SAM (sterile alpha motif) domains are protein-protein interaction modules found in a large number of regulatory proteins. Byr2 and Ste4 are two SAM domain-containing proteins in the mating pheromone response pathway of the fission yeast, *Schizosaccharomyces pombe*. Byr2 is a mitogen-activated protein kinase kinase kinase (MAP3K) that is regulated by Ste4. Tu et al. (Tu, H., Barr, M., Dong, D. L., and Wigler, M. (1997) Mol. Cell. Biol. 17, 5876–5887) showed that the isolated SAM domain of Byr2 binds a fragment of Ste4 that contains both a leucine zipper (Ste4-LZ) domain as well as a SAM domain, suggesting that Byr2-SAM and Ste4-SAM may form a hetero-oligomer. Here, we show that the individual SAM domains of Ste4 and Byr2 are monomeric at low concentrations and bind to each other in a 1:1 stoichiometry with a relatively weak dissociation constant of 56 ± 3 μM. Inclusion of the Ste4-LZ domain, which determines the oligomeric state of Ste4, has a dramatic effect on binding affinity, however. We find that the Ste4-LZ domain is trimeric and, when included with the Ste4-SAM domain, yields a 3:1 Ste4-LZ-SAM:Byr2-SAM complex with a tight dissociation constant of 19 ± 4 nM. These results suggest that the Ste4-LZ-SAM protein may recognize multiple binding sites on Byr2-SAM, indicating a new mode of oligomeric organization for SAM domains. The fact that high affinity binding occurs only with the addition of an oligomerization domain suggests that it may be necessary to include ancillary oligomerization modules when searching for binding partners of SAM domains.

Oligomerization-dependent Association of the SAM Domains from *Schizosaccharomyces pombe* Byr2 and Ste4*

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SAM domains (also known as Pointed, SPM, and HLH domains) are frequently found in eukaryotic regulatory proteins ranging from receptor tyrosine kinases to transcription factors (1–3). Structures of several SAM domains reveal a common tertiary fold but show a diverse array of oligomeric states and binding schemes (4–11). Some SAM domains, such as that from the Ets family transcription factor TEL, can self-associate to form an open-ended polymeric structure (10), whereas the closely related Ets-1, GABPα, and Erg SAM domains are monomeric (4, 12, 13). The SAM domains from Eph receptor tyrosine kinases can either be monomeric or dimeric or may possibly form an extended oligomeric structure (7, 9, 14). SAM domains have also been described in interactions with non-SAM domain-containing proteins. For example, the SAM domain of BAR (46), a protein involved in the regulation of apoptosis, associates with both Bcl-2 and Bcl-XL (13). Cdk10, a member of the Cdc2 family of kinases, binds the SAM domain of Ets-2 and thereby regulates the activity of this transcription factor (15). The mitogen-activated protein kinase Erk2 docks on the SAM domain of Ets-1, enhancing the kinetics of phosphorylation at an adjacent N-terminal target site within this transcription factor (16). Although several complexes between nonidentical SAM domains have been described like TEL/TEL2 (17–23), and Yan/Mae (24) Scm/ph (3, 11, 25), their recognition mechanisms have not yet been characterized. Here we investigate one example of a hetero-SAM domain interaction that occurs between the Byr2 and Ste4 proteins in the fission yeast *Schizosaccharomyces pombe*.

Sexual differentiation in *S. pombe* is controlled via a mitogen-activated protein kinase pathway that includes Ste4 and Byr2 (26). Byr2 is a mitogen-activated protein kinase kinase kinase that is activated by interactions with both Ras1 and Ste4 (27–29). The SAM domain of Byr2 has previously been shown to bind to the N-terminal 160 amino acids of Ste4, a region containing a SAM domain followed immediately by a putative leucine zipper (Ste4-LZ) domain. A speculative model of Byr2 activation has therefore emerged in which Byr2 and Ste4 interact via their SAM domains, leading to oligomerization of Byr2 by virtue of the Ste4 leucine zipper domain (30). Here we find that although the two SAM domains do bind to each other, the role of the Ste4-LZ domain is not to oligomerize Byr2. Instead, the leucine zipper domain of Ste4 trimerizes, thereby displaying three SAM domains that together bind a single Byr2-SAM domain with high affinity.

**EXPERIMENTAL PROCEDURES**

*Ste4 and Byr2 Constructs*—The region of the Byr2 gene encompassing its SAM domain (amino acids 1–70; SPBC1D7.05 in the *S. pombe* GeneDB, www.genedb.org/genedb/pombe/index.jsp) was PCR-amplified from a *S. pombe* cDNA library and cloned into a modified pET-3c (Novagen) expression vector containing a C-terminal six-histidine tag. The expressed protein sequence comprised amino acids 1–70 of Byr2, followed by RDHHHHHHH. The DNA sequences encoding the SAM domain (amino acids 9–72) and the Ste4-LZ domain (amino acids 83–152) of Ste4 (SPAC1565.04c in the *S. pombe* GeneDB) were cloned similarly into the pET-3c vector with the same C-terminal His6 sequence, plus a MEKTR leader sequence. Two different Ste4 constructs...
were prepared containing both the Ste4-LZ domain and the SAM do-
main. The Ste4-LZ-SAM-A construct in the pTrcHisB (Invitrogen) vec-
tor encoded the sequence MGDSDDSY and then amino acids 1–152 of
Ste4, followed by RDHHHHHHHH. The Ste4-LZ-SAM-B, consisting of
residues 1–157, was also PCR-amplified from a *S. pombe* cDNA library and
cloned into pET28a with no added purification tags. The GST-Byr2
construct was made by subcloning amino acids comprising 1–66 of the
Byr2-SAM domain into a pGEX-3X vector (Amersham Biosciences). The
expressed protein was GST, Byr2 amino acids 1–66, followed by
RDHHHHHHH.

**Protein Purification**—Byr2-SAM, Ste4-SAM, Ste4-LZ, and Ste4-LZ-
SAM-B were all expressed in BL21(DE3) pLy8s cells (31). The Ste4-
LZ-SAM-A and the GST-Byr2 fusion proteins were expressed in ARIB14
cells (32). The cultures were typically grown at 37 °C in LB medium
containing 100 μg/ml ampicillin or 35 μg/ml kanamycin to an *A*∞
0.0 of 0.8 and then induced by the addition of 1 mM isopropyl-1-thio-β-
galactopyranoside for 4–5 h. In the case of His₆-tagged proteins, cells from
a 1-liter culture were resuspended in 10 ml of 50 mM Tris, 200 mM NaCl,
300 mM imidazole, pH 8.0, and lysed by sonication. The protein in the
soluble extract was applied to a 1-ml column of nickel-nitrotriacetic
acid-agarose (Qia_gen) and washed extensively in the same buffer.
The bound protein was then eluted with 10 ml of 50 mM Tris, 200 mM NaCl,
and 300 mM imidazole, pH 8.0. Ste4-LZ-SAM-B was purified using Q-
Sepharose anion exchange chromatography in 50 mM Tris, pH 7.5,
and an increasing NaCl gradient. The protein eluted at 0.3 M NaCl and
was further purified using Sephacyr S-100 size exclusion chromatogra-
phy in 50 mM NaCl, 20 mM potassium phosphate, pH 7.0. Concentra-
tions of the expressed proteins were determined based upon their pre-
dicted molar absorptivity values (33).

**Purification of the Ste4-LZ-SAM-A/Byr2-SAM Complex**—A molar ex-
cess of Byr2-SAM was combined with Ste4-LZ-SAM-A, and the mix-
ture was applied to a Superdex-75 HR/10/30 (Amersham Biosciences)
gel filtration column. The proteins were eluted with 25 mM Tris, pH 7.8,
200 mM NaCl, and 10 mM β-mercaptoethanol (βME) at a flow rate of 1
ml/min. The peak corresponding to the complex was pooled and concen-
trated using a Centriprep followed by a Centricon (YM-3) concentrator
(Millipore).

**GST Fusion Protein Binding Assay**—40 μl of the supplied slurry of
glutathione-Sepharose 4B beads (Amersham Biosciences) was equili-
brated in assay buffer (50 mM Bis-Tris-propane, pH 7.5, 150 mM NaCl,
and 10 mM βME), 500 μl of ~1 mg/ml of the GST fusion proteins were
incubated with the beads for 1 h at 4 °C. The beads were washed twice
with 500 μl of the assay buffer and then incubated with 300 μl of ~0.5
mg/ml of the untagged proteins in the same buffer for 1 h at 4 °C. The
beads were washed three times with 500 μl of the assay buffer, followed
by elution of the bound proteins with 60–80 μl of sample buffer (Tris/SDS,
pH 6.5, glycerol, DTT, and Coomassie Blue G-250). The proteins were
separated using a 15% Tris-Tricine-SDS-PAGE.

**Native Gel Shift Assay**—The native gel binding assay was carried out
by incubating various concentrations of Ste4-LZ-SAM-B with 10 μM Byr2-
SAM in 50 mM NaCl, 20 mM potassium phosphate, pH 7.0, resulting in
Ste4-LZ-SAM-Byr2-SAM molar ratios ranging from 1:3 to 8:1 with
respect to the protein monomers. The proteins were separated under
native conditions in a 15% acrylamide (29:1) gel containing 125 mM
glycine, pH 7.5.

**Analytical Gel Filtration**—A Bio-Silect SEC 125-5 analytical size
exclusion column (Bio-Rad) was equilibrated with 25 mM Tris, pH 8.0,
200 mM NaCl, 15 μl of a standard protein mixture or 50 μl of a 22 mg/ml
solution of the Ste4-LZ protein was applied to the column and eluted
with buffer buffer at a flow rate of 1 ml/min. The flow rate was 5 μl/min over both sample and reference flow cells. Immobiliza-
tion of the Byr2-SAM domain was carried on the sample cell only using
EDC/NHS amine coupling chemistry. Excess cross-linking agent was
washed away with ethanolamine to obtain about 300 response units of
Byr2-SAM immobilized on the sample flow cell.

For equilibrium binding measurements using Ste4-LZ-SAM-A, we
used a modification of the procedure described by Myszka et al. (34).
The instrument was equilibrated with HPS buffer at a flow rate of 50
μl/min until a steady base line was reached. Base-line data were col-
lected for at least an hour before starting the equilibrium measure-
ments. Ste4-LZ-SAM-A concentrations of 1.9, 3.8, 7.5, 15, 30, 60, 120,
and 240 μM in HPS buffer were pumped sequentially over the sensor
surface. Responses were collected until they reached an equilibrium
value. As a second test for equilibrium conditions, 7.5 μM Ste4-LZ-
SAM-A was reapplied to the chip after the highest concentration to
verify that the response returned to the same value as that measured
initially with this concentration of protein (data not shown). Because of
the extended periods over which data were collected for this experi-
ment, it was advantageous to prime the system with the next higher
analyte concentration. In contrast, Myszka et al. (34) increased the
analyte concentration as a step gradient without priming the sys-
tem. This modified method may be used in experiments with the Bio-
core-X or the Biacore 2000 instrument for high affinity interactions
requiring measurements extending over several days. The equilibrium
response value (*R* *)_∞* for each concentration was determined once
the binding response stabilized. *R* *)_∞* values were then plotted against ana-
lyte concentrations, and the dissociation constant (*K* *)_d* was determined
by fitting the data to a hyperbolic binding equation describing the
formation of a 1:1 complex:

\[
R_{\text{eq}} = R_{\text{max}} \times \left(1 + K_d [A]\right)
\]

where *R* *)_eq* is the response at saturating concentration of analyte
and *A* refers to the analyte. Fits were performed using the program Kalei-
daGraph (version 3.09), treating *R* *)_eq* and *K* *)_d* as adjustable parame-
ters.

For equilibrium binding measurements with Ste4-SAM, 25 μl of
Ste4-SAM domain at concentrations of 0.75, 1.5, 3, 6, 12, 24, 48, and 96
μM were injected at a flow rate of 50 μl/min. The response values at
saturation were obtained by averaging initial and final values ranging
between 5 and 20 s in the saturation phase of the binding curve for each
concentration. The experiment was repeated three times, using fresh
dilutions of the Ste4-SAM domain and injecting each in random order
of concentration. The binding data were fit to a hyperbolic binding equa-
tion as presented above. The reported *K* *)_d* is an average of three
experiments.

**Equilibrium Sedimentation**—Equilibrium sedimentation runs were
performed at 4 °C (for the isolated SAM domains) or 20 °C (for all other
proteins) in a Beckman Optima XL-A analytical ultracentrifuge. Con-
centrations ranged from 0.1 to 4 mg/ml for the separate SAM domains,
from 0.35 to 3 mg/ml for the Ste4-LZ domain, and less than 0.7 mg/ml
for all other proteins. The samples were examined in 3-mm double-
sector, 12-mm double sector, and 12-mm six sector cells at appropriate
wavelengths (228, 250, 295, or 300 nm) to ensure the absorption was
sufficient to give a good signal-to-noise ratio, and the maximum absorb-
bance was within the linear range of the instrument (less than 1.35 OD).
Buffer conditions were 25 mM Tris, 200 mM NaCl, pH 7.8, for Byr2-
SAM; 25 mM Tris, 200 mM NaCl, 5 mM βME, pH 7.8, for Ste4-SAM; 5
mM Bis-Tris-propane, pH 7.0, for Ste4-LZ-SAM-A; and 5 mM Bis-
Tris-propane, pH 7.0, for the purified Ste4-LZ-SAM/Byr2-SAM complex.
Partial specific volumes were calculated from the amino acid compositions of the proteins (35) and corrected to the appropriate temperature (36). Individual scans were least squares fit to an exponential equation for a single ideal
species using the Beckman Origin-based software (version 3.01). To
estimate equilibrium dissociation constants, the Beckman global analy-
ysis software (the “multifit” option) was used to analyze multiple scans
simultaneously. Data sets consisted of a minimum of four scans for two
samples, 10-fold different in concentration, run at two speeds with the
ratio of *ω*² greater than 2.

**RESULTS**

**Ste4-SAM Binding to Byr2-SAM**—Ste4 and Byr2 are multi-
domain proteins (Fig. 1). Previous work by Tu et al. (30) showed
that the first 160 amino acids of Ste4, which include a SAM
domain, associates with the SAM domain of Byr2 (residues
1–70). Because SAM domains are known protein-protein inter-
action modules, we first tested whether the isolated SAM do-
mains can bind to each other. In initial binding studies, pull-
down experiments were performed using a GST-tagged version
of Byr2-SAM. As shown in Fig. 2 (lane 6), the Ste4-SAM do-

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1 The abbreviations used are: GST, glutathione S-transferase; βME, β-mercaptoethanol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RA, Ras-associating.
main is able to bind to the GST-Byr2-SAM, whereas no association is observed with control SAM domains from Polyhomoletic (Ph) or Sex comb on midleg (Scm) (3, 11, 25).

To establish the stoichiometry of binding, we used equilibrium sedimentation. As shown in Fig. 3, Ste4-SAM behaves as a single species with a molecular mass of 9,400 Da, very close to that of the calculated monomer molecular weight of 9,183 Da. At the lowest concentration and highest speed used, the Byr2-SAM yielded a molecular weight of 10,500 Da when the concentration distribution was fit to a single species. This value is significantly higher than the calculated monomer molecular weight for Byr2-SAM of 9,462 Da. The apparent molecular weight was found to increase with higher initial protein concentration and slower centrifugation speeds, however, suggesting that Byr2-SAM is weakly self-associating. Assuming a monomer-dimer equilibrium, a group fit of runs at different concentrations and speeds yielded a dissociation constant of $K_d = 0.2$ mM. When Byr2-SAM and Ste4-SAM were mixed together...
at a 1:1 molar ratio, we found a maximum molecular weight of 19,500 Da at the highest concentration and lowest speed, which compares favorably to the calculated molecular weight of 18,645 for the heterodimer. Thus, the sedimentation results indicate that 1) Ste4-SAM is monomeric; 2) Byr2-SAM is predominantly monomeric at low concentrations but weakly self-associates; and 3) Ste4-SAM and Byr2-SAM form a 1:1 complex.

Fig. 4 shows surface plasmon resonance experiments that were carried out to determine the binding affinity between Byr2-SAM and Ste4-SAM domains. Because of their relatively weak interaction, the association and dissociation rates were too fast to measure conventionally. Thus, we were restricted to measuring the equilibrium response for each concentration of Ste4-SAM pumped over immobilized Byr2-SAM. As shown in Fig. 4, the equilibrium response values are well fit by a hyperbolic binding curve with a dissociation constant of 56 ± 3 μM and a stoichiometry of 1:1 between Ste4-SAM and Byr2-SAM. Together, these experiments indicate that the isolated SAM domains are at least partly responsible for the association between the Byr2 and Ste4 proteins.

Leucine Zipper Domain of Ste4 Is Trimeric—Ste4 contains a leucine zipper domain C-terminal to its SAM domain that may be involved in homo-oligomerization. To determine the oligomeric state of this predicted coiled-coil domain, Ste4-LZ and Ste4-LZ-SAM-A were expressed and purified. Equilibrium sedimentation measurements indicate that both the Ste4-LZ and Ste4-LZ-SAM-A proteins are trimeric. For Ste4-LZ we observed a molecular weight of 29,100 Da, which is comparable with the calculated trimer molecular weight of 29,073 Da. For Ste4-LZ-SAM-A we found a molecular weight of 57,500 Da, in agreement with a calculated trimer molecular weight of 57,648 Da (Fig. 5A). Given that Ste4-SAM is monomeric, we attribute the oligomerization of Ste4-LZ-SAM-A to the presence of the Ste4-LZ domain.

As shown in Fig. 5B, the 29 kDa Ste4-LZ trimer elutes from an analytical gel filtration column at a volume consistent with an extended coiled-coil structure.

Ste4-LZ-SAM/Byr2-SAM Binding—We next sought to deter-
mine how the trimeric presentation of the Ste4 SAM domain affects the interaction with Byr2-SAM. Ste4-LZ-SAM-A binding to Byr2-SAM was first confirmed in GST pull-down experiments shown in Fig. 2 (lane 5). Consistent with the yeast two-hybrid results observed previously (37), Ste4-LZ-SAM-A binds tightly to the GST-fused Byr2-SAM and not to the control proteins. To determine the stoichiometry of this interaction, we purified the Ste4-LZ-SAM-A/Byr2-SAM complex by gel filtration chromatography. As shown in Fig. 6A, a molecular weight of 67,300 Da was obtained for the purified complex by equilibrium sedimentation. This value is comparable with the calculated molecular weight of 67,108 Da for a 3:1 ratio of Ste4-LZ-SAM-A to Byr2-SAM.

Given the 1:1 stoichiometry of binding for the isolated SAM domains, the 3:1 ratio in the presence of the trimeric Ste4-LZ domain is unexpected. We therefore obtained an independent measure of the ratio of the subunits in the complex using a native gel shift assay under stoichiometric binding conditions, i.e. at a Byr2-SAM concentration well above the $K_d$ (see below). Ste4-LZ-SAM-B and Byr2-SAM were mixed in various ratios, and the bound and free forms of Ste4-LZ-SAM-B were separated by native gel electrophoresis. As shown in Fig. 6B, the binding sites on Ste4-LZ-SAM-B were saturated at a 2.8:1 ratio of Ste4-LZ-SAM-B to Byr2-SAM. This experiment is consistent with the stoichiometry and molecular weight determination described above.

To determine the affinity of the Ste4-LZ-SAM/Byr2-SAM association, we used surface plasmon resonance experiments. Byr2-SAM was immobilized, and Ste4-LZ-SAM-A was present in the mobile phase. Binding was readily detected, but it proved impossible to obtain adequate fits to the kinetic binding data using a variety of kinetic models. Apparently the binding and/or dissociation mechanisms are relatively complex. We therefore turned to equilibrium binding measurements, where a mechanistic model is not required. Responses to various concentrations of Ste4-LZ-SAM-A were monitored until a stable value was obtained. As shown in Fig. 7, the equilibrium response versus concentration is well described by a hyperbolic binding equation. In two independent experiments, we obtained dissociation constants of 22 and 15 nM, or an average of 19 ± 4 nM, for the 3:1 complex between the Ste4-LZ-SAM-A and Byr2-SAM proteins. Thus, combining the trimeric Ste4-LZ with the Ste4-SAM domain enhances binding affinity for Byr2-SAM over 2000-fold, compared with that measured with the individual Ste4-SAM domain.

**DISCUSSION**

Complex Formation between Byr2 and Ste4—Our results indicate that the primary interaction between Byr2 and Ste4 is mediated by their individual SAM domains. However, trimerization of the Ste4-SAM domain by the adjacent Ste4-LZ domain dramatically increases the affinity of this interaction,
albeit with an unexpected stoichiometry of 3 Ste4-LZ-SAM to 1 Byr2-SAM. Although we cannot rule out direct contributions to binding from the Ste4-LZ region within the context of the Ste4-LZ-SAM construct, Ste4-LZ alone does not bind to Byr2-SAM detectably. Alternatively, the presence of the coiled-coil region immediately adjacent to the Ste4-SAM domain may indirectly influence binding to Byr2-SAM by altering the structure or dynamics of the Ste4-SAM domain. However, the far UV circular dichroism spectrum of Ste4-SAM indicates a well folded protein, with a helical content similar to that observed for other SAM domains. Also, we observe only a minor increase in the thermal stability of Ste4-LZ-SAM-B as compared with the isolated Ste4-SAM domain alone (not shown). Thus, it appears most likely that the dramatic enhancement in binding affinity is a result of multivalent interactions between three Ste4-SAM domains with a Byr2-SAM monomer. According to this model, shown schematically in Table I, the isolated Ste4-LZ and Ste4-SAM domains form a 1:1 heteromeric complex with modest affinity ($K_d = 56 \mu M$) between primary binding surfaces on each protein. Secondary, lower affinity sites become significant when three Ste4-SAM domains are held in proximity by the trimeric and presumably parallel leucine zipper domain, because the entropic cost of uniting Ste4 and Byr2 in the complex has to be paid only once. This results in a lowering of the $K_d$ for binding to 19 nM.

Multivalent binding requires that identical Ste4-SAM domains recognize different sites on the Byr2-SAM domain in an asymmetric manner. Although unexpected, breakdown of symmetry in molecular interactions is certainly not unprecedented. For example, a dimeric growth hormone receptor binds to a monomeric growth hormone using different binding surfaces on the growth hormone (38). Moreover, SAM domains are known to utilize multiple binding surfaces to generate polymeric structures (10, 11).

**Biological Implications**—There is a clear requirement for the interaction of Ste4 with Byr2 in the *S. pombe* mating process. The sterile phenotype of *S. pombe* harboring deletions or other mutations in either the Ste4 or Byr2 SAM domain indicate that SAM domains are needed for activation of the pheromone response pathway (29, 30, 37, 39). In addition, removal of the Ste4-LZ region in Ste4 produces a marked decrease in sporulation (39), consistent with our results demonstrating that the

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**Fig. 5. Ste4-LZ and Ste4-LZ-SAM are elongated trimers.** A, equilibrium sedimentation of Ste4-LZ. The experiment was carried out at 20 °C, a starting concentration of 35 μM, and a speed of 24,000 rpm. The apparent molecular weight is consistent with a trimeric homo-oligomer. B, apparent molecular weight determination by gel filtration chromatography. The elution volume versus molecular weight is plotted in the open circles for a set of standard proteins: thyroglobulin (670 kDa), immunoglobulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The arrow indicates the elution position of the Ste4-LZ protein, which corresponds to an apparent molecular weight of 70 kDa. C, equilibrium sedimentation results for the Ste4-LZ-SAM-A protein. The experiment was carried out at 20 °C, a starting concentration of 38 μM, and a speed of 10,000 rpm. In A and C, the solid curves in the lower panels show the best fit of the absorbance versus radius profile, whereas residual errors are presented in the upper panels.
isolated Ste4-SAM domain has only a weak interaction with Byr2 in the absence of trimerization. However, the mechanism by which binding of Ste4 to Byr2 affects these signaling pathways remains murky.

Earlier work has shown that Byr2 recognizes membrane-associated Ras-1 in its active GTP bound state via a Ras-
and Polyheal form open-ended helical polymers (10, 11). The interaction of Byr2 and Ste4, however, is the first well characterized example of a complex involving discrete, closed SAM domain oligomers. This diversity in interaction modes demonstrates the versatility of SAM domains in mediating protein-protein interactions in biological systems.

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TABLE I
Summary of molecular masses and dissociation constants for proteins and protein complexes

| Proteins                        | Experimental MW (Da) | Calculated MW (Da) | Oligomeric State | Stoichiometry | Schematic |
|--------------------------------|----------------------|--------------------|------------------|---------------|-----------|
| Ste4-SAM                        | 9,460                | 9,183              | Monomer          | -             |           |
| Byr2-SAM                        | 10,500               | 9,462              | Monomer/Dimer    | -             |           |
| Ste4-SAM +                     | 10,470               | 18,043             | Dimer            | 1/1           | (Kd = 66 μM) |
| Byr2-SAM                       |                      |                    |                  |               |           |
| Ste4-LZ                         | 29,100               | 29,073             | Dimer            | -             |           |
| Ste4-LZ-SAM +                   | 57,500               | 57,216             | Dimer            | -             |           |
| Byr2-SAM +                     | 67,300               | 67,600             | -                | 3/1           | (Kd = 19 nm) |

* The apparent molecular mass was concentration- and speed-dependent due to self-association. This value is from equilibrium sedimentation at 10 μM Byr2-SAM, the lowest concentration used.

binding domain (28, 40). Through Ras-1 association and interactions with other proteins such as Shk1, Byr2 may become converted to an “open conformation,” thereby relieving the autoinhibition of its kinase activity (30). By analogy to other kinase pathways, it was previously speculated that Ste4 could oligomerize Byr2 in this open conformation, leading to Byr2 autophosphorylation and further catalytic activation (30). This model seems unlikely in view of our current results indicating that Ste4-LZ-SAM does not alter the oligomerization state of Byr2-SAM, although it cannot be ruled out for the full-length proteins in their native cellular contexts.

The C-terminal region in Ste4 has also been shown to be a key factor in the pheromone response pathway. Deletion of this region renders S. pombe sterile (39), and it is possible that the C-terminal region could play a scaffolding role in assisting the recruitment of Byr2 to additional components of the mating pathway. In particular, sequence analysis reveals a distant relation to Ras-associating (RA) domains (41, 42), suggesting that Ste4 could play a role in binding to Ras-1. This exact function seems unlikely, however, for two reasons. First, the structures of the Byr2-Ras-binding domain/Ras complex and the structure of a Rap-RA/Rap1A complex indicate that the binding sites on Ras-1 would overlap (43, 44). This would lead to competition for the Ras-1 binding site rather than synergy. Second, the interaction of Byr2 and Ste4 is apparently not required for Ras-1 stimulated recruitment of Byr2 to the cell membrane (28, 40). This suggests that Byr2 can bind to Ras-1 in the absence of the interaction with Ste4. Thus, the RA domain may have evolved a different function in Ste4 or may bind another small GTPase that has yet to be identified.

Implications for Other SAM Domains—The fact that high affinity binding between the SAM domains of Byr2 and Ste4 only occurs in the context of the Ste4 oligomer serves as a cautionary note. Although SAM domains are widely distributed protein-protein interaction modules, the binding partners of only a few SAM domains are known. The relative dearth of information may arise because two-hybrid screening or other means of identifying binding partners are not effective with isolated SAM domains. Rather, an appropriate oligomerization module may need to be included. For example, the SAM domain of the EphB1 receptor has been implicated in binding to a protein tyrosine phosphatase (45), but this interaction only occurs upon receptor clustering. Similar oligomerization-dependent interactions may occur in other systems as well.

Prior work demonstrated that the SAM domains from TEL and Polyheal form open-ended helical polymers (10, 11). The interaction of Byr2 and Ste4, however, is the first well characterized example of a complex involving discrete, closed SAM domain oligomers. This diversity in interaction modes demonstrates the versatility of SAM domains in mediating protein-protein interactions in biological systems.
Oligomerization-dependent SAM Domain Association

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