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A Domain Shared by the Polycomb Group Proteins Scm and ph Mediates Heterotypic and Homotypic Interactions

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The formation of body structures along the anterior-posterior (A-P) axis in Drosophila flies is controlled by the homeotic transcription factors (31, 35). The homeotic proteins are deployed in restricted domains along the embryonic A-P axis that correspond with their realms of function (11, 30, 60). Restricted homeotic expression requires a set of transcriptional repressors, collectively known as the Polycomb group (PcG) proteins. Mutations that inactivate individual PcG proteins trigger homeotic gene expression in inappropriate positions along the A-P axis (37, 55, 57).

So far, approximately 15 PcG repressors have been identified in Drosophila (see references 44 and 52 for reviews). Several of these PcG proteins localize at specific sites in chromosomes, including the homeotic loci (10, 19, 36, 47). However, little is known about the molecular mechanisms used by PcG proteins for transcriptional repression. Although the molecular cloning and sequence determination of seven of the fly PcG proteins have been completed (7, 9, 13, 24, 28, 36, 43, 49, 53), the predicted primary sequences provide few clues about prior (A-P) axis in Drosophila flies. The Scm and ph proteins share a homology domain with 38% identity over a length of 65 amino acids, termed the SPM domain, that is located at their respective C termini. Using the yeast two-hybrid system and in vitro protein-binding assays, we show that the SPM domain mediates direct interaction between Scm and ph. Binding studies with isolated SPM domains from Scm and ph show that the domain is sufficient for these protein interactions. These studies also show that the Scm-ph and Scm-Scm domain interactions are much stronger than the ph-ph domain interaction, indicating that the isolated domain has intrinsic binding specificity determinants. Analysis of site-directed point mutations identifies residues that are important for SPM domain function. These binding properties, predicted α-helical secondary structure, and conservation of hydrophobic residues prompt comparisons of the SPM domain to the helix-loop-helix and leucine zipper domains used for homotypic and heterotypic protein interactions in other transcriptional regulators. In addition to in vitro studies, we show colocalization of the Scm and ph proteins at polytene chromosome sites in vivo. We discuss the possible roles of the SPM domain in the assembly or function of molecular complexes of PcG proteins.
Ets family of transcription factors (21, 29) and yeast proteins required for mating (46). We will refer to the high-homology domain subgroup that includes the Scm and ph versions as the SPM domain, and we will refer to the extended domain family as the SAM domain (46).

One of the more well-characterized SAM domains is present in the human TEL oncoprotein, an Ets class transcription factor, where it has been referred to as a helix-loop-helix (HLH) domain (23, 51). Recent studies have shown that this domain mediates self-binding and oligomerization of TEL protein and of other fusion protein derivatives (21, 29).

If the SPM domain in Scm and ph has similar biochemical properties to the related domain in TEL, then it might be used for binding interactions of these two PEG proteins. In this study, we used the yeast two-hybrid system and in vitro binding assays to test for direct protein-protein interactions between Scm and ph. We found that the SPM domain mediates both self-binding and Scm-ph cross-association and that the isolated domain is sufficient for binding interactions. Deletion mutations were used to assess the SPM domain role in full-length protein interactions, and site-directed point mutations were used to test how individual residues contribute to binding activity. Immunoexperiments showing colocalization of the Scm and ph proteins on larval polytene chromosomes. Taken together, the in vitro and in vivo data suggest that the Scm and ph proteins participate in PEG complexes as direct binding partners.

MATERIALS AND METHODS

Generation of yeast two-hybrid constructs. Two-hybrid fusion protein constructs were made with the pEG202 bait and pG4-5 prey vectors (20, 25) as follows. Scm constructs were derived from the S9 cDNA (7), and ph constructs were derived from the c4-11 cDNA (13), which corresponds to the proximal of the two ph genomic copies (12).

(i) lexScm767–877. For construct lexScm767–877, a 0.6-kb NgoMI-Ncol cDNA fragment was inserted into the Smal site of pBluescript KS+ (Stratagene). A 0.6-kb EcoRI-Ncol fragment was isolated from this clone and inserted into pEG202.

(ii) ACTScm767–877. For construct ACTScm767–877, a 0.6-kb EcoRI Xhol fragment from lexScm767–877 was inserted into pG4-5.

(iii) lexScm797–877. For construct lexScm797–877, PCR was used to create a 0.5-kb fragment, spanning the SPM domain and part of the adjacent 3’ untranslated region. This fragment, which contains a primer-derived EcoRI site preceding codon 797, was digested with EcoRI and NotI and inserted into pBluescript KS+ to create pmSphM. The same EcoRI-NotI fragment was then inserted into pEG202.

(iv) lexScm797–877. For construct lexScm1–877, PCR was used to create a 0.5-kb fragment spanning the N-terminal Scm coding region. This fragment, which contains a primer-derived EcoRI site immediately preceding the start codon, was inserted into pBluescript. The remainder of the Scm coding region was then inserted as a 2.8-kb NotI-SacI fragment to construct pRS3.1, which contains the complete open reading frame. A 2.9-kb EcoRI-NotI fragment, derived from this reconstructed clone, was then inserted into pEG202.

(v) ACTScm1–877. For construct ACTScm1–877, a 2.4-kb EcoRI Clal fragment was isolated from pRS3.1 and was inserted into EcoRI-ClaI cut ACTScm767–877.

(vi) lexScm1–797. For construct lexScm1–797, PCR was used to create a 150-bp BglII-SacI fragment spanning Scm amino acids 746 to 797. The PCR introduced a TAA stop codon and Xhol and SacI sites immediately after amino acid 797. This fragment was then inserted into BglII-SacI cut pRS3.1 to create pRS3.1 Scm, which contains the open reading frame. A 2.9-kb EcoRI-NotI fragment, derived from this reconstructed clone, was then inserted into pEG202.

(vii) ACTScm1–797. For construct ACTScm1–797, the same 1.3-kb Xhol fragment described above was inserted into Xhol-cut ACTScm1–877.

(viii) ACTph1–1589. For construct ACTph1–1589, PCR was used to create a 250-bp EcoRI Xhol fragment spanning the ph N terminus and containing a primer-derived EcoRI site just upstream of the start codon. This fragment was then used to replace the N-terminal EcoRI Xhol fragment in the c4-11 DNA. A 4.7-kb EcoRI-BamHI fragment spanning amino acids 1 to 1586 was isolated from this clone and inserted into pEG202 to create lexph1–1586. A 0.5-kb BamHI fragment containing the C-terminal three ph codons and flanking 3’ UTR sequence was inserted into BamHI cut lexph1–1586 to make lexph1–1589.

A 5.2-kb EcoRI fragment containing the complete ph coding region was isolated from this clone and inserted into pG4-5.

(ix) ACTph1–1418. For construct ACTph1–1418, lexph1–1586 was cut with NotI, which cleaves at codon 1418 and at a downstream polylinker site. After ligation, which creates a clone with a C-terminally truncated ph fusion protein, a 4.2-kb EcoRI Xhol fragment was isolated and inserted into pG4-5.

(x) ACTph1–522. For ACTph1–522, lexph1–1586 was cut with Sall, which cleaves at codon 522 and at a downstream polylinker site. After ligation, a 1.6-kb EcoRI Xhol fragment was isolated and inserted into pG4-5.

(xi) ACTph1297–1589. For ACTph1297–1589, PCR was used to generate a 0.2-kb fragment containing EcoRI and BamHI sites upstream of amino acids 1297 and 1577, respectively. This EcoRI-BamHI fragment was inserted into pEG202 to create lexph1297–1577. This clone was cut with Xhol, and a 1.2-kb Xhol fragment from cDNA c4-11, containing the ph C terminus, was inserted to create lexph1297–1589. A 1.3-kb EcoRI fragment was then isolated from this clone and inserted into pG4-5.

PCR amplifications were performed with Vent polymerase (New England Biolabs). All DNA segments included in these constructs that resulted from PCR amplification were sequenced by dideoxy chain termination to verify wild-type Scm and ph sequences.

Yeast two-hybrid tests. Yeast strains for two-hybrid tests were constructed from the base strain EGY48 (MATa his3-1 leu2-3,112 ade2-101 LEU2) (20). Plasmids were introduced into yeast by lithium acetate transformation (27) with 1 to 5 µg of plasmid DNA in 45% polyethylene glycol–0.1 M LiAc–10 mM Tris (pH 8)–1 mM EDTA as the transformation buffer. Two-hybrid tests were performed by patching or streaking strains onto indicator plates supplemented with 2% galactose (20% raffinose) and ph fusion protein expression. Activation of the chromosomal lacZ reporter in the absence of a prey protein. Each bait protein also did not repress lacZ from the reporter pkJ100 (20), which has LexA binding sites upstream of UASgal.

Generation of GST fusion constructs. Glutathione-S-transferase (GST)-Scm and GST-ph fusion protein constructs were generated with the pGEX series of vectors (Pharmacia) as follows:

(i) GSTScm1–797. For construct GSTScm1–797, a 2.7-kb EcoRI-Ncol fragment, containing open reading frame of the silencing domain, was isolated from pRS3.1 (see above) and inserted into the Smal site of pGEX-3X.

(ii) GSTScm1–797. For construct GSTScm1–797, a 2.4-kb EcoRI-SacI fragment was isolated from pRS536 (see above) and was inserted into the Smal site of pGEX-3X.

(iii) GSTScm1–797. For construct GSTScm1–797, a 0.4-kb EcoRI-MscI fragment was isolated from pMinSphM (see above) and was inserted into the Smal site of pGEX-3X.

(iv) GSTph1511–1576. For GSTph1511–1576, PCR was used to generate a 0.2-kb ph cDNA fragment containing a primer-derived EcoRI site immediately upstream of residue 1511 and a primer-derived TGA stop codon and Xhol site immediately after residue 1576. The resulting EcoRI-Xhol fragment was inserted into pGEX-4T-1.

Production of GST fusion proteins. Cultures of Escherichia coli DH5α harboring the fusion constructs described above were grown at 37°C in LB broth plus 200 µg of ampicillin per ml. When cultures reached an optical density of 0.550 of 1, fusion protein expression was induced by addition of 0.1 mM IPTG (iso-propyl-β-D-thiogalactopyranosidase). Unfused GST control protein was induced in cells containing pGEX-2T. After 45 min of further incubation at 37°C, cells were collected by centrifugation, washed once with 10% sucrose–20 mM Tris (pH 8.0)–25 mM EDTA, and resuspended in lysis buffer (10% sucrose, 40 mM Tris [pH 7.5], 0.2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride). The cell suspensions were flash frozen in liquid nitrogen, thawed, and then treated with 0.4 mg of lysozyme per ml for 1 h on ice. The cell lysates were sonicated twice for 30 s and then subjected to an additional freeze-thaw cycle. Insoluble material was pelleted by centrifugation for 30 min at 15,000 × g. After collection of the supernatant, the pellet was resuspended in 0.5 M and sodium dodecyl sulfate (SDS) to 0.03% SDs-polyacrylamide gel electrophoresis (PAGE) analysis of the soluble and insoluble fractions showed that greater than 75% of the smaller fusion proteins (GST, GSTScm1–797, and GSTph1511–1576) was recovered from the insoluble fraction. The larger GSTScm1–877 and GSTScm1–797 proteins was distributed in the soluble fraction.

GST fusion proteins were bound to glutathione-agarose beads (Sigma) as follows:...
Site-directed mutations in Scm were made by one- or two-step PCR strategies. These strategies used a wild-type forward end primer, 5'-GGCGGAATTCAGCGTCGAT-3' (for the K49A mutation). Clones containing the desired mutations were identified by DNA sequencing. A 0.2-kb PCR fragment was each inserted as a RI site adjacent to ph codon 1576 followed by an engineered RI site. The W1A mutation was constructed by one-step PCR with the forward end primer 5'-CGCTCGAGTACCTATGAGCCGTCGAT-3' and the wild-type reverse end primer. The 163D mutation was made by one-step PCR with the wild-type forward end primer and the reverse end primer 5'-CG CCTTGGAGTCATCTGAGCCGTCGAT-3' and 5'-GGCCCAAGGGGCTCTGGTCTGAAGGAG-3'. The L34A, L42A, and 531A mutations were made with specific forward and reverse primers containing the mutations. Each mutant primer was first used in a PCR with the appropriate wild-type forward or reverse end primer to generate two PCR products that overlap at the site of the mutation. Pairing of products were gel purified, combined, and used as a template in a second PCR with the wild-type forward and reverse end primers. The mutagenic primers used to make these three mutations are as follows: L34A, 5'-GGCCCAAGGGGCTCTGGTCTGAAGGAG-3' and 5'-GAGCCACAGAAGCCGTCCTGGTCTGAAGGAG-3'; L42A, 5'-GTTCAAGGAGAAGCCGTCCTGGTCTGAAGGAG-3' and 5'-GGCCCAAGGGGCTCTGGTCTGAAGGAG-3'; and G51A, 5'-GGCGGAATTCAGCGTCGAT-3' and G51A, 5'-GGCGGAATTCAGCGTCGAT-3'. The mutant 0.2 kb PCR products were each inserted as EcoRI-Xhol fragments into pGEX-4T-1 for use in binding assays.

Immunostaining of polytene chromosomes. Affinity-purified rabbit polyclonal Scm antibody was generated with a GST fusion protein encompassing Scm amino acids 323 to 877 as an immunogen. The production and characterization of this antibody will be presented elsewhere (7b). The specificity of the antibody for Scm protein is demonstrated by (i) detection of a single, major band of approximately the expected molecular mass (95 kDa) on Western blots of embryo extracts, (ii) elimination of this reacting species in extracts from Scm null mutant embryos, and (iii) depletion experiments that show complete loss of chromosome staining after preincubation of the antibody with the Scm immunogen. In parallel depletion experiments, chromosome immunostaining is retained after preincubation with fusion proteins containing the SPAM domain from either Scm (GSTScm767-1576) or pGSt1511-1576). The affinity-purified rabbit polyclonal antibody used for chromosome staining has been described previously (13, 19).

Polytene chromosomes were isolated from third instar larval prior to spiracle evisceration. Immunostaining with Scm antibody was performed with a modified version of protocols described previously (47, 62). Chromosomes were fixed in 45% acetic acid-1% formaldehyde for 2 to 4 min. After chromosome spreading and unmounting, the slides were washed in phosphate-buffered saline (PBS: 0.15 M NaCl, 10 mM NaPi [pH 7.2]) and then incubated in blocking buffer (PBS plus 5% BSA, 5% nonfat dry milk, 0.4% Tween 20) for 30 min at room temperature. Slides were incubated with anti-Scm antibody (1/50) in blocking buffer overnight at 4°C. Signals were developed with the secondary antibody and HRP detection system from the Vectastain ABC kit (Vector Laboratories). The signal was intensified by addition of 0.008% NBT and 0.008% CoCl2 to the HRP reaction mixture. Immunostaining of chromosomes with ph antibody was performed as described previously (13).

RESULTS

Tests for Scm protein self-binding in the two-hybrid system. A series of two-hybrid fusion protein constructs were generated with the LexA DNA binding domain in the vector pEG202 and the acidic activation domain in the vector pG4-5 (20, 25). Results of selected pairwise tests on X-Gal indicator plates are shown in Fig. 1, and a compilation of the two-hybrid data is presented in Fig. 2. The coding region for a C-terminal portion of the Scm protein was inserted into the bait and prey vectors to create constructs lexScm767-877 and ACTScm767-877, respectively. This portion of Scm consists of 65 amino acids of the SPAM domain plus 45 amino acids of the region immediately upstream. As shown in Fig. 1, a yeast strain harboring both of these plasmids and a lexAop-lacZ reporter gene pro-
The SPM homology domain spans amino acids 806 to 870 in Scm protein (7). To more precisely test whether self-interaction is mediated by the SPM domain, we pared down the LexA fusion protein to contain only Scm amino acids 797 to 877. As shown in Fig. 1, lexScm797–877 also produces a strong positive signal in the two-hybrid test. Additional constructs were generated to assess the role of the SPM domain in the context of full-length Scm protein (Fig. 2). Figure 1 shows that the isolated SPM domain interacts with full-length Scm protein and that full-length Scm protein also contains full-length Scm lacking only the SPM domain. Two-hybrid tests of these truncated proteins against either full-length Scm or isolated SPM domain do not show interaction (Fig. 1 and 2). Taken together, the two-hybrid data show that Scm protein is capable of self-interaction and they suggest that the SPM domain is necessary and sufficient for this interaction.

**Tests for Scm-ph interaction in the two-hybrid system.** The SPM domain in polyhomeotic protein spans amino acids 1513 to 1576 at the extreme C terminus of the protein (7, 13). To test for cross-interaction between Scm and ph through their C-terminal regions, the construct ACTph1297–1589 (Fig. 2) was generated. Figure 1 shows that there is a strong positive signal when ACTph1297–1589 is tested for interaction with lexScm767–877. In addition, lexScm767–877 interacts with full-length ph protein, and it fails to interact with deletion derivatives of ph protein that lack the C terminus (Fig. 1 and 2). These data, as well as additional tests summarized in Fig. 2, are consistent with physical interaction mediated through the respective SPM domains of the Scm and ph proteins.

In contrast to the strong interactions observed with many Scm-ph combinations (Fig. 1 and 2), we found that there was little or no signal when the two full-length proteins were tested for interaction on X-Gal indicator plates. This result could not be easily explained by failure of production or nuclear entry of either full-length protein, since each showed a strong interaction when tested against smaller Scm-containing fusion proteins (Fig. 2). We considered whether this result might reflect the inability of an Scm-ph multimer, with a minimum molecular mass of about 300 kDa, to specifically activate the lacZ reporter used in these assays. Examples of promoter bias exhibited by particular bait-prey combinations in the two-hybrid system have been reported (16). To address this possibility, we tested the full-length lexScm1–877 and ACTph1–1589 fusion constructs for activation of a lexAop-LEU2 reporter. This LEU2 reporter contains a different promoter and it is integrated into the yeast chromosome rather than present on a high-copy plasmid (20). We found that this full-length protein combination does activate the LEU2 reporter as measured by growth on LEU– media (data not shown). In addition, the reciprocal full-length bait-prey combination, with lexph1–1589 and ACTScm1–877, also yielded growth on LEU– media. Growth was galactose dependent in these tests, indicating that it required expression of the respective prey proteins. We suggest that the full-length Scm and ph proteins do interact in yeast but that the resulting fusion protein complex is not configured for efficient activation of the lacZ reporter. The full-length Scm-ph protein interaction was further tested with an in vitro protein binding assay (see below).

In addition to ph interaction, Scm protein was tested for possible interaction with several other PcG proteins in the two-hybrid system. We found that both full-length and C-terminal Scm fusion proteins fail to interact with the Pc, esc, Psc, or Su(z)2 proteins (data not shown).

**Scm and ph protein interactions in vitro.** The two-hybrid data provide evidence for specific interactions between domains of the Scm and ph proteins. However, we cannot exclude the possibility that endogenous yeast proteins contribute to the interactions detected in the system. We also wished to perform independent tests of the full-length protein interactions, since the two-hybrid signals with these larger proteins were among the weakest detected. Consequently, we tested for direct interactions by using an in vitro protein binding assay: GST pull-
down assays (58). Briefly, 35S-radiolabelled proteins were produced by in vitro translation and then tested for binding to GST fusion proteins purified from *E. coli* and immobilized on glutathione-agarose beads (see Materials and Methods).

We first tested the ability of the isolated SPM domain to interact with itself in vitro. The minimal SPM domain from Scm (amino acids 797 to 877) was synthesized in radiolabelled form and tested for binding to GST fusion proteins containing the minimal domain from either Scm or ph (amino acids 1511 to 1576). The pulldown assay data shown in Fig. 3 and subsequent figures consist of results from supernatant (S) samples, which contain a fraction of the unbound material, and pellet (P) samples, which correspond to the bound material. Figure 3A shows that the minimal SPM domain does not bind to the negative control, GST alone (lane 2). However, there is substantial binding to GST fusion proteins containing the minimal domain from either Scm or ph (lanes 8 and 10). Since the GSTScm797–877 and GSTph1511–1576 bead preparations contain equivalent amounts of protein, it appears that the isolated SPM domain from Scm binds about equally well to itself and to the SPM domain from ph. Figure 3A also shows that this isolated SPM domain binds to full-length Scm-GST (lane 4) and that the signal is reduced to background levels if the SPM domain is specifically deleted from the full-length protein (lane 6).

Figure 3B shows the result for self-binding with the isolated SPM domain from ph. We find that there is ph-ph binding activity (lane 4), but that it is dramatically weaker than the Scm-Scm or Scm-ph isolated domain interaction (Fig. 3A). As a further comparison, the same radiolabelled ph1511–1576 protein preparation used in self-binding (Fig. 3B) was tested for cross-binding to GSTScm797–877. This test, shown in Fig. 3B, lane 6, is similar to the test shown in Fig. 3A, lane 10, except the radiolabelled and GST forms of the ph and Scm partners have been reversed. The side-by-side comparison (Fig. 3B, lanes 4 and 6) confirms the dramatic difference in strengths of the ph-ph and Scm-ph domain interactions. This comparison also shows that the relatively weak ph-ph self-interaction is not due to some technical problem with the folding or behavior of the ph fusion proteins used. Rather, we suggest that the qualitative difference in ph-ph and Scm-ph binding affinities reflects intrinsic properties of the different versions of the SPM domain.

We next tested for binding interactions using the Scm and ph proteins in their respective full-length forms. Figure 4A shows that radiolabelled Scm1–877 binds to GSTScm1–877 and to the minimal SPM domain in GSTScm797–877, but not to GST alone. Similarly, radiolabelled ph1–1589 binds to full-length Scm attached to beads (Fig. 4B, lane 4) and to the minimal domain (lane 6). Thus, although the Scm-ph full-length protein interaction was not detected with the *lacZ* reporter in the two-hybrid system (Fig. 2), these tests confirm that Scm-ph binding does occur between the full-length proteins.

The contribution made by the SPM domains to the full-length protein interactions was assessed in the experiment shown in Fig. 4C. In this experiment, deletion derivatives of ph (ph1–1417) and Scm (Scm1–797) that specifically lack the SPM domains were used. In contrast to the binding seen between full-length radiolabelled ph and GST-Scm (lane 2), binding is greatly reduced or eliminated when the SPM domain is specifically removed from either or both proteins tested (lanes 4, 6, and 8). In summary, these results show that the SPM domain is sufficient for binding interactions in vitro and that the domain is required in both partners to mediate the Scm-ph full-length protein interaction.
Effects of SPM domain point mutations upon binding in vitro. To begin to investigate the mechanism and specific residues used for SPM domain protein contact, we tested the effects of site-directed mutations in either the Scm or ph domains (see Fig. 6) upon binding in vitro. The mutations were targeted at residues that are highly conserved in alignments of proteins with similar domains. Our point mutations (Fig. 6) fall into two classes: those that target conserved residues in the extended SAM domain family (1, 46) and those that target residues conserved only in the high-homology SPM subgroup.

Three site-directed mutations were generated in the SPM domain of Scm (Fig. 6). The G31S mutation alters a residue that is absolutely conserved in all 23 compiled versions in the extended domain family (1, 46). This mutant was tested in the context of radiolabelled full-length Scm protein for binding to the minimal Scm and ph domains. Figure 5A shows that both Scm-Scm and Scm-ph interactions are greatly reduced in vitro. Consistent with the residual binding activity seen in the G31S lanes, we find that this mutant also mediates a reduced but still detectable interaction in the two-hybrid system (data not shown).

The L35S L36S double mutation and the K49A mutation affect residues conserved in the high-homology subgroup but not in the extended domain family. Figure 5A shows that both Scm-Scm and Scm-ph interactions are greatly reduced in vitro. Consistent with the residual binding activity seen in the G31S lanes, we find that this mutant also mediates a reduced but still detectable interaction in the two-hybrid system (data not shown).

Five site-directed mutations were generated in the SPM domain of ph (Fig. 6). All five mutations alter residues that are highly conserved in the extended domain family. These mutations were inserted into the context of the minimal GST-ph151-1576 fusion protein and then tested for binding to the minimal Scm radiolabelled domain. Figure 5B shows that the W1A and G51A ph mutations cause significant reductions in binding activity to Scm. In contrast, Fig. 5B shows that mutations in the conserved hydrophobic residues, L34A, L42A, and I63D, have little effect upon in vitro Scm-ph interaction.

Colocalization of ph and Scm at chromosomal sites. The two-hybrid and GST pulldown assays show that the Scm and ph proteins can bind each other directly and that their respective SPM domains mediate qualitatively strong interactions. However, these experiments do not address whether the Scm and ph proteins are partners at sites of action in vivo. To assess association in vivo, we compared the Scm and ph distributions on wild-type polytene chromosomes. In addition, we tested for colocalization at an engineered chromosomal site containing an isolated segment of homeotic gene regulatory DNA.

FIG. 5. In vitro binding with Scm and ph mutant proteins. (A) Binding reactions were performed with minimal GST-Scm (lanes 1 to 8) or minimal GST-ph (lanes 9 to 16) fusion proteins. Radiolabelled protein was full-length wild-type Scm or full-length Scm containing the indicated amino acid substitutions. (B) Binding reactions were performed with either wild-type minimal GST-ph fusion protein (lanes 1 and 2) or minimal GST-ph fusion proteins harboring the indicated amino acid substitutions (lanes 3 to 12). Radiolabelled protein was wild-type Scm1-877. The weak signals in the supernatant lanes in panel B reflect the large percentage of radiolabelled protein that binds and is recovered in the pellet sample in the minimal Scm-minimal ph binding reaction. Longer exposure of the gel in panel B (not shown) reveals intact radiolabelled protein in the supernatant lanes as in Fig. 5A, lane 9.

FIG. 6. Mutations in the SPM domains. Alignments of the primary amino acid sequences of the SPM domains from Scm (top) and ph (bottom) are shown. Arrows indicate site-directed mutations analyzed in this work. Scm mutations are shown above the sequence, and ph mutations are indicated below the sequence. Boldface letters indicate amino acid positions that are highly conserved in the extended SAM domain family (1, 46). The positions and extents of potential α-helices in Scm derived from secondary structure prediction with the PHDsec program (48) are shown.
Polytene chromosome immunostaining experiments have shown that ph protein accumulates at its two most well-characterized target loci, the Antennapedia (ANT-C) and bithorax (BX-C) homeotic gene complexes (13, 19). In addition, ph protein is associated with approximately 100 other sites in the genome. Figure 7A shows immunolocalization of ph protein at the BX-C site as well as at five flanking sites on chromosome 3R. Figure 7B shows the same section of chromosome stained with antibody against Scm protein. There is strong signal at the BX-C locus, and the Scm distribution on flanking sites is identical to the ph distribution. Comparison of Fig. 7C and D shows that the ph and Scm protein distributions in the ANT-C region are also identical. Since the antibodies used in these studies are both rabbit polyclonal antibodies, we did not perform double-staining experiments to determine if all approximately 100 ph and Scm sites are identical. However, comparison of the Scm sites on the five major chromosome arms with the ph sites described previously (13) indicates that there is at least 90% overlap in the distributions of these two proteins on polytene chromosomes.

To compare ph and Scm association with an additional site of action in vivo, we tested for colocalization at a site containing regulatory DNA isolated from a homeotic gene. The germ line transformant, 85-39, contains a 14-kb segment from the bxd regulatory region of the BX-C complex (54) inserted near the tip of chromosome 3L at cytological location 62A. Previous work has shown that this transformed DNA segment creates a novel site of ph protein accumulation (13) and that expression programmed by this 14-kb DNA segment is regulated by ph and Scm in vivo (54). Figure 7F shows that Scm protein accumulates at the insertion site of this bxd regulatory DNA. Thus, the Scm and ph proteins are both recruited to an engineered chromosomal site containing an in vivo regulatory target. This result, together with the coincidence of the ph and Scm proteins at many wild-type chromosomal sites, provides evidence for association of these proteins in vivo.

**DISCUSSION**

**The SPM domain mediates protein-protein interaction.** The shared SPM domains in the Scm and ph proteins (7) comprise a potential common link in the biochemical roles of these two proteins in PcG repression. The present data, from both two-hybrid and in vitro assays, show that the SPM domain is used for protein-protein contact. Our studies with minimal constructs of either Scm or ph, encoding as little as 65 to 70 amino acids, show that the isolated domain is sufficient for protein interaction. Although these binding assays do not allow direct determination of binding constants, the signals observed suggest that the domain mediates a moderate- to high-affinity interaction (16).

The isolated SPM domain from Scm binds both to itself and to the domain from ph (Fig. 3). Thus, this domain is designed both for self-recognition and for heterotypic interaction with a related partner. These properties are reminiscent of the HLH domains in transcription factors (see reference 40 for review), which mediate both self- and cross-interactions among related family members. However, there are functional and predicted structural differences between these two classes of self-binding domains. The HLH proteins typically contain a basic region located adjacent to the HLH region that provides sequence-specific DNA-binding activity. The Scm and ph proteins lack an adjacent basic region, and in vitro tests have so far failed to reveal sequence-specific binding activity for either protein (8a). From a structural standpoint, HLH domains contain two major \( \alpha \)-helices separated by a loop (15, 17), whereas secondary structure predictions suggest that the SPM domain and the extended SAM domain family (1, 46) consist of a series of three to five \( \alpha \)-helices.

The site-directed mutations shown in Fig. 6 were constructed to identify residues important for the fold or function of the SPM domain. Figure 6 also depicts the locations and extents of four putative \( \alpha \)-helices that are derived from secondary structure prediction (48). Although a three-dimen-
of protein domains is not yet available, the effects of site-directed mutations can be interpreted in the context of a predicted multihelical bundle with the locations and extents of helices as shown. Severe reductions in binding activity are seen with two glycine substitutions, G31S in Scm and G51A in ph. Given their location bordering predicted helices, these G residues may play structural roles in promoting tight turns that connect these helices. Alternatively, they may lie in regions where the absence of side chains is needed to prevent steric conflicts. The absolute conservation of the G31 residue in SAM domains from functionally diverse proteins (1, 46) is consistent with a role in the basic fold of the domain. We also found that mutation of another absolutely conserved residue, W1, causes reduced binding in vitro (Fig. 5). This hydrophobic residue lies adjacent to the first predicted helix. In contrast, mutations in conserved, hydrophobic residues within the predicted α-helices (for example, the leucine residues in helix III) have little effect upon binding. A possible explanation for this result is that multiple hydrophobic interactions contribute to the fold or are used directly for partner contact and that elimination of single interactions in the context of many has little consequence. We note that none of our single residue mutations completely eliminates the strong Scm-Scm or Scm-ph domain interactions. These may also reflect the use of multiple contacts in the binding mechanism.

Biological role of related domains in Ets oncoproteins. Members of the Ets family of transcription factors, which include the human proteins TEL, ETS-1, and ERG-2 (see reference 59 for review) and the fly proteins YAN and PNT (32, 34), contain the SAM homology domain in their respective N-terminal regions. The homology domain in these proteins has also been variously termed the B domain (8), the pointed domain (32), or the HLH domain (23, 51). The most detailed information about this domain in Ets proteins has been derived from studies of the TEL oncoprotein.

The TEL gene was identified by its association with chromosome translocation breakpoints that lead to leukemia in humans (22, 23). A common property of these translocations is that they create novel fusion proteins that contain the N-terminal portion of TEL, including the SAM domain. Fusions of TEL to either of two tyrosine kinas, the platelet-derived growth factor receptor β or the ABL tyrosine kinase, have been described previously (21, 23). Studies of the TEL–platelet-derived growth factor receptor β and TEL–ABL fusions have shown that a key step in transformation is activation of the respective kinase activities by oligomerization of the fusion proteins (21, 29). These workers have shown that the self-binding is mediated specifically by the SAM homology domain from TEL. A third type of leukemia involves TEL fusion to the AML1 transcription factor (22). In this case, TEL-AML fusion converts the transcriptional activator into a repressor (26). This change requires the SAM domain and correlates with oligomerization of the TEL-AML fusion protein (38). Thus, the common link among the TEL fusion proteins is the SAM domain, which by triggering oligomerization alters the biochemical activities of the proteins and contributes to the disease state.

Differential binding specificities of the Scm and ph domains. Binding assays with isolated SPM domains show that the Scm-Scm and Scm-ph interactions are much stronger than the ph-ph interaction (Fig. 3). Although we have not precisely measured the difference in affinities, the fractions of radiolabeled proteins bound in these experiments suggest that the difference is at least 10-fold. Since these experiments use minimal domain fusions, and since the same minimal ph1511–1576 protein binds avidly to the domain from Scm, but weakly to itself, we conclude that the binding specificity is an intrinsic property of the domain. We suggest that specific amino acid residues that differ between the Scm and ph domains impart the interaction specificities. Further mutational studies will be needed to identify these specificity residues.

Studies of the SAM domains from TEL and related Ets-proteins also provide information about interaction specificities. Although the TEL domain mediates self-interaction, the comparable homology domains from ETS-1, ERG-2, and GAP43 lack self-binding activity in vitro (29). In addition, the ETS-1 protein has been reported to be monomeric in solution (29). Our Scm-ph binding studies establish that a single version of the domain can participate in both homotypic and heterotypic interactions. Taken together, these results show that specificity is built into different versions of the SAM domain so that only certain combinations can form oligomers.

Several families of transcription factors that share a common self-binding domain, such as the basic HLH leucine zipper (bHLH-LZ) domain (3, 6 [see reference 2 for review]) or the POZ (also known as BTB) domain (5), have been described. In these cases, differential binding between specific family members can have important consequences for regulation in vivo. For example, within the bHLH-LZ subfamily that includes Myc, Max, Mad, and Mxi-1, the Myc/Max heterodimer functions as a transcriptional activator (3), whereas Mad/Max and Mxi-1/Max dimers contribute to repression (4, 50). Thus, the distribution of Max into different heterodimeric forms serves as a control point for the biological effects of this group of proteins. It will be important to identify forms of Scm-ph oligomers that occur in vivo and to determine how SPM domain binding specificity influences formation or function of these oligomers. We note that the number of possible oligomeric forms is increased by the presence of distinct ph proteins encoded by the two gene copies in Drosophila (12).

ph and Scm function and PcG multimeric complexes. Cytological and biochemical studies suggest that the fly ph protein works together in multimeric protein complexes with other PcG proteins in embryonic extracts (1). These ph data, together with our ph-Scm binding studies, suggest that direct contact between the ph and Scm proteins contributes to the formation and/or function of PcG protein complexes. In addition, the co-localization of Scm and ph on polytene chromosmes (Fig. 7) provides evidence for association of these proteins at sites of action in vivo.

The ability of the SPM domain to mediate both homo- and hetero-oligomerization of these proteins could supply a variety of contacts used in assembly or regulation of PcG complexes. The specific removal of this domain in the cases of Xf24D1 alleles provides evidence for association of these proteins at sites of action in vivo.

Several of the other highly conserved domains in PcG proteins have also been implicated in protein-protein contacts that contribute to complex formation (see reference 52 for review). For example, the chromodomain from Pc protein is required for its own distribution in chromatin (39) and for the distribution of ph protein (18). In addition, the Pc chromodomain is sufficient to target other PcG proteins to ectopic chromatin
PcG proteins shows that many of the components that control fly homologs, ph and Psc (33). The remarkable conservation of PcG protein (1), and similar interactions occur between their constituents and their stoichiometries are not known. Further biochemical studies will be needed to assess if and how Scm-ph provide potential sites for interactions with other PcG proteins, much about the biochemical nature of multi-partners remains to be determined.

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