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Yeast Ribosomal/Cytochrome *c* SET Domain Methyltransferase Subfamily

*IDENTIFICATION OF Rpl23ab METHYLATION SITES AND RECOGNITION MOTIFS**

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Ribosomal protein L23ab is specifically dimethylated at two distinct sites by the SET domain-containing enzyme Rkm1 in the yeast *Saccharomyces cerevisiae*. Using liquid column chromatography with electrospray-ionization mass spectrometry, we determined that Rpl23ab purified from the Δ*rkm1* deletion strain demonstrated a loss in mass of ~56 Da when compared with Rpl23ab purified from the wild type strain. When Rpl23ab was proteolyzed, using proteinase ArgC or CNBr, and the peptides derived were analyzed by tandem mass spectrometry, no sites of methylation were found in Rpl23ab purified from the Δ*rkm1* deletion strain, whereas two sites of dimethylation were observed in the wild type strain at lysine residues 105 and 109. We show that both Rpl23a and Rpl23b are expressed and methylated *in vivo* in yeast. Using polysomal fractionation, we demonstrate that the deletion of *RKM1* has no effect on ribosomal complex formation. Comparison of SET domain methyltransferase substrates in yeast reveal sequence similarities around the lysine methylation sites and suggest that an (Asn/Pro)-Pro-Lys consensus sequence may be a target for methylation by subfamily 2 SET domain methyltransferases. Finally, we show the presence of Rkm1 homologs in fungi, plants, and mammals including humans.

SET domain methyltransferases are a new family of methyltransferases known to specifically methylate lysine residues in a variety of proteins (1, 2). The family was named after the *Drosophila* genes in which it was first discovered, Su(var), Enhancer of zeste, and Trithorax (3), all of which were later shown to encode histone lysine methyltransferases (4–6). The SET domain methyltransferases differ from other methyltransferases with known structures in that its catalytic core forms a knot-like structure with a conserved tyrosine residue in the active site necessary for catalysis (6–12). SET domain methyltransferases have been shown to modify a variety of proteins including Rubisco[3] (13, 14), cytochrome *c* (15), and most notably histones (16). Currently, no physiological effect has been observed for the lysine methylation of Rubisco or cytochrome *c* (14, 15). However, in histones lysine methylation has been shown to play an important role in the activation or repression of transcription through modulating the structure of chromatin (16).

Histone tails are highly decorated by lysine methylation reactions and the majority of these modifications occur in close proximity and can exhibit opposite effects. The most well characterized biology involves the interplay between the methylation of lysine 4 and lysine 9 of mammalian histone H3, catalyzed by distinct SET domain methyltransferases (16). Methylation of lysine 4 is correlated with active transcription and euchromatin, whereas methylation of lysine 9 is associated with repressed transcription and heterochromatin (17–20). In yeast, histone H3 methylation has also been actively studied and a similar interplay has been observed, although the distance between the methylated sites is greater. Histone H3 lysine 4 methylation by Set1 is associated with areas of active transcription, whereas lysine 36 methylation by Set2 is associated with the repression of transcription (21–23).

The involvement of SET methyltransferases in translation mediated by the methylation of ribosomal proteins has been studied to a much lesser extent. Currently in yeast only two SET ribosomal protein lysine methyltransferases have been identified. We previously demonstrated that ribosomal lysine (K) methyltransferase 1 (Rkm1) is able to dimethylate the ribosomal protein L23ab at two distinct sites (24). We also identified a second SET domain methyltransferase Rkm2 that is responsible for trimethylating the ribosomal protein L12ab (Rpl12ab) at lysine 10 (25). In the study of Rpl12ab we identified a second site of lysine methylation at position 3, which we postulate is added by an as yet unidentified methyltransferase (25). It is interesting to note that in Rpl12ab, as in the histone H3 tail, the methyl lysine modifications occur in close proximity to each other.

Ribosomal proteins are also methylated at arginine residues in reactions similar to those that occur in histone tails (16). In *Saccharomyces cerevisiae*, the arginine methyltransferase 2

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[3] The abbreviations used are: Rubisco, ribulose-bisphosphate carboxylase/oxygenase; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; Rkm1, ribosomal lysine (K) methyltransferase 1; Rkm2, ribosomal lysine (K) methyltransferase 2; LC-MS+, liquid chromatography with electrospray-ionization mass spectrometry and fraction collection; MSMS, tandem mass spectrometry; HPLC, high performance liquid chromatography.

(Rmt2) enzyme catalyzes the δ-monomethylation of arginine 66 in Rpl12ab (26). In the fission yeast, *Schizosaccharomyces pombe*, the Prmt3 homolog (Rmt3) was found to asymmetrically dimethylate an arginine residue in the ribosomal small subunit protein S2 (27). The exact role the modification plays is still unknown, although it does appear to be important for small subunit stability, and in the absence of Rmt3 ribosome biogenesis is increased (27, 28). The mammalian PRMT3 was also shown to asymmetrically dimethylate S2 (29). How all of these modifications, both at lysine and arginine residues, act cooperatively or antagonistically to regulate the function of the ribosome in translation remains to be determined.

In this study, the Rkm1 substrate Rpl23ab was analyzed by mass spectrometry to demonstrate that the sites of dimethylation are located at lysine residues 105 and 109. In addition, we demonstrate that both isoforms are equally methylated and that deleting the *RKM1* gene does not have an apparent effect on ribosomal complex stability. Future studies will be necessary to determine the role Rpl23ab lysine methylation plays in translation.

EXPERIMENTAL PROCEDURES

Yeast Ribosomal Large Subunit Purification—Strains used in this study are listed in Table 1. Large ribosomal subunits derived from wild type and Δ*rkm1 S. cerevisiae* were purified as described previously (25).

Liquid Chromatography with Electrospray-Ionization Mass Spectrometry and Fraction Collection (LC-MS+)—Dried ribosomal proteins purified as described in the previous section were resuspended in 100 μl of water and fractionated by reverse-phase liquid chromatography. As the effluent eluted it was directed to a "T" flow splitter that divided ~50% of the column eluent to a fraction collector and the other 50% was directly injected into an electrospray-ionization mass spectrometer as described previously (25, 30). For fractionation by reverse-phase HPLC, a PLRP-S polymeric column was used that had a pore size of 300 Å, a bead size of 5 μm, and dimensions of 150 × 2.1 mm (Polymer Laboratories, Amherst, MA). The column was initially equilibrated with solvent A (0.1% trifluoroacetic acid in water) at 95% and solvent B (0.1% trifluoroacetic acid in 1% water, 99% acetonitrile) at 5% and the column was maintained at 40 °C. Elution of the ribosomal proteins was performed using a program of 5 min at 5% B, followed by a 25-min gradient from 5 to 40% B and ending with a 120-min gradient from 40 to 100% B, all at a flow rate of 100 μl/min. The API III+ mass spectrometer (PE Sciex) used was tuned and calibrated as described previously (31) to yield a mass accuracy of 0.01% (±1.0 Da at 10 kDa). The 50-μl fractions collected during the fractionation were stored at −20 °C for latter analysis by proteolytic digestion. The data obtained were analyzed using the BioMultiview 1.3.1 software (Applied Biosystems).

Digestion of Rpl23ab with ArgC or CNBr—Half of the Rpl23ab-containing fraction (25 μl) collected during the LC-MS+ analysis was diluted on ice by adding 45 μl of 0.5 M ammonium bicarbonate. To the mixture, 200 ng of ArgC (endoproteinase ArgC from mouse submaxillary gland; suitable for protein sequencing, lyophilized powder; Sigma product P6056) dissolved in 5 μl of 0.5 M ammonium bicarbonate, was added to commence proteolysis. The reaction was allowed to proceed for 3 h at 37 °C. The reaction was then quenched by lyophilization and stored at −20 °C. The other half of the sample was digested with CNBr (Sigma product 481432). The CNBr was dissolved in acetonitrile to a concentration of 1 g/ml. Once the CNBr solution was made, 2.5 μl was added to 25 μl of the Rpl23ab containing fraction and the cleavage reaction was allowed to proceed for 5 h at room temperature in the dark. The reaction was quenched by lyophilization and stored at −20 °C. The peptide samples obtained from each digestion procedure were then individually analyzed by μLC-MSMS mass spectrometry using a QSTAR instrument as described below.

TABLE 1
Strains
These strains were prepared by the Saccharomyces Genome Deletion Project and obtained through Invitrogen Corp.

Strain	Genotype
BY4742	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0
Δ*rkm1*	BY4742, Δ*yp1208w*::*Kan*^{r}
Δ*rp123a*	BY4742, Δ*yb1087c*::*Kan*^{r}
Δ*rp123b*	BY4742, Δ*yer117w*::*Kan*^{r}

Identification of the Methylated Site by Micro-Liquid Chromatography Tandem Mass Spectrometry (μLC-MSMS)—Samples were dissolved in 10 μl of 0.1% formic acid and 5% acetonitrile (v/v) and then analyzed by μLC-MSMS with data-dependent acquisition as described previously (25).

In Vivo Labeling of Rpl23ab Knockouts with S-Adenosyl-L-[methyl-^{3}H]Methionine ([^{3}H]AdoMet)—Yeast strains in which the genes encoding Δ*rpl23a* or Δ*rpl23b* were deleted were obtained from Invitrogen. Genotypes of these strains are described in Table 1. Cell growth, labeling, and protein analysis by SDS-PAGE was performed as described previously (24).

Polysomal Analysis of Δrkm1 Deletion Strain—Polysomal analysis was performed after the method of Zanchin *et al.* (32) with the modifications described previously (25).

RESULTS AND DISCUSSION

Intact Mass Analysis of Rpl23ab Derived from the Wild Type and Δrkm1 Deletion Strains—In a previous study we demonstrated that the ribosomal protein Rpl23ab (L23ab) was dimethylated at two sites by the SET domain-containing ribosomal lysine methyltransferase Rkm1 (24). The sites at which the dimethylation occurred remained to be identified. In Fig. 1 we show, using LC-MS+, the loss of ~56 Da in Rpl23ab from the Δ*rkm1* strain compared with protein from the wild type strain, confirming that the protein is modified by two dimethylation events (one dimethyl group is 28 Da). Large subunit proteins were purified as described under "Experimental Procedures" and then separated using liquid column chromatography. As the effluent eluted from the column half of the fraction was collected for later proteolytic digestion, whereas the other half was directed to an electrospray ionization mass spectrometer. The UV chromatograph obtained during the reverse phase HPLC purification of Rpl23ab demonstrates that the protein elutes in a prominent peak at around 39.9 min using the gradient we developed (Fig. 1*A*). When the material in this peak was analyzed by the mass spectrometer we observed that the major species eluting is Rpl23ab with only minor contaminants. The

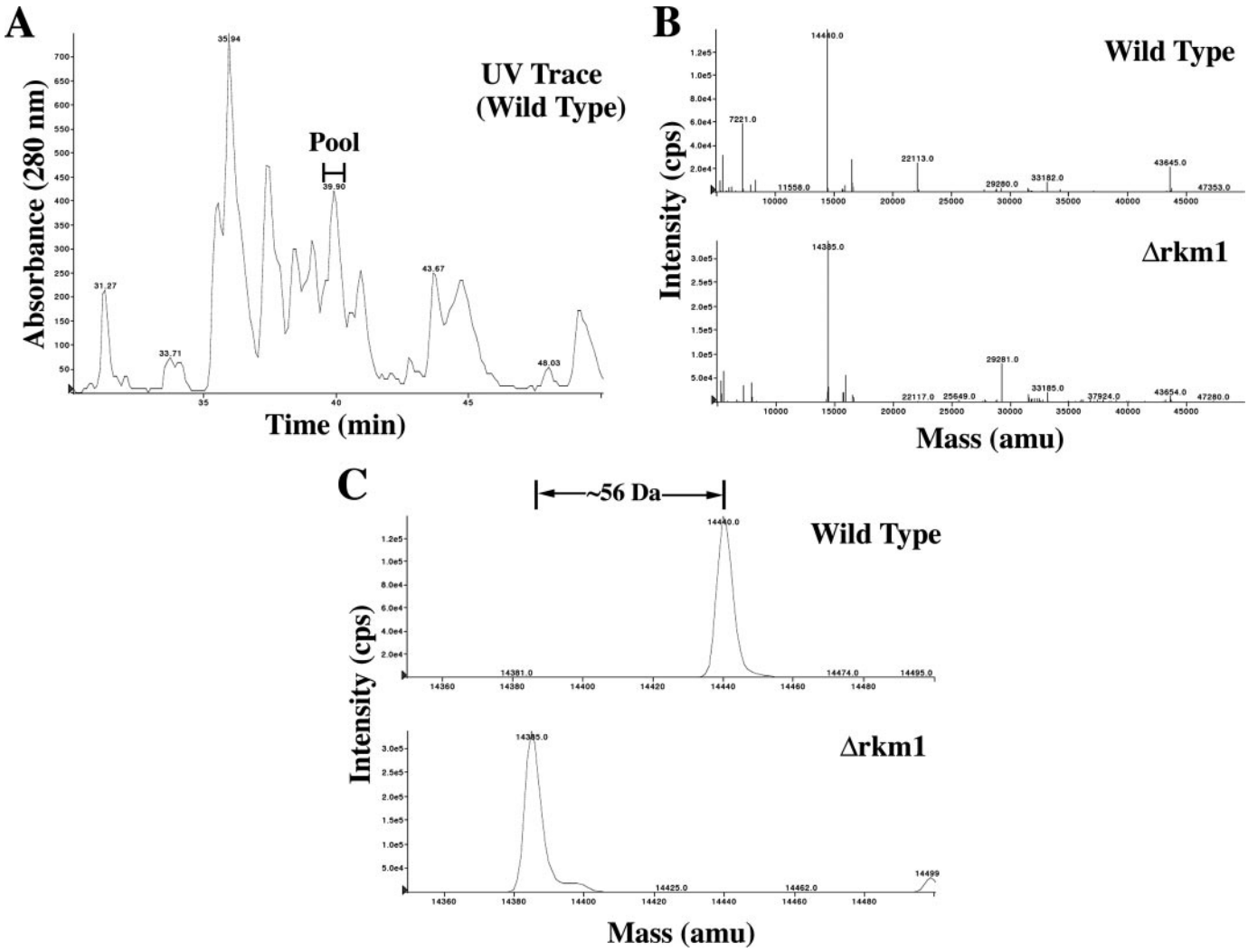

FIGURE 1. **Analysis of Rpl23ab by LC-MS+.** Large subunit proteins were purified from both the wild type and the Δ*rkm1* strain and fractionated by reverse phase HPLC as described under "Experimental Procedures." The column effluent was split; one-half was directly injected into a quadruple electrospray ionization mass spectrometer for intact mass determination, whereas the second half was collected in 1-min 50-μl fractions. *A* shows the UV absorbance of the preparation of wild type large ribosomal subunit proteins as they were fractionated by reverse phase HPLC; *pool* designates the fractions that contain Rpl23ab. A similar elution profile was obtained for the Δ*rkm1* strain (data not shown). *B* shows the deconvoluted MS spectrum in the range of 0–50,000 Da for the proteins that elute in the region designated *pool*. In all the strains analyzed the major species observed in this area corresponded to Rpl23ab. In the wild type strain we obtained a molecular mass of 14,440 ± 0 Da, whereas in the Δ*rkm1* strain we observed a molecular mass of 14,384 ± 1 Da (average ± S.D. from three experiments). *C* is an enlarged view of the region that spans from 14,350 to 14,500 Da for both the wild type and Δ*rkm1* strain included to demonstrate that the methylated variants are completely absent in the Δ*rkm1* strain. All the masses reported for the LC-MS+ analysis are average masses based on natural isotopic abundance. *amu*, atomic mass unit.

deconvoluted spectra for both the wild type and Δ*rkm1* strains in the mass range of 0–50,000 Da are depicted in Fig. 1*B*. In the Rpl23ab derived from the wild type parent strain we observed an intact mass of 14,440 ± 0 Da, corresponding to the mass of the Rpl23ab polypeptide with four methyl groups, the loss of the initiator methionine residue, and one acetyl group. In Rpl23ab derived from the Δ*rkm1* strain we observed an intact mass of 14,384 ± 1 Da, which corresponds to the mass of the Rpl23ab polypeptide without the methyl groups. An amplified view of the area surrounding the Rpl23ab species is shown in Fig. 1*C* to demonstrate that the +56 Da species corresponding to the presence of two dimethylated residues is completely absent in protein purified from the Δ*rkm1* strain. In Rpl23ab prepared from either the Δ*rkm1* or wild type strain, we find no evidence of peaks corresponding to partial methylation events. Specifically, species of mass 14,398 (one methyl group), 14,412 (two methyl groups), or 14,426 (three methyl groups) are not observed for the wild type or mutant protein in Fig. 1*C*.

Determining the Sites of Dimethylation in Rpl23ab—LC-MS+ fractions containing purified Rpl23ab derived from both the wild type and Δ*rkm1* strain were digested and analyzed by μLC-MS to sequence the peptides produced. In Rpl23ab derived from the wild type strain we observed two peptides with single sites of lysine dimethylation, which accounts for the additional ~56 Da observed in the wild type strain. One peptide observed was composed of amino acid residues 74–108 and had a dimethylated lysine at position 105 (Fig. 2*A*, Table 2), whereas a second site of dimethylation was observed at lysine 109 in a peptide composed of amino acid residues 109–136 (Fig. 2*B*, Table 2). In Rpl23ab derived from the Δ*rkm1* deletion strain we observed a peptide that spans amino acid residues 86–109, completely devoid of methylation (Fig. 3, Table 2).

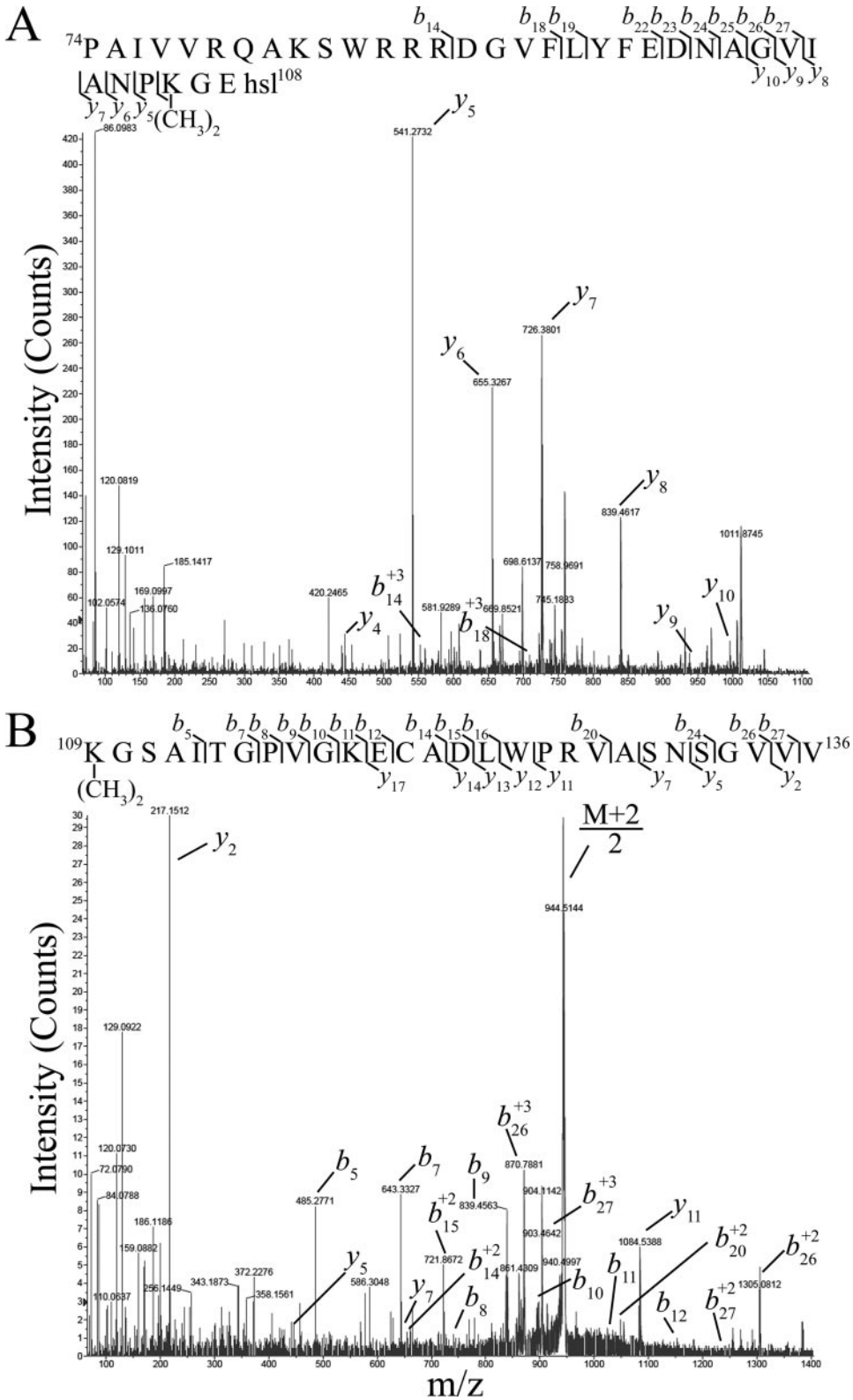

FIGURE 2. **μLC-MS identification of methylated peptides in the wild type strain.** Half of the fraction (25 μl) collected during the LC-MS run that contained Rpl23ab derived from the wild type strain was digested with CNBr as described under "Experimental Procedures." After digestion the peptides produced were analyzed by μLC-MS on a QSTAR mass spectrometer. The MSMS spectrum obtained for the peptide that spans amino acid residues 74–108 and contains a dimethylation at lysine 105 is depicted in *A*. The letters "*hsl*" denotes the presence of a homoserine lactone derivative produced during the CNBr treatment. *B* depicts the MSMS spectrum obtained for the peptide that spans C-terminal residues 109–136 and contains a dimethyl group on lysine 109. The *y* and *b* ions observed within the region illustrated are marked for both peptides. A fragmentation map is also included to demonstrate the location of fragmentation.

N-terminal peptides were also obtained confirming the presence of an N-terminal acetyl group and the loss of the initiator methionine residue in Rpl23ab prepared from both the wild type and Δ*rkm1* deletion strain (data not shown). We looked for, but did not detect, ArgC or CNBr fragments containing Lys-105 or Lys-109 residues that were unmethylated or monomethylated in Rpl23ab derived from wild type cells. These results confirm our findings from the intact mass studies that no partially methylated species occur in Rpl23ab.

These modifications of Rpl23ab are only four residues apart and are located near the C terminus. On the yeast large ribosomal protein Rpl12ab, two lysine methylation sites occur seven residues apart from each other, but in this case at the N terminus (25). In histones H3 and H4, lysine modifications occur at multiple, often closely spaced, sites on their N-terminal tails, allowing for their association with specific proteins to regulate the activation or repression state of chromatin (16, 18, 19). These modifications at lysine residues create specific recognition sites for protein-protein interactions, including ones mediated by Tudor, MBT, chromo, and PHD domains (33, 34).

Identifying the Methylated Isoform—Because the Rpl23 protein in yeast is encoded by two genes that when translated produce two identical polypeptides as isoform a and isoform b, we wanted to determine which of the two isoforms was methylated by Rkm1. Yeast strains in which either the Δ*rpl23a* or Δ*rpl23b* genes had been deleted were *in vivo* labeled with [^{3}H]AdoMet and the cell lysate was analyzed by SDS-PAGE and fluorography. Fig. 4*A* shows the Coomassie-stained gel and demonstrates that in the absence of one isoform the second isoform compensates and that the migration of both isoforms is identical. The autoradiograph in Fig. 4*B* demonstrates that when lysates obtained from either the ^{3}H-methylated Δ*rpl23a* or

TABLE 2

MSMS ions for methylated Rp123ab peptides in the wild type strain and unmodified Rp123ab peptide in the Δ*rkm1* strain

Values are listed as monoisotopic uncharged masses.

Peptide (hsl is homoserine lactone)	Ion	Observed mass	Theoretical mass[a]	Mass error[b]
		Da		*ppm*
Wild type strain				
PAIVVRQAKSWRRRDGVFLYFEDNAGVIANPKGEhsl	*b*14	1704.007	1704.017	−6.1
$(CH_3)_2$	*b*18	2122.378	2122.202	82.7
	*b*19	2235.395	2235.286	48.5
	*b*22	2674.389	2674.461	−26.9
	*b*23	2789.457	2789.488	−11.1
	*b*24	2903.508	2903.531	−7.8
	*b*25	2974.699	2974.568	44
	*b*26	3031.693	3031.589	34.2
	*b*27	3130.685	3130.658	8.8
	*y*2	216.143	216.149	−28.3
	*y*4	443.250	443.240	21.8
	*y*5	540.270	540.293	−41.1
	*y*6	654.330	654.336	−8.7
	*y*7	725.379	725.373	8.9
	*y*8	838.459	838.457	2.5
	*y*9	937.545	937.525	20.9
	*y*10	994.534	994.547	−12.2
KGSAITGPVGKECADLWPRVASNSGVVV	*b*5	484.268	484.301	−68.8
$(CH_3)_2$	*b*7	642.316	642.370	−83.9
	*b*8	739.423	739.423	0.1
	*b*9	838.446	838.491	−54.3
	*b*10	895.483	895.513	−33.3
	*b*11	1023.522	1023.608	−83.5
	*b*12	1152.539	1152.650	−96.3
	*b*14	1326.624	1326.697	−54.8
	*b*15	1441.707	1441.723	−11.2
	*b*16	1554.807	1554.808	−0.1
	*b*20	2093.010	2093.109	−47.6
	*b*24	2452.214	2452.253	−16.0
	*b*26	2608.160	2608.343	−70.2
	*b*27	2707.264	2707.412	−54.6
	*y*5	459.241	459.271	−65.8
	*y*7	660.300	660.346	−70.2
	*y*11	1083.599	1083.606	−5.8
	*y*12	1269.683	1269.685	−1.6
	*y*13	1382.696	1382.769	−52.5
	*y*14	1497.725	1497.796	−47.4
	*y*17	1800.802	1800.885	−46.0
Δ*rkm1* strain				
RRDGVFLYFEDNAGVIANPKGEMK	*b*3	427.204	427.229	−59.4
	*b*5	583.331	583.319	19.7
	*b*6	730.405	730.387	24.2
	*b*7	843.417	843.471	−65.1
	*b*8	1006.518	1006.535	−16.7
	*b*9	1153.622	1153.603	16.3
	*b*10	1282.681	1282.646	27.3
	*b*11	1397.688	1397.673	11.2
	*b*12	1511.705	1511.716	−7.3
	*b*13	1582.751	1582.753	−1.2
	*b*14	1639.625	1639.774	−91.3
	*b*16	1851.819	1851.927	−58.3
	*b*18	2036.936	2037.007	−34.9
	*y*2	277.144	277.148	−16.1
	*y*4	463.194	463.212	−39.1
	*y*5	591.321	591.307	23.9
	*y*6	688.366	688.360	9.1
	*y*7	802.360	802.403	−53.7
	*y*8	873.352	873.440	−99.9
	*y*9	986.473	986.524	−51.0
	*y*11	1142.589	1142.614	−21.6

[a] The theoretical masses given were obtained using the Prosight proteomic server (prosightptm.scs.uiuc.edu/) taking into consideration the post-translational modification.
[b] Error was calculated using the Prosight server.

Δ*rpl23b* deletion strains are analyzed, a radiolabeled species is detected at the expected migration position of Rpl23ab. These results indicate that both isoforms of this protein are methylated and that both isoforms are expressed. Lysates obtained from the wild type and Δ*rkm1* deletion strains were loaded in adjacent lanes as controls to demonstrate the position where the loss of signal is observed when the gene encoding the methyltransferase is deleted.

Interactions of Methylated Lysine Residues with RNA and Ribosomal Complex Stability in the Δrkm1 Deletion Strain—In a previous study (24), we showed no growth defects of the Δ*rkm1* strain under a variety of conditions. However, loss of the Rmt3-catalyzed protein arginine methylation of the S2 small ribosomal protein in *S. pombe* has been shown to disrupt the interaction of small and large subunits due to an apparent loss in small subunit stability (27, 28). In the case of *S. cerevisiae*

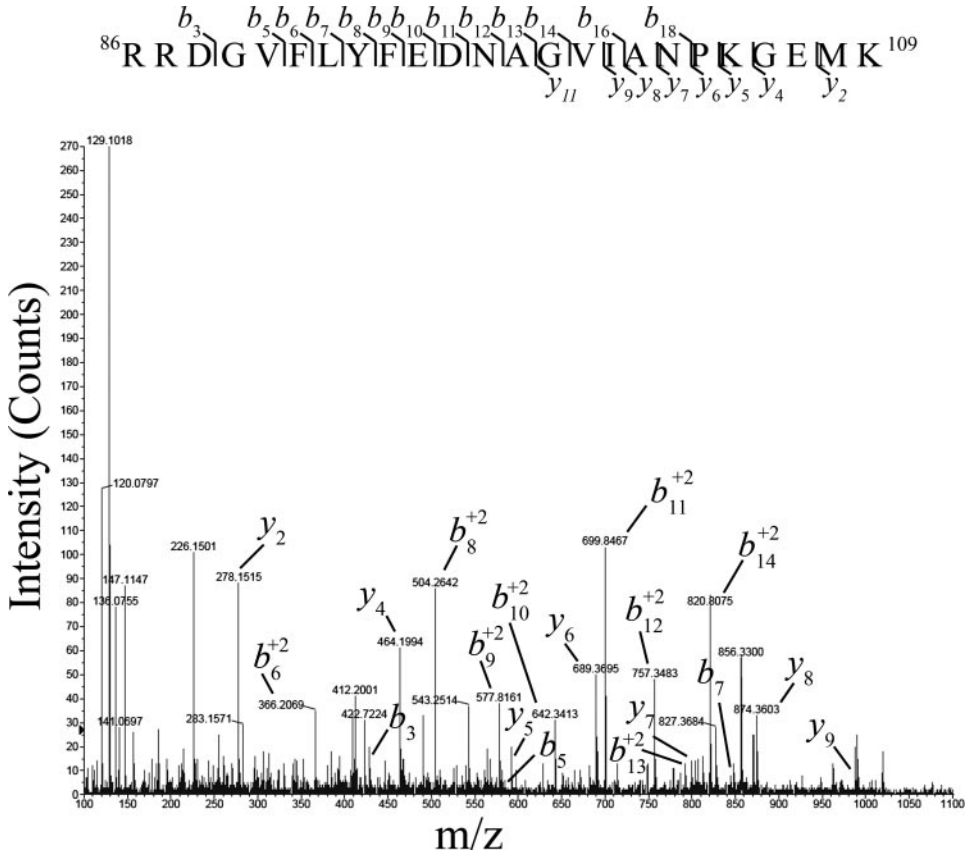

FIGURE 3. **Analysis of the unmethylated peptide in Rpl23ab derived from the Δ*rkm1* strain.** Half of the fraction collected during the large subunit analysis of the Δ*rkm1* strain, which contained Rpl23ab was digested with the proteinase ArgC. Following digestion, the peptides were sequenced on a QSTAR mass spectrometer. The MSMS spectrum obtained for the unmodified peptide that spans amino acid residues 86–109 is depicted in the figure. The location of the *y* and *b* ions observed in the region of 0–1100 are indicated.

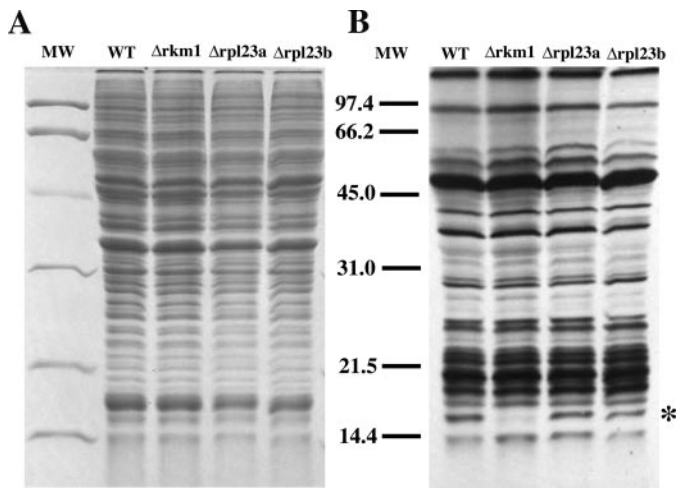

FIGURE 4. **Identifying the methylated isoform of Rpl23ab.** Yeast cells containing a deletion in either the *rpl23a* or *rpl23b* genes were *in vivo* labeled with [^{3}H]AdoMet and the lysate recovered was analyzed by SDS-PAGE and fluorography as described previously (24). *A* is the Coomassie-stained gel to illustrate total protein loaded. *B* is the autoradiograph (3 days exposure) to demonstrate the fractionated ^{3}H-methylated species. The *asterisk* denotes the point of migration of Rpl23a and Rpl23b.

Rpl23ab, analysis of the cryo-EM reconstruction of the yeast 80 S ribosome shows that this protein is located at the intersubunit face and makes contacts with helices 14 and 44 of the 18 S rRNA on the small subunit (35). Furthermore, it has been suggested that the region of the homologous *Bacillus stearothermophilus* L14 protein corresponding to yeast Rpl23ab residues 102–111 is a contact region for RNA (36). We thus wanted to test whether the methyl lysine modifications on Rpl23ab at residues 105 and 109 are important for the interaction between the large and small subunit by analyzing ribosomal complex stability in the Δ*rkm1* mutant.

Yeast lysates obtained from the wild type and Δ*rkm1* deletion strains were fractionated on a low salt sucrose gradient that preserved large ribosomal protein complexes. When the Δ*rkm1* strain was analyzed we observed no disruption in the ratio of 40 S to 60 S subunit, as compared with the wild type strain (Fig. 5). Ribosomal complex formation was also not altered. Analysis of the Δ*rkm1* strain demonstrated the presence of the 80 S ribosomal complex and progressive polysomal complexes (2–5) in ratios similar to what was observed in the wild type parent strain. The lack of an observable phenotype in the Δ*rkm1* strain suggests that the modification is not essential for stability or that the absence of methylation on a single ribosomal protein might not be sufficient to induce a measurable change. The identification of additional ribosomal lysine methyltransferases will allow us to study the effect of the loss of multiple methyl lysine modifications.

Structural reconstruction studies of the *S. cerevisiae* 80 S ribosome (35, 37) and the *Escherichia coli* 70 S ribosome (38) have positioned the methylated residues of Rpl23ab and the corresponding region of the prokaryotic L14 homolog making contacts with the yeast 25 S or bacterial 23 S large subunit ribosomal RNA. These contacts would be expected to shield the methylated residues from interactions with other ribosomal proteins or cytosolic factors. These results suggest that methylation may function to correctly position the Rpl23ab/L14 protein within the large subunit. Interestingly, the position of Rpl23ab and its bridges to the small subunit RNA change during the translation cycle (37, 38). Further studies will be needed to assay the effect of methylation on translational efficiency. However, it is clear that the interactions of methylated lysine residues in Rpl23a occur with RNA rather than with proteins. A similar situation is found for the methylated lysine residues of Rpl12ab (25, 35, 37). Thus, it appears that the interaction of methylated lysine residues in these ribosomal proteins with RNA is clearly different from the interaction of methylated lysine residues in histones with proteins (16, 18, 19, 33, 34).

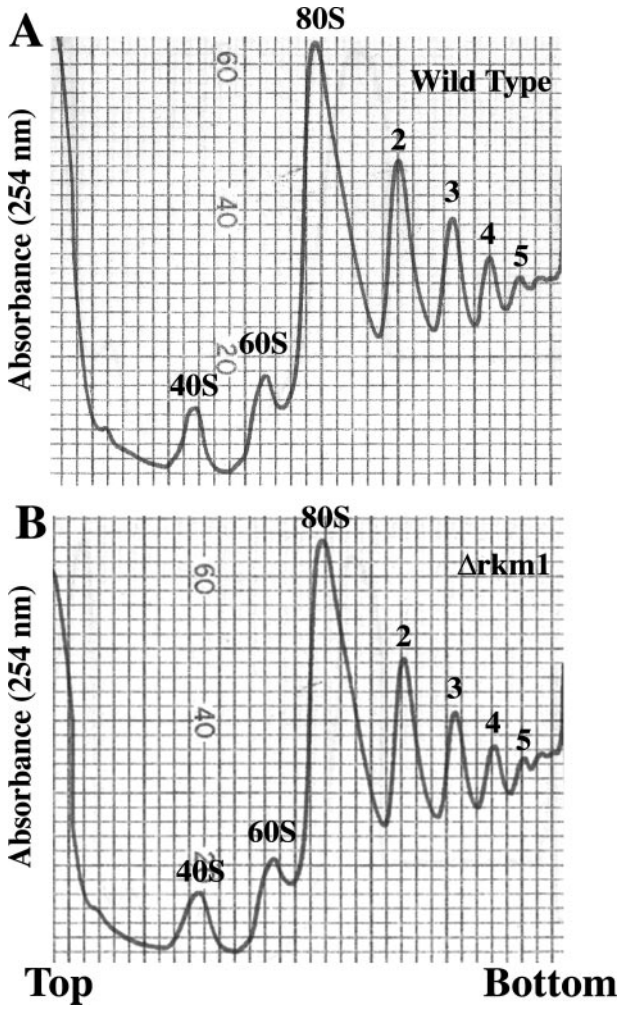

FIGURE 5. **Ribosomal complex stability in the Δ*rkm1* deletion strain.** The stability of ribosomal complex formation was studied in the Δ*rkm1* strain (*B*) and compared with the wild type (*A*) strain. Whole cell lysates from the two strains were fractionated in sucrose gradients made under low salt and the elution was monitored by UV at 254 nm using an ISCO UA6 apparatus as described previously (32). The gradient was pumped through the UV monitor by injecting a high-density solution through the bottom of the tube. The positions of the ribosomal protein complexes are indicated including the small (40 S) and large (60 S) ribosomal subunits, the 80 S ribosomes, and the various polysomal complexes (*2–5*).

Comparing Lysine-methylated Sites in Yeast—The sequences of known SET methyltransferase subfamily 2 substrates were compared to identify motifs that may be recognized by SET methyltransferases. The entire sequences of Rpl12ab, the substrate of Rkm2 (25), Rpl23ab, the substrate of Rkm1 (24), and Cyc1, the substrate of Ctm1 (15), were first compared and analyzed by BLASTP and Clustal method. Although there was no significant homology found between these species, we did note several similar short sequences in common. For example, a Val-Gly-Ala sequence is found at residues 21–23 of Rpl12ab and residues 19–21 of Rpl23ab; a Thr-Val-(Lys/Glu)-Lys-Gly sequence is found at residues 60–64 of Rpl23ab and 24–29 of Cyc1. Whether these similarities reflect a common origin is not clear; pairwise comparison of the yeast Cyc1 structure (PDB code 1CRI) with the L14 protein from *Bacillus stereothermophilus* structure (PDB code 1WHI; the yeast Rpl23ab homolog (36)) revealed no similarity using the Dalilight program of the European Bioinformatics Institute. When we directly compared the regions adjacent to the sites of methylation in these proteins and in yeast histone H3, we found that the Lys-3 methylation site of Rpl12ab was preceded by a Pro-Pro sequence, whereas the Lys-105 methylation site of Rpl23ab and the Lys-79 methylation site of Cyc1 were both preceded by an Asn-Pro sequence. Because it is possible that methyltransferases evolved to recognize lysine residues in distinct portions of these proteins, we suggest that the (Asn/Pro)-Pro-Lys sequence might be a recognition motif for SET methyltransferase subfamily 2 members. A second possible SET methyltransferase recognition sequence, (Gly/Asn)-(Gly/Glu)-(Val/Met)-Lys, is suggested by the comparison of the second methylation site in the ribosomal proteins and by the lysine 36 site in histone H3. The sequence preceding the Lys-36 site of methylation in yeast histone H3 (Gly-Gly-Val) was also found to have some similarity to the Gly-Glu-Met sequence preceding the Lys-109 methylation site of Rpl23ab and the Asn-Glu-Val sequence preceding the Lys-10 methylation site of Rpl12ab. The identification of additional SET domain methyltransferase substrates will aid in further establishing the SET methyltransferase recognition sequences.

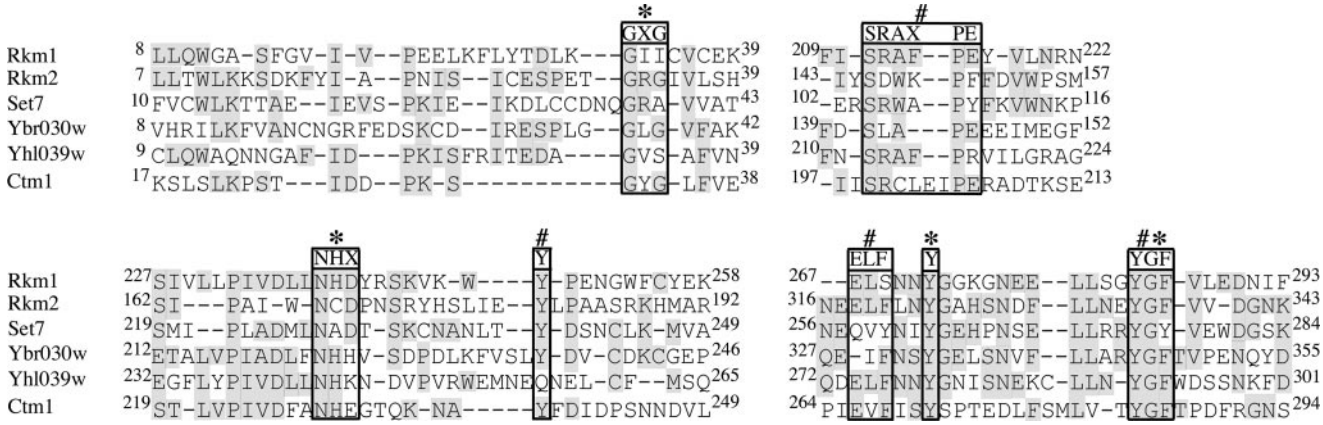

FIGURE 6. **Sequence comparison of yeast SET domain subfamily 2 methyltransferases.** The sequences of subfamily 2 members Rkm1, Rkm2, Set7, Ybr030w, Yhl039w, and Ctm1 were compared over their entire length. The alignment was made by comparing results obtained using the Megalign program choosing the Clustal method of alignment and BLAST alignment results (www.ncbi.nlm.nih.gov/BLAST/)). Regions of closest similarity are depicted including *boxed* regions that have been suggested to be important for AdoMet binding (*) and lysine substrate binding (#) from the structures of other SET domain methyltransferases (39) and Dirk *et al.* (14)). *Gray* shading indicates identities. Consensus motifs are indicated in *bold lettering* in the *boxes* where at least three residues are identical. Yeast SET domain subfamily 2 members were defined by Porras-Yakushi *et al.* (25).

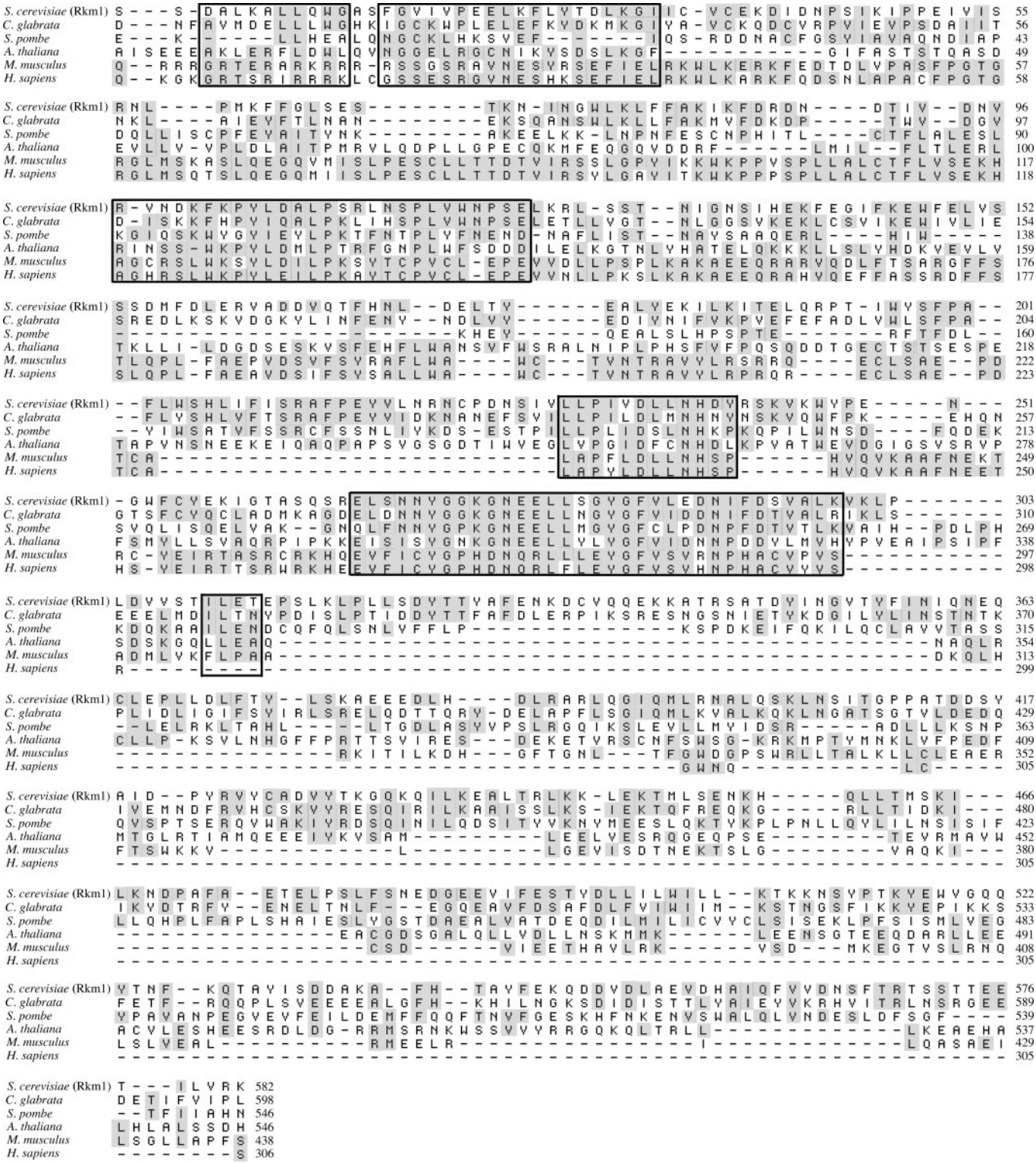

FIGURE 7. **Yeast Rkm1 homologs are present in other fungi, plants, and mammals.** BLAST searches were performed against the yeast Rkm1 sequence to identify homologs in other organisms (www.ncbi.nlm.nih.gov/BLAST/). Listed are the sequences derived from the search for an unknown protein in *C. glabrata* (accession number XP_445936), a protein annotated as a histone lysine *N*-methyltransferase in *S. pombe* (accession number NP_595446), an unknown protein in *Arabidopsis thaliana* (accession number NP_001030933), the SET domain-containing 4 protein (*Setd4* gene product) in *Mus musculus* (accession number NP_663457), and the SET domain-containing 4 isoform c (SETD4 gene product) in *Homo sapiens* (accession number NP_001007260). The sequence alignment was constructed using the Megalign/Clustal program as described in the legend to Fig. 6. *Gray* shading indicates sites where at least two residues are identical. Areas with high sequence similarity have been *boxed*.

Sequence Comparison of SET Methyltransferase Subfamily 2 Members—When the SET methyltransferase subfamily 2 members Rkm1, Rkm2, Set7, Ybr030w, Yhl039w, and Ctm1 were compared over the entire length of the protein, the greatest similarities were found in the regions previously established to be involved in AdoMet and lysine substrate binding (Fig. 6) (14,

39). Importantly, we find conserved sequences in adjacent positions such as LL*XX*YGF(V/T) or PI(V/A)DLL that are not well conserved in the SET subfamily 1 members, suggesting their possible role in ribosomal/cytochrome *c*-specific recognition. These regions are also found in the Rubisco SET lysine methyltransferases (14).

Identifying Rkm1 Homologs in Other Organisms—A BLAST search was performed against the Rkm1 sequence in an attempt to identify homologs in other organisms. The highest sequence similarity was found in other fungi such as *Candida glabrata*, *S. pombe*, higher plants, and mammals (Fig. 7). Interesting, the lysine residues corresponding to yeast Rpl23ab sites 105 and 109 are present in the human placenta ribosomal protein L23 but appear to be unmodified (40). These results suggest that the human Rkm1 homolog has another methyl-accepting substrate or that it is not expressed in placenta. In rat, the L29 large ribosomal protein is differentially methylated at Lys-4 in liver, brain, and thymus (41). It will be of interest to determine the methylation state of mammalian L23 in non-placental tissues.

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