Structure of the Sterile α Motif (SAM) Domain of the *Saccharomyces cerevisiae* Mitogen-activated Protein Kinase Pathway-modulating Protein STE50 and Analysis of Its Interaction with the STE11 SAM

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The sterile α motif (SAM) is a 65–70-amino acid domain found in over 300 proteins that are involved in either signal transduction or transcriptional activation and repression. SAM domains have been shown to mediate both homodimerization and heterodimerization and in some cases oligomerization. Here, we present the solution structure of the SAM domain of the *Saccharomyces cerevisiae* protein, Ste50p. Ste50p functions as a modulator of the mitogen-activated protein kinase (MAPK) cascades in *S. cerevisiae*, which control mating, pseudohyphal growth, and osmo-tolerance. This is the first example of the structure of a SAM domain from a MAPK module protein. We have studied the associative behavior of Ste50p SAM in solution and shown that it is monomeric. We have examined the SAM domain from Ste11p, the MAPK kinase that associates with Ste50p in vivo, and shown that it forms dimers with a self-association constant of ~0.5 mM. We have also analyzed the interaction of Ste50p SAM with Ste11p SAM and the effects of mutations at Val-37, Asp-38, Pro-71, Leu-73, Leu-75, and Met-99 of Ste50p on the heterodimerization properties of Ste50p SAM. We have found that L73A and L75A abrogate the Ste50p interaction with Ste11p, and we compare these data with the known interaction sites defined for other SAM domain interactions.

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules found across the eu-karyota. A typical MAPK module consists of three levels of protein kinases, a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) that transduce signals via sequential activation of these kinases by phosphorylation. *Saccharomyces cerevisiae* has five discrete MAPK modules that transduce extracellular stimuli, e.g. mating pheromone, nitrogen starvation, hyperosmolarity, perturbations in cell wall integrity, and signals that control the synthesis of spore walls (1). The mating pheromone response controls conjugation and mating. The pheromone binds to a receptor (Ste2p or Ste3p) and activates a heterotrimeric G protein (Gα, Gβ, Gγ, and Ste6p, and Gγ, Ste18p). The Gβγ dimer is responsible for transducing the signal to the MAPKKK Ste11p via a path that involves Ste50p, Ste20p (a PKA-related kinase), and Ste5p (a scaffold protein). The MAPKKK Ste11p then phosphorylates the MAPKK Ste7p, which goes on to activate the MAPK, Fus3p (2). Activation of the transcription factor Ste12p then leads to the transcription of pheromone inducible genes, e.g. *FUS1*. Ste50p is thought to be a modulating protein that is found constitutively bound to Ste11p and that functions to sustain and prolong the pheromone-induced signaling response (3).

The formation of pseudohyphae and invasive growth in response to nitrogen starvation also involves a MAPK cascade involving Ste11p and Ste50p (4). Starvation signals are routed through Ras2p, which, acting via Cdc42p (a Rho family small G protein), Ste20p and Ste5p, activates Ste11p. In this cascade the MAPKKK is Ste7p, and the MAPK is Kss1p. Kss1p then goes on to activate the Tec1p-Stec1p2 transcription complex, which in conjunction with Flo8p co-transactivates genes, e.g. *FLO11*, that are essential for pseudohypha formation.

The response to osmotic stress involves two MAPK cascades. The osmosensor Snl1p/Ypd1/Skk1p activates the MAPKKKs Ssk2p and Ssk22p that activate the MAPKK Pbs2p and finally the MAPK Hog1p. The Sho1p osmosensor on the other hand activates Ste11p with the cooperation of Ste50p, which then activates Pbs2p and Hog1p. Thus, Pbs2p/Hog1p can be activated independently by two separate osmotic sensors. Although Ste50p was originally identified as a modulator of the pheromone response pathway in *S. cerevisiae* (5), it has since been shown to have only a moderate effect on mating. It has, however, been demonstrated that Ste50p is absolutely required for the Sho1p/Ste11p control of the HOG (high osmolarity glycerol) pathway and that Ste50p may be an integral part of the Ste11p MAPKKK (6).

The interaction between Ste50p and Ste11p is mediated by the N-terminal regions of these proteins (6), each of which includes a sterile α motif (SAM) domain. The SAM domain was first identified as a 65–70-residue domain found in 14 proteins including Ste50p, Ste11p, and their *Schizosaccharomyces pombe* homologues, Ste4 and Byr2 (7). Refined searches iden-
tified 60 further SAM domain-containing proteins (8) including the Ehp family of receptor tyrosine kinases. SAM domains are now known to exist in over 300 proteins. The protein set that contains SAM domains suggested that the SAM domain is a conserved protein-protein interaction domain found in proteins involved in development and signal transduction. Extensive work on many SAM-containing proteins has shown that the SAM domains do indeed mediate homodimerization and heterodimerization. Proteins known to dimerize through SAM domain interactions now include not only receptors such as the Ehp family but also many transcription factors, e.g., the Ets family (9), where the domain is known as the pointed (Pnt) domain, the polyhomeotic proteins, and the p63/p73 members of the p53 family of tumor suppressor proteins (10). SAM domains have also been shown to bind other non-SAM proteins and protein motifs, e.g., protein-tyrosine phosphatases (11, 12), PDZ domains (13), and SH2 domains (14).

Ste11p appears to be under complex regulatory control. The protein has an N-terminal SAM domain (residues 14–86) that binds to the Ste50p SAM domain (residues 27–109). Residues 133–335 of Ste11p have two different functions: to negatively regulate the C-terminal kinase domain and to mediate the interaction between Ste11p and Ste50p (15). Binding of Ste50p SAM to the Ste11p SAM appears to alleviate the negative intramolecular regulation of residues 133–335 on the Ste11p kinase domain. It is tempting to speculate that binding of the SAM domain induces structural changes in the regulatory domain of Ste11p allowing activation of the kinase domain. However, it appears that Ste11p and Ste50p are constitutively bound in vivo, so binding per se, although necessary for activation of Ste11p, is not sufficient (15). In fact, it seems that a C-terminal region of Ste50p is also required for a productive interaction with Ste11p. Although this C-terminal region has no identifiable domains, it is notable that it has a high degree of homology to the C-terminal region of its homologue in S. pombe Ste4. This region in Ste4 is also required for correct function (16). It seems that Ste11p undergoes different levels of regulation in the distinct pathways in which it is involved. In the HOG pathway Ste50p is absolutely required and is sufficient to activate Ste11p. However, in the mating response where Ste50p is important but not essential, activation of Ste11p requires interactions with both Ste50p and Ste55p (15).

Here we present the structure of the SAM domain from the STE50 protein, the first SAM domain structure to be solved for a protein functionally involved in a MAPK cascade. Despite low sequence similarity to the other SAM domains whose structures have been solved (9, 17–23), the structure of Ste50p SAM shows a similar fold to these proteins. The self-association of the Ste50p SAM domain has been investigated using analytical ultracentrifugation and gel filtration, which show that the domain from Ste50p is monomeric; this is supported by the NMR data. The SAM domain from the S. cerevisiae MAPKKK Ste11p by contrast is shown to be dimeric. The Ste50p SAM domain is shown to bind to the Ste11p SAM domain in an in vitro assay, and two separate mutations in the Ste50p SAM domain were found that inhibit this interaction. We examine these results in light of the proposed function of the Ste50p SAM domain as a hetero-oligomerization motif.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The SAM domains from Ste50p (residues 27–108) and Ste11p (residues 14–86) were amplified by PCR from a preparation of genomic yeast DNA. Restriction enzyme sites were added into the PCR primers used to facilitate cloning into expression vectors. Primer sequences used were: Ste50p SAM, 5’-CGCGATATCGCATGTTGAGAAAAAGAC-3’ (forward) and 5’-CGCGAATTCGGAATCGCAGTACCACTCATCTCTTG-3’ (reverse); and Ste50p SAM, 5’-CGCGGATATCGCATGTTGAGAAAAAGAC-3’ (forward) and 5’-CGCGAATTCGGAATCGCAGTACCACTCATCTCTTG-3’ (reverse). Bold type indicates restriction endonuclease sites. Ste50p SAM and Ste11p SAM were expressed in E. coli (BL21) as GST fusion proteins using the pGEX-2T vector (Amerham Biosciences) and purified using glutathione agarose (Sigma) by standard protocols (Amerham Biosciences). The proteins were either used directly for pull-down assays or cleaved with thrombin restriction protease (Merck). Unlabeled and labeled SAM samples were prepared to 35 μM by growing the E. coli cells in 2TY or MOPS medium supplemented with 5% Cetone (Spectra Stable Isotopes), respectively. The SAM domains were then purified by size exclusion chromatography (16/80 S75; Amerham Biosciences). The protein samples for NMR spectroscopy consisted of ~1.0 mM protein in 16 mM Na2HPO4, 5 mM NaH2PO4, 10 mM NaCl, 5 mM EDTA, 50% D2O, 0.5 mM NaN3, pH 6.0, 0.5 M NaCl. The sample of Ste50p SAM was generated by mixing a 1:1 ratio of unlabeled:13C,15N-labeled protein and adding 8 μg guanidine hydrochloride to a final concentration of 6 M. The protein was then refolded by rapid dilution (50-fold) into NMR buffer and subjected to size exclusion chromatography to remove the residual guanidine hydrochloride. The sample was then concentrated to 500 μl (~1 mM protein) for NMR.

**NMR Spectroscopy**—NMR experiments were recorded at 35 °C on Bruker DRX spectrometers operating at 500 and 800 MHz, equipped with H/C/N triple resonance probes with actively shielded z-gradients. Finally, 100 structures were calculated, and the 25 with the lowest energy were selected for further analysis. Backbone φ angle restraints were measured using a method from an HNHA experiment. Backbone ψ angles were calculated from the backbone chemical shifts using the program TALOS (27) and included as loose restraints with errors of ±30° or twice the standard deviation, whichever was greater.

**Structure Calculation**—The Ste50p SAM domain structures were calculated iteratively using the programs ARIA 1.2 (25) and CNS 1.0 (26). Eight iterations were calculated, with 20 structures, iteration, of which seven were used for analysis of the ambiguous restraints. Finally, 100 structures were calculated, and the 25 with the lowest energy were selected for further analysis. Backbone φ angle restraints were measured using a method from an HNHA experiment. Backbone ψ angles were calculated from the backbone chemical shifts using the program TALOS (27) and included as loose restraints with errors of ±30° or twice the standard deviation, whichever was greater.

**Ste11p SAM-Ste50p SAM Titration**—A titration was performed by adding aliquots of unlabeled Ste50p SAM domain to 500 μl of 0.66 mM 15N-labeled Ste11p SAM and then concentrating the sample back to 500 μl. Ste50p SAM was added in 0.25, 0.5, 0.75, and 1.0 μl molar equivalents. An 15N HSQC and size exclusion chromatography were run at each titration point. Analytical gel filtration was performed on a SMART system using a Superdex 75 PC 3/20 column (Amerham Biosciences). A similar titration was performed by adding aliquots of unlabeled Ste50p SAM to 550 μl of 0.6 mM 15N-labeled Ste11p SAM and then concentrating the sample back to 550 μl. Unlabeled Ste50p SAM was added in 0.5, 1.0, 1.5, 2.0, and ~11 molar equivalents. An 15N HSQC and size exclusion chromatography were run at each titration point.

**Analytical Ultracentrifugation**—Sedimentation equilibrium analysis was performed using a Beckman Optima XL-I analytical ultracentrifuge. For both Ste11p SAM and Ste50p SAM, three samples were run simultaneously, with protein concentrations of 1, 2, and 3 mg/ml, respectively. The protein samples were 90 μl with 100 μl of reference buffer in the second sector of the two sector cells. The buffer used was identical to that used for the NMR experiments. All of the experiments were run at 4 °C. The data were collected at equilibrium for three to five cycles at angular velocities of 40,000, 50,000, and 60,000 rpm. Data collection was performed both by measuring absorbance at 280 nm and by using the Rayleigh interference optics. Because of problems arising from the differential oxidation of dithiothreitol in the sample and reference cells, all of the data analysis reported uses interference data. Data analysis was performed using WinNonlin (29).

**Site-directed Mutagenesis**—Site-directed mutagenesis of the pGEX-2T Ste50p SAM expression construct encoding residues 27–108 was performed using the QuikChange site-directed mutagenesis kit (Stratagene). The sequence of the Ste50p SAM coding region of all mutants was verified using an automated DNA sequencer (Applied Biosystems Inc.).

**GST Pull-down Assays**—To ensure that the concentration of the GST fusion was sufficient, ~0.5 mg of 50% glutathione agarose bead slurry was used to clear the fusion protein from 1 liter of culture cell extract. The fusion proteins were not digested with thrombin; instead the beads were washed a further three times with phosphate-buffered saline, 0.1% Triton. The concentration of the
visually from the strength of the bands on the SDS-PAGE gels to be at a concentration of the immobilized GST-SAM domains was estimated (Invitrogen). The supernatant was removed, and the beads were washed three times using 50% slurry of glutathione-agarose beads with a GST-SAM fusion protein bound was discarded if in conflict with restraints provided by the HNHA.

**RESULTS**

Structure Determination—Backbone resonances were assigned using standard three-dimensional HNCA, HN(CO)CA, HNCACB, and CBCA(CO)NH experiments. The spectra recorded were of sufficient quality to achieve a complete backbone assignment for all residues (27–108), with the exception of Val-66 and Leu-75, whose backbone amide resonances were missing. Side chain assignments were made using three-dimensional H(CC)(CO)NNH and (HCC)(CO)NNH experiments, along with a three-dimensional HCHC total correlation spectroscopy. The side chain assignment was completed for the same range of residues. Analysis of the $^{15}$N- and $^{13}$C-separated NOE spectra generated a total of 1,498 unambiguous and 791 ambiguous distance restraints. These were translated by ARIA into 1,013 unambiguous and 566 ambiguous, nondegenerate restraints. The final number of restraints, after eight ARIA iterations, was 1,354 unambiguous and 304 ambiguous (Table I). Assignment of the HNHA spectrum provided 35 torsion angle restraints, whereas analysis of the chemical shifts using the TALOS program gave a further 108 torsion angle restraints. The latter restraints were used loosely and discarded if in conflict with restraints provided by the HNHA.

In the case of the HNHA, the torsion angles were set to $\phi = -120 \pm 20^\circ$ for $^{3}$J$_{\text{NH}}$-H < 8.0 Hz and $\phi = -57 \pm 20^\circ$ for $^{3}$J$_{\text{NH}}$-H > 6.0 Hz.

No long range nuclear Overhauser effects were observed for residues 27–31 and 100–108, and the line widths for these resonances were considerably narrower than those in the remainder of the domain. These residues are therefore not structured and have been omitted from all figures and the structural statistics. The structure of residues 31–100 is well ordered and has good covalent geometry (Fig. 1 and Table I). There were no distance restraints violated by more than 0.1 Å and no dihedral restraints violated by greater than 5°.

**Ste50p SAM Domain Structure**—The Ste50p SAM consists of six helices, which form a compact, globular fold (Figs. 1 and 2). The N terminus forms a single turn of $3_{10}$ helix from residues 32–54 (helix A'). This leads into two short $\alpha$-helices, residues 37–46 (helix A) and 55–62 (helix B), followed by another short $\alpha$-helix at residues 75–81 (helix D), and the domain ends with a long C-terminal $\alpha$-helix comprising residues 86–101 (helix E). Helices A and B form an anti-parallel helical hairpin on one side of a helical bundle. The other side of the bundle is made up of helix E, which packs against helices C and D in an anti-parallel manner, with one half of the coil being made up of helices C and D, interrupted by the loop. The two sides of the bundle are approximately orthogonal. The first helix, helix A', is at the bottom of the bundle and packs in an anti-parallel fashion against the N-terminal half of helix C.

**The Ste50p SAM Domain Is a Monomer in Solution**—Because other SAM domains are known to form homodimers or higher order oligomers, we examined the Ste50p SAM domain for any evidence of homotypic interaction. NMR can be used to solve the structures of homodimers if a sample can be produced that contains a 1:1 mixture of monomer with different labeling patterns. We produced such a sample of Ste50p SAM, in which $6 \mu$ guanidine hydrochloride was used as a denaturant prior to mixing unlabeled and $^{13}$C,$^{15}$N-labeled proteins. The mixture was refolded by rapid dilution, and two NMR experiments were recorded to look for intermolecular interactions: a $^{13}$C-separated, $^{13}$C-rejected NOE experiment and a $^{15}$N,$^{13}$C-separated NOE recorded without carbon decoupling in the indirect dimension. In neither experiment was there any evidence for intermolecular nuclear Overhauser effects, suggesting that the Ste50p SAM domain is monomeric. To exclude the possibility that the absence of intermolecular contacts was due to low sensitivity in the NMR experiments, we then sought to confirm this result by other means.

| TABLE I | Experimental restraints and structural statistics |
|---|---|
| (SA)$^a$ represents the average RMS deviations for the ensemble. (SA)$_c$$^b$ represents values for the structure that is closest to the mean. |
| Number of experimental restraints | |
| Unambiguous | 1354 |
| Ambiguous | 304 |
| Dihedral restraints | 137 |
| Coordinate precision | |
| RMS deviations of backbone atoms (31–100) (Å) | 0.53 ± 0.11 |
| RMS deviations of all heavy atoms (31–100) (Å) | 0.98 ± 0.08 |
| RMS deviations | |
| From the experimental restraints | |
| Nuclear Overhauser effect distances (Å) | 0.009 ± 0.002 |
| Ramachandran analysis (%) | |
| Residues in most favored regions | 74.3 |
| Residues in additionally allowed regions | 23.8 |
| Residues in generously allowed regions | 1.4 |
| Residues in disallowed regions | 0.5 |

$^a$ The Lennard-Jones potential was not used at any stage in the refinement.

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**Fig. 1. The structure of the Ste50 SAM domain.** On the left is a superimposition of the 25 lowest energy structures of 100 calculated. The ribbon representation on the right is the closest structure to the mean. This figure was produced using Molscript (37) and Raster3D (38).
Given that the $K_d$ values published for other SAM domain homo-oligomers were relatively high (in the region of 0.5–5 mM), we performed analytical ultracentrifugation using samples of the Ste50p SAM domain at concentrations of 1.0, 2.0, and 3.0 mg/ml (−0.1, 0.2, and 0.3 mM, respectively). The sedimentation equilibrium data for the Ste50p SAM domain are shown in Fig. 3b, and the goodness of fit to a monomeric model for the data sets is shown in Fig. 3a. As can be seen the majority of the residuals are randomly distributed at approximately 0, but there is a slight upward trend at higher values of the radius, $r$. This is diagnostic of an aggregating protein, although given that this upward trend is restricted to the faster rotor speeds with the highest protein concentrations, it is likely to be a very small effect. The data are consistent with a molecular mass of 11.0 kDa for the Ste50p SAM domain. The Ste50p SAM domain is calculated to have a mass of 10.0 kDa. The calculated molecular mass obtained from the calculation was 12.75 kDa, which exceeds the actual mass by more than 40%. In addition, the model only described the data poorly as judged by the pattern of the residuals. Fitting to a monomer to dimer equilibrium still produced a poor fit. However, simultaneous fitting of the $K_a$ and the second virial coefficient gave residuals that distribute well at approximately 0 (Fig. 4a). The values obtained in fringe units were $\ln K_a = -2.00$ and $B = -6.4 \times 10^{-3}$. Using an approximate conversion factor of 3.3 fringes (g/l) this gives an estimated $K_d$ of 0.5 mM.

The Ste50p SAM Domain Interacts with the Ste11p SAM Domain in Vitro—GST pull-down experiments were used to test the ability of Ste11p and Ste50p SAM domains to interact homotypically and heterotypically in vitro (Fig. 5). Ste50p SAM domain does not self-associate, in agreement with all the previous data (lanes 11 and 12). Addition of free Ste11p SAM to immobilized Ste50p SAM (lanes 9 and 10) shows that these two SAM domains can interact with each other. The reciprocal experiment, where Ste11p is immobilized on beads, was also performed. When free Ste50p SAM was added, an interaction between the two SAM domains was again visible (lanes 5 and 6). In addition, free Ste11p SAM was added to immobilized Ste11p SAM (lanes 7 and 8). Despite the apparent decrease in the amount of input Ste11p, it can still be seen that free Ste11p SAM can self-associate with the immobilized Ste11p, thus confirming the dimerization of Ste11p observed by analytical ultracentrifugation. It should be noted that although GST fusion proteins are known to be dimers, using the SAM domains as GST fusion proteins in these assays does not appear to hamper their abilities to homo- or heterodimerize.
Analysis of the Oligomerization State of the Ste50p SAM-Ste11p SAM Complex—In an attempt to analyze the Ste11p SAM-Ste50p SAM interaction by NMR, unlabeled Ste11p SAM was titrated into 15N-labeled Ste50p SAM, and the changes in the 15N HSQC and 15N TROSY spectra were monitored (data not shown). The peaks became very broad during the titration in the HSQC spectra, with most disappearing completely during the course of the titration. A few new peaks appeared that were also very broad and weak. Interestingly, the broadening was mirrored in the TROSY spectra, indicating that it was not due to an increase in the correlation time expected for a Ste11p SAM-Ste50p SAM complex but rather must originate from chemical exchange at an intermediate rate on the chemical shift timescale.

A similar experiment was performed using unlabeled Ste50p SAM and 15N-labeled Ste11p SAM, and the resultant resonances were similarly broad. Adjustments in pH, buffer composition, ionic strength, and the addition of nondenaturing detergents only led to minor improvements in the spectra and affected all of the resonances in a similar manner. A similar observation was made upon varying the temperature from 25 to 35 °C, where the increase led to a modest sharpening of all the signals equally. Unfortunately, the majority of the resonances from the complex were too broad to observe, even when 15N TROSY HSQC was performed.

We then analyzed the titration points by gel filtration to investigate the oligomeric state of the complex formed. Fig. 6 shows the gel filtration traces for free Ste11p SAM, free Ste50p SAM, and four points from the titration where gradually increasing amounts of Ste50p SAM (0.25, 0.5, 0.75, and 1 molar equivalents) were titrated into the free Ste11p SAM sample. By comparing the retention times of the peaks to standards (Fig. 6, inset), the apparent molecular masses of the various species were calculated. Ste11p runs at a retention time equivalent to a molecular mass of 16.73 kDa. Since Ste11p SAM has a calculated molecular mass of 8687 kDa and our AUC data show it to be a dimer, a mass by gel filtration of 16.73 kDa is very close to the value that would be expected (17.37 kDa). In contrast, Ste50p SAM runs with a retention time equivalent to a molecular mass of 20.64 kDa. As all other data indicate that the Ste50p SAM is monomeric, this domain runs aberrantly through a gel filtration column, either because it deviates significantly from a spherical shape or because it interacts with the matrix. (The former explanation is possible, even though the domain is relatively spherical (Fig. 1), because the Ste50p SAM that we used has unstructured residues at either end that could affect migration in a gel.) The addition of a 0.25 molar equivalent of Ste50p SAM into Ste11p SAM results in a peak at a retention time equal to a molecular mass of 33.90 kDa and a minor peak at the position of free Ste11p SAM. This Ste11p SAM-Ste50p SAM complex peak is roughly equivalent to the additive molecular masses of free Ste11p SAM and free Ste50p SAM measured by gel filtration (37.37 kDa). Thus, this species is most likely a trimeric complex formed between dimeric Ste11p SAM and monomeric Ste50p SAM. The addition of a 0.5 molar equivalent of Ste50p SAM also results in the trimeric complex peak, but the free Ste11p SAM peak has disappeared, and a small amount of free Ste50p SAM is observed. This confirms that the oligomeric species forming is an asymmetric trimer because the Ste11p SAM is saturated at a 0.5 molar equivalent of Ste50p SAM. Further additions of 0.75 and 1 molar equivalents of Ste50p SAM result in an increase in the free Ste50p SAM peak.
Identification of Residues Mediating Hetero-oligomerization in Ste50p SAM by Mutagenesis—The broadened resonances observed in the 15N HSQC spectra meant that it was not possible to map the binding interface on Ste50p for Ste11p using NMR. Thus, we investigated residues in Ste50p SAM that are important for binding to Ste11p using a mutagenesis strategy. A sequence alignment of those SAM domains whose structures have been solved is shown in Fig. 2. As no heterodimeric SAM domain structures have been solved, we looked at homodimer interfaces for potentially important residues. Seven residues have been described in Eph A4 SAM that, when mutated, result in disruption of the dimer (17). Four analogous residues in Ste50p were chosen for mutation: Val-37, Asp-38, Pro-71, and Met-99. Met-99 also aligns with Ile-1574 of the polyhomeotic (ph) SAM domain, which, importantly, in binding studies had been shown to affect both its homo- and heterodimerization properties (30). The function of the other three residues identified in the EphA4 SAM dimer interface was not so clear in Ste50p SAM. These lie in and near helices 3 and 4. This region is important for EphA4 SAM dimerization, because it forms part of the N-terminal arm exchange interface (also seen in the EphB2 oligomer). This region has also been implicated as important for p63 SAM function (31). Leu-940 in EphA4 SAM aligns with Gly-67 that lies in a linker region between helices 3 and 4, as such it was deemed unsuitable for mutation in case it was required for the flexibility of this loop. The oligomeric structure of the EphB2 SAM domain identified a second possible interaction face that participates in a “back-to-back” dimer, utilizing Leu-955 and Lys-961 of helix 5 and Met-945 from the loop between helices 3 and 4. Arg-970 and Asn-974 also lie in the back-to-back interface. The analogous residue to Asn-974 in Ste50p SAM lies outside the structured region of the SAM domain, because helix 5 in EphB2 SAM is longer than in Ste50p SAM. Leu-75 was chosen for mutagenesis because it aligns with Met-945 from EphB2 SAM and has no long range nuclear Overhauser effects, thus any mutation would be unlikely to disrupt the packing of the Ste50p SAM domain. Leu-75 was also identified as a mutation that could disrupt back-to-back dimerization. Thus, residues Val-37, Asp-38, Pro-71, Leu-73, Leu-75, and Met-99 of Ste50p...
Structure of the SAM Domain of STE50

Fig. 7. Gel filtration analysis of wt Ste11p SAM in complex with wt and mutant Ste50p SAM. A, wt Ste11p SAM in complex with wt Ste50p SAM and four mutant Ste50p SAMs: V37A, D38A, P71A, and M99A. B, Ste50p SAM, Ste11p SAM, and wt Ste11p SAM in complex with wt Ste50p SAM and two Ste50p SAM mutants: L73A and L75A. Elution positions for free wt Ste50p SAM, free wt Ste11p SAM, and the wt Ste50p SAM-wt Ste11p SAM are indicated by dashed lines. The samples were run on a Superdex 75 column (Amersham Biosciences) attached to an analytical SMART system (Amersham Biosciences).

Fig. 8. Structures of the SAM domains. A, Structure of the SAM domain monomer, solved by NMR, is found to be very similar to the structures of other SAM domains and consists of five helices forming a compact fold. Of particular interest is the ability of SAM domains to mediate dimerization and oligomerization and how this property relates to their function. The EphA4 ephrin receptor SAM domain forms a homodimer (17), whereas the EphB2 SAM domain crystal structure has been solved in both monomeric and oligomeric forms (18, 19). Two interaction faces were identified in the crystal structure of the EphB2 oligomeric SAM domain and one in the EphA4 dimeric SAM domain (Fig. 9C). These proteins use a common interaction surface; both monomers self-associate via the exchange of the N-terminal peptide arm, in which a conserved N-terminal hydrophobic residue inserts into a cavity in the adjacent monomer. This interaction is asymmetric and buries 1900 Å² of total surface area. The second interface in the Eph B2 structure (the back-to-back interface) involves contacts between helix 5 and loop 3 in both monomers in a symmetrical interaction (Fig. 9C), which buries 1250 Å² of total surface area. The oligomer formed consists of eight monomeric units in the crystal; however, the contacts described could be used to produce a polymer of any length in vivo. The fold of the EphB2 SAM in the monomeric form differs from the oligomeric form, in that the N-terminal peptide arm, including the conserved hydrophobic residue Tyr-907, is solvent-exposed (19). Thus, the position of this arm may affect the oligomerization properties of the domain. Two further structures of SAM domains also suggest that this domain is a potential oligomerization module with the ability to control the formation of large multisubunit protein complexes involved in transcription repression (23). Oligomers were seen with the SAM domains from the transcriptional repressor TEL and in the polycomb protein polyhomeotic. Oligomerization occurs through two interacting surfaces on each SAM domain. These have been designated the mid-loop (ML) and end helix (EH) surfaces (Fig. 9) and are different from the N-terminal arm surfaces (Fig. 9) and are different from the N-terminal arm replacement site (24). Thus, the position of this arm may affect the oligomerization properties of the domain. Two further structures of SAM domains also suggest that this domain is a potential oligomerization module with the ability to control the formation of large multisubunit protein complexes involved in transcription repression (23). Oligomers were seen with the SAM domains from the transcriptional repressor TEL and in the polycomb protein polyhomeotic. Oligomerization occurs through two interacting surfaces on each SAM domain. These have been designated the mid-loop (ML) and end helix (EH) surfaces (Fig. 9) and are different from the N-terminal arm exchange seen with the Eph receptor SAM domains.

In the Ste50p SAM domain the N-terminal arm is well defined (Fig. 1) and is held against the body of the domain. The structure-based sequence alignment (Fig. 2) shows that the conserved hydrophobic residue that is buried in the arm exchange interface in the EphB2 and EphA4 SAM domain oligomers is not conserved in the Ste50p SAM. There are two other hydrophobic residues in the N-terminal region of Ste50p SAM, Phe-32 and Trp-35. These residues are both buried and therefore unavailable to mediate homodimerization via an N-terminal exchange mechanism. The N-terminal region of the Ste50p SAM is, however, strikingly similar to the same region in complexes were produced as a 1:1 molar mix of Ste50p SAM: Ste11p SAM, and therefore free Ste50p SAM is visible in all traces. It was also observed that D38A Ste50p SAM is less well behaved than the other variants, because a distinct high molecular mass shoulder is seen in this complex, indicative of aggregation.

Two mutants with different properties were chosen for further analysis by GST pull-down assays. L75A was examined as an example of a mutation that disrupts Ste50p/Ste11p SAM binding, and M99A was chosen as a control for a nonactive mutation. M99A Ste50p SAM retains its ability to interact with wt Ste11p SAM (Fig. 8, top panel, lane 7) and is unchanged in its inability to homodimerize; however, L75A Ste50p SAM can no longer heterotetramerize with wt Ste11p SAM (Fig. 8, bottom panel, lane 7). Thus, it seems that L73A Ste50p and L75A Ste50p are thermodynamically involved in the Ste50p/Ste11p SAM interface.

DISCUSSION

The structure of the Ste50p SAM domain monomer, solved by NMR, is found to be very similar to the structures of other SAM domains and consists of five helices forming a compact fold. Of particular interest is the ability of SAM domains to mediate dimerization and oligomerization and how this property relates to their function. The EphA4 ephrin receptor SAM domain forms a homodimer (17), whereas the EphB2 SAM domain crystal structure has been solved in both monomeric and oligomeric forms (18, 19). Two interaction faces were identified in the crystal structure of the EphB2 oligomeric SAM domain and one in the EphA4 dimeric SAM domain (Fig. 9C). These proteins use a common interaction surface; both monomers self-associate via the exchange of the N-terminal peptide arm, in which a conserved N-terminal hydrophobic residue inserts into a cavity in the adjacent monomer. This interaction is asymmetric and buries 1900 Å² of total surface area. The second interface in the Eph B2 structure (the back-to-back interface) involves contacts between helix 5 and loop 3 in both monomers in a symmetrical interaction (Fig. 9C), which buries 1250 Å² of total surface area. The oligomer formed consists of eight monomeric units in the crystal; however, the contacts described could be used to produce a polymer of any length in vivo. The fold of the EphB2 SAM in the monomeric form differs from the oligomeric form, in that the N-terminal peptide arm, including the conserved hydrophobic residue Tyr-907, is solvent-exposed (19). Thus, the position of this arm may affect the oligomerization properties of the domain. Two further structures of SAM domains also suggest that this domain is a potential oligomerization module with the ability to control the formation of large multisubunit protein complexes involved in transcription repression (23). Oligomers were seen with the SAM domains from the transcriptional repressor TEL and in the polycomb protein polyhomeotic. Oligomerization occurs through two interacting surfaces on each SAM domain. These have been designated the mid-loop (ML) and end helix (EH) surfaces (Fig. 9) and are different from the N-terminal arm exchange seen with the Eph receptor SAM domains.

In the Ste50p SAM domain the N-terminal arm is well defined (Fig. 1) and is held against the body of the domain. The structure-based sequence alignment (Fig. 2) shows that the conserved hydrophobic residue that is buried in the arm exchange interface in the EphB2 and EphA4 SAM domain oligomers is not conserved in the Ste50p SAM. There are two other hydrophobic residues in the N-terminal region of Ste50p SAM, Phe-32 and Trp-35. These residues are both buried and therefore unavailable to mediate homodimerization via an N-terminal exchange mechanism. The N-terminal region of the Ste50p SAM is, however, strikingly similar to the same region in...
the ph SAM (Fig. 2B). The ph SAM domains homo-oligomerize via two separate interfaces, which include some residues that are conserved in Ste50p SAM (Fig. 2). Despite this conservation, our data show that the Ste50p SAM domain does not homo-oligomerize.

In contrast, we have shown that the Ste11p SAM domain self-associates with a $K_d$ of $0.5 \text{ mM}$. Other data have suggested the self-association of Ste11p by yeast two-hybrid analysis (32) and by co-immunoprecipitation (4). Although this interaction is relatively weak, it is similar to the affinities measured for the self-association of Eph A4 SAM (0.5–5 mM) (17) and Eph B2 SAM (0.16–0.39 mM) (33). The dissociation constants measured for other SAM domains are quite variable; mutants of Tel SAM and ph SAM that force them to dimerize rather than oligomerize have affinities of 1.7 and 190 nM, respectively (21, 23). The Byr2 SAM-Ste4 SAM heterodimer was shown to have an affinity of 56 nM; however, the addition of the Ste4 leucine zipper domain increased the affinity to 19 nM (34). It therefore appears that the interactions between SAM domains may be functional at many different absolute affinities. In the absence of information concerning the local concentrations of these proteins in vivo it is difficult to speculate on the likelihood of these complexes occurring in the cell. However, in the case of Ste11p-Ste50p, the signaling complexes occur on the Ste5 scaffold protein (35), making local concentrations quite high, thus favoring the interaction despite relatively low affinities.

The quality of the spectra of the Ste50p SAM-Ste11p SAM complex precluded a detailed analysis of the interaction by NMR, even when using techniques typically suitable for higher molecular mass proteins. As Ste11p SAM dimerizes, it is likely that the Ste50p SAM-Ste11p SAM complex is a heterotrimer.
This was also suggested by the NMR titrations, because the quality of the spectra dramatically deteriorated at the point when the ratio of Ste11p SAM:Ste50p SAM was 2:1, i.e. when a Ste11p dimer binds a Ste50p monomer. Further confirmation of trimer formation came from size exclusion chromatography. When a complex of the same ratio, 2:1, Ste11p SAM:Ste50p SAM, was passed down a S75 gel filtration column, there was no free Ste11p SAM detectable. The chromatogram showed two peaks, a major peak with a calculated molecular mass of 33.90 kDa and a minor peak at 20.64 kDa. These correspond to a Ste11p SAM-Ste50p SAM trimer and a trace of free Ste50p SAM, respectively. The trimer is presumably composed of two Ste11p domains and a single Ste50p domain. A recent investigation of the interaction between the SAM domains of Byr2 and Ste4, the S.pombe homologues of Ste50p and Ste11p (34), suggests a somewhat similar scenario in this system. Although the isolated Ste4 SAM and Byr2 SAM domains interact to form a simple dimer, when the Ste4 construct was extended to include the juxtaposed leucine-zipper domain, a tetramer formed consisting of three Ste4 SAM-LZ domains and a single Byr2 SAM domain. Although the Ste11p SAM construct used in our studies contains the minimal SAM domain and Ste11p contains no other known protein-protein interaction domains, the Byr2-Ste4 work provides a precedent for multiple, possibly asymmetric interactions between SAM domains.

We have shown that the solvent-exposed hydrophobic residues Leu-73 and Leu-75 are thermodynamically important for the interactions of the Ste50p SAM domain; mutation of these residues abolishes heterotrimization with the Ste11p SAM domain. Comparison of the location of these residues with the other interfaces in the EphB2 oligomers shows that Leu-73 and Leu-75 lie close to the back-to-back interface, which involves residues from helix 5 and from the loop between helices 3 and 4 (Fig. 9). The residues equivalent to Leu-73 and Leu-75 are also involved in the ML and EH interfaces in the Tel oligomeric structure (Figs. 2 and 9). Thus, the Ste50p SAM-Ste11p SAM interaction may be similar to the Tel ML or EH interface. Residues equivalent to Asp-38, Pro-71, and Met-99, mutation of which did not affect the Ste50p SAM-Ste11p SAM interaction, are not in close contact in the Tel complex (Fig. 2).

Thus, our mutagenesis data do not enable us to determine whether the Ste50p SAM-Ste11p SAM is a back-to-back-type interaction or an ML/EH-like interaction. However, both of these interfaces involve a similar region of the SAM domain and may reflect evolution from a common ancestor interface. An absolute definition of the binding interface between Ste11p SAM and Ste50p SAM awaits either the structure determination of the complex or a more extensive mutagenesis study. It can be seen from Fig. 2 that different regions of residues 50–70 (Ste50p SAM numbering) are exclusively involved in the ML/EH interface in EphB2 and may provide a site for discriminating between these different modes of interactions.

In conclusion, the growing data on SAM domains indicate a correlation between their capacity to multimerize and their biological function. Helical polymers have been described for both the TEL SAM and the ph SAM (21, 23). Both of these transcriptional repressors could utilize homopolymerization to aid their biological activity of repressing gene expression in higher order chromatin. Homodimerization has been observed by the SAM domains of both EphA4 and the EphB2, whereas (17, 18, 36) higher order polymers have been described for EphB2 (18, 36). Receptor multimerization is a well defined aspect of the function of these proteins. The SAM domains of the transcription factor Ets-1 and the transcriptional activators p73 and p73α have, however, been demonstrated to be monomeric (9, 20, 22). We have shown that a MAPKKK modulator protein (Ste50p) also contains a monomeric SAM domain, whereas its cognate MAPKKK (Ste11p) includes a SAM capable of dimerization. The ability of the kinase Ste11p to dimerize through its SAM domain could play an essential role in its activation, for example via transautophosphorylation. Binding of Ste50p SAM domain to the Ste11p SAM domain may modulate the structure of Ste11p to allow full activation of the kinase, possibly by maintaining it in the active conformation and allowing the signal to be sustained. The ability of one Ste50p SAM to bind to two Ste11p SAM domains fits with this activation scenario.

REFERENCES

1. Banuett, F. (1998) Microbiol. Mol. Biol. Rev. 62, 249–274
2. Marsh, L., Neiman, A. M., and Herekowitz, I. (1991) Annu. Rev. Cell Biol. 7, 699–728.
3. Xu, G., Jansen, G., Thomas, D. Y., Hollenberg, C. P., and Rad, M. R. (1996) Mol. Microbiol. 20, 773–783.
4. Rad, M. R., Jansen, G., Buhring, F., and Hollenberg, C. P. (1998) Mol. Gen. Genet. 259, 29–38.
5. Rad, M. R., Xu, G., and Hollenberg, C. P. (1999) Mol. Gen. Genet. 263, 145–154.
6. Pass, W., Witten, E. A., and Saito, H. (1998) Mol. Cell. Biol. 18, 5788–5796.
7. Ponting, C. P. (1995) Protein Sci. 4, 1928–1930.
8. Schultz, J., Ponting, C. P., Hofmann, K., and Bork, P. (1997) Protein Sci. 6, 249–253.
9. Slupsky, C. M., Gentile, L. N., Donaldson, L. W., Mackereth, C. D., Seidel, J. J., Graves, B. J., and McIntosh, L. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12129–12134.
10. Thoors, C. D., and Bowie, J. U. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1708–1710.
11. Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., Van Etten, R. L., and Daniel, T. O. (1998) Genes Dev. 12, 667–678.
12. Sierra-Pages, C., Kedersha, N. L., Fazikas, L., Medley, Q., Debant, A., and Streuli, M. (1995) EMBO J. 14, 2827–2838.
13. Heck, B., Bohme, B., Karr, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Pawan, T., Ruehissen-Waigmann, H., and Strebel, K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9779–9784.
14. Stein, E., Cerretti, D. P., and Daniel, T. O. (1996) J. Biol. Chem. 271, 23588–23593.
15. Wu, C. L., Leberer, E., Thomas, D. Y., and Whiteway, M. (1999) Mol. Biol. Cell 10, 2425–2440.
