Characterization of the Yeast Trimeric-SAS Acetyltransferase Complex*

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The yeast SAS2 (Something About Silencing 2) gene encodes a member of the MYST protein family of histone acetyltransferases (HATs) and is involved in transcriptional silencing at all silent loci (HML, HMR, telomeres, and rDNA) in Saccharomyces cerevisiae. Sas2 is the catalytic subunit of a yeast histone acetyltransferase complex termed SAS complex. The enzymatic activity of SAS complex on free histones has been reported, but nucleosomal HAT activity has not yet been documented. Here we show that the native yeast SAS complex is a small trimeric protein complex composed solely of Sas2, Sas4, and Sas5 with a molecular mass of about 125 kDa. It is capable of acetylating both free histones and nucleosomes, although the nucleosomal HAT activity of SAS complex is very weak when compared with that of NuA4. We also demonstrate that the putative acetyl-CoA binding motif in Sas2 is essential for both the in vivo silencing function and the enzymatic activity of SAS complex. Unlike NuA4, which acetylates all four available lysines at the N-terminal tail of histone H4, SAS complex exclusively acetylates lysine 16 of histone H4 in vitro and is required for the bulk of H4 lysine 16 acetylation in vivo. This specific lysine preference corresponds to the role of SAS complex in antagonizing the spreading of Sir proteins at silent loci in S. cerevisiae.

In eukaryotic nuclei, chromosomal DNA is packaged into a compact structure with histones to form chromatin. Chromatin can be an effective silencer of gene expression. Specifically, heterochromatin consists of highly condensed chromatin and is thought to contain primarily transcriptionally silenced genes. In yeast Saccharomyces cerevisiae, this type of transcriptional silencing occurs at several genomic locations including the HML and HMR mating-type loci, telomeres, and rDNA (1). Many proteins contribute to the establishment and maintenance of silenced chromatin. Among these proteins, Sir (silent information regulator) proteins 1–4 have been shown to play crucial roles at all silent loci (2). The Something About Silencing 2 (SAS2) gene was first isolated in a genetic screen scoring for a mutant that can enhance the epigenetic silencing defects at HML in sir1Δ background (3). It has also been reported that SAS2 regulates silencing differently at different loci. SAS2 functions as a positive regulator at HML and telomeres (3); its deletion leads to loss of silencing at these loci. Oppositely, deletion of SAS2 enhances rDNA silencing (4) and restores the silencing defects at HMR with a mutated silencer (5). Notably, loss of SAS2 does not affect silencing at wild-type HMR mating locus. The exact mechanisms for Sas2-mediated silencing at different loci remain unclear. Genetic evidence showed that two other genes, termed SAS4 and SAS5, share the same regulatory functions in silencing with SAS2 and may function in the same genetic pathway with SAS2 (6, 7).

Sas2 is a member of the MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) protein family of histone acetyltransferases (HATs),1 Proteins in this family include human MOZ (monocytic leukemia zinc finger protein), MORF (MOZ-related factor), Tip60, and HBO1, Drosophila MOF (males absent on the first), and Chm, and yeast Esa1, Sas2, and Sas3 (8). The MYST-related proteins all share a highly conserved acetyl-CoA binding motif that is essential for their HAT enzymatic activities (9). Many members in the MYST family are found to be the catalytic subunit of a multiprotein HAT complex (Sas3p in NuA3 and Esa1p in NuA4), and Sas2 is no exception. Recent biochemical studies indicate that Sas2, Sas4, and Sas5 exist as part of a 230–450 kDa protein complex in yeast (4, 10). Unlike Sas2, Sas4 is unique in that it does not share homology to any known protein. Sas5, on the other hand, shares a conserved YEATS (YaF9-ENL-FA9-Taf14-Sas5) domain with several proteins including human leukemogenic proteins AF9/ENL/Gas41, yeast Taf14, and YaF9. Although the exact function of the YEATS domain is unknown, the YEATS triple knock-out yeast strain (yaF9Δ, taf14Δ, sas5Δ) is not viable (11), suggesting that the YEATS domain is essential for viability in S. cerevisiae. The predicted molecular masses of Sas2 (39 kDa), Sas4 (55 kDa), and Sas5 (29 kDa) add up to only 123 kDa, suggesting that the SAS complex may contain additional subunits that have not been identified.

The yeast SAS complex, either native or recombinant, has intrinsic HAT activity on free histones (12), but nucleosomal HAT activity has not yet been reported. In studies with recombinant SAS complex, it is clear that both Sas4 and Sas5 are required for optimal Sas2 HAT activity. It has been demonstrated that mutations in the conserved acetyl-CoA binding domain of Sas2 disrupt silencing at HML and telomeres (4, 10)

* This work is supported by NIGMS, National Institutes of Health Grant R37-GM-047867. 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.

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1 The abbreviations used are: HAT, histone acetyltransferase; TAP, tandem affinity purification; CBP, calmodulin-binding protein; MudPIT, multidimensional protein identification technology; WT, wild type; SWR, Swiz/Snf2-related.
Trimeric SAS HAT Complex

Genotypes of all yeast strains used in this study are shown.

| Strain         | Genotype                  | Source |
|----------------|---------------------------|--------|
| UWJS001        | a Sas3–13Myc::HIS3MX6     | Ref. 10|
| UWJS002        | a sac25::kanMX3           | Open Biosystems* |
| UWJS004        | a sac43::kanMX3           | Open Biosystems* |
| UWJS005        | a sac53::kanMX3           | Open Biosystems* |
| UWJS053        | a ESA1-TAP::His3MX6       | Open Biosystems* |

* Strains were purchased from Open Biosystems.

and that the recombinant SAS complex with mutant Sas2 loses its HAT activity (12). These results suggest a link between Sas2 HAT activity and its regulatory roles in gene silencing.

There is also a functional link between SAS complex and chromatin deposition activity. The histone deposition proteins Asf1 (anti-silencing function 1) and CAF-1 (chromatin assembly factor-1) both associate with the SAS complex (4, 10, 13). Asf1 and CAF-1 contribute to chromatin assembly by depositing newly synthesized histone H3 and H4 into nucleosomes (14–16). How SAS complex-mediated histone acetylation is involved in the chromatin assembly is unclear.

It has been reported previously that a point mutation at lysine 16 in the N-terminal tail of histone H4 phenocopies the silencing effects caused by deletion of SAS2 (4). This H4 K16R mutation weakens the silencing at HML and telomere but enhances the silencing at rDNA and restores the silencing at the mutant HMR. We and others have shown that SAS complex is a histone H4 lysine 16-specific histone acetyltransferase complex (12, 17). The lysine preference of SAS complex plays a direct role in preventing the further spreading of Sir proteins into subtelomeric regions by antagonizing the deacetylation function of Sir2 (17, 18). The same anti-silencing function of SAS complex has also been observed at HMR silent locus, at which the SAS complex assists a unique tRNAThr gene to establish a heterochromatic barrier (19, 20). Together, these data suggest that SAS complex plays an important role in blocking the propagation of heterochromatin.

In this report, we show that the native yeast SAS complex is a small trimeric protein complex consisting solely of Sas2, Sas4, and Sas5. Contrary to previous findings, we show that the molecular mass of SAS complex is about 125 kDa. In addition, we demonstrate that SAS complex has novel in vitro nucleosomal HAT activity that is dependent on the acetyl CoA binding motif of Sas2. Unlike NuA4, SAS complex exclusively acetylates lysine 16 of histone H4. All three subunits of the complex are required to maintain the overall levels of acetylation at H4 lysine 16 in yeast cells.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The genotypes of all strains used in this study are described in Table I. Chromosomal-tagged or deleted strains were generated by one-step PCR-based strategy as described previously (21, 22). Standard yeast manipulations were performed as described (23). All knock-out strains were confirmed by PCR. The expressions of tagged proteins were verified by Western blots. Plasmids for expression of galactose-induced Sas proteins (pS116, pS128, and pS129) were published previously (10). To make Sas2-TAP expression vectors, pRS426-TAP (pS225) was first made by subcloning a PCR fragment containing HindIII-TAP-Spel into pRS426 (pS23). Wild-type or mutant SAS open reading frames with 1 kb of upstream sequence were amplified by PCR from pS126, pS137, and pS138 (10). The PCR products containing a SAS gene with a 5′ Xhol site and a 3′ HindIII site were then subcloned into pRS426-TAP (pS223) to generate plasmids pS224 to pS227. All plasmids generated were further verified by sequencing.

Protein Expression and Purification—Whole cell extracts were prepared by following a previously published procedure (24). Purification of SAS complex (from YJW458, YJW475, and YJW476) and NuA4 (from UWJS053) from a 12-liter culture by the tandem affinity purification method were essentially performed as described elsewhere with minor changes (25). Whole cell extracts (5 mg) and SAS complex (purified from a 4-liter culture) from YJW458 were fractionated in a Superose 6 HR 10/30 gel filtration column using AKTApurifier system (Amersham Biosciences) (10). TAP-purified SAS complex (purified from 2 liters of YJW458) was also fractionated by 10–40% glycerol gradient sedimentation in TAP elution buffer at 300,000 × g for 2 h. SAS complex was loaded on a 4.5-ml gradient and collected in 100-μl fractions. Gelfiltration calibration kits (Amersham Biosciences) were used to estimate the molecular weight of the protein complex. To check the overall in vivo histone H4 acetylation level (see Figs. 5A and 6A), whole cell extracts were prepared as described (17).

MudPIT—MudPIT mass spectrometry analyses were carried out as described previously (25–28).

Immunoprecipitations, Western Blots, and Antibodies—For immunoprecipitations of Myc epitopes (see Fig. 2), 1 mg of whole cell extracts from each strain were mixed with 1 μl of α-Myc (clone 9E10, Roche Applied Science) at 4 °C overnight. 1 μl of washed, packed Protein G-Sepharose beads (Amersham Biosciences) were added to the mixture and incubated at 4 °C for 2 h. The beads were washed four times with the extraction buffer, eluted by boiling with SDS-PAGE loading buffer, and then run in 10% SDS-PAGE for Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma). Fractions obtained from size exclusion chromatography and glycerol gradient centrifugation were assayed by Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma). Fractions obtained from size exclusion chromatography and glycerol gradient centrifugation were assayed by Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma). Fractions obtained from size exclusion chromatography and glycerol gradient centrifugation were assayed by Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma). Fractions obtained from size exclusion chromatography and glycerol gradient centrifugation were assayed by Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma). Fractions obtained from size exclusion chromatography and glycerol gradient centrifugation were assayed by Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma). Fractions obtained from size exclusion chromatography and glycerol gradient centrifugation were assayed by Western blot analysis using peroxidase-conjugated α-Myc (clone 9E10, Roche Applied Science) and α-M2 (Sigma).
were used in all HAT assays described above. Recombinant yeast histones (29) as histone sources and were used as the experiments in Fig. 4 SDS-PAGE and assayed for acetylated histone H4 by Western blots. For the experiments in Fig. 4, we took advantage of the tandem affinity purification system. All fractions were analyzed by Western blots probing for Sas2-TAP or Sas4-Myc. Molecular mass standards were used to estimate the size of the protein complex. Fr, fraction.

**Table II**

| Protein | SAS complex (SAS4-Myc, SAS2-TAP) | Mock (SAS4-Myc) | Molecular size (kDa) | % coverage (spectrum count) |
|---------|---------------------------------|-----------------|----------------------|---------------------------|
| Sas5    | 59.7 (84)                       | 29              | Sas5                 | 29                        |
| Sas4    | 57.8 (273)                      | 55              | Sas4                 | 55                        |
| Sas2    | 44.1 (88)                       | 39              | Sas2                 | 39                        |
| Sas10   | 13.1 (3)                        | 23              | Taf10                | 23                        |
| Taf6    | 7.6 (6)                         | 58              | Taf6                 | 58                        |
| Bpl10   | 4.5 (1)                         | 25              | Bpl10                | 25                        |
| Pbi1    | 3.5 (3)                         | 72              | Pbi1                 | 72                        |
| Ybr007c | 3.1 (3)                         | 82              | Ybr007c              | 82                        |
| Bmh2    | 2.9 (1)                         | 31              | Bmh2                 | 31                        |
| Mkt1    | 13.3 (20)                       | 94              | Mkt1                 | 94                        |
| Mt1     | 7.8 (5)                         | 50              | Mt1                  | 50                        |
| Bvb1    | 6.2 (2)                         | 52              | Bvb1                 | 52                        |
| Leb3    | 5.3 (2)                         | 49              | Leb3                 | 49                        |
| Hsp60   | 4.4 (2)                         | 61              | Hsp60                | 61                        |
| Pma1    | 2.1 (1)                         | 100             | Pma1                 | 100                       |

**Fig. 1.** Gel filtration chromatography analysis suggests that the native yeast SAS complex is about 450 kDa in size. The native yeast SAS complex was TAP-purified from the strain (YJW458) expressing both C-terminal Myc-tagged Sas4 and TAP-tagged Sas2. Both whole cell extracts (WCE) and TAP-purified SAS complex were subjected to gel filtration chromatography on a Superose 6 column using AKTA purifier system. All fractions were analyzed by Western blots probing for Sas2-TAP or Sas4-Myc. Molecular mass standards were used to estimate the size of the protein complex. Fr, fraction.

**Fig. 2.** SAS complex is a trimeric protein complex. Yeast strains with or without chromosomal Myc-tagged SAS subunit were transformed with plasmid expressing FLAG-tagged SAS subunit (top, YJW274, W303a + pSAS2-FLAG; YJW428, SAS2-Myc + pSAS2-FLAG; middle, YJW481, W303a + pSAS4-FLAG; YJW479, SAS4-Myc + pSAS4-FLAG; bottom, YJW482, W303a + pSAS5-FLAG; YJW480, SAS5-Myc + pSAS5-FLAG). Yeasts were grown in minimal medium with 2% galactose to induce the expression for FLAG-tagged subunit. Whole cell extracts were made, and 1 mg of whole cell extracts were immunoprecipitated with α-Myc antibody followed by Western blots against either Myc-tagged or FLAG-tagged proteins (lanes 1 and 4, 5% input (In); lanes 2 and 5, 5% supernatant (Sup); lanes 3 and 6, 20% bead (Be)). Lanes 1–3 serve as a negative control for Myc immunoprecipitation. Lane 6 shows that no FLAG-tagged subunit can be pulled down by the same subunit with Myc tag.

SDS-PAGE and assayed for acetylated histone H4 by Western blots. For the experiments in Fig. 4, nucleosome arrays were assembled using recombinant yeast histones (29) as histone sources and were used as substrates in HAT assays. Approximately 2 pmol of SAS complexes were used in all HAT assays described above.

**RESULTS**

*The Native Yeast SAS Complex Is a 125-kDa Trimeric Complex*—Many proteins in the MYST family of HATs are components of multiprotein complexes (30–32). In budding yeast *S. cerevisiae*, two well-studied examples from this family are Esa1 in NuA4 and Sas3 in NuA3. We and others have previously shown that Sas2, Sas4, and Sas5 form the SAS histone acetyltransferase complex and that Sas2 is the catalytic subunit (4, 10, 12). Size exclusion chromatography analysis indicated that the SAS complex was between 230–450 kDa in size based on fractionation of whole cell extracts and complex purified from cells overexpressing Sas2. This molecular weight of the SAS complex, coupled with mass spectrometry analysis, suggested additional subunits besides the three known components. We were unable, however, to identify any bona fide additional subunits from the SAS complex purified from cells overexpressing Sas2. Thus, we sought to purify the native complex from cells with endogenous levels of Sas2.

To purify the native and endogenously expressed SAS complex, we took advantage of the tandem affinity purification method (22). We generated a yeast strain with a TAP tag in Sas2 and a Myc tag in Sas4. Both tags were integrated into the 3' end of the gene of interest to ensure that expression was driven by the endogenous promoter. Yeast whole cell extracts were prepared from these double-tagged strains and subjected to the two-step affinity purification procedure to isolate SAS complex. We then fractionated both whole cell extracts and TAP-purified SAS complex by Superose 6 size exclusion chromatography. Fractions were analyzed by Western blots probing for Sas2-TAP and Sas4-Myc in purified complex (Fig. 1). In both cases, we observed one protein peak at fraction 16 corresponding to 450 kDa in size, suggesting that we did not disrupt the integrity of the SAS complex during the purification procedures. This result using native SAS complex is identical to our previously published findings (10) using overexpressed SAS complex. Therefore, it remained a possibility that there were additional proteins in the complex besides Sas2, Sas4, and Sas5.

To gain further insight into the detailed protein composition of SAS complex, we analyzed the TAP-purified SAS complex by MudPIT mass spectrometry analysis (Table II). A mock TAP purification from a yeast strain expressing Sas4-Myc was performed as a negative control. The three known subunits, Sas2,
Sas4, and Sas5, were identified as the top three proteins in MudPIT analysis based on the percent coverage and the number of unique peptide hits. Additional proteins identified in the mass spectrometry analysis fell into two groups: those also found in the negative control sample and those found only in the purified SAS complex sample. Among the proteins that were recovered only from TAP-purified SAS complex, however, none had significant coverage or peptide hits (when compared with Sas2, Sas4, and Sas5) to suggest that they were bona fide components of the SAS complex. We also tested whether any of these proteins interact with the SAS complex in vivo by coimmunoprecipitation experiments; none of these proteins coimmunoprecipitated with the SAS complex (data not shown). Based on these findings, we conclude that Sas2, Sas4, and Sas5 are the sole components of the SAS complex. These data presented us with an interesting paradox since our size exclusion chromatography analysis indicated a 450-kDa complex, a number that is not the additive molecular weights of Sas2, Sas4, and Sas5.

One possible answer to this problem may be that the SAS complex contains multiple copies of one or more subunits. To test this hypothesis, we transformed a plasmid expressing Sas2-FLAG into a yeast strain with chromosomally tagged SAS2-Myc. If there was more than one copy of Sas2 in the SAS complex, coimmunoprecipitations from whole cell extracts with α-Myc antibody should be able to pull down FLAG-tagged Sas2. In fact, we were not able to detect any Sas2-FLAG signal in the α-Myc immunoprecipitation although the expression of Sas2-FLAG was robust (Fig. 2, top panel, lane 6). Myc immunoprecipitations were determined to be efficient based on the fact that Sas2-Myc was dramatically reduced in supernatant (lane 5 compared with Input lane 4) and enriched on the beads (lane 6). We performed the same experiments with Sas4 and Sas5 with similar results (Fig. 2, middle and bottom panels). As a negative control, immunoprecipitations were carried out in the absence of Myc-tagged SAS subunit (lanes 1–3). Reciprocal immunoprecipitations with α-FLAG were performed with the same outcome (data not shown). Collectively, these data indicate that the native yeast SAS complex is a trimeric protein complex consisting of single copies of Sas2, Sas4, and Sas5 respectively.

In gel filtration chromatography analysis, protein complexes will migrate according to their respective molecular weights only if they are globular. Therefore, this method should not be applied to determine the molecular weight of non-globular proteins. Accordingly, another possible explanation of our paradox is that the SAS complex forms a non-globular shape. To test this theory, we fractionated the SAS complex by glycerol gradient sedimentation, which separated proteins primarily on the basis of their mass and to a lesser extent on their density and shape (33). The SAS complex purified from the SAS2-TAP, SAS4-Myc double-tagged strain was fractionated by 10–40% glycerol gradient sedimentation. Fractions were then analyzed by Western blots probing for Sas2-CBP and Sas4-Myc (Fig. 3). The SAS complex signal peaked at fractions 9 and 10 in our analyses. After calibrating the fraction profile with molecular mass standards, we estimated that the native yeast SAS complex was actually 150 kDa in size, approximately the sum of Sas2-CBP (44 kDa), Sas4-Myc (75 kDa), and Sas5 (29 kDa). If we subtract the contribution of the CBP (5 kDa) and Myc (20 kDa) tags from this value it indicates a molecular mass of the untagged complex of 125 kDa, very near the sum of the predicted molecular mass of Sas2, Sas4, and Sas5 (123 kDa). Therefore, we concluded that the SAS complex is a small (125 kDa), trimeric (Sas2, Sas4, and Sas5) protein complex consisting of single copies of each subunit.

SAS Complex Has Weak, but Real, Nucleosomal HAT Activity—Several groups have suggested that the HAT activity of the SAS complex is responsible for counteracting the Sir2-mediated deacetylation at telomeres and HML mating locus, thereby preventing the propagation of Sir proteins into euchromatin (17–20). Thus, one possible function for the SAS complex in vivo is nucleosome acetylation. We had previously reported, however, that recombinant SAS complex was able to acetylate free histones but not nucleosomes in vitro. A very similar acetylation pattern was obtained when we tested the HAT activity of the native SAS complex (12). Although the HAT activity of the native SAS complex was above background, the results were not significant. Since those studies were published, we have optimized our in vitro HAT assays by adding histone deacetylase inhibitors, lowering salt content to 80 mM total, and using more concentrated, TAP-purified SAS complex. These new conditions allowed us to detect significant SAS nucleosomal HAT activity for the first time (Fig. 4A, lane 5). Using similar amounts of histones, we found stronger SAS HAT activity on free histones when compared with that on nucleosomes (Fig. 4A, compare lanes 2 and 5). We also compared the nucleosomal HAT activity of SAS complex to NuA4 and found that even with an excessive amount of SAS complex (2 pmol of SAS versus 0.1 pmol of NuA4), nucleosomal HAT activity of SAS complex is still much weaker than NuA4 (Fig. 4, compare lanes 5 and 6). To rule out the possibility that the SAS nucleosomal HAT activity was contributed by the contaminated free histones in the reaction, we used immobilized nucleosomal arrays as substrates that allowed us to wash away any free histones in the reaction. Again, the nucleosomes were significantly acetylated by SAS complex (Fig. 4B). Therefore, we conclude that the native SAS complex has real, although weak, nucleosomal HAT activity.

Members of the MYST family share a conserved acetyl CoA binding motif. Mutations at the acetyl CoA binding domain of Sas3 cause the loss of HAT activity in Nua3 (9). We have previously shown that the mutations in the Sas2 acetyl CoA binding domain (M1, 219GLG to AAA; M2, 216QR to AA; M3, 224LI to AA) resulted in the disruption of silencing at the telomere and the HML mating locus. Among those mutations tested, SAS2-M1 mutation showed the most severe silencing defects (10). In addition, recombinant SAS complex containing...
Sas2-M1 lost its ability to acetylate histones (12). It has been demonstrated that loss of \( \text{SAS2} \) will cause a dramatic reduction of \textit{in vivo} acetylation at histone H4 lysine 16 (17). To further clarify the importance of the acetyl CoA binding domain of Sas2 to its enzymatic activity, we checked the overall acetylation level at histone H4 lysine 16 in the presence of mutant \( \text{SAS2} \) and found that bulk H4 lysine 16 acetylation levels were indeed lowered \textit{in vivo} (Fig. 5A). Next, we TAP-purified the native SAS complex with either wild-type \( \text{SAS2} \) or mutant \( \text{SAS2} \) (M1). The purification results were visualized by silver staining, and no difference could be observed between wild-type and mutant SAS complex (data not shown). However, the mutant SAS complex was no longer able to acetylate nucleosomes (Fig. 5B).

Our data indicate that the acetyl CoA binding domain of \( \text{SAS2} \) is not only essential for its silencing function but also crucial for the HAT activity of the SAS complex. This result also confirms that the detected weak nucleosomal HAT activity was indeed due to Sas2 and not to any minor contaminants in the preparation.

**Fig. 4. SAS complex has weak, but real, nucleosomal HAT activity.** A, \textit{in vitro} HAT assay using TAP-purified SAS (lanes 2 and 5, from YJW458) and NuA4 (lanes 3 and 6, from YWJS953) as histone acetyltransferases, HeLa core histones (lane 1–3), and HeLa nucleosomes (lanes 4–6) as substrates. Half of the reactions were spotted on Whatman P-81 filters for quantification. The other half was loaded on an 18% SDS-PAGE to separate individual histones. Gel was then stained with Coomassie Blue and subjected for fluorography. B, immobilized nucleosome (Nuc.) arrays assembled from recombinant yeast histones were incubated with SAS complex. After stringent washing, acetylated nucleosomes were directly subjected for quantification by liquid counting assay. Data shown are the average of at least three separate experiments. Error bars represent standard deviation.
Histone H4 Lysine 16 Is the Exclusive Histone Substrate for the SAS Complex—We and others have shown that SAS complex specifically acetylates lysine 16 of histone H4 and that loss of SAS2 is directly responsible for the reduction in overall acetylation level at histone H4 lysine 16 (12, 17). Here we showed that SAS4 and SAS5 are also essential for maintaining the acetylation specifically at lysine 16 of histone H4 (Fig. 6A). Deletion of either subunit in the SAS complex caused the equal reduction of acetylation at histone H4 lysine 16. Therefore, these results indicate that the SAS complex can function only if all three subunits are present.

We next examined the in vitro substrate specificity of the native SAS complex by using yeast recombinant histone H4 as acetylation substrate. The same experiments were also performed with NuA4 as a comparison. The amount of SAS complex and NuA4 used in the HAT assays were normalized based on the HAT activity obtained from a liquid counting assay using 3H-labeled acetyl CoA. After the HAT reaction, we analyzed the results by Western blots probing for various mono-acetylated H4 isoforms (Fig. 6B). Unlike NuA4, which acetylates all available lysines at the H4 N-terminal tail, the native SAS complex exclusively acetylates lysine 16 on H4. This result further demonstrates that the native SAS complex is an H4 lysine 16-specific HAT complex that correlates with its antisilencing function in antagonizing the Sir2-mediated deacetylation of H4 lysine 16.

**DISCUSSION**

We have presented evidence in this report that the yeast SAS complex is a small trimeric histone acetyltransferase complex that is capable of acetylating nucleosomes. Although the composition of the SAS complex is simple (Sas2, Sas4, and Sas5), each subunit is vital for HAT activity both in vivo and in vitro. Sas2, as the catalytic subunit, is essential for the enzymatic
Sas4 does not show significant homology to any known protein, but it may play a role in physically linking the SAS complex to chromatin assembly since it interacts directly with histone deposition protein Asf1 (13). Sas5 is a member of the YEATS protein family. In *S. cerevisiae*, YEATS domain-containing proteins are all components of chromatin-related complexes (Sas5 in SAS; Yaf9 in NuA4 and SWR complex; Taf14 in NuA3, TFIID, TFIIF, SRB (suppressor of RNA polymerase B mutations) mediator complex, and SWI/SNF) (9, 34–37). Deletion of all three YEATS-containing proteins (yaf9Δ, taf14Δ, sas5Δ) from yeast results in lethality (11). Individually, Sas5 is required for the full HAT activity of the SAS complex (12). Deletion of Yaf9 shows a similar phenotype to deletion of certain NuA4 or SWR components (11, 38). It appears that a major role for the YEATS-containing complexes is anti-silencing. One possible mechanism for YEATS protein function could be to target their respective complex to specific chromatin boundaries, perhaps through histone binding and recognition.

We present here novel nucleosomal HAT activity for the SAS complex. We confirmed that the SAS complex can acetylate nucleosomes after optimizing reaction conditions. There is no question that the SAS complex is responsible for the specific acetylation of histone H4 lysine 16 in living cells. The fact that SAS can easily acetylate free histones, but not nucleosomes made from them, rules out the possibility that other histone post-translational modifications are a prerequisite for SAS-mediated acetylation (39). It is still not clear, however, why free histones serve as better in vitro substrates for SAS complex when compared with nucleosomes. One obvious explanation for this discrepancy is that we are missing some associated factors in our in vitro system that are required for full activity of the SAS complex. These factors may not necessarily co-purify with Sas2 but may be required for optimal SAS complex function on nucleosomes. Specifically, nucleosome remodeling may play a role in facilitating the SAS complex function by making histones more accessible for acetylation. Indeed, Gas41, a human homolog of Sas5, was found to interact with the Snf5 subunit of SWI/SNF (40).

As proposed by Ehrenhofer-Murray (41), SAS complex may play a role in reestablishing the euchromatin mark following replication-coupled nucleosome deposition. Histone deposition proteins, Asf1 and/or CAF-I, incorporate newly synthesized histone H3 and H4 on DNA in a replication-coupled manner (14, 16, 42). Interestingly, Asf1 and CAF-I are associated with the SAS complex (4, 13, 43). It is possible that SAS complex is recruited by Asf1 or CAF-I following histone deposition to acetylate nucleosomes at histone H4 lysine 16 and reestablish the euchromatin mark. As discussed above, other chromatin-modifying complexes may play a role in this process. For example, Asf1 has been shown to interact with *Drosophila* SWI/SNF (Brahma complex) (44).

Yeast with sas2Δ or mutated Sas2 all show silencing defects and loss of enzymatic HAT activity, implying a direct link between these two functions. It has been suggested that the SAS complex is involved in the formation of a barrier that prevents spreading of heterochromatin from silenced regions into euchromatin. Specifically, this mechanism has been termed anti-silencing and can be found at the telomeres and HMR mating locus boundaries (17–20). The acetylation of histone H4 lysine 16 by SAS complex may provide a barrier to Sir proteins that would prevent adjacent euchromatic regions from being transcriptionally inactivated. It would be interesting to discover whether the SAS complex is involved in anti-silencing at other loci.

Recent studies regarding H2A.Z, a histone H2A variant, have revealed an interesting link between H4 lysine 16 acetylation and histone variant replacement, suggesting another mechanism for the establishment of silencing barriers by SAS complex. It is believed that SWR complex replaces H2A with H2A.Z at subtelomeric regions (29, 45, 46) and that this substitution plays a key role in blocking the propagation of Sir proteins. Interestingly, many genes near telomeres are dependent on Sas2 for activation based on microarray analysis (17), and the same results have been reported on many components of SWR complex, including SWR1, BDF1, HTZ1, and YAF9 (11, 29, 45–48). This overlap in function suggests a possible cooperation between SAS complex and SWR complex in the establishment of silencing barriers. Perhaps acetylation of histone H4 lysine 16 by the SAS complex not only antagonizes the NAD-dependent deacetylation by Sir2 but also signals the SWR complex for H2A.Z incorporation.

**Acknowledgments**—We are grateful to Bing Li for providing recombinant nucleosome arrays used in HAT assays and Samantha Pattenden for critical reading of the manuscript.

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J. Biol. Chem. 2005, 280:11987-11994.
doi: 10.1074/jbc.M500276200 originally published online January 18, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M500276200

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