Expression of proteins with dimethylarginines in *Escherichia coli* for protein–protein interaction studies

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(RECEIVED November 16, 2006; FINAL REVISION February 1, 2007; ACCEPTED February 22, 2007)

Abstract

Protein arginine methylation often modulates protein–protein interactions. To isolate a sufficient quantity of proteins enriched in methyl arginine(s) from natural sources for biochemical studies is laborious and difficult. We describe here an expression system that produces recombinant proteins that are enriched in ω-N^G,N^G-asymmetry dimethylarginines. A yeast type I arginine methyltransferase gene (*HMT1*) is put on a plasmid under the control of the *Escherichia coli* methionine aminopeptidase promoter for constitutive expression. The protein targeted for post-translational modification is put on the same plasmid behind a T7 promoter for inducible expression of His_6-tagged proteins. Sbp1p and Stm1p were used as model proteins to examine this expression system. The 13 arginines within the arginine-glycine-rich motif of Sbp1p and the RGG sequence near the C terminus of Stm1p were methylated. Unexpectedly, the arginine residue on the thrombin cleavage site (LVPRGS) of the fusion proteins can also be methylated by Hmt1p. Sbp1p and Sbp1p/hmt1 were covalently attached to solid supports for the isolation of interacting proteins. The results indicate that arginine methylation on Sbp1p exerts both positive and negative effects on protein–protein interaction.

Keywords: protein expression; protein arginine methylation; protein–protein interactions; Sbp1p; Stm1p

Supplemental material: see www.proteinscience.org.

The arginine guanidino nitrogen atoms can undergo post-translational methylation. The products after modification are: δ-monomethylarginine (δ-MMA), ω-monomethylarginine (ω-MMA), asymmetric ω-N^G,N^G-dimethylarginine (aDMA), and symmetric ω-N^G,$N^{G'}$-dimethylarginine (sDMA).

A family of isozymes, protein arginine *N*-methyltransferases (PRMTs), carries out these reactions (Gary and Clarke 1998; Niewmierzycka and Clarke 1999).

There are two major types of PRMTs (Gary and Clarke 1998). The type I enzymes catalyze the formation of ω-MMA and aDMA. The type II enzymes perform the conversion of arginine to ω-MMA and sDMA. In budding yeast, Hmt1p is a type I enzyme responsible for most of the arginine N-methylation reactions (Gary et al. 1996). Hsl7p was initially reported as a type II enzyme (Lee et al. 2000). A recent report suggests that it is a type III enzyme that synthesizes ω-MMA (Miranda et al. 2006). There is also a type IV enzyme (RMT2) in budding yeast that is accountable for the formation of δ-MMA (Niewmierzycka and Clarke 1999; Chern et al. 2002).

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Abbreviations: PRMTs, protein arginine N-methyltransferases; aDMA, asymmetric ω-N^G,N^G-dimethylarginine; sDMA, symmetric ω-N^G,$N^{G'}$-dimethylarginine; ω-MMA, ω-monomethylarginine; [^{3}H]AdoMet, S-Adenosyl-L-[*methyl*-^{3}H]methionine; CHCA, α-cyano-4-hydroxy-cinnamic acid; Sbp1p/hmt1, Sbp1p coexpressed with *HMT1*; Stm1p/hmt1, Stm1p coexpressed with *HMT1*.

Article and publication are at http://www.proteinscience.org/cgi/doi/10.1110/ps.062667407.

The substrates of PRMTs participate in various cellular functions such as RNA processing, transcriptional regulation, signal transduction, and DNA repair (Bedford and Richard 2005). Methylation of arginine residue(s) on these proteins often modulates protein–protein interactions. For instance, methylation of Sam68 inhibits its binding to the SH3 (Src homology 3) domains of $p59^{fyn}$ and phospholipase Cγ-1 (Bedford et al. 2000). In contrast, SMN (survival of motor neurons protein) preferentially interacts with methylated SmB and SmD (Friesen et al. 2001).

Purification of methylated proteins from natural sources for biochemical studies can be a difficult task. The target protein might not be abundantly expressed. Furthermore, the methyl groups on the protein might not exist in stoichiometric amounts (Chern et al. 2002; Ostareck-Lederer et al. 2006). The isolated proteins can be a mixture of methylated and unmodified polypeptides. Thus, characterization of a particular form of the protein is difficult.

We circumvent this difficulty by coexpressing the protein to be methylated with Hmt1p. Sbp1p and Stm1p were used as model proteins to test this expression system. Sbp1p is a 33-kDa, single-strand nucleic acid-binding protein. It has a large arginine-glycine-rich domain that includes 1 RGR, 5 RGG, and 3 RGRGG repeats (totaling 13 arginines from residues 125–167). It has been suggested as a substrate of Hmt1p (Frankel and Clarke 1999). Stm1p binds quadruplex and triple helical nucleic acids (Frantz and Gilbert 1995; Nelson et al. 2000). It has been implicated in telomere structure maintenance (Hayashi and Murakami 2002) and signaling of apoptosis-like cell death (Ligr et al. 2001). Stm1p has a single RGG motif near the C terminus of the protein (^{234}NNRRGGRGARK^{244}). We report here the characterization of the coexpressed proteins, Sbp1p/hmt1 and Stm1p/hmt1. Furthermore, Sbp1p and Sbp1p/hmt1 were used as baits in pull-down experiments. Results suggest Nop13p, Mas1p, and Rpl16Ap interact primarily with Sbp1p/hmt1.

Results

Expression of recombinant proteins

The expression plasmid for the production of Sbp1p/hmt1 is presented in Figure 1. The gene encoding the type I arginine methyltransferase, *HMT1*, is put on a plasmid under the control of the *Escherichia coli* methionine aminopeptidase promoter for constitutive expression. Thus, the modification enzyme is readily available for the methylation reaction before the biosynthesis of the target proteins. The gene encoding the protein to be modified is put under the control of a strong T7 promoter for inducible expression. The recombinant proteins have an N-terminal His_6-tag for affinity purification. With this construct, we routinely obtain over 20 mg of purified Sbp1p/hmt1 from a liter of cell medium.

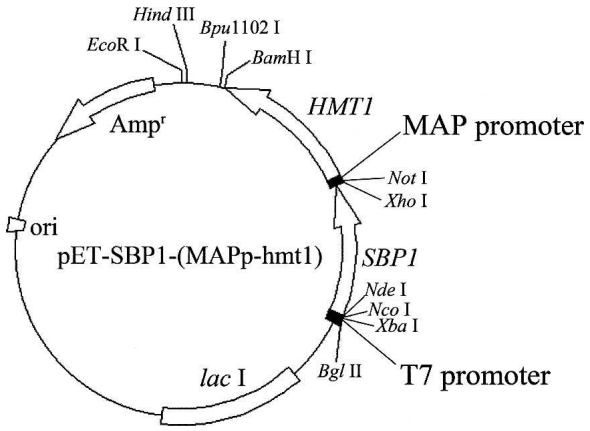

Figure 1. Plasmid constructed for the expression of proteins with dimethylarginines. Sbp1p is the protein targeted for modification. The restriction sites and the promoters are labeled.

In vitro methylation of recombinant proteins expressed with and without HMT1

Purified recombinant proteins were incubated with Hmt1p and [^{3}H]AdoMet in an in vitro assay. Proteins that have been methylated in vivo will not be labeled with tritium. In this set of experiments, Sbp1p was readily tritiated (Fig. 2B). A small amount of radioisotopes can also be detected on Sbp1p/hmt1 (Fig. 2B). The fluorogram was exposed for 24 h. According to the relative intensity of the tritium labels on the fluorogram, we can conclude confidently that most, but not all of the arginines available for methylation have been modified in vivo.

To ensure our system can produce methylated proteins other than Sbp1p/hmt1, Stm1p/hmt1 was used as substrate in the in vitro methylation assay. Stm1p has a single RGG motif near the C terminus of the protein (^{234}NNRRGGRGARK^{244}). By site-directed mutagenesis, we have affirmed that Arg237 is the only residue on Stm1p that can be modified by Hmt1p in vitro (see below). The His_6-tagged Stm1p is ~32 kDa in size, but migrates close to 37 kDa on a denaturing polyacrylamide gel (Fig. 2A). Stm1p is prone to degradation during purification. Since the N-terminal His_6-tagged Stm1p was purified with metal-affinity beads, polypeptides <28 kDa should not contain the methylation site. As shown on the fluorogram (exposed for 43 d), Stm1p was methylated, while Stm1p/hmt1 was not labeled (Fig. 2B). The

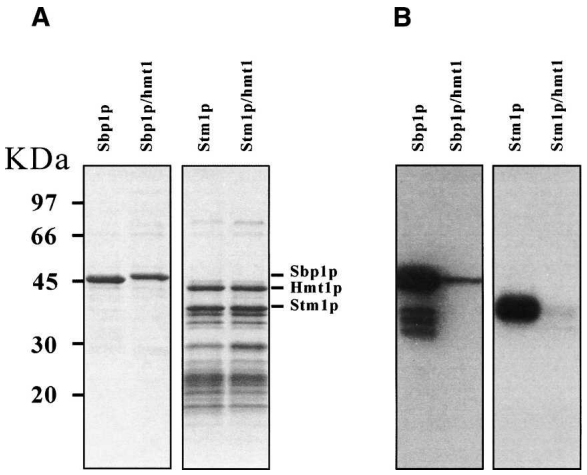

Figure 2. In vitro methylation of Sbp1p, Sbp1p/hmt1, Stm1p, and Stm1p/hmt1. Recombinant proteins after in vitro methylation assays were separated by SDS-PAGE and visualized by dye staining (*A*) or fluorography (*B*). The positions of Sbp1p, Hmt1p, and Stm1p on the gel are labeled.

result implies that Stm1p/hmt1 was methylated in *E. coli* cells. Therefore, the expression system we present here is not specific for Sbp1p; it can methylate proteins with RGG motif in general.

Mass spectrometric analysis of recombinant proteins

The purified recombinant proteins were analyzed with mass spectrometry. We obtained an average molecular mass of 34,998 ± 3 Da (calculated molecular mass 34,994 Da, Fig. 3A, peak a) for Sbp1p. The recombinant Sbp1p/hmt1 has an average mass of 35,365 ± 3, an increase of 367 Da (Fig. 3B, peak a′). The result implies an addition of 26 methyl groups onto Sbp1p. However, components with mass higher than that of the unmodified Sbp1p but lower than that represented by peak a′ can be detected on the mass spectrum (Fig. 3B, denoted with asterisks). Probably, they represent Sbp1p/hmt1 carrying <26 methyl groups.

Peaks that are 178 ± 2 Da higher in mass than that of Sbp1p or Sbp1p/hmt1 are detected on the deconvoluted mass spectra (Fig. 3A,B, peaks b and b′, respectively). His_6-tagged Sbp1p or Sbp1p/hmt1 was digested with *Achromobacter protease* I. The N-terminal fragments were isolated for MALDI-TOF mass spectrometric analysis. The spectrum for peptides derived from Sbp1p/hmt1 is presented in Figure 3C. A similar spectrum was obtained for the Sbp1p sample (data not shown). Two peptides (N and N′, m/z = 1741.1 and 1919.1) with mass difference of 178 Da can be detected on the mass

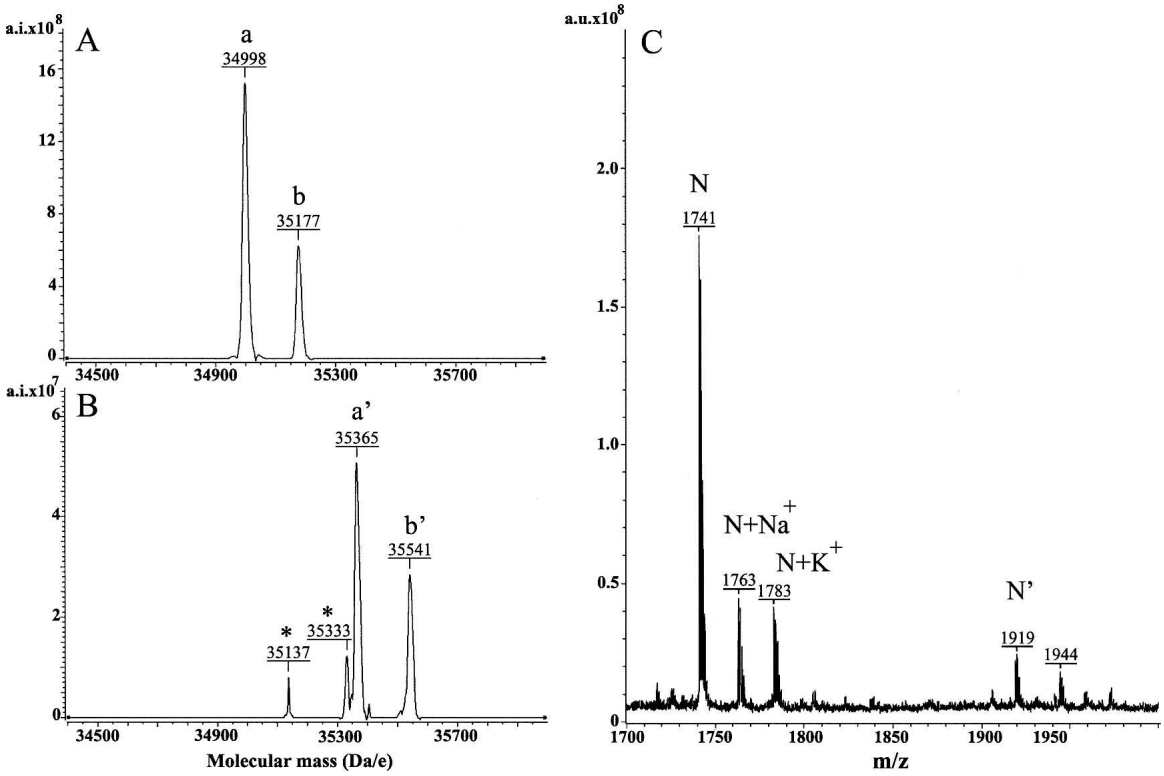

Figure 3. Mass spectrometric analyses of Sbp1p, Sbp1p/hmt1, and their N-terminal fragments. Deconvoluted mass spectra of Sbp1p (*A*) and Sbp1p/hmt1. (*B*) obtained on a FT-ICR mass spectrometer equipped with an electrospray ionization source. (*C*) MALDI-TOF mass spectrum of the N-terminal fragments of Sbp1p/hmt1 isolated from an *Achromobacter* protease 1 digests.

spectrum. The calculated m/z for the N-terminal fragment of the recombinant Sbp1p (GSSHHHHHHSSGLVPK) is 1740.8, which is represented by peak N in Figure 3C. Therefore, the His_6-tag of recombinant Sbp1p and Sbp1p/hmt1 are partially modified, and this modification is not attributed to the presence of the coexpressed Hmt1p. Furthermore, the mass difference between peaks b and b′ is 364 Da. The result is in close agreement with that of the mass difference observed for peaks a and a′ (Fig. 3A,B). Hence, proteins represented by peak b′ possibly have 26 methyl groups added.

The affinity-purified Stm1p and Stm1p/hmt1 were further separated from its degraded products on a C_{18} reverse-phase column. The full-length proteins were collected for mass spectrometric analysis. Unlike Sbp1p, the N-terminal of the recombinant Stm1p was not partially modified. We obtained an average molecular mass of 32,001 ± 2 Da (calculated average molecular mass 31,999 Da) for Stm1p. Stm1p/hmt1 has an average molecular mass of 32,027 ± 2 Da. The results imply an addition of two methyl groups onto Stm1p.

Amino acid analysis of Sbp1p/hmt1

To ensure the recombinant proteins have the expected arginine methylation, Sbp1p/hmt1 was subjected to amino acid analysis. The chromatograms of the phenylthiocarbamyl amino acids are presented in Figure 4. As expected, Sbp1p/hmt1 carries aDMA (Fig. 4B), while recombinant Sbp1p lacks this residue (Fig. 4C). A small amount of MMA can also be detected in the Sbp1p/hmt1 sample (Fig. 4B). Contrary to that reported by Lim et al. (1998), we observed consistently that aDMA was eluted from the HPLC column in front of sDMA. The discrepancy was possibly due to the different columns used in the amino acid separation.

Methylation sites on Sbp1p

Sbp1p has an arginine-glycine-rich domain encompassing residues 125–167 (^{119}TPGQMQRGGFRGRGGFRGRGG FRGGFRGGYRGGFRGRGNFRGRGGARGGF^{168}). Sbp1p and Sbp1p/hmt1 were digested with chymotrypsin, and the resulting peptides were analyzed on a MALDI-TOF mass spectrometer. The mass spectra representing the region from m/z 425 to m/z1125 are shown in Figure 5. Six ion peaks possibly representing chymotryptic peptides originated from the arginine-glycine-rich region of Sbp1p can be detected (Fig. 5A). The m/z value of these peaks and the sequences of the inferred peptides are summarized in Table 1. These peptides cover the whole arginine-glycine-rich region. Ions that can be observed in the Sbp1p/hmt1, but absent from the Sbp1 sample, are labeled in Figure 5B. They possibly present modified peptides. The results

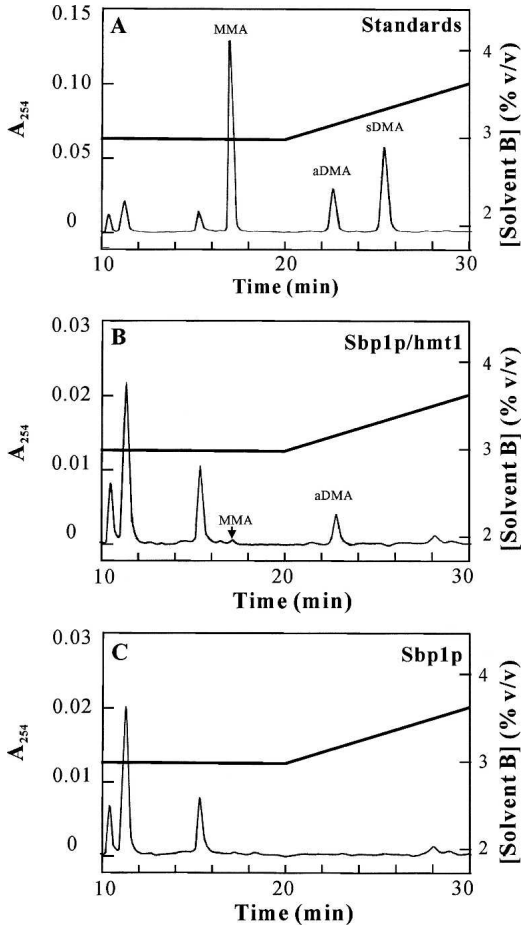

Figure 4. Amino acid analysis of Sbp1p and Sbp1p/hmt1. The elution profile of amino acid standards (*A*), acid hydrolysates from Sbp1p/hmt1 (*B*), and Sbp1p (*C*) are presented with the phenylthiocarbamyl MMA, aDMA, and sDMA peaks labeled.

are summarized in Table 1 and imply that arginines within this region were heavily dimethylated. However, some of the arginine-containing peptides were not methylated (m/z 649.3 and m/z 1078.4) or carried only monomethyl arginines (m/z 691.3, m/z 1060.5, and m/z 1092.4).

Methylation site on Stm1p

Purified His_6-Stm1p or its mutants (R236K, R237K, R240K, and R243K) were incubated with recombinant Hmt1p and [^{3}H]AdoMet. These recombinant His_6-tagged proteins have intact thrombin cleavage site. After the in vitro methylation assay, the proteins were isolated on gels and the methylated proteins were visualized by fluorography. As shown on Figure 6B, Hmt1p can methylate Stm1p (lane 1) and mutants with Arg236 (lane 2), Arg240 (lane 4), or Arg243 (lane 5) to lysine substitution. A mutant with Arg237 to lysine (R237K, lane 3) substitution drastically reduced the amount of methylated proteins,

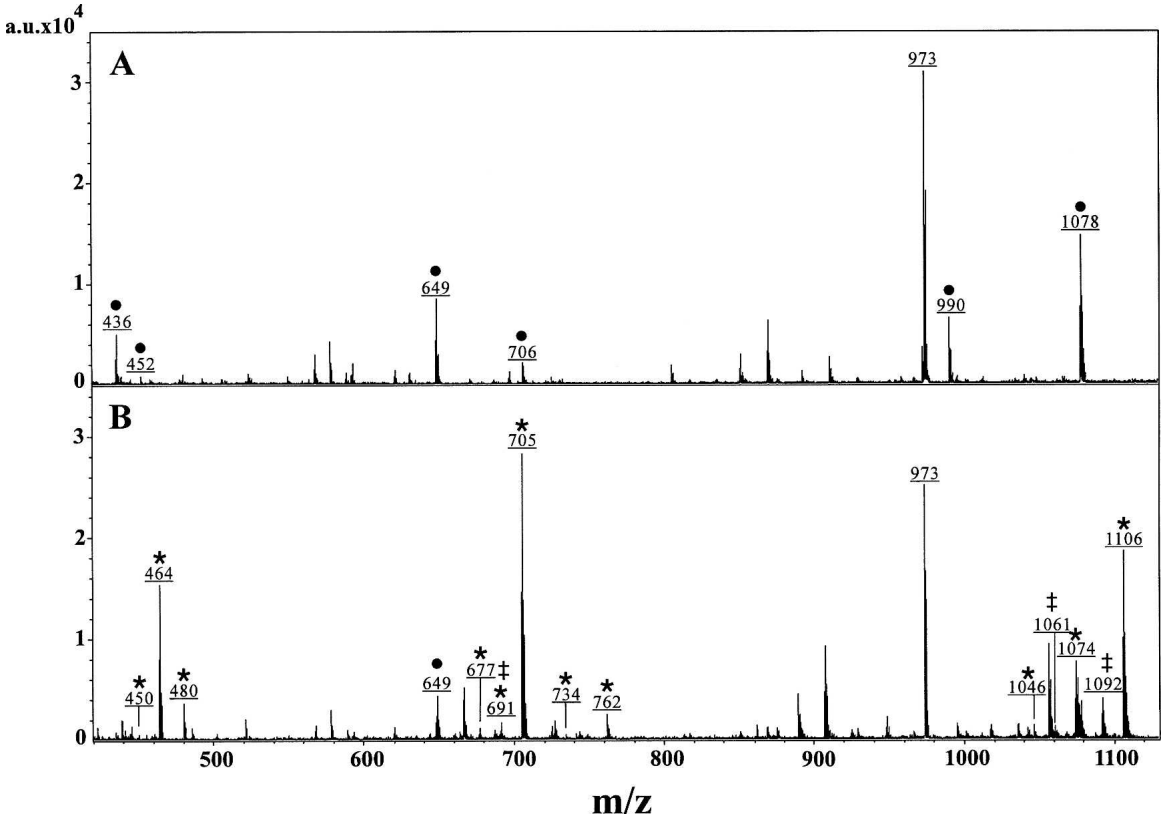

Figure 5. MALDI-TOF mass spectra of Sbp1p (*A*) and Sbp1p/hmt1 (*B*) chymotrypsin digests. Ions with m/z value match with those deduced from the arginine-glycine-rich region of Sbp1p are marked (●). Ions that are possibly derived from peptides with mono- and dimethylarginine(*s*) are labeled (‡) and (*), respectively.

suggesting that Arg237 is the major methylation site. The His_6-Stm1p R237K mutant with an additional arginine to lysine replacement at the thrombin cleavage site (LVPRGS) is no longer a substrate of Hmt1p (Fig. 6A,B, lanes 6). This is the first report that the thrombin cleavage site on recombinant proteins can be modified by Hmt1p.

We coexpressed the Stm1p R236K, R237K, R240K, or R243K mutant with Hmt1p, and the proteins were purified for mass spectrometric analysis. We obtained an average molecular mass of 31,972 ± 2 Da for the R237K mutant (calculated average molecular mass 31,971 Da). Experimentally, the R236K, R240K, and R243K mutants have an average molecular mass of 32,000 ± 1 Da. The results show clearly that among the four mutants, only R237K was not methylated.

Proteins interact with Sbp1p or Sbp1p/hmt1

Sbp1p and Sbp1p/hmt1 were covalently linked to solid supports for pull-down assays. The interacting proteins were resolved on a denaturing polyacrylamide gel and presented in Figure 7. Noticeably, the Sbp1p sample has several bands (2, 3, 6, 11, 12, and 13) with higher dye-binding intensity than that of the Sbp1p/hmt1 sample, while band four of the Sbp1p/hmt1 sample is more intense than that of the Sbp1p sample. Apparently, methylation of Sbp1p changes its affinity, positively or negatively, toward several proteins. The protein patterns on Figure 7 are highly reproducible. The pull-down experiments have been performed four times, and we could not detect significant differences in protein patterns among the gels.

Protein bands as designated in Figure 7 were subjected to in-gel digestion and peptide mapping analysis on a MALDI-TOF instrument. Proteins identified after database search are summarized in Table 2. We identified 17 proteins that interact with Sbp1p. With the exception of Mam33p, all of them also interact with Sbp1p/hmt1. However, seven of the proteins (Imd3p, Tef1p, Hmt1p, Rps8Bp, Rps9Bp, Rpl7Ap, and Rpl13Bp) show decreased interacting affinity with Sbp1p/hmt1, the methylated protein. Furthermore, Sbp1p/hmt1 binds three additional proteins: Nop13p (band 3), Mas1p (band 4), and Rpl16Ap (band 13). The mass spectra representing the peptide digests from bands 3, 4, and 13 are provided as Supplemental materials (Supplemental Figs. S1–S3).

Table 1. *Mass of peptides from the arginine-glycine-rich domain of Sbp1p and Sbp1p/hmt1 after chymotrypsin digestion*

			Observed m/z	
Residues	Sequence	Calculated m/z	Sbp1p	Sbp1p/hmt1
141–144;	RGGF	436.2	436.2	*ND*
149–152	+2 Me	464.2	*ND*	464.2
145–148	RGGY	452.2	452.2	*ND*
	+ 2 Me	480.2	*ND*	*480.2*
129–134;	RGRGGF	649.3	649.3	649.3
135–140	+2 Me	677.3	*ND*	677.3
	+ 3 Me	691.3	*ND*	691.3
	+ 4 Me	705.3	*ND*	705.3
153–158	RGRGNF	706.4	706.3	*ND*
	+ 2 Me	734.4	*ND*	734.4
	+ 4 Me	762.4	*ND*	762.3
159–168	RGRGGARGGF	990.5	990.5	*ND*
	+ 4 Me	1046.5	*ND*	1046.5
	+ 5 Me	1060.5	*ND*	1060.5
	+ 6 Me	1074.5	*ND*	1074.5
119–128	TPGQMQRGGF	1078.5	1078.5	1078.5
	+ 1 Me	1092.5	*ND*	1092.4
	+ 2 Me	1106.5	*ND*	1106.4

(*ND*) not detected; (Me) methyl group.

Discussion

We present here a method to prepare recombinant proteins with methylated arginine(s). The method circumvents the difficult task of isolating a particular form of protein (unmodified or methylated) from natural sources. It has long been recognized that Hmt1p modifies proteins with RGG and RXR sequences (Smith et al. 1999). Proteins with single (Stm1p) and multiple (Sbp1p) methylation sites were selected to test our expression system.

The recombinant Sbp1p and Sbp1p/hmt1 were analyzed by mass spectrometry. According to the mass difference between Sbp1p and Sbp1p/hmt1, the modified proteins can carry up to 26 methyl groups (Fig. 3). Most of these methyl groups are added onto the 13 arginine residues within the arginine-glycine-rich domain of the protein, as demonstrated by the peptide maps of the chymotrypsin digests (Fig. 5; Table 1). Some of the His_6-tags on the recombinant proteins have been modified with an addition of 178 Da (Fig. 3C). Geoghegan et al. (1999) have reported the isolation of modified His-tags from several recombinant proteins expressed in *E. coli*. The α-amino group was blocked with a gluconoyl moiety and an addition of 178 Da. Possibly, some of the recombinant Sbp1p has an α-*N*-D-gluconoyl-His_6-tag.

Stm1p and Stm1p/hmt1 are unstable and fragmented easily during the purification process. The full-length proteins for mass measurement can be obtained with an additional chromatographic step on a reverse-phase column. The difference in mass between Stm1p (32,001 ± 3 Da) and Stm1p/hmt1 (32,027 ± 2 Da) implies an addition of two methyl groups. However, repeated peptide-mapping experiments on a MALDI-TOF instrument were unable to confirm the methylation site on Stm1p/hmt1.

Stm1p has a RGG motif near the C terminus of the protein (^{233}FNNRRGGRGARKG^{245}) and Arg237 within this motif is the most probable methylation site. We generated single mutants by replacing the arginines between residues 233 and 245 with lysine. The proteins were used as substrates in an in vitro methylation assay. The Stm1p R237K mutant is a poor substrate for Hmt1p (Fig. 6A,B, lanes 6). We can abolish the methylation reaction by replacing the arginine on the thrombin cleavage site of the R237K mutant with lysine (Fig. 6A,B, lanes 6). Therefore, Arg237 on Stm1p is the methylation site.

Even though the thrombin cleavage site (LVPRGS) does not contain an RGG or RXR sequence, it can be methylated by Hmt1p (Fig. 6B, lanes 3, 6). The same modification has been observed on recombinant Hmt1p with N-terminal His_6-tag fusion (T.-C.S. Tam, L.-F. Liu, and M.F. Tam, unpubl.). The modified proteins are resistant to thrombin cleavage.

To affirm the Stm1p methylation site, the R236K, R237K, R240K, and R243K mutants were coexpressed with Hmt1p. The proteins were isolated for mass analysis. As expected, the R237K mutant (31,972 ± 2 Da) is ~28 Da lighter than the other three mutants (32,000 ± 1 Da). Hence, Arg237 is the methylation site on Stm1p.

Sbp1p is a 33-kDa, single-strand nucleic acid-binding protein involved in RNA metabolism. Gavin et al. (2006) screened for protein complexes in *Saccharomyces cerevisiae*, and Sbp1p was detected in seven different complexes that are composed of at least 82 proteins. We identified 20 Sbp1p and Sbp1p/hmt1 interacting proteins, and 13 of them are among the list (Rpl4Ap, Rpl7Ap, Rpl8Bp, Rpl13Bp, Rpl16Ap, Rpl20Bp, Rps4Ap, Rps8Bp, Rps9Bp, Imd3p, Tef1p, Nop13p, and Nop1p) provided by Gavin et al. (2006).

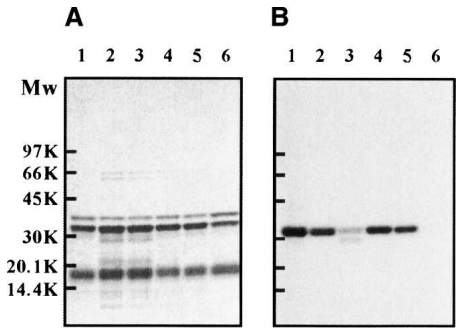

Figure 6. Identification of the major methylation site on Stm1p. Recombinant wild-type Stm1p (lanes *1*), the Stm1p R236K (lanes *2*), the Stm1p R237K (lanes *3*), the Stm1p R240K (lanes *4*), the Stm1p R243K (lanes *5*) mutants, and the Stm1p R237K mutant with an additional thrombin cleavage site arginine to lysine substitution (lanes *6*) were subjected to in vitro methylation assay. The resulting mixtures were subjected to SDS-PAGE analysis and the proteins were visualized with Coomassie dye staining (*A*) or fluorography (*B*). The fluorogram represents a 20-h exposure.

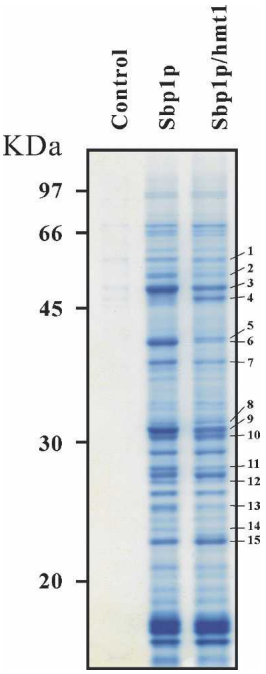

Figure 7. SDS-PAGE analysis of Sbp1p and Sbp1p/hmt1 interacting proteins. Proteins eluted from an Affi-Gel 10 column without proteins covalently attached were used as blank. Bands selected for in-gel digestion and mass spectrometric analysis are numbered.

Mam33p was detected only in the Sbp1p sample, together with Rps4Ap and Rpl2Bp (Fig. 6, band 9; Table 2). It is an acidic protein of the mitochondrial matrix and participates in oxidative phosphorylation (Seytter et al. 1998). Mam33p also binds Tef1p (Gavin et al. 2006), which has a higher affinity toward Sbp1p than Sbp1p/hmt1 (Fig. 7, band 2). We speculate that Mam33p does not interact directly with Sbp1p. Possibly, Mam33p was pulled down together with Tef1p.

Sbp1p is an in vivo substrate of Hmt1p. Sbp1p isolated from wild-type BY4741 cells carries arginine methylation. This modification is absent from Sbp1p isolated from BY4741 with *HMT1* deletion (C.H. Hsieh, L.F. Liu, and M.F. Tam, in prep.). We observed a strong interaction between these two proteins (Fig. 7, band 6). Interestingly, Sbp1p/hmt1 is no longer a Hmt1p substrate, and this protein–protein interaction is abolished almost completely.

Nop13p is a nucleolar protein of unknown function (Wu et al. 2001) and detected only in the Sbp1p/hmt1 sample (Fig. 7, band 6; Supplemental Fig. S1). Nop13p can interact with Imd3p (Ho et al. 2002). Since the amount of Imd3p in the Sbp1p/hmt1 sample is significantly less than that in the Sbp1p sample (Fig. 7, band 6), we propose that Nop13p interacts with Sbp1p/hmt1 without the participation of Imd3p.

The extra protein band (band 4) in the Sbp1p/hmt1 sample represents the recombinant Sbp1p and Mas1p (Supplemental Fig. S2). Sbp1p and Sbp1p/hmt1 are dimeric in solution. Sbp1p/hmt1 dimers dissociate more readily than Sbp1p dimers under acid condition (data not shown). Probably, a minor population of Sbp1p/hmt1 did not have both subunits covalently bound to the solid supports. The dimers dissociated during the elution step in the presence of trifluoroacetic acid and mixed with the interacting proteins.

Mas1p is the smaller subunit of the mitochondrial processing protease (Witte et al. 1988). It also interacts with Rpl16Ap, another protein detected only in the Sbp1p/hmt1 sample (Fig. 7, band 13; Table 2). Further experiments are needed to determine whether Mas1p interacts directly with Sbp1p/hmt1.

There are 11 and 12 ribosomal proteins that interact with Sbp1p and Sbp1p/hmt1, respectively. Among them Rps8Bp, Rps9Bp, Rpl7Ap, and Rpl13Bp (Fig. 7, bands 11, 12, 13) have decreased affinity while Rpl16Ap (Fig. 6, band 13; Supplemental Fig. S3) can only be found in the Sbp1p/hmt1 sample. Additional experiments are needed to elucidate whether Sbp1p arginine methylation would affect ribosome assembly/processing.

Protein arginine methylation can exert either a positive or negative effect on protein–protein interactions. For example, arginine methylation inhibits the interaction of Npl3p with the TREX protein complex that participates in mRNA transport (Yu et al. 2004). Conversely, methylation of NIP45 promotes its interaction with NF-AT (nuclear factor of activated T cell), resulting in elevated cytokine production (Mowen et al. 2004).

Both positive and negative effects on protein interaction can be observed for the methylated Sbp1p (Fig. 7). For most proteins reported, the interacting proteins are regulated either positively or negatively by arginine methylation (Bedford et al. 2000; Friesen et al. 2001; Mowen et al. 2004; Yu et al. 2004). In this study, we used either completely unmodified or proteins highly enriched with arginine methylation in our experiments. Thus, our data are easier for interpretation.

In summary, we report here an expression system for the preparation of proteins with enriched methyl arginines. These recombinant proteins can be used as baits in pull-down experiments and identify proteins that interact preferentially with either the methylated or unmodified form of the query proteins. This simple expression system can be applied to other proteins with arginine methylation for protein–protein interaction or biochemical studies.

Materials and Methods

Protein expression and purification

The coding sequence of *SBP1* and *STM1* were obtained by PCR using the *S. cerevisiae* genomic DNA as template. The PCR

Table 2. *Identity of proteins interact with Sbp1p and Sbp1p/hmt1*

	Sbp1p				Sbp1p/hmt1			
Band[a]	Interacting protein	Number of matched fragments	Sequence coverage (%)	Mascot score	Interacting protein	Number of matched fragments	Sequence coverage (%)	Mascot score
1	Npl3p	7	22	54	Npl3p	10	33	61
2	Imd3p	12	31	104	Imd3p	7	17	54
3	Tef1p	14	38	137	Tef1p	13	34	102
					Nop13	12	27	82
4	N.D.				Sbp1p	14	44	119
					Mas 1p	11	30	89
5	Rpl4Ap	9	35	63	Rpl4Ap	13	51	118
6	Hmt1p	17	43	168	Hmt1p	16	45	173
7	Nop1p	11	31	65	Nop1p	10	26	81
8	Rps6Ap	15	42	179	Rps6Ap	9	36	94
9	Rps4Ap	11	39	96	Rps4Ap	7	33	128
	Rp12Bp	15	63	146	Rp12Bp	10	52	56
	Mam33p	9	39	86				
10	Rpl8Bp	15	47	164	Rpl8Bp	14	42	124
11	Rps8Bp	13	51	146	Rps8Bp	11	40	152
	Rp17Ap	10	39	96	Rp17Ap	9	37	82
12	Rpl13Bp	18	62	181	Rpl13Bp	13	52	174
13	Rps9Bp	14	58	105	Rps9Bp	11	40	148
					Rp116Ap	8	29	63
14	Rpl17Bp	13	57	121	Rpl17Bp	11	51	111
15	Rpl20Bp	10	45	76	Rpl20Bp	10	39	74

[a] Bands are numbered according to Figure 7.
(N.D.) Negative results from database search.

products have NdeI and XhoI cutting sites at the 5′ and 3′ ends, respectively. The fragments were restricted and inserted into pET-15b to generate the pET-SBP1 and pET-STM1 plasmids for the expression of His_6-tagged Sbp1p and Stm1p.

HMT1 is available in the laboratory (Chern et al. 2002). It was inserted into pBS-MP (Hwang et al. 1999) to replace the coding region without changing the promoter sequence of the *E. coli* methionine aminopeptidase (MAP) gene (pBS-MAP_p-hmt1). The *HMT1* gene with the MAP promoter was then amplified from pBS-MAP_p-hmt1 and inserted into the NotI site of a pGEX-STM1 plasmid, which is 11 nucleotides 3′ to the stop codon of *STM1*. The *STM1* and *HMT1* genes in tandem were then amplified with 5′ and 3′ primers carrying NdeI and BamHI sites, respectively. The PCR product starts with the initiating codon of *STM1* at the 5′ end and inserted into the pET-15b vector to generate pET-STM1-(MAP_p-hmt1). The sequence on the pET-15b vector encoding the His_6-tag is between the NcoI and NdeI sites. Hence, *STM1* is expressed as an N-terminal His_6-tag fusion protein under the control of a T7 promoter. Finally, the *STM1* on pET-STM1-(MAP_p-hmt1) was replaced with *SBP1* for Sbp1p/hmt1 production (pET-SBP1-[MAP_p-hmt1], Fig. 1). In addition, the arginine residue on the thrombin cleavage site of all of the expression plasmids mentioned above (pET-SBP1, pET-STM1, pET-STM1-[MAP_p-hmt1] and pET-SBP1-[MAP_p-hmt1]) was replaced with a lysine residue. Site-directed mutagenesis of the thrombin cleavage site and generation of the Stm1p R236K, R237K, R240K, and R243K mutants were carried out with QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

E. coli BL21(DE3) cells were used as host for protein expression. Cells transformed with plasmids were grown in Lauia broth at 30°C until the culture reached an absorbance of 0.6 at 600 nm. Isopropyl β-D-1-thiogalactopyranoside was then added (IPTG, 1 mM final concentration) and cells were cultured for 4 h before harvesting. After cell lysis, the recombinant proteins were affinity purified with Ni^2 chelating beads (Novagen) according to the manufacturer's instructions.

Amino acid analysis

Purified proteins were hydrolyzed in the gas phase (1 h at 150°C) with 6 N HCl. The hydrolysates were derivatized with phenylisothiocyanate according to the Pico-Tag amino acid analysis handbook (Waters Corporation). The phenylthiocarbamyl amino acids were separated on a reverse-phase column as reported by Lim et al. (1998), with modifications. Briefly, a Pico-Tag column was maintained at 43°C and equilibrated with 2.5% (v/v) acetonitrile in 70 mM sodium acetate (pH 6.5), and 3.6 mM triethylamine (Solvent A) at a flow-rate of 1 mL/min. After sample loading, the column was washed sequentially with 10 mL of Solvent A and 10 mL of 3% Solvent B (15% [v/v] methanol and 45% acetonitrile [v/v] in water). The methylated arginines were then eluted from the column with a shallow gradient going from 3% to 4% Solvent B in 15 min.

In vitro methylation assay

Reactions were carried out in 50 mM sodium phosphate (pH 7.2), at a final volume of 20 μL. The reaction mixture contained 50 pmols of protein substrates and 0.65 μM (0.83 μCi) of

S-Adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; 63.3 Ci/mmol, 0.55 mCi/mL). The enzyme (Hmt1p) to substrate ratio (mole/mole) was set at 1:2 for Stm1p or Stm1p/hmt1, and 1:20 for Sbp1p or Sbp1p/hmt1. Incubation was carried out at 30°C for 30 min. SDS-PAGE sample buffer was added to stop the methylation reaction and proteins were separated on precast Novex 4%–20% polyacrylamide gel (Invitrogen). Proteins were visualized with Blue-silver stain (Candiano et al. 2004), then dried and subjected to fluorography.

Preparation of yeast extracts

Yeast strain BY4742 with *SBP1* deletion was obtained from Research Genetics. Cells were grown with constant shaking at 30°C in YPD medium (1% [w/v] yeast extract, 2% [w/v] bactopeptone, and 2% [w/v] D-glucose) to 1 O.D. at 600 nm. Cells were collected and resuspended in 50 mM sodium phosphate (pH 7.5), 0.1 M NaCl, and 10% glycerol. Cell breakage was induced by passing the suspension through an EmulsiFlex-C5 homogenizer (Avestin). IGEPAL CA-630, a nonionic detergent, was added to the cell lysates to a final concentration of 1%. The mixture was incubated at 4°C for 30 min with gentle swirling before cell debris and organelles were removed by ultracentrifugation (400,000*g*, 30 min). Proteins in the extracts were quantified by the dye-binding method of Bradford (1976) and used immediately for the pull-down assay.

Pull-down assay

Sbp1p and Sbp1p/hmt1 were covalently linked to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions. The amount of proteins bound onto Affi-Gel 10 was estimated by quantifying the proteins remaining in the coupling solution after the reaction. Equal amounts of Affi-Gel 10 beads with the functional groups blocked were used as controls in the assays. Cell lysates at 50-fold (w/w) excess of linked proteins were incubated with the beads for 16 h at 4°C. The gel slurries were transferred into columns and washed with 10 column volumes of 50 mM sodium phosphate (pH 7.0) and 100 mM NaCl. The salts on the columns were then removed with two bed volumes of water and eluted immediately with 1% trifluoroacetic acid. The acid was removed in vacuo and the proteins were analyzed by SDS-PAGE.

Enzyme digestion

Sbp1p and Sbp1p/hmt1 were digested with *Achromobacter* protease 1 or chymotrypsin. *Achromobacter* protease 1 digestions were carried out overnight in 50 mM NH_4HCO_3 (pH 9.0), 10% (v/v) acetonitrile, and 10% (v/v) glycerol at 42°C, and an enzyme to substrate ratio (w/w) of 1:50. The protein N-terminal fragments were recovered with Ni^{2+} chelating beads and then desalted on $ZipTip_{C18}$ pipette tips (Millipore Corp.) before MALDI-TOF mass spectrometric analysis.

Chymotrypsin digestions were carried out in 100 mM Tris-HCl (pH 7.8), and 10 mM $CaCl_2$. The enzyme to substrate ratio was set at 1:40. Incubation was carried out at 25°C for 2.5 h. The reaction was stopped by lowering the pH of the mixture to four with the addition of 2% trifluoroacetic acid. The mass of the resulting peptides were determined on a MALDI-TOF mass spectrometer.

In-gel digestion of Sbp1p interacting proteins with trypsin was carried out essentially with the accelerated digestion protocol of Havlis et al. (2003), except that the digestion temperature was set at 50°C (Finehout et al. 2005).

Mass spectrometry

The molecular masses of recombinant proteins were determined on a Bruker (Bruker-Daltonics) Apex-IV 7.0e instrument with an Apollo electrospray source. The samples were desalted on $ZipTip_{C4}$ pipette tips and then infused into the instrument at 1.5 μL/min and spectra were recorded in broad-band mode. The instrument was mass calibrated externally with an electrospray calibrant solution (G2421A) purchased from Agilent Technologies immediately prior to sample infusion. Spectra deconvolution was performed with the XMASS software package included with the instrument.

Peptide mass analysis of Sbp1p and Sbp1p/hmt1 chymotrypsin digests and mass determination of the protein N-terminal His_6-tags were performed on a Bruker REFLEX II time-of-flight mass spectrometer equipped with a Scout source and delayed extraction. Samples were prepared on an AnchorChip with α-cyano-4-hydroxycinnamic acid (CHCA) and nitrocellulose as matrix (Wu et al. 2006). Ions from Angiotensin II (1046.54) and CHCA ion clusters (855.07, 644.05, 568.14, and 379.09) or from a mixture of Angiotensin II and peptides generated from Lys-C digestion of horse heart myoglobin (1360.75, 1502.67, and 2859.49) were used as external calibration standards. Detection was set in positive reflector mode, with each mass determination being the average of 100 spectra.

Mass spectra for in-gel digests were acquired in reflector mode on a Bruker Autoflex time-of-flight instrument. Each sample was acquired separately with CHCA/nitrocellulose (Wu et al. 2006) and CHCA/pyridine as matrices. Data from both spectra were combined for database search. The CHCA/pyridine matrix is an acetonitrile solution containing 5 mM pyridine, 2 mg/mL of CHCA, and 0.1% (v/v) trifluoroacetic acid. The preparation is a modification of that reported by Zabet-Moghaddam (2006) for metal sample targets. Equal volumes of sample and matrix were mixed and deposited onto a 600-mm/384 well AnchorChip sample target for data acquisition. Spectra were acquired in positive-ion mode with ions from trypsin fragments (842.51, 1045.56, and 2211.10) as internal standards. Peptide masses obtained with signal to noise (S/N) ratio higher than seven were searched against the NCBInr database using the MASCOT program (http://www.matrixscience.com) for all taxonomy with 100 ppm peptide mass tolerance, one missed cleavage, and oxidized methionine as variable modification.

Electronic supplemental material

Supplemental material includes mass spectra representing tryptic digests of bands 3, 4, and 13 of Figure 7.

Acknowledgments

This work is supported by the National Science Council (NSC 94-2311-B-001-010 to M.F.T.) and Academia Sinica, Taiwan, Republic of China.

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