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Holocarboxylase synthetase is a chromatin protein and interacts directly with histone H3 to mediate biotinylation of K9 and K18☆

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Abstract

Holocarboxylase synthetase (HCS) mediates the binding of biotin to lysine (K) residues in histones H2A, H3 and H4; HCS knockdown disturbs gene regulation and decreases stress resistance and lifespan in eukaryotes. We tested the hypothesis that HCS interacts physically with histone H3 for subsequent biotinylation. Co-immunoprecipitation experiments were conducted and provided evidence that HCS co-localizes with histone H3 in human cells; physical interactions between HCS and H3 were confirmed using limited proteolysis assays. Yeast two-hybrid (Y2H) studies revealed that the N-terminal and C-terminal domains in HCS participate in H3 binding. Recombinant human HCS was produced and exhibited biological activity, as evidenced by biotinylation of its known substrate, recombinant p67. Recombinant histone H3.2 and synthetic H3-based peptides were also good targets for biotinylation by recombinant HCS (rHCS) *in vitro*, based on tracing histone-bound biotin with [^{3}H]biotin, streptavidin and anti-biotin antibody. Biotinylation site-specific antibodies were generated and revealed that both K9 and K18 in H3 were biotinylated by HCS. Collectively, these studies provide conclusive evidence that HCS interacts directly with histone H3, causing biotinylation of K9 and K18. We speculate that the targeting of HCS to distinct regions in human chromatin is mediated by DNA sequence, biotin, RNA, epigenetic marks or chromatin proteins.

Keywords: Biotin; Chromatin; Histone H3; Holocarboxylase synthetase

1. Introduction

Epigenetic marks such as DNA methylation and various histone modifications form the basis for gene regulation [1,2]. For example, trimethylation of lysine (K)-4 in histone H3 (H3K4me3) is associated with transcriptional activation of surrounding DNA, whereas dimethylation of K9 (H3K9me2) is associated with transcriptional silencing [2–4]. While much well-deserved attention has been paid to gene regulation by histone acetylation and methylation [3,4], other histone modifications also play important roles in chromatin biology [2]. For example, it has been demonstrated that histones contain covalently bound biotin [5–7]. The following 11 biotinylation sites have been identified in human histones: lysine (K)-9, K13, K125, K127 and K129 in histone H2A [8]; K4, K9, K18 and perhaps K23 in histone H3 [9,10]; and K8 and K12 in histone H4 [7]. Preliminary evidence suggests that K5 and K16 in histone H4 might also be biotinylated [7,11].

Evidence is emerging for important biological functions of histone biotinylation. It has been demonstrated that K12-biotinylated histone H4 (H4K12bio) is enriched in pericentromeric alpha satellite repeats and represses the *interleukin-2* gene [12]. Moreover, H4K12bio participates in signaling DNA double-strand breaks [13], in the repression of the *SMVT* biotin transporter gene [14] and in the repression of retrotransposons to promote genome stability [15].

Two putative histone biotinyl ligases have been identified in humans: biotinidase and holocarboxylase synthetase (HCS). While the nuclear localization of biotinidase [8,16,17] and its exact role in histone biotinylation [18,19] are controversial, the situation is less ambiguous for HCS. Both HCS and its microbial ortholog BirA have

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Gal4 AD, Gal4 activation domain; Gal4 BD, Gal4 binding domain; GFP, green fluorescent protein; HCS, holocarboxylase synthetase; H3K4bio, histone H3, biotinylated at lysine-4; H3K9bio, biotinylated at lysine-9; H3K18bio, biotinylated at lysine-18; H3K4me3, histone H3, trimethylated at lysine-4; H3K9me2, histone H3, dimethylated at lysine-9; H3K9me3, histone H3, trimethylated at lysine-9; H4K12bio, histone H4, biotinylated at lysine-12; K, lysine; rHCS, recombinant human holocarboxylase synthetase; Y2H assay, yeast two-hybrid assay.

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histone biotinyl ligase activity [10,20]. HCS enters the nuclear compartment [20], where it is associated with chromatin [14,15,19]. HCS does not contain a DNA-binding motif that would explain its binding to chromatin. Studies in cells derived from HCS-deficient individuals, human HCS knockdown cell lines and HCS knockdown *Drosophila melanogaster* consistently showed decreased levels of histone biotinylation and abnormal patterns of gene regulation [9,14,15,19].

HCS-mediated biotinylation of proteins requires ATP and proceeds in two steps [21]. In the first step, HCS catalyzes the synthesis of biotinyl-5′-AMP. In the second step, the biotinyl moiety is conjugated to distinct lysine residues in target proteins. Recently, it was proposed that, for biotinylation of histone H2A, the second step may occur in the absence of HCS, if synthetic biotinyl-5′-AMP is provided [22]. In this study, we tested the hypothesis that HCS interacts physically with histone H3 to mediate binding to chromatin and subsequent biotinylation. Histone H3 was used as a model, because it is known to be a good target for biotinylation [9].

2. Materials and methods

2.1. Cell cultures

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and penicillin and streptomycin. Jurkat cells were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI 1640) supplemented with 10% bovine calf serum and penicillin and streptomycin. The cells were grown at 37°C in the presence of 5% CO_2 in a humidified incubator.

2.2. Co-immunoprecipitation assays

Transgenic HEK293 cells were generated, which overexpress HCS fused to green fluorescent protein (GFP); these cells were denoted HEK293 HCS-GFP. Briefly, a clone coding for full-length human HCS was obtained from Yoichi Suzuki (Tohoku University, Sendai, Japan) [23] and PCR amplified with primers 5′-ATTTCTCGAGATGCATCACCATCACCATCACGAAGATAGACTCCACATGG-3′ and 5′-ATTTGAATTCGCCGCCGTTTGGGGAG-3′. The PCR product was digested with *Xho*I and *Eco*RI and ligated into vector pEGFP-N1 (Clontech, Mountain View, CA, USA) to produce plasmid HCS-GFP (Fig. 1A). HEK293 cells were transfected with HCS-GFP using TurboFect reagent (Fermentas, Glen Burnie, MA, USA), and stably transformed cells were selected in medium containing 20 mg/L of G418 for 4 weeks. Overexpression of HCS was confirmed by Western blot analysis, using mouse anti-GFP (Promega, Madison, WI, USA) as probe (data not shown).

For immunoprecipitation experiments, 1.5×10^8 HEK293 HCS-GFP cells were collected by centrifugation and suspended in 7 ml of cell lysis buffer. Samples were centrifuged and the chromatin solution was pre-cleared by overnight incubation with ~7 μl of mouse IgG (Santa Cruz, Santa Cruz, CA, USA) at 4°C; IgG was removed by incubation with 1.4 ml of settled protein A beads (4°C for 2 h). A 300-μl aliquot was saved as input control (see below), and the remaining sample was split into two 3-ml aliquots for subsequent immunoprecipitations. One of the 3-ml aliquots was incubated overnight with 490 μl of mouse anti-GFP (Promega) at 4°C; the second 3-ml aliquot was incubated with 3 μl of mouse IgG (Santa Cruz) at 4°C. Proteins were precipitated at 4°C for 2 h using 0.6 ml of settled protein A beads. Samples were lyophilized and dissolved in 30 μl of H_2O. Precipitated proteins were boiled in sample loading buffer and resolved by gel electrophoresis; transblots were probed using goat anti-human histone H3.2 (Santa Cruz). Input control and recombinant human histone H3.2 (NewEngland Biolabs, Ipswich, MA, USA) were used as positive controls, whereas the sample precipitated with nonspecific IgG was used as negative control.

Regular HEK293 cells were used in an additional control experiment. About 4×10^8 HEK293 cells were collected by centrifugation and suspended in 15 ml cell lysis buffer, containing 5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Igepal CA-630 (vol/vol), protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 100 mM PMSF. Suspensions were incubated on ice for 10 min with occasional vortexing. Nuclei were collected by centrifugation (2880×g, 5 min) and resuspended in 15 ml nuclei buffer containing 50 mM Tris (pH 8.1), 10 mM EDTA and 35 mM lauryl sulfate on ice. The sample was sonicated on ice, using three 30-s pulses at 60% amplitude with 60 s between pulses. Samples were centrifuged and the chromatin solution was precleared by using about 1 ml of protein A beads (Pierce, Rockford, IL, USA) at 4°C for 2 h. A 750-μl aliquot was saved as input control (see below), and the remaining sample was split into two 7-ml aliquots for subsequent immunoprecipitations. One of the 7-ml aliquots was incubated overnight with 490 μl of anti-serum to the C-terminus in human HCS [8] at 4°C; the second aliquot was incubated without anti-HCS. Proteins were precipitated at 4°C for 2 h using 1.4 ml of protein A beads. Samples were lyophilized, dissolved in 1 ml H_2O and precipitated again at 4°C for 2 h using 400 μl protein A beads to decrease nonspecific binding. Precipitated proteins were boiled in sample loading buffer and resolved by gel electrophoresis; transblots were probed using goat anti-human histone H3.2 (Santa Cruz). Nuclear extract before precipitation (input control) was used as positive controls.

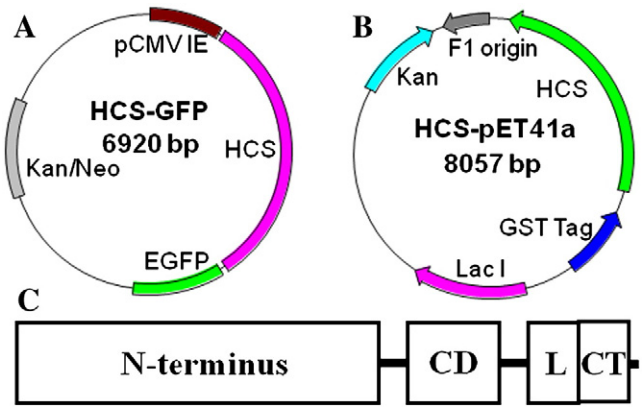

Fig. 1. Schematic presentation of HCS expression plasmids and HCS domains. (A) Plasmid HCS-GFP; (B) plasmid HCS-pET41a; and (C) diagram of HCS domains. CD=Central domain; L=linker domain; CT=C-terminal domain.

2.3. Recombinant HCS

*Eco*RI and *Xho*I restriction sites were inserted into the human HCS clone by PCR, using forward primer 5′-GTCCGAATTCGGGGAAGATAGACTCCACATGGATAATG-3′ and reverse primer 5′-ATTTCTCGAGCCCGCCGTTTGGGGAG-3′. The PCR product was digested with *Eco*RI and *Xho*I and cloned into vector pET41a (Novagen, Madison, WI, USA), fusing glutathione S-transferase (GST), S·tag and a 6× his·tag to the N-terminus of HCS; this construct also contains a 6× his·tag at the C-terminus of HCS. The plasmid was named "HCS-pET41a" and codes for a fusion protein of 114.6 kDa (Fig. 1B); its identity was verified by sequencing. ArcticExpress (DE3) competent cells (Stratagene, La Jolla, CA, USA) were transformed with HCS-pET41a and grown overnight at 37°C in 1 ml LB broth containing 20 mg/L gentamycin and 50 g/L kanamycin. Cultures were expanded to 150 ml and grown at 30°C for about 4 h to an optical density of 0.3 to 0.6 at 600 nm. Isopropyl-beta-D-thiogalactopyranoside was added for a final concentration of 1 mM, and cultures were continued for 24 h at 12°C. Cell pellets were collected by centrifugation at 2700×g for 10 min and resuspended in 15 ml of phosphate-buffered saline with protease inhibitor cocktail (Sigma). Samples were sonicated on ice two times for 5 s at maximal output energy (Branson 250 Digital Sonifier, Danbury, CT, USA) with a 1-min break between pulses. Cell debris was removed by centrifugation (2700×g at 4°C for 15 min), and HCS fusion protein was purified using High-Affinity GST Resin (Genescript, Piscataway, NJ, USA) according to the manufacturer's instructions. HCS in the column fractions was identified by gel electrophoresis, Coomassie blue staining, anti-his·tag antibody (Novagen) and an antibody to the C-terminus in human HCS [8].

2.4. Limited proteolysis assays

In limited proteolysis assays, interactions between two proteins slow their proteolytic degradation to an extent that is detectable by Western blot analysis. Limited proteolysis experiments were conducted as described [24] to confirm that HCS interacts with histone H3. Briefly, 3 μg recombinant human histone H3.2 (New England Biolabs) was incubated with or without 15 μg recombinant HCS (rHCS) in 50 μl of 75 mM Tris acetate buffer (pH 7.5), containing 0.3 mM DTT and 45 mM $MgCl_2$, at 37°C for 2 h. Note that the concentrations of H3.2 and HCS are approximately equimolar in this sample. Ten microliters of the mixture (Time 0 sample) was collected before addition of 40 ng trypsin (Sigma) to the remaining solution. Incubation was continued at 37°C, and 10-μl aliquots were collected after 5, 10 and 15 min. Proteolysis was stopped by adding Tris-Glycine sample buffer (Invitrogen, Carlsbad, CA, USA) and heating at 95°C for 10 min. Proteins were resolved using 3–18% Tris-Glycine gels. Histone H3.2 was probed by using Coomassie blue and goat anti-human histone H3.2 (Santa Cruz). Protection of histone H3.2 by GST in the HCS-GST fusion protein was formally excluded by incubation of tryptic digestion of a mixture of recombinant H3.2 and GST. GST was purified from ArcticExpress (DE3) cells transformed with vector pET41a.

2.5. Yeast two-hybrid assays

Lysine residues in the N-terminus of histone H3 are the primary target for biotinylation by ligases [9,10]; to maintain maximal flexibility of the N-terminus in yeast two-hybrid (Y2H) assays, a Gal4 activation domain was fused to the C-terminus of histone H3 to produce the prey vector. Briefly, plasmid pGADT7 (Clontech) containing two *Hin*dIII restriction sites was digested with *Hin*dIII. The

segment coding for Gal4 activation domain (Gal4 AD) was PCR amplified using 5′-CGTGGAGAGCGGGCTATGGATAAAGCGGAATTAATTCC-3′ (forward) and 5′-AAGAAGTCCAAAGCTTCTGAATAAGCCCTCGTAATATAT-3′ (reverse). A sequence coding for histone H3.2 was PCR amplified using plasmid H3-EGFP and primers 5′-GAATTGGGTACCGCCATGGCCCGTACTAAGCAGAC-3′ (forward) and 5′-AGCCCGCTCTCCACG-3′ (reverse). To maintain the nuclear localization signal in the final construct, primers 5′-AATCAACTCCAAGCTTTGCAAAGATGGATAAAGCG-GAATTAATTCCCGAGCCTCCAAAAAAGAAGAGAAAGGTCGAATTGGGTACCGCC-3′ and 5′-TTAGTTGAGGTTCGAAACGTTTCTACCTATTTCGCCTTAATTAAGGGCTCG-GAGGTTTTTTCTTCTCTTTCCAGCTTAACCCATGGCGG-3′ were annealed before proceeding to the ligation. Equimolar amounts of PCR-amplified Gal4 AD and histone H3.2 sequences, and annealed primers were mixed and ligated using the In-fusion system (Clontech) according to the manufacturer's instructions; the final plasmid was denoted "H3-pGADT7" and its identity was confirmed by sequencing in the DNA core facility of the University of Nebraska-Lincoln. Full-length human HCS contains four putative domains, i.e., N-terminal domain (M_1–F_{446}), biotin transfer/ATP binding (central) domain (F_{471}–S_{575}), linker domain (T_{610}–V_{668}) and C-terminal domain (H_{669}–R_{718}) (Fig. 1C) [25]. Full-length HCS and sequences coding for individual HCS domains were fused to a Gal4 binding domain using the pGBKT7 vector as described [25] to produce the following bait vectors: pGBKT7-HCS (full-length HCS), pGBKT7-$HCS_{1–446}$ (N-terminus), pGBKT7-$HCS_{471–575}$ (central domain, containing biotin transfer/ATP binding sites), pGBKT7-$HCS_{610–668}$ (linker domain) and pGBKT7-$HCS_{669–718}$ (C-terminus). Expression of individual constructs was confirmed by Western blot analysis [26], using mouse monoclonal antibodies against Gal4BD (Abcam). In previous studies, we demonstrated that human HCS is bioactive in yeast [25]. Y2H assays were conducted as described earlier, using two reporter genes [25]; the secretion of α-galactosidase in response to *GAL4* activation was monitored directly on the selection plates [27,28].

2.6. Antibodies to biotinylated histone H3

Polyclonal rabbit anti-human antibodies to K4-biotinylated histone H3 (H3K4bio), K9-biotinylated histone H3 (H3K9bio) and K18-biotinylated histone H3 (H3K18bio) were generated in our previous studies [9]. Target specificity of anti-H3K9bio and anti-H3K18bio was confirmed in an extensive series of testing [9], using chemically pure synthetic peptides, bulk extracts of human histones and biotin-depleted histones as described [7]; unlike some commercial antibodies [29], these antibodies do not cross-react with acetylation marks, judged by probing synthetic acetylated peptides with antibodies to biotin marks. As reported previously [9], anti-H3K4bio cross-reacts with H3K9bio and H3K18bio and was excluded from this study.

2.7. Biotinylation of proteins by rHCS

The polypeptide p67 comprises the 67 C-terminal amino acids in human propionyl-CoA carboxylase, including the biotin-binding site K669, and is a substrate for biotinylation by HCS [30,31]. Biotin-free, recombinant p67 was prepared as described [10]. The biological activity of recombinant HCS was tested by incubating 0.3 μg biotin-free p67 with 0.45 μg rHCS in 50 μl of 75 mM Tris acetate buffer (pH 7.5), containing 0.3 mM biotin, 0.3 mM DTT, 7.5 mM ATP and 45 mM $MgCl_2$ at 37°C for 2 h. Biotinylation of histone H3 and a synthetic, H3-based peptide by rHCS was tested with the following minor modifications. Ten micrograms of recombinant human histone H3.2 (New England Biolabs, Ipswich, MA, USA) or 20 μg of peptide $N_{1–25}$, which comprises the 25 N-terminal amino acids in H3 [9], was substituted for p67; the amount of rHCS in reaction mixtures was increased to 0.9 μg, and incubations were conducted for 12 h unless noted otherwise. Reactions were terminated by adding Tris-Glycine (for rH3 and p67) or Tricine (for $N_{1–25}$) loading buffer (Invitrogen) and heating at 95°C for 10 min. Proteins were resolved using 16% Tris-Glycine gels (for rH3 and p67; Invitrogen) and Tricine gels (for $N_{1–25}$), and protein-bound biotin in transblots was probed with horseradish peroxidase-conjugated streptavidin (Pierce), anti-biotin (Abcam, Cambridge, MA, USA), anti-H3K9bio and anti-H3K18bio.

Finally, [^{3}H]biotin (PerkinElmer, Boston, MA, USA) was used to track biotinylation of recombinant histone H3.2. Experiments were carried out as described above, but cold biotin was replaced with [^{3}H]biotin. For experiments with [^{3}H]biotin, 40 μl of the reaction mixtures was spotted onto P81 phosphocellulose paper (Whatman, Kent, UK). Filters were washed four times with 50 ml of 0.75% (wt/vol) phosphoric acid, followed by a final wash with 50 ml acetone at room temperature [32]. Filters were air dried and tritium was quantified in a liquid scintillation counter. Blanks were prepared by omitting all proteins and were subtracted from protein-containing samples. [^{3}H] Biotinylation of p67 was used as positive control; samples incubated in the absence of rHCS were used as negative controls.

3. Results

3.1. HCS/histone H3 interactions

If HCS-GFP fusion protein was precipitated with anti-GFP, the precipitated complex contained histone H3, as judged by probing with anti-H3 (Fig. 2A); 64.3×10^6 HEK293 HCS-GFP cells were used in this pulldown. In contrast, if nuclear extracts were precipitated with non-specific IgG, no histone H3 was detectable (negative control). Positive controls (input sample and recombinant histone H3.2) produced the expected results, i.e., H3 bands were easily detectable in those samples.

In additional specificity tests, nuclear proteins from regular HEK293T cells were precipitated with anti-human HCS and purified with protein A beads. A histone H3 band was clearly detectable, if these samples were probed with anti-H3 (Fig. 2B); 186.7×10^6 HEK293 cells were used in this pulldown. Data analysis by gel densitometry suggested that <5% of the H3 in input chromatin was precipitated with anti-HCS. The similar intensity of the H3 band in HCS overexpression cells and HCS wild-type cells (compare Fig. 2A and B) can be explained by the three times greater number of wild-type cells used in immunoprecipitation experiments. Nuclear extract (before precipitation) was used as positive control and produced a

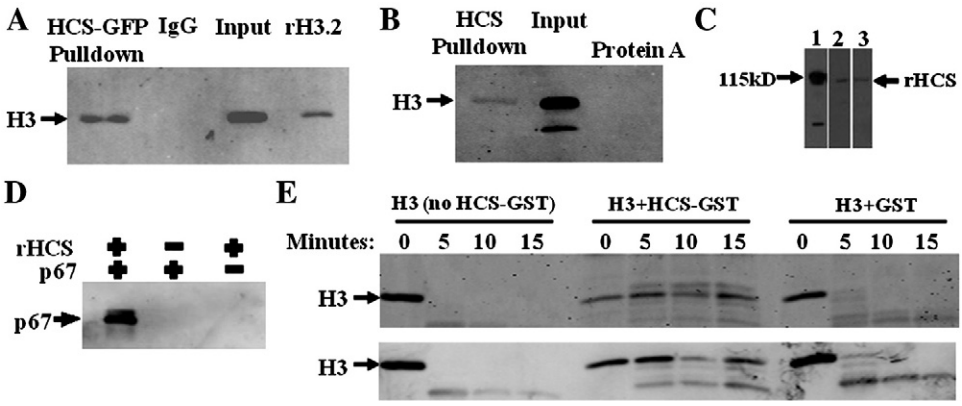

Fig. 2. HCS interacts physically with histone H3.2 in HEK293 cells. (Panel A) Nuclear extracts from HEK293 HCS-GFP cells were precipitated with anti-GFP and probed with anti-histone H3. Nuclear extracts precipitated with non-specific IgG were used as negative control. IgG precipitates (without anti-GFP) were probed with anti-histone H3 ("IgG"); nuclear extracts without antibody treatment (input) and 0.1 μg of recombinant human histone H3.2 (rH3.2) were used as positive controls. (Panel B) Nuclear extracts from HEK293 cells were precipitated with anti-HCS and probed with anti-histone H3 ("HCS pulldown"). As positive control, nuclear extracts collected before treatment with anti-HCS were probed with anti-histone H3 ("Input"); as negative control, protein A precipitates (without anti-HCS) were probed with anti-histone H3 ("Protein A"). (Panel C) Purified rHCS was probed with anti-human HCS (Lane 1), anti-poly·his tag (Lane 2) and Coomassie blue (Lane 3). (Panel D) rHCS was incubated with p67 and cofactors for enzymatic biotinylation; negative controls were generated by omission of individual compounds from reaction mixtures. p67-bound biotin was probed using streptavidin. (Panel E) Preincubation of H3 with HCS protects H3 against proteolysis by trypsin in limited proteolysis assays. Left=Recombinant histone H3.2 alone; middle=H3.2 pre-incubated with HCS-GST; right=H3.2 pre-incubated with GST alone. H3 was probed with Coomassie blue (top panel) and anti-histone H3.2 (bottom panel).

strong H3 signal; no H3 band was detectable if nuclear proteins were precipitated with protein A beads in the absence of anti-HCS.

HCS/histone H3 interactions were confirmed by limited proteolysis assay. First, recombinant human HCS was expressed and purified. A his- and GST-tagged protein of the expected size (114.6 kDa) was detected by using anti-his and anti-HCS (Fig. 2C). Proper folding and biological activity of rHCS were confirmed by successful biotinylation of recombinant p67 (Fig. 2D). If histone H3 was pre-incubated with rHCS-GST at equimolar concentrations and treated with dilute trypsin, the rate of proteolytic degradation of H3 was slowed compared with rHCS-free controls (Fig. 2E). Theoretically, histone H3 could have been protected by interacting with GST rather than with HCS in the HCS-GST fusion protein. Therefore, a control experiment was conducted in which H3 was pre-incubated with GST in the absence of HCS. Pre-incubation of H3 with GST alone did not slow the proteolytic degradation of H3 (negative control). The results from our limited proteolysis assay are consistent with the theory that HCS interacts directly with histone H3. Co-immunoprecipitation data were confirmed in at least three separate experiments.

Y2H assays were conducted and produced results consistent with direct interactions between HCS and histone H3 (Fig. 3). Full-length HCS and individual domains were expressed by yeast cells, as confirmed by Western blot analysis using anti-Gal4BD (data not shown). If full-length HCS was used as bait, colonies became visible on plates within 14–15 days after mating, consistent with transient interactions typically observed for enzymes and their substrates [28,33]. This theory is supported by previous Y2H assays, which revealed that yeast colonies became visible no earlier than 14–15 days after mating of full-length HCS bait with p67 as prey [25]. Co-transforming AH109 yeast strain with the following plasmids did not produce visible colonies, suggesting that auto-activation of reporter genes, non-specific interaction of HCS with Gal4 activation domain (Gal4 AD) and non-specific interaction of histone H3.2 with Gal4 binding domains (Gal4 BD) are not meaningful confounders: full-length HCS *x* empty Gal4 AD, and H3.2 *x* empty Gal4 BD, respectively (not shown). Of the four HCS domains tested (compare Fig. 1C), N-terminus, linker domain and C-terminus produced detectable interactions with histone H3 (Fig. 3). The C-terminal domain $HCS_{669–718}$ interacted with H3 more strongly than full-length HCS or any other individual domain. In response to transformation with pGBKT7-$HCS_{669–718}$, yeast colonies became visible on plates after only 4–5 days of culture. In response to transformation with the N-terminal bait pGBKT7-$HCS_{1–466}$, yeast colonies became visible on plates after 8–9 days of culture; for the linker domain $HCS_{610–668}$, colonies became visible after 15–16 days. The comparably strong interactions of N- and C-terminal domains relative to full-length HCS might be due to ready access for histone H3 to critical amino acid residues in constructs coding for individual domains.

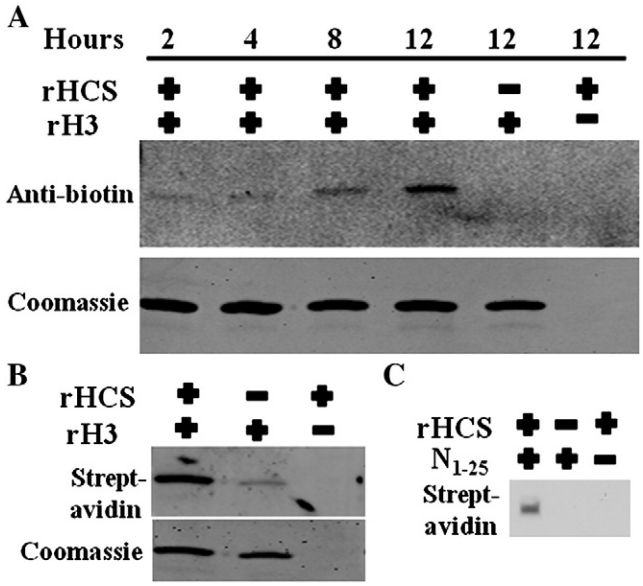

Fig. 4. Recombinant human HCS catalyzes biotinylation of histone H3.2. (Panel A) rHCS was incubated with recombinant human histone H3.2 and cofactors for enzymatic biotinylation for up to 12 h; negative controls were generated by omission of rHCS and H3.2. Samples were collected at timed intervals, and histone-bound biotin was probed using anti-biotin. Equal loading of histone H3.2 was confirmed by staining with Coomassie blue. (Panel B) rHCS was incubated with recombinant human histone H3.2 and cofactors for enzymatic biotinylation for 12 h; negative controls were generated by omission of rHCS and H3.2. Histone-bound biotin was probed using streptavidin. Staining with Coomassie blue was used as loading control. (Panel C) Same as Panel B, but a synthetic peptide representing amino acids 1 to 25 ($N_{1–25}$) in human histone H3.2 was used as substrate. Negative controls were generated by omission of rHCS and peptide.

3.2. Biotinylation of histone H3

In previous studies, human rHCS was expressed in regular *E. coli* systems to demonstrate biotinylation of histones [20,22]. In our hands, these systems produced unsatisfactory amounts of bioactive rHCS, presumably because of improper folding of rHCS. We increased the yield of bioactive rHCS by using the ArcticExpress expression system described in Materials and Methods.

rHCS exhibited catalytic activity to mediate biotinylation of histone H3. First, rHCS was incubated with recombinant human histone H3.2 and cofactors for up to 12 h, and histone-bound biotin was probed at timed intervals by using anti-biotin. Biotinylation of H3.2 by rHCS was readily detectable after 8 to 12 h of incubation (Fig. 4A, upper panel); no biotinylation was detectable if rHCS or H3 was omitted. Gels were stained with Coomassie blue to confirm equal

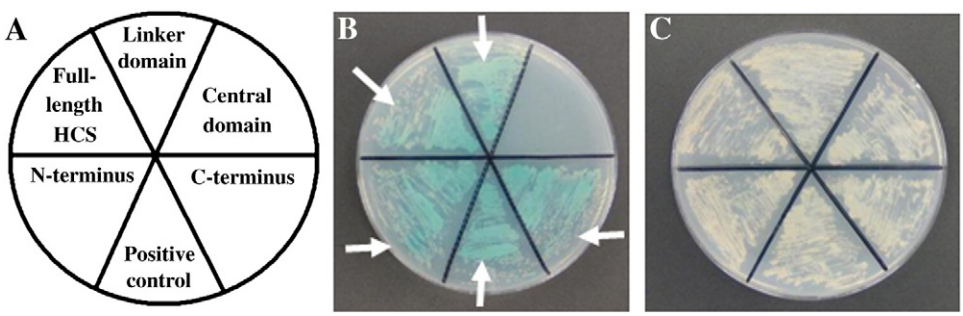

Fig. 3. The N-, C- and the linker domains in human HCS interact with histone H3.2 in Y2H assays. (A) Plate layout of HCS interactions; the interaction between p53 and T antigen was used as positive control; (B) activation of reporter genes and secretion of α-galactosidase, mediated by HCS–H3 interactions (arrows); and (C) successful co-transformation of test plasmids was verified by growing AH109 host strain on SD/−Leu, −Trp, +Kan plates.

loading of histone H3.2 (Fig. 4A, lower panel). Biotinylation of histone H3 by HCS was confirmed using streptavidin as a probe (Fig. 4B). Second, rHCS was incubated with H3-based peptide N_{1-25} and cofactors. Peptide-bound biotin was probed using streptavidin (Fig. 4C). Biotinylation of H3.2-based peptides by rHCS was readily detectable, whereas negative controls without rHCS or peptide produced no detectable signal. Third, biotinylation of histone H3 was traced by using [^{3}H]biotin. If rHCS was incubated with histone H3, [^{3}H]biotin and co-factors, binding equaled 0.33±0.06 pmol of [^{3}H] biotin/(500 pmol of H3×12 h), quantified by liquid scintillation counting; the background radioactivity in samples without rHCS was 0.11±0.01 pmol of [^{3}H]biotin/(500 pmol of H3×12 h). Binding of [^{3}H] biotin to p67 was about 75 times greater than binding to histone H3: 24.6±3.7 pmol of [^{3}H]biotin/(500 pmol of p67×12 h); the background radioactivity in samples without rHCS was 1.0±0.1 pmol of [^{3}H]biotin/(500 pmol of p67×12 h). These observations suggest that HCS catalyzes biotinylation of both histone H3 and p67, and that p67 is a better substrate for biotinylation than histone H3 in a chromatin-free context.

HCS exhibited enzymatic activity to catalyze biotinylation of K9 and K18 in histone H3. If recombinant human histone H3.2 was incubated with rHCS and cofactors, biotinylation of K9 and K18 in H3 was apparent in Western blots probed with anti-H3K9bio and anti-K18bio, respectively (Fig. 5A and B). Equal loading of histone H3 was confirmed by staining with Coomassie blue (not shown). If rHCS was omitted from reaction mixtures, no biotinylation signal was detectable. Comparable results were obtained if peptide N_{1-25} was substituted for recombinant histone H3.2 in biotinylation reactions (Fig. 5C and D).

4. Discussion

To the best of our knowledge, this is the first report of a direct physical interaction between human HCS and histone H3. Importantly, this article also provides conclusive evidence for biotinylation of K9 and K18 in histone H3 by HCS. These observations bear biological significance, given the known roles of HCS-mediated biotinylation of histones in gene regulation [12,14] and repression of transposable elements [15] in humans; phenotypes caused by HCS knockdown include decreased lifespan and stress resistance in *D. melanogaster* [19]. Importantly, a series of HCS mutations have been identified in humans that decrease both enzymatic activity [34] and biotinylation of histones [9,20]. This study offers novel mechanistic insights into histone biotinylation processes.

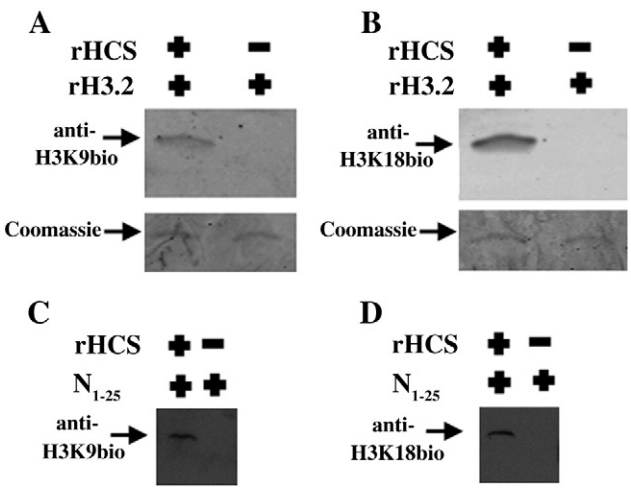

Fig. 5. Recombinant human HCS catalyzes biotinylation of K9 and K18 in human histone H3. Recombinant human histone H3 was incubated with rHCS and cofactors; negative controls were prepared by omission of rHCS. Transblots of histone were probed with anti-H3K9bio (Panel A) and anti-H3K18bio (Panel B). Histone H3-based peptide N_{1-25} was incubated with rHCS and cofactors; negative control was prepared by omission of rHCS. Transblots of peptide were probed with anti-H3K9bio (Panel C) and anti-H3K18bio (Panel D).

Biotinylation of histones is a fairly novel epigenetic mark [5–7]. So far, 11 biotinylation sites have been identified in human histones [7–10]. In the early days of histone biotinylation studies, streptavidin was the probe of choice, despite its cross-reactivity with some compounds other than biotin [35]. Recently, it has been proposed that streptavidin cross-reacts with non-biotinylated histones and, therefore, is not a good probe for biotinylated histones [36]. In this study, a very clean and robust array of experiments were used to demonstrate that K9 and K18 in histone H3 are biotinylated by HCS; probes for histone biotinylation included streptavidin, anti-biotin, [^{3}H]biotin, anti-H3K9bio and anti-H3K18bio, and produced similar results. Notwithstanding our observations, proper caution should be exercised when working with streptavidin as a probe, and site-specific antibodies should be used as the gold standard in studies of histone biotinylation.

We propose that the N-terminal tail of histone H3 is important for permitting interactions between HCS and histone H3, based on the following observations. In our studies, the two proteins did not interact if the histone H3 N-terminus was masked by fusion to Gal4 AD in the Y2H assay (data not shown); interactions were apparent only for the C-terminal fusion. This observation is consistent with analysis of nucleosomes by X-ray crystallography. These previous X-ray studies revealed that the N-terminal tails of histones protrude from the nucleosomal surface [37] and carry the majority of the posttranslational histone modifications [2], including biotinylation marks [38]. Our preliminary data revealed that HCS also strongly interacts with histone H4, but to a much weaker extent with histone H2A and H2B, suggesting that the H3H3H4H4 tetramers rather than the H2AH2B dimers are the primary docking sites for HCS in nucleosomes. This is the first report to provide an explanation for the binding of HCS to chromatin.

We cannot formally exclude the possibility that some biotinylation of histones may occur without direct participation of HCS. In a first such scenario, HCS might catalyze the formation of biotinyl-5′-AMP, followed by HCS-independent diffusion of biotinyl-5′-AMP to target sites for biotinylation, as described for histone H2A [22]. One could still consider biotinylation of histone H2A an HCS-dependent mechanism, given that the synthesis of biotinyl-5′-AMP depends on HCS. Please note that biotinylation marks are more abundant in histone H3 than in histone H2A, judged by using anti-biotin as probe [6]. This observation suggests that the direct physical interaction between HCS and its substrate H3 described in this article creates an environment that is favorable for biotinylation.

In a second such scenario, the enzyme biotinidase might contribute to biotinylation of histones. It was shown repeatedly that biotinidase has the ability to mediate biotinylation of histones *in vitro* [5,7–9]. However, knockdown studies in *D. melanogaster* suggest that HCS is more important than biotinidase for histone biotinylation *in vivo* and that phenotypes of HCS deficiency are more severe that those of biotinidase deficiency [19]. Comparable observations were made in studies using fibroblasts from human patients with mutations in genes coding for HCS and biotinidase [9].

Notwithstanding the novelty and mechanistic significance of this study, a few uncertainties remain. First, while previous studies suggest a role for H4K12bio in the repression of genes and transposable elements [12,14,15], the biological functions of H3K9bio and H3K18bio are currently unknown. Studies of possible biological functions are part of ongoing research efforts in our laboratory. Second, previous studies suggest that inhibitors of DNA methylation decrease the abundance of H4K12bio at adjacent loci.

This crosstalk may be of less importance for H3K9bio and H3K18bio, given that HCS maintained biotinyl ligase activity towards histone H3 in our DNA-free assay system. The importance of this observation is currently being investigated. It is thinkable that DNA methylation promotes biotinylation of histone H3, if H3 is presented in the nucleosomal context as opposed to naked H3. Third, our observation that HCS directly interacts with histone H3 does not explain the dispersed distribution of HCS in chromatin and its recruitment to distinct loci that we observed in previous studies [14,19]. We speculate that the targeting of HCS to distinct regions in human chromatin is mediated by DNA sequence, epigenetic marks, biotin, RNA or chromatin proteins. Our laboratory already provided preliminary evidence for interactions between HCS and proteins that might participate in the nuclear translocation of HCS and its enrichment in distinct genomic loci [39]. A full account of these interactions will be provided in future publications.

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