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SYT Associates with Human SNF/SWI Complexes and the C-terminal Region of Its Fusion Partner SSX1 Targets Histones*

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Hiroyuki Kato‡§¶, Agneta Tjernberg‖, Wenzhu Zhang‖, Andrew N. Krutchinsky‖, Woojin An‡, Tamotsu Takeuchi, Yuji Ohtsuki**, Sumio Sugano‡‡, Diederik R. de Bruijn§§, Brian T. Chait‖, and Robert G. Roeder‡**

*From the ‡Laboratory of Biochemistry and Molecular Biology and ‖Laboratory of Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, New York 10021, the §National Institute of Infectious Diseases, Musashimurayama, Tokyo 208-0011, Japan, the **Department of Pathology, Kochi Medical School, Kochi 783-8505, Japan, the ‡‡Laboratory of Genome Structure Analysis, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan, and the §§Department of Human Genetics, University Hospital, Nijmegen, The Netherlands*

A global transcriptional co-activator, the SNF/SWI complex, has been characterized as a chromatin remodeling factor that enhances accessibility of the transcriptional machinery to DNA within a repressive chromatin structure. On the other hand, mutations in some human SNF/SWI complex components have been linked to tumor formation. We show here that SYT, a partner protein generating the synovial sarcoma fusion protein SYT-SSX, associates with native human SNF/SWI complexes. The SYT protein has a unique QPGY domain, which is also present in the largest subunits, p250 and the newly identified homolog p250R, of the corresponding SNF/SWI complexes. The C-terminal region (amino acids 310–387) of SSX1, comprising the SSX1 portion of the SYT-SSX1 fusion protein, binds strongly to core histones and oligonucleosomes *in vitro* and directs nuclear localization of a green fluorescence protein fusion protein. Experiments with serial C-terminal deletion mutants of SSX1 indicate that these properties map to a common region and also correlate with the previously demonstrated anchorage-independent colony formation activity of SYT-SSX in Rat 3Y1 cells. These data suggest that SYT-SSX interferes with the function of either the SNF/SWI complexes or another SYT-interacting co-activator, p300, by changing their targeted localization or by directly inhibiting their chromatin remodeling activities.

The chromatin structure of active eukaryotic genes is subject to dynamic change by chromatin modifiers such as ATP-dependent chromatin remodeling factors (reviewed in Refs. 1–5). Homologs of a yeast prototype ATP-dependent remodeling complex, SNF/SWI, appear to be widely present in eukaryotes from yeast to humans (6–8). Functions of the subunits of the SNF/SWI complexes (9, 10) were first demonstrated by genetic studies in *Saccharomyces cerevisiae*, which showed that *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, and *SNF5* are required for the expression of a set of genes that include the *HO*, *GAL1*, *SUC2*, and *ADH2* genes (11–13). In *Drosophila melanogaster*, the *SWI2/SNF2* homolog *brm* was originally identified as a suppressor of *Polycomb* mutations (14). The *Drosophila* complex has been isolated, and some of the subunits have been characterized (15, 16), revealing that the SNF/SWI complexes are highly conserved in subunit composition and in primary structure among yeast, fruit fly, and human. A number of studies have described various biochemical properties of the human and yeast SNF/SWI complexes, as well as other ATP-dependent chromatin remodeling complexes (reviewed in Refs. 1–3, 17). For instance the SNF/SWI complexes are recruited by transcriptional activators to nucleosomal templates (18–23), perturb nucleosome positioning, and facilitate binding of activator proteins to nucleosomes (24–26). Mechanisms for this perturbation have been proposed to involve interconversion between two different nucleosomal states (27), sliding of histone octamers (28) or a change of DNA topology (29, 30).

The human complexes are composed of at least nine subunits that include apparent homologs to SWI2/SNF2 (hbrm/hSNF2α, BRG-1/hSNF2β) (31–33), SWI3 (BAF170 and BAF155) (34, 35), and SNF5 (INI1/hSNF5) (36, 37), as well as non-homologous proteins (38). Functions of the human SWI/SNF complexes have been variously described in relation to cell cycle regulation, malignant transformation, and signal transduction. The human SWI2/SNF2 homologs, hbrm/hSNF2α and BRG1/hSNF2β, bind to the retinoblastoma protein (Rb)[1] and lead to cell cycle arrest (39, 40) and repression of E2F-dependent transcription (41). A recent study suggested that the Rb-SNF/SWI complex differentially regulates expression of cyclin E and cyclin A, depending on the association with a histone deacetylase (HDAC1), and thereby controls progression from G_1 into S phase or exit from S phase (42). Activities of the SNF/SWI complexes themselves are also regulated in a cell cycle-dependent manner, because both hbrm and BRG-1 are inactivated by phosphorylation at the G_2/M transition (43, 44) and because hbrm but not BRG-1 is degraded by mitogenic stimulation and ectopic RAS expression (45). It has also been shown that some SNF/SWI components physically interact with other cell cycle regulators (46, 47) and that BRG-1 represses transcription of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF219114 and AF259792.

¶ To whom correspondence should be addressed: National Institute of Infectious Diseases, 4-7-1, Gakuen, Musashimurayama, Tokyo 208-0011 Japan. Tel.: 81-42-561-1960; Fax: 81-42-563-2392; E-mail: hykato@nih.go.jp.

[1] The abbreviations used are: Rb, retinoblastoma protein; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MS, mass spectrometry; CBB, Coomassie Brilliant Blue; nt, nucleotide(s); GST, glutathione *S*-transferase; EGFP, enhanced green fluorescence protein; SSX1C, SSX1 78-amino acid C-terminal region.

the c-*fos* gene (48). Moreover, phosphatidylinositol 4,5-biphosphate, a major signal mediator in lymphocytes, induces translocation of SNF/SWI complexes to chromatin (49). Finally, involvement of the SWI/SNF proteins in malignant transformation has been highlighted by the discovery that the *INI1/hSNF5* gene is frequently mutated in rhabdoid tumors (50). A number of studies have reported alterations of the genes encoding SNF/SWI subunits in various human cancers (51, 52).

Synovial sarcomas are typified by a unique chromosomal translocation t(X;18)(p11.2;q11.2) that results in fusion of the *SYT* gene on chromosome 18 with the *SSX1 or SSX2* gene in Xp11.2 and, consequently, production of the chimeric SYT-SSX proteins (53). SYT is a ubiquitously expressed protein with a QPGY domain, whereas the SSX proteins carry KRAB-like domains and are expressed almost exclusively in the testis (54). The SYT and SSX proteins localize in distinct nuclear domains. Localization of the SYT-SSX fusion proteins does not appear to be completely identical to that of either SYT or SSX, although variability has been reported (55–58). Interestingly, the SYT-SSX and SYT proteins appear to localize in particular nuclear speckles where hbrm protein is present (59). Thus, altered localization of the SYT-SSX proteins and their associated proteins may account for the underlying mechanisms of synovial sarcoma formation.

Here we report that the SYT protein in fact is present in native human SNF/SWI complexes and shares the QPGY domain with the largest subunits of these complexes. Interestingly, the C-terminal 78-amino acid region of SSX1 binds strongly to core histones and oligonucleosomes. Deletion analysis reveals that this activity correlates both with the nuclear localization of the SSX1 C-terminal domain and with the transforming activity of SYT-SSX in rat 3Y1 cells (60). We hypothesize that the forced mislocalization or dysfunction of the SYT-associated chromatin remodeling factors, such as the SNF/SWI complexes or the SYT-interacting co-activator p300, by SYT-SSX is the cause of the malignant transformation.

EXPERIMENTAL PROCEDURES

Purification of Human SNF/SWI Complexes—Human SNF/SWI complexes were affinity-purified on anti-FLAG antibody (M2)-conjugated agarose (Kodak/IBI) from nuclear extracts prepared from FLAG-tagged INI1-expressing HeLa (F-Ini1-HeLa) cells essentially as described by Sif *et al.* (44). Complexes were washed extensively (five times) with buffer D (20 mM Tris-HCl, pH 7.9, 10% glycerol, 1 mM dithiothreitol (DTT), 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Nonidet P-40) containing 500 mM KCl (300 mM in some preparations) prior to elution with FLAG peptide. For mass spectrometric protein identification, the complexes were further purified and concentrated by batch-adsorption onto SP-Sepharose (Amersham Biosciences, Inc.) and elution with buffer D, 300 mM KCl. The eluate was directly applied onto an SDS-polyacrylamide gel (Novex/Invitrogen).

Protein Identification by Mass Spectrometry—The SNF/SWI complexes were separated by 4 to 20% gradient SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (CBB) R-250 or by negative staining with zinc sulfate and imidazole (Bio-Rad). The excised bands were destained and processed by in-gel digestion with trypsin (61). Molecular masses of the peptides were determined by MS analysis with matrix-assisted laser desorption/ionization quadrupole-time-of-flight mass spectrometry (61), and the assignment was confirmed by the subsequent MS/MS analysis of selected ion species. Proteins were identified by using the search engines ProFound (62) and PepFrag (63).

Molecular Cloning of the p250 and p250R cDNAs—C-terminal and N-terminal B120 fragments (64) were used to screen a human fetal brain cDNA library in λZAPII (Stratagene) for p250 cDNAs. The most abundant spliced variant in the 5′-end sequence was selected from among cDNAs isolated by screening as well as from the NCBI data base. The resulting p250 cDNA is a composite of human fetal brain cDNAs (nucleotides (nt) 1–430 and 2571–6042) and B120 cDNA (nt 431–2570). The p250R cDNA was isolated by screening the human fetal brain phage library with a probe from GenBank™ cDNA (AA191300). The 5′-terminal sequence was obtained from HeLa cDNA by using the oligo-capping cloning method as described previously (65). Briefly, the PCR template was synthesized with reverse transcriptase and an oligo-dT primer from HeLa cell mRNA 5′-capped with an oligo RNA (5′-AGCAUCGAGUCGGCCUUGUUGGCCUACUCC-3′). A nested PCR reaction was performed with cap primers (5′-AGCATCGAGTCGGCCTTGTTGGC-3′ and 5′-GAGTCGGCCTTGTTGGCCTACTC-3′) and p250R internal primers corresponding to nt 2024–2001 and 1925–1902. The p250R cDNA is a composite of HeLa (nt 1–2590) and the fetal brain (nt 2591–5123) cDNAs.

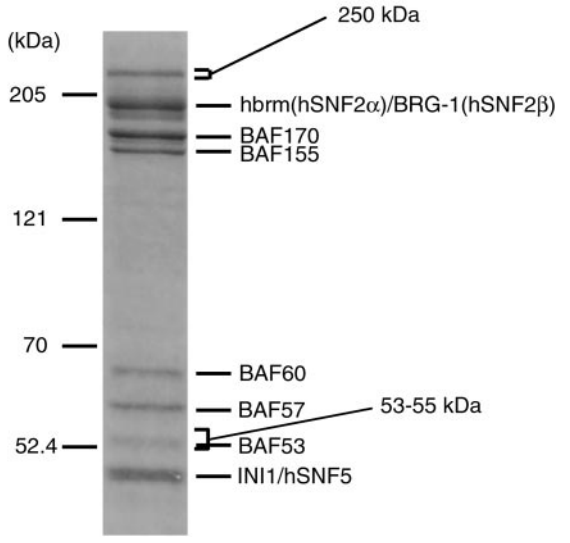


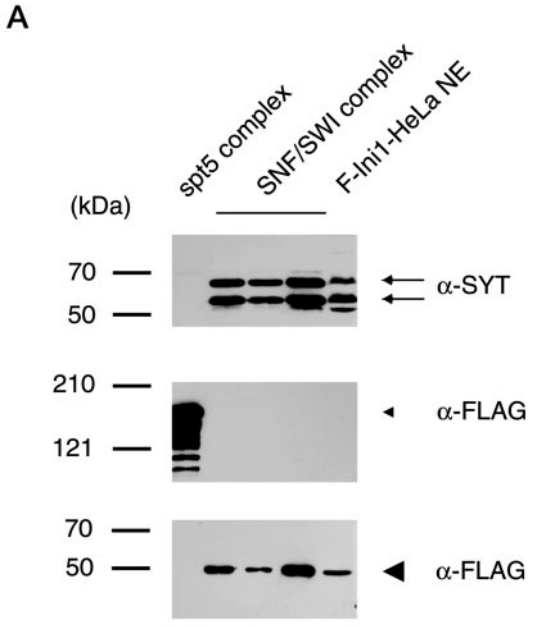
FIG. 1. **Subunit composition of the human SNF/SWI complexes.** The FLAG-tagged Ini1-containing HeLa SNF/SWI complexes (44), electrophoresed on 10% SDS-polyacrylamide gel and stained with CBB R-250, are shown. The reported subunits as well as gel slices containing the p250 band or a region of 53–55 kDa used for mass spectrometric analyses are indicated.

Antisera and Immunopurification of p250 and p250R Complexes—Rabbit antisera (Covance, Denver, PA) to p250 and p250R were raised against a glutathione *S*-transferase (GST)-p250 (amino acids 640–806) fusion protein containing the ARID domain and a GST-p250R (amino acids 21–274) fusion protein, respectively. Antibodies were affinity-purified on corresponding GST fusion proteins coupled to CNBr-activated Sepharose 4B (Amersham Biosciences, Inc.). Purified antibodies were cross-linked to CNBr-activated Sepharose 4B and used for purification of the SNF/SWI complexes from HeLa nuclear extract. Antibody coupling and subsequent washing of the cross-linked matrix were performed under conditions recommended by the manufacturer. Immune complexes were washed extensively (four to five times) with buffer D containing 500 mM KCl and eluted with an acidic solution. Antisera to SYT (C44) and SSX2 (B39), generously provided by D. de Bruijn, were used for coupling on Protein G-Sepharose 4FF (Amersham Biosciences, Inc.) with dimethyl pimelimidate dihydrochloride (Sigma Chemical Co.) at a final concentration of 60 mM for 30 min at room temperature. Monoclonal antibody to FLAG epitope (M2) and M2 conjugated to Sepharose were purchased from Sigma.

Immunoblot Analysis—Affinity-purified proteins or extracts were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Inc.), incubated first with an antiserum or a purified antibody and then with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit for all polyclonal antibodies or anti-mouse for anti-FLAG antibody), and detected by chemiluminescence (ECL detection reagents, Amersham Biosciences, Inc.). Antisera and the M2 antibody were used in 1000-fold dilution and at a final concentration of 4 μg/ml, respectively.

GST Pull-down Assay—All of the GST fusion proteins were expressed in BL21(DE3)pLysS (Novagen), and extracts were prepared by sonication in lysis buffer (20 mM Tris-HCl (pH 7.9), 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Native core histones and oligonucleosomes were purified from HeLa cells as described (66). Recombinant full-length and tailless histones were expressed in *Escherichia coli* and purified.[2] GST proteins (~3–6 μg) bound to glutathione-Sepharose beads were incubated with HeLa nuclear extract (5 mg) or recombinant H2A, H2B, H3, and H4 histones (~0.5 μg each) and

[2] W. An and R. G. Roeder, unpublished data.



Fragment ion	Observed m/z	Calculated m/z
b5	596.31	596.24
b6	724.35	724.29
b8	909.15	909.37

FIG. 2. **Detection of SYT protein in SNF/SWI complexes.** *A*, immunoblotting with α-SYT antiserum. Three different preparations of the purified SNF/SWI complexes (*left* and *right*, washed with 0.3 M KCl; *middle*, washed with 0.5 M KCl) in combination with a purified SPT5 complex and F-Ini1-HeLa nuclear extract were electrophoresed on SDS-polyacrylamide gels (*upper and lower panels*, 10%; *middle panel*, 5%) and analyzed by immunoblotting with α-SYT or α-FLAG as the first antibody. Anti-rabbit (for anti-SYT antibody) and anti-mouse (for anti-FLAG antibody) secondary antibodies conjugated with horseradish peroxidase were used. *Arrows* indicate two specific α-SYT-reactive bands of 53 and 65 kDa. The *small* and *large arrowheads* indicate the 160-kDa SPT5 protein and the 49-kDa FLAG-tagged Ini1 protein, respectively. *B*, immunoprecipitation of Ini1 protein. The association between Ini1 and SYT was tested by immunoprecipitation with α-SYT and control antisera. Antisera cross-linked on Sepharose beads were incubated with F-Ini1-HeLa nuclear extract and analyzed by immunoblotting with α-FLAG monoclonal antibody (M2). F-Ini1 (49 kDa) is indicated by the *arrow*. *C*, analysis by MS/MS. Gel slices obtained from the 53- to 55-kDa region in Fig. 1 were analyzed by MS and MS/MS spectrometry as described under "Experimental Procedures." Trypsin-digested protein samples were analyzed by mass spectrometry. A candidate peptide from the 53- to 55-kDa sample matching the C-terminal peptide of the SYT protein was further analyzed by the MS/MS (61). The observed and calculated *m/z* values of the fragment ions from the candidate peptide are shown.

washed four times with buffer D containing 300 mM KCl. Bound proteins were either eluted with 0.1% sodium deoxycholate or directly suspended in SDS sample buffer and analyzed by 4 to 20% gradient SDS-PAGE.

Analysis of Subcellular Localization—Enhanced green fluorescence protein (EGFP) fusion proteins with the SSX1 C-terminal regions were constructed with pEGFP-C2 vector (CLONTECH) and transfected into Vero cells by using LipofectAMINE (Invitrogen). Twenty-four hours after transfection, fluorescence was directly detected by a high-performance digital cooled charge-coupled device camera (AxioCam, Carl Zeiss) interfaced with a computer (OptiPlex, Dell) and processed by an analysis system (AxioVision, Carl Zeiss). The obtained images were edited by using software (Photoshop 5.0, Adobe).

RESULTS

SYT Protein Is Present in Native SNF/SWI Complexes—Thaete *et al.* (59) reported co-localization of SYT with hbrm in characteristic nuclear speckles. However, the presence of endogenous SYT protein in native SNF/SWI complexes has not been demonstrated. To investigate this possibility, the SNF/SWI complexes (Fig. 1) purified from FLAG-tagged Ini1-expressing HeLa cell nuclear extract (44) by an immunoaffinity precipitation method using anti-FLAG monoclonal antibody (M2)-conjugated agarose beads were analyzed by Western blotting. An anti-SYT serum (C44) detected two strong bands of 53 and 65 kDa in F-Ini1-HeLa nuclear extract and in all three preparations of the purified SNF/SWI complexes but not in the control SPT5 complex isolated from a FLAG-tagged SPT5-expressing cell line (Fig. 2*A*, *upper panel*). M2 monoclonal antibody detected a band of ~160 kDa and degradation products in the SPT5 complex[3] (*middle panel*), as well as an F-Ini1 protein of 49 kDa both in the SNF/SWI complexes and in F-Ini1-HeLa nuclear extract (*lower panel*). Because the *in vitro* translated SYT protein has been detected as a 53-kDa band, the 65-kDa band may represent either a modified or an alternatively spliced or translated product (56). It appears that most (more than 50%) of the SYT protein associates with the SNF/SWI complexes, because signal ratios of SYT to F-Ini1 between the purified SNF/SWI complexes and the F-Ini1-HeLa nuclear extract are equivalent (Fig. 2*A*).

To further demonstrate an association between SYT and SNF/SWI complexes, we tested whether the anti-SYT serum immunoprecipitates F-Ini1 protein in the extract. An anti-SYT antibody covalently cross-linked to protein G-Sepharose was incubated with F-Ini1-HeLa nuclear extract, and the resulting pull-down protein was analyzed by immunoblotting with anti-FLAG M2 monoclonal antibody. The anti-SYT serum clearly (Fig. 2*B*, *lane 1*) was at least as efficient as the anti-Ini1 serum (*lane 4*) in immunoprecipitating F-Ini1; however, two other control antisera (anti-SSX2 and anti-p73L) were not able to significantly precipitate FLAG-Ini1 protein (*lanes 2* and *3*). To investigate the abundance of the SYT-containing SNF/SWI complexes in the F-Ini1-containing SNF/SWI complexes, anti-SYT and anti-FLAG immunoprecipitates were analyzed by Western blot for relative amounts of SYT and F-Ini-1. The percentage of SYT-containing SNF/SWI complexes was estimated as at least 3.4% of the total F-Ini1-containing SNF/SWI complexes (data not shown).

To rule out the possibility that the signals were due to cross-reaction of the anti-SYT serum with some of the enriched

[3] M. Estable and R. G. Roeder, unpublished data.

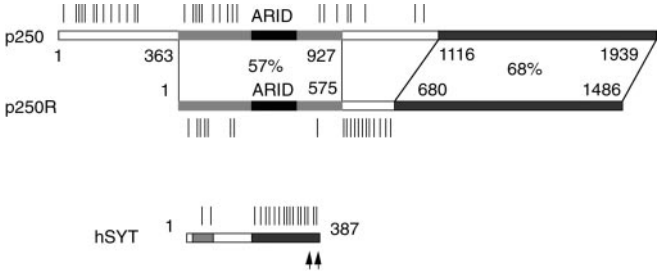


FIG. 3. **Comparison of the p250, p250R, and SYT proteins.** Comparison of primary sequences of the p250, p250R, and SYT proteins is presented. *Dark* and *light shades* in the p250 and p250R protein bars denote C-terminal and N-terminal conserved regions (68 and 57% identity), respectively. *Closed bars* indicate the ARID domains. *Dark* and *light shades* in the human SYT denote the QPGY and SNH domains, respectively. *Vertical lines* indicate the positions of the QPGY motifs. *Vertical arrows* indicate two common fusion points in SYT-SSX recombination.

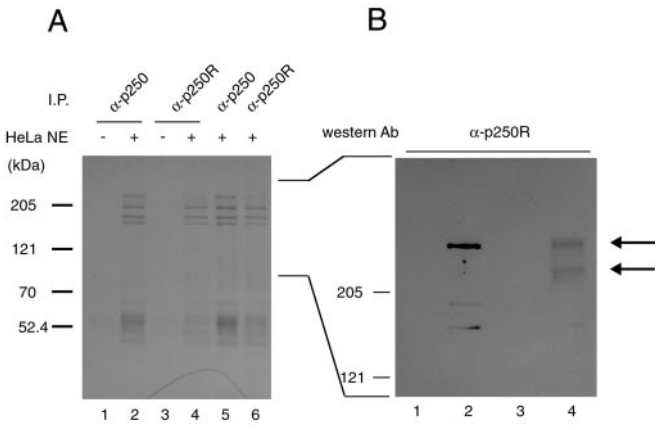


FIG. 4. **SNF/SWI complexes containing p250 or p250R.** *A*, immunoaffinity isolation of p250- or p250R-containing complexes. Proteins were immunoprecipitated from HeLa cell nuclear extract by using affinity-purified α-p250 (*lane 2*) or α-p250R antibody (*lane 4*). After extensive washing, immune complexes were resolved by 10% SDS-PAGE and stained with CBB R-250. For side-by-side comparison, the same samples as in *lanes 2* and *4* were loaded in *lanes 5* and *6*, respectively. *B*, immunoblotting with α-p250R antibody. To obtain higher resolution of high molecular weight proteins, the same immune complexes as in *lanes 1–4* of *panel A* were separated by 5% SDS-PAGE and then transferred and reacted with α-250R antibody. *Arrows* indicate two major immune-reactive bands with apparent molecular masses of 220 and 260 kDa. Note that the *upper band* (260 kDa) is slightly larger than p250 in *lane 2*.

SNF/SWI components, we performed a mass spectrometric analysis of tryptic peptides obtained from several SDS-polyacrylamide gel slices in the 53–55 kDa range (Fig. 1). In one of these slices, we detected a peak at *m/z* 1680.68 that exactly matches the calculated *m/z* value for the SYT peptide PYGY-DQGQYGNYQQ. Subsequent MS/MS spectrometric analysis of the *m/z* 1680.68 peptide confirmed this identification (Fig. 2*C*). From the relatively low observed signal intensities, we suspect that SYT is a sub-stoichiometric component of the complex. This is the first demonstration of SYT association with native SNF/SWI complexes.

p250 and a Related Protein, p250R, Are Intrinsic Components of Human SNF/SWI Complexes—In a separate experiment, we analyzed the approximate 50-kDa bands in the SNF/SWI complexes and noticed that a short amino acid motif is commonly present in both SYT and 250-kDa proteins. We therefore became interested in the relationships between these proteins with respect to structure and complex formation. Mass spectrometric analyses of the 250-kDa protein isolated from the SWI/SNF complexes (Fig. 1) revealed that a number of derived peptide sequences matched those in the previously described B120 protein (64). Peptide sequences attributed to p250 correspond to amino acids 236–261, 262–274, 674–685, 699–708, 714–732, 821–840, 842–862, 877–901, 902–915, 975–1009, 1010–1022, 1141–1150, 1243–1251, and 1804–1822. Notably, TPQPSSPMDQMGK (amino acids 236–261) enabled us to distinguish a particular cDNA encoding this sequence from cDNAs encoding a number of splice variants. Screening of a phage cDNA library led to isolation of a cDNA encoding a 1939-amino acid protein, referred to as p250, with an N-terminal half that is almost identical to B120 and a novel C-terminal half. A noted difference between the N termini of p250 and B120 is due to splicing variation. At least several additional splice variants with the same acceptor site have been found (data not shown). Cloning of cDNAs corresponding to p250 (designated p270 or BAF250) has been recently reported by others, and further suggests that p250 has additional N-terminal amino acids (67, 68). By searching the GenBank™ expressed sequence tag sequences, we found expressed sequence tag sequences with close similarity to *p250* cDNA. Screening of a phage cDNA library for *p250* cDNA and identification of the 5′ end by the oligo-capping cloning method (65) revealed the presence of a close homolog of p250, referred to as p250R, that is composed of 1486 amino acids and shows strong identity to p250 throughout the entire coding sequence (Fig. 3). p250R lacks a region corresponding to the p250 N terminus. Because the initiator ATG is preceded by a termination codon in the *p250R* 5′-terminal cDNA obtained by the oligo-capping method, this ATG may be the *bona fide* initiator. Alternatively, other potential splice variants may have a further N-terminal sequence. The p250 protein shows strong sequence homology to the *Drosophila* Osa/eyelid protein. *Osa*/*eyelid*, originally identified as a *trithorax* group member (69), has organ-specific genetic interactions with *brm*, *mor*, and *snr1* (homologs of *SNF2*/*SWI2*, *SWI3*, and *SNF5*, respectively) and antagonizes wingless signaling (70, 71). Both of the p250 and p250R proteins have a highly conserved region, referred to as the ARID domain (72), that has been found in more than a dozen other proteins (Fig. 3). These include putative matrix-associated region-binding proteins, the *Drosophila* Dead ringer (73) and Bright (74) proteins and the putative homologs of p250 and p250R, Osa/eyelid and yeast SWI1. Another remarkable feature also common to p250 and p250R is the presence of a number of peptide motifs containing tyrosine residues surrounded by several proline (P), glutamine (Q), and glycine (G) residues (Fig. 3). Similar QPGY motifs, comprising a QPGY domain, have been reported for the SYT protein (59). The SYT-SSX fusion proteins found in synovial sarcomas (53) are typically composed of the first 379 amino acids of SYT (excluding only the C-terminal 8 amino acids) and the C-terminal 78 amino acids of SSX (SSXC). The SYT-SSX proteins retain most of the QPGY motifs. This motif is tentatively defined in this report as *XXYXX*, where a tyrosine residue is surrounded by at least three *X* residues are either P, Q, or G. p250, p250R, and SYT have 31, 20, and 18 motifs, respectively (Fig. 3).

We next analyzed p250- and p250R-containing complexes by immunoprecipitation with specific affinity-purified antibodies. Both anti-p250 and anti-p250R antibodies precipitated complexes with a subunit composition similar to those of F-Ini1-containing complexes (Fig. 4*A*, *lanes 5* and *6*). However, the largest subunits, presumed to be p250 and p250R, were found to be somewhat different, because the latter migrated as a broad band. When these samples were separated by 5% SDS-PAGE and analyzed by Western blotting with the anti-p250R antibody, p250 appeared as a sharp 250-kDa band and p250R appeared as two broad bands around 260 and 220 kDa (Fig. 4*B*). These results indicate that the two antibodies specifically

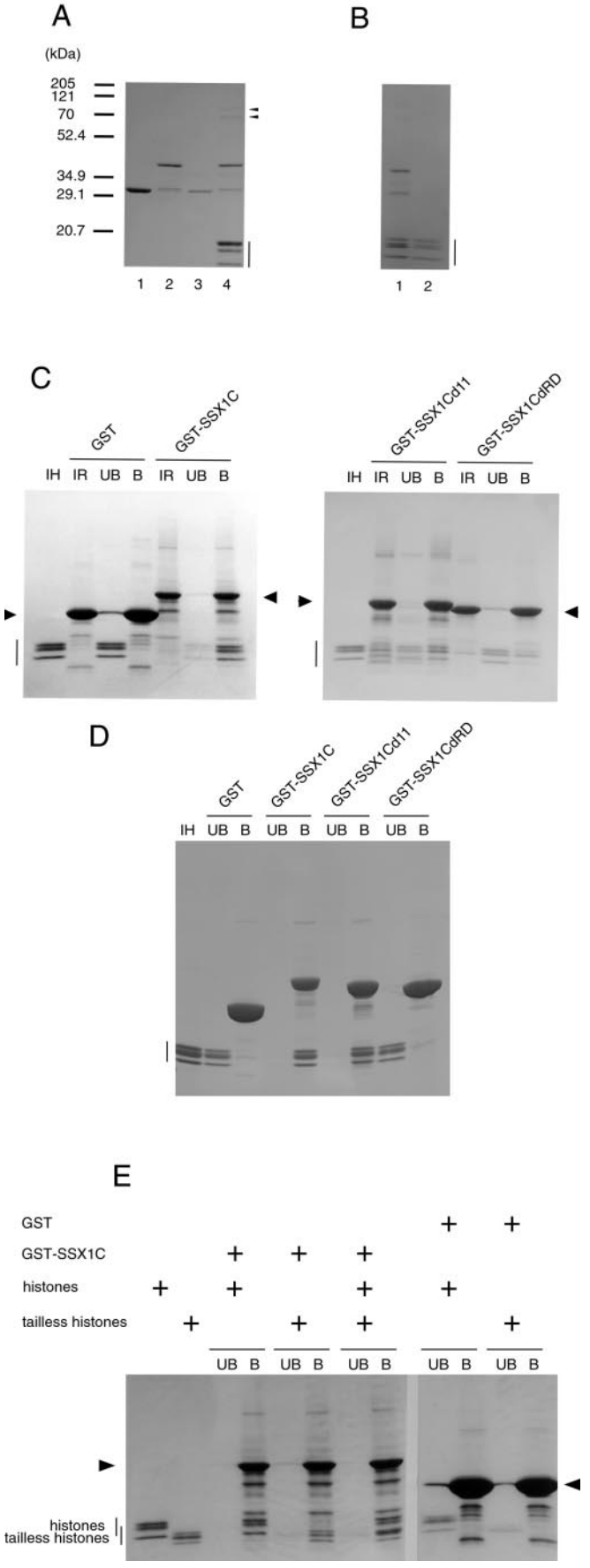


FIG. 5. **Target proteins of the SSX1 C-terminal region.** *A*, GST pull-down experiment. GST (*lanes 1* and *3*) and GST-SSX1C fusion protein (*lanes 2* and *4*) were incubated with buffer D (*lanes 1* and *2*) or HeLa nuclear extract (*lanes 3* and *4*). The pull-down fractions were eluted with buffer D containing 0.1% sodium deoxycholate and analyzed by 10% SDS-PAGE and staining with CBB R-250. Specific low molecular weight proteins are marked by the *vertical line*. Two specific high molecular weight bands are indicated by *arrowheads*. *B*, side-by-side comparison with the native core histones. The same sample as in *lane 4* of *panel A* (*lane 1*) was separated by 4 to 20% gradient SDS-PAGE with the core histones (*lane 2*) purified from HeLa cells. *C*, GST-SSX1C mutants with serial deletions were tested for binding to the purified core histones. GST-SSX1Cd11 and GST-SSX1CdRD have 11- and 34-amino acid deletions from the SSX1 C terminus, respectively. For quantitative comparison, bound fractions were directly suspended in SDS sample buffer and analyzed by 4 to 20% SDS-PAGE. *IH*, *IR*, *UB*, and *B* denote input core histones, input recombinant GST fusion protein, unbound fraction, and bound fraction, respectively. *Arrowheads* indicate GST fusion proteins. *D*, binding to purified oligonucleosomes was analyzed as in *C*. *E*, binding to tailless histones. Full-length and tailless recombinant core histones were used for the pull-down assay. *Arrowheads* indicate GST and GST-SSX1C.

precipitated different complexes containing corresponding p250 and p250R antigens without cross-reaction. After being denatured, however, both antigens are recognized by the anti-p250R antibody, probably because of the similarity between p250 and p250R (43% identical in the corresponding region). Similarly, anti-p250 antibody also detected these bands (data not shown). Thus we conclude that p250 and p250R are intrinsic and mutually exclusive components of human SNF/SWI complexes.

The C-terminal Region of SSX1 Binds to Core Histones—The observation that SYT is associated with the native SNF/SWI complexes led us to investigate the molecular mechanisms that enable SYT-SSX proteins to eventually cause malignant tumors. We hypothesized that the SSX C-terminal domain provides that property by interacting with protein targets. To address this question, we used an affinity-purification method involving GST pull-downs. Sepharose beads containing GST fusion proteins were incubated with HeLa nuclear extract, washed, and eluted. Associated proteins were analyzed on a 10% polyacrylamide-SDS gel (Fig. 5*A*). Proteins pulled down by a fusion protein containing the 78-amino acid C-terminal region of SSX1 (SSX1C) showed specific bands of low molecular masses (<20 kDa) and two specific bands of high molecular masses (70–90 kDa) (*lane 4*). Because the low molecular mass proteins have a pattern similar to core histones, they were compared with purified native core histones in a side-by-side manner (Fig. 5*B*). Both the pulled-down proteins and the core histones showed indistinguishable sets of four bands (corresponding to H3, H2B, H2A, and H4 from *upper* to *lower*) on 4 to 20% gradient polyacrylamide-SDS gel (compare *lane 1* with *lane 2*). A mass spectrometric analysis revealed that two high molecular mass bands are identical to Ku70 and Ku80 (data not shown). We suspect that Ku70 and Ku80 were precipitated through binding to DNA ends of nucleosomes, because it is likely that at least some fractions of the core histones present in the nuclear extract exist as oligonucleosomes.

To know whether SSX1C binds directly to core histones and to determine the region in SSX1C required for this binding, a set of C-terminally truncated SSX1C mutants were tested for interaction with purified DNA-free core histones (Fig. 5*C*). Most of the core histones were retained on GST-SSX1C (compare *B* with *UB* in GST-SSX1C). A somewhat smaller fraction of histones was co-precipitated with the 11-amino acid truncation mutant (GST-SSX1Cd11). However, a further deletion that removes the C-terminal 34 amino acids severely affected, but did not completely abolish, the binding (compare *UB* with *B* in GST-SSX1CdRD). These results indicate that the C-terminal SSX1 domain, the SSX part of the SYT-SSX1 fusion protein, in fact binds to core histones and that the interaction

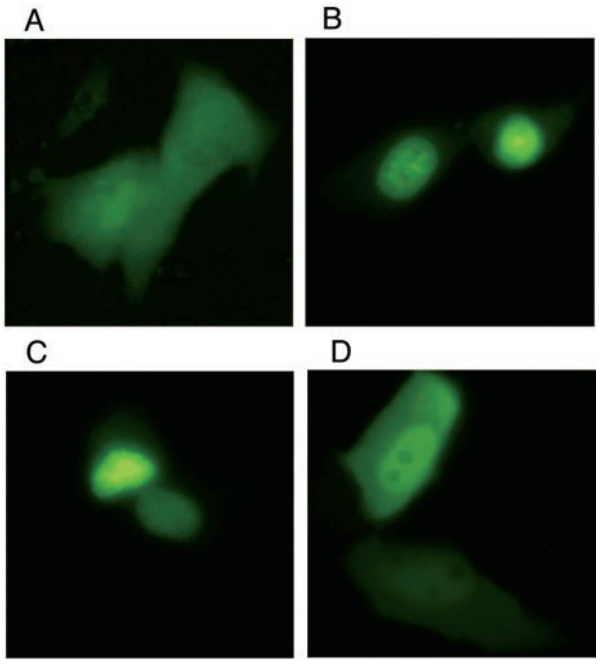


FIG. 6. **Localization activity of the truncated SSX1 C-terminal regions.** Plasmids expressing EGFP (*A*) or EGFP fusion proteins with SSX1C (*B*), SSX1Cd11 (*C*), or SSX1CdRD (*D*) were introduced into Vero cells. The fluorescence images, which were processed by a digital charge-coupled device camera-interfaced analysis system, are shown. To visualize preferential nuclear localization against high levels of fluorescence, images with somewhat lower density were prepared by linearly lowering the density with a computer program.

is not merely charge-dependent. SSX1C may have complex histone-interaction interfaces. Similar histone-binding properties were also observed in experiments with oligonucleosomes (Fig. 5*D*), suggesting that SSX1C may target chromatin *in vivo*. Because covalent modifications of the N-terminal tails of some core histones are likely related to transcriptional regulation, a requirement of the N termini for this interaction was next investigated by using tailless core histones expressed in *E. coli*. GST-SSX1C clearly, yet less efficiently, bound tailless histones (Fig. 5*E*), suggesting that the major interaction interface is in the core region and that the N termini may stabilize the interaction. The histone-binding activity and the presence of the C-terminal acidic amino acid stretch in SSX1 are reminiscent of properties displayed by nucleosome assembly factor NAP-1 (75).

SSX1C Region Required for Nuclear Localization—The observation that the SSX1C region strongly binds both to core histones and to oligonucleosomes prompted us to ask about the physiological relevance. A recent report showed that the SSX2 C-terminal region is required for nuclear localization (58). To test the possibility that the histone-binding activity correlates with the nuclear localization activity, cellular localization of the truncated mutants has been investigated following fusion with EGFP. EGFP-SSX1C clearly was localized in nuclei (Fig. 6*B*) and EGFP-SSX1Cd11 still exhibited an indistinguishable nuclear localization pattern (Fig. 6*C*). In contrast, EGFP-SSX1CdRD spread over the whole cell body with preferential staining of nuclei compared with EGFP alone (Fig. 6, *A* and *D*). In sum, a 44-amino acid region (amino acids 111–154) of SSX1 is sufficient for weak nuclear localization, whereas a region (amino acids 155–177) that includes the C-terminal end is required for full nuclear localization. Therefore, the histone-binding activity and the nuclear localization activity appear to be closely related to each other.

DISCUSSION

Numerous ATP-dependent chromatin remodeling complexes have been reported and shown to possess similar chromatin-disruption activities (24, 25, 76–82). Genetic studies in yeast have revealed that functions of the remodeling factors are likely to be distinct in some cases and partially redundant in others (77, 82). However, molecular mechanisms to explain the apparent differences in mutant phenotypes have not been well elucidated. A possible explanation is that these complexes physically and functionally interact with different regulatory molecules and/or are recruited to different chromosomal target sites by protein-protein interactions. In the case of mammals, determination of the primary structures of the subunits, identification of associated proteins, and findings of connections with phenotypes of genetic diseases or of mutants generated by genetic engineering have helped predict their physiological function (7). In this context, further identification and characterization of the stoichiometric components and the associated proteins could provide insights into SNF/SWI function.

SYT Is an Intrinsic Component of SNF/SWI Complexes—A remarkable feature of the p250 and p250R proteins is the presence of numerous QPGY motifs, comprising a QPGY domain, as has been reported for the SYT protein (Fig. 3). The QPGY domain is not evident in other proteins in the data base (data not shown). Because the SYT QPGY domain has a transactivation activity (59), these QPGY domains may serve as interaction interfaces with common components such as one of the SNF/SWI subunits or other transactivators. However, this possibility has not been addressed in this article. Motif sharing between p250/p250R and SYT is intriguing in light of the co-localization of SYT with hbrm in characteristic nuclear speckles (59). We have shown here that SYT is present in at least a subpopulation of purified native SNF/SWI complexes by Western blot and mass spectrometry analyses (Fig. 2, *A–C*). Nagai *et al.* (60) recently reported that rat fibroblast 3Y1 cells expressing SYT-SSX1, but neither SYT nor SSX1 alone, exhibit increased growth rate, anchorage-independent growth in soft agar, and tumor formation in nude mice, presumably through interaction with hBRM/hSNF2α. Consistent with this observation, simple C-terminal truncation mutants of SYT in synovial sarcomas have not been reported so far. These data strongly suggest that the SSX C-terminal region confers a novel ability to cause malignant transformation on SYT. Very interestingly, Eid *et al.* (83) recently showed that SYT associates with the histone acetyltransferase p300 and promotes cell adhesion to a fibronectin matrix, although the effect of SYT-SSX on p300 has not been investigated. A recent study has suggested that an ATP-dependent chromatin remodeling factor and a histone acetyltransferase activate the chromatin template in a sequential manner (84). Taken together, one could anticipate that SYT plays a common role in the function of both SNF/SWI complexes and p300 and that SYT fused with the SSX C terminus interferes with one or more steps of chromatin activation achieved by these apparatuses (84). We therefore sought to identify target proteins (most likely interacting proteins) of the SSX C-terminal region.

Presence of Multiple SNF/SWI Complexes Containing Alternative Large Subunits—We have characterized a large subunit of the mammalian SNF/SWI complexes, p250, and a closely related protein, p250R. Both p250 and p250R are intrinsic and stoichiometric components of corresponding complexes. These proteins share a highly conserved ARID domain. Recently, other groups also reported the isolation and characterization of p250 (p270 or BAF250) and showed that the ARID domain of p250 exhibits nonspecific or pyrimidine-preferred DNA binding activity (67, 68). We have also failed to detect clear sequence-

specific DNA-binding activity with GST-p250 and GST-p250R proteins *in vitro* (data not shown). Collins *et al.* (71) showed that the *Drosophila* Osa protein (potential homolog of p250/p250R) also has nonspecific DNA-binding activity and distributes over the entire length of all the polytene chromosomes. Nevertheless, judging from an experiment showing that ectopic expression of an Osa ARID domain-VP16 activation domain fusion protein provides some Osa function, it appears that the Osa ARID domain has functional specificity (*i.e.* targeting specific promoters) (71). These results, combined with the fact that the ARID domain is the only sequence apparently conserved between SWI1 and Osa or p250/p250R, suggests that the ARID domain may have unidentified target-recognition activity.

The subunit compositions of the complexes isolated via antibodies directed against p250 and p250R are indistinguishable except for the p250 and p250R bands (Fig. 4*A*). The p250-containing complex has a clear stoichiometric band of 250 kDa, whereas the p250R-containing complex has broad bands of 220 and 260 kDa. The SNF/SWI complexes were originally reported to be chromatographically separable into complexes A and B (24). A recent report showed that complex A (BAF) contains p250 (BAF250), whereas complex B (PBAF) contains a 180-kDa protein (BAF180) that is unrelated to p250 in primary structure and carries multiple bromodomains and two BAH regions (85). The authors suggested that PBAF is slightly different from the originally reported complex B and more closely related to the yeast RSC complex than to the yeast SNF/SWI complex. It is possible that the p250R-containing complex could represent the original complex B or that unseparated p250- and p250R-containing complexes could represent complex A. Our data thus extend structural, and potentially functional, complexity to the SNF/SWI complexes. Interestingly, the *Drosophila* SNF/SWI complexes may also have multiple alternative subunits to Osa, because the complexes precipitated by α-Brm contain additional high molecular weight bands other than those corresponding to Osa (71). The presence of multiple alternative subunits and the observed transcriptional regulation by p250/BAF250 (68) and Osa (71) imply that these subunits somehow regulate the SNF/SWI activities and that they may interfere with one another.

An interesting possibility is that SYT is physically or functionally related to p250 and p250R. A Western blot analysis showed that p250- and p250R-containing complexes clearly contain SYT, suggesting that SYT is not an alternative subunit to p250 or p250R (data not shown). Eid *et al.* (83) reported that, in contact-inhibited or adhesion cells, SYT becomes tyrosine-phosphorylated, possibly in the QPGY domain, and forms complexes with p300 and at least two other proteins (83). The QPGY domains of p250 and p250R may also serve as residues for tyrosine phosphorylation and protein-protein interactions.

Correlation between Histone Binding and Nuclear Localization—Our finding, based on deletion studies, that the histone-binding activity of SSX1C parallels its nuclear localization activity could be related to the previous observations that the SSX1 and SSX2 proteins localize in the nucleus and associate with mitotic chromosomes (56–58). It is thus likely that SSX proteins associate with chromatin throughout the cell cycle via direct binding of SSXC to core histones. Here it is noteworthy that an SYT-SSX1 protein, with the same truncation of the SSX1 C-terminal 34 amino acids as SSX1CdRD, exhibited weaker yet still significant anchorage-independent colony formation activity in soft agar (60). Thus it appears that binding to core histones, nuclear localization, and anchorage-independent growth all approximately correlate with one another, indicating the possibility that the histone-binding and nuclear localization activities contribute to malignant transformation.

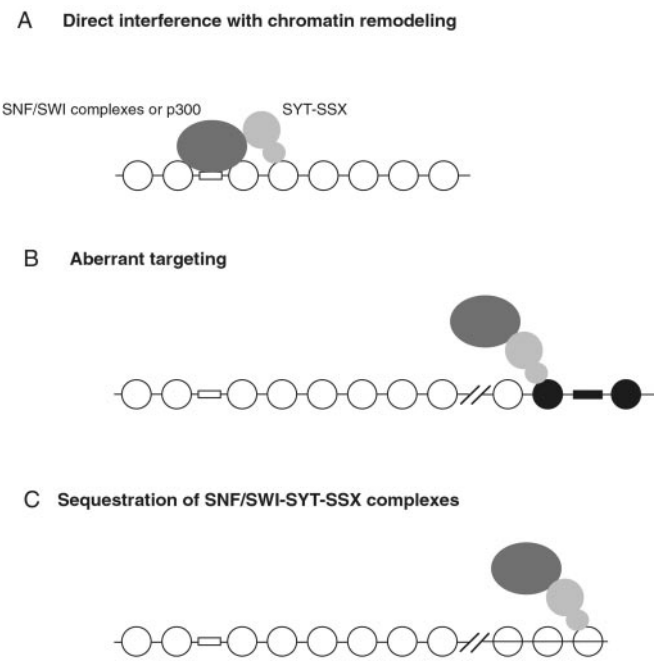


FIG. 7. **Possible mechanisms of SYT-SSX actions.** Three possible functional interactions between chromatin and SNF/SWI complexes or p300 that are caused by SYT-SSX are depicted. Regular (*open circles*) and specifically modified or structured (*closed circles*) chromatin as well as physiologically (*open bar*) and aberrantly (*closed bar*) targeted promoters are shown. *A*, direct inhibition of the chromatin remodeling activity of the SNF/SWI complexes. *B*, aberrant targeting by preferential association with an unidentified modified nucleosome. *C*, sequestration of the SNF/SWI complexes from target promoters.

Possible Molecular Mechanisms of Malignant Transformation by the SYT-SSX Fusion Protein—These results, in conjunction with the observation that several of the SNF/SWI components are potentially negative growth regulators or tumor suppressors (50, 51, 86, 87), have led us to propose the following working hypotheses to explain the molecular mechanisms of the SYT-SSX action in tumor formation (Fig. 7). First, the histone-binding activity of SSX1C directly inhibits the SNF/SWI remodeling activity by stabilizing or changing the nucleosome structure or, alternatively, by blocking the interaction between the SNF/SWI complexes and the core histones (Fig. 7*A*). This model may also explain mechanisms of potential interference with p300 activity by SYT-SSX (23). Second, SSX1C recruits the SNF/SWI complexes to aberrant target sites by interacting with modified chromatin (*e.g.* containing acetylated or methylated histones or a specific configuration) (Fig. 7*B*). The fact that the SSX1C-histone interaction requires the N-terminal tails of the core histones for maximum affinity suggests that the interaction may be influenced by histone modifications. Because bromodomains of several nuclear proteins specifically interact with a histone H3 or H4 tail in an acetylation-dependent manner (reviewed in Ref. 88), the association of SNF/SWI complexes and p300 (both hbrm/BRG-1 and p300 are bromodomain-containing proteins) with SYT-SSX may alter target specificity. Third, SYT-SSX1C sequesters the SNF/SWI complexes from the regular target site to other sites by forced binding (Fig. 7*C*). Because the SYT-SNF/SWI complexes appear to be a minor population among the total SNF/SWI complexes, sequestration by SYT-SSX would be from SYT-specific target sites. These possibilities are currently being investigated.

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