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Hasim, Sahar; Tati, Swetha; Madayiputhiya, Nandakumar; Nandakumar, Renu; and Nickerson, Kenneth W., "Histone biotinylation in *Candida albicans*" (2013). *Kenneth Nickerson Papers*, 2.

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Histone biotinylation in Candida albicans

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Abstract

Candida albicans is an opportunistic fungal pathogen in humans. It is a polymorphic fungus: it can live as yeasts, hyphae, or pseudohyphae. Biotin is required for cell growth and fatty acid metabolism because it is used as a cofactor for carboxylases such as acetyl-CoA carboxylase, and pyruvate carboxylase. In addition, we have discovered that biotin is used to modify histones in C. albicans. Biotinylation was detected by Western blots using a monoclonal antibiotin HRP-conjugated antibody as well as with qTOF and LC/MS/MS mass spectrometry. As a precaution, the antibiotin antibody was dialyzed against neutravidin prior to use. During this study, we observed that three histones, H2A, H2B, and H4, were biotinylated at many lysine residues in an apparently nonsite-specific manner. Roughly, equivalent levels of acetylation, methylation, and phosphorylation were found in histones from biotin-replete and biotin-starved cells, but histone biotinylation was only observed for cells grown in excess biotin. The function of histone biotinylation in C. albicans is still unknown but, because C. albicans is a natural biotin auxotroph, a storage reservoir for biotin is attractive. Techniques used to detect histone biotinylation in C. albicans did not detect any histone biotinylation in Saccharomyces cerevisiae.

Introduction

Candida albicans is a common fungal human pathogen. It is an opportunistic organism, which lives in multiple morphological forms such as yeasts, hyphae, pseudohyphae, and chlamydospores, and these morphological forms are important for the pathogenicity of C. albicans (Saville et al., 2003). Candida albicans is naturally auxotrophic for biotin. In mammals, five different carboxylases: acetyl-coenzyme A (CoA) carboxylase (I and II isoforms), pyruvate carboxylase, methylcrotonyl-CoA carboxylase, and propionyl-CoA carboxylase are dependent on biotin, and the biotin protein ligase (BPL), which attaches biotin to those enzymes, is itself an essential enzyme. Biotin is also a coenzyme for five enzymes in Saccharomyces cerevisiae and C. albicans: acetyl coenzyme A carboxylase (ACC), both cytoplasmic and mitochondrial forms (Sheridan et al., 1990; Hoja et al., 2004), two isoforms of pyruvate carboxylase (Stucka et al., 1991; Walker et al., 1991; Brewster et al., 1994), and a urea-degrading enzyme–urea amidolyase (DUR 1, 2) (Roon & Levenberg, 1972; Navarathna et al., 2010). Methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase are absent in S. cerevisiae and probably in all of the hemiascomycetes (Navarathna et al., 2010). Interestingly, in S. cerevisiae, biotin is also incorporated into a prevalent but nonessential 43-kDa tRNA-binding protein, Arc1p (Kim et al., 2004).

In eukaryotes, histones are basic proteins that bind to the DNA, participate in chromosome structure, and through extensive post-translational modification participate in bulk aspects of gene regulation. The histone–DNA complexes are arranged as nucleosomes with eight histones per nucleosome, including two molecules each of histone 2A, histone 2B, histone 3, and histone 4. Candida albicans SC5314 (Inglis et al., 2012) contains one copy of the H1 gene (Ca 19.5137.1), two copies of the H2A gene (Ca 19.6924 and Ca 19.1051), two copies of the H2B gene (Ca 19.6925 and Ca 19.1052), two copies of
the H3 gene (Ca 19.1853 and Ca 19.6791), and two copies of the H4 gene (Ca 19.1059 and Ca 19.1854). Thus, the biotinylated histones are easily distinguished from the five biotin-requiring enzymes, which are all > 129 kDa.

For human histones, one of the post-translational modifications is the covalent attachment of the vitamin biotin (Zempleni, 2005) catalyzed by the enzymes biotinidase and holocarboxylase synthetase (HCS). Biotinidase removes biotin from biocytin, the biotin-lysine adduct, and makes it available for reuse by other enzymes like holocarboxylase synthetase, which catalyzes the ATP-dependent attachment of biotin to apocarboxylases or histones. The following sites are biotinylated in human histones: K9, K13, K125, K127, and K129 in histone H2A (Chew et al., 2006), K4, K9, and K18 in histone H3 (Camporeale et al., 2004), and K8 and K12 in histone H4 (Sarah et al., 2004). Biotinylation of histones has been reported to play a role in the regulation of gene expression (Gralla et al., 2008), cell proliferation (Stanley et al., 2001; Narang et al., 2004), and the cellular response to DNA damage (Peters et al., 2002; Kothapalli & Zempleni, 2004). However, the functional significance of histone biotinylation has been questioned (Healy et al., 2009) along with the suggestion that histones could be biotinylated in vitro but they are not present in native histones (Healy et al., 2009). We have reexamined this issue in the yeast C. albicans. In this study, we found prevalent biotinylation of the histones in C. albicans. Because C. albicans is a natural auxotroph for biotin, a storage function is attractive.

Materials and methods

Strains, media, and growth conditions

The clinical isolate of C. albicans SC5314 was provided by Dr. Alexander Johnson, University of California at San Francisco. Saccharomyces cerevisiae BHY10 was provided by Dr. Daniel Nickerson, University of Washington. The genome of C. albicans SC5314 has been sequenced, see the Candida Genome Database, http://www.candidagenome.org/ (Inglis et al., 2012).

YPD medium (10 g of yeast extract, 5 g of peptone, and 20 g of glucose L⁻¹) at 30 °C was used for growth and maintenance of C. albicans. For YPDB, the YPD was supplemented with 1.2 mg biotin L⁻¹. The defined glucose–phosphate–proline (GPP) and glucose–salts–biotin (GSB) media followed Hornby et al. (2001). Thus, the final biotin concentrations are 0, 4, 0.1, and 0.1 μM for YPD, YPDB, GPP, and GSB, respectively. Modified GSB (mGSB) was supplemented with 1% peptone. The biotin-starved cells were grown in defined media, either GS (15 g glucose, 2 g KH₂PO₄, 1 g (NH₄)₂SO₄, 0.1 g MgSO₄·7H₂O, 50 mg of CaCl₂·2H₂O L⁻¹ of double-distilled water, pH 5.6) or mGSB (15 g glucose, 10 g peptone, 2 g KH₂PO₄, 1 g (NH₄)₂SO₄, 0.1 g MgSO₄·7H₂O, 50 mg CaCl₂·2H₂O L⁻¹ of double-distilled water, pH 5.6).

Growth of biotin-deprived cells

SC5314 cells were grown overnight at 30 °C in 50 mL of YPD, whereupon the cells were harvested, washed 3× with PBS, and inoculated (OD₆₀₀ = 0.1) into biotin-free GPP, which had supplemented with either no additions, biotin (100 nM), or neutravidin (15 μg). The cultures were grown at 30 °C for 40 h with shaking at 225 rpm. Cell density was measured with a Klett colorimeter at 660 nm.

Total protein extraction

The procedure was as described by Atkin et al. (1995). An overnight culture of C. albicans SC5314 was inoculated into 40 mL of YPDB at 1 × 10⁷ cells mL⁻¹ and grown at 30 °C with shaking (200 rpm) to an OD₆₀₀ of 0.6. The cells were harvested at 3000 g for 10 min, washed at 4 °C in 10 mL of lysis buffer (5 mM EDTA, 250 mM NaCl, 0.1% NP-40, and 50 mM Tris pH 7.5). The pellet was resuspended in 0.4 mL of lysis buffer with a protease inhibitor cocktail P8215, Sigma, St. Louis), whereupon the cells were lysed by adding 0.1 mL of acid-washed glass beads (0.1 mm) and vortexing for 3 min at 4 °C. Following centrifugation at 3000 g for 20 min at 4 °C, the protein content of the supernatants was quantified by the Bradford assay, and samples (20 μg) were analyzed by glycine SDS-PAGE (4–20% BioRad TGX gels).

Histone extraction

Cells were grown in 10 mL of YPD medium overnight. Approximately 1 × 10⁷ cells from the overnight cultures were inoculated into 100 mL YPDB and grown to an OD₆₀₀ of 0.4–0.6. The cells were harvested at 3000 g for 5 min, washed twice in cold water, and the cell pellets (0.5 g) were resuspended in 1 mL of spheroplasting buffer [1 M sorbitol, 25 mM Tris-HCl (pH 7), 100 mM dithiothreitol (DTT), 10 mM PMSF (phenylmethanesulfonylfluoride), 25 mM EDTA, and 0.01%(v/v) β-mercaptoethanol] for 30 min at room temperature. The cells were then centrifuged at 3000 g, 4 °C for 10 min. Cell pellet was treated with 1 mL of spheroplasting buffer containing yeast cell-wall-degrading enzymes: 3.5 mg of zymolase for 3–4 h at 30 °C with gentle shaking. These cells were centrifuged at 850 g for 10 min at 4 °C, whereupon the pellets (spheroplasts) were treated...
with 1 mL of histone extraction buffer (0.25 M sucrose, 60 mM KCl, 3 mM MgCl2, 15 mM Pipes pH 6.8, 0.8% Triton X-100, and protease inhibitor cocktail), overnight at 4 °C with gentle rocking. The tubes were centrifuged at 9000 g in a microfuge at 4 °C for 20 min, whereupon the pellets were resuspended in 1 mL of 0.4 M H2SO4, and centrifuged at 13 000 g for 5 min. The supernatants were transferred to a new tube, and 12 volumes of cold acetone were added to precipitate the proteins overnight. The tubes were then centrifuged at 6000 g for 15 min, the pellets resuspended in 100 µL of 4 M urea, and protein content was determined using the Bradford assay.

Histones and total cell lysates, prepared as described by Atkin et al. (1995) were separated using glycine- or tricine SDS-PAGE and then either stained with Coomassie blue R-250 or subjected to Western blot analysis. The tricine SDS-PAGE (10%) gels were as described by Schagger (2006) except that only the cathode buffer was used for the running buffer.

Antibody dialysis and Western blotting

For Western blotting, the proteins were transferred to ice-cold 0.2-µm nitrocellulose membranes for 1 h at 150 volts. The membranes were blotted with an antibiotin mab conjugated to HRP (1 : 200 in TBST, A0185 Sigma, St. Louis) using the Super Signal chemiluminescent substrate (Thermo Scientific #34079) and Kodak Biomax XAR film. To ensure batch-to-batch reproducibility for the antibiotin antibody, any biotin already bound to the antibiotin antibody was removed by overnight dialysis at 4 °C in 3 kDa cutoff tubing against 1 L of 1 µM neutravidin (Thermo Scientific #31000) in TBST. The dialyzed antibody was then used directly in the Western blots. The Western for Fig. 2 was also washed twice with TBST and then reprobed with an anti-histone H3 pAb (1 : 1000 in TBST, Ab 1791 Abcam, Cambridge, MA) and an anti-rabbit HRP-coupled secondary antibody.

Analysis of intact histones by q-TOF mass spectroscopy

Analysis of intact histones was performed using a q-TOF mass spectrometer (Q star XL, Applied Biosystems) integrated with a UPLC (Accela UPLC, Thermo Fisher Scientific). The mass spectrometer was equipped with an electrospray ion source (ESI). The spray voltage was set to 5200. Data were acquired in positive ion mode and scanned from m/z 100–2000. Whole protein samples were diluted with HPLC-grade water (JT Baker) containing 0.1% formic acid (mobile phase A) to a final concentration of 150 µg µL⁻¹. The separation was carried out in reverse-phase mode employing C18 column (Hypersil gold, dimension, 50 × 3, particle size 3 microns, Thermo Fisher Scientific). The protein samples were loaded in mobile phase A and eluted by a linear zero to 95% gradient of acetonitrile (Sigma, St. Louis) containing 0.1% formic acid (mobile phase B) in 23 min followed by re-equilibrating the column with mobile phase A for a total running time of 30 min. Analyst QS software (Applied Biosystem) was used to generate the intact mass spectra for histones during elution and later deconvoluted using Mag-Tran deconvolution software for intact molecular weight determinations.

Analysis of biotinylation and other modifications by LC/MS/MS

For the LC/MS/MS experiments, in-gel trypsin digestion was performed using the protocol from Shevchenko et al. (2007). Figure 1 presents a flow sheet describing this histone sample preparation and analysis. The selected gel bands were excised, washed, destained, reduced with TCEP, and alkylated with iodoacetamide. The proteins were then digested in 50 mM ammonium bicarbonate (pH8) with trypsin and Glu-C at 4 °C for 45 min followed by 25 °C for 4 h and overnight digestion at 37 °C. The peptides were extracted and subjected to LC/MS/MS analysis.

Two LC/MS/MS protocols were employed. All earlier experiments, including experiments #1 and #2, used a fully automated, online one-dimensional LC/MS/MS with a 3000 Dionex nano LC system (Dionex) integrated with LCQ Fleet Ion Trap mass spectrometer (Thermo Fisher Scientific) equipped with a nano-source. The method included an online sample preconcentration and desalting step using a monolithic C18 trap column (Pep Map, 300 µm LD, 5 µm, 100A, 1 mm monolithic C18 column, Dionex USA). Loading and desalting of the sample on the trap column was conducted using a loading pump with mobile phase A (water plus 0.1% formic acid) at a flow rate of 40 µL min⁻¹. The desalted peptides were then eluted and separated on a C18 Pep Map column (Dionex 75 µm I.D × 15 cm, 3 µm, 100A, Dionex) with a 0–95% gradient of mobile phase B, acetonitrile plus 0.1% formic acid. The total run time was 90 min including 25-min re-equilibration at a flow rate of 300 µL min⁻¹. The eluted peptides were directly introduced into the mass spectrometer using a nanosource in online fashion. The LCQ fleet mass spectrometer was operated with the following parameters: nanospray voltage (2.0 kV), heated capillary temperature of 200 °C, full scan m/z range 400–2000. The LCQ was operated in a data-dependent mode with 35% collision energy for
collision-induced dissociation (CID). Note that this first protocol was for CID only.

When the more sensitive 3000 RSLC system, capable of both CID and ETD, became available, we switched to the second protocol for experiments #3 and 4. The extracted peptides were subjected to nanoLC/MS/MS analysis using LTQ Velos Pro ion trap integrated with ETD (Thermo Fisher Scientific) connected to a Dionex U3000 RSLC system with C18 trap (dionex monolithic) and PicoFrit 15-cm C18 nano analytical column (New Objective). All columns were packed in-house. The peptide samples in mobile phase A were loaded in the trap with an injection volume of 10 µL at a flow rate of 40 µL min⁻¹ using the loading pump. Elutions used the nanopump at a flow rate of 200 nL min⁻¹ with a 60-min run time: mobile phase A for 5 min, 0–95% mobile phase B for 30 min, mobile phase B for 10 min, and mobile phase A for 15 min to re-equilibrate before the next injection. All MS methods for the LTQ Velos Pro ETD were set up in the data-dependent acquisition mode. After the survey scan, the most intense precursors were selected for subsequent fragmentation using optimal settings for each activation technique. The normalized collision energy was set to 35% for CID, with an isolation width of 3.0 activation time of 10 ms with a default charge state of 4.0. Supplemental activation was enabled for ETD, and the activation time was set to 100 ms, isolation width of 3.0. The signal threshold for triggering an MS/MS event for both CID, and ETD was set to 500 counts. Dynamic exclusion was disabled in both.

The acquired MS/MS raw data were searched against C. albicans protein sequence database (NCBI) using MASCOT (Matrix Sciences, UK) bioinformatics software to identify the protein and further biotinylation analysis of peptides. This database uses gene sequences from C. albicans strain WO-1, not strain SC5314. However, the amino acid sequences for all the histones are identical for strains WO-1 and SC5314. The MASCOT search parameters are as follows for both CID and for ETD. Carbamidomethyl was used as the fixed modification and biotinylation (+226 Da) at residue K and other modifications as the variable modifications. Additional database searches were performed using MASCOT specifying the following post-translational modifications as variable modifications: Acetylation K (+42 Da), phosphorylation of serine, threonine, or tyrosine (+80 Da), and methylation K (+14 Da). ESI-TRAP was set as instrument for identifying CID fragments, and ESI-ETD was used for identifying ETD fragments. MS tolerance was set to 1.5, and ms/ms tolerance was set at 1.0 for both searches.

**Results**

*Candida albicans* protoplasts

Histone purification protocols generally require spheroplast formation prior to histone extraction. However, a standard protocol recommended for *S. cerevisiae* (Active Motif, Carlsbad, CA) gave only 15–20% spheroplasts with *C. albicans*. Accordingly, we first modified these proto-
cols, so that exponentially growing cultures of *C. albicans* gave ≥ 80% spheroplasts (Fig. 1). Key modifications included replacing 50 mM K$_2$PO$_4$ (pH 6.5) with 25 mM Tris-HCl (pH 7.0) and adding 25 mM EDTA, 10 mM PMSF, 0.1 M DTT, and 3.5 mg mL$^{-1}$ zymolyase to the spheroplasting buffer. We had previously employed very similar modifications for making protoplasts of *Ceratocystis ulmi* (McNeel et al., 1983).

**Histones are biotinylated in *C. albicans***

*Candida albicans* is a natural biotin auxotroph (Odds, 1988). Thus, we were curious whether *C. albicans* uses biotin to modify its histones and if so to identify and characterize those histones. For this purpose, we isolated histones from wild-type *C. albicans* SC5314 grown aerobically in a rich medium (YPDB) at 30 °C and 225 rpm. Biotin (the B in YPDB) is present at 4.9 μM. This is the same concentration of biotin as in the commonly used Lee’s medium (Lee et al., 1975). Western blot analyses (Fig. 2) were performed on 4–20% gels with both total cell extracts (lane A) and crude histone extracts (lane B) using an antibiotin HRP-conjugated monoclonal antibody, which had first been dialyzed in TBST buffer for 6–8 h at 4 °C against neutravidin to remove any residual-free biotin. Histone preparations (lane B) showed a broad band of biotinylation between 12 and 15 kDa with no other visible bands. The high-MW carboxylases, pyruvate carboxylase (129, 731 Da), and acetyl-CoA carboxylase (253, 392 Da), were only detected in the total cell extracts (lane A). In neither gel did we detect any bands near 43.6 kDa equivalent to Arc1p, the biotinylated tRNA-binding protein found in *S. cerevisiae* by Kim et al. (2004). The 12- to 15-kDa band visible in lanes A and B makes it unlikely that histone H1 is biotinylated, but it does not help distinguish among the remaining histones. However, the histone bands were much better resolved when we switched to tricine SDS gels (compare lanes D and E), and we detected biotinylated bands at both 11–12 and 14 kDa (lane D). When the membranes were stripped and reprobed with anti-histone H3 antibodies (lane C), the expected H3 band was observed at c. 15 kDa (lane C). Thus, it appears, based on the antibiotin Western blots, that only histones H2A/B and H4 were biotinylated. To confirm which histones are biotinylated, and where, we switched to mass spectrometry.

**Biotinylation of intact histones**

Using UPLC–ESI mass spectrometry, we were able to elute and identify some of the intact histones. Figure 3 shows the deconvoluted spectrum of the protein peak eluting at 33.5 min which is histone H4. Time-of-flight (TOF-MS) mass spectrometry detected both the unmodified histone H4 (11, 736.2 Da) and the doubly biotinylated histone H4 (12, 188.1 Da). The two peaks (Fig. 3) differ by 451.9 (2 × 226) Da, and the doubly biotinylated peak area is c. 5-times that of the unmodified histone H4. No singly biotinylated proteins were detected (Fig. 3). However, because of the deconvolution process, we cannot rule out the possible presence of singly or triply biotinylated H4 proteins. Similarly, we cannot reliably estimate the percentage of H4 proteins that have been biotinylated because a large percentage of those proteins could be acetylated, methylated, phosphorylated, or otherwise modified. We could not deconvolute any of the other histones clearly due to adduct formation and peak shouldering.

**Identification of biotinylated peptides**

In the next set of experiments (Fig. 4), nanoLC/MS/MS analysis was carried out to identify individual biotinylated histones and their sites of biotinylation. We used an
in-gel digestion strategy with trypsin (Roche; cleaves after K and R) to elucidate all the biotinylated peptides (Fig. 1). Database searches against C. albicans sequences using the MASCOT search engine identified peptides from histones H2A, H2B, H3, and H4, and the variable modification search (mass difference of 226) identified multiple biotinylated peptides from histones H2A, H2B, and H4 and a few from histone H3. In each case, the attachment was at a lysine side chain but in no case was the lysine part of an MKM sequence, as would be expected for biotinylation of a carboxylase (Samols et al., 1988). For instance, the search resulted in a histone H4 peptide, MSGTGRGKGGK, for which the stepwise fragmentation masses indicated biotins attached to both K8 (Fig. 4 lower) and K11 (Fig. 4 upper). Peptides were observed with a mass of 1260.6067 (one biotin) and

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1486.6837 (two biotins) where the nonbiotinylated peptide was 1034.5291. Thus, the mass difference per biotin was 226, illustrating biotinylation of the MSGTGRGKGGK peptides. Detecting singly biotinylated peptides is, of course, compatible with a multiply biotinylated histone.

**The biotinylated peptides from histones H2B and H4**

Peptide patterns from four biological replicates are presented for histones H2B (Table 1) and H4 (Table 2). For histone H2B experiment #1, LC/MS/MS detected positions K17, K18, and K31 as biotin attachment sites (Table 1). Because of the very minor sequence differences between histones H2B.1 and H2B.2, we cannot distinguish whether these biotinylated peptides came from H2B.1, H2B.2, or both. Reproducibility is the essence of science. However, when we conducted a biological replicate (experiment #2), the general picture was the same, but the particulars were different. Experiment #2 found that H2B was biotinylated at positions K8, K12, K18, K22, K32, and K90 (Table 1). This detection heterogeneity between biological replicates is often seen in mass spectrometric analysis of post-translation modifications. Likely causes include: loss of the modification following electron impact during the mass spectrometry, variation in abundance of the tryptic peptides containing the modified site, that is, poor peptide fragmentation and protein sequence coverage, suppression of ionization in the presence of other unmodified peptides, and reduced fragmentation efficiency during collision-induced dissociation (CID). Alternatively, our results are consistent with histone biotinylation occurring in a nonsite-specific or poorly site-specific manner.

This sequence of experiments was continued with a dual pressure ion trap LTQ Velos Pro LC/MS/MS. This instrument has much higher scan speed and c. 20-fold better sensitivity and coverage. Also, it uses two different methods of peptide fragmentation to create nontryptic peptides, collision-induced dissociation (CID), and electron transfer dissociation (ETD). Both methods can detect the same peptide multiple times, thus increasing the confidence level that peptide is in fact biotinylated. ETD fragmentation is better for detecting large peptides, which may have been missed by CID. The biotinylated peptides from histone H2B detected in CID and ETD mode are shown in Table 1 experiments #3 and #4, respectively. Using the same protocols, eight biotinylated peptides were detected from histone H4 (Table 2). These peptides had biotins attached to the lysine side chains for K8, K11, K15, K19, K47, K62, K82, and K94. Again, each of the four experiments detected histone H4 biotinylated peptides, but they were different peptides with different positions for biotinylation (Table 2).

**Table 1.** Sequence of Histone H2B (Accession #CAWT_02708). The biotinylated peptides are observed after trypsin digestion followed by nanoLC/MS/MS analysis. The sites of biotinylation are in bold. The sequence is for H2B.2 (Ca.19.6925). H2B.1 (Ca 19.1052) differs in only two amino acids, T79S and S130N.

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<th>Peptide</th>
<th>Biotinylated residue</th>
<th>Protein</th>
<th>MW of peptide + biotin</th>
<th>No. of biotinylations</th>
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*Number of times this biotinylated peptide was detected.
We were both intrigued and perplexed by how many of the biotinylated lysines occurred at the C-terminus of the peptide detected, 10 of 12 for histone H2B (Table 1) and 5 of 7 for histone H4 (Table 2). Trypsin does not usually cut at modified lysines (Klotz, 1967), and in the few cases studied, it did not cut at biotinylated lysines (Guo et al., 2001; Lehman et al., 2008). We can only suggest that the tryptic peptides are further fragmented during the collision-induced dissociation and that having a lysine side chain biotinylated somehow weakens the peptide bond on the carboxyl side of that lysine.

**Total histone modifications in biotin-starved and biotin-enriched C. albicans cultures**

The MASCOT search engine was also used to identify modified H2A/B and H4 peptides that had been acetylated, methylated, and phosphorylated (Table 3). Biotin-replete yeast cells were inoculated into defined GPP medium with biotin either omitted (Table 3A) or supplemented (Table 3B). Extensive modification by acetylation, methylation, and phosphorylation was detected for both growth conditions, and the extent of acetylation, methylation, and phosphorylation was roughly equivalent for the biotin-starved and biotin-supplemented cells. However, biotinylation was only found in the biotin-replete cultures.

**No biotinylation of histones in S. cerevisiae or with biotin-starved C. albicans**

Western blotting with an antibiotin monoclonal antibody was used to confirm the absence of histone biotinylation in biotin-starved cells (Fig. 5). Histones were extracted from C. albicans SC5314 cells that had been grown in two other defined media without biotin, GS, and mGS. There was no histone biotinylation with either of the biotin-starved cells (Fig. 5 lanes G and H), whereas histone biotinylation was readily detected in the cells grown with biotin (lanes E and F). Similarly, when histones were extracted from S. cerevisiae strain BHY10, there were no detectable bands (Fig. 5 lanes A and C), whereas histone biotinylation was readily detected for C. albicans cells grown in the same media (lanes B and D). Finally, nanoLC/MS/MS analysis of trypsin-digested histones from S. cerevisiae did not detect any biotinylation peptides (date not shown).

**Cell growth with and without added biotin**

*Candida albicans* is a biotin auxotroph (Odds, 1988), but cultures of *C. albicans* can achieve c. 60% of their normal
cell yield when grown without added biotin (Fig. 6), even with neutravidin (15 μg/50 mL) present in the growth medium (Fig. 6). This suggestion of an internal biotin reservoir was confirmed by two further cycles of growth in biotin-deficient media, which achieved 40% and 20% of the normal cell yield, respectively (data not shown). These results were obtained with C. albicans SC5314, but we previously obtained very similar data with C. albicans A72, for which the cell yield with biotin-deficient GS was 60% of that with GSB (K.W. Nickerson and P.A. Sullivan, unpublished data).

**Discussion**

In this report, we show a novel post-translational histone modification in that the histones from C. albicans are biotinylated. Histone biotinylation was detected by TOF-MS, LC/MS/MS (both CID and ETD), and reactivity with an antibiotin antibody in Western blots. For antibody detection, we routinely dialyzed the antibody against neutravidin prior to use. Biotin targets ε-amino groups of lysine side chains of protein molecules (Zempleni, 2005). Lysines at K8, K11, K15, K19, K47, K62, K82, and K94 were biotinylated in histone H4 (Table 2) as well as K8, K12, K17, K18, K22, K31, K32, K50, and K90 in histone H2B (Table 1). Thus, 9 of the 21 lysines in H2B and 7 of the 11 lysines in H4 can be biotinylated. It is likely that further experiments would have extended the coverage to include further lysine side chains. There appears to be little if any specificity with regard to which lysine side chains are biotinylated, and there are no MKM sequences.

It is unlikely that histone H1 is biotinylated because the broad western band (Fig. 2) covers only 11–15 kDa, whereas histone H1 is 18.47 kDa. For histone H4, both TOF-MS analysis of the intact protein (Fig. 3) and LC/MS/MS analysis of the peptide fragments (Fig. 4) indicated that a high percentage of the H4 protein chains were biotinylated, but we could not quantify the percent biotinylation more precisely. However, Table 3B indicates that 8 of the 29 peptides from histones H2A/B and H4 were biotinylated. This value of 28% is, of course, only a very rough estimate, but it is clearly far higher than the situation in humans where ≤0.01% of the histones are biotinylated (Stanley et al., 2001). This disparity suggests that histone biotinylation may serve different functions in humans and C. albicans (Kothapalli et al., 2005) and accentuates the fact that we do not yet know the physiological function of histone biotinylation in C. albicans.

Because histone biotinylation is clearly optional (Fig. 5), one possible role for histone biotinylation is to serve as a storage reservoir for scarce biotin. Such a storage role would not rule out any other gene regulatory roles. The evidence supporting a biotin storage role includes: (A) C. albicans is a biotin auxotroph (Odds, 1988) as are many strains of S. cerevisiae (Hall & Dietrich, 2007). According to the Saccharomyces Genome Database, c. 50 000 protein carboxylases (acetyl-CoA carboxylase and pyruvate carboxylases 1 and 2) are present per cell, and we assume that equivalent levels are needed for C. albicans. Thus, a biotin reservoir would be useful. (B) None of the histones in C. albicans contains an MKM sequence, and at present, we have no evidence...
that specific lysines need to be biotinylated. (C) *Candida albicans* achieved c. 60% of its normal cell yield when grown in biotin-free media both with and without added neutavidin (Fig. 6). Maintaining 60–70% of the biotin-free cell yields even with added neutavidin is consistent with an internal source of stored biotin. (D) *Candida albicans* exhibits equivalent cell growth in defined GPP media with biotin levels ranging from 10 to 5000 nM (data not shown). In contrast, biotinylated histones were found when the cells were grown with 4 μM biotin (Table 3B and Fig. 5E and F) but not when the cells were grown without added biotin or with only 100 nM added biotin (Table 3A and Fig. 5G and H). Thus, excess biotin is needed for histone biotinylation. (E) Taken together, Figs 5 and 6 comprise a one-step kinetic experiment in which the inoculated cells have abundant biotinylated histones, but they have disappeared following 24-hr growth in biotin-free media. (F) Growth in biotin-free media leads to the disappearance of biotinylated histones (Table 3A), but the levels of acetylated, methylated, and phosphorylated histones remained unchanged (Table 3A). (G) Biotinylation of histones is not seen in *S. cerevisiae*, a close relative of *C. albicans*, J. Zempleni (pers. commun.) and our data on strain BHY10 (Fig. 5A and C). Instead, we suggest that *S. cerevisiae* uses Arc1p in similar storage capacity. Kim et al. (2004) demonstrated that: (1) biotinylation of Arc1p was mediated by yeast biotin protein ligase Bpl1p; (2) the extent of biotinylation increased with BPL1 overexpression; (3) Arc1p lacks the MKM consensus sequence for carboxylase biotinylation (Samols et al., 1988); and (4) biotinylation of Arc1p was not essential for its activity. A storage role for the biotinylation of Arc1p (YGL105W) would be consistent with its high copy number of 57,700 per cell (Ghaemmaghami et al., 2003).

We have shown that *C. albicans* contains biotinylated histones and that this biotinylation occurs primarily on histones H2A, H2B, and H4 when the cells are grown on media, which contains excess (4–5 μM) biotin. Further study of these modifications is required to find the physiological and pathogenic significance of histone biotinylation in *C. albicans*. With regard to the physiological importance of biotinylation, we believe that a storage function is part of the story. Our future research will have four directions. First, more accurately quantifying the percentage of histone molecules that are biotinylated. Second, identifying whether those biotinylated histones are cytoplasmic or nuclear or both. Third, determining whether biotin protein ligase (Bpl1p), the enzyme that biotinylates all carboxylases as well as Arc1p in *S. cerevisiae*, also modifies the histones in *C. albicans*. Significantly, Bpl1p from *C. albicans* is only 59% similar to the *S. cerevisiae* enzyme, even though Bpl1p is considered to be a highly conserved enzyme (Pendini et al., 2008). And fourth, determining whether histone biotinylation is involved in any of the developmental changes exhibited by *C. albicans*, for example, yeast-mycelial dimorphism, chlamydospore formation, white-opaque switching, biofilm formation, or the multicellular structure that extends aerially from colony surfaces, which is called the ‘finger’ (Daniels et al., 2012).

**Acknowledgements**

We thank Janos Zempleni and Zoya Avranova for helpful suggestions and discussions. This work was supported by Ann L. Kelsall and the Farnesol and *Candida albicans* Research Fund, University of Nebraska Foundation.

**References**


