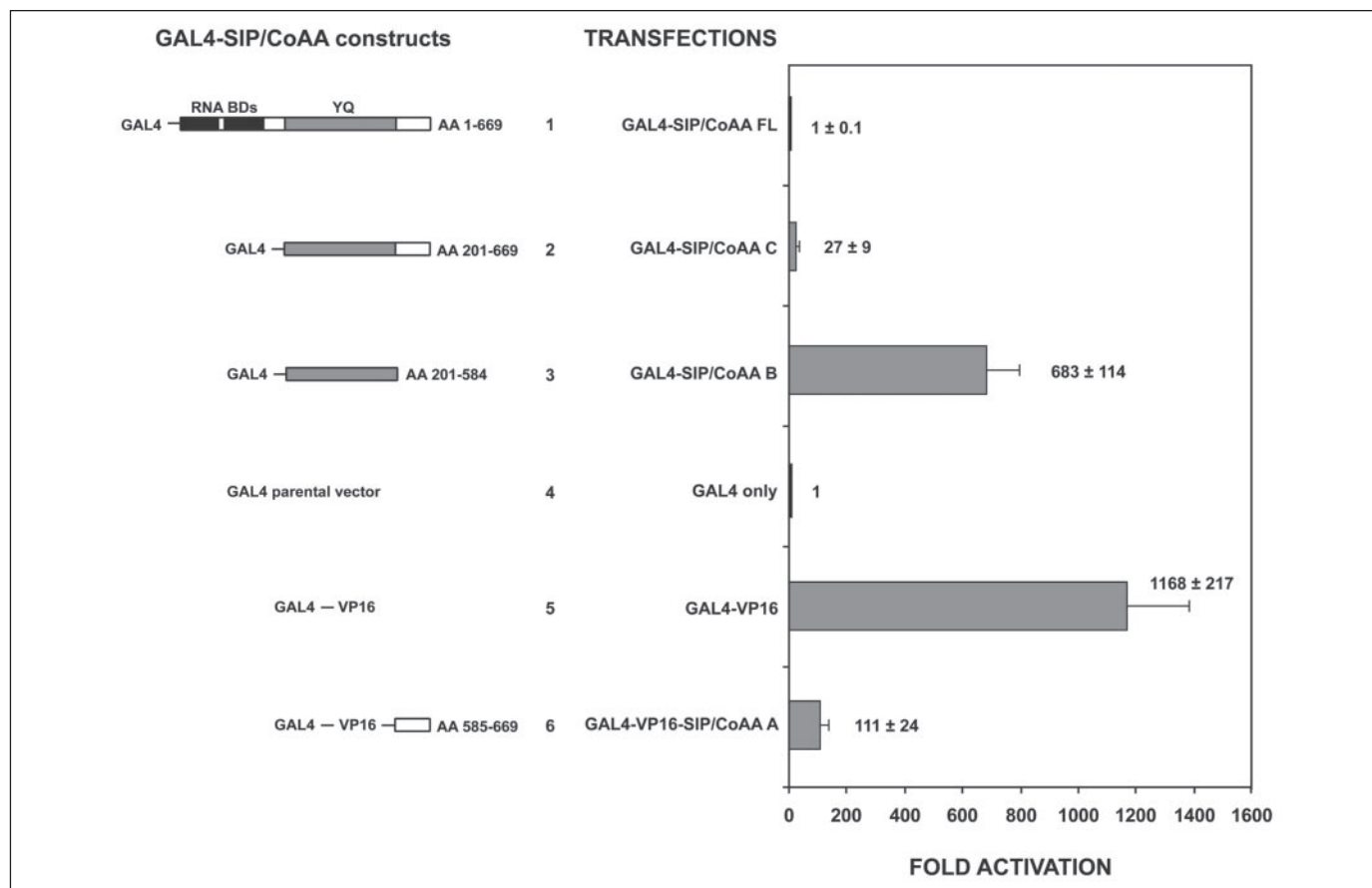
FIGURE 4. **The YQ domain of SIP/CoAA can activate transcription although the last 85 amino acids at the C-terminal end cis-repress YQ transactivation.** On the *left* are schematic diagrams of the expression constructs GAL4-SIP/CoAA FL, GAL4-SIP/CoAA C, and GAL4-SIP/CoAA B, GAL4-VP16, and GAL4-VP16-SIP/CoAA A. In the diagrams, *shaded rectangles* represent different domains in SIP/CoAA proteins. The name of each construct and the amino acid (*AA*) length are indicated beside the histogram. The SIP/CoAA domains are the two N-terminal RNA binding domains (*RNA BDs*) and the C-terminal YQ domain. The GAL4 DNA binding domain of the fusion proteins is indicated in the constructs. On the *right* fold activations of the luciferase reporter are represented as a *histogram*. The indicated expressor constructs were transfected into COS7 cells together with the pG5-*luc* reporter. Fold activation of the reporter was calculated by dividing the normalized *firefly* luciferase figures of the expressors by that of the (low) background average of the *firefly* luciferase value of the unfused GAL4 parental vector (transfection 4). The background has been designated as 1 activation unit.

A and reporter construct pG5-*luc* were co-transfected into COS7 cells. The VP16-induced transcriptional activation of the luciferase reporter was strongly repressed by the addition of the C-terminal SIP/CoAA 85 amino acids (11-fold repression, compare transfection 5 to 6) similarly to what we observed in transfection 2 where the amino acids 585–669 cis-repress YQ activation (25-fold repression, compare transfection 2 to 3). Thus, our results suggest that the C-terminal end of SIP/CoAA can down-modulate YQ-induced transcriptional activation and act as a general repressor domain. Transfection efficiency was normalized using constitutively expressed *Renilla* luciferase gene integrated in the GAL4 vector. Western blots showed the same level of protein expression in each transfection (data not shown).

The YQ Repeat Region and the C-terminal Domain in SIP/CoAA Interact with the QPGY Repeat Region of SYT —Full-length and deletion constructs of SYT and SIP/CoAA were fused, respectively, to the VP16 activation domain and the GAL4 DNA binding domain (Fig. 5, *a* and *b*). The combined VP16 and GAL4 fusion constructs (with the appropriate controls) were co-transfected in COS7 cells together with the reporter vector *firefly* pG5-*luc,* and constitutively expressed *Renilla* luciferase was used as an internal control. Enhancement of *firefly* luciferase expression level was observed when full-length GAL4-SIP/CoAA (GAL4-SIP/CoAA FL) was transfected with full-length VP16-SYT (VP16-SYT FL) (73.2-fold), SYT A (36.8-fold), and B (22.2-fold) (Fig. 5*a*, *transfections 1–3*). There was no activation of the reporter gene with

VP16-SYT D where the QPGY domain of SYT was partially removed (Fig. 5*a*, *transfection 4*). These results confirmed the yeast two-hybrid interaction in a mammalian reporter assay. Most interestingly, a lack of luciferase expression was also observed when VP16-SYT-SSX2 FL was co-transfected with GAL4-SIP/CoAA FL (transfection 5). Finally, to investigate whether it is the deletion of SYT amino acids 380–387 that may account for the loss of luciferase expression in transfection 5, we co-transfected GAL4-SIP/CoAA with VP16-SYTΔ8 (transfection 6), the latter expressing SYT without the last eight amino acids that are lost in the SYT-SSX translocation fusion. GAL4-SIP/CoAA still binds VP16-SYTΔ8 indicating that it is the SSX region in the fusion, not the lack of amino acids 380–387, which affects the loss of luciferase expression.

Mammalian two-hybrid assays were then carried out with VP16-SYT FL in parallel with GAL4-SIP/CoAA FL and deletion constructs GAL4-SIP/CoAA C, B, and A (Fig. 5*b*). Interaction with the SYT protein is shown using construct C containing the C-terminal region of SIP/CoAA (37.7-fold) (transfection 2), with B expressing the YQ domain (8.8-fold) (transfection 3) and with A where only the last 85 amino acids of SIP/CoAA are expressed in the fusion (9.8-fold) (transfection 4). Lower fold activation in transfections 3 and 4 may suggest that the interaction interfaces are within the YQ domain but also involve the C-terminal end of SIP/CoAA. Western blots showed the same level of protein expression in each transfection (data not shown).
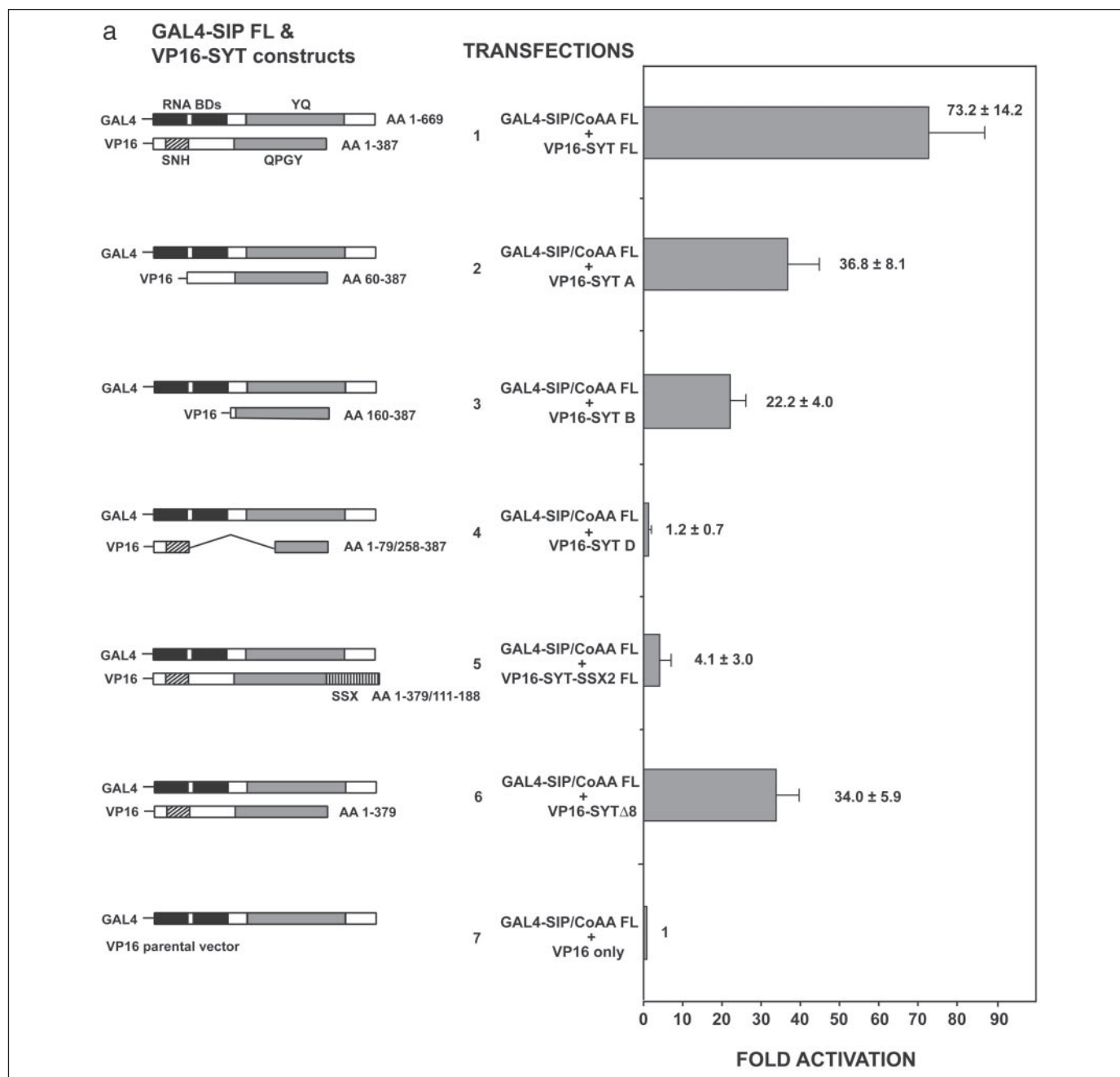
FIGURE 5. **The QPGY domain of SYT binds the transactivator YQ domain and the repressing domain at the C-terminal end of SIP/CoAA protein.** *a*, on the *left* are schematic diagrams of the expression constructs GAL4-SIP/CoAA FL, VP16-SYT fusion proteins, and VP16-SYT-SSX2 FL. In the diagrams, *shaded rectangles* represent different domains in SIP/CoAA and SYT proteins. The name of each construct and the amino acid (*AA*) length are indicated beside the histogram. The domains in SIP/CoAA from *left* to *right* are as follows: two RNA binding domains (*RNA BDs*) and the YQ domain; in SYT, SNH domain and QPGY domain. GAL4 and VP16 are indicated in the constructs. On the *right* fold activations of the luciferase reporter are represented as a *histogram*. The indicated expressor constructs were transfected into COS7 cells together with the pG5-*luc* reporter. Fold activation of the reporter was calculated by dividing the normalized *firefly* luciferase figures of the paired expressors by that of the (low) background average of the *firefly* luciferase value of GAL4-SIP/CoAA FL co-transfected with the unfused VP16 parental vector (*transfection 6*). The background has been designated as 1 activation unit. *b*, on the *left* are schematic diagrams of the expression constructs GAL4-SIP/CoAA fusion proteins and VP16-SYT FL. On the *right* fold activations of the luciferase reporter are represented as a *histogram*. Fold activation of the reporter was calculated by dividing the normalized *firefly* luciferase figures of the paired expressors by that of the average of the *firefly* luciferase values of GAL4-SIP/CoAA FL, C, B and A co-transfected with the unfused VP16 parental vector (data not shown).

In conclusion, the YQ repeats and the adjacent C-terminal domain of SIP/CoAA interact with the QPGY domain of SYT. Removal of the RRMs of SIP/CoAA and the SNH conserved domain of SYT has only a small effect.

SIP/CoAA Interacts in Vitro with the Proto-oncoprotein SYT and with the Oncoprotein SYT-SSX2, which Represses Transcription in a Mammalian Two-hybrid Assay—Interestingly, in Fig. 5*a* (*transfection 5*), there is no activation of the luciferase reporter when co-transfecting

VP16-SYT-SSX2 FL with GAL4-SIP/CoAA FL. We have reported previously a similar result when VP16-SYT-SSX2 FL was expressed together with GAL4-hBRM FL (10). The SYT-SSX2 protein was able to bind hBRM by *in vitro* pull-down and *in vivo* in colocalization studies (9, 10), but there was no activation of luciferase expression when we used a mammalian two-hybrid assay. The lack of activation in the two-hybrid assay was therefore likely to be due to the repression activity of the SSX region in the fusion rather than the lack of interaction. Here we inves-
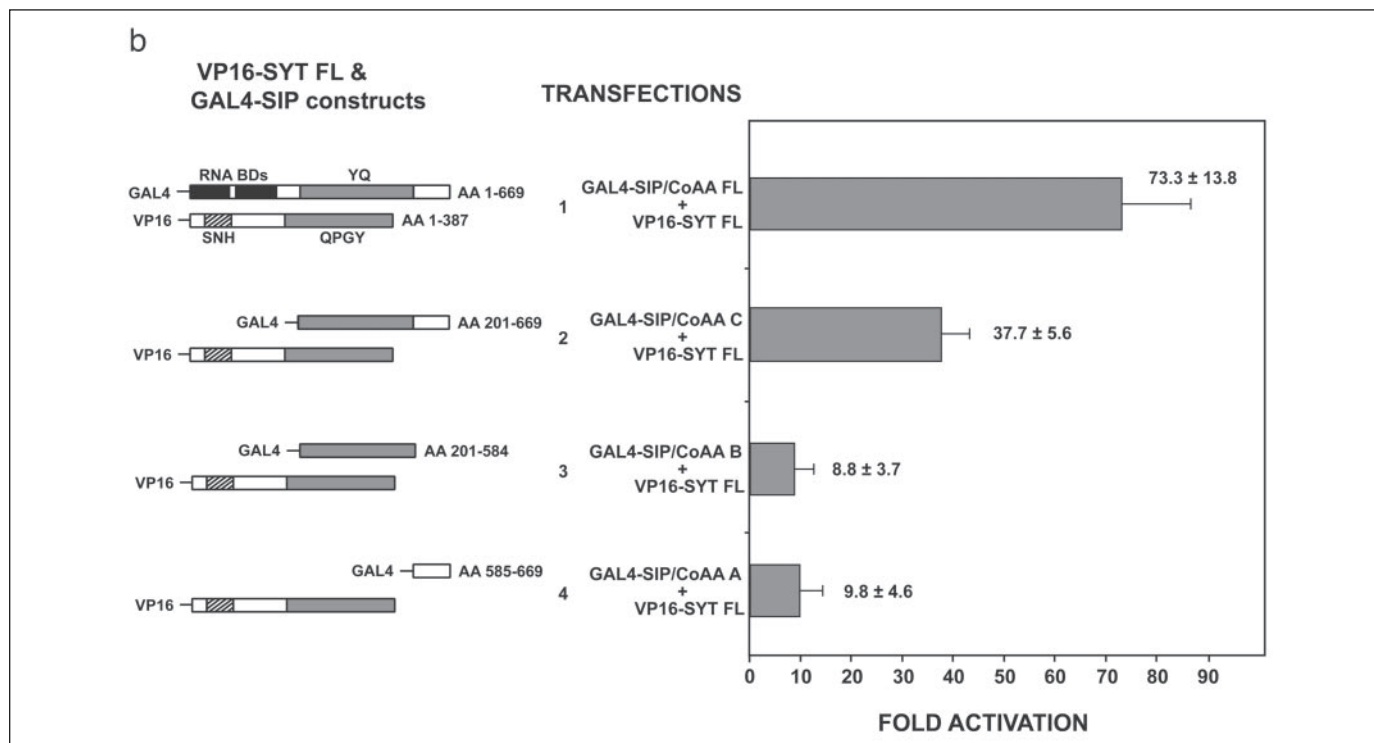
FIGURE 5—*continued*

tigated if SIP/CoAA FL also interacts *in vitro* with SYT-SSX2 FL. FLAG-SIP/CoAA FL, VP16-SYT FL, and VP16-SYT-SSX2 FL were separately expressed in mammalian COS7 cells (Fig. 6*a*, *lane 1*, for cell lysate expressing FLAG-SIP/CoAA FL; Fig. 6*b*, compare untransfected cell lysate in *lane 1* with *lanes 2* and *3* for transiently expressed SYT FL and SYT-SSX2 FL). Proteins were expressed separately and first immuno-purified before pull-down assays to obtain similar protein levels for SYT and SYT-SSX2. This allowed us to estimate the efficiency of SIP/CoAA binding with SYT and SYT-SSX2. The endogenous level of SYT would need to be taken into consideration if the proteins were co-expressed, which would have made it difficult to compare the pull-down results. The proteins were immunopurified using VP16 and FLAG tag antibod-ies. FLAG-SIP/CoAA FL was then eluted from the conjugated protein G beads (Fig. 6*a*, compare *lane 2* with immunodepleted cell lysate with *lane 3* with eluted immunopurified FLAG-SIP/CoAA FL). The immu-nopurified FLAG-SIP/CoAA FL was incubated with the VP16-SYT FL or VP16-SYT-SSX2 FL beads by using *in vitro* binding condition buffer. *In vitro* co-precipitation was then performed through the VP16 tag. After washing, the bead-bound proteins were analyzed by Western blot. The immunoprecipitation results show that FLAG-SIP/CoAA FL is successfully co-precipitated with VP16-SYT FL or VP16-SYT-SSX2 FL (Fig. 6, *a* and *b*, *lanes 4* and *5*). Negative controls were carried out using a VP16 protein immunoprecipitate with FLAG-SIP/CoAA FL to ensure specificity (Fig. 6, *a* and *b*, *lane 6*) and with buffer only for background levels (*lane 7*). This result is in agreement with what we have observed previously with SYT-SSX2 and hBRM proteins (9, 10). In the presence of SYT-SSX, the repression activity of the SSX domain present in the fusion may be responsible for abolishing the *firefly* luciferase expression in a mammalian two-hybrid assay.

SYT and SIP/CoAA Activate Hormone-response Elements in an hBRM and/or BRG1-dependent Manner—It is well established that ATP-dependent chromatin remodeling complexes are involved in transcrip-tional regulation by nuclear hormone receptors (42–44). For example,

the estrogen (ER) and glucocorticoid (GR) receptor co-activators and their requirement for hBRM or BRG1, the core ATPase proteins of the SWI/SNF complexes in human, are well documented (45–56). Cell lines that are deficient in hBRM and BRG1 show a reduced activation of estrogen and glucocorticoid receptors, which can be rescued through restoration of BRG1 (46, 47, 54). More recently, it was shown that SIP/CoAA associates with nuclear receptors as a novel ribonucleoprotein transcription co-activator (25), but it was not known whether this co-activation is dependent on chromatin remodeling complexes. We inves-tigated the possibility that SYT might potentiate the ability of SIP/CoAA to activate hormone-response elements, and this might be associated with the interaction of hBRM and/or BRG1. The SW13 cell line is deficient in both hBRM and BRG1 and is known to lack SWI/SNF activity (46, 57). These cells provide the opportunity to analyze the importance of hBRM and/or BRG1 in relation to SYT and SIP/CoAA co-activation. To examine this hypothesis, SYT and SIP/CoAA were co-transfected into the SW13 cell line along with luciferase reporters containing estrogen- (ERE) or glucocorticoid-response elements (GRE). In Fig. 7*a*, SW13 cells were transfected with plasmids encoding the estrogen receptor (ERα), the pERE-*luc* reporter construct, and the pRL-SV40 *Renilla* luciferase used as an internal control for transfection effi-ciency. In Fig. 7*b*, the ERα and the pERE-*luc* vector were substituted with the GR and the pGRE-*luc* reporter construct. Different combina-tions of SYT, SIP/CoAA, and full-length hBRM or BRG1 were used to study their ability, with or without hormone stimulation, to activate the two reporters containing the indicated response elements. The results show that SYT and SIP/CoAA co-activate the estrogen and glucocorti-coid elements and in a hormone-dependent manner (Fig. 7, *a* and *b*, *transfections 8* and *12*). In addition, SYT and SIP/CoAA co-activation is dependent on hBRM or BRG1 (compare transfections 5–12, expressing hBRM or BRG1, with transfections 1–4 without hBRM or BRG1). In the absence of hBRM/BRG1 (transfections 1–4), the reporter is activated between 5- and 10-fold probably because of hBRM/BRG1-independent

FIGURE 6. **Mammalian expressed FLAG-SIP/CoAA interacts with VP16-SYT and VP16-SYT-SSX2 in a similar manner.** Interactions of immunopurified proteins FLAG-SIP/CoAA and VP16-SYT or VP16-SYT-SSX2 from separately transfected COS7 cells are shown. FLAG-SIP/CoAA, VP16-SYT, and VP16-SYT-SSX2 were immunopurified from the cell lysates using anti-FLAG and anti-VP16-agarose-conjugated mouse monoclonal antibodies. *Lanes 1a, 2b,* and *3b* are Western blots of cell lysates following transfection with the indicated constructs. *Lanes 2a* and *3a* represent the purification steps of FLAG-SIP/CoAA. FLAG-SIP/CoAA protein was first expressed in COS7 cells (*lane 1a*) and then eluted from the beads (compare *lane 2a* to *lane 3a*) and added to the purified full-length VP16-SYT (VP16-SYT FL) and VP16-SYT-SSX2 (VP16-SYT-SSX2 FL) proteins bound to the protein G-beads. Expression levels of VP16-SYT FL and VP16-SYT-SSX2 FL are shown in *lanes 2b* and *3b,* respectively. Proteins bound to the beads were analyzed by protein electrophoresis as shown in *lanes 4–7.* The Western blot was probed with rabbit anti-SIP/CoAA 907 and anti-SYT 903 antibodies. *Lane 1a,* cell lysate from FLAG-SIP/CoAA transfection; *lane 1b,* untransfected cell lysate. *Lane 2a,* cell lysate after FLAG-SIP/CoAA immunodepletion; *lane 2b,* cell lysate from VP16-SYT transfection; *lane 3a,* eluted FLAG-SIP/CoAA; *lane 3b,* cell lysate from VP16-SYT-SSX2 transfection. *Lane 4, in vitro* co-precipitation of FLAG-SIP/CoAA with VP16-SYT using anti-VP16 monoclonal antibody. *Lane 5, in vitro* co-precipitation of FLAG-SIP/CoAA with VP16-SYT-SSX2 using anti-VP16 monoclonal antibody. *Lane 6, in vitro* co-precipitation of FLAG-SIP/CoAA with VP16 only using anti-VP16 monoclonal antibody. *Lane 7, in vitro* co-precipitation with buffer only using anti-VP16 monoclonal antibody.
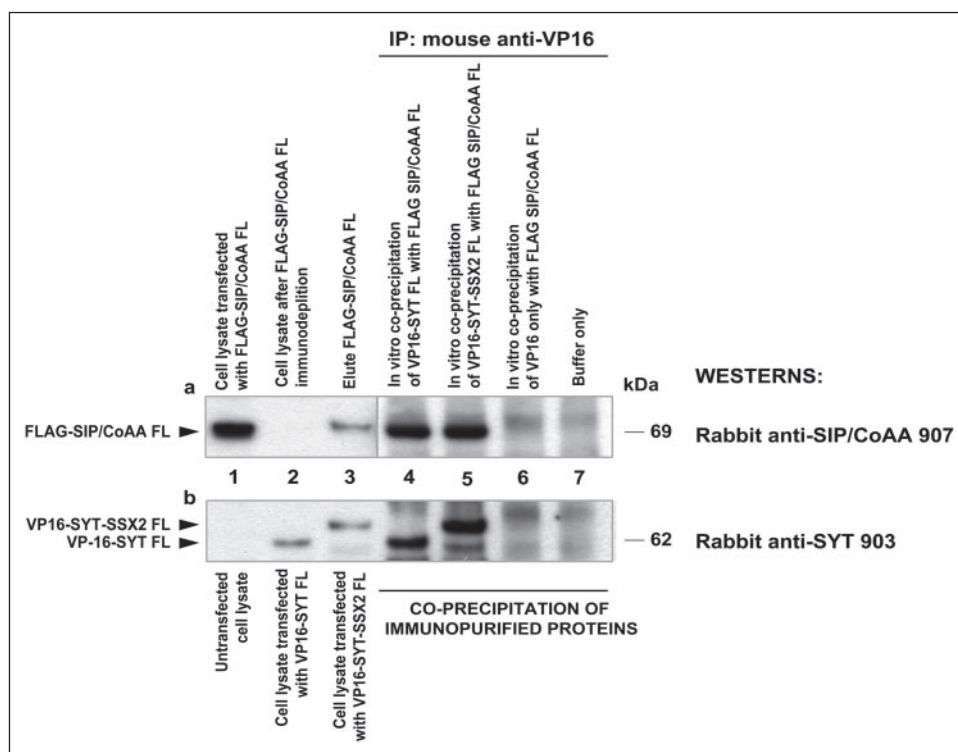
transcriptional activation stimulated by hormone addition. This hBRM/BRG1-independent activation is only minimally activated by SYT-SIP/CoAA in the case of the estrogen reporter and not at all with the glucocorticoid reporter (compare transfection 1 with transfections 2–4). No significant differences in transcriptional activation were revealed between hBRM and BRG1, which are highly homologous proteins (compare transfections 5–8, expressing hBRM, with transfections 9–12, expressing BRG1). Western blots showed similar levels of exogenous protein expression (data not shown). This observation is in agreement with our previous results that SYT binds with the same affinity to the hBRM and BRG1 SNF11 binding domain (SNF11 BD) located at the N-terminal ends of the two proteins. The SNF11 BD binds *in vivo* and *in vitro* the proto-oncoprotein SYT and the translocated proteins SYT-SSX2 (10, 40). Thus, SYT and SIP/CoAA co-activate the estrogen- and glucocorticoid-response elements. The ability to activate transcription fully is hormone-dependent and requires either hBRM or BRG1.

The N-terminal Domain of BRG1 Is Necessary for SYT and SIP/CoAA to Activate Transcription—Previous data showed that the interaction between the proto-oncoprotein SYT and hBRM/BRG1 is through the N-terminal SNF11 BD (10, 40). Here we investigated if this region was also important for the SYT and SIP/CoAA co-activation of hormone-response elements. We constructed a truncated form of BRG1 (BRG1ΔSNF11) missing amino acids 1–211, which removes the SNF11 BD at the N-terminal end. Results show that BRG1ΔSNF11 transfected instead of BRG1 FL strongly reduces the ability of SIP/CoAA (Fig. 8a) or SIP/CoAA and SYT (Fig. 8b) to activate transcription (compare transfection 2 with transfection 1) above the BRG1 level of activation (transfection 3). Transcriptional activation of the reporter due to BRG1 alone expression in transfection 3 could be attributed to the endogenous levels of SYT and SIP/CoAA expressed in SW13 cell line (data not shown) and/or through different mechanisms that may not require SYT and SIP/CoAA protein binding, *e.g.* the recruitment by the hormone of the histone acetylases, methyltransferases, and mediator complexes. West-

ern blots showed the same level of protein expression in each transfection (data not shown). Thus, the ability of SIP/CoAA (Fig. 8a) or both SIP/CoAA and SYT (Fig. 8b) to activate transcription as nuclear hormone receptor co-activators is dependent on the presence of the N-terminal region of hBRM/BRG1, which encompasses the SNF11 BD (amino acids 156–211).

DISCUSSION

The proto-oncoprotein SYT is a protein involved in the generation of synovial sarcomas as a results of the t(X,18) chromosomal rearrangement. Two domains have been identified in the proto-oncoprotein SYT as follows: (i) the SNH domain at the N terminus (amino acids 15–75), and (ii) the transactivating QPGY region at the C terminus of the protein (amino acids 187–387). The SNH domain is known to interact with the chromatin remodeling proteins hBRM and BRG1 (9, 10, 40) and with the acute leukemia-associated protein AF10 (a fusion partner of MLL) (21), and p300 binds the N-terminal end of SYT in G_1-arrested confluent cells (22). To date, no transcription factors are known to interact with the QPGY domain of SYT.

The goal of this work was to investigate the transactivating QPGY region of SYT and to isolate possible interacting partners targeting this domain. In the synovial sarcoma chromosomal translocation, the activation domain of the SYT protein is retained in the SYT-SSX fusions (9), and it is therefore assumed to play a role in the oncogenic process. The yeast two-hybrid technique was used to screen a library made from the synovial sarcoma cell line SS255 to identify new proteins, which may interact with this domain. The work described here identifies SIP/CoAA as the first protein interacting with the transactivating QPGY domain of the proto-oncoprotein SYT.

The protein sequence revealed that SIP/CoAA is closely related to members of the EWS and TLS/FUS family of proteins in that it has RRM-type RNA binding domains and multiple repeats of a short sequence containing tyrosine and glutamine that resemble those found

FIGURE 7. **SYT and SIP/CoAA activate ERE and GRE.** The activation is hBRM/BRG1 and ligand-dependent. *a,* SW13 cells were seeded in steroid-free media and co-transfected with expression plasmid for ERα (150 ng), pERE*luc firefly* luciferase reporter (150 ng), and pRL-SV40 *Renilla* luciferase for internal normalization (150 ng) together with different combinations of human pCMV-Tag2-SYT FL (400 ng), pCMV-Tag2-SIP/CoAA FL (400 ng), pCMV-Tag2-hBRM FL (400 ng), and pCMV-Tag2-BRG1 FL (400 ng) expressor plasmids as indicated. Total amounts of DNA for each well were equalized with additional pCMV-Tag2 parental vector. The cells were allowed to recover for 24 h and stimulated with either vehicle (0.1% EtOH) or 17β-estradiol (*E2*, 10 nM) as indicated. 24-h post-stimulation cells were harvested, lysed, and monitored for dual-luciferase activities. Basal luciferase activity (transfection 1, 0.1% EtOH) was set to "1" and fold activation is shown. *b,* SW13 cells were seeded in steroid-free media and co-transfected with expression plasmid for GR (150 ng), pGRE*luc firefly* luciferase reporter (150 ng), and pRL-SV40 *Renilla* luciferase for internal normalization (150 ng) together with different combinations of the expressor plasmids pCMV-Tag2-SYT FL (400 ng), pCMV-Tag2-SIP/CoAA FL (400 ng), pCMV-Tag2-hBRM FL (400 ng), and pCMV-Tag2-BRG1 FL (400 ng) as indicated in figure. Total amounts of DNA for each well were equalized with additional pCMV-Tag2 parental vector. The cells were allowed to recover for 24 h and stimulated with either vehicle (0.1% EtOH) or dexamethasone (*DEX*, 100 nM) as indicated. 24-h post-stimulation cells were harvested, lysed, and monitored for dual-luciferase activities. Basal luciferase activity (transfection 1, 0.1% EtOH) was set to 1, and fold activation is shown.
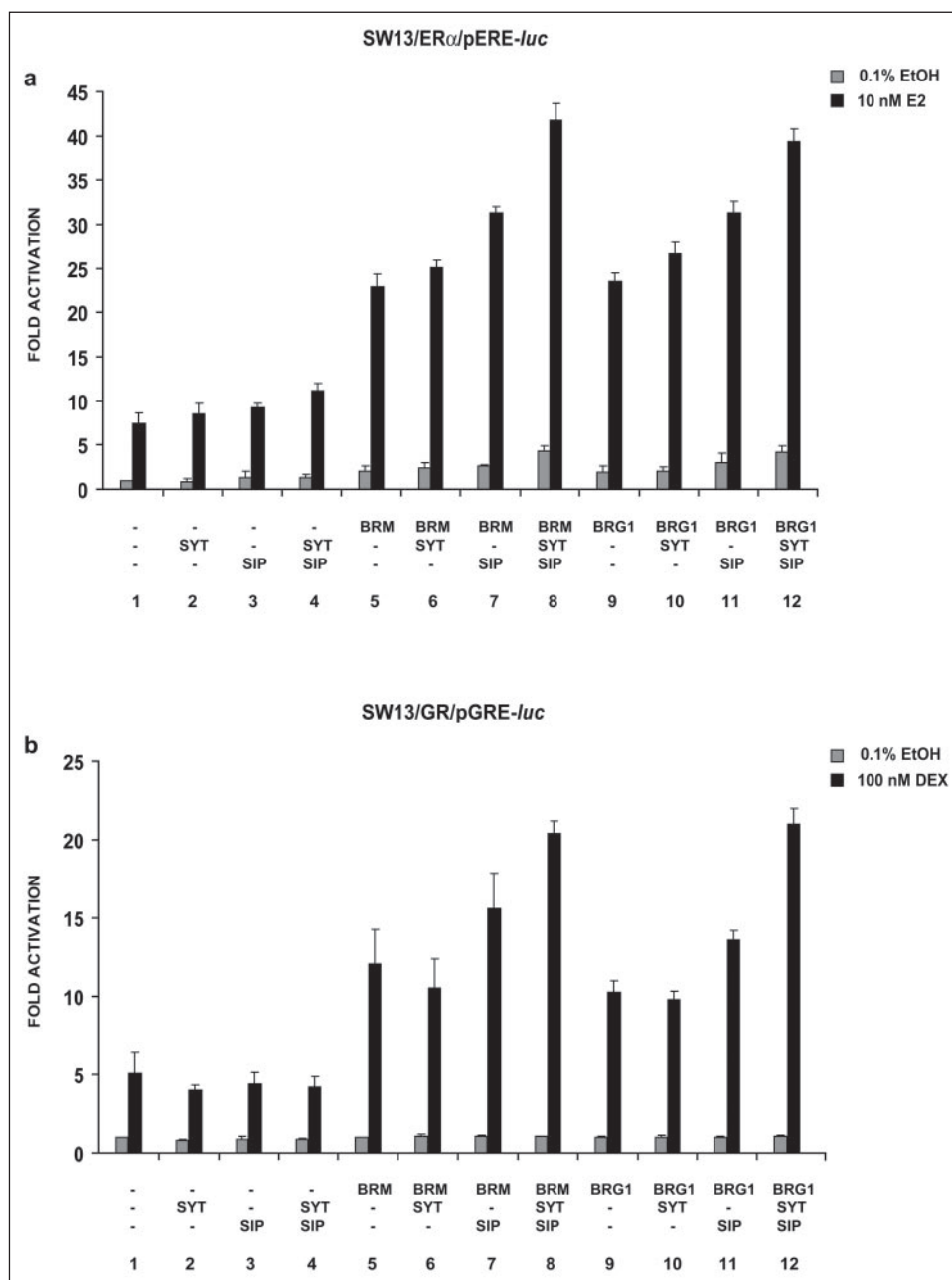
in the EWS and TLS/FUS proteins (Fig. 3, *a–d*). However, SIP/CoAA differs from the EWS and TLS/FUS in having two RNA binding domains, whereas the EWS and TLS/FUS proteins have only one, and they also contain additional nucleic acid-binding RGG motifs absent in SIP/CoAA (Fig. 2). EWS and TLS/FUS family were first discovered as fusion partners in chromosomal translocations in human cancer at a time when the functions of the wild type forms were still unknown (23, 35, 36). There are now several lines of evidence that describe these proteins as having a dual role in transcription and mRNA processing. The N-terminal repeat region of EWS and TLS/FUS associates with different components of the basal transcription factor complex TFIID, and the repeat region of EWS has been found to interact with the human RPB3 subunit of the RNA polymerase complex (58, 59). Furthermore, the same domain binds to hyperphosphorylated RNA polymerase II (60) and to the transcriptional co-activator CBP (61). EWS and TLS/FUS also recruit several splicing factors through their C-terminal RNA rec-ognition motifs and RGG-rich regions (60, 62, 63). Similarly, the multiple repeat region of SIP/CoAA is involved in transcriptional regulation by co-activating transcription mediated by multiple hormone-response elements and acting synergistically with the thyroid hormone receptor-binding protein (TRBP) and with the cAMP-response element-binding protein (CBP) (25). The same domain also interacts with both TRBP and p300 *in vitro* (25). In addition, SIP/CoAA is a hormonally recruited co-regulator that directly participates in alternative RNA splicing decisions in a promoter-preferential manner mediated through the two RRMs (26, 27).

It has been shown previously that the characteristic repeat motifs at the N terminus of EWS and at the C-terminal QPGY domain of SYT are able to activate transcription when fused to a GAL4 DNA binding domain in transient transfection experiments (9, 10, 41). Similarly, we showed here that the YQ domain of SIP/CoAA activates transcription in the same manner (Fig. 4). In addition, transactivation by EWS and

FIGURE 8. **SYT and SIP/CoAA activate hormone-response elements in presence of functional hBRM/BRG1 chromatin remodeling proteins.** *a* and *b,* SW13 cells were seeded in steroid-free media and co-transfected with expression plasmid for ERα (150 ng), pERE*luc firefly* luciferase reporter (150 ng), and pRL-SV40 *Renilla* luciferase for internal normalization (150 ng) together with different combinations of human pCMV-Tag2-SYT FL (400 ng), pCMV-Tag2-SIP/CoAA FL (400 ng), pCMV-Tag2-hBRG1 FL (400 ng), and pCMV-Tag2-BRG1ΔSNF11 (400 ng, amino acids Δ1–211) expressor plasmids as indicated in figure. Total amounts of DNA for each well were equalized with additional pCMV-Tag2 parental vector. The cells were allowed to recover for 24 h and stimulated with either vehicle (0.1% EtOH) or 17β-estradiol (*E2*, 10 nM) as indicated. 24-h post-stimulation cells were harvested, lysed, and monitored for dual-luciferase activities. Basal luciferase activity (transfection 1, 0.1% EtOH) was set to 1, and fold activation is shown.
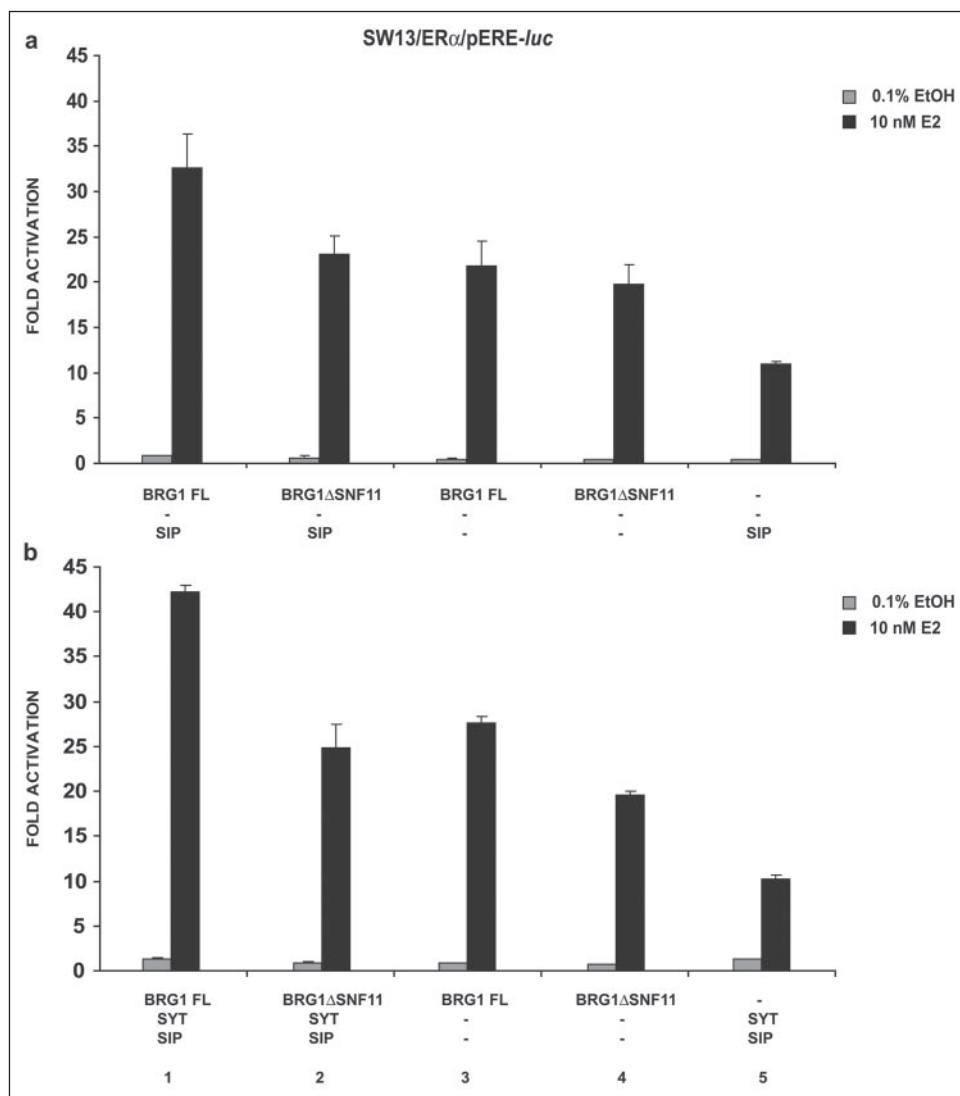
SYT as well as SIP/CoAA is much stronger when the regions flanking the domains are removed. The ability to activate transcription with the full-length EWS and SYT proteins has been reported previously to be very poor (9, 10, 64). To investigate further the significance of this observation for SIP/CoAA, we showed that the last 85 amino acids at the C-terminal end of the protein can strongly repress transactivation by the YQ domain. A similar observation has been reported also for EWS where regions of the RNA binding domain, including the multiple RGG motifs, but not the RRM motif (see Fig. 1), repress transactivation by the EWS activation domain (64). Here we have identified that the specific interaction between SIP/CoAA and SYT proteins involves the YQ repeat region plus the adjacent C-terminal repressing domain of SIP/CoAA and the QPGY domain of SYT. It is of interest that SYT also homo-oligomerizes with itself through its QPGY domain (10). Thus, these similar repeat domains, which activate transcription, may also serve as contact interfaces for protein-protein interactions. In this context SYT may activate SIP/CoAA by interacting through the YQ repeat region together with blocking the recruitment of repressors by binding the C terminus of SIP/CoAA.

It has been shown previously that endogenous SYT is an intrinsic component of the two SWI/SNF complexes in human cells (20). There are several lines of evidence demonstrating the importance of SWI/SNF acting as transcriptional activator complexes in concert with activated nuclear receptors recruited to hormone-response elements (45, 46, 52–56, 65). Our previous work (9, 10) together with that of Nagai *et al.* (40) showed that the proto-oncoprotein SYT and the SYT-SSX translocated fusions bind both hBRM and BRG1. There is no difference in binding affinity when hBRM and BRG1 have been compared *in vitro* in their ability to bind SYT (10). In this paper we have investigated the functional significance of SWI/SNF complexes recruited to hormone-response elements together with the proto-oncoprotein SYT and SIP/CoAA. In particular, we were interested to see if both SYT and SIP/CoAA activate hormone-response elements, and if this activation was hBRM/BRG1-dependent. Here we showed that SYT, in conjunction with SIP/CoAA, was able to activate to higher levels promoter elements containing estrogen and glucocorticoid-response elements when hBRM or BRG1 was restored in the SW13 cell line (57). Also important was the ability of SYT to activate transcription in a hormone-dependent manner together with SIP/CoAA. The binding to the BRG1 N-terminal SNF11 binding domain is important for SYT and SIP/CoAA to activate transcription because deletion of the first BRG1 211 amino acids strongly reduced SYT and SIP/CoAA co-activation. Thus, the ability of SYT and SIP/CoAA proteins to activate transcription requires functional SWI/SNF complexes and in particular the SNF11 binding domain of hBRM and BRG1. In this context we have shown SIP/CoAA in the role of a nuclear receptor co-activator, but further investigation will be
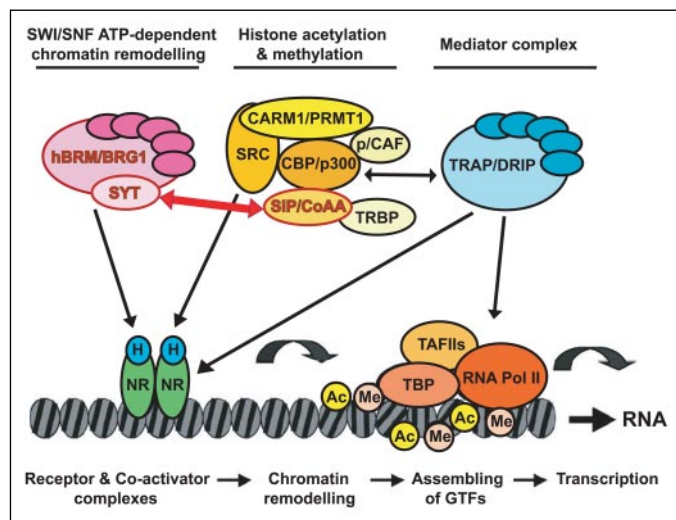
FIGURE 9. **A hypothetical mechanism of action of SYT and SIP/CoAA proteins in normal cells.** SYT protein activates transcription by binding to the ATP-chromatin remodeling complex, via the hBRM/BRG1 interaction, and recruiting histone acetylases and methylases via SIP/CoAA binding. SIP/CoAA works synergistically with SYT to activate transcription. Schematically represented are the ATP-dependent chromatin remodeling complex in *shaded pink,* the p160 steroid receptor co-activator (*SRC*) family of proteins with its interactions to CBP/p300, CARM1/PRMT1, and p/CAF proteins in *shaded yellow,* and the mediator complex in *shaded blue.* The mediator complex transduces both positive and negative regulatory information from co-activators/co-repressors to the core transcriptional machinery (71, 73).

required to determine whether association with SYT also modulates splicing decisions and what genes are regulated in this perspective. However, it should be pointed out that the luciferase reporter constructs used in our experiments do not have introns. We were unable to show a direct binding between hBRM and SIP/CoAA proteins. In mammalian three-hybrid assays, constructs expressing GAL4-hBRM and VP16-SIP/CoAA specifically activate transcription only with co-transfected GFP-SYT but not in presence of the green fluorescent protein control vector alone. *In vitro* co-precipitations also failed to show binding the two proteins (data not shown).

We now know that SYT-SSX as well as SYT binds to both hBRM/BRG1 (10) and SIP/CoAA (Fig. 6). More importantly, hBRM/BRG1 binds to the N terminus of SYT whereas SIP/CoAA binds SYT at the C-terminal half. Knowing that SIP/CoAA is a general co-activator that associates with the histone acetyltransferases p300/CBP and with TRBP (25), it is possible that SYT is an important link between the ATP-dependent chromatin remodeling complexes SWI/SNF on the one side and the histone acetylation/methylation complex on the other side (Fig. 9). In support of this hypothesis, while our manuscript was being reviewed, Iwasaki and co-workers (66) showed that in reporter gene assays CoAA, SRC-1 (steroid receptor co-activator-1), and TRBP synergistically activate transcription with SYT suggesting that, at least in part, SYT may stimulate transcription via SRC-1-containing histone acetyltransferase complexes. Initiation of transcription in eukaryotic cells is a process involving a large number of cofactors in a complicated multistep process, but how nuclear receptors recruit these multiple cofactors, in which order, and through how many alternative ways is not completely clear. Data in the literature show that SWI/SNF can be recruited directly by glucocorticoid and estrogen receptors (45–52, 54, 55, 67) but also indirectly through p300 (68), which itself is recruited onto promoters by the steroid receptor co-activator family of co-activators (69, 70). Thus, in the light of this work, SWI/SNF-SYT-SIP/CoAA interactions (Fig. 9) may suggest a link between SWI/SNF and p300/CBP and therefore a new indirect way by which SWI/SNF is

recruited to target promoters through the SYT-SIP/CoAA-p300/CBP interactions. Histone acetylation then exerted by p300/CBP may provide additional anchors to further stabilize, through the hBRM/BRG1 bromodomain, the recruitment of SWI/SNF. To complete a general picture, the recruitment of the mediator complex (TRAP-DRIP) is believed to transduce regulatory information from gene-specific regulatory proteins to the core transcriptional apparatus of eukaryotes (see also Fig. 9) (68, 71).

From a functional point of view, SYT binds to and acts with SIP/CoAA in stimulating hormone receptor-dependent transcriptional activation, and SYT-SIP/CoAA transcriptional activation is fully functional only when SYT can bind the N terminus of hBRM/BRG1. The removal of the N-terminal region from hBRM/BRG1, which is known to interact specifically with SYT *in vivo* and *in vitro*, almost eliminates SYT and SIP/CoAA transcriptional activation above BRG1 alone. Thus, SYT and SIP/CoAA co-activation functions in an hBRM/BRG1-dependent manner, and their role is hormone-regulated. We observed only little synergistic interaction between SYT and SIP/CoAA in the activation of the estrogen- and glucocorticoid-response elements. However, Iwasaki and co-workers (66) have shown a stronger synergistic effect using different promoters as well as different cell lines that can account for this difference.

Finally, with regard to synovial sarcoma, SYT-SSX fusion proteins interact with hBRM/BRG1 and SIP/CoAA similarly to the wild type SYT, eliminating the hypothesis that the SSX repressor domain in the fusions may abolish or alter the binding to hBRM/BRG1 and SIP/CoAA. In synovial sarcomas, the t(X;18) translocation leads to the addition of the C-terminal region of SSX1, SSX2, or SSX4 to the SYT protein, which loses only the last eight amino acids in the most common translocation point. We showed here (Fig. 5a, *transfection 6*) that the removal of the last eight amino acids of SYT is not responsible for the loss of activation detected in the mammalian assay. Thus, the presence of SSX in the fusion has to account for the transcriptional repression of the reporter. In this scenario the SSX domains may redirect the SYT-SSX fusion oncoprotein alone or together with wild type SYT and the natural SYT interacting partners to different target genes. This could result in activation of genes normally repressed by the proteins SSX and/or down-regulation of SYT-responsive genes. Alternatively, the SYT-SSX fusion proteins may remain on the SYT-regulated promoters, binding to natural SYT-interacting partners but also with SSX C-terminal interacting proteins, which may repress promoter activation. It is known from the literature that the C-terminal domains of the SSX proteins are able to bind to histones (20) and also to interact with repressors such as the polycomb group complex (72). In this context, the SYT-SSX fusion may stabilize the nucleosome structure and inhibit SWI/SNF remodeling activity. Gene transcription would be repressed where normally it is activated. The consequence of both events would be altered target specificity and gene expression. Clarification of the SSX C-terminal interactions with possible SSX protein interacting partners is required to further elucidate these mechanisms, and progress in the field is significantly dependent on identifying the target genes that are controlled by these nuclear proteins.

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