THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 42, Issue of October 18, pp. 39585–39593, 2002
Printed in U.S.A.

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

Received for publication, July 19, 2002
Published, JBC Papers in Press, August 8, 2002, DOI 10.1074/jbc.M207273200

Ranjini Ramachander‡§, Chongwoo A. Kim‡§, Martin L. Phillips‡, Cameron D. Mackereth¶, Christopher D. Thanos‖, Lawrence P. McIntosh¶, and James U. Bowie‡ ‡‡**

From the ‡Department of Chemistry and Biochemistry, Molecular Biology Institute, and the UCLA-DOE Laboratory of Structural Biology and Molecular Medicine, University of California, Los Angeles, California 90095, the ¶Departments of Biochemistry and Chemistry and the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and ‖Sunesis Pharmaceuticals, San Francisco, California 94080

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 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.

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 RDHHHHHH. 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 His_6 sequence, plus a MEKTR leader sequence. Two different Ste4 constructs

* This work was supported by National Institutes of Health Grant RO1CA81000 (to J. U. B.) and by the National Cancer Institute of Canada with funds from the Canadian Cancer Society (to L. P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.
** Canadian Institutes of Health Research Scientist.
‡‡ Leukemia and Lymphoma Society Scholar. To whom correspondence should be addressed: Boyer Hall, UCLA, 611 Charles E. Young Dr. E., Los Angeles, CA 90095-1570. Tel.: 310-206-4747; Fax: 310-206-4749; E-mail: bowie@mbi.ucla.edu.

were prepared containing both the Ste4-LZ domain and the SAM domain. The Ste4-LZ-SAM-A construct in the pTrcHisB (Invitrogen) vector encoded the sequence MGDSDDSY and then amino acids 1–152 of Ste4, followed by RDHHHHHH. 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[1] 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 RDHHHHHH.

Protein Purification—Byr2-SAM, Ste4-SAM, Ste4-LZ, and Ste4-LZ-SAM-B were all expressed in BL21(λDE3) pLysS cells (31). The Ste4-LZ-SAM-A and the GST-Byr2 fusion proteins were expressed in ARI814 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_{600} of 0.8 and then induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4–5 h. In the case of His_6-tagged proteins, cells from a 1-liter culture were resuspended in 10 ml of 50 mM Tris, 200 mM NaCl, 30 mM imidazole, pH 8.0, and lysed by sonication. The protein in the soluble extract was applied to a 1-ml column of nickel-nitrilotriacetic acid-agarose (Qiagen) 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 Sephacryl S-100 size exclusion chromatography in 50 mM NaCl, 20 mM potassium phosphate, pH 7.0. Concentrations of the expressed proteins were determined based upon their predicted molar absorptivity values (33).

Purification of the Ste4-LZ-SAM-A/Byr2-SAM Complex—A molar excess of Byr2-SAM was combined with Ste4-LZ-SAM-A, and the mixture 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 concentrated 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 equilibrated 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 ~1 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 6.8, 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 mixing 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 Tris, pH 7.0, and a running buffer consisting of 25 mM Tris, 19 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 the equilibration buffer at a flow rate of 1 ml/min.

Surface Plasmon Resonance Measurements—Equilibrium surface plasmon resonance experiments were carried out using a Biacore-X. Byr2-SAM was cross-linked to CM5 research grade chips (Biacore) by first equilibrating the instrument at 20 °C in running buffer containing HPS (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.005% polysorbate 20) at a flow rate of 10 μl/min until a steady base line was obtained. 35 μl of 7.5 μg/ml Byr2-SAM in 10 mM Hepes, pH 7.5, was injected at a flow rate of 5 μl/min over both sample and reference flow cells. Immobilization 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 collected for at least an hour before starting the equilibrium measurements. Ste4-LZ-SAM-A concentrations of 1.9, 3.8, 7.5, 15, 30, 60, 120, and 240 nM 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 nM 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 experiment, we found it 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 system. This modified method may be used in experiments with the Biacore-X or the Biacore 2000 instrument for high affinity interactions requiring measurements extending over several days. The equilibrium response value (R_{eq}) at each concentration was determined once the binding response stabilized. R_{eq} values were then plotted against analyte 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.

$$R_{eq} = R_{max} \times (1/(1 + K_d/[A])) \quad \text{(Eq. 1)}$$

where R_{max} is the response at saturating concentration of analyte and *A* refers to the analyte. Fits were performed using the program KaleidaGraph (version 3.09), treating R_{max} and K_d as adjustable parameters.

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 equation as presented above. The reported K_d is an average of three experiments.

Equilibrium Sedimentation—Sedimentation equilibrium 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. Concentrations 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, 280, 295, or 300 nm) to ensure the absorbance was sufficient to give a good signal-to-noise ratio, and the maximum absorbance 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 Tris, 5 mM Bis-Tris propane, 125 mM NaCl, 1 mM βME, pH 7.8, for mixtures of the SAM domains; 25 mM Tris, 100 mM NaCl, pH 7.8, for Ste4-LZ; 25 mM Tris, 150 mM NaCl, 1 mM βME, pH 7.8, for Ste4-LZ-SAM-A; and 25 mM Tris, 200 mM NaCl, 1 mM βME, pH 7.8, for the purified Ste4-LZ-SAM-A/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 analysis 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 ω2 greater than 2.

RESULTS

Ste4-SAM Binding to Byr2-SAM—Ste4 and Byr2 are multidomain 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 interaction modules, we first tested whether the isolated SAM domains 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-

[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.

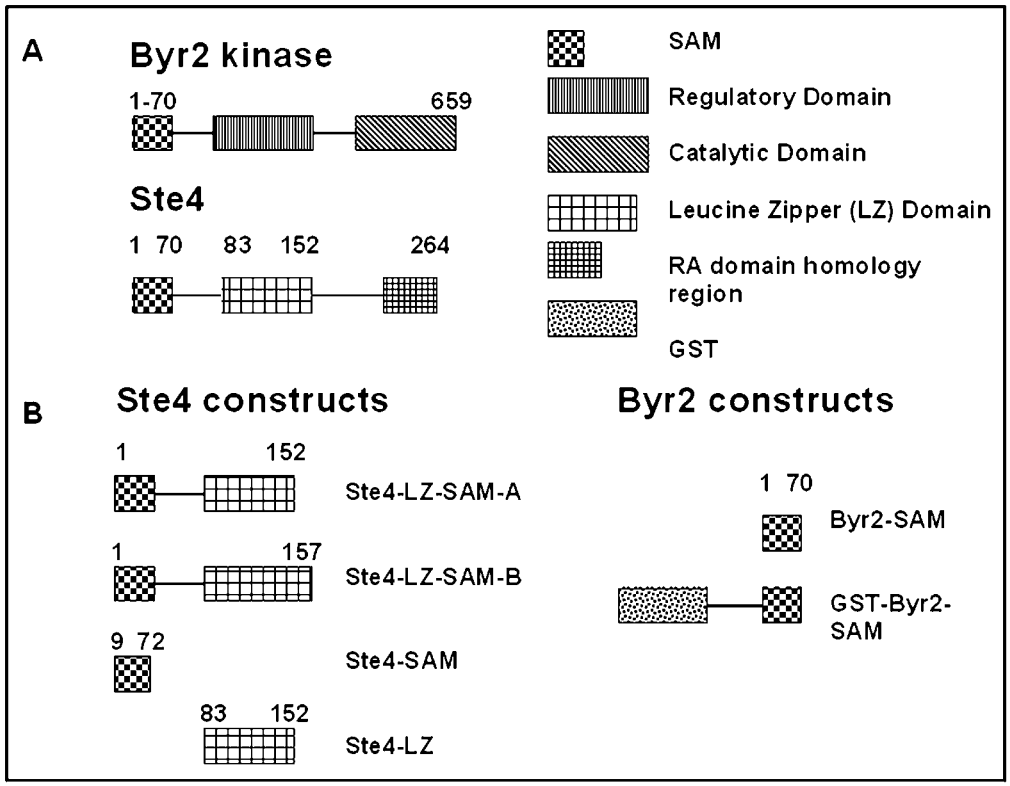

FIG. 1. **Domain structure of Byr2 and Ste4 proteins and constructs used in this work.** *A*, Byr2 kinase possesses an N-terminal SAM domain, a regulatory domain (which includes both a Ras1 binding domain and a kinase inhibitory domain), and a kinase catalytic domain. Ste4 also has an N-terminal SAM domain, followed by a leucine zipper (Ste4-LZ) domain and a C-terminal RA domain homology region. *B*, Ste4-SAM contains only the SAM domain of Ste4, whereas Ste4-LZ contains the isolated leucine zipper domain. Ste4-LZ-SAM-A and Ste4-LZ-SAM-B possess the Ste4-SAM and the Ste4-LZ domains. Byr2-SAM contains only the SAM domain of Byr2, and GST-Byr2-SAM is a fusion of GST and Byr2-SAM. Additional N- and C-terminal sequences, including a His_6 purification tag, are described under "Experimental Procedures."

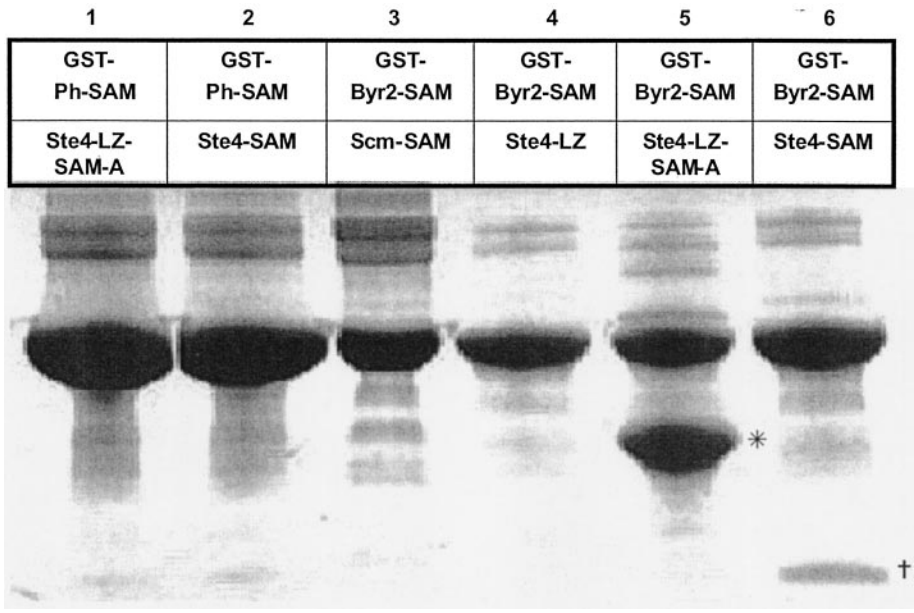

FIG. 2. **GST pull-down assays.** *Lane 1*, no binding is detected for GST-Ph-SAM mixed with Ste4-LZ-SAM-A. GST-Ph-SAM is an unrelated SAM domain from the protein Polyhomeotic (25). *Lane 2*, no binding is detected for GST-Ph-SAM mixed with Ste4-SAM. *Lane 3*, no binding is detected for GST-Byr2 mixed with Scm-SAM. Scm-SAM is an unrelated SAM domain from the protein Scm (3). *Lane 4*, no binding is detected for GST-Byr2-SAM mixed with Ste4-LZ. *Lane 5*, GST-Byr2-SAM mixed with Ste4-LZ-SAM-A. The presence of the *lower band* corresponding to Ste4-LZ-SAM-A (labeled by the *asterisk*) indicates that the trimeric Ste4-LZ-SAM-A (see "Results") protein binds tightly to Byr2-SAM. *Lane 6*, GST-Byr2-SAM mixed with Ste4-SAM. The presence of the faint *lower band* corresponding to Ste4-SAM (labeled by †) indicates weak binding between the two SAM domains. The pronounced band in each lane arises from the GST-labeled protein, whereas higher molecular weight species correspond to minor contaminants.

main is able to bind to the GST-Byr2-SAM, whereas no association is observed with control SAM domains from Polyhomeotic (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 ~0.2 mM. When Byr2-SAM and Ste4-SAM were mixed together

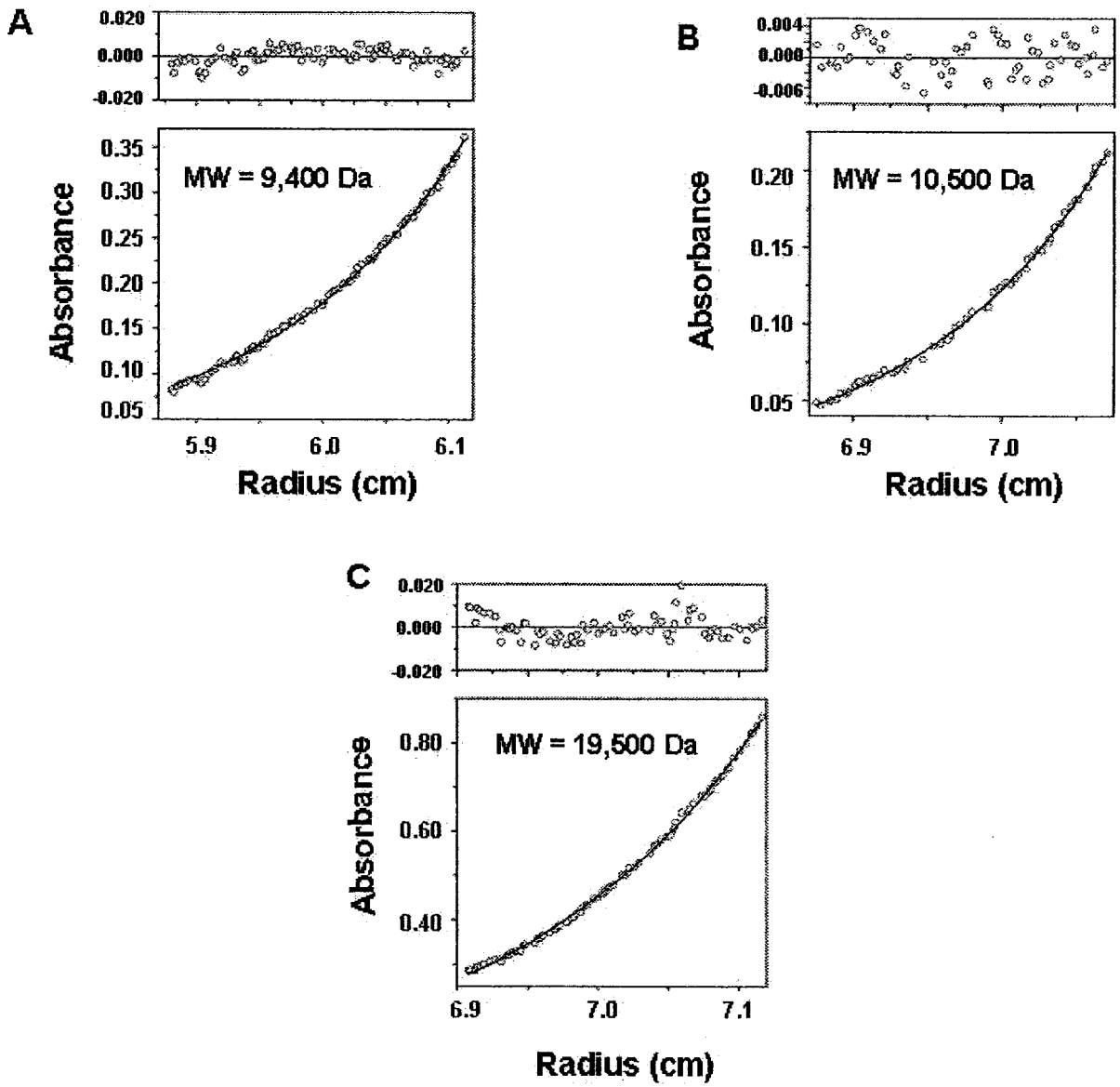

FIG. 3. **Equilibrium sedimentation of SAM domains.** *A*, Ste4-SAM at 4 °C, a starting concentration of 70 μM, and a speed of 28,000 rpm. *B*, Byr2-SAM at 4 °C, a starting concentration of 10 μM, and a speed 28,000 rpm. *C*, a mixture of Byr2-SAM and Ste4-SAM at 4 °C. The starting concentration of each protein was 0.4 mM, and the rotor speed was 17,000 rpm. In each case, 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*.

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 $\pm$ 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 57,500 Da, in agreement with a calculated trimer molecular weight of 57,648 Da (Fig. 5*A*). 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. 5*B*, the 29 kDa Ste4-LZ trimer elutes from an analytical gel filtration column at a volume consistent with a 70-kDa protein. This anomalously high apparent molecular weight suggests that the Ste4-LZ domain is rod-like, as might be expected for an extended coiled-coil structure.

Ste4-LZ-SAM/Byr2-SAM Binding—We next sought to deter-

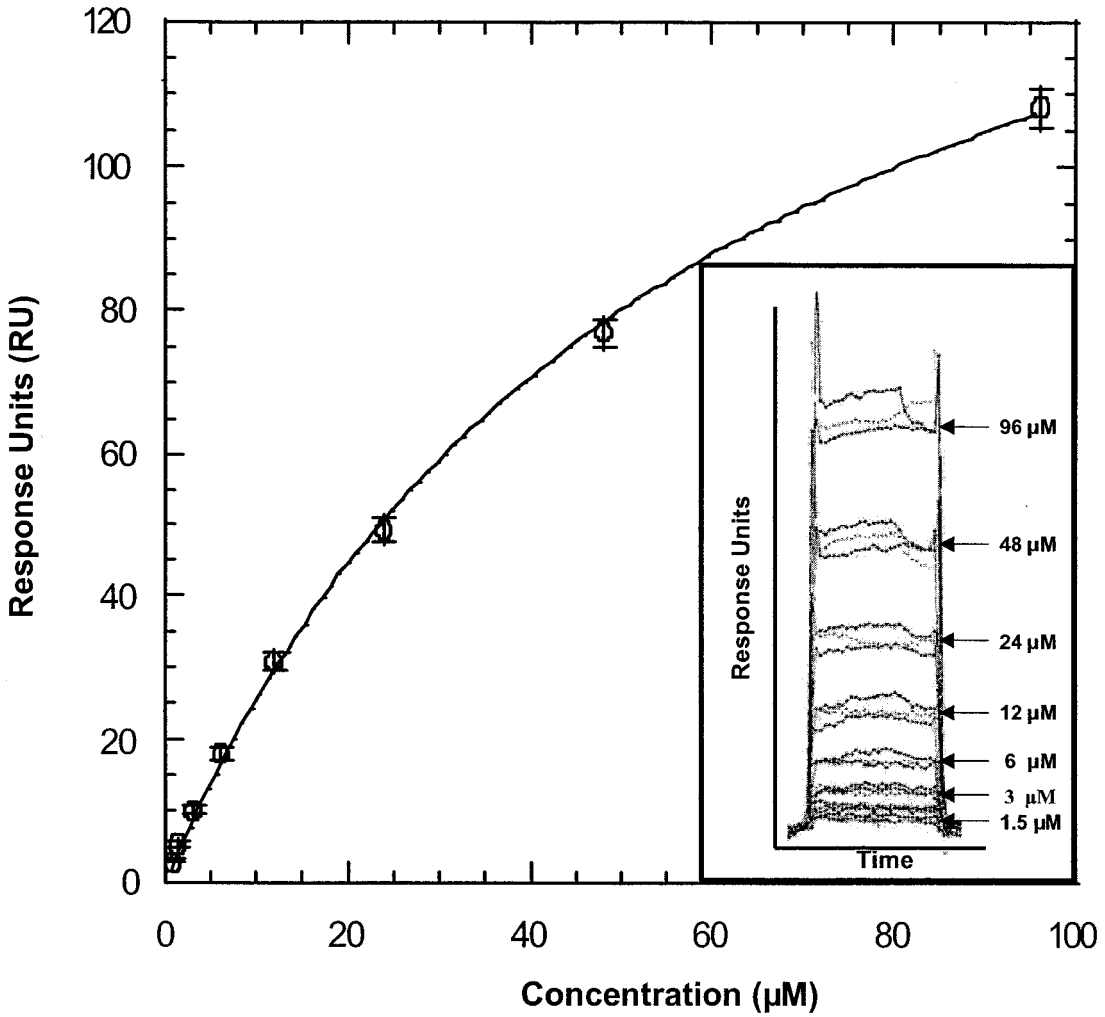

FIG. 4. **Equilibrium binding measurements by surface plasmon resonance.** Byr2-SAM was cross-linked to the Biacore chip as described under "Experimental Procedures." Equilibrium response units *versus* applied Ste4-SAM concentration are plotted. A dissociation constant of 56 ± 3 μM was determined by a fit to a hyperbolic equation describing a 1:1 binding equilibrium (*solid line*). The *inset* shows raw data (response units as a function of time) obtained from the instrument upon titrating with various concentrations of Ste4-SAM.

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. 6*A*, 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. 6*B*, 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,

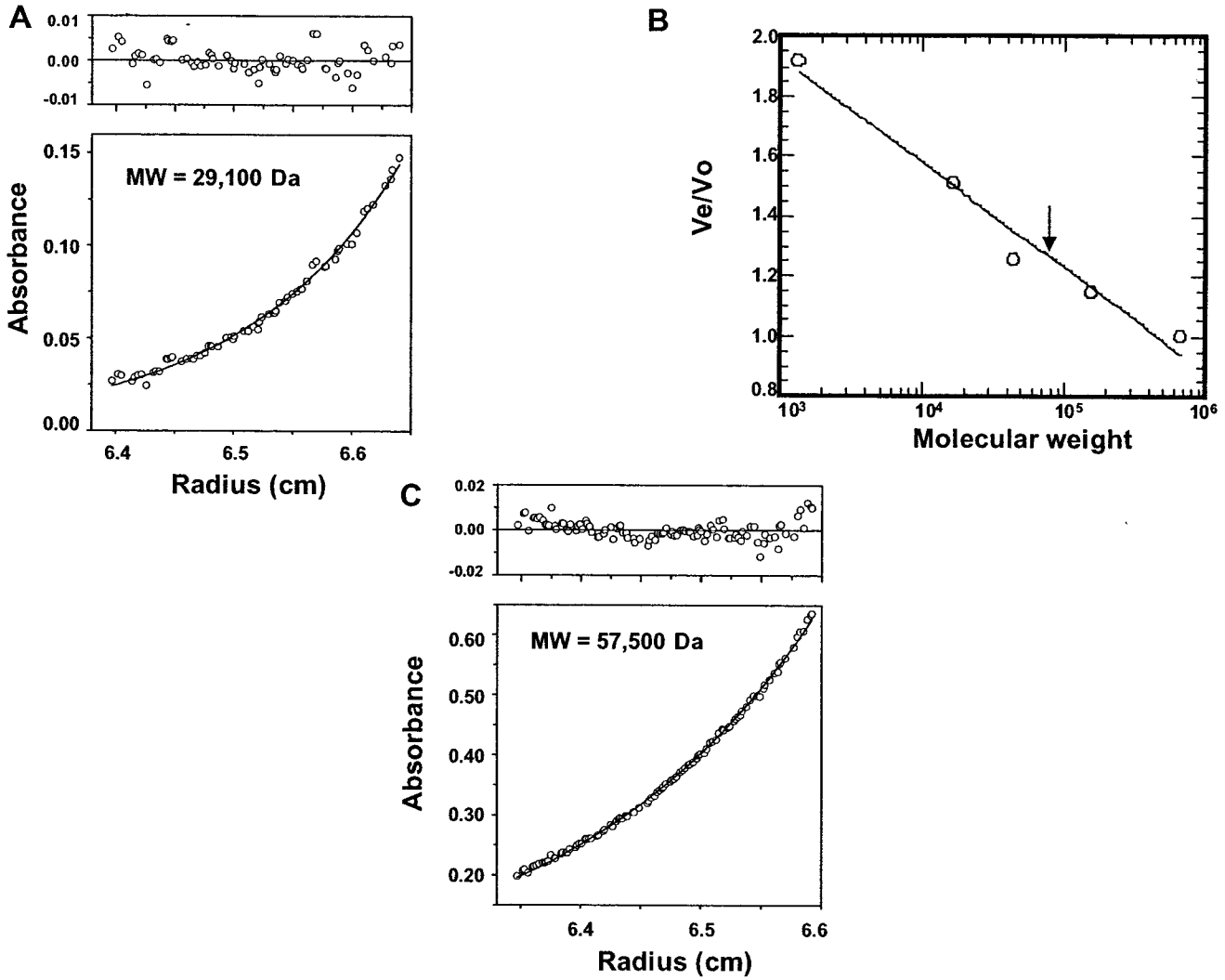

FIG. 5. **Ste4-LZ and Ste4-LZ-SAM are elongated trimers.** *A*, equilibrium sedimentation of Ste4-LZ. The experiment was carried out at 20 °C, a starting concentration of 35 μM, and a speed of 24,000 rpm. The apparent molecular weight is consistent with a trimeric homo-oligomer. *B*, apparent molecular 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 B_{12} (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*.

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 Byr2-SAM and Ste4-SAM domains form a 1:1 heteromeric complex with modest affinity (K_d = 56 μM) 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

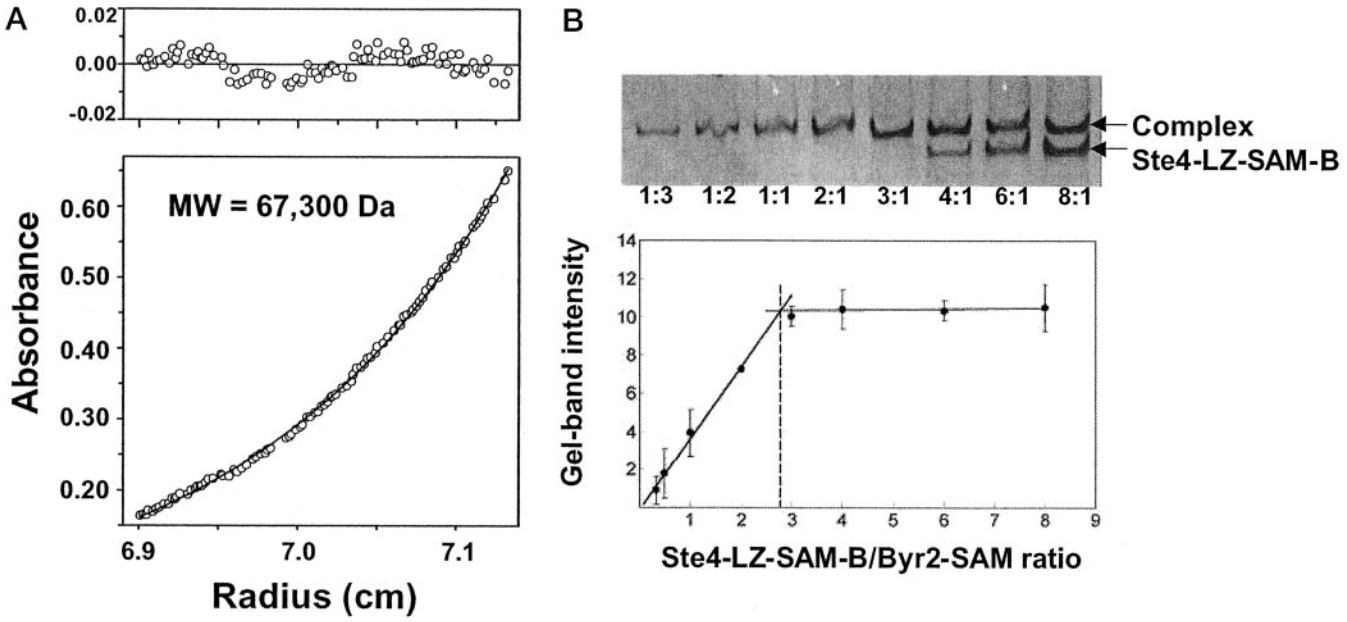

FIG. 6. **Ste4-LZ-SAM and Byr2-SAM bind in a 3:1 complex.** *A*, equilibrium sedimentation results for the purified complex of Ste4-LZ-SAM-A and Byr2-SAM. The experiment was carried out at 20 °C, a starting concentration of 0.3 mg/ml, and a speed of 10,000 rpm. The *solid curve* in the *lower panel* shows the best fit of the absorbance *versus* radius profile to a molecular weight of 67,300 Da, whereas residual errors are presented in the *upper panel*. *B*, native gel shift assay. 10 μM Byr2-SAM was titrated with increasing concentrations of Ste4-LZ-SAM-B polypeptide chains, and the mixture of bound and free Ste4-LZ-SAM-B were separated by native gel electrophoresis (see "Experimental Procedures"). Under these conditions, no free Byr2-SAM was observed (not shown). The graph shows the integrated band intensity of the complex as a function of molar ratios of the two proteins (Ste4-LZ-SAM-B to Byr2-SAM). Note that this ratio refers to the mole ratio of protein monomers. The *error bars* correspond to the range of values obtained from three experiments.

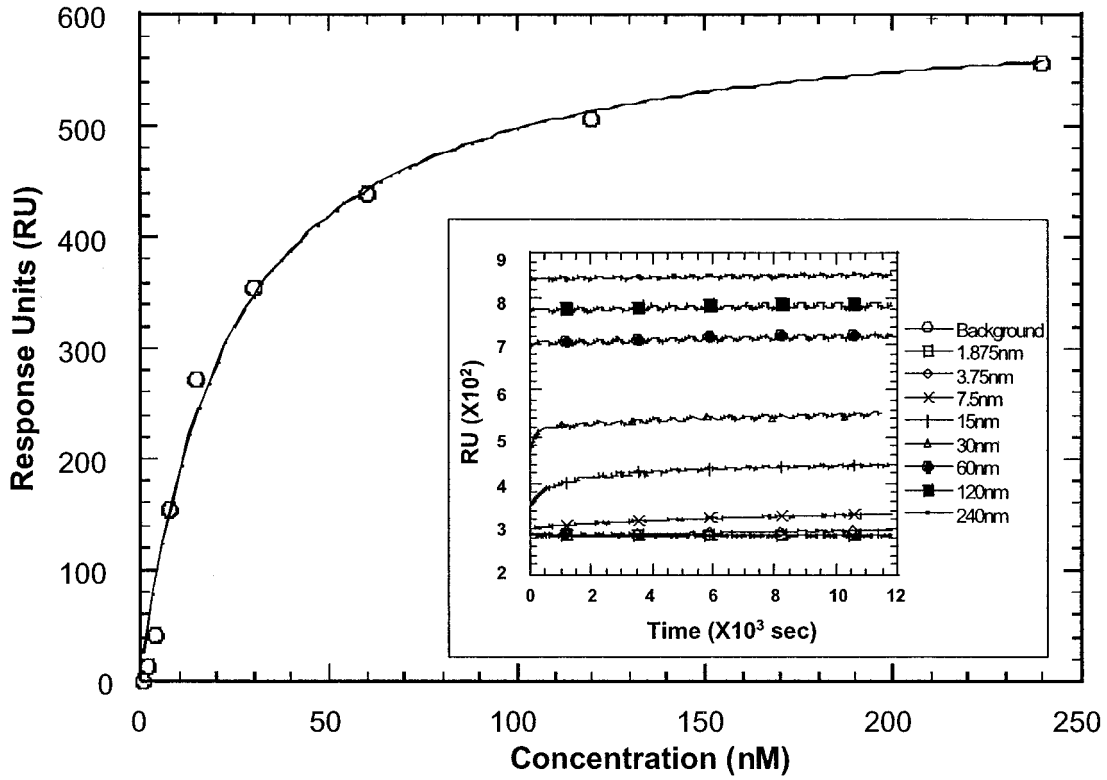

FIG. 7. **Byr2-SAM/Ste4-LZ-SAM-A binding isotherm.** Byr2-SAM was cross-linked to the Biacore chip as described under "Experimental Procedures." Equilibrium response units *versus* concentration of Ste4-LZ-SAM-A polypeptide monomers are plotted. The dissociation constant was determined by fitting the data to a hyperbolic binding equation describing the equilibrium between free and bound forms of Ste4-LZ-SAM-A with monomeric Byr2-SAM. The *solid line* shows the fitted curve. The *inset* shows raw data (response units as a function of time in min) obtained from the surface plasmon resonance instrument upon titrating with increasing concentrations of Ste4-LZ-SAM-A.

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-

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 + Byr2-SAM	19,470	18,643	Dimer	1:1 (K_d ~ 56 μM)	
Ste4-LZ	29,100	29,073	Trimer	-	
Ste4-LZ-SAM-A	57,500	57,216	Trimer	-	
Ste4-LZ-SAM-A + Byr2-SAM	67,300	67,600	-	3:1 (K_d ~ 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 by 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 Raf-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 Polyhomeotic 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.

Acknowledgments—An *S. pombe* cDNA library was generously provided by Sean Hemmingsen at the NRC in Saskatoon, Canada. We thank A. Chamberlain, H. Tran, M. Mathews, D. Grosfeld, F. Qiao, and E. Bare for helpful comments on the manuscript.

REFERENCES

1. Ponting, C. P. (1995) *Protein Sci.* **4,** 1928–1930
2. Schultz, J., Ponting, C. P., Hofmann, K., and Bork, P. (1997) *Protein Sci.* **6,** 249–253
3. Kyba, M., and Brock, H. W. (1998) *Dev. Genet.* **22,** 74–84
4. 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
5. Chi, S. W., Ayed, A., and Arrowsmith, C. H. (1999) *EMBO J.* **18,** 4438–4445
6. Smalla, M., Schmieder, P., Kelly, M., Ter Laak, A., Krause, G., Ball, L., Wahl, M., Bork, P., and Oschkinat, H. (1999) *Protein Sci.* **8,** 1954–1961
7. Stapleton, D., Balan, I., Pawson, T., and Sicheri, F. (1999) *Nat. Struct. Biol.* **6,** 44–49
8. Thanos, C. D., and Bowie, J. U. (1999) *Protein Sci.* **8,** 1708–1710
9. Thanos, C. D., Goodwill, K. E., and Bowie, J. U. (1999) *Science* **283,** 833–836
10. Kim, C. A., Phillips, M. L., Kim, W., Gingery, M., Tran, H. H., Robinson, M. A., Faham, S., and Bowie, J. U. (2001) *EMBO J.* **20,** 4173–4182
11. Kim, C. A., Gingery, M., Pilpa, R. M., and Bowie, J. U. (2002) *Nat. Struct. Biol.* **9,** 453–457
12. Slupsky, C. M., Gentile, L. N., and McIntosh, L. P. (1998) *Biochem. Cell Biol.* **76,** 379–390
13. Mackereth, C. D., Schärpf, M., Gentile, L. N., and McIntosh, L. P. (2002) *J. Biomol. NMR*, in press
14. Thanos, C. D., Faham, S., Goodwill, K. E., Cascio, D., Phillips, M., and Bowie, J. U. (1999) *J. Biol. Chem.* **274,** 37301–37306
15. Kasten, M., and Giordano, A. (2001) *Oncogene* **20,** 1832–1838
16. Seidel, J. J., and Graves, B. J. (2002) *Genes Dev.* **16,** 127–137
17. Carroll, M., Tomasson, M. H., Barker, G. F., Golub, T. R., and Gilliland, D. G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14845–14850
18. Golub, T. R., Goga, A., Barker, G. F., Afar, D. E., McLaughlin, J., Bohlander, S. K., Rowley, J. D., Witte, O. N., and Gilliland, D. G. (1996) *Mol. Cell. Biol.* **16,** 4107–4116
19. Jousset, C., Carron, C., Boureux, A., Quang, C. T., Oury, C., Dusanter-Fourt, I., Charon, M., Levin, J., Bernard, O., and Ghysdael, J. (1997) *EMBO J.* **16,** 69–82
20. Lacronique, V., Boureux, A., Valle, V. D., Poirel, H., Quang, C. T., Mauchauffe, M., Berthou, C., Lessard, M., Berger, R., Ghysdael, J., and Bernard, O. A. (1997) *Science* **278,** 1309–1312
21. Kwiatkowski, B. A., Bastian, L. S., Bauer, T. R., Jr., Tsai, S., Zielinska-Kwiatkowska, A. G., and Hickstein, D. D. (1998) *J. Biol. Chem.* **273,** 17525–17530
22. Poirel, H., Lopez, R. G., Lacronique, V., Della Valle, V., Mauchauffe, M., Berger, R., Ghysdael, J., and Bernard, O. A. (2000) *Oncogene* **19,** 4802–4806
23. Potter, M. D., Buijs, A., Kreider, B., van Rompaey, L., and Grosveld, G. C. (2000) *Blood* **95,** 3341–3348
24. Baker, D. A., Mille-Baker, B., Wainwright, S. M., Ish-Horowicz, D., and Dibb, N. J. (2001) *Nature* **411,** 330–334
25. Peterson, A. J., Kyba, M., Bornemann, D., Morgan, K., Brock, H. W., and Simon, J. (1997) *Mol. Cell. Biol.* **17,** 6683–6692
26. Hughes, D., and Yamamoto, M. (1993) *Signal Transduction: Prokaryotic and Simple Eukaryotic Systems* (Kuriyan, J., and Taylor, B. L., eds) Academic Press Inc., New York
27. Wang, Y., Xu, H. P., Riggs, M., Rodgers, L., and Wigler, M. (1991) *Mol. Cell. Biol.* **11,** 3554–3563
28. Masuda, T., Kariya, K., Shinkai, M., Okada, T., and Kataoka, T. (1995) *J. Biol. Chem.* **270,** 1979–1982
29. Bauman, P., and Albright, C. F. (1998) *Biochimie* (*Paris*) **80,** 621–625
30. Tu, H., Barr, M., Dong, D. L., and Wigler, M. (1997) *Mol. Cell. Biol.* **17,** 5876–5887
31. Dubendorff, J. W., and Studier, F. W. (1991) *J. Mol. Biol.* **219,** 45–59
32. Schatz, P. J., Cull, M. G., Martin, E. L., and Gates, C. M. (1996) *Methods Enzymol.* **267,** 171–191
33. Fodor, S. P. A., Copeland, R. A., Grygon, C. A., and Spiro, T. G. (1989) *J. Am. Chem. Soc.* **111,** 5509
34. Myszka, D. G., Jonsen, M. D., and Graves, B. J. (1998) *Anal. Biochem.* **265,** 326–330
35. Cohn, E. J., and Edsall, J. T. (1943) *Proteins, Amino acids and Peptides as Ions and Dipolar Ions*, pp. 370–381, Reinhold Publishing, New York
36. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, pp. 90–125, The Royal Society of Chemistry, Cambridge, UK
37. Barr, M. M., Tu, H., Van Aelst, L., and Wigler, M. (1996) *Mol. Cell. Biol.* **16,** 5597–5603
38. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255,** 306–312
39. Okazaki, N., Okazaki, K., Tanaka, K., and Okayama, H. (1991) *Nucleic Acids Res.* **19,** 7043–7047
40. Bauman, P., Cheng, Q. C., and Albright, C. F. (1998) *Biochem. Biophys. Res. Commun.* **244,** 468–474

41. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5857–5864
42. Letunic, I., Goodstadt, L., Dickens, N. J., Doerks, T., Schultz, J., Mott, R., Ciccarelli, F., Copley, R. R., Ponting, C. P., and Bork, P. (2002) *Nucleic Acids Res.* **30,** 242–244
43. Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) *Nature* **375,** 554–560
44. Scheffzek, K., Grunewald, P., Wohlgemuth, S., Kabsch, W., Tu, H., Wigler, M., Wittinghofer, A., and Herrmann, C. (2001) *Structure* **9,** 1043–1050
45. 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
46. Zhang, H., Xu, Q., Krajewski, S., Krajewska, M., Xie, Z., Fuess, S., Kitada, S., Godzik, A., and Reed, J. C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2597–2602