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Inhibition of Chromatin Remodeling by Polycomb Group Protein Posterior Sex Combs Is Mechanistically Distinct from Nucleosome Binding[†]

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ABSTRACT: Polycomb Group (PcG) proteins are essential regulators of development that maintain gene silencing in *Drosophila* and mammals through alterations of chromatin structure. One key PcG protein, Posterior Sex Combs (PSC), is part of at least two complexes: Polycomb Repressive Complex 1 (PRC1) and dRING-Associated Factors (dRAF). PRC1-class complexes compact chromatin and inhibit chromatin remodeling, while dRAF has E3 ligase activity for ubiquitylation of histone H2A; activities of both complexes can inhibit transcription. The noncovalent effects of PRC1-class complexes on chromatin can be recapitulated by PSC alone, and the region of PSC required for these activities is essential for PSC function *in vivo*. To understand how PSC interacts with chromatin to exert its repressive effects, we compared the ability of PSC to bind to and inhibit remodeling of various nucleosomal templates and determined which regions of PSC are required for mononucleosome binding and inhibition of chromatin remodeling. We find that PSC binds mononucleosome templates but inhibits their remodeling poorly. Addition of linker DNA to mononucleosomes allows their remodeling to be inhibited, although higher concentrations of PSC are required than for inhibition of multinucleosome templates. The C-terminal region of PSC (amino acids 456−1603) is important for inhibition of chromatin remodeling, and we identified amino acids 456−909 as being sufficient for stable nucleosome binding but not for inhibition of chromatin remodeling. Our data suggest distinct mechanistic steps between nucleosome binding and inhibition of chromatin remodeling.

PcG[1] genes were initially identified in *Drosophila* (*1*, *2*) and are conserved throughout metazoans (*3*). They play key roles in *Hox* gene expression during development (*4*) and other cellular processes such as X-inactivation (*5*), genomic imprinting (*6*), cell cycle progression, and self-renewal of embryonic and adult stem cells (*7*). PcG proteins form several multiprotein complexes that are thought to maintain gene silencing through interaction with chromatin (reviewed in ref *8*), including covalent modifications of histone proteins (*7*) and noncovalent changes in chromatin (*9*−*12*) and DNA (*13*) structures.

PRC1 was the first PcG complex to be purified from *Drosophila* (*11*, *14*) and contains four main PcG proteins [Posterior Sex Combs (PSC), Polyhomeotic (Ph), dRING, and Polycomb (Pc)] as well as substoichiometric levels of Sex Comb on Midleg (SCM) (*15*). PRC1 inhibits chromatin remodeling by the human Swi/Snf (hSwi/Snf) ATP-dependent chromatin remodeling complex (*11*, *14*) and also inhibits *in vitro* transcription (*10*). Subsequent studies in mammalian cells identified a PRC1-like complex with a similar constellation of PcG proteins and activities (*16*). Reconstitution of *Drosophila* PRC1 indicated that the four stoichiometric PcG proteins in this complex [PRC1 Core Complex (PCC)] or a subcomplex of PSC, Pc, and dRING is sufficient for its activities toward chromatin (*14*, *17*). PSC alone can recapitulate most of the activities of PRC1-class complexes, although it is slightly less efficient than PCC (*14*).

The importance of the interaction of PSC with chromatin *in vivo* was evidenced by the analysis of a series of *Psc* alleles that encode C-terminal truncations (*18*). Proteins encoded by alleles that produce severe phenotypes *in vivo* assemble into PCC but do not inhibit chromatin remodeling or transcription *in vitro* (*18*). PSC truncations were also tested for their ability to compact chromatin, and the same sequences required for inhibition of chromatin remodeling and transcription are required for chromatin compaction (*9*). Thus, chromatin compaction mediated by the C-terminal region of PSC (aa 456−1603) is likely responsible for inhibition of chromatin remodeling and transcription. This region is predicted to be unstructured (*19*), and the mechanism by which it interacts with chromatin is unknown. Other unstructured proteins function by assuming structured conformations when they contact their ligand(s); it is possible that the C-terminal region of PSC assumes a structured conformation when bound to chromatin. A homolog of PSC, Suppressor 2 of zeste [Su(z)2], resides adjacent to PSC in the *Drosophila* genome and can functionally substitute for some PSC activities *in vivo* (*20*, *21*). Su(z)2 has *in vitro* activities similar to those of PSC (*22*), but the C-terminal region of Su(z)2, which mediates its inhibition of chromatin remodeling activity, is not homologous to that of PSC. Instead, the two C-terminal regions share a unique amino acid composition (*23*). Thus, the precise ordering of the primary

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[1]Abbreviations: PcG, Polycomb Group; PSC, Posterior Sex Combs; PRC1, Polycomb Repressive Complex 1; dRAF, dRING-Associated Factors; Ph, Polyhomeotic; Pc, Polycomb; SCM, Sex Comb on Midleg; PCC, PRC1 Core Complex; N, nucleosome; EMSA, electrophoretic mobility shift assay; REA, restriction enzyme accessibility assay; STEM, scanning transmission electron microscopy; aa, amino acid.

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sequence may not be important for the function of this region, but further work is needed to understand how it exerts its effects on chromatin. For example, it is not known whether different parts of the large C-terminal region of PSC (aa 456−1603) have different activities.

In addition to PRC1, PSC is part of a second complex in *Drosophila*, dRING-associated factors (dRAF) (*24*). dRAF contains both PSC and dRING, as well as other subunits not found in PRC1, but not PRC1 component Pc or Ph. dRAF functions as an E3 ligase for ubiquitylation of histone H2A, an activity that also seems to be essential for PcG silencing but cannot be recapitulated by PSC alone and is carried out poorly by PCC (*24*). Therefore, it seems that PSC contributes two key functions to PcG silencing: noncovalent modification of chromatin structure that requires its C-terminal, unstructured region (*19*) and ubiquitylation of histone H2A that involves the N-terminal RING domain (*25*−*27*) and other conserved sequences. The N-terminal region is also involved in assembly of PSC into both complexes.

Although extensive data support the significance of noncovalent modification of chromatin structure by PSC, exactly how this protein interacts with chromatin is not yet known. Initial work indicated that PSC inhibits remodeling of multinucleosome arrays but not of mononucleosomes (*14*); however, it is unclear if this substrate discrimination is at the level of binding or a subsequent step. To understand the mechanism of PSC, the substrate and protein requirements for PSC binding to nucleosomes and inhibiting chromatin remodeling were investigated.

MATERIALS AND METHODS

Proteins and Templates. FLAG epitope-tagged PSC and truncations were expressed in Sf9 cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen) and purified as described previously (*14*). hSwi/Snf was purified from nuclear extracts from HeLa cells expressing FLAG-Ini-1 as described previously (*28*). Active fractions of PSC and truncation preparations were determined using DNA in excess of protein (*14*) and were typically between 20 and 30%, except for PSC^{1-572}, which was <1% active. All stated concentrations refer to active concentrations unless otherwise indicated.

DNA templates contain one or tandem repeats of the *Xenopus laevis* 5S nucleosome positioning sequence (*29*) or the 601 nucleosome positioning sequence (*30*). Plasmids and details are available on request. Different linker orientations were achieved by placing the positioning sequence in different locations relative to the DNA ends. Mononucleosome and dinucleosome (1N and 2N, respectively, where N stands for nucleosome) DNA templates were prepared by polymerase chain reaction (PCR) followed by gel purification on acrylamide gels, while 6N and 12N DNAs were prepared by restriction enzyme digestion of plasmids followed by purification on a Sephacryl S-1000 gel filtration column (170 cm × 1.5 cm). In some cases, 1N and 2N templates were prepared with Cy5- or Cy3-labeled primers for visualization. Mononucleosome templates were assembled from the purified DNA fragments and HeLa histone octamers by step dialysis as described previously (*31*, *32*), followed by purification by sedimentation through 10 to 30% glycerol gradients. Nucleosomal templates greater than 1N were assembled by salt gradient dialysis with HeLa histone octamers as described previously (*32*). Nucleosome assembly was tested by an electrophoretic mobility shift assay (EMSA) or, for multinucleosome templates, by *Eco*RI digestion (*Eco*RI cuts between each 5S repeat) followed by EMSA (*33*). Mononucleosomes were heated at 50 °C for at least 1 h prior to being used for REAs to position the nucleosomes. All experiments were conducted with at least two preparations of mononucleosomes except for the 1N-100 template, which was from a single nucleosome assembly.

To confirm mononucleosome positioning, nucleosomes were first analyzed on native acrylamide gels. End-positioned nucleosomes migrate more rapidly than middle-positioned ones (Figure 1A of the Supporting Information). Mononucleosome positions were further confirmed by micrococcal nuclease digestion followed by purification of the protected, 150 bp DNA fragment [conducted by addition of SDS to a final concentration of 0.1% and PCR cleanup kit (Macherey-Nagel)] and digestion with *Hha*I. If nucleosomes are positioned over the positioning sequence, then the restriction digest of the protected fragments should be identical, irrespective of the length or configuration of the template (Figure 1B−D of the Supporting Information). This was observed for 601 (Figure 1D of the Supporting Information) but not for 5S templates (Figure 1C of the Supporting Information).

Restriction Enzyme Accessibility Assay (*REA*). REAs were conducted as described previously (*14*), with nucleosomes at 1 nM. In some cases, mononucleosomes were also tested at 5 nM. Concentration in all cases refers to the concentration of mononucleosome-sized DNA (so that reaction mixtures with 12N or 1N templates contain the same amount of DNA). Standard reaction conditions are as follows: 12 mM Hepes (pH 7.9), 0.12 mM EDTA, 12% glycerol, 60 mM KCl, 2 mM ATP, 0.025% NP40, and either 4 mM (multinucleosome templates) or 6 mM $MgCl_2$ (1N templates). *Pst*I or *Hha*I (New England Biolabs), which is unique in each 1N, 2N, or 6N template and is covered by a nucleosome, was used for restriction digestion of 5S templates. For 601 mononucleosomes, only *Hha*I is present, so all experiments were conducted with *Hha*I. DNA was visualized with SYBR Gold or SYBR Green I stain (Invitrogen), or Cy5 or Cy3 fluorescence on a Typhoon Imager. hSwi/Snf was titrated to ensure that it was present at saturating concentrations for nucleosome remodeling with each template. The percent (%) inhibition was calculated from the percent (%) uncut using the following equation:

$$\%\ \text{inhibition} = \frac{(\%\ \text{uncut with hSwi/Snf and PSC}) - (\%\ \text{uncut with hSwi/Snf})}{(\%\ \text{uncut without hSwi/Snf}) - (\%\ \text{uncut with hSwi/Snf})} \times 100\%$$

Electrophoretic Mobility Shift Assay (*EMSA*). Reactions were conducted under the conditions described for REAs except without ATP. Simple binding reaction mixtures contained 1 or 5 nM nucleosomes, and competition binding reaction mixtures contained 1 nM PSC, 0.5 nM nucleosomes of labeled substrates, and up to 10 nM nucleosomes of unlabeled competitors. Samples were resolved in 0.6 or 0.8% 0.5×-TBE agarose gels at 4 V/cm for 2−4 h and visualized with SYBR Gold stain or by Cy3 or Cy5 fluorescence on a Typhoon Imager. All experiments were conducted at least three times with two different preparations of PSC. EMSAs were quantified in ImageQuant as indicated in Figure 5 of the Supporting Information.

Co-Immunoprecipitation. Nuclear extracts of Sf9 cells infected with viruses encoding FLAG-PSC, HA-PSC, or both were prepared as described previously (*34*) except that extracts were not dialyzed after the high-salt extraction. M2 anti-FLAG agarose (Sigma) used for immunoprecipitation was blocked with

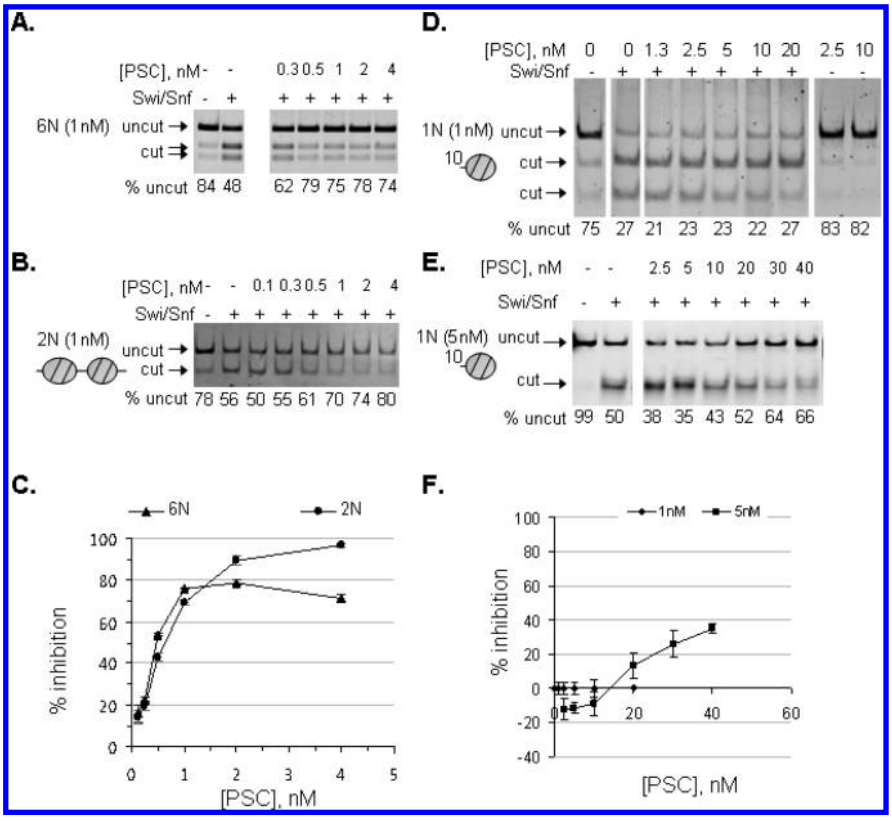

FIGURE 1: PSC inhibits remodeling of mononucleosomes poorly. Restriction enzyme accessibility (REA) assays were carried out with PSC at the indicated concentrations and hSwi/Snf. (A and B) Representative gels of REAs with 6N (A) and 2N (B) templates. (N refers to nucleosomes, so 6N is a 6-nucleosome template; all of these templates are composed of repeats of the 5S nucleosome positioning sequence.) (C) Summary of REAs with 6N and 2N templates. (D and E) Representative gel of REA with mononucleosomes assembled on a 157-bp (10-1N) 5S template at 1 nM (D) or 5 nM (E). Note that *Pst*I was used for digestion in the experiment with 1 nM nucleosomes, while *Hha*I was used with 5 nM nucleosomes. Both enzymes have single digestion sites in the template but produce slightly different digestion patterns (*Hha*I digests the template into 77 and 80 bp fragments, which are not resolved, while *Pst*I digests it into 99 and 52 bp fragments). Both enzymes were used in these assays and they produce very similar results. The experiments in 1E were visualized with a 5′ Cy5 label, so only one product is visible. (F) Summary of REAs with a mononucleosome template. Error bars denote the standard error of the mean.

500 ng/μL BSA (NEB) in BC buffer [20 mM Hepes (pH 7.9) and 0.2 mM EDTA with 20% glycerol, 300 mM KCl, and 0.05% NP40] and protease inhibitors for 30 min at 4 °C. Nuclear extracts were incubated with anti-FLAG agarose at 4 °C for 2 h. Agarose beads were washed five times with blocking buffer and boiled in SDS−PAGE sample buffer. Samples were resolved via SDS−PAGE and immunoblotted with M5 anti-FLAG or HA-7 anti-HA antibodies (Sigma). Copurification was conducted in two independent experiments.

Chemical Cross-Linking. PSC (150−450 nM total protein) was diluted into a final reaction mix of 12 mM Hepes (pH 7.4), 0.12 mM EDTA, 0.5 mM PMSF, and 2 mM $MgCl_2$ and allowed to equilibrate at 30 °C for 15 min followed by addition of up to 0.25 mM 1,8-bis-maleimidodiethylene glycol (BM) or 2.5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Thermo Fisher) at room temperature for 1−2 h. Cross-linked products were resolved by SDS−PAGE and immunoblotted with M5 anti-FLAG antibody. Experiments were conducted at least three times with two different preparations of PSC.

Glycerol Gradient Sedimentation. Glycerol gradients from 5 to 35% (280 μL) were created in BC buffer with 60−900 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and 0.05% NP40. Proteins were adjusted to the appropriate KCl concentration and to a total protein concentration of 25−100 mM in BC buffer with 0.5 mM DTT, 0.2 mM PMSF, and 0.05% NP40 and incubated for 15 min at 30 °C before being loaded onto gradients. Gradients were centrifuged for 3 h at 55000 rpm and 4 °C in rotor TLS-55 (Optima mini-ultracentrifuge) and fractionated into 40 μL fractions by pipeting from the top. Fractions were analyzed by SDS−PAGE followed by Western blotting. Similar results were obtained on larger gradients (2.2 mL), in that PSC and PCC migrate as distinct peaks but PSC migrates much further into the gradient than PCC.

RESULTS

PSC Inhibits Nucleosome Remodeling of 2N Substrates More Effectively Than That of 1N Substrates. To determine the chromatin substrate requirements for PSC, inhibition of remodeling was compared on templates with different numbers of nucleosomes. Remodeling of templates consisting of two or six nucleosomes (termed 2N or 6N, respectively) was 50% inhibited at PSC concentrations between 0.5 and 1 nM, a PSC:nucleosome ratio of 0.5−1, and inhibition was saturated at 2 nM PSC (Figure 1A−C). (All concentrations refer to the active concentration unless otherwise indicated; see Materials and Methods for an explanation.) This is the same concentration range previously demonstrated for inhibition of remodeling of 12N templates (*14*). In contrast, but consistent with previous work (*14*), remodeling of a 157 bp mononucleosome template (10-1N) is inhibited poorly (Figure 1D,F). Increasing the concentration of mononucleosomes to 5 nM allowed some inhibition of remodeling, although inhibition did not reach 50% at a 40-fold higher concentration than needed to reach 50% inhibition of nucleosomal arrays (Figure 1E,F).

Remodeling of a Nucleosome with Linker DNA Is Inhibited by PSC. 2N templates differ from 1N templates in two ways: an additional nucleosome and the presence of linker DNA. Because PSC binds tightly to naked DNA [K_d = 0.2 nM for the 155 bp fragment (*14*)], linker DNA might be important for the interaction of PSC with mononucleosomes. A series of mononucleosomes was created with linker DNA on one or both sides, and the ability of PSC to inhibit Swi/Snf-mediated chromatin remodeling of them was tested. We refer to these templates according to the length of the linker DNA and its orientation. Thus, 50-1N-50 templates should have a nucleosome positioned in the middle of a 247 bp fragment with 50 bp linkers on each side, while 1N-100 has a 100 bp linker 3′ to the nucleosome positioning sequence. We initially prepared these templates using the 5S nucleosome positioning sequence for direct comparison with our polynucleosome template results. However, although the nucleosome positioning was sufficient to block restriction enzymes present in the 5S sequence (data not shown), detailed analysis of positioning indicated that the longer templates all had multiple nucleosome positions (Figure 1 of the Supporting Information and data not shown). Thus, we prepared a second series of mononucleosome templates using the artificial 601 nucleosome positioning sequence (*30*) (Figure 1 of the Supporting Information). We were able to obtain well-positioned populations of mononucleosomes after purification by glycerol gradient sedimentation. We note, however, that all 601 assemblies onto 247 bp fragments produced two positions, one in which the nucleosome is likely at one end of the template and the other of which positions it in the middle of the fragment. These positions could be separated on glycerol gradients.

To compare inhibition of chromatin remodeling on different mononucleosome templates, it is important that all of the substrates can be remodeled similarly. We therefore characterized the kinetics of remodeling by hSwi/Snf on each of our mononucleosome templates (Figure 2 of the Supporting Information). We find that two different 5S templates (10-1N and 50-1N-50) and two 601 mononucleosomes (100-1N and 50-1N-50) are all remodeled with similar kinetics (Figure 2A,B of the Supporting Information); the rate constants are also similar to those previously measured for mononucleosomes (between 0.07 and 0.14 min^{-1}) (*35*). In contrast, two of the 601 mononucleosomes (10-1N and 1N-100) were remodeled 3−14 times slower than the other templates (rate constants of 0.01−0.02 min^{-1}) (Figure 2C−E of the Supporting Information). We do not understand the basis of this effect. Previous studies have demonstrated that RNA polymerase II transcription is differentially affected in the forward and reverse orientation on 601 mononucleosomes (*36*), so it is possible that our data reflect these differences. However, the two mononucleosomes that are remodeled slowly have linkers in the opposite orientation (for 10-1N, the linker protrudes from the 5′ end of the 601 sequence, while for 1N-100, it protrudes from the 3′ end), so that a simple orientation effect seems unlikely. Nevertheless, we chose to compare inhibition of remodeling on the rapidly remodeled nucleosomes (i.e., 5S 10-1N, 5S 50-1N-50, 601 100-1N, and 601 50-1N-50), because we were concerned that the slow remodeling might enhance inhibition by PSC. The 5S 1N-10 mononucleosome is well-positioned (Figure 1A,C of the Supporting Information) and so can be compared with the 601 templates with longer linkers.

Addition of 100 bp of linker DNA to one side or 50 bp to both sides of 601 mononucleosomes enhanced inhibition of chromatin remodeling by PSC; 50% inhibition was reached between 8 and 10 nM PSC. (Table I summarizes all of the templates used and the results of REAs.) This PSC concentration is ~10-fold higher than that required for inhibition on 2N templates (Figure 2 and Table I). We also tested 5S mononucleosomes with 50 bp linkers on both sides and found that their remodeling is also inhibited by PSC but requires ~2-fold higher concentrations than the 601 mononucleosomes. Because our initial mononucleosome experiments suggested that PSC can inhibit 10-1N templates when the templates are used at 5 nM instead of 1 nM, we repeated the analysis of the mononucleosomes with linkers using a template at 5 nM (Figure 3 of the Supporting Information and Table I). PSC inhibited remodeling more efficiently when then template was used at 5 nM; 50% inhibition was reached between 7.5 and 15 nM PSC for templates with two 50 bp linkers or one 100 bp linker. This is a ratio of 1.5−3 PSCs per nucleosome, which is lower than the ratio of 8−10 PSCs per nucleosome required for inhibition using 1 nM substrate but is still higher than the amount of PSC required for 50% inhibition of a 2N template [less than 1 PSC per nucleosome (Figure 1C and Table I)]. These experiments indicate that linker DNA contributes to PSC inhibition of chromatin remodeling but is not sufficient to explain the difference between 1N and 2N templates.

Table I: Summary of REA Results

			50% inhibition		
template	type	[N]	nMPSC	PSC:N	Figure
6N	5S	1	0.5−1	0.5−1	1
2N	5S	1	0.5−1	0.5−1	1
10-1N	5S	1	>20	>20	1
	5S	5	>40	>8	1
100-1N	601	1	8−10	8−10	2
		5	7.5	1.5	S3
50-1N-50	601	1	8−10	8−10	2
		5	15	3	S3
	5S	5	20	20	2

Previously, we found that PSC does not inhibit the background restriction enzyme accessibility of polynucleosomal templates (*14*). However, because the mononucleosome experiments involve using high concentrations of PSC, we repeated these experiments with mononucleosome templates. We find that even low concentrations of PSC inhibit background restriction enzyme access (Figures 1D and 2A−C). This does not seem likely to account for inhibition of hSwi/Snf-induced chromatin remodeling because it occurs even at low concentrations of PSC that do not block hSwi/Snf-induced restriction enzyme accessibility. To further test the effect of PSC on restriction enzyme access, we incubated PSC with naked DNA under the same conditions that were used for the REA. Under these conditions, PSC does not inhibit restriction enzyme access, although some template loss was observed at higher concentrations of PSC (Figure 2D and Figure 3D of the Supporting Information). Thus, inhibition of restriction enzyme activity seems unlikely to account for the effect on accessibility observed in chromatin remodeling reactions.

Mononucleosome experiments were conducted with 4 mM free $MgCl_2$, while experiments with polynucleosome templates, which can be compacted by $MgCl_2$, were conducted with 2 mM free $MgCl_2$. To confirm that this difference in reaction conditions did not explain the less efficient inhibition of remodeling of mononucleosomes, a small number of experiments were conducted with mononucleosome templates with 2 mM free $MgCl_2$ (Figure 4 of the Supporting Information). Results from these

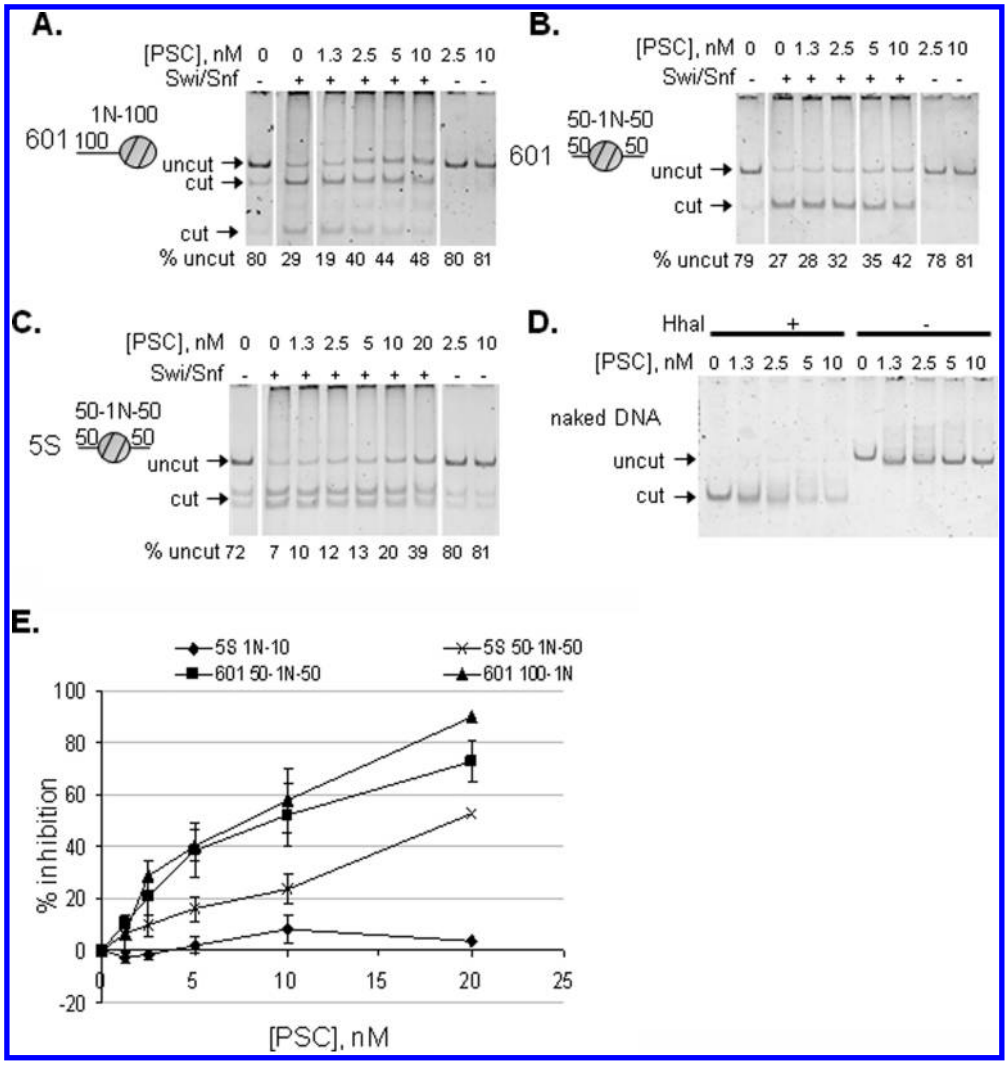

FIGURE 2: PSC inhibits mononucleosomes with one long linker or two linkers better than mononucleosomes with one short linker. (A−C) Representative REAs on a series of mononucleosome templates. The three templates are all 247 bp long. (D) PSC does not inhibit restriction enzyme digestion. Naked DNA at 1 nM was incubated with PSC under identical conditions as in the remodeling assays either with or without the restriction enzyme *Hha*I. (E) Summary of REAs on the various mononucleosome templates. Note that even though remodeling of templates with linkers can be inhibited by PSC, higher concentrations are required than for 2N templates (compare with Figure 1C). Error bars denote the standard error of the mean.

experiments were very similar to those with 4 mM free $MgCl_2$ in that remodeling of the 10-1N template was inhibited poorly by PSC while remodeling of the 50-1N-50 template was inhibited by high concentrations of PSC.

PSC Binds Mononucleosomes under Conditions Where It Does Not Inhibit Their Remodeling. One explanation for the poor ability of PSC to inhibit mononucleosome templates without linkers is that it binds these templates poorly. This would also be consistent with the observation that increasing the template concentration by 5-fold improves inhibition of remodeling (compare Figure 2 and Figure 3 of the Supporting Information). To test this idea, we conducted EMSAs using mononucleosome templates. PSC shifts the mobility of all of the mononucleosomes tested, indicating that it binds to them (Figure 3A). Quantification of EMSAs (see Figure 5 of the Supporting Information for an explanation of quantification) indicates that 50% binding occurs between 0.9 and 1.5 nM for all three templates (Figure 3B). These numbers represent an upper estimate of the K_d for the interaction between PSC and mononucleosomes because the template concentration used here (1 nM) is too high to allow measurement of the true K_d. (Nucleosomes are not stable at concentrations below 1 nM, so different methods will be required to measure the true K_d.) Nevertheless, these data indicate that, under the conditions used for REAs, binding to all of the templates (as determined by EMSA) is very similar.

For all of the nucleosomes, two bound species were observed. The first appeared at low concentrations of PSC. The second species migrated slowly in agarose gels and in some cases was retained in the well. Both species were included in the quantification of the fraction bound. However, when the second bound species is quantified separately, the 10-1N templates require higher concentrations of PSC to produce the slow mobility complex (bound species 2) (Figure 3C); 50% of the template formed the second bound species on the longer templates at ~2 nM PSC, while less than 40% of the 10-1N template formed the second bound species at 5 nM PSC. When nucleosomes were used at 5 nM PSC, the 10-1N template formed the second bound species (Figure 3F of the Supporting Information) with 10 nM PSC. The concentration of PSC required to form the second bound species was higher when 50-1N-50 mononucleosomes at 5 nM were used as the template. This suggests that the ratio of

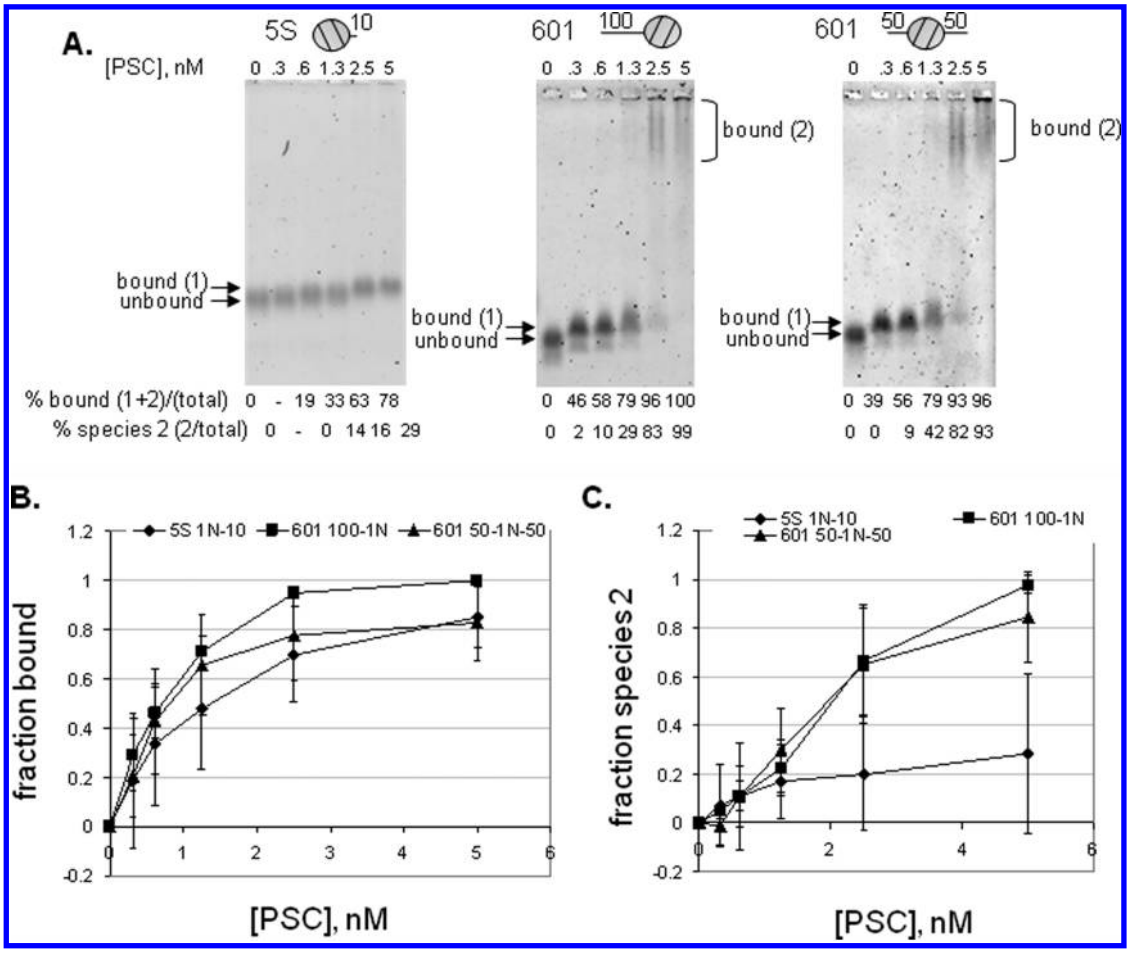

FIGURE 3: PSC binds all mononucleosome templates at low concentrations that do not inhibit chromatin remodeling. (A) EMSAs with various mononucleosome substrates at 1 nM. Details of quantification of EMSAs are presented in Figure 5 of the Supporting Information. At low concentrations, PSC binds mononucleosomes [bound (1)], while at higher concentrations, it forms a second, slowly migrating species [bound (2)]. (B) Quantification of fraction bound (bound 1 + 2) for each template. (C) Quantification of slowly migrating species [bound (2)] only. Error bars denote the standard deviation.

PSC to nucleosomes might be important for forming this species, because at both 1 and 5 nM template the second species forms at a ratio of two or more PSCs per nucleosome.

Inhibition of nucleosome remodeling occurs at the PSC concentrations that produced the slowly moving species (compare Figure 3C with Figure 2E). However, formation of this species is not sufficient because 100% of the 50-1N-50 or 100-1N template forms the second bound species at 5 nM PSC, but remodeling is inhibited by ~40% (Figure 3 of the Supporting Information). This bound species may be necessary for inhibition of nucleosome remodeling, because the 10-1N template at 1 nM formed little of this species, and no inhibition of nucleosome remodeling was observed. In contrast, in experiments with 5 nM template, the second species was formed and some inhibition of remodeling was observed (although at PSC concentrations 2-fold higher than that required for formation of the second bound species). Thus, PSC binds to mononucleosomes under conditions where it does not inhibit their remodeling, and inhibition of remodeling correlates with formation of higher-order PSC−nucleosome complexes, although the precise nature of these complexes is not known.

PSC Binds Less Stably to Mononucleosomes Than to Dinucleosomes. EMSA suggests that binding of PSC to mononucleosomes occurs at concentrations where remodeling of polynucleosomes is inhibited but does not result in inhibition of mononucleosome remodeling. To determine whether PSC preferentially binds polynucleosome templates, binding to mononucleosomes was compared to binding to 2N templates in the presence of competitor nucleosomes. PSC was incubated with a mixture of Cy3- or Cy5-labeled 1N (50-1N-50) or 2N templates (probe) and increasing amounts of each unlabeled competitor, and binding was analyzed by EMSA (Figure 4A). In these experiments, PSC was used at a concentration of 1 nM, a concentration that does not form the slowly migrating species. Under these conditions, binding to the 50-1N-50 template is prevented by excess 50-1N-50, or 2N. In contrast, binding to a 2N template is prevented by a 2N competitor, but not a 10-fold excess of 50-1N-50.

The experiments described above address partitioning of PSC among different templates when binding to all of them is initiated simultaneously. To probe the stability of binding of PSC to the two templates, PSC was prebound to either template, and binding was challenged by the addition of excess unlabeled competitor. In this case, after incubation with the competitor for 15 min, PSC remained bound to the 50-1N-50 and 2N templates in the presence of an excess of either competitor (Figure 4B). Thus, PSC binds more tightly to 2N than 50-1N-50 templates, which might explain how it is able to inhibit remodeling of 2N templates more effectively than 1N ones. Nevertheless, once PSC is bound to the 50-1N-50 template, the complex is stable for at least 15 min.

PSC Self-Interacts. The correlation between inhibition of chromatin remodeling and formation of the slowly migrating species that might represent interaction between PSC-bound

nucleosomes and the requirement for two nucleosomes for an efficiently used substrate suggests that PSC may bring nucleosomes together to inhibit their remodeling. This could occur if PSC has two binding sites or if the protein interacts to form dimers or multimers. Several strategies were used to determine whether PSC can interact with itself. First, PSC was epitope tagged with either FLAG or HA and expressed in Sf9 cells. When both versions of PSC were coexpressed, they could be co-immunoprecipitated with anti-FLAG beads (Figure 5A). Second, treatment of PSC with two different chemical cross-linking reagents results in formation of high-molecular mass complexes (Figure 5B). In the cross-linking experiments, an initial high-molecular mass complex is observed, which could be a dimer, but with increasing cross-linking, additional higher-molecular mass species are formed. Third, PSC was analyzed by glycerol gradient sedimentation. The peak of PSC migrated further in the gradient than immunoglobulin (150 kDa) but not as far as ferritin (440 kDa) (Figure 5C). PSC also migrates further into the gradient than PCC (composed of PSC, Pc, and dRING), which has a predicted molecular mass of 261 kDa. Previous scanning transmission electron microscopy (STEM) measurements suggest that PCC is a monomeric complex (*9*). A subcomplex composed of PSC and dRING migrates like PSC. PSC, PCC, and PSC with dRING migrate as discrete peaks at KCl concentrations from 300 to 900 mM, although at lower KCl concentrations, variable levels of aggregates were observed in the pellet (data not shown). These results are consistent with PSC forming salt-stable dimers that are prevented by assembly into PCC. Efforts to confirm that PSC is indeed the size of a dimer using size exclusion chromatography were not successful. Under all conditions tested, PSC migrates in the void volume and as an extended peak in the earliest eluting fractions from a Superose 6 column (date not shown). Thus, we conclude that PSC can self-interact but confirming the stoichiometry of the interaction will require additional methods. The behavior of PSC in size exclusion chromatograph could be explained by its predicted unstructured C-terminal region that may adopt an extended, flexible shape.

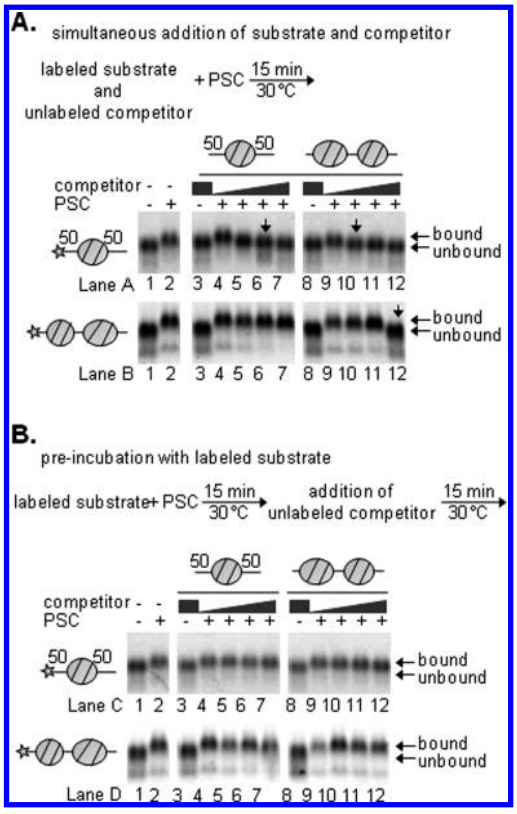

FIGURE 4: Competition binding assays demonstrate that PSC binds 2N templates preferentially over 1N templates but that binding to both templates is stable. (A) Competition assays in which competitor was added simultaneously with labeled substrates, as indicated. (B) Competition assays in which competitor was added after a 15 min preincubation with the labeled substrate. PSC was used at a concentration of 1 nM and labeled nucleosomes at a concentration of 0.5 nM. Competitor was used at a concentration of up to 10 nM. Vertical arrows indicate concentrations at which loss of binding was observed.

Inhibition of Chromatin Remodeling Requires PSC Sequences That Are Not Essential for Nucleosome Binding. Previous data indicate that the N-terminal region of PSC (aa 1−572) can assemble into PCC but does not inhibit chromatin remodeling or compact chromatin (*9*, *18*). In contrast, the C-terminal half of the protein (aa 456−1603) has both activities. To narrow the region of PSC required for inhibition of chromatin remodeling, a series of PSC constructs truncated from the

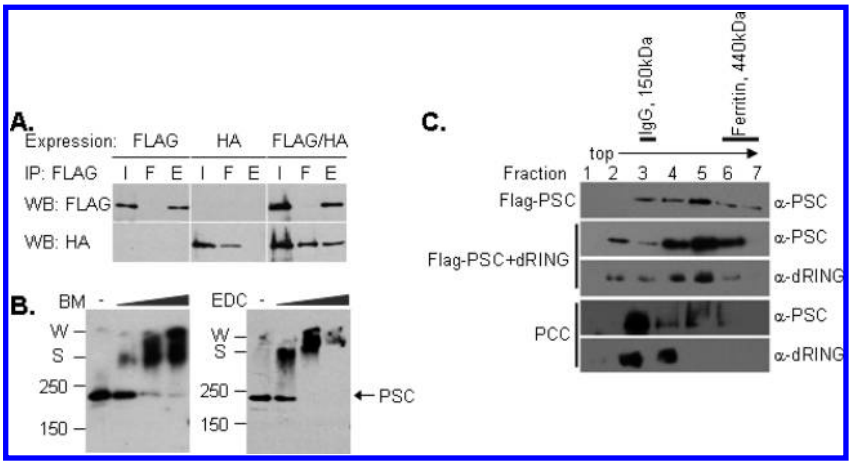

FIGURE 5: PSC interacts with itself likely to form dimers. (A) FLAG- and HA-tagged PSC were expressed individually or together, and FLAG-PSC immunoprecipitated. When FLAG-PSC and HA-PSC were coinfected (third panel), HA-PSC copurifies with FLAG-PSC. (B) Cross-linking with BM (left) or EDC (right) followed by Western blot analysis. PSC cross-links into high-molecular mass species when treated with either cross-linker. (C) Glycerol gradient sedimentation of PSC, PSC with dRING, or PCC (PSC, dRING, and Pc) followed by Western blot analysis. The peak of FLAG-PSC is in fraction 5, as is the peak for PSC with dRING, while the peak of PCC is in fraction 3. The molecular mass of FLAG-PSC is 171 kDa. Gradients were run in 900 mM KCl, although similar results were obtained at 300 and 600 mM KCl. S, stacking gel boundary. W, well.

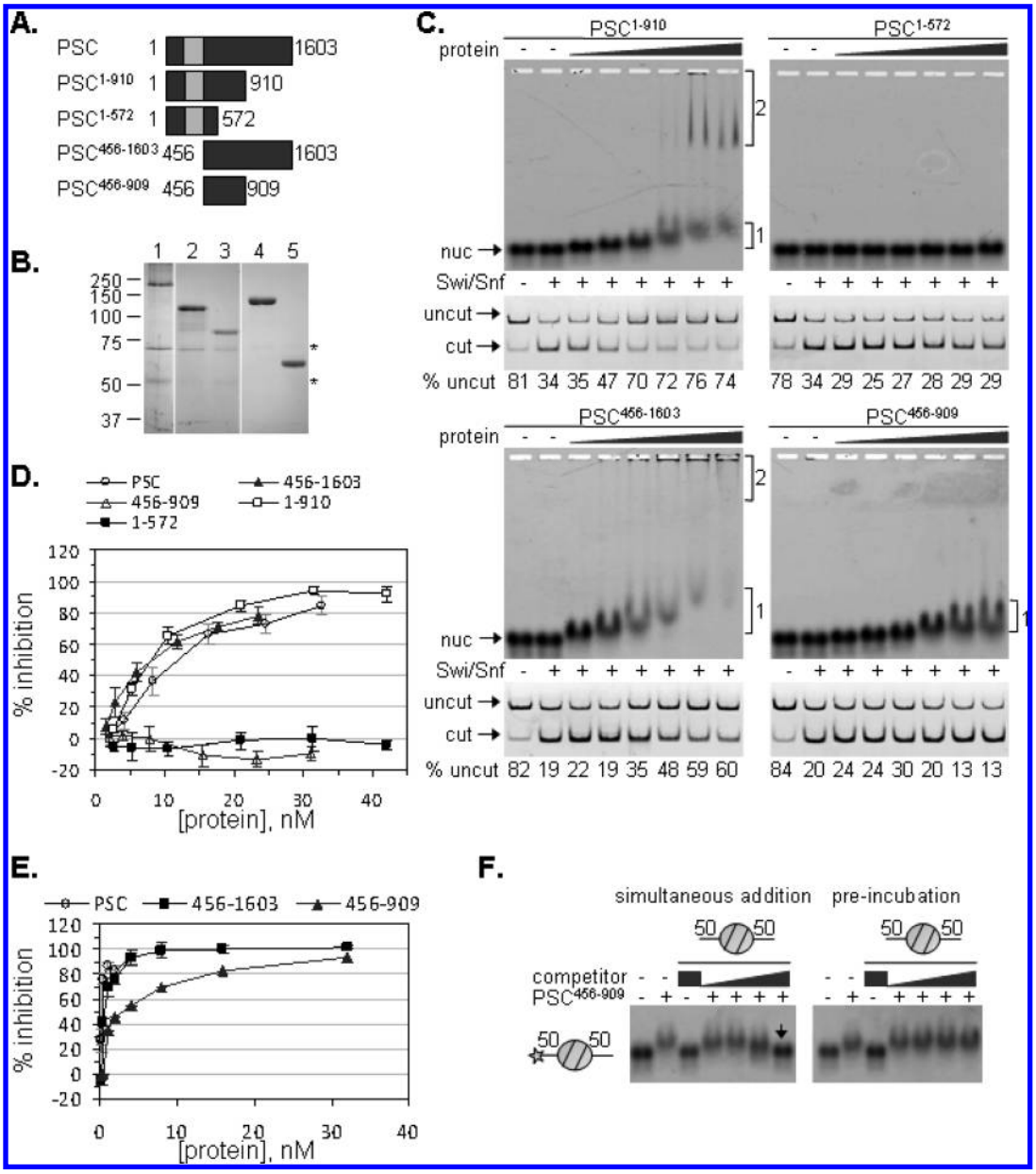

FIGURE 6: Inhibition of chromatin remodeling requires sequences in PSC not required for stable nucleosome binding. (A) Schematic diagram of PSC truncations. Note that some of these truncations have been previously characterized (*9*, *18*). (B) Colloidal Blue-stained gel of representative preparations of PSC and PSC truncations (1−5: PSC, PSC^{1-910}, PSC^{1-572}, $PSC^{456-1603}$, $PSC^{456-909}$). Asterisks indicate contaminants that are likely Hsc70 (~70 kDa) and β-tubulin (~55 kDa). (C) EMSA and REA of each truncation with a 50-1N-50 mononucleosome (two linkers). The two shifted species are indicated next to the gels in cases where they form. (D) Summary of REAs on the two-linker 1N substrate with PSC and the various truncations. (E) $PSC^{456-909}$ was tested for inhibition of remodeling of a 12N array. Full-length PSC and $PSC^{456-1603}$ were included for comparison. (F) $PSC^{456-909}$ was tested for stable binding to the two-linker 1N substrate, using competition assays as seen in Figure 4.

N-terminus, the C-terminus, or both (Figure 6A,B) was tested for inhibition of binding to and chromatin remodeling of a mononucleosome substrate with two linkers (Figure 6C). These experiments were conducted with 5S 50-1N-50 mononucleosomes at 5 nM. PSC^{1-572} did not bind mononucleosomes or inhibit their remodeling, but both PSC^{1-910} and $PSC^{456-1603}$ bind to mononucleosomes, induce formation of the slowly migrating species, and inhibit nucleosome remodeling. $PSC^{456-909}$, which encompasses the overlap between the two active truncations, binds the mononucleosomes but does not induce formation of the slowly migrating species or inhibit nucleosome remodeling. Thus, this truncation separates nucleosome binding activity of PSC from inhibition of chromatin remodeling. The activity of $PSC^{456-909}$ was also tested on 601 50-1N-50 mononucleosome templates at 1 nM; despite binding to these templates, it does not inhibit their remodeling (not shown).

To further investigate the activity of $PSC^{456-909}$, its ability to inhibit remodeling of 12N arrays was compared with the abilities of PSC and $PSC^{456-1603}$ (Figure 6E). $PSC^{456-909}$ was able to inhibit remodeling of a 12N array but required at least 10-fold higher concentrations than PSC or $PSC^{456-1603}$. $PSC^{456-909}$ was also tested for stable binding to the 5S 50-1N-50 mononucleosome in the two competition assays used in Figure 4 (Figure 6F). $PSC^{456-909}$ behaved like PSC in this assay: its binding is stable to competitor added after preincubation. Thus, inhibition of chromatin remodeling and formation of the slowly migrating mononucleosome−PSC species require additional sequences in PSC that are not necessary for stable nucleosome binding.

DISCUSSION

The protein and substrate requirements for nucleosome binding and inhibition of chromatin remodeling by PSC were investigated. The key conclusions are that (1) PSC binds mononucleosomes under conditions where it does not inhibit their remodeling, (2) mononucleosomes with two 50 bp linkers or a single 100 bp linker are better substrates for inhibition of remodeling than mononucleosomes with a single 10 bp linker, (3) a region of PSC sufficient for chromatin binding does not inhibit chromatin remodeling, and (4) full-length PSC interacts with itself. The analysis of substrate and PSC sequence requirements thus reveals that inhibition of chromatin remodeling requires additional steps beyond nucleosome binding.

Relationship between Nucleosome Binding by PSC and Inhibition of Remodeling. Our data indicate that while two nucleosomes are required for full inhibition of chromatin remodeling at low concentrations of PSC, remodeling of mononucleosomes with DNA linkers can be inhibited more than 50%, albeit at higher PSC concentrations. We consider two models for how this can occur. The first is based solely on differences in PSC affinity for different substrates, while the second suggests that inhibition of nucleosome remodeling requires that nucleosomes be brought together to form a structure that is more difficult to remodel.

Our data indicate that PSC binds nucleosomes and interacts with itself, suggesting a simple explanation for higher-affinity binding of PSC to 2N than 1N templates: two PSC molecules could bind 2N templates (one per nucleosome) and interact with each other. Similar interactions may occur when PSC-bound mononucleosomes are brought together, which seems to occur in the slowly migrating species observed via EMSA. The data in Figure 4B, as well as unpublished results with multinucleosome templates, suggest that once PSC is bound, little exchange is observed for at least 15 min with either 1N or 2N templates. In REAs, PSC is prebound to the template prior to the addition of hSwi/Snf. The amount of inhibition may therefore reflect the fraction of the templates that are bound by PSC in the initial binding step, which remain stably bound and inhibited over the entire reaction. Although our stability experiment does not provide evidence of this, it is possible that in the 1 h mononucleosome remodeling experiments, PSC binds and falls off nucleosomes during the reaction, so that the level of inhibition observed reflects an ongoing competition between PSC and hSwi/Snf for substrate binding. Another possibility is that hSwi/Snf itself is able to displace PSC from 1N templates more effectively than from polynucleosome templates.

A second model for inhibition of chromatin remodeling by PSC suggests an additional step beyond nucleosome binding in which nucleosomes are brought together to form a structure that is refractory to binding or remodeling by hSwi/Snf. This interaction is by definition intermolecular for 1N templates but intramolecular (and therefore more efficient) for 2N ones. The finding that inhibition of remodeling of mononucleosomes occurs at lower ratios of PSC to nucleosomes when nucleosomes are at 5 nM versus 1 nM is consistent with the idea that the concentration of PSC-bound mononucleosomes is important for inhibition of chromatin remodeling. These data also suggest that inhibition does not involve nonspecific aggregation of PSC, because this would occur at the same PSC concentration when mononucleosomes are used at either 1 or 5 nM, but inhibition is more effective with 5 nM template. The finding that inhibition of chromatin remodeling is correlated with the formation of the slowly migrating PSC-1N species by EMSA also suggests that PSC-bound nucleosomes can interact, likely through PSC−PSC interactions. These interactions could be important for inhibition of remodeling and could be analogous to the interactions that occur on polynucleosome templates and lead to chromatin compaction (*9*), although we cannot entirely rule out the possibility that the slowly migrating species are nonspecific aggregates whose formation is facilitated by the extra DNA present on mononucleosome templates with linkers. Bringing nucleosomes together could inhibit chromatin remodeling either by decreasing the affinity of hSwi/Snf for its substrate, which was suggested by the exclusion of hSwi/Snf from PSC-bound multinucleosome arrays (see Figure 6 of ref *14*), or by allowing binding but not remodeling. It is difficult to distinguish these two possibilities at present, in part because we do not yet know if hSwi/Snf and PSC can simultaneously bind mononucleosomes (as would need to occur if PSC binding alone does not inhibit remodeling).

Mapping the Minimal Sequence Requirements for Inhibition of Chromatin Remodeling. The data with PSC truncations (Figure 6) further support the idea that nucleosome binding is not sufficient for inhibition of chromatin remodeling because $PSC^{456-909}$ binds stably to 1N templates but does not inhibit their remodeling (Figure 6). $PSC^{456-909}$ also does not promote formation of the slowly migrating species in EMSA, suggesting that additional regions of PSC mediate this activity. $PSC^{456-1097}$ was also tested for inhibition of remodeling of nucleosomal arrays (data not shown) and behaves like $PSC^{456-909}$. Thus, sequences required for formation of the slowly migrating species in EMSA and inhibition of remodeling may reside between 1097 and 1603. Because PSC^{1-910}, which lacks these sequences, can form the slowly migrating species and inhibit chromatin remodeling, sequences between aa 1 and 456 may functionally replace the C-terminal sequences in this setting. *Psc* alleles truncated at aa 910, 1075, or 1098 have milder phenotypes than those truncated at aa 521 but nevertheless have phenotypes, indicating that the truncated region of the protein (aa 1098−1603) is important for PSC function *in vivo* (*21*). The proteins encoded by these alleles do not have defects *in vitro* (*18*), and it seems that either an intact N-terminal or C-terminal domain, in conjunction with the sequences from base pair 456 to 909, is sufficient for chromatin compaction and inhibition of chromatin remodeling *in vitro*.

PSC Self-Interacts. Our data indicate that PSC can interact with itself; the idea of self-interaction is consistent with previously observed intragenic complementation of *Psc* alleles in *Drosophila* (*20*). In mammalian systems, Mel-18 (a homolog of PSC) was demonstrated to form homodimers (*37*), even though most other analyses of PSC homologs have not detected homodimers. Computation and structural analysis suggest that both PSC and dRING have a RING domain and a predicted ubiquitin fold domain. The ubiquitin fold in the C-terminal region of Ring1b (mammalian homolog of dRING) can form homodimers (*38*, *39*), although Ring1b preferentially forms heterodimers with Bmi-1. The RING domains of Bmi-1 and Ring1b form heterodimers (*25*, *26*), while full-length Bmi-1 and Ring1b form heterotetramers. Our analysis of a PSC−dRING subcomplex (Figure 5C) indicates that it migrates like a likely PSC dimer on a glycerol gradient; this complex may be a heterotetramer whose migration is dominated by the behavior of PSC. Our finding that PSC may form stable dimers suggests the *Drosophila* proteins differ from their mammalian homologs in their interactions.

Our glycerol gradient data also suggest that PSC assembled into PCC does not form salt-stable multimers. This is consistent with our previous STEM analysis of PCC, which measured the mass of PCC as that expected for a monomer (*9*). It is also consistent with structural analysis of Ring1b, which indicates that dimerization is blocked by interaction with the C-terminal region of a Pc homolog (*40*). Thus, addition of Pc may be the key to inhibiting PSC and dRING dimerization. It is interesting in this regard that Pc is missing from the dRAF complex that mediates histone ubiquitylation (*24*). Perhaps PSC dimerization is important in this complex. Because PSC can inhibit chromatin remodeling on its own or in PCC, it seems that dimerization is not central to inhibition of chromatin remodeling or chromatin compaction. Although our glycerol gradient data are consistent with PSC forming dimers, it should be noted that this stoichiometry has not been rigorously confirmed. It will be interesting to determine the stoichiometry of PSC−PSC interactions in the presence and absence of mononucleosome substrates.

In summary, we have dissected the protein and substrate requirements for the Polycomb protein PSC to inhibit chromatin remodeling. Inhibition of chromatin remodeling and chromatin compaction may reflect the same activity because all PSC truncations that are active in chromatin remodeling can compact chromatin. Taken together, our results suggest inhibition of chromatin remodeling, and likely chromatin compaction, by PSC involve at least two distinct molecular events: nucleosome binding and interactions among PSC-bound nucleosomes.

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SUPPORTING INFORMATION AVAILABLE

Analysis of nucleosome positioning of the mononucleosome templates used in this study (Figure 1), anlysis of the kinetics of remodeling of the mononucleosome templates used in this study (Figure 2), binding and inhibition of chromatin remodeling of mononucleosomes by PSC using 5 nM substrate rather than 1 nM substrate (Figure 3), inhibition of nucleosome remodeling by PSC at a lower $MgCl_2$ concentration (2 mM free Mg^{2+} instead of the 4 mM Mg^{2+} used in the main figures) (Figure 4), and depiction of how EMSA experiments were quantified (Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

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