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Holocarboxylase Synthetase-dependent Biotinylation of Histone H4

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HOLOCARBOXYLASE SYNTHETASE-DEPENDENT BIOTINYLATION OF HISTONE H4

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Holocarboxylase synthetase (HCS) catalyzes the binding of biotin to lysine (K) residues in histones H3 and H4. Histone biotinylation marks play important roles in the repression of genes and retrotransposons. Preliminary studies suggest that K16 in histone H4 is a target for biotinylation by HCS. Here we tested the hypothesis that H4K16bio is a real histone mark in human chromatin, and that H4K16bio is overrepresented in repressed gene loci and repeat regions. Polyclonal rabbit anti-human H4K16bio was generated and affinity purified. Using anti-H4K16bio and chromatin immunoprecipitation assays, we demonstrated that H4K16bio is overrepresented in repeat regions (pericentromeric alpha satellite repeats; long terminal repeats (LTR) compared with euchromatin promoters. H4K16bio was also enriched in the repressed *interleukin-2* gene promoter. The enrichment at LTR22 and promoter 1 of the sodium-dependent multivitamin transporter (SMVT) depended on biotin supply; the enrichment was greater in biotin-supplemented cells compared with biotin-normal and biotin-deficient cells. The

enrichment of H4K16bio at LTR15 and SMVT promoter 1 was significantly lower in fibroblasts from an HCS-deficient patient compared with an HCS wild-type control. We conclude that H4K16bio is a real phenomenon and that this mark, like other biotinylation marks, plays a role in the transcriptional repression of repeats and genes.

HCS catalyzes the covalent binding of biotin to carboxylases, in addition to its role as a histone biotinyl ligase. Biotinylated carboxylases play crucial roles in the metabolism of fatty acids, amino acids, and glucose. HCS null individuals are not viable whereas HCS deficiency is linked to developmental delays and phenotypes such as short life span and low stress resistance. Here, we developed a 96-well plate assay for high-throughput analysis of HCS based on the detection of biotinylated p67 using IRDye-streptavidin and infrared spectroscopy. We demonstrated that the catalytic activity of rHCS depends on temperature and time, and proposed optimal substrate and enzyme concentrations to ensure ideal measurement of rHCS activity and its kinetics. Additionally, we demonstrated that this assay is sensitive enough to detect biotinylation of p67 by endogenous HCS from Jurkat lymphoid cells.

CHAPTER 1

LITERATURE REVIEW

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Biotin

Biotin, also known as vitamin B7 or H, is a water-soluble vitamin involved in many metabolic pathways in mammals (1). Chemically, biotin is a monocarboxylic acid consisting of a ureido group, thiophane heterocyclic ring, and a valeric acid side chain (1-3) (Fig. 1).

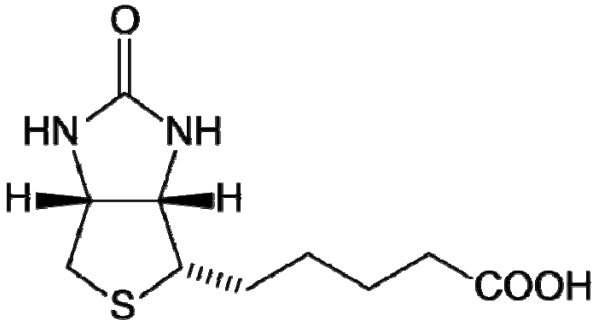

Figure 1. Biotin structure

Humans cannot synthesize biotin but depend on dietary biotin supply (4). Biotin can be found in high concentration in egg yolk, cow milk, liver and some vegetables (5). For absorption of dietary biotin, the protein-bound biotin in foods has to be converted to free biotin by the action of gastrointestinal proteases, peptidases, and biotinidase (1, 5). The uptake of free biotin by the cells is mediated by the sodium-dependent multi-vitamin transporter (SMVT) which also transports pantothenic acid and lipoate (5, 6). Monocarboxylate transporter 1 (MCT1) has also been reported to participate in the transport of biotin, but its function is restricted to lymphoid cells (7, 8) (Fig. 2).

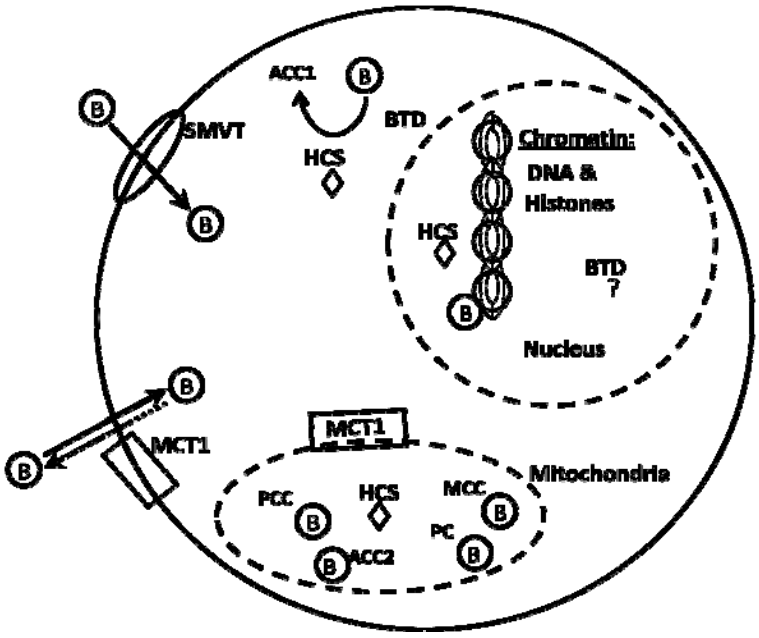

Figure 2. Biotin transport and metabolism, modified from (9). B: biotin, SMVT: sodium multivitamin transporter, MCTI: monocarboxylate transporter 1, PCC: propionyl-CoA carboxylase, ACC1: cytoplasmatic acetyl-CoA carboxylase α, ACC2: mitochondrial acetyl-CoA carboxylase β, PC: pyruvate carboxylase, MCC: and 3-methylcrotonyl-CoA carboxylase.

Biotin plays important roles in the metabolism of glucose, amino acids and fatty acids due to its role as a coenzyme in the metabolism of propionyl-CoA carboxylase (PCC), cytoplasmatic acetyl-CoA carboxylase 1 (ACC1), mitochondrial acetyl-CoA carboxylase 2 (ACC2), pyruvate carboxylase (PC) and 3-methylcrotonyl-CoA carboxylase (MCC) (10, 11). Additionally, it has been shown that biotin is involved in gene regulation by its attachment to histones in the chromatin (10).

Holocarboxylase Synthetase (HCS)

HCS functions as a biotin-ligase for carboxylases and histones (Fig. 3). This enzyme is a 726 amino acid protein with an estimate molecular weight of 81 kDa (12). Four domains in human HCS have been identified: the N-terminal domain, C-terminal domain, central domain, and linker domain (13) (Fig-3). The N-terminal and C-terminal domains have been proposed to participate in substrate recognition while central domain has the binding sites for ATP and biotin (13). The linker domain has been associated with the flexibility of HCS to interact with other substrates for biotinylation (13). It has been reported that HCS localizes in mitochondria and cell nuclei; however due to HCS lacks of nuclear localization sequence and DNA binding motif (14) its localization in the nucleus is still controversial (15, 16).

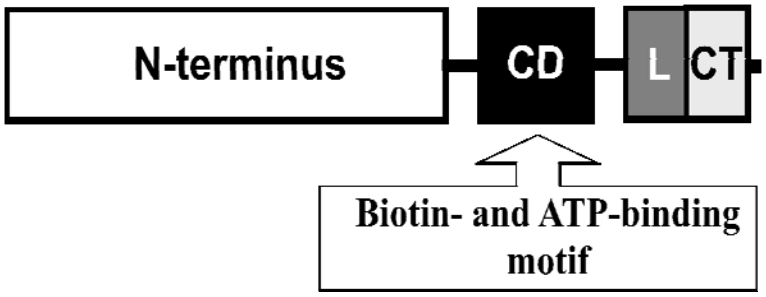

Figure 3. Four domains in human HCS: N-terminal domain, central domain (CD) containing biotin and ATP binding sites, linker domain (L), and C-terminal domain (CT) (13).

HCS catalyzes biotinylation of carboxylases and histones in a two-step ATP-dependent reaction (17). In the first step, biotinyl 5'-AMP (B-AMP) is generated; in the second step, biotin is transferred from B-AMP to the lysine residue in the apocarboxylases and histones (15). It has been demostrated that HCS acts as its ortholog BirA ligase found in *Escherichia coli*. BirA ligase participates in the biotinylation of biotinoyl carboxyl carrier protein (BCCP) and its amino acid sequence is 21% identical with HCS (18-21).

The important role of HCS in biotinylation of histones has been confirmed in various studies (22, 23). Camporeale et al. compared the ability of SMVT mutants and HCS-deficient *Drosophila melanogaster* to biotinylate histones. The results demonstrated that HCS knockdown is associated with a decrease in biotinylation of histones due to the low biotin-ligase activity, rather than the low levels of biotin in the cells (22). Importantly, Baolong et al. recently reported that HCS interacts physically with histone H3 to mediate binding to chromatin and subsequent biotinylation of K9 and K18 in histone H3 (24).

For the study of the activity of HCS, a peptide spanning C-terminal 67 amino acids of PCC, known as p67, has been used as substrate for biotinylation by HCS in previous studies (25-27). p67 contains the biotin-binding consensus sequence (AMKM) for biotin binding (26, 27). In this thesis p67 was used as a substrate for the development of the 96-well plate assay for high-throughput screening of HCS activity (see Chapter 3).

Biotinidase (BTD)

BTD belongs to the nitrilase superfamily of enzymes which consists of 12 families of amidases, N-acyltransferases, and nitrilases (28). BTD shares significant sequence similarities with some members of the nitrilase superfamily such as vanins -1, -2 and -3 (29). In mammalian cells, 26% of the cellular biotinidase activity resides in the nucleus (30) although its nuclear localization has been disputed subsequently (31). The BTD gene localizes in chromosome 3p25 and its structure has been determined (32). The *BTD* gene contains four exons: $A_{125\text{-}44}$, $B_{45\text{-}309}$, $C_{310\text{-}459}$ and $D_{460\text{-}1961}$. Exons B through D code for the mature protein (31) while the 5'-flanking region of exon 1 contains a CCAAT element, three initiator sequences, an octamer sequence, three methylation consensus sites, two GC boxes, and one HNF-5 site but lacks a TATA element (32).

The classical role of BTD in metabolism is to hydrolyze biocytin (biotinyl- ε -lysine) generated in the breakdown of biotin-dependent carboxylases (33). Thus, free biotin is released and recycled in holocarboxylase synthesis and used in other processes (10). The role of BDT in biotinylation of histones has been demonstrated based on *in vitro* studies with purified histones (33). It was proposed that BTD might mediate the binding of biotin to histones by the hydrolysis of biocytin forming a biotinyl-thioester intermediate (cysteine-bound biotin) near its active site. Thus, biotin is bound to the biotinyl moiety from the cysteine residue and transfer to the ε-amino group of the lysine in histones (20, 33).

Evidence has been provided that BTD might catalyze debiotinylation of histones (34). It was proposed that variables such as microenviroment in chromatin, posttranscriptional modifications and alternative splicing might determine whether BTD acts as a biotinyl histone transferase or histone debiotinylase (1). For example covalent modifications such as glycosylation might influence the enzyme activity to biotinylate or debiotinylate histones (34, 35). In the same way, the presence of cofactors such as high concentrations of biocytin might favor and increase the rate of biotinylation of histones (34).

Apocarboxylases

Biotin serves as a covalently bound coenzyme for propionyl-CoA carboxylase (PCC), cytoplasmatic acetyl-CoA carboxylase 1 (ACC1), mitochondrial acetyl-CoA carboxylase 2 (ACC2), pyruvate carboxylase and 3-methylcrotonyl-CoA carboxylase (MCC) (10, 11). Biotin is covalently attached to the ε-amino group of a distinct lysine residue in holocarboxylases (inactive enzyme) by holocarboxylase synthetase (HCS) (15, 25, 36).

In the cell, PCC localizes in the mitochondria and is required for catabolism of the amino acids isoleucine, threonine, methionine, valine, and odd chain fatty acids (10). It is also involved in the carboxylation of propionyl-CoA to D-methylhalonyl-CoA (37, 38). The last 67 amino acids of PCC C-terminus domain has been used as an important tool for study of the bioactivity of HCS because its properties as a well-established HCS

substrate (25, 26). This polypeptide, known as p67, contains the biotin-binding consensus (AMKM) sequence (26, 27).

ACC1 and ACC2 catalyze the binding of bicarbonate to acetyl CoA, generating malonyl CoA (10). These two enzymes share approximately 70% of the amino acid sequence but differ in cellular localization (10, 39, 40). ACC1 participates in fatty acid synthesis in cytoplasm, while ACC2 participates in the regulation of fatty acid oxidation in mitochondria (10).

PC participates in the carboxylation of pyruvate to oxalacetate in gluconeogenesis (10, 41), and MCC participates in the catabolism of the leucine by converting 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA (42).

Biotin and Gene expression

Interleukin-2

Human lymphoid cells normally secret cytokines in response to stimulation of the immune system (43, 44). Interleukin-2 (IL-2) is a cytokine secreted when a cell surface receptor interacts with its specific ligand molecule causing a rapid hydrolysis of inositol phospholipids to diacylglicerol and inositol phosphates by phospholipase C (PLC) (43, 44). Diacylglycerol then activates protein kinase C (PKC) and inositol phosphate, thus, triggering the release and mobilization of Ca++. This process results in additional

cellular responses that mediate T-cell activation, and therefore IL-2 production and secretion (43, 44).

In vitro, phorbol 12-myristate 13-acetate (PMA) has been used to stimulate production of IL-2. PMA has an analogous structure to diacylglycerol, thus, it activates PKC and T-cells to stimulate IL-2 production. A combination of PMA with phytohemaglutinin (PHA), a co-stimulator that binds non-specifically to the cell surface receptor complex, can be used to increase IL-2 expression (43, 44).

Previous studies have provided evidence that biotin plays an important role in immune function (45-50). For instance, biotin supplementation has been reported to decrease the secretion of interleukin-2 and interleukin-1β by PBMC (51). In addition, the association of biotin with repression of the gene coding for IL-2 (52, 53) was reported by Camporeale at el when they demonstrated that biotinylation of K12 in histone H4 represses *interleukin-2* gene (52). Likewise, Pestinger at el, reported similar observations with the biomarks H3K9bio, H3K18bio and H4K8bio (53).

Transposable elements: Long terminal repeats (LTR)

Transposable elements are sequences in the DNA that can change their positions within the genome of a single cell in a process called transposition (54-56). Transposition has been associated with mutations and abnormal patterns in gene expression (57, 58). In mammals, transposable elements type I constitute approximately 42% of the human

genome and are divided into two groups: the long terminal repeats (LTR) and the long interspersed nucleotide elements (LINE) (59). Additionally, LTRs are grouped in intact retrotransposons LTR and solitary LTR (59). In the intact retrotransposons LTR, the viral genes *gag*, *pol* and *enw* are flanked by two repeat regions: 5'-LTR and 3'-LTR, and the expression of retroviral genes is regulated by 5'-LTR (59, 60). In the case of solitary LTR, the retroviral genes have been deleted by recombination of the LTR ; (54, 57-60), and therefore they are unable to produce viral proteins. However, they may have retained their promoter activity; which could cause abnormal patterns of host gene expression (57, 58).

Previous studies have suggested that biotinylation plays an important role in the silencing of genes coding for retrotransposons (59). It was found, that biotinylation of lysine 12 in histone H4 (H4K12bio) and lysine 9 in histone H2 (H2AK9bio) represses retrotransposon transcription in human and mouse cell lines (59). Additionally, biotinylation of lysines 9 and 18 in histone H3 (H3K9bio and H3K18bio), and lysine 8 in histone H4 (H4K8bio) were associated with LTR repression in human lymphoid cells (53).

Biotin Deficiency

Symptoms of biotin deficiency can be found in individuals with insufficient dietary intake; mutations in BTD, HCS, SMVT or carboxylases (10), and in individuals that consume large amounts of raw egg white, lipoic acid, excessive alcohol, or are on term-long drugs treatments (10, 61, 62). Symptoms can go from feeding difficulties, hypotonia, vomiting, alopecia, ataxia, conjunctivitis, periorifitial dermatitis and skin infections to critical progressive loss of consciousness, coma and death (12, 62, 63). Its diagnosis is suggested by the increase in the excretion of organic acids such as lactate and 3-hydroxyisovaleric acid in the urine, and its treatment consists in the administration of pharmacological oral doses of biotin (15).

Mutations in HCS lead to the inability to form active holocarboxylases resulting in multiple carboxylases deficiency (MCD) (10, 62); while the defects of BTD has effects in the recycling of biotin from degraded carboxylases and the release of biotin from dietary proteins (10). Importantly, previous studies suggested that half of the pregnant women in USA experience marginal biotin deficiency (64-68); however, its impact as teratogenic effect has not been demonstrated in humans. On the contrary, studies with mice showed fetal malformations such as cleft palate and micromelia (69, 70).

Studies with *Drosophila melanogaster* suggest that biotin deficiency caused by mutant HCS resulted in a decrease of life span (up to 32%) and stress survival (22, 71). Moreover, it was demonstrated that these effects were caused by the deficiency of histone biotinylation rather than carboxylases. In addition, it was proposed that HCS deficiency

affected gene expression in these cells due to chromatin modifications. The results showed a 50% decrease in the expression of 77 genes associated with defense response, and a 100% increase of 124 genes involved in signal transduction, transport and cell death (22, 71).

Histones and Chromatin

Histones are small basic proteins (11-22 kDa) involved in the folding of the DNA into chromatin (72). They are modified by the covalently binding of biotin to lysine residues by holocarboxylase synthetase (73, 74). There are five major classes of histones in chromatin: H1, H2A, H2B, H3 and H4 (74). Histones H2A, H2B, H3 and H4 form an octamer of core histones with one H3-H3-H4-H4 tetramer and two H2A-H2B dimers while histone H1 is the linker histone bound to the DNA in between two nucleosomes (Fig. 4) (75). Importantly, histones are positively charged proteins due to the presence of a large number of lysine and arginine residues (20-25% of the amino acids). The electrostatic interaction between the negative charge of DNA and the positive charge of histones stabilizes the formation of the nucleosomal core particle (74).

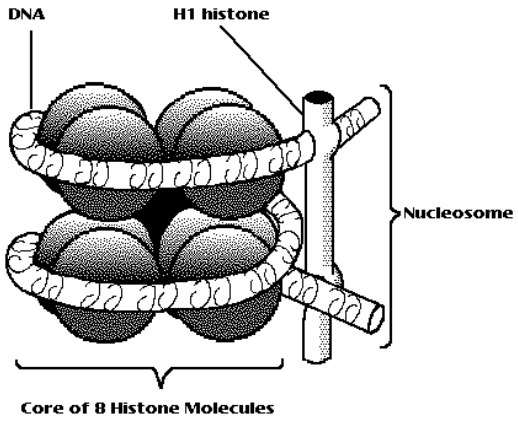

Figure 4. Nucleosome structure. Octamer of histones.
http://www.statemaster.com/encyclopedia/Nucleosome

Covalent histones modifications

Histones are target for reversible covalent modifications such as acetylation, methylation, phosporylation, poly (ADP-ribosylation) and biotinylation. These modifications have distinct functions in gene expression, chromatin remodeling, transcriptional regulation, and DNA repair (15, 76-78). For example, acetylation of lysine residues has been associated with transcriptional activity of genes (79). Acetylation neutralizes the charges of lysine residues allowing dissociation of histones from DNA. This dissociation allows RNA polymerase to access the promoter region and begin transcription (79). In contrast, hypoacetylation has been associated with repression (79). Methylation, may be associated with transcriptional activation or repression. For instance, methylation of K9 in histone H3 is associated with heterochromatin (inactive genes), whereas methylation of Lys K4 in histone H3 is associated with active

transcription (80). Phosphorylation and poly (ADP-ribosylation) are associated with signaling and DNA repair (81).

Biotinylation of Histones

In previous studies, 11 biotinylation sites in histones have been identified: K9, K13, K125, K127 and K129 in histone H2A (82); K4, K9, K18 and perhaps K23 in histone H3 (72); and K8 and K12 in histone H4 (83) (Fig. 5).

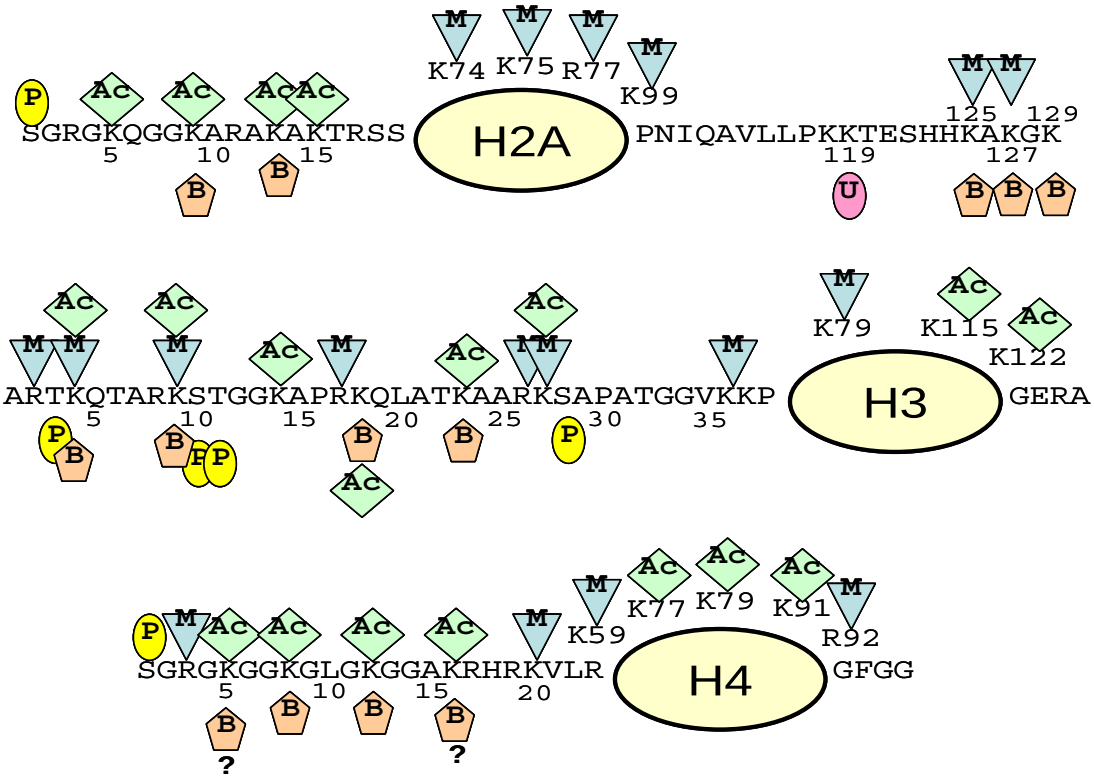

Figure 5. **Covalent modifications in human histones**, modified from (2). Abbreviations: A: acetate, B: biotin, M: methyl, P: phosphate, U: ubiquitin.

Biotinylation of histones plays an important role in the cellular response to DNA damage (84), cell proliferation (73), and mitotic condensation of chromatin (85). In fact, H4K12bio has been associated with gene silencing (52) and repression of transponsable elements (86).

The use of specific antibodies for studies of histone biotinylation has become an important tool for studying their biological functions (82, 83, 87, 88). In previous studies specific antibodies to H4K12bio were generated to investigate its relative enrichment in repeat regions such as pericentromeric alpha satellite repeats (52) and telomeres (89) , as well as its association with the repression of SMVT transporter gene and retrotransponsons (59, 90). Pestinger et al. also used specific antibodies to report the enrichment of H3K9bio, H3K18bio and H4K8bio in pericentromeric alpha satellite repeats, long terminal repeats, and promoter 1 of the *SMVT* gene (53). The biological functions of the other biotinylation sites are still unknown. Mass spectrometry studies provided preliminary evidence for the existence of a novel biotinylation site, H4K16bio, in addition to the 11 known biotinylation sites, but with inconclusive results (87).

This master thesis focused on two major projects. First, I discovered a new histone biotinylation site, K16-biotinylated histone H4, and generated first insights into its biological functions in gene regulation and chromatin structure (see chapter 2). Second, I developed a 96 well-plate assay for high-throughput screening of HCS (see chapter 3).

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CHAPTER 2

K16-BIOTINYLATED HISTONE H4 IS OVERREPRESENTED IN REPEAT REGIONS AND PARTICIPATES IN THE REPRESSION OF TRANSCRIPTIONALLY COMPETENT GENES IN HUMAN JURKAT LYMPHOID CELLS

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Introduction

Histones mediate the folding of DNA into chromatin (1). Amino acid residues in the N-terminal tails and, to a lesser extent, other domains of histones are targets for covalent modifications such as acetylation and methylation (2). These posttranscriptional modifications play important roles in gene regulation, chromatin remodeling, mitosis, and DNA repair. For example, lysine (K)-9 acetylated histone H3 (H3K9ac) and K4-trimethylated histone H3 (H3K4me3) are overrepresented in transcriptionally active euchromatin, whereas hypoacetylated histones and K9-dimethylated histone H3 (H3K9me2) are overrepresented in heterochromatin and repressed genes.

Wolf and co-workers provided evidence that histones are also modified by covalent attachment of the vitamin biotin, based on *in vitro* studies with purified histones and the histone biotinyl ligase, biotinidase (3). Subsequently, biotinylated histones were detected in chromatin from human and other metazoans by using radiotracers, streptavidin, anti-biotin, biotinylation site-specific antibodies, and mass spectrometry {(4-9), T. Kuroishi et al., unpublished}. The majority of biotinylation takes place at K4, K9, K18, and perhaps K23 in histone H3 (10, 11), and K8 and K12 in histone H4 (6), but a weak biotinylation signal can also be detected in histone H2A (4, 12). These previous studies also suggest that holocarboxylase synthetase (HCS) is more important than biotinidase for catalyzing biotinylation of histones *in vivo* (7, 11, 13). Phenotypes of HCS knockdown include a short life span and low heat survival in *Drosophila melanogaster* (7) and aberrant gene regulation in humans (14, 15).

Biotinylation of histones is a fairly rare event, i.e., less than 0.1% of histones in bulk extracts are biotinylated in samples of human origin {(16), T. Kuroishi et al., unpublished}. However, the comparably low level of global histone biotinylation is not representative of the level of biotinylation in confined regions of chromatin. For example, about one of three histone H4 molecules in telomeric repeats is biotinylated at K12 (17). Biotinylation of histones has important functions in gene repression and genome stability. Previous studies suggest that K9- and K18-biotinylated histone H3 (H3K9bio, H3K18bio) and K8- and K12-biotinylated histone H4 (H4K8bio, H4K12bio) are enriched in repeat regions and participate in gene repression (14, 15, 17-19). The abundance of histone biotinylation marks depends on both biotin supply and HCS activity (14, 15, 19); low abundance of H4K12bio in biotin- or HCS-deficient humans, mice, and flies de-represses retrotransposable elements, thereby increasing genome instability and possibly cancer risk (14).

Preliminary mass spectrometry studies provided evidence that histone H4 is also biotinylated at K16 (H4K16bio) (12). Here, we tested the hypotheses that H4K16 is a true biotinylation mark in human chromatin and that H4K16bio, like other biotinylation signatures, is overrepresented in repeat regions and participates in the repression of transcriptionally competent chromatin.

Materials and Methods

Cell culture:

Jurkat human lymphoma cells (ATCC, Manassas, VA) were cultured in commercial RPMI-1640 (Thermo Scientific, Waltham, MA) using standard cell culture techniques (20). Regular commercial RPMI-1640 contains 820 nmol/l biotin, which is >3,000 times the biotin concentration in human plasma (21). In some experiments, cells were cultured in biotin-deficient (0.025 nmol/l biotin), biotin-physiological (0.25 nmol/l biotin), and biotin- pharmacological (10 nmol/l biotin) media for 12 d prior to analysis (20). These concentrations represent levels observed in plasma from biotin-deficient individuals, biotin-normal individuals, and individuals taking over-the-counter biotin supplements (21, 22). Biotin-defined media were prepared using customized RPMI-1640 and biotin-depleted fetal bovine serum (20). Efficacy of treatment was confirmed by probing biotinylated carboxylases with IRDye-streptavidin (19). Where indicated, expression of interleukin-2 (IL-2) was stimulated by treating Jurkat cells with phorbol-12-myristate-13-acetate (PMA) and phytohemaglutinin (PHA) (20) for 3 h.

Human WG2215 HCS-deficient skin fibroblast (Montreal Children's Hospital Cell Repository, Montreal, Canada) were cultured in Dulbecco's modified Eagle's medium containing 10 nmol/l biotin to investigate effects of HCS deficiency on histone biotinylation (19); human HCS wild-type IMR-90 fibroblasts (ATCC, Manassas, VA)

were used as controls. HCS activity in IMR-90 and WG2215 fibroblasts was assessed by using biotinylated carboxylases as markers in streptavidin blots (19).

Antibodies:

Rabbit anti-human H4K16bio was generated as described (6). Briefly, an H4-based, K16-biotinylated synthetic peptide (GGAK(bio)RHRKVLRD, Anaspec; San Jose,CA) was conjugated to keyhole limpet hemocyanin (KLH; Pierce, IL) and used as antigen for injection into New Zealand rabbits. Specificity tests revealed that the anti-serum cross-reacted with K16-acetylated histone H4, using the synthetic peptide GGAK(ac)RHRKVLRD (H4K16ac) as a target (data not shown). To eliminate cross-reactivity with H4K16ac, the antiserum was affinity purified by using immobilized peptide H4K16ac and HiTrap™ NHS-Activated Sepharose™ High Performance Column (cat.# 17-0717-01, GE Healthcare; Piscataway, NJ) following the manufacturer's protocol. Identity and purity of all synthetic peptides were confirmed by mass spectrometry and HPLC, respectively, by the manufacturer.

Biotinylation site specificity of anti-H4K16bio was confirmed by using synthetic histone-based peptides, histone bulk extracts, biotin-depleted histones, and competition studies as described before (6). Equal loading and transfer was confirmed by staining membranes with Ponceau S (19).

Polyclonal rabbit anti-human antibodies to H4K16bio, H4K12bio and H3K9me2 were generated in a commercial facility (Cocalico Biologicals, Reamstown PA) as

described (6, 10, 19), while antibodies to the C-terminus in histone H3 (ab1791) and H3K9ac (ab10812) were purchased from Abcam (Cambridge, MA). H3K9me2 and H4K12bio were used as gene repression and heterochromatin marks, whereas H3K9ac was used as a euchromatin mark. Rabbit polyclonal anti-human pyruvate carboxylase (PC) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and PC in transblots was probed as described before (19).

Micro chromatin immunoprecipitation (µChIP) assay:

The enrichment of H4K16bio in distinct loci in human chromatin was assessed by micro chromatin immunoprecipitation (µChIP) assay as described (19, 23) with the following modifications. Chromatin form Jurkat cells was precipitated using 14µl of anti-H4K12bio and anti-H3K9me2, and 28 µl of anti-H4K16bio sera.

Quantitative real-time PCR (qRT-PCR):

The abundance of DNA in immunoprecipitated chromatin was quantified by qRT-PCR using Perfecta SYBR Green FastMix ROX (VWR; West Chester, PA) and PCR primers for long terminal repeats (LTR) 15 and 22, pericentromeric alpha satellite repeats in chromosome 4, and promoters in genes coding for IL-2, sodium-dependent multivitamin transporter (SMVT), aldehyde dehydrogenase (ADH) 5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19). Amplicons were quantified

using the cycle threshold values method (24). GAPDH was used to normalize qRT-PCR data for amplification efficiency. The relative enrichment of histone marks in precipitated chromatin was calculated as percent of input DNA (25, 26); enrichment data were normalized for nucleosomal occupancy by precipitating samples with an antibody to the (non-modified) C-terminus in histone H3. Non-specific rabbit IgG (Santa Cruz, Santa Cruz, CA) was used as a control for background noise and precipitated negligible amounts of chromatin (<5% of compared with anti-H4K16bio), consistent with previous observations (19).

The abundance of mRNA coding for IL-2, LTR22, and SMVT was quantified using Absolute QPCR SYBR Green fluorescein mix (ABgene; Rochester, NY) as described (6, 19).

Statistical analysis:

Bartlett's test was used to test for homogeneity of variances (27). Data were log transformed if variances were heterogeneous. Significance of differences among more than two groups was tested by one-way ANOVA and Fisher's Protected Least Significant Difference procedure for post hoc testing. Student's paired t-test was used for pairwise comparisons of data from Jurkat cells before and after stimulation with PMA and PHA. μChIP data from WG2215 and IMR-90 fibroblasts were not normally distributed and were analyzed by using the Mann-Whitney U test. StatView 5.0.1 (SAS Institute, Cary NC, USA) was used to perform all calculations. Differences were considered statistically significant if $P < 0.05$. Data are expressed as mean ± S.D.

Results

Specificity of anti-H4K16bio:

Affinity-purified anti-H4K16bio is specific for its designated target, based on the following lines of observations. In a first series of experiments we compared the affinity of anti-H4K16bio for the synthetic peptides H4K16bio and H4K16ac (**Fig. 1A**). Purified anti-H4K16bio produced a strong signal with peptide H4K16bio (lane 1), but not signal with peptide H4K16ac (lane 2). The presence of biotinylation and acetylation marks was confirmed by probing transblots with IRDye-streptavidin (lanes 3 and 4) and anti-H4K16ac (lanes 5 and 6). Equal loading and transfer was confirmed by staining transblots with Ponceau S (lanes 7and 8). Note the non-specific signal in the Ponceau S-stained membrane, caused by interference of the dye in the sample loading buffer (denoted "L").

In a second series of experiments, we assessed the biotinylation site specificity of anti-H4K16bio by comparing its binding to synthetic peptides biotinylated at K8 (N_{6-15}bioK8), K12 (N_{6-15}bioK12), or K16 (N_{13-24}bioK16), where subscripts denoted amino acid residues in histone H4; the non-biotinylated peptide N_{1-19} was used as negative control. Anti-H4K16bio produced a strong signal with peptide H4K16bio, whereas the signals with peptides H4K8bio and H4K12bio were hardly detectable (Fig. 1**B**, lanes 1-3); no signal was detectable with non-biotinylated peptide (lane 4). Equal biotinylation of synthetic peptides was confirmed by probing transblots with IRDye-streptavidin (Fig. 1**C**, lanes 1-3); equal loading of the non-biotinylated peptide N_{1-19} was confirmed gravimetrically.

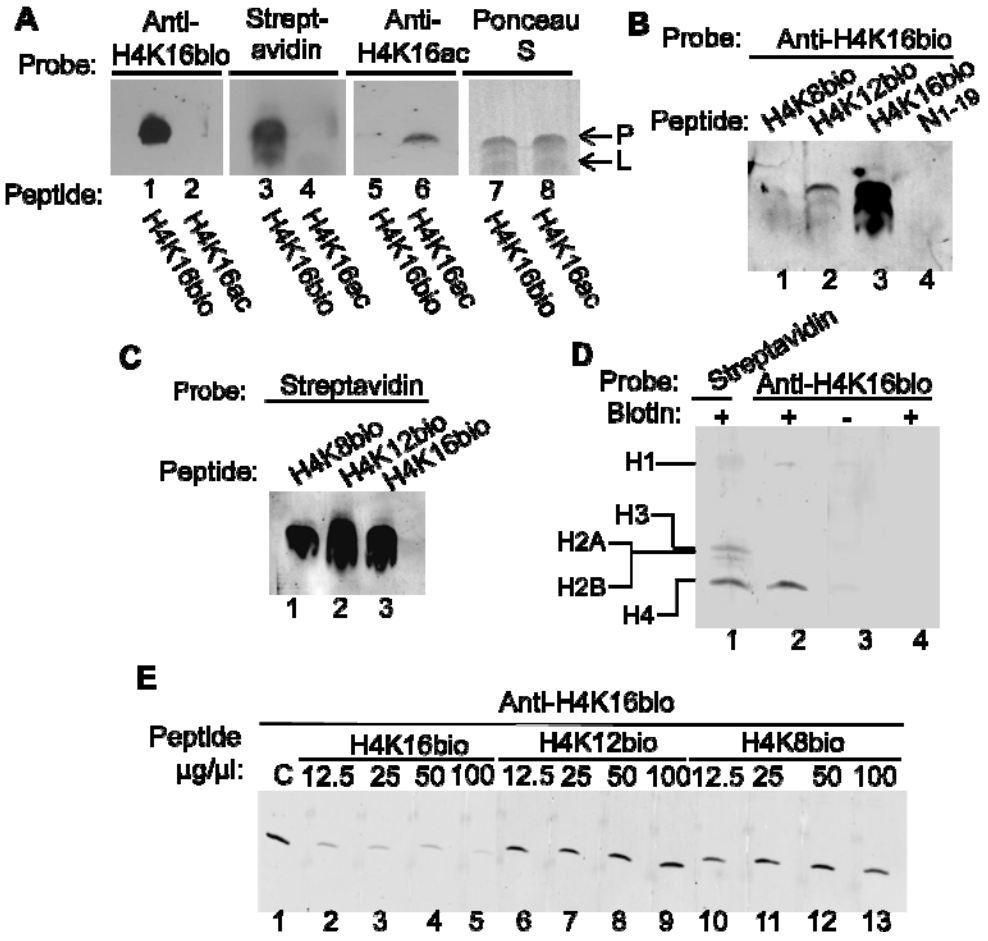

Figure 1. Validation of anti-H4K16bio. (A) K16-biotinylated and –acetylated peptides were probed with anti-H4K16bio, IRDye-streptavidin, anti-H4K16ac, and Ponceau S. (B) Synthetic H4-based peptides biotinylated at K8 ($N_{6\text{-}15}$bioK8), K12 ($N_{6\text{-}15}$bioK12), and K16 ($N_{13\text{-}24}$bioK16), and non-biotinylated peptide $N_{1\text{-}19}$ were probed with anti-H4K16bio. (C) Peptides H4K8bio, H4K12bio, and H4K16bio from panel B were probed with IRDye-streptavidin. (D) Bulk extracts of Jurkat cell histones were probed with IRDye-streptavidin, anti-H4K16bio and pre-immune serum; "-" denotes biotin-depleted histone samples. (E) Bulk extracts of Jurkat cell histones were probed with anti-H4K16bio in the presence of increasing amounts of synthetic peptides H4K16bio, H4K12bio, and H4K8bio. The control sample ("C") was assayed in the absence of peptide competitors. Some gels were electronically re-arranged to facilitate comparisons. Abbreviations: L, loading buffer; P, peptide.

In a third series of experiments, we identified H4K16bio in histone bulk extracts from Jurkat cells. First, we probed histones with streptavidin to demonstrate that the majority of biotinylation marks resides in histones H3 and H4, and that biotinylation of histones H1, H2A, and H2B is quanitatively minor (Fig. 1**D**, lane 1), consistent with our previous results (4). Equal loading and integrity of proteins was confirmed by staining with Commassie blue (not shown). When histones were probed with anti-H4K16bio the antibody produced a signal only with histone H4, but not with other classes of histones (lane 2). This signal was specifically caused by the biotinylated fraction of histone H4, because biotin-depleted histones did not produce a signal (lane 3). When transblots were probed with pre-immune serum, no signal was detectable (lane 4).

In a fourth and final series of antibody testing, bulk extracts of Jurkat cell histones were probed with anti-H4K16bio in the presence of increasing amounts of synthetic peptides. As expected, peptide H4K16bio outcompeted histone H4 for binding by anti-H4K16bio compared with peptide-free control (Fig. 1**E**, lanes 1-5). In contrast, peptides H4K12bio (lanes 6-9) and H4K8bio (lanes 10-13) did not compete for binding.

Relative enrichment of H4K16bio at selected genomic loci:

H4K16bio is overrepresented in pericentromeric alpha satellite repeats (heterochromatin) in chromosome 4 compared the promoters of the two euchromatin genes *GAPDH* and *ADH5* in Jurkat cells (**Fig. 2A**); the enrichment of H4K16bio in the promoters of the two euchromatin genes *GAPDH* and *ADH5* produced comparable

signals. The known repression marks H4K12bio and H3K9me2 (18, 19) were used as positive controls and showed a pattern similar to that of H4K16bio (Fig. 2**B** and **C**). In contrast, the known activation mark H3K9ac (negative control) was greatly enriched in euchromatin promoters compared with pericentromeric alpha satellite repeats (Fig. 2**D**).

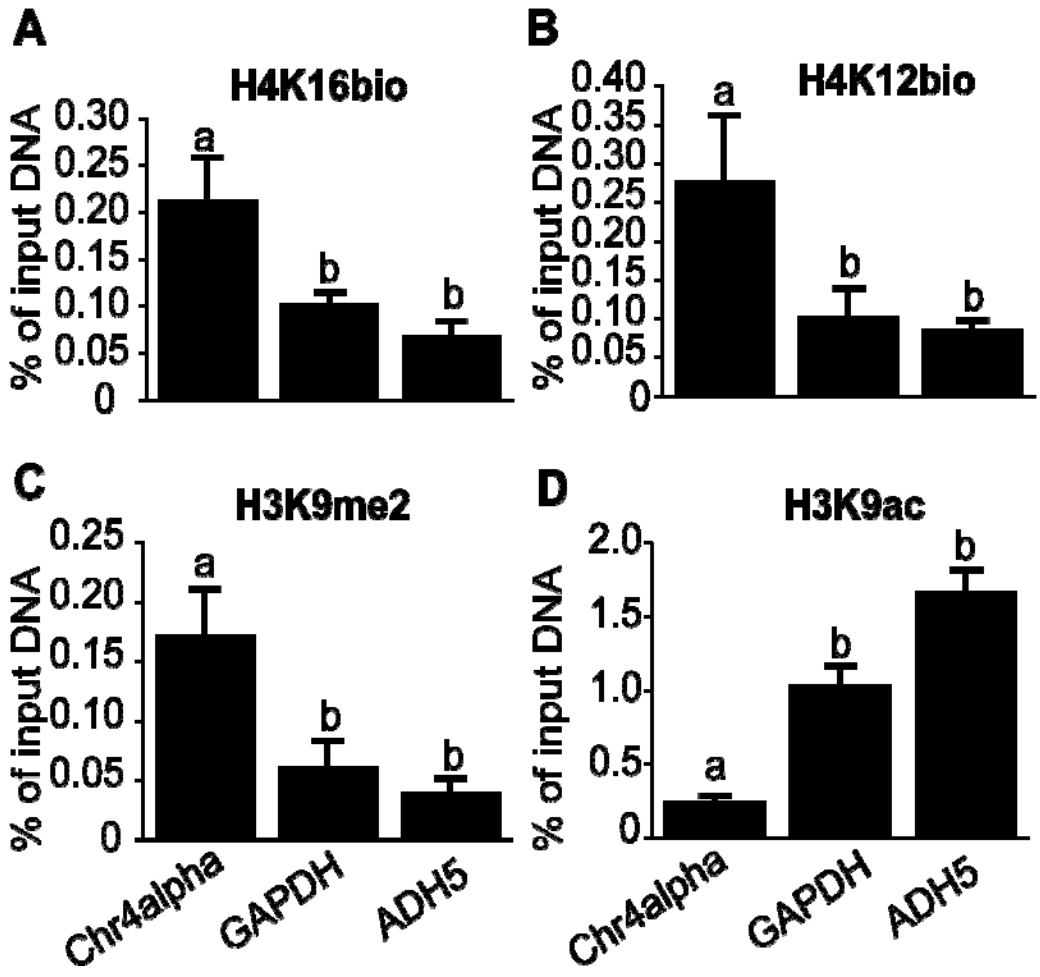

Figure 2. H4K16bio is overrepresented in pericentromeric alpha satellite repeats (heterochromatin) compared with promoters in euchromatin. Chromatin was immunoprecipitated using antibodies to H4K16bio, H4K12bio, H3K9me2, and H3K9ac, and qRT-PCR was used to quantify the relative enrichment of alpha satellite repeats in chromosome 4 (Chr4alpha), and promoters of the *GAPDH* and *ADH5* genes. Bars without a common letter are significantly different (n = 4, $P < 0.05$)

H4K16bio was overrepresented in the transcriptionally repressed promoter in the IL-2 gene prior to stimulation with PMA and PHA compared with 3 h after activation with PMA and PHA (**Fig. 3A**). qRT-PCR was used to confirm efficacy

of de-repression of the *IL-2* gene by treatment with PMA and PHA (arbitrary units): 1.0 ± 0.2 before stimulation vs. 28 ± 2.7 after stimulation (n=4; $P < 0.05$). The controls showed the expected pattern. While the repression marks H4K12bio and H3K9me2 were removed from the IL-2 gene promoter in response to stimulation with PMA and PHA (Fig. 3**B** and **C**), the relative enrichment of the activation mark H3K9ac was greater after than before gene activation (Fig. 3**D**).

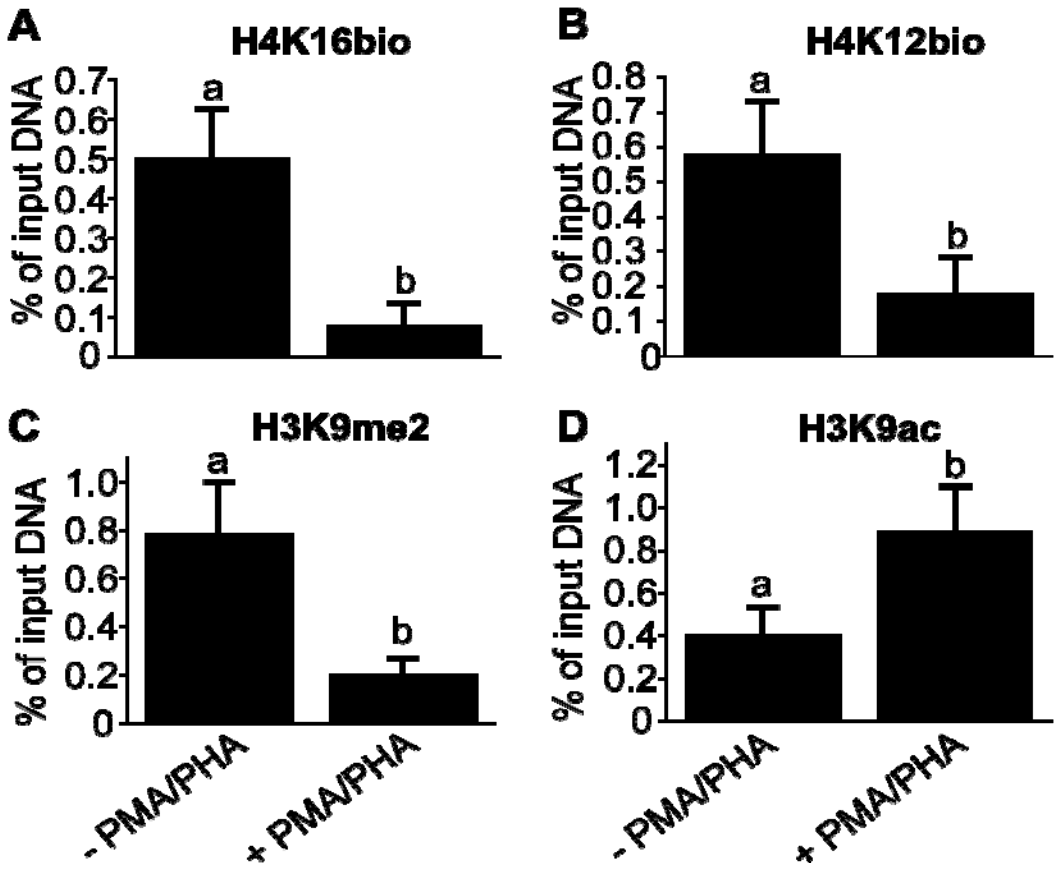

Figure 3. Differential enrichment of histone marks in the *IL-2* gene promoter in Jurkat cells. Jurkat cells were stimulated with PMA/PHA for 3h. Chromatin was immunoprecipitated with antibodies to H4K16bio, H4K12bio, H3K9me2, and H3K9ac before and after stimulation with phorbol-12-myristate-13-acetate (PMA) and phytohemagglutinin (PHA). qRT-PCR was used to quantify the relative enrichment of *IL-2* promoter sequences. Bars without a common letter are significantly different (n = 5, $P < 0.05$)

Biotin dependence:

Previous studies suggest that the abundance of H3K9bio, H3K18bio, H4K8bio, and H4K12bio at LTR15, LTR22, and *SMVT* promoter 1 depends on the concentration of biotin in culture media and dietary biotin intake in various model organisms including healthy adults (14, 15, 19). Here, we observed a similar scenario for H4K16bio. The H4K16bio mark was 13-fold and 4-fold more abundant in the LTR22 locus in cells cultured in medium containing 10 nmol/l compared with cells cultured in 0.025 nmol/l, and 0.25 nmol/l biotin, respectively (**Fig. 4A**). Similar patterns were observed for the repression marks H4K12bio (Fig. 4**B**), and H3K9me2 (Fig.4**C**), while the enrichment of H3K9ac was not affected by biotin (Fig. 4**D**). Consistent with previous observations (14), biotin deficiency (i.e., low abundance of H4K16bio at LTRs) de-repressed retrotransposons. The abundance of LTR transcripts originating in the U5 region (14) was 2.9 ± 0.6 in cells cultured in medium containing 0.025 nmol/l, 0.9 ± 0.06 in cells cultured in medium containing 0.25 nmol/l, and 0.2 ± 0.02 in cells cultured in medium containing 10 nmol/l (arbitrary units, n=4, $P < 0.05$ among all treatment groups).

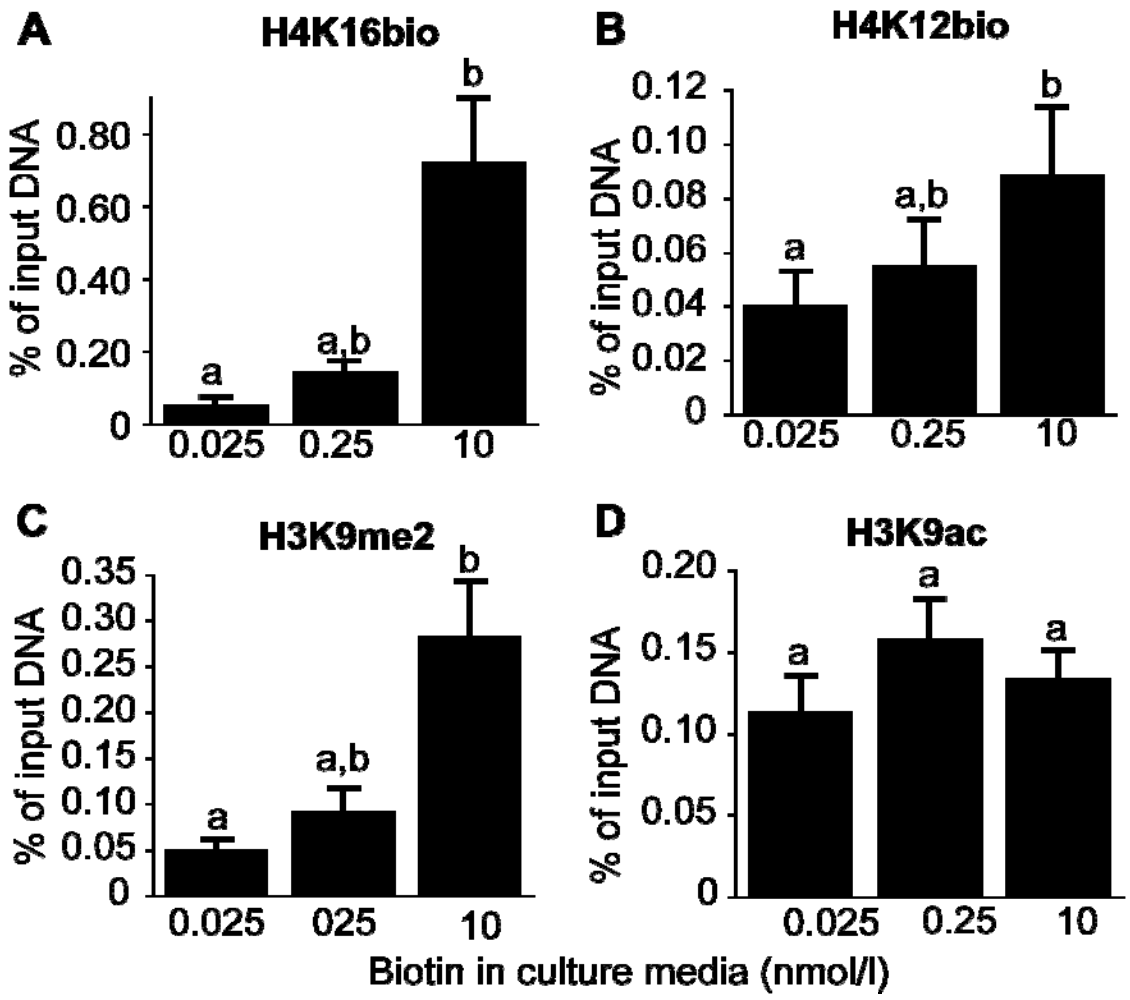

Figure 4. The abundance of H4K16bio at the LTR22 locus depends on the concentration of biotin in culture media. Cells were cultured in biotin-defined media for 12 d, and chromatin was precipitated with antibodies to H4K16bio, H4K12bio, H3K9me2, and H3K9ac; qRT-PCR was used to quantify the relative enrichment of *LTR22* sequences. Bars without a common letter are significantly different (n = 4-5, $P < 0.05$).

The enrichment of H4K16bio, H4K12bio, and H3K9me2 at the *SMVT* promoter 1 locus was comparable to that at LTR22, e.g., the H4K16bio mark was 1.7-fold and 1.4-fold more abundant in cells cultured in medium containing 10 nmol/l compared with cells

cultured in 0.025 nmol/l, and 0.25 nmol/l biotin, respectively. However, the differences among the different biotin concentrations did not reach statistical significance for the SMVT promoter ($P = 0.21$).

The efficacy of biotin treatment was confirmed by probing biotinylated holocarboxylases with IRDye-streptavidin (**Fig. 5**, upper gel). Clearly, the abundance of holocarboxylases depended on the concentration of biotin in culture media. Equal loading of lanes was confirmed by using anti-PC as a probe for total PC (holo & apo; Fig. 5, lower gel).

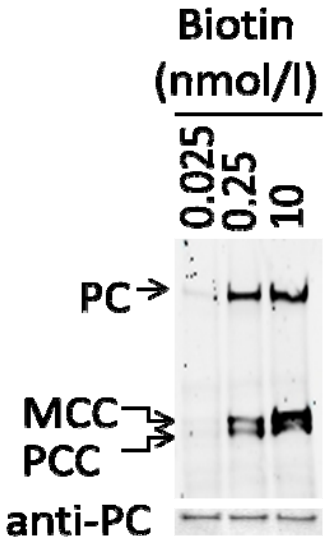

Figure 5. Biotinylation of carboxylases depends on the concentration of biotin in culture media. Jurkat cells were culture in biotin-defined media for 12 d, and carboxylase-bound biotin was probed with IRDye-streptavidin in pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase (MCC), and propionyl-CoA carboxylase (PCC) (upper gel). Equal loading of lanes was confirmed by using anti-PC as a probe (lower gel).

HCS deficiency:

Consistent with a previous report (19), the enrichment of H4K16bio and the repression marks H4K12bio and H3K9me2 decreased at the *LTR22* locus in HCS-deficient fibroblasts (WG2215) compared with HCS-normal fibroblasts (IMR-90) (**Fig. 6. A-C**). The same pattern was obtained if H4K16bio was studied at *SMVT promoter 1* and *LTR15* loci (data not shown). HCS activity in both types of fibroblasts was confirmed by probing transblots of biotinylated carboxylases with IRDye-streptavidin (Fig. 6**D**, upper gel). Biotinylated carboxylases were easily detectable in IMR-90 fibroblasts, whereas no signal was detectable in WG2215 fibroblasts. Anti-PC was used to confirm that the decrease of biotinylation signal in WG2215 cells was due to the inability of mutant HCS to biotinylate carboxylases rather than low apocarboxylases expression or differences in the loading or integrity of the proteins (Fig. 6D, lower gel).

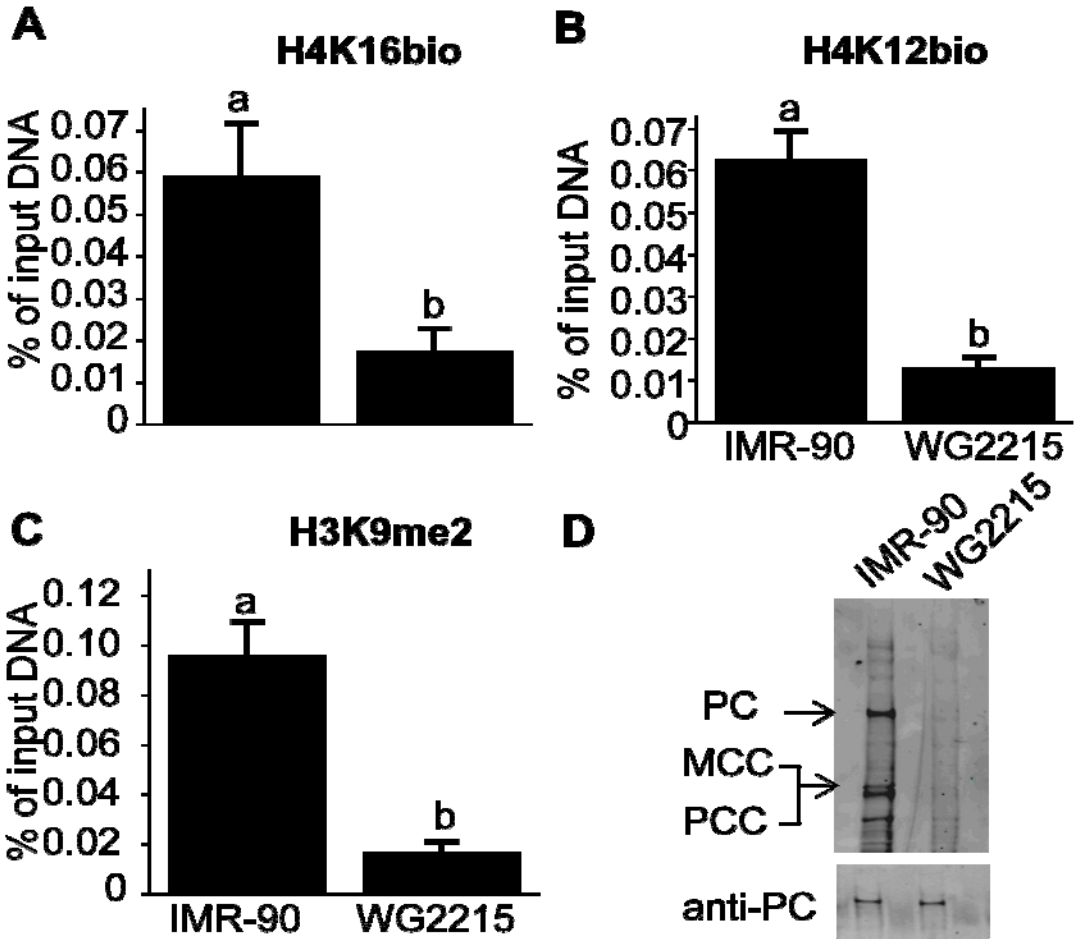

Figure 6. The enrichment of H4K16bio at LTR22 depends on HCS in primary fibroblasts. (A-C) HCS-normal fibroblasts (IMR-90) and HCS-deficient fibroblasts (WG2215) were cultured in medium containing 10 nmol/l biotin. Chromatin was precipitated with antibodies to H4K16bio, H4K12bio, and H3K9me2, and qRT-PCR was used to quantify the relative enrichment of LTR22. Bars without a common letter are significantly different (n = 3-4, $P < 0.05$). (D) Biotin in PC, MCC, and PCC was probed with IRDye-streptavidin (upper gel); equal loading was confirmed by using anti-PC as a probe (lower gel).

Discussion

Here, we demonstrate that K16 in human histone is a target for covalent biotinylation, and we provide first insights into possible biological functions of H4K16bio in pericentromeric heterochromatin and gene repression. A novel antibody was generated and its specificity for H4K16bio was demonstrated in an extensive series of testing. Importantly, we report that the abundance of the H4K16bio mark depends on both biotin supply and HCS activity.

H4K16bio seems to play an important role in gene regulation, based on the following observations. First, the enrichment of H4K16bio in the *IL-2* gene promoter greatly decreases in response to gene activation with phorbol ester and mitogen; H4K16bio depletion co-incides with an increase in the abundance of IL-2 transcript. Second, the enrichment of H4K16bio at the retroelement LTR22 depends on the concentration of biotin in culture media; H4K16bio depletion co-incides with an increase in the abundance of LTR transcript. This observation is particularly important given that previous in-depth studies of retroelement repression suggest an increase in chromosomal abnormalities and, therefore, cancer risk in biotin-depleted cells (14). Third, the enrichment of H4K16bio in the *SMVT* gene promoter increases in response to biotin supplementation. In previous studies we provided evidence that increased biotinylation of histones in the SMVT promoter is an important regulatory mechanism to repress the expression of the SMVT gene in biotin-supplemented cells (15). It appears that the roles of histone biotinylation in gene repression are not specific for distinct biotinylation sites, as all biotinylation sites studies to date are overrepresented in transcriptionally repressed chromatin (18, 19).

A recent report suggests that some of the commercial antibodies to biotinylated histones cross-react with acetylated histones (28). In response to that report, we re-examined our in-house antibodies to H3K9bio, H3K18bio, H4K8bio, and H4K12bio and did not detect any cross-reactivity with acetylation marks {(13), T. Kuroishi et al., in preparation}. We propose that these conflicting results are caused by the targets used for specificity testing. Healy et al. used recombinant histones that were enzymatically acetylated or biotinylated (28); the extent acetylation and biotinylation and the actual modification sites (e.g., K8 vs. K12) remained unknown. In contrast, in our studies we used synthetic peptides as targets, where both the extent and location of the modification were unambiguous {(13), T. Kuroishi et al., in preparation}. However, in contrast with the other in-house antibodies, the batch of polyclonal anti-H4K16bio tested here showed some cross-reactivity with H4K16ac. It seems prudent to individually validate each batch of polyclonal antibodies to biotinylated histones.

Our studies of histone biotinylation in HCS mutant fibroblasts lend further strength to the notion that histone biotinylation is a real phenomenon. The abundance of biotinylation marks was substantially lower in HCS mutant WG2215 fibroblasts compared with IMR-90 fibroblasts. Similar observations were made in previous studies (10, 14, 15), along with the observation that recombinant human HCS and its microbial ortholog BirA biotinylate histones in vitro (11, 13). There is still some level of uncertainty as to whether effects of HCS on gene regulation are mediated by biotinylation of histones or biotinylation of proteins such as carboxylases. However, the phenotypes of HCS knockdown are vastly different than phenotypes of carboxylase knockdown (7, 29),

suggesting that aberrant biotinylation of histones in HCS-deficient cells impairs gene regulation.

Two recent reports suggest that histone biotinylation is rare, occurring in <0.1% of histones (16) or that biotinylation of histones might be an in vitro artifact (28). We agree that histone biotinylation is rare, but stress that the abundance of any epigenetic mark must not be confused with its importance. For example, only ~3% of cytosines are methylated, but the role of DNA methylation in gene regulation is undisputed (30). Likewise, phosphorylation of serine-14 in histone H2B and poly(ADP-ribosylation) of histones are detectable only after induction of apoptosis and major DNA damage, respectively, but the role of these epigenetic marks in cell death is clear (31-33). Moreover, low abundance of an epigenetic mark in bulk histone extracts and high enrichment at specific loci are not mutually exclusive. Evidence suggests that about one out of three histone H4 molecules might be biotinylated at K12 in telomeric repeats (17).

We conclude that biotinylation of histones H3 and H4 plays important roles in gene regulation and genome stability. Further mechanistic studies in whole organisms await the arrival of a conditional HCS knockout mouse, which is an ongoing effort in our laboratory.

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CHAPTER 3

A 96-WELL PLATE ASSAY FOR HIGH-THROUGHPUT ANALYSIS OF HOLOCARBOXYLASE SYNTHETASE ACTIVITY

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Introduction

Holocarboxylase synthetase (HCS, E.C. 6.3.4.10) was identified by Suzuki and co-workers (1, 2); it catalyzes the covalent attachment of biotin to distinct lysine residues in carboxylases (3). Mammalian cells express the following five carboxylases: acetyl-CoA carboxylase 1 in the cytoplasm, and acetyl-CoA carboxylase 2, 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, and 3-methyl-crotonyl-CoA carboxylase in mitochondria. Biotinylated carboxylases play essential roles in the metabolism of fatty acids, amino acids, and glucose (4, 5).

HCS also catalyzes the binding of biotin to lysines in histones (6). Histones H3 and H4 are better targets for biotinylation than other histones (7, 8), but biotinylation sites have been identified in H2A, H3, and H4 (9-11). Biotinylation of histones plays essential roles in gene repression and genome stability (12-14).

Consistent with the important role for HCS as protein biotinyl ligase, the monomeric enzyme is widely distributed throughout cells and has been detected in cytoplasm, mitochondria, and nuclei in mammalian cells (1, 15, 16). Three splicing variants of HCS have been identified in human placenta with molecular weights of 86, 82, and 76 kDa (15, 17) but additional variants might exist (1). Nuclear HCS is a chromatin protein (18) and appears to be enriched near the nuclear lamina (16).

To date, no living HCS *null* individual has been identified, suggesting embryonic lethality. HCS knockdown studies (~30% residual activity) produced phenotypes such as decreased life span and heat resistance (18) and increased frequency of retrotransposition

events in *Drosophila melanogaster* (13). Numerous mutations in the human *HCS* gene have been identified and characterized at both the enzymatic and clinical level (19, 20). Unless diagnosed and treated early, HCS deficiency appears to be uniformly fatal (21). In most individuals clinical signs improve if treatment with pharmacological doses on biotin is initiated early (19-22). In addition to mutations, about 2,200 single nucleotide polymorphisms have been mapped in the *HCS* locus but their importance for human health is unknown (23).

The prevention of adverse effects of HCS deficiency depends on the early diagnosis of enzyme activity. As of today, analysis of HCS activity depends on indirect assays that quantify the abundance of biotinylated carboxylases ("multiple carboxylase deficiency"). These assays are cumbersome and do not lend themselves to the analysis of a large number of samples, as required for studies of enzyme kinetics in HCS-deficient individuals. Moreover, our laboratory is interested in the identification of synthetic and natural HCS inhibitors that specifically target distinct subcellular structures. Such inhibitors would provide useful tools to distinguish biological effects mediated by biotinylation of cytoplasmic and mitochondrial carboxylases from those mediated by biotinylation of nuclear histones. Here we took an important step towards these goals and developed a 96-well assay for high-throughput analysis of both purified HCS and HCS in biological samples.

Materials and Methods

Principle of assay

The assay is based on the HCS-dependent biotinylation of a polypeptide named p67, followed by detection and quantification of p67-bound biotin in a 96-well plate format using IRDye-streptavidin and infrared spectroscopy. p67 comprises the 67 C-terminal amino acids in human propionyl-CoA carboxylase, including the biotin-binding site lysine-669, and is a widely accepted substrate in studies of HCS (24). Briefly, a mixture (50 µl) of 75 mmol/l Tris-acetate (pH 8.0; all concentrations are final), 7.5 mmol/l ATP, 0.3 mmol/l dithiothreitol, 0.3 mmol/l biotin, 45 mmol/l $MgCl_2$, 6 nmol/l recombinant HCS (rHCS), and 8 µmol/l recombinant p67 was incubated for two hours at 37°C, unless noted otherwise ("Results"). Recombinant proteins were stored for up to 6 months without appreciable loss of activity; dithiothreitol and ATP were stored in small aliquots at -20°C. Reactions were terminated by mixing 40 µl of sample with 60 µl of coating buffer containing 50 mmol/l sodium carbonate (pH 9.6) and 200 mmol/l sodium EDTA in black 96-well plates (Corning, New York, NY; catalog number 3601); plates were incubated over night at 4°C on a rotating shaker. The coating buffer was removed, wells were washed twice with distilled water, and blocked with 200 µl of blocking solution {0.1% bovine serum albumin (wt./vol) and 0.05% Tween-20 (vol./vol) in phosphate-buffered saline} for at least 4 h at 4°C. The blocking solution was discarded, and plates were washed twice with phosphate-buffered saline and incubated with 100 µl of IRDye®-800CW-streptavidin for 1 h on a rotator at room temperature. IRDye-

streptavidin was prepared by diluting a commercial stock solution (LI-COR, Lincoln, NE; catalog number 926-32230; 2.4 moles of IRDye 800 CW/mole of streptavidin, 1 mg/ml) 4,000-fold in 0.1% Tween 20 in phosphate-buffered saline. Plates were washed four times with 0.1%-Tween-20 in phosphate-buffered saline. IRDye fluoresence was quantified using the 800CW channel in an infrared imaging system (Odyssey LI-COR; Lincoln, NE). HCS- or p67-free samples were used as negative controls.

Recombinant HCS (rHCS)

A clone of human HCS fused to glutathione S-transferase, S·tag, and 6x his·tag was expressed and purified as described (6) with the following modifications. Cells were centrifuged at 4ºC (3,000 g for 30 minutes) and precipitated cells were resuspended in 15 ml of binding buffer {PBS, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM $KH2PO_4$, pH 7.3)} with protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were sonicated on ice (Branson 250 Digital Sonifier, Danbury, CT) for 7 min with a 10-s burst (30% amplitude) alternated with 20-s resting periods. The lysate was centrifuged at 10,000 g for 10 min at 4ºC. The supernatant was collected and rHCS fusion protein was purified by using GSTrap FF columns on an AKTA protein purification system (GE Health care; Piscataway, NJ). Protein purity was confirmed by gel electrophoresis and staining with coomassie blue; bioactivity of rHCS was tested using gel electrophoresis with recombinant p67 as substrate as described (6). p67-free and HCS-free mixes were

used as negative controls. Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL).

Expression and purification of p67

Recombinant p67 was used as substrate for HCS activity assays. His-tagged p67 was produced and purified using His Trap FF columns on an AKTA protein purification system (GE Healthcare, Piscataway, NJ) as described (8); purity was confirmed by using gel electrophoresis and staining with coomassie blue. The microbial HCS ortholog BirA is known to biotinylate an appreciable fraction of recombinant p67 (8); hence, the biotinylated fraction of recombinant p67 was removed using avidin columns as described (8). Protein concentrations were determined using the bicinchoninic acid method.

Assay calibration

Assays were calibrated by preparing dilution series of up to 0.8 nmoles/l of IRDye-streptavidin in phosphate-buffered saline. One molecule of streptavidin can bind up to four molecules of biotin (25), but the true extent of biotin binding in this assay is unknown. Hence, the assay was calibrated by assuming that 2 molecules of biotin were bound per molecule of streptavidin. Infrared absorbance of IRDye-streptavidin was measured at 800 nm (channel 800CW) in the Odyssey imaging system. Non-linear

regression analysis was used to calculate K_m and V_{max} of HCS for p67 (GraphPad Prism 5.00; La Jolla, CA).

Analysis of HCS in human cells

Whole cell extracts from 150 * 10^6 Jurkat human lymphoid cells were prepared using 500 µl of cold lysis buffer in the presence of protease inhibitors (26). Cells were sonicated on ice (Branson 250 Digital Sonifier, Danbury, CT) with a 5-s burst (30% amplitude). Complete lysis was confirmed by visual inspection under a light microscope. Debris was removed by centrifugation at 1,600 g at 4ºC for 5 min; the supernatant contained 17.3 µg/µl total protein. Activity of endogenous HCS was quantified using the plate assay by incubating 23 µl of supernatant (~400 µg protein) with cofactors and recombinant p67 in a final volume of 50 µl for 2 h at 37°C. Negative controls were prepared by omitting supernatant and by supernatants for 2 min at 99°C.

Statistical analysis

Bartlett's test was used to test for homogeneity of variances (27). Significance of differences among more than two groups was tested by one-way ANOVA and Fisher's Protected Least Significant Difference procedure for post hoc testing. Student's paired t-test was used for pairwise comparisons. StatView 5.0.1 (SAS Institute, Cary NC, USA)

was used to perform all calculations. Differences were considered statistically significant if $P < 0.05$. Data are expressed as mean ± S.D.

Results

Recombinant HCS and p67

Purified rHCS produced a protein of the expected size (114.6 kDa due to tags) and was >90% pure as judged by staining with coomassie blue (**Fig. 1A,** lane 1). Likewise, his-tagged recombinant p67 produced a chemically pure protein of the expected size, judged by staining with coomassie blue (Fig. 1A, lane 2). When rHCS was incubated with p67 and cofactors, a strong biotinylation signal was detected in p67 using streptavidin as probe (Fig. 1A, lane 3), consistent with the notion that rHCS was bioactive. Importantly, we confirmed our previous observation that recombinant p67 is biotinylated by BirA in *E. coli* (Fig. 1**B**, lane 1) and therefore requires affinity purification before use. When recombinant p67 was purified by using avidin columns, the vast majority of biotinylated p67 was removed (Fig. 1B, lane 2). For all assays described below, we used biotin-depleted p67.

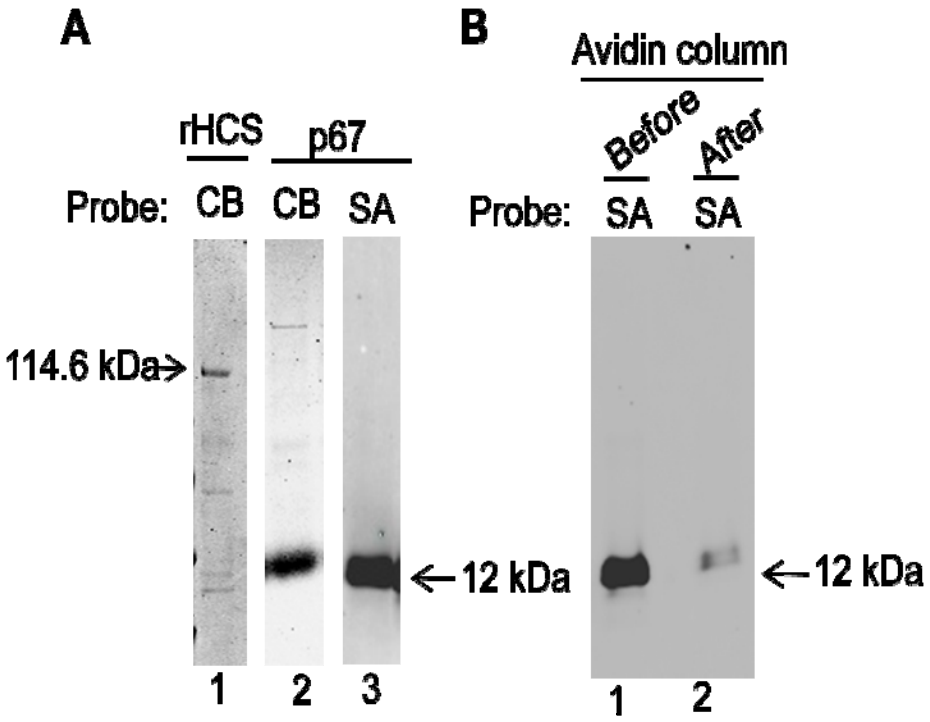

Fig 1. Recombinant HCS and p67. (A) Recombinant HCS (lane 1) and p67 (lane 2) were purified using affinity chromatography and stained with coomassie blue (CB); p67 was incubated with rHCS and co-factors, and biotin was probed with streptavidin (SA, lane 3). (B) Biotinylation of recombinant p67 by microbial BirA was probed with streptavidin before and after purification of p67 by avidin chromatography.

Temperature and time

The catalytic activity of rHCS depended on temperature and time. When various amounts of rHCS were incubated with p67 and cofactors at either 4°C or 37°C, enzyme activities were significantly higher at 37°C (**Fig. 2**); representative examples (2.2 nmol/l and 5.8 nmol/l rHCS) are depicted. At 4°C, enzyme activities were just above

background noise. This is consistent with enzyme-mediated rather than non-enzymatic catalysis. All subsequent experiments were conducted at 37°C.

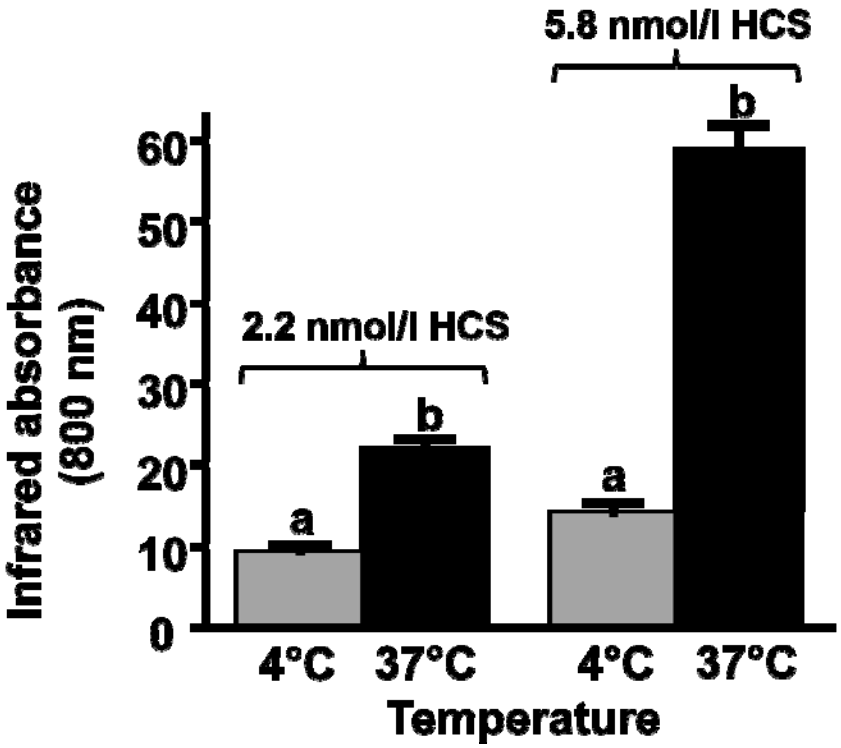

Fig. 2. Temperature dependency of rHCS. Various amounts of rHCS were incubated with p67 and cofactors at 4˚C and 37˚C. Enzyme activities were measured by infrared spectroscopy. Bars without a common letter are significantly different for the same concentration of rHCS (n=3, $P < 0.05$).

When various amounts of rHCS were incubated with p67 and cofactors for 1, 2, or 3 h, enzyme activities increased linearly for up to 2 h, but leveled off thereafter. For example, enzyme activities (7.2 nmol/l rHCS) were 12.6 ± 2.2 of infrared absorbance after 1 h, 28.8 ± 2.9 after 2 h, and 34.1 ± 2.2 after 3 h (**Fig. 3**). Values are expressed in

arbitrary units of infrared absorbance (800 nm) until assay optimization and calibration was achieved (see below). All subsequent experiments were conducted using 2-h incubations.

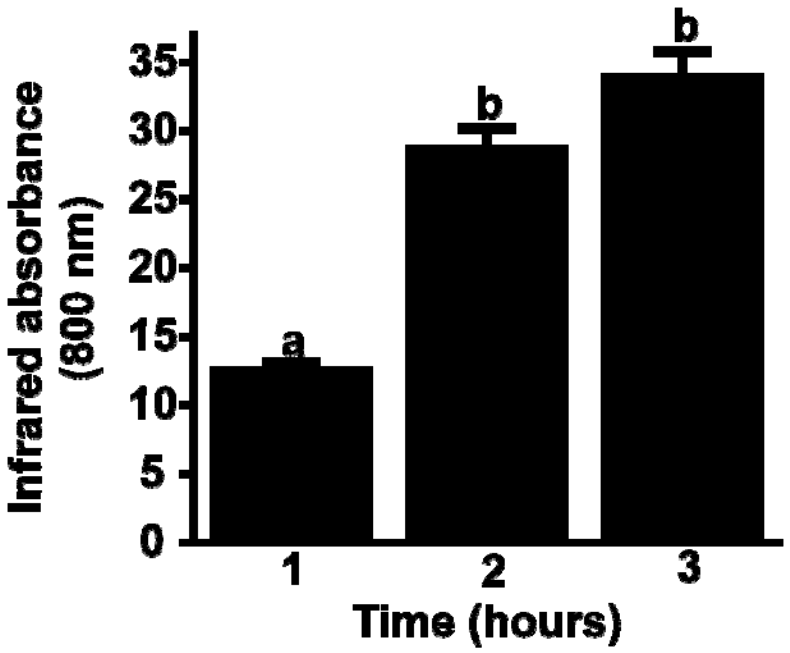

Fig. 3. Effect of incubation time on rHCS activity. rHCS was incubated with p67 and cofactors for 1, 2 or 3 h at 37°C. Enzyme activity was measured by infrared spectroscopy. Bars without a common letter are significantly different (n = 3, $P < 0.05$)

Concentrations of HCS and p67

The amount of p67 that was biotinylated in plate assays increased linearly up to a concentration of rHCS of 8 nmol/l, and leveled off thereafter (**Fig. 4**). Subsequent experiments were conducted using 6 nmol/l HCS to ensure assay linearity. Furthermore, an rHCS dilution series was conducted to determine detection limit of rHCS in this assay. At a concentration of 1.08 nmol/l HCS, biotinylation of p67 was significantly greater

than in negative controls without rHCS (n = 3, $P < 0.05$), whereas 0.54 nmol/l HCS were not significantly different from background controls (data not shown).

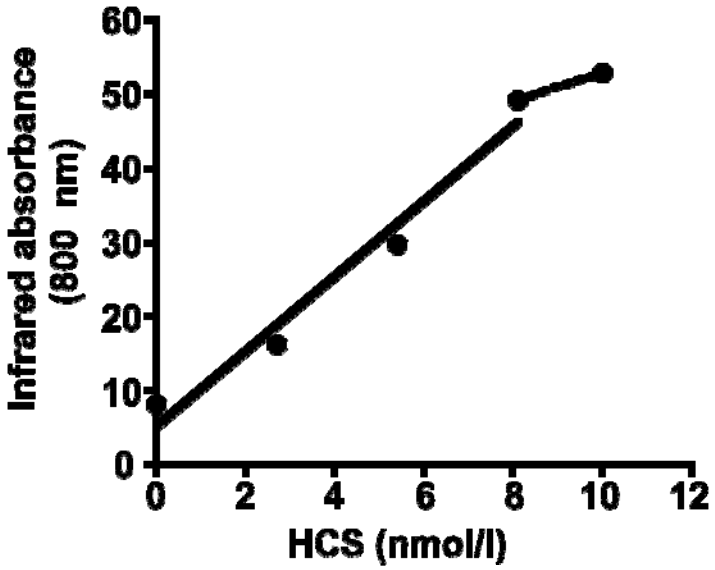

Fig. 4. rHCS concentration. Recombinant p67 was incubated with various concentrations of rHCS and cofactors at 37°C for 2 h. Biotinylation of p67 was quantified by IRDye-streptavidin and infrared absorbance.

Dose-response studies were conducted with varying amounts of p67 to determine a concentration where substrate availability is no longer a limiting factor in this plate assay. A linear dose-response was obtained for up to 5.2 µmol/l p67, while p67 was no longer rate limiting beyond that concentration (**Fig. 5**). These findings were further corroborated by enzyme kinetics studies. The apparent K_m of rHCS for p67 equaled 4.1 ± 1.5 µmol/l and the apparent V_{max} equaled 34.9 ± 4.1 units of infrared absorbance (**Fig.6**). For subsequent experiments, p67 was used at a concentration of 8 µmol/l.

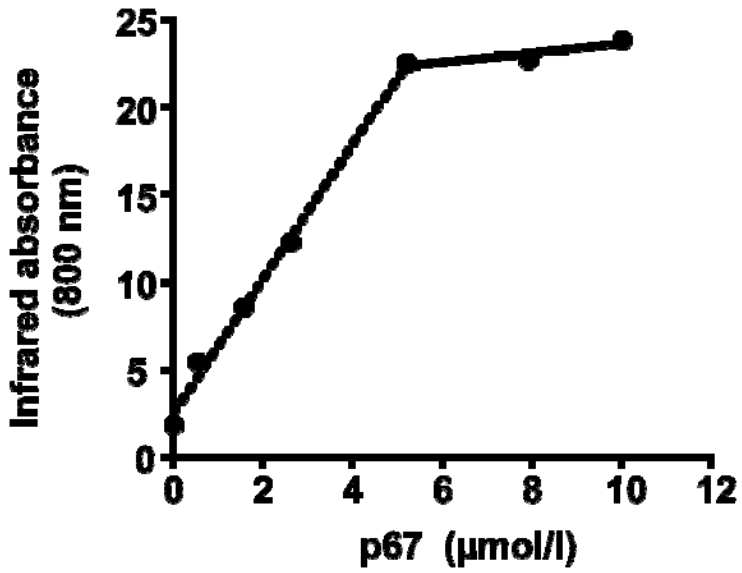

Fig. 5. p67 concentration. Various amounts of recombinant p67 were incubated with 6 nmol/l rHCS and cofactors at 37°C for 2 h. Biotinylation of p67 was quantified by IRDye-streptavidin and infrared absorbance.

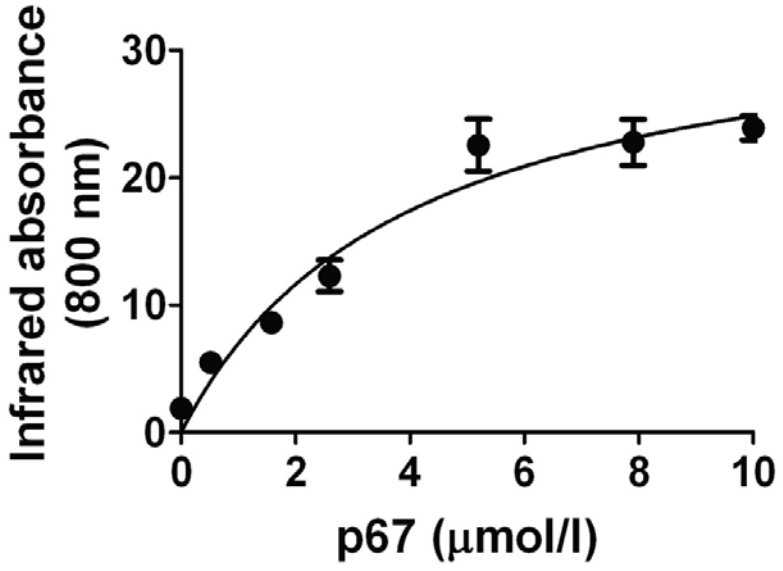

Fig 6. Kinetics of p67 biotinylation by rHCS. The line was fitted to the Michaelis-Menten equation by nonlinear regression analysis (n = 3).

Assay calibration

A dilution series of IRDye-streptavidin was prepared to calibrate the HCS assay. One molecule of streptavidin can bind up to four biotin molecules (25); we assumed that each molecule of IRDye-streptavidin bound, on average, two molecules of biotin under the conditions of this assay. The dilution series of IRDye-streptavidin produced a linear response across all concentrations tested (**Fig. 7**), and the assay system was re-calibrated daily. A typical sample of rHCS under the conditions described in the preceding sections produced a signal of about 36 units of infrared absorbance, suggesting that 0.16 µmol of p67 were biotinylated when incubated with 6 nmol/l HCS for 2 h at 37°C. We define one unit of HCS activity as 0.08 µmol of biotinylated p67 formed x $nmol/l^{-1}$ HCS x h^{-1} at 37°C.

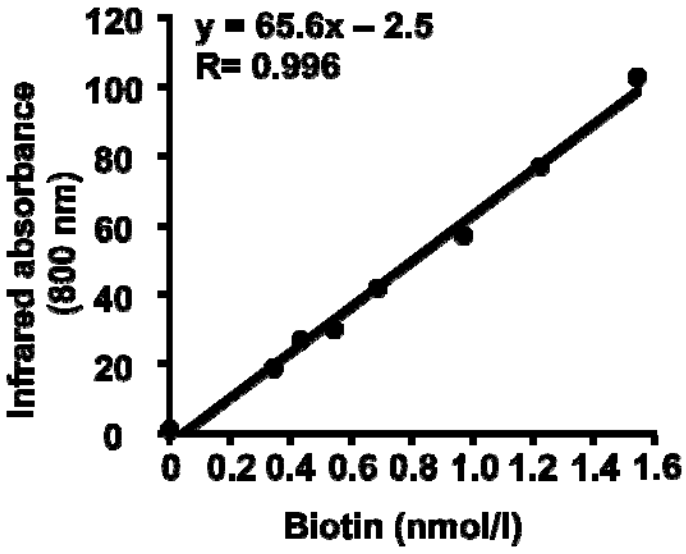

Fig. 7. Assay calibration. A standard curve was produced by quantifying the infrared absorbance of IRDye-streptavidin (up to 0.8 nmol/well) at 800 nm. Note that 0.8 nmoles of IRDye-streptavidin were considered to be the equivalent of 1.6 nmol of biotin/well (n = 3).

HCS activity in Jurkat cells

Supernatants from Jurkat cells contained 0.14 ± 0.02 µmol of biotinylated p67 produced per hour in 400 µg of total protein; no signal was detected in cell-free controls and heat-treated protein extracts (negative controls).

Discussion

Here, we present a 96-well plate assay for high-throughput screening of HCS activity based on the detection of biotinylated p67 using IRDye-streptavidin and infrared absorbance. Previous assays of HCS activity are based on the biotinylation of p67 in a gel electrophoresis-based format. While we acknowledge that the gel-based assay is slightly more sensitive than the plate assay (not shown), we also note that the plate assay has the advantages that large numbers of samples (including replicates) can be analyzed rapidly and cost-efficiently. Despite its lower sensitive, analysis of HCS in cell extracts by plate assay is feasible, as evidenced by successful quantification of enzyme activity in Jurkat cell extracts. Moreover, the plate assay does not require the use of radiochemicals, unlike some of the other assays of HCS deficiencies in patients with multiple carboxylase deficiency (19). We envision two possible applications for the plate assay. First, the assay can be used in the analysis of enzyme kinetics for mutant or variant human HCS, including newborn screening. Second, the assay could be particularly useful when screening libraries of synthetic or naturally occurring compounds for potential HCS

inhibitors in a high-throughput format. With the newly discovered HCS-dependent biotinylation of histones and its role in gene regulation and genome stability (12-14, 28), the need for robust HCS assays can only increase.

This study confirmed our previous observations that particular attention needs to be paid to the protocols for preparing recombinant p67 and HCS. Recombinant p67 contains biotin and requires affinity purification prior to use in HCS assays (8), otherwise artificially high activities may be recorded. The yield of bioactive rHCS is low, if produced following protocols published in the 1990s, at least in our laboratory. We suspect that the low yield of bioactive rHCS by previous protocols is caused by improper folding of the protein and devised a protocol tailored towards maximizing the yield of rHCS (6).

One obstacle to the broad use of this 96-well plate assay is its apparent dependence on the availability of an infrared spectrophotometer, which may not be available in clinical laboratories. If no infrared spectrophotometer is available, we propose modifying the assay by substituting streptavidin-peroxidase for IRDye-streptavidin in a standard plate reader format at 450 nm. We conclude that a robust method was developed for a robust assay of HCS activities in both clinical and research settings.

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