Heterochromatin at yeast telomeres and silent mating (HM) loci represses adjacent genes and is formed by the binding and spreading of silencing information regulators (SIR proteins) along histones. This involves the interaction between the C terminus of SIR3 and the N terminus of histone H4. Since H4 is hypoacetylated in heterochromatin we wished to determine whether acetylation is involved in regulating the contacts between SIR3 and H4. Binding of H4 peptide (residues 1–34) acetylated at lysines Lys-5, Lys-8, Lys-12, and Lys-16 to an immobilized SIR3 protein fragment (residues 510–970) was investigated using surface plasmon resonance. We find that acetylation of H4 lysines reduces binding (Kc) of H4 to SIR3 in a cumulative manner so that the fully acetylated peptide binding is decreased ~50-fold relative to unacetylated peptide. Thus, by affecting SIR3-H4 binding, acetylation may regulate the formation of heterochromatin. These data help explain the hypoacetylated state of histone H4 in heterochromatin of eukaryotes.

In yeast, the silent mating loci (HMLa and HMRa) and sub-telomeric regions have many features of higher eukaryotic heterochromatin and can silence genes transposed to their vicinity in an epigenetic manner (1, 2). Repression of these regions is believed to be initiated through the interaction of silencing information regulators (SIR) proteins with RAP1 (3) and the histone H3 and H4 N termini (4–6). SIR2, SIR3, and SIR4 proteins then spread from the initiation site along chromatin (7, 8). Direct binding, genetic studies, and chromatin immunoprecipitation experiments argue that the formation and spreading of heterochromatin involves the interaction of SIR3 and SIR4 with the histone H3 and H4 N-terminal tails. Overexpression of SIR3 alone spreads the silent state appreciably (9) and single mutations of the H4 tail strongly disrupt SIR3 binding (6, 7). Therefore, the dominant interaction is likely to be that between SIR3 and the H4 tail.

Lysine residues on the N-terminal tails of all four core histones, including H4, are hypoacetylated in yeast heterochromatin even at H4 Lys-12 a site previously thought to be preferentially acetylated in yeast heterochromatin (10, 11). Genetic disruption of heterochromatin by deletion of SIR genes results in increased acetylation at all core histone sites. This was shown recently using chromatin immunoprecipitation and highly specific antibodies to acetylation sites. However, it is not known whether hypoacetylation is a cause or result of the heterochromatic state. For example, the underlying DNA in heterochromatin is refractory to a number of molecular probes such as nuclease and dam methylase (12). Thus, heterochromatin may prevent access to certain histone-modifying proteins and thereby persist in a hypoacetylated state. Interestingly, mutations in histone deacetylases such as HDA1 and RPD3 result in improved rather than decreased silencing of heterologous genes by heterochromatin (13–15). However, since these deacetylases are known to affect the expression of heterochromatin proteins (16) it is possible that their role in silencing heterochromatin is indirect.

In this paper we have directly addressed the function of acetylation in regulating SIR3-H4 interactions by analyzing the binding of SIR3 to unacetylated and acetylated H4 peptides in vitro. The interaction between SIR3 and H4 involves two distinct regions at the histone H4 N terminus. Residues 16–29 represent an essential site of interaction since non-conservative substitutions within this region strongly decrease HM and telomeric silencing (17, 18) and SIR3 binding to HM and telomeric heterochromatin in vivo (7, 8). Also the H4 sequence 15–34 that contains the major silencing domain has been shown to interact directly with SIR3 residues 623–910 in vitro (5).

H4 residues between amino acids 4–14 also influence silencing albeit less strongly. Mutation or deletion of acetylatable lysines Lys-5, Lys-8, and Lys-12 has little effect on HM silencing or SIR3 binding at HM loci (7). However, these residues have a measurable effect on telomeric silencing that is more sensitive to genetic disruption than that of the HM loci (6).

Moreover, conservative mutations at Lys-5, Lys-8, and Lys-12 can suppress in part even the HM silencing defect caused by a non-conservative H4 K16Q mutation (5). Also, neutral amino acid substitutions at Lys-5, Lys-8, or Lys-12 can relieve the deleterious effect on HM silencing that occurs when all four lysine residues are replaced by arginines (10). Finally, residues within 4–14 can bind SIR3 in vitro even in a K16Q mutant (5). These data all argue for the importance in silencing of residues 16–29 and, to a lesser extent, residues 4–14.

Non-conservative substitution at H4 Lys-16 disrupts silencing of HM loci, while conservative substitution has little effect (18, 19). However, while these data argue for the importance of the charge at Lys-16 they do not address the role of acetylation directly. In direct binding experiments histone H4 acetylation was found to improve binding to bromodomains of proteins p300 and TAFII250 as measured by calorimetry (20, 21). In contrast, mono- or diacetylated H4 was shown to bind the repressor TUP1 in far-Western assays while tri- and tetracety-
Histone H4 Acetylation Reduces SIR3 Binding

RESULTS AND DISCUSSION

To determine whether acetylation of the histone H4 N terminus affects its binding to SIR3, we measured the affinity of various acetylated H4 peptides for SIR3 by SPR. The regions of histone H4 (residues 1–34) and SIR3 (residues 510–970) used for SPR binding analysis are shown boxed in Fig. 1. The H4 peptides contain the acetylation sites (Lys-5, Lys-8, Lys-12, and Lys-16) as well as the rest of the genetically determined silencing domain (residues 16–29). These were interacted with the region of SIR3 (residues 510–970) that includes residues 623–910, a domain that has been shown to bind histone H4 residues 15–34 in vitro (5).

Peptides containing single acetylated sites as well as combinations of two, three, or all four sites were analyzed for their affinity for GST-SIR3 (503–970) bound to a Biacore B1 sensor chip and compared with a control chip containing GST alone (Fig. 2). The association (k_a) and dissociation (k_d) rate constants, the association equilibrium constant (K_a) and standard free-energy changes (ΔG^0) are shown in Table I. We found that ΔG^0 for the association of unacetylated H4 peptide with the SIR3 C terminus is −49.1 kJ mol⁻¹ while the ΔG^0 for the association of fully acetylated H4 peptide with SIR3 is −33.2 kJ mol⁻¹. This stabilization free-energy (ΔΔG^0 = −9.75 kJ mol⁻¹) favors the interaction between hypoacetylated H4 and SIR3 and may contribute to the requirement for a hypoacetylated state at heterochromatic regions like the silent HM loci and at yeast telomeres (24).

We have found, in particular, that significant decreases in the association rate constant were associated with increasing levels of H4 acetylation. A comparison of k_a values reveals that monoacetylated H4 peptides associated with SIR3 only 60–70% as well as unmodified peptide, di-acetylated peptide 40%, tri-acetylated peptides 14–22%, and tetra-acetylated peptide 2.7% relative to unacetylated peptide. These data indicate that the acetylation-induced disruption of H4-SIR3 binding is cumulative (Fig. 3). There is considerable redundancy in the ability of acetylated lysines to prevent SIR3 binding since similar results are obtained irrespective of which lysine (Lys-5, Lys-8, Lys-12, or Lys-16) is acetylated. Acetylation of lysines
Histone H4 Acetylation Reduces SIR3 Binding

**Table I**

Summary of the acetylation dependence of $k_a$ (M$^{-1}$ s$^{-1}$), $k_d$ (s$^{-1}$), $K_a$ (M$^{-1}$) and $\Delta G^\circ$ (kJ·mol$^{-1}$) of various H4 peptides binding to a SIR3-GST fusion at 150 mM NaCl and standard deviation for $k_a$ and $k_d$.

| Amino acids 1-34 | $k_a$ | % Standard deviation $k_a$ | $k_d$ | % Standard deviation $k_d$ | $K_a$ | $\Delta G^\circ$ |
|------------------|-------|---------------------------|-------|---------------------------|-------|---------------|
| H4 unacetylated   | 5.21  | 12.6                      | 1.76  | 15.7                      | 2.96  | -42.9         |
| H4 Lys-5-Ac       | 3.63  | 1.80                      | 1.88  | 3.40                      | 1.93  | -41.8         |
| H4 Lys-8-Ac       | 3.13  | 14.6                      | 1.92  | 24.5                      | 1.72  | -41.6         |
| H4 Lys-12-Ac      | 3.79  | 17.3                      | 2.18  | 4.90                      | 1.73  | -41.6         |
| H4 Lys-16-Ac      | 3.57  | 20.3                      | 1.97  | 11.8                      | 1.81  | -41.7         |
| H4 Lys(-12,-16)-Ac| 2.21  | 19.8                      | 1.70  | 16.4                      | 1.30  | -41.9         |
| H4 Lys(-5,8,-12)-Ac| 1.01  | 22.6                      | 2.25  | 25.1                      | 0.45  | -38.2         |
| H4 Lys(-5,8,-16)-Ac| 1.16  | 23.3                      | 1.90  | 7.40                      | 0.61  | -39.0         |
| H4 Lys(-8,12,-16)-Ac| 0.73  | 39.1                      | 1.89  | 17.8                      | 0.39  | -37.8         |
| H4 Lys(-8,-12,-16)-Ac| 0.14  | 46.0                      | 2.35  | 27.2                      | 0.06  | -33.3         |

**Fig. 3.** Acetylation of lysines in histone H4 more strongly affects association rate rather than dissociation for SIR3-GST binding. Data was collected as indicated under “Experimental Procedures” for two independent assays for multiple analyte concentrations (“Experimental Procedures”), and values were calculated for $k_a$, $k_d$, and $K_a$. Binding is for peptide analytes to a SIR3 (residues 503-970)-GST ligand as indicated under “Experimental Procedures”. A, rates of association ($k_a$) for each peptide analyte. These data demonstrate that acetylation has a cumulative negative effect on H4-SIR3 binding. B, rates of dissociation ($k_d$) for each peptide analyte. $k_d$ are relatively unaffected by changes in H4 acetylation. C, dissociation equilibrium constants ($K_a$) for each peptide analyte. A $K_a$ value of 35 nM is observed for the unacetylated H4-SIR3 interaction as compared with 1.68 µM for fully acetylated H4. **D**, a plot of $\Delta G^\circ$ versus H4 peptide acetylation. The interaction of unacetylated H4 peptide is more favorable than that of acetylated peptide.

Lys-5, Lys-8, and Lys-12 has a similar effect on $K_a$ as does acetylation at Lys-5, Lys-8, Lys-16, or at Lys-8, Lys-12, Lys-16 (ranging from 3.9 $\times$ 10$^7$ M$^{-1}$ to 6.1 $\times$ 10$^9$ M$^{-1}$). The most dramatic reduction in association occurs when all four lysines are acetylated resulting in a $K_a$ value of 5.9 $\times$ 10$^7$ M$^{-1}$. This is a 50-fold decrease in $K_a$ compared with the unacetylated H4-SIR3 interaction ($K_a = 2.96$ $\times$ 10$^9$ M$^{-1}$). While acetylation of H4 causes a decrease in association rates ($k_a$), disassociation rates ($k_d$) ranging from 1.7 $\times$ 10$^{-3}$ s$^{-1}$ to 2.35 $\times$ 10$^{-3}$ s$^{-1}$ are only modestly perturbed by changes in acetylation. If we extrapolate from these in vitro interactions to those occurring in vivo, hypoacetylation of H4 may promote the formation of heterochromatin but may have little effect on its maintenance. Also, during the process of yeast aging, SIR proteins are released from telomeres and the silent mating loci and are transferred to the rDNA locus (14, 25). Histone H4 at the rDNA locus is likely to be deacetylated by the SIR2 enzyme. The favorable dissociation rate ($k_d$) for SIR3 binding to hypoacetylated H4 may facilitate the relocalization event.

It is possible that the observed binding of the unacetylated peptide to SIR3 is due predominantly to ionic interactions and that neutralization of the positive charge on lysine via acetylation causes the reduced binding. If charge effects alone are responsible for H4-SIR3 binding then increasing the ionic strength of the solvent would be expected to reduce overall binding. To address this possibility, we analyzed the binding of unacetylated H4 residues 1-34 to SIR3 at various ionic strengths ranging from 100 to 250 mM NaCl. We found that the most dramatic reduction in association occurs when all four lysines are acetylated resulting in a $K_a$ value of 5.9 $\times$ 10$^7$ M$^{-1}$. This is a 50-fold decrease in $K_a$ compared with the unacetylated H4-SIR3 interaction ($K_a = 2.96$ $\times$ 10$^9$ M$^{-1}$). While acetylation of H4 causes a decrease in association rates ($k_a$), disassociation rates ($k_d$) ranging from 1.7 $\times$ 10$^{-3}$ s$^{-1}$ to 2.35 $\times$ 10$^{-3}$ s$^{-1}$ are only modestly perturbed by changes in acetylation. If we extrapolate from these in vitro interactions to those occurring in vivo, hypoacetylation of H4 may promote the formation of heterochromatin but may have little effect on its maintenance. Also, during the process of yeast aging, SIR proteins are released from telomeres and the silent mating loci and are transferred to the rDNA locus (14, 25). Histone H4 at the rDNA locus is likely to be deacetylated by the SIR2 enzyme. The favorable dissociation rate ($k_d$) for SIR3 binding to hypoacetylated H4 may facilitate the relocalization event.

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tion. It is also possible that the steric bulk of the acetyl moiety and the propensity for acetylated histone tails to form α-helical structures (28) decrease interactions between acetylated H4 and SIR3.

In vivo, substitution of Lys-16 with glutamine has a strong disruptive effect on silencing while similar substitutions at Lys-5, Lys-8, and Lys-12 have much weaker effects. Also, bulk monoacetylated H4 is acetylated predominantly at Lys-16, Lys-5, Lys-8, and Lys-12 have much weaker effects. Also, bulk disruptive effect on silencing while similar substitutions at H4 Lys-16 acetylation is a unique trigger for the acetylation of further regulating SIR3-H4 interactions. This could occur if lysine residues has similar effects on reducing SIR3-H4 binding in vivo. If so, acetylation or non-conservative substitution of Lys-16 in vivo may lead to acetylation of the other H4 sites thereby disrupting heterochromatin. This would lead to the hyperacetylation of all histone sites. Conversely, since SIR2 heterochromatin protein is a deacetylase with specificity for H4 Lys-16 (14, 29, 30), deacetylation of the other H4 sites thereby disrupting heterochromatin. This suggests that the in vivo context is important in further regulating SIR3-H4 interactions. This could occur if H4 Lys-16 acetylation is a unique trigger for the acetylation of the remaining H4 sites in vivo. If so, acetylation or non-conservative substitution of Lys-16 in vivo may lead to acetylation of the other H4 sites thereby disrupting heterochromatin.

In conclusion, yeast heterochromatin is formed as part of a complex that includes RAP1, SIR3, and histone H4 among other proteins (7). The interaction between SIR3 and H4 is crucial for the stable formation of this complex. While the in vitro experiments described here utilize only relevant portions of H4 and SIR3, it is clear that acetylation of H4 strongly reduces H4-SIR3 binding in this system. Therefore, hypoacetylation of H4 is likely to favor the formation of heterochromatin in vivo in the context of contributions from other proteins in the silencing complex and the presence of DNA. Given that SIR2 is a histone H4 deacetylase, our data support a model in which deacetylation of H4 by SIR2 promotes SIR3-H4 binding and by inference the subsequent formation of heterochromatin (29, 30).

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