Chromatin Assembly Factor 1 Interacts with Histone H3 Methylated at Lysine 79 in the Processes of Epigenetic Silencing and DNA Repair†

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Received October 16, 2005; Revised Manuscript Received December 16, 2005

ABSTRACT: In eukaryotic cells, chromatin is classified into euchromatin, which is active in transcription, and heterochromatin that silences transcription. Histones in these two domains contain distinct modifications. Chromatin assembly factor 1 (CAF-1) is a highly conserved protein that functions in DNA replication, DNA repair, and heterochromatin silencing. CAF-1 binds histones H3 and H4 and deposits histones onto DNA to form nucleosomes. However, modifications on H3 and H4 associated with CAF-1 are not known. Here, we have purified a complex containing CAF-1 and H3 and H4 from yeast cells and determined the modifications present on these histones using linear ion trap FT-ICR mass spectrometry. H4 that copurified with CAF-1 was a mixture of isoforms acetylated at lysines 5, 8, 12, and 16, whereas an H3 peptide methylated at lysine 79 and an H3 peptide acetylated at lysine 56 were detected. In yeast cell extracts, these two H3 modifications peaked in the late S phase with different kinetics. Moreover, the association of CAF-1 with H3 methylated at lysine 79 appeared to occur in the late S phase. Finally, cells lacking both Dot1p, the methyltransferase that methylates H3 lysine 79, and Cac1p, the large subunit of CAF-1, exhibited a dramatic loss of telomeric silencing and increased sensitivity to DNA damaging agents. Together, these data indicate that CAF-1 interacts with H3 methylated at lysine 79 during the processes of epigenetic silencing and DNA repair.

In eukaryotic cells, genomic DNA is packaged into chromatin, an organized complex of DNA and protein. Chromatin is organized into functional domains such as euchromatic and heterochromatic regions. The basic unit of chromatin is the nucleosome. A nucleosome consists of 146 base pairs of DNA wrapped around a histone octamer containing a tetramer of H3 and H4 and two dimers of H2A and H2B (*1*). These core histones are subject to a variety of modifications, including acetylation, phosphorylation, methylation, and ubiquitination (*2*), which, when present in distinct combinations, are required for establishment and maintenance of different chromatin states (*3*, *4*). Because distinct chromatin states are important in regulation of gene expression and maintenance of genomic integrity, these chromatin states, once established, must be inherited during the S phase of the cell cycle.

The inheritance of chromatin structure during the S phase is carried out immediately after DNA replication by two distinct pathways (*5*, *6*). The first pathway, known as parental histone segregation, involves the transfer of preexisting nucleosomal histones onto the two nascent chromatids behind the replication fork. It is still unclear whether parental histones are deposited via a semiconserved mechanism where two H3−H4 dimers from one parental H3−H4 tetramer are deposited separately onto the two nascent chromatids (*7*) or via a mechanism whereby parental histones are deposited randomly onto the two nascent chromatids (*8*). Moreover, it is unclear if transfer of parental histones to the two nascent chromatids is assisted by histone chaperones. The second pathway, known as de novo nucleosome assembly, involves the deposition of newly synthesized, and generally hyperacetylated, histones onto replicating DNA and, in contrast to the transfer of parental histones, is known to require histone chaperone complexes. Histone chaperones are a group of proteins that bind to histones and promote nucleosome formation during DNA replication, repair, and transcription (*9*).

Chromatin assembly factor 1 (CAF-1)[1] is a highly conserved histone chaperone. In both yeast and human cells, CAF-1 consists of three subunits (Cac1p, Cac2p, and Cac3p in yeast and p150, p60, and p48 in human cells) (*10*−*12*). Moreover, in both yeast and human cells, CAF-1 forms a complex with histones H3 and H4 and promotes nucleosome

† This work is supported by a grant from NIH (GM72719) and a grant from the Fraternal Order of Eagles Award for Cancer Research (Z.Z.). We gratefully acknowledge support from the Mayo Clinic College of Medicine, and D.C.M. thanks the W. M. Keck Foundation for their generous support.

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[1] Abbreviations: CAF-1, chromatin assembly factor 1; LTQ-FT-ICR, linear ion trap Fourier transform ion cyclotron resonance mass spectrometer; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TAP, tandem affinity purification; KLH, keyhole limpet hemocyanin.

10.1021/bi0521083 CCC: $33.50
Published on Web 02/07/2006

formation using replicated DNA (*12*, *13*). Furthermore, CAF-1 from both yeast and mammalian cells associates with proliferating cell nuclear antigen (PCNA), a component essential for DNA replication and DNA repair (*14*−*16*). Finally, both yeast and mammalian CAF-1 are involved in chromatin-mediated silencing through formation and/or maintenance of heterochromatin (*12*, *17*−*19*). Thus, it appears that the role of CAF-1 in DNA replication, repair, and heterochromatin formation and/or maintenance is conserved from yeast to mammalian cells.

Because distinct modifications on histones are required for maintaining different chromatin states, CAF-1 may associate with histones that are modified in distinct ways to facilitate inheritance of distinct chromatin structures during the S phase. Indeed, H4 copurified with CAF-1 from mammalian cells was found to be a mixture of unacetylated and mono-, di-, and triacetylated isoforms (*13*). It was proposed that this acetylation pattern might facilitate CAF-1's ability to deposit histones onto replicated DNA. Surprisingly, deleting the H3 and H4 N-termini containing the acetylatable lysine residues did not affect the ability of CAF-1 to bind H3 and H4 or to deposit these histones onto replicated DNA for nucleosome formation (*20*). Thus, acetylation of the N-terminal lysine residues in H4 is not necessary for CAF-1 binding or CAF-1-mediated nucleosome formation during the S phase of the cell cycle. However, other H3 and H4 modifications may mediate CAF-1−histone binding as well as other CAF-1 functions.

Recently, it has been shown that mutations of the H4 N-terminal lysine residues impair its import into the nucleus by Cac1p (*21*) in yeast cells. Because the CAF-1−histone complex has never been purified from yeast cells, it is not known what modifications are present on histones associated with CAF-1 and what potential functions these histone modifications might mediate. Therefore, in an effort to identify modifications on H3 and H4 copurified with CAF-1 and to understand the functions of these modifications, we purified the CAF-1−histone complex from yeast cells and identified modifications present on copurified H3 and H4 using mass spectrometry. We found that the copurified H4 was a mixture of different acetylated isoforms, similar to what was found in mammalian cells. More importantly, two H3 modifications were also detected, acetylation of lysine 56 and methylation of lysine 79. Acetylation of lysine 56 associated with CAF-1 has very recently been reported (*22*). However, conflicting results were reported on whether acetylation of lysine 56 was cell cycle regulated (*22*, *23*). Here, we focused mainly on characterizing the methylation of H3 lysine 79. We found that methylation of lysine 79 and acetylation of lysine 56 were regulated during the cell cycle with different kinetics and dynamics. Methylation of lysine 79 increased late in the S phase when acetylation of lysine 56 peaked. In addition, the association of CAF-1 with H3 methylated at lysine 79 occurred predominantly in the late S phase. Furthermore, cells lacking both methylation of lysine 79 and Cac1p exhibited a dramatic loss of telomeric silencing and a significant reduction in the association of Sir proteins, the structural component of yeast heterochromatin, at telomeric heterochromatin. Finally, while *cac1*Δ *dot1*Δ double mutant cells grew normally, they were sensitive to a variety of DNA damaging agents. On the basis of these results, we suggest that CAF-1 utilizes H3 modified at lysine 79 to mark chromatin for inheritance during the S phase or during DNA repair.

EXPERIMENTAL PROCEDURES

Yeast Strains. The yeast strains used in this study are isogenic to W303, and standard yeast media and genetic manipulations were used. Standard procedures were followed to tag Cac2p, the second subunit of CAF-1, with TAP (tandem affinity purification) (*24*). Briefly, two PCR primers, cac2(TAP)f (5′AGCGATAGTAAAAAGAGGCGCATACATCCTACGCCAGTCGATTTGTCCATGGAAAAGAGAAG3′) and cac2(TAP)r (5′AACTAGATTAGGCATTCTTATGTACCGCATTAAATATATTAAAAATACGACTCACTATAGGG) were used to amplify plasmid pBS1479, and the resulting PCR products were transformed into a wild-type yeast strain. To identify yeast strains expressing the Cac2p-TAP fusion protein, we prepared whole cell extracts from each candidate transformant and performed Western blot analysis on each strain using peroxidase anti-peroxidase antibodies (PAP, Sigma).

Purification of CAF-1 for Silver Staining and Western Blot Analysis. Exponentially growing yeast cells (12 L for mass spectrometry analysis and 0.3 L for Western blot analysis) were harvested by centrifugation and washed once with 10% glycerol. The volume of yeast cells was estimated and an equal volume of buffer A (25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.01% NP40, 1 mM DTT) with protease inhibitors (l mM PMSF, 1 mM benzamidine, 1 mM Pefablock) and 15 KU/mL DNase I was used to resuspend yeast cells. The resulting cell suspension was frozen in liquid nitrogen. To make yeast cell extracts, the frozen yeast cells were ground with dry ice in coffee grinders for 12−16 min. After evaporation of the dry ice, the yeast lysate was centrifuged at 10000*g* for 10 min, and the resulting supernatant was incubated on ice with different concentrations of ethidium bromide (normally 75 μg/mL) for 30 min. The resulting lysate was cleared by centrifugation at 100000*g* for 1 h, and the supernatant was incubated with IgG−Sepharose beads (Pharmacia) for 2 h. After the beads were washed extensively, first with buffer A and then with TEV cleavage buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 0.01% NP-4, 1 mM DTT), the proteins were eluted by incubating the beads with TEV cleavage buffer containing TEV protease at 16 °C for 2 h. For large-scale purification of CAF-1, the eluted proteins were incubated with calmodulin beads for 1−2 h at 4 °C. After the calmodulin beads were extensively washed, bound proteins were either eluted with calmodulin elution buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.01% NP40, 1 mM MgOAc, 1 mM imidazole, 20 mM EGTA, 5 mM β-mercaptoethanol) or eluted directly with SDS sample buffer. For analysis of CAF-1−histone interactions, proteins eluted from the IgG beads were precipitated with trichloroacetic acid and then dissolved in SDS sample buffer. Purified proteins were resolved by SDS−PAGE and either visualized by silver staining or detected by Western blot.

Determination of Modifications on Histones by Mass Spectrometry. The silver-stained ID gel bands were prepared for mass spectrometry analysis using the following procedures. The bands were destained with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (*25*), reduced

and alkylated with DTT and iodoacetamide, and digested overnight at 37 °C with trypsin (0.004 μg/μL; Promega Corp., Madison, WI). Peptides were extracted with 2% trifluoroacetic acid and acetonitrile, dried on a SpeedVac, and resuspended in 0.1% formic acid for nano-LC-MS/MS analysis using a ThermoElectron linear ion trap Fourier transform ion cyclotron resonance (LTQ-FT-ICR) mass spectrometer (ThermoElectron, Bremen, Germany). The LTQ-FT-ICR was coupled with a Michrom Paradigm MS4 MDLC system (Michrom BioResources Inc., Auburn, CA) using trap loading onto a 300 μm × 1.0 mm Vydac C18 trap (LC-Packings/Dionex, Sunnyvale, CA) and separated with a ProteoPep C18 PicoFrit column (75 μm × 5.0 cm; ProteoPep, Burn, MA) running a gradient of 5% acetonitrile and 0.1% formic acid to 60% acetonitrile and 0.1% formic acid for 50 min. The LTQ-FT-ICR mass spectrometer was operated in a data-dependent mode similar to that described previously (*26*), consisting of a low resolving power (25000 fwhm at *m*/*z* 400) and FT full scan (*m*/*z* 375−1600), followed by a high resolving power (50000 fwhm at *m*/*z* 400) and FT SIM scan plus linear ion trap CID fragmentation and analysis on the top three most abundant ions. The targets for the FT full scan and SIM scan were set at 3×10^6 and 1×10^5, respectively, and the ion trap target was set to 1×10^5 with the normalized collision energy set at 35%. The MS/MS raw data were converted to DTA files using Bioworks 3.1 and correlated to theoretical fragmentation patterns using both Sequest and Mascot (Matrix Sciences, London, U.K.) search algorithms with tryptic peptide sequences from the NCBI nr and Swissprot databases (*27*, *28*). All searches were conducted with variable or differential modifications allowing +16 for methionine sulfoxide, +57 for carboxamidomethylcysteines, and +14, +28, and +42 for methyl-, dimethyl-, and trimethyl- or acetyllysines, respectively. The Swissprot database searches were initially restricted to trypsin-generated peptides from all species and then narrowed to yeast, allowing for four, five, and six missed cleavages. The accurate mass measurements were derived from the SIM scan, and the MS/MS data were used to determine peptide identity and modification.

Antibodies and Western Blot Analysis. Antibodies against Cac2p and acetylated H3 lysine 56 were generated in rabbits. To generate antibodies against Cac2p, GST-Cac2p was expressed in *Escherichia coli*, purified to near homogeneity, and used to inject rabbits for antibody production. To generate antibodies against H3 acetylated at lysine 56, an acetylated peptide [51-IRRFQK(acetylated)STEL(C)-60] was coupled to KLH and injected into rabbits for antibody production. Antibodies against H3 were either purchased from Abcam (ab1791) or kindly provided by Dr. Alain Verreault. Antibodies against H3-meK79 were purchased from Upstate (New York). Chromatin immunoprecipitation assays were performed as previously described (*29*).

Silencing Assays and Sensitivity to DNA Damaging Agents. To assay telomeric silencing, 10-fold serial dilutions of freshly grown yeast cells were spotted onto nonselective YPD media, to assay cell viability, and synthetic media lacking uracil or media containing the drug FOA, to assay silencing. To analyze the sensitivity of cells to DNA damaging agents, such as hydroxyurea (HU) and methylmethane sulfate (MMS), 10-fold serial dilutions of yeast cells were spotted onto YPD plates containing different concentrations of HU (50, 100, and 150 mM) or MMS (0.001%, 0.003%, and 0.01%). Yeast cells spotted onto YPD plates were subjected to ultraviolet (UV) radiation using a cross-linker. These plates were incubated at 30 °C for 3 days and photographed using a digital camera (Cannon).

RESULTS

Purification of the CAF-1−Histone Complex from Yeast Cells. To determine modifications on histones H3 and H4 associated with yeast CAF-1, we decided to purify the CAF-1−histone complex from yeast cells, which has not been reported before, using affinity chromatography and determine the modifications present on copurified H3 and H4 by mass spectrometry. We followed a standard procedure to fuse the C-terminus of the coding sequence of the *CAC2* gene with a DNA sequence coding for the TAP (tandem affinity purification) tag, which consists of a calmodulin binding peptide, a TEV cleavage site, and an IgG binding domain of *Staphylococcus aureus* protein A, using homologous recombination (*24*). The resulting Cac2-TAP fusion protein was found to be fully functional in silencing (data not shown). Therefore, we performed affinity purification using Cac2p-TAP strains. Because CAF-1 is a chromatin binding protein, the interactions of Cac2p with its potential binding partners could be affected by DNA. As ethidium bromide could disrupt protein−protein interactions mediated by DNA (*30*), we first tested the effect of ethidium bromide concentrations on the binding of Cac2p with its interacting partners. Briefly, Cac2p was purified by sequential affinity purification in the presence of increasing concentrations of ethidium bromide, and the copurified proteins were resolved on SDS−polyacrylamide gels followed by either silver staining (Figure 1A) or Western blot analysis (Figure 1B). As shown in Figure 1A, in the presence of increasing amounts of ethidium bromide, the CAF-1 complex (Cac1p−Cac2p−Cac3p) remained intact; however, the amounts of Cac1p and Cac3p isolated were slightly reduced. This suggests that association of the three subunits of the CAF-1 complex was not greatly affected by increasing amounts of ethidium bromide. In contrast, the amounts of PCNA, a known CAF-1 interacting partner (*15*), and H3 that copurified with Cac2p were reduced in the presence of increasing amounts of ethidium bromide. No PCNA was detected in the presence of 150 μg/mL ethidium bromide (Figure 1B). Because the amount of H3 copurified with CAF-1 did not decrease significantly when the concentration of ethidium bromide reached above 50 μg/mL, in most of the following experiments, CAF-1 was purified from cell extracts containing 75 μg/mL ethidium bromide.

The above experiments showed that CAF-1 copurified with PCNA and H3. We have previously shown that PCNA binds directly to Cac1p, the large subunit of CAF-1 (*15*). It is not known which subunits of CAF-1 interact with histones and whether the interaction between CAF-1 and histones requires an intact CAF-1 complex. Therefore, we purified Cac2-TAP from wild-type and *cac1*Δ mutant yeast cells and analyzed the copurified proteins using silver staining (Figure 1A) or Western blot (Figure 1C). As shown in Figure 1A,C and Supporting Information Figure 1, H3 and H4, PCNA, Cac1p, and Cac3p were copurified with Cac2p from wild-type but not from the *cac1*Δ strain. As Cac1p is necessary for formation of the CAF-1 complex (Figure 1A), this observa-

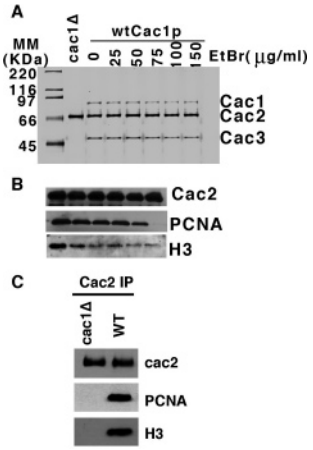

FIGURE 1: Yeast histones H3 and H4 can be copurified with CAF-1. (A) Purification of CAF-1 in the presence of different concentrations of ethidium bromide using Cac2-TAP. CAF-1 was purified using Cac2p-TAP and sequential affinity chromatography, and copurified proteins were detected by silver staining. The molecular mass of Cac1p, Cac2p, and Cac3p was about 90, 60, and 48 kDa, in agreement with the published apparent molecular mass of each subunit (*12*). As a control, Cac2p was purified from the *cac1*Δ strain, and only Cac2p, not Cac1p and Cac3p, was detectable. (B) Western blot analysis of Cac2p, PCNA, and H3 that copurified with CAF-1. (C) The association of CAF-1 with histones depends on the presence of Cac1p. Cac2p-TAP was used to purify proteins from wild-type or *cac1*Δ mutant cells. The copurified proteins were detected by Western blot. Note that the amounts of H3 and H4 copurified with CAF-1 were low compared to the amounts of CAF-1 (Supporting Information, Figure 1).

tion suggests that the interaction between CAF-1 and histones requires an intact CAF-1 complex and is not mediated solely through the Cac2p subunit in yeast cells. Furthermore, because disruption of the CAF-1 complex appears to prevent copurification of the CAF-1−histone complex, this indicates that histones are copurified with CAF-1 through protein−protein interactions. The interaction between CAF-1 and histones could be regulated by other proteins in cells because it is known that the interaction between Asf1p, another histone chaperone, and histones is mediated by the checkpoint kinase Rad53 (*31*, *32*).

Identification of Modifications on H3 and H4 Copurified with CAF-1 by Mass Spectrometry. As we had determined that we could copurify H3 and H4 with CAF-1 from yeast cells using TAP-tagged Cac2p and this interaction seemed to be specific (Figure 1 and Figure 1 in Supporting Information), we next proceeded to analyze the modifications present on the copurified H3 and H4 by mass spectrometry. The bands corresponding to molecular masses of H3 and H4 were excised from the gel, digested with trypsin, and subjected to analysis with a LTQ-FT-ICR mass spectrometer. This hybrid mass spectrometer provides both high mass accuracy (better than 2 parts per million externally calibrated) and collision-induced dissociation (CID) fragmentation capability for identification of peptides as well as modifications present on peptides (Figure 2). Attempts were made to look for acetylated and methylated lysine residues by allowing for up to six missed cleavages as well as phosphorylated serine and threonine residues. In addition to several unmodified H4 peptides (Table 1), we observed that one peptide, 4-GKGGKGLGKGGAK-16, was acetylated in multiple combinations, namely, diacetylated at lysines 5 and 12, triacetylated at lysines 5, 8, and 12, and tetraacetylated at

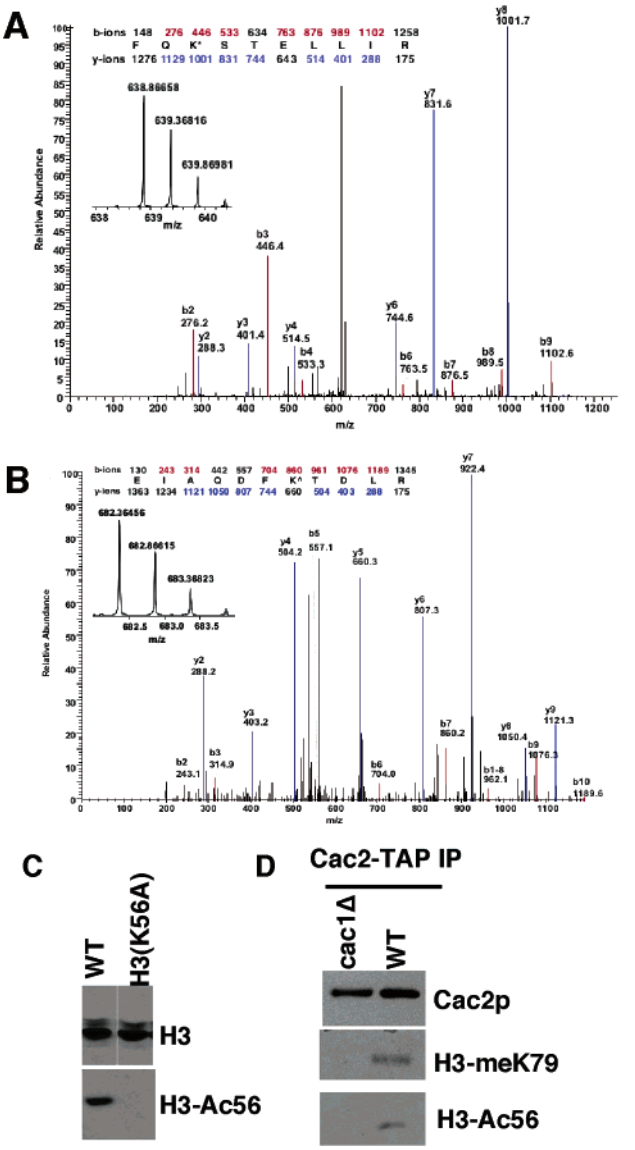

FIGURE 2: CAF-1-associated H3 is acetylated at lysine 56 and methylated at lysine 79. (A) LTQ-MS/MS spectrum of the $[M + 2H^+]^{2+}$ ion for peptide 73-EIAQDFK(dimethyl)TDLR-83. (B) LTQ-MS/MS spectrum of the $[M + 2H^+]^{2+}$ ion for the peptide 54-FQK-(acetyl)STELLIR-63. The observed b-type ions are shown in red and the y-type ions are shown in blue. The accurate mass measurement from the high resolving power LTQ-FT SIM scan is highlighted in the insert. (C) Antibodies against H3 acetylated at lysine 56 are specific. H3 antibodies (upper panel) or antibodies against H3 acetylated at lysine 56 (lower panel) were used to detect H3 or H3 acetylated at lysine 56 in yeast whole cell extracts prepared from cells expressing wild-type H3 or an H3 mutant with lysine 56 replaced by alanine. (D) Western blot analysis revealed that H3 in the CAF-1 complex was acetylated at lysine 56 and methylated at lysine 79. Cac2-TAP was precipitated from wild-type or *cac1*Δ mutant cells, and copurified proteins were detected by Western blot using antibodies against H3 methylated at lysine 79 (purchased) or antibodies against H3 acetylated at lysine 56.

lysines 5, 8, 12, and 16. The unacetylated form of this peptide was not detected, probably due to low abundance of the unmodified form and/or digestion of the unmodified peptide by trypsin to smaller peptides. Nonetheless, these results clearly indicate that CAF-1-associated H4 is a mixture of different acetylated forms, similar to what has been found in mammalian cells (*13*).

In addition to the detection of acetylated lysine residues in H4 peptides, several H3 peptides containing modified

Table 1: H4 Peptides Detected by LTQ-FT-ICR Mass Spectrometry[a]

A

Sequence	Mascot Score	Sequest X_{corr}	Calculated *m/z* $(M+2H^+)^{2+}$	Observed *m/z* $(M+2H^+)^{2+}$	Mass accuracy (ppm)
80-TVTSLDVVYALKR-92	71	3.63	732.92466	732.92456	**-0.1446**
79-KTVTSLDVVYALK-91	107	4.52	718.92159	718.92267	**1.4995**
23-DNIQGITKPAIR-35	64	3.5	663.38042	663.38043	**0.0060**
80-TVTSLDVVYALK-91	94	3.25	654.87411	654.87439	**0.4276**
46-ISGLIYEEVR-55	73	4.03	589.82442	589.82434	**-0.1373**
68-DSVTYTEHAK-77	53	3.08	575.77238	575.77155	**-1.4502**
60-SFLESVIR-67	53	2.42	475.76891	475.76895	**0.0694**

B

Sequence	Mascot Score	Sequest X_{corr}	Calculated *m/z* $(M+2H^+)^{2+}$	Observed *m/z* $(M+2H^+)^{2+}$	Mass accuracy (ppm)
4-G**K***GG**K***GLG**K***GGAK-16	59	3.33	620.85404	620.85413	**0.1385**
4-G**K***GG**K***GLG**K***GGA**K***R-17	66	4.21	719.90988	719.91034	**0.6376**
4-G**K***GG**K**^GLG**K***GGA**K**^R-17	68	3.8	705.89423	705.89435	**0.1686**
4-G**K***GG**K***GLG**K***GGA**K**^R-17	63	3.68	712.90205	712.90247	**0.5807**
4-G**K***GG**K***GLG**K**^GGAK-16	53	3.21	613.84622	613.84705	**1.3538**

C

Sequence	Mascot Score	Sequest X_{corr}	Observed *m/z* $(M+2H^+)^{2+}$	Mass accuracy (ppm) (dimethyl-K)	Mass accuracy (ppm) (formyl-K)
80-TVTSLDVVYAL**K**^R-92	70	2.94	746.92261	-23.7047	**0.6520**
56-AVL**K**^SFLESVIR-67	70	3.27	695.40924	-25.3139	**0.8455**
24-DNIQGIT**K**^PAIR-35	73	3.26	677.38098	-22.2853	**4.5720**[b]

D

1 SGR**GKGGKGL GKGGAKR**HRK ILR**DNIQGIT KPAIR**RLARR GGVK**RISGLI**
51 **YEEVRAVLKS FLESVIRDSV TYTEHAK**R**KT VTSLDVVYAL KR**QGRTLYGF
101 GG

[a] Panel A lists the unmodified H4 peptides detected by mass spectrometry. The sequence, Mascot and Sequest scores, and peptide mass accuracy of each peptide determined from the double charged precursor ion are shown. Panel B lists the observed acetylated forms of the H4 N-terminal peptide. * indicates acetylation of the lysine residue; ∧ indicates formylation of the lysine residue. Lysine formylation was likely formed through a reaction with formaldehyde used in silver staining (*45*). Panel C lists other H4 peptides that contained a +28 mass on the lysines highlighted in bold. A comparison of peptide mass accuracy with either the dimethyl- or formyllysine modification is shown. In panel D, the distribution and coverage (71%) of H4 peptides detected are shown in red. [b] The higher ppm value for this peptide was due to multiple unresolved masses at *m/z* 677.

lysines were also scored. In this case, both acetylation and methylation of lysine residues were observed (Figure 2 and Table 2). In addition, these two modifications occurred on lysine residues present in the core domain of H3. For instance, peptide masses representing the mono-, di-, and trimethylated H3 peptide 73-EIAQDFKTDLR-83 were identified (Table 2B). The highest scoring MS/MS spectra were matched to the methyl- and dimethyllysine 79 peptides (Table 2A,B), whereas the trimethyl peptide gave poor quality spectra (data not shown). Interestingly, this H3 peptide in the unmethylated form was not detected. This suggests that unmodified lysine 79 in the CAF-1 complex, if present, was in low abundance because we did not detect the peptide 73-EIAQDFK-79, which could arise from the unmethylated peptide 73-EIAQDFKTDLR-83 after tryptic digestion. We also detected another histone H3 modification, acetylation of lysine 56 (Figure 2B and Table 2).

To ascertain that lysine 56 is acetylated and lysine 79 is methylated on H3 associated with CAF-1, we prepared antibodies that recognized H3 acetylated at lysine 56 (H3-AcK56) (Figure 2C) and purchased antibodies that recognized H3 methylated at lysine 79 (H3-meK79) and performed Western blot analysis on proteins copurified with CAF-1. As shown in Figure 2D, H3 copurified with CAF-1 was acetylated at lysine 56 and methylated at lysine 79, supporting our MS data indicating that lysine 56 and lysine 79 were acetylated and methylated, respectively. Acetylation of H3 lysine 56 in yeast cells was very recently reported by other groups (*22*, *23*, *33*), supporting our method of analysis of modifications present on histones associated with CAF-1.

The Association of CAF-1 with H3-meK79 Is Not Mediated by Nucleosomes or DNA. Over 90% of bulk H3 is methylated at lysine 79 (*34*); thus, it was possible that H3-meK79 copurified with CAF-1 was from contaminating nucleosomes. Because nucleosomes and CAF-1−histone complexes migrate at different positions along a glycerol gradient, we asked whether H3-meK79 comigrated with CAF-1. Briefly, the purified CAF-1−histone complex was loaded onto a 15−40% glycerol gradient (5 mL) and spun at 200000*g* for 18 h, and 200 μL fractions were collected and analyzed by silver staining to detect the CAF-1 complex (Figure 3A) or Western blot to detect Cac2p, PCNA, and H3-meK79 (Figure 3B). As shown in Figure 3A, the CAF-1 complex peaked at fraction 11 with an apparent molecular mass of 158 kDa, similar to what has been detected for the CAF-1−histone complex isolated from human cells (*13*). Under the same conditions, mononucleosomes purified from Hela cells peaked at fraction 21 (Figure 3A). More importantly, H3-

Table 2: H3 Peptides Identified by LTQ-FT-ICR Mass Spectrometry[a]

A

Sequence	Mascot Score	Sequest X_{corr}	Observed *m/z* $(M+2H^+)^{2+}$	Mass accuracy (ppm) acetyl-K	Mass accuracy (ppm) trimeth-K
54-FQKSTELLIR-63	47	2.46	638.86658	-0.0626	-26.9250
53-RFQKSTELLIR-63	45	4.03	716.91754	0.5091	-24.8669

B

Sequence	Mascot Score	Sequest X_{corr}	Calculated *m/z* $(M+2H^+)^{2+}$	Observed *m/z* $(M+2H^+)^{2+}$	Mass accuracy (ppm)
73-EIAQDFK*TDLR-83	41	2.7	675.35662	675.35699	0.5538
(K* = methyl-K)					
73-EIAQDFK#TDLR-83	55	3.03	682.36444	682.36456	0.1744
(K# = dimethyl-K)					
73-EIAQDFK^TDLR-83	25	1.68	689.37227	689.37231	0.0638
(K^ = trimethyl-K)					
41-YKPGTVALR-49	25	1.61	502.79801	502.79813	0.2406

C

1 ARTKQTARKSTGGKAPRKQLASKAARKSAPSTGGVKKPHR**YKPGTVALR**E
51 IR**RFQKSTELLIR**KLPFQRLVR**EIAQDFKTDLR**FQSSAIGALQESVEAYL
101 VSLFEDTNLAAIHAKRVTIQKKDIKLARRLRGERS

[a] Peptides listed in this table were identified as described in Table 1. In panel A, two H3 peptides containing modified lysine 56 were detected by mass spectrometry. Peptide mass accuracy when lysine 56 was modified either by acetylation or by trimethylation is shown. In panel B, three different methylated forms of the H3 peptide EIAQDFKTDLR are shown. The low Mascot and Sequest scores for the trimethyl-modified peptide and the unmodified peptide, 41-YKPGTVALR-49, were attributed to poor quality MS/MS spectra. In panel C, the distribution of H3 peptides detected is shown in red. Note the overall coverage is low (22%), probably due to tryptic peptides being too small or large for the chromatography and mass spectrometry parameters. Because of the low coverage, other modified peptides are likely missing.

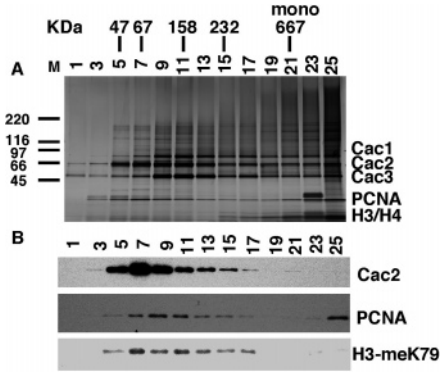

FIGURE 3: CAF-1 and H3-meK79 comigrate on a glycerol gradient. Odd fractions of a glycerol gradient (15−40%) were resolved using SDS−PAGE. Proteins were detected by silver staining (A) or Western blot (B) using antibodies against Cac2p, PCNA, or H3-meK79. The peak fractions for protein standards and mononucleosomes (mono) are indicated at the top.

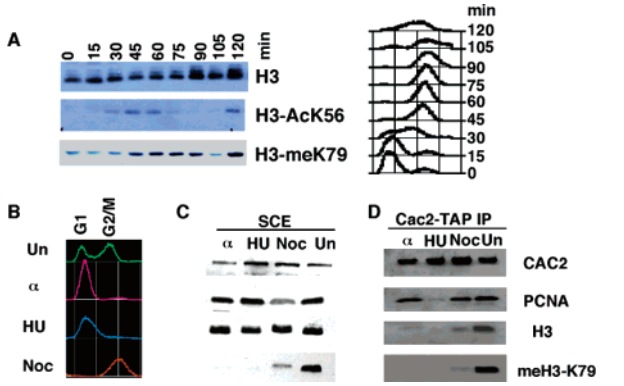

FIGURE 4: Cell cycle regulation of acetylation of H3 lysine 56, methylation of H3 lysine 79, and the association of CAF-1 with H3-meK79. (A) Acetylation of H3 lysine 56 and methylation of lysine 79 is cell cycle regulated. Whole cell extracts were prepared from wild-type cells (*MATa*) released from G1 arrest using α factor at the times indicated, and H3, H3 acetylated at lysine 56, or H3 methylated at lysine 79 was detected by Western blot using specific antibodies against H3 or against each H3 modification. (B) FACS analysis of the DNA content of unsynchronized cells (Un) and cells arrested by α-factor (a), hydroxyurea (HU), or nocodazole (Noc). (C) Methylation of H3-K79 in soluble extracts is cell cycle regulated. Cac2p, PCNA, H3, or H3 methylated at lysine 79 was detected by Western blot in soluble cell extracts (SCE) prepared from unsynchronized cells or from cells arrested as in (B). (D) The association of CAF-1 with histones is cell cycle regulated. The CAF-1 complex was purified with Cac2-TAP from cells at different stages of the cell cycle, as in (B), and copurified proteins were detected by Western blot.

meK79 and PCNA comigrated with the CAF-1 complex (Figure 3B). These results suggest that H3-meK79 copurified with CAF-1 is not due to nucleosome contamination and that a CAF-1−histone complex containing H3-meK79 occurs in yeast cells.

Acetylation of Lysine 56, Methylation of Lysine 79, and the Association of CAF-1 with H3 Are Regulated during the Cell Cycle. The expression of Dot1p, the enzyme that methylates H3 lysine 79, peaks at the G1/S transition of the cell cycle (*35*). This suggests that methylation of H3 lysine 79 in yeast cells might start in the S phase. Moreover, there are two conflicting reports on whether acetylation of H3 lysine 56 is cell cycle regulated (*22*, *23*). We therefore determined when acetylation of H3 lysine 56 and methylation of H3 lysine 79 occurred during cell cycle progression. Briefly, wild-type *MATa* cells were arrested at G1 using α-factor, a mating pheromone, for 3 h and then released into fresh media to allow cells to progress through the cell cycle. A fraction of cells was removed to prepare whole cell extracts or to analyze DNA content by FACS (Figure 4A, right panel). With the progression of the cell cycle, acetylation of H3 lysine 56 occurred at the onset of the S phase (30 min after release) and peaked in the late S phase (45 min after release) (Figure 4A). Strikingly, acetylation of lysine 56 was

significantly reduced at the G2 and M phase and reappeared in the next cell cycle (120 min). These data agreed with data published by Masumoto et al. that acetylation of lysine 56 occurred in the S phase (*22*).

In contrast to acetylation of H3 lysine 56 that was barely detectable in cells immediately after release from α-factor block, methylation of lysine 79 could be detected in whole cell extracts. Interestingly, methylation of H3 lysine 79 increased significantly late in the S phase (45 min after release) when acetylation of lysine 56 peaked. Moreover, methylation of lysine 79 remained constant when cells progressed into the G2 or M phase. These results indicate that acetylation of H3 lysine 56 and methylation of H3 lysine 79 are regulated in the S phase but with distinct kinetics and dynamics.

CAF-1 is involved in the deposition of histones onto newly replicated DNA, which occurs during the S phase. We therefore tested whether the association of CAF-1 with H3-meK79 might be cell cycle regulated. To test this idea, we arrested yeast cells (*MATa*, *cac2-TAP*) at the G1 phase of the cell cycle using α-factor, at the early S phase using hydroxyurea (HU), a ribonucleotide reductase inhibitor that blocks the synthesis of dNTPs required for DNA replication, and at mitosis or the M phase using nocodazole, an inhibitor of microtubule assembly (Figure 4B). We prepared soluble protein extracts from either arrested cells or unsynchronized cells and used Western blot analysis to detect the levels of Cac2p, PCNA, H3, and H3-meK79 present in cells at different stages of the cell cycle. As shown in Figure 4C, there were no significant differences in the amounts of Cac2p and PCNA in the soluble extracts prepared from cells arrested at the G1, early S, or M phase compared to those from unsynchronized cells, suggesting that the protein level of Cac2p and PCNA may not be cell cycle regulated. Interestingly, we also did not detect any significant changes in the amount of soluble H3 present at the different stages of the cell cycle compared to unsynchronized cells, even though it is known that histone transcription is limited to the S phase and decreases rapidly in cells arrested by HU or α-factor (*36*). In agreement with the results that methylation of H3 lysine 79 increased in whole cell extracts in the late S phase, methylation of H3 lysine 79 in soluble extracts was barely detectable in α-factor- or HU-arrested cells (early S phase) and was significantly reduced in M phase cells in comparison to unsynchronized cells because of the fact that these cells contain an appreciable portion in the late S phase. As the level of H3-meK79 in soluble extracts is cell cycle regulated, we next wanted to determine whether the association of CAF-1 with H3-meK79 was cell cycle regulated. Therefore, we purified the CAF-1 complex using the TAP method from extracts of yeast cells arrested at different cell cycle stages and detected the amount of PCNA, H3, and H3-meK79 by Western blot analysis. As shown in Figure 4D, copurification of H3 with CAF-1 was barely detectable when cells were arrested in the G1 or early S phase and was significantly reduced when cells were arrested in the M phase compared to unsynchronized cells. The association of H3-meK79 with CAF-1 followed the same pattern as that of H3. Thus, these results indicate that both methylation of lysine 79 of H3 and the association of CAF-1 with H3-meK79 are regulated during the cell cycle and probably occur predominantly in the late S phase.

The cac1Δ dot1Δ Double Mutant Cells Exhibit a Significant Loss of Telomeric Silencing and Reduced Binding of Sir4p at Telomeric Silent Chromatin. Dot1p is the only histone methyltransferase that methylates lysine 79 of H3 in yeast and mammalian cells (*34*, *37*, *38*). Therefore, to gain insight into the interactions between CAF-1 and H3-meK79, we tested whether the *CAC1* gene genetically interacts with the *DOT1* gene. In yeast cells, loss of H3 lysine 79 methylation by deleting the Dot1p methyltransferase results in reduced silencing at telomeres and the *HM* loci (*34*). Cells lacking each subunit of CAF-1 also exhibit a partial loss of telomeric silencing. We therefore tested how the *cac1*Δ *dot1*Δ double mutant cells affected telomeric silencing using a standard telomeric silencing assay (*39*, *40*). In this assay, the *URA3* gene, integrated at the left end of chromosome VII, is silenced in a fraction of wild-type cells, allowing these silenced wild-type cells to grow efficiently in medium containing FOA, a drug that kills cells expressing the *URA3* gene. As expected, the *cac1*Δ or *dot1*Δ single mutant cells displayed a partial loss of telomeric silencing compared to wild-type cells. Interestingly, the *dot1*Δ *cac1*Δ double mutant cells reduced telomeric silencing to a more significant degree than either *cac1*Δ or *dot1*Δ single mutant cells (Figure 5A), suggesting that CAF-1 genetically interacts with *DOT1* to promote silencing. We also attempted to determine whether an H3 lysine 79 mutant where lysine 79 was replaced by alanine [H3(K79A)] synergistically reduced silencing in the absence of Cac1p. However, this H3 mutant reduced telomeric silencing so dramatically and much more than the *dot1*Δ mutant alone as reported (*34*), probably due to alteration in chromatin structure by this H3 mutant, which precluded analysis of the effect of the *cac1*Δ *H3*(*K79A*) double mutant on telomeric silencing (data not shown). Nonetheless, because methylation of lysine 79 solely depends on Dot1p in yeast cells, and Dot1p is only known to methylate H3 lysine 79 (*34*), it is reasonable to assume that the synergistic effect of the *dot1*Δ mutant on telomeric silencing in the absence of Cac1p is likely due to a loss of methylation of lysine 79.

In the yeast *Saccharomyces cerevisiae*, silencing requires four silent information regulator (Sir) proteins that function as the structural component of yeast silent chromatin. We, therefore, tested whether *cac1*Δ *dot1*Δ double mutant cells affected Sir4p binding to the telomeric heterochromatin using chromatin immunoprecipitation assays. Briefly, after cross-linking protein−DNA using formaldehyde, a reversible cross-linker, chromatin was sheared into small fragments (0.5−1 kb in length) by sonication. Antibodies against Sir4p were then used to precipitate Sir4p-bound chromatin fragments, and DNA coprecipitated with Sir4p was detected by multiplex PCR. Two sets of PCR primers were used, one annealed to the yeast silent chromatin (0.77 kb DNA from the right end of chromosome VI) and the other primer annealed to adjacent euchromatin (20 kb from the telomeric end of chromosome VI). In agreement with published results (*29*), Sir4p bound specifically to the DNA fragment 0.77 kb away from the telomeric end compared to the DNA fragment 20 kb away from the telomeric end (Figure 5B,C). The *cac1*Δ or *dot1*Δ single mutant cells showed a slight reduction of Sir4p binding to silent chromatin. By contrast, the *cac1*Δ *dot1*Δ double mutant cells exhibited a significant reduction in Sir4p binding to the silent chromatin when compared to

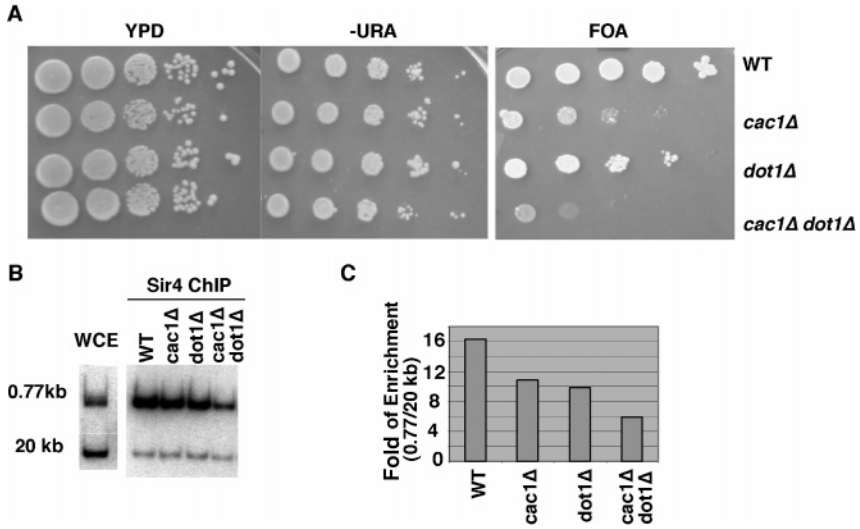

FIGURE 5: CAF-1 interacts genetically with Dot1p in silencing. (A) The *cac1*Δ mutant reduced telomeric silencing dramatically in the absence of Dot1p. Tenfold serial dilutions of yeast cells were spotted onto YPD medium to assay cell growth and −URA and FOA media to assay silencing. (B, C) Sir4p binding to telomeric silent chromatin is reduced in the *cac1*Δ *dot1*Δ double mutant cells compared to cells from either of the single mutants. A chromatin immunoprecipitation assay was performed using antibodies against Sir4p, and coprecipitated DNA was detected by PCR. The band intensity ratio (PCR products 0.77 kb away from the telomeric end over PCR products 20 kb away from telomeric end) was calculated and expressed as fold of enrichment.

either the *cac1*Δ or *dot1*Δ single mutant cells. We also observed that Sir3p binding to the silent chromatin was reduced significantly in the *cac1*Δ *dot1*Δ double mutant cells (data not shown). These results suggest that loss of silencing in the *cac1*Δ *dot1*Δ double mutant cells is due to reduced binding of Sir proteins to silent chromatin.

*The cac1*Δ *dot1*Δ *Double Mutant Cells Are Sensitive to DNA Damaging Agents*. Recently, it has been shown that cells lacking Dot1p are sensitive to ionizing radiation (*41*). In addition, *cac1*Δ mutant cells are also sensitive to DNA damaging agents such as UV and MMS (*12*, *15*). Since we detected a genetic interaction between the *CAC1* gene and the *DOT1* gene in silencing, we wanted to test whether there was an interaction between these genes in response to DNA damaging agents. Therefore, we tested whether the *cac1*Δ *dot1*Δ double mutant cells were more sensitive to DNA damaging agents than either single mutant cells. Compared to the *cac1*Δ or *dot1*Δ single mutant cells, which were only slightly sensitive to DNA damaging agents, such as HU, MMS, and UV radiation, the *cac1*Δ *dot1*Δ double mutant cells were much more sensitive to these DNA damaging agents. It was possible that the sensitivity of the *cac1*Δ *dot1*Δ double mutant cells toward to these DNA damaging agents was due to a global alteration in chromatin structure in the double mutant cells. However, the *cac1*Δ *dot1*Δ double mutant strain grew at the same rate as the wild-type strain (data not shown; see Figure 5A), and the double mutant cells progressed normally through the cell cycle as compared to wild-type cells (Figure 6B). Thus, we suggest that CAF-1 and Dot1p cooperate to facilitate the DNA repair process in yeast cells.

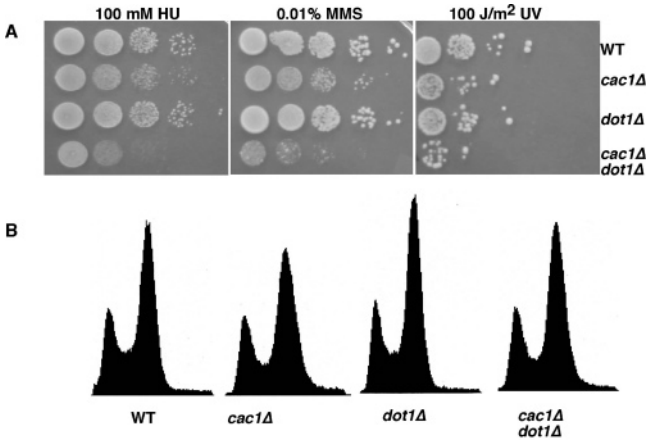

FIGURE 6: CAF-1 genetically interacts with Dot1p in response to DNA damaging agents. (A) The *cac1*Δ *dot1*Δ double mutant cells are very sensitive to DNA damaging agents. Tenfold serial dilutions of yeast cells were spotted onto YPD media or YPD media containing either 100 mM hydroxyurea (HU) or 0.01% methylmethane sulfate (MMS) and were grown for 2 days at 30 °C. To assay sensitivity to UV radiation, cells spotted onto YPD media were irradiated with UV (100 J/m^2). (B) The *cac1*Δ *dot1*Δ double mutant cells progress through the cell cycle normally compared to wild-type cells. Exponentially growing yeast cells from wild type or various mutants were collected, and the DNA content was analyzed by FACS.

DISCUSSION

CAF-1 is a highly conserved histone H3 and H4 chaperone that promotes nucleosome formation during DNA replication and DNA repair. To identify modifications on histones H3 and H4 associated with CAF-1 and study what functions these modifications might mediate, we have purified a CAF-1−H3−H4 complex from yeast cells using affinity chromatography and identified modifications on the copurified H3 and H4 using LTQ-FT-ICR mass spectrometry, which provides both accurate mass (better than 2 parts per million externally calibrated) and CID fragmentation for peptide identification. Using this method, we found that H4 was a mixture of acetylated isoforms, whereas H3 was acetylated at lysine 56 and methylated at lysine 79. Thus, this form of mass spectrometry allowed us to differentiate unambiguously acetylated peptides from trimethylated peptides and formylated peptides from dimethylated peptides. The identity of H3 acetylated at lysine 56 and methylated at lysine 79 was confirmed by Western blot analysis. Therefore, these studies demonstrate the potential value of using this high mass

accuracy and high resolving power mass spectrometry to identify modifications on histones as well as other proteins.

While CAF-1-associated H4 was detected as a mixture of acetylated isoforms, H3 copurified with CAF-1 was found to be acetylated at lysine 56 and methylated at lysine 79. Currently, it is not known whether these two modifications are present on one histone molecule or on different histone molecules. While this paper was in preparation, three other groups reported that lysine 56 of H3 is acetylated (*22*, *23*, *33*). Of these reports, two conflicted as to whether acetylation of lysine 56 was cell cycle regulated or not (*22, 23*). We have shown here that acetylation of lysine 56 occurred in the S phase and disappeared in the G2 phase with similar kinetics as described by Masumoto et al. (*22*). Thus, we conclude that acetylation of H3 lysine 56 is likely to be regulated during the cell cycle.

Compared to acetylation of H3 lysine 56, methylation of H3 lysine 79 increased in the late S phase when acetylation of lysine 56 peaked in whole cell extracts. Moreover, methylation of H3 lysine 79 remained constant in cells in the G2 or M phase when acetylation of lysine 56 disappeared. This suggests that the dynamics or regulation of methylation of H3 lysine 79 is different than that for acetylation of lysine 56. The functional significance of the difference in dynamics of these two H3 modifications in the CAF-1−histone complex remains to be investigated.

We have provided evidence supporting the idea that CAF-1 associates with H3-meK79 in yeast cells. We have shown that the association of CAF-1 with H3-meK79 depends on an intact CAF-1 complex, and the interaction is neither mediated by DNA nor due to nucleosome contamination. As the interaction between CAF-1 and histones occurred predominantly in unsynchronized cells compared to cells arrested in the G1 or early S phase, this suggests that the CAF-1−histone interaction is likely to be regulated in the late S phase when methylation of H3 lysine 79 increases. CAF-1 is known to bind newly synthesized H3 and H4. On the other hand, Dot1p, the histone methyltransferase that methylates H3 lysine 79, is known to preferentially methylate nucleosomal histones (*34*, *38*). Therefore, it is surprising that lysine 79 is methylated on H3 copurified with CAF-1. Recently, it has been shown that yeast Dot1p methylates recombinant H3 in the presence of short oligonucleotides (*42*). In addition, the expression of the *DOT1* gene peaks at the G1/S transition, suggesting that yeast Dot1p plays a role in the S phase. Thus, methylation of H3 lysine 79 by Dot1p may occur on newly synthesized H3 before or during the deposition of H3 and H4 onto DNA by CAF-1. Supporting this idea, acetylation of H3 lysine 56 has been shown very recently to occur on newly synthesized H3 during the S phase (*22*).

Because the association of CAF-1 with histones occurred predominantly in the late S phase when methylation of lysine 79 of H3 increased, it is possible that H3 methylated at lysine 79 determines the S phase association of CAF-1 with H3. We could not, however, detect any significant reduction in the amount of H3 associated with CAF-1 in *dot1*Δ mutant cells compared to wild-type cells (data not shown), indicating that methylation of H3 lysine 79 is not a primary determinant that regulates the S phase association of CAF-1 with H3. This is not surprising because CAF-1 deposits histones onto euchromatic regions, where H3-meK79 is enriched, and heterochromatic regions, where H3 unmodified at lysine 79 is present. Thus, it is conceivable that CAF-1 may deposit histones with different modifications during euchromatin and heterochromatin replication. In yeast cells, methylation of lysine 79 at euchromatin prevents Sir proteins from promiscuous association with euchromatin (*34*, *43*). Therefore, we suggest that CAF-1 deposits H3-meK79 onto euchromatic DNA such that Sir proteins will be blocked from association with histones during replication of euchromatic regions.

The *cac1*Δ *dot1*Δ double mutant cells exhibited significant reduction in telomeric silencing compared to either *cac1*Δ or *dot1*Δ mutant cells alone, suggesting that the role of CAF-1 in silencing is not solely dependent on its ability to deposit H3-meK79 onto replicating DNA. Human CAF-1 is known to interact with heterochromatin protein 1 (HP1) (*19*), the functional homologue of Sir proteins, and this interaction is believed to deliver HP1 to the sites of heterochromatin replication (*44*). In yeast cells, CAF-1 is known to interact with Sir1p (*46*). We also observed that CAF-1 interacts with Sir3p and Sir4p (Zhang et al., unpublished observation). These observations suggest that CAF-1 may have multiple roles in silent chromatin formation and inheritance.

ACKNOWLEDGMENT

We thank Kristi Simmons for editing and proofreading the manuscript. Special thanks to Dr. Heather M. Thompson for critical editing of the manuscript. We thank Dr. A. Verreault for antibodies against H3, Dr. S. Dent for histone plasmids, and Drs. R. Kamakaka and J. Svejstrup for yeast strains. We also thank Dr. Leemor Joshua-Tor for TEV protease and Dr. Bruce Stillman for advice.

SUPPORTING INFORMATION AVAILABLE

One figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI0521083