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Six lysine residues on c-Myc are direct substrates for acetylation by p300

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Abstract

The c-Myc oncoprotein (Myc) functions as a transcription regulator in association with an obligatory partner, Max, to control cell growth and differentiation. The Myc:Max complex regulates specific genes by recognizing "E-box" DNA sequences and promoter-bound factors such as Miz-1. Myc recruits histone acetyltransferases (HATs) to modify chromatin and is, itself, acetylated in mammalian cells by several of these HATs including p300/CBP, GCN5, and Tip60. The Myc residues that are directly modified by these different HATs remain unknown. Here, we have analyzed the acetylation of recombinant Myc:Max complexes by purified p300 HAT in vitro by using MALDI-TOF and LC-ESI-MS/MS mass spectrometry. These analyses identify six lysine residues in human Myc (K143, K157, K275, K317, K323, and K371) as direct substrates for p300. Our results further indicate that p300 can acetylate DNA-bound Myc:Max complexes and that acetylated Myc:Max heterodimers efficiently interact with Miz-1.

Keywords: Acetylation; Mass spectrometry; c-Myc; Histone acetyltransferase; p300

Myc regulates cell growth, proliferation, and cell death, and is essential for embryonic development. Myc overexpression is oncogenic and its deregulated expression has been associated with many types of cancer. Myc regulates specific gene transcription in association with an obligatory protein partner called Max. Myc and Max heterodimerize and bind to specific E box DNA sequences through their bHLHZip domains [1]. While Max lacks a transcription regulatory domain, Myc has an N-terminal transcription activation domain (TAD), also involved in transcription repression, which recruits the HATs GCN5 [2,3] and Tip60 [4] as part of the distinct multiprotein complexes STAGA [5,6] and TIP60 [7], respectively. Thus, Myc appears to activate the transcription of target genes in part by increasing acetylation of chromatin. Myc also binds to promoters that lack E-box elements through protein-protein interactions with other sequence-specific transcription factors such as Miz-1. Myc binding to Miz-1 requires residues within its bHLHZip domain that are "exposed" in Myc:Max heterodimers and results in transcription repression of the cell cycle arrest genes *p15/INK4B* and *p21/CIP1* [8–10].

Recently, GCN5, Tip60, and p300/CBP have been shown to acetylate Myc and to regulate its turnover in mammalian cells ([11,12] and F. Faiola and E. Martinez, unpublished). However, the residues on Myc that are direct substrates for these different HATs remain to be identified and whether acetylation affects specific functions of the Myc:Max complex remains unclear. In this study, we use mass spectrometry to map Myc residues that are direct substrates for p300-mediated acetylation in vitro. Our analyses also suggest that p300 can acetylate DNA-bound Myc:Max complexes and that acetylation might not affect Myc:Max association with specific promoters.

Materials and methods

Recombinant proteins and acetylation assays in vitro. Recombinant human Myc:Max complexes were reconstituted as described previously [13]. Flag-p300 HAT domain (p300-HAT) was expressed in BL21-

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CodonPlus(DE3)-RIL bacteria (Stratagene) and affinity-purified with anti-Flag M2 agarose (Sigma) as described [5]. Both Flag peptide-eluted (soluble) p300-HAT and M2 agarose-bound p300-HAT (p300 resin) were used where indicated. Recombinant p300 (full-length) was a gift of Dr. Elena Lymar. In vitro acetylation reactions were performed with the indicated amounts of Myc:Max and Max:Max complexes and p300-HAT, and 5 μM [^{3}H]acetyl-CoA (3.8 Ci/mmol, 0.25 mCi/ml, Amersham) in HAT buffer (50 mM Tris–HCl, pH 8.0, 14% glycerol, 70 mM KCl, 0.2 mg/ml BSA, 0.09% NP-40, 10 mM sodium butyrate, 0.14 mM EDTA, 8 mM 2-mercaptoethanol, and 0.3 mM PMSF) for 1 h at 30 °C and analyzed by SDS–PAGE, Coomassie staining, and fluorography [5].

Mass spectrometry. Myc:Max complex (4 μg) was incubated with 550 ng of immobilized p300-HAT and 50 μM acetyl-CoA (Amersham) in HAT buffer (without BSA), for 1 h at 30 °C with constant mixing. The p300 resin was spun down and the supernatant was incubated with fresh p300 resin and acetyl-CoA, as above. This step was repeated once more, the resin was then removed by centrifugation, and acetylated Myc and Max were resolved by SDS–PAGE and stained with Colloidal blue (Invitrogen). Myc protein bands were cut from the gel, minced into small pieces, destained with 25 mM NH_4HCO_3 in 50% CH_3OH, incubated with CH_3OH (45%) and acetic acid (10%) for 6 h, washed twice for 15 min each with H_2O and once for 30 min in 50 mM NH_4HCO_3, dried in a Speed-Vac, resuspended in 25 mM NH_4HCO_3, and digested with trypsin for 6–9 h at 37 °C. Tryptic peptides were extracted twice with 50–100 μl of 100% acetonitrile, dried in a Speed-Vac, dissolved in 3 μl of 0.1% TFA, and resolved by reverse-phase HPLC (Agilent, Waldbronn, Germany) chromatography at 6 μl per minute with a gradient from 2 to 65% mobile-phase B over 65 min, 65% B for 10 min, and 65% B to 90% B over 10 min. Mobile-phase A was 0.1% TFA in water and mobile-phase B was 0.1% TFA in acetonitrile. Peptide fractions were collected in 0.5 ml siliconized Eppentorf tubes. For MALDI-TOF analysis, 0.5 μl peptide fraction was mixed with 0.5 μl of α-cyano-4-hydroxycinnamic acid matrix solution (5 mg in a solution containing 500 μl acetonitrile, 500 μl water, and 1 μl formic acid). Monoisotopic masses of all peptides were measured by MALDI using a voyager DE-STR Biospectrometry Workstation (ABI Biosystems) with delayed extraction operating in the reflection mode. High-accuracy mass measurements of acetylated peptides were carried out by re-calibration of the spectrum using the known theoretic mono-isotopic masses of Myc tryptic peptides. For nano-ESI tandem mass spectrometric analyses (ESI-MS/MS), HPLC fractions containing acetylated peptides were analyzed on a QTOF-Ultima-Global mass spectrometer (Micromass, UK) with an external nano-electrospray ion source. Mass accuracy was at least 0.02 Da with external calibration. Conventional mass spectra were obtained to measure mass values of precursor ions and to assign their charge states from stable isotope spacing before selection and analysis by MS/MS.

DNA-binding and GST pull-down assays. DNA-binding and electrophoretic mobility shift assays (EMSA) were essentially as described previously [13]. The vector pGEX4T1-Miz-1(637–803) was a gift from Dr. John Capone. Immobilized GST-Miz-1(637–803) (15 μg) was incubated for 3–5 h at 4 °C with Myc:Max or Max:Max (2 μg) in buffer BC100 (20 mM Tris–HCl, pH 7.9, 20% glycerol, 100 mM KCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.2 mM PMSF, and 0.1% NP-40). Bound complexes were washed extensively with BC400 (same as above but with 400 mM KCl), and bound proteins were analyzed by SDS–PAGE and fluorography or by Western blot with anti-Myc (N-262) and anti-Max (C-17) antibodies (Santa Cruz Biotech.).

Results and discussion

Identification of p300-acetylated Myc residues by MALDI-TOF and ESI-MS/MS

To identify Myc residues that are direct substrates for acetylation by p300, a recombinant Myc:Max complex (Fig. 1A, lane 1) was used in acetylation assays in vitro with soluble and immobilized recombinant Flag-tagged human p300 HAT domain (p300-HAT and p300 resin, respectively, lanes 2 and 3). As shown in Fig. 1B, similar amounts of soluble and immobilized p300-HAT acetylated Myc to a similar extent (right panel, fluorography, lanes 3 and 5) and Max was also acetylated, consistent with our recent findings (F. Faiola and E. Martinez, unpublished). As expected, p300-HAT was auto-acetylated (lanes 3–7). For mass spectrometric analyses, Myc:Max complexes were acetylated with p300-HAT immobilized on M2 agarose, the p300 resin was then removed by centrifugation and acetylated Myc proteins were isolated from a SDS–PAGE

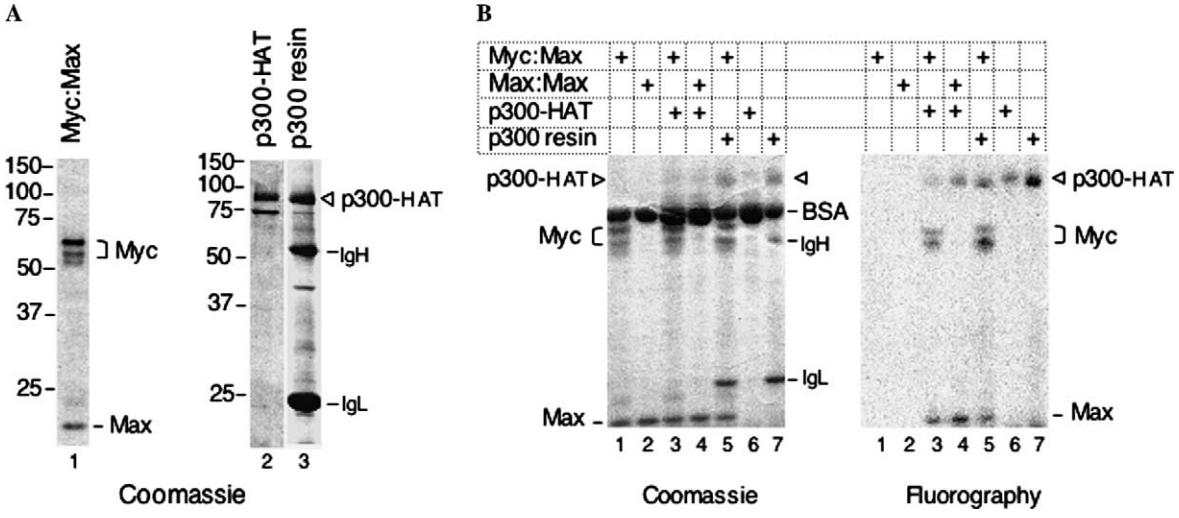


Fig. 1. Acetylation of recombinant Myc:Max complex by p300-HAT in vitro. (A) Coomassie-stained SDS–PAGE gels of recombinant Myc:Max (lane 1), soluble p300-HAT domain (lane 2), and p300-HAT immobilized on anti-Flag M2 agarose (p300 resin, lane 3). IgH and IgL indicate the heavy and light chains of the anti-Flag monoclonal antibody. (B) Acetylation of Myc:Max and Max:Max complexes (4 pmol) by p300-HAT and p300 resin (about 125 ng each) in the presence of [^{3}H]acetyl-CoA, as indicated. Left panel: Coomassie-stained SDS–PAGE gel; right panel: fluorographic image of the same gel indicating acetylated p300-HAT, Myc, and Max.

gel, digested with trypsin, and tryptic peptides were purified by reverse-phase HPLC chromatography, as described in Materials and methods. A total of 14 HPLC fractions were collected and each fraction was first analyzed by MALDI-TOF mass spectrometry. A total of 34 Myc tryptic peptides were detected in the 14 fractions (Fig. 2) which covered about 46% of the protein and included 17 of the 25 lysine residues present in human Myc (Fig. 3A). Significantly, 7 peptides had a mass that suggested acetylation at six lysine residues (Figs. 2 and 3A, and see below).

A high accuracy method for mass measurement by MALDI-TOF mass spectrometry [14] was used to further analyze the identified Myc acetylated peptides. The spectra of the above peptides containing tentatively acetylated Myc lysines were calibrated with the calculated monoisotopic masses of the co-eluted non-acetylated tryptic peptides. This allowed high accuracy mass determination of the acetylated tryptic peptides with less than 5 ppm deviation from their theoretical masses (Fig. 3B) and confirmed acetylation of six lysine residues: K143, K157, K275, K317, K323, and K371 (Figs. 3A and B).

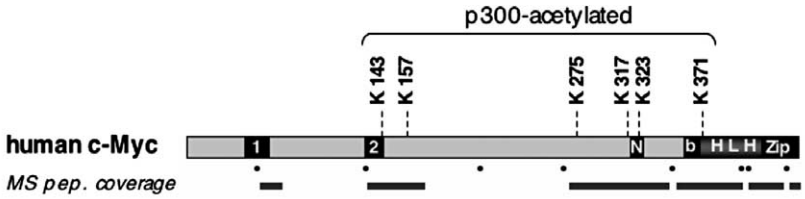
Acetylation of K157 and K323 was also verified by tandem mass spectrometry (ESI-MS/MS). An HPLC fraction containing a double-charged precursor ion at m/z 535.8 equivalent to the single-charged ion at m/z 1070.5 observed in MALDI (peptide 21 in Fig. 2), was analyzed by MS/MS. The MS/MS spectrum of this precursor ion (Fig. 4) shows a signature immonium ion at m/z 126.1 indicating an acetylated lysine [15] and a series of C-terminal and N-terminal fragmentation ions (“y” and “a, b” ions), and internal fragmentation ions, which correspond to the peptide KacDSGSPNPAR having an acetylated lysine residue at the N-terminus (i.e., K157). Similarly, an HPLC fraction containing a double-charged precursor ion at m/z 495.8, which is equivalent to the single-charged ion at m/z 990.5 observed in MALDI (peptide 16 in Fig. 2), was analyzed by MS/MS. The series of “y” ions matched the peptide sequence KDYPAAKacR with a mass increment of 42 U at lysine 323 indicating acetylation at this site (Fig. 5).

These results demonstrate that Myc is directly acetylated by p300 at six lysine residues, of which five are within or in close proximity to functionally important Myc domains (Fig. 3A). These include (i) K143 and K157 located next to the essential Myc box 2 (MB2), which is required for most transcription regulatory functions of Myc and for Myc protein destabilization, (ii) K317 and K323 that are

Peptide	Mass	Residues	Sequence
1	659.4 (M)	368-372	NELKR
2	698.4 (M)	271-276	QAPG**Kac**R
3	701.4 (M)	368-372	NEL**Kac**R
4	714.4 (M)	335-340	pyro-QISNNR
5	731.4 (M)	335-340	QISNNR
6	740.4 (M)	373-378	SFFALR
7	792.4 (M)	317-323	KDYPAAK
8	816.5 (M)	325-331	VKLDSVR
9	820.4 (M)	318-324	DYPAAKR
10	862.4 (M)	318-324	DYPAA**Kac**R
11	868.5 (M)	358-364	THNVLER
12	879.5 (M)	149-156	LASYQAAR
13	900.4 (M)	158-166	DSGSPNPAR
14	923.5 (M)	429-435	HKLEQLR
15	926.5 (M)	365-371	pyro-QRRNELK
16	990.5 (M)	317-324	KDYPAA**Kac**R
17	1008.4 (M)	347-355	SSDTEENVK
18	1028.4 (M)	157-166	KDSGSPNPAR
19	1024.6 (M)	357-364	RTHNVLER
20	1032.5 (M)	317-324	**Kac**DYPAA**Kac**R
21	1070.5 (M)	157-166	**Kac**DSGSPNPAR
22	1087.6 (M)	413-421	LISEEDLLR
23	1099.6 (M)	382-340	VLRQISNNR
24	1180.7 (M)	356-364	RRTHNVLER
25	1215.7 (M)	413-422	LISEEDLLRK
26	1437.8 (M)	53-65	FELLPTPPPSPSR
27	1550.8 (M)	399-412	ATAYILSVQAEEQK
28	1552.8 (M)	379-392	DQIPELGNNEKAPK
29	1565.9 (M)	52-65	KFELLPTPPPSPSR
30	1722.0 (M)	52-66	KFELLPTPPPSPSRR
31	1976.9 (M)	300-316	CHVSTHQHNYAAPPSTR
32	2156.1 (M)	277-298	SESGSPSAGGHSKPPHSPLVLK
33	2274.2 (M)	373-392	SFFALRDQIPELGNNEKAPK
34	2540.2 (E)	127-148	NIIIQDCMWSGFSAAA**Kac**LVSEK

Fig. 2. Peptides identified by MALDI-TOF (M) mass spectrometry. For each HPLC-purified peptide, the measured mass, position in human c-Myc sequence (residues), and the amino acid sequence are shown. Kac indicates an acetylated lysine and (E) is a peptide identified by ESI.

A



B

Measured MH^+	Calculated MH^+	Error ppm	Peptide Sequence	Kac position
698.3941	698.3949	-1.1	QAPG$\mathbf{K_{ac}}$R	**275**
701.3973	701.3946	3.8	NEL$\mathbf{K_{ac}}$R	**371**
862.4445	862.4423	2.7	DYPAA$\mathbf{K_{ac}}$R	**323**
990.5357	990.5373	-1.6	KDYPAA$\mathbf{K_{ac}}$R	**323**
1032.5430	1032.5478	-4.6	$\mathbf{K_{ac}}$DYPAA$\mathbf{K_{ac}}$R	**317, 323**
1070.5209	1070.5231	-2.1	$\mathbf{K_{ac}}$DSGSPNPAR	**157**
2540.2544	2540.2529	0.6	NIIIQDCMWSGFSAAA$\mathbf{K_{ac}}$LVSEK	**143**

Fig. 3. (A) Diagram illustrating the location of p300-acetylated Myc lysine residues. Myc box 1 (1), Myc box 2 (2), a nuclear localization signal (N), and the bHLHZip domain are indicated. Below the Myc structure: dots (·) represent lysine residues not covered by MS peptides and gray bars are sequences covered by MS peptides (MS pep. coverage). (B) High accuracy MALDI-TOF mass measurement of acetylated peptides (see text for details). Measured and theoretical (calculated) monoisotopic ion (MH+) masses are shown. Acetylated lysines (K_{ac}) are in bold.

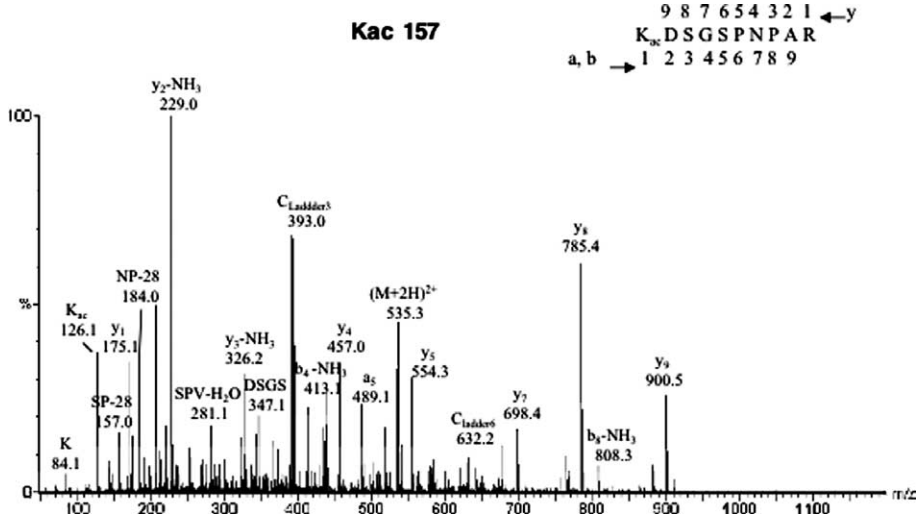


Fig. 4. The MS/MS spectrum of the double-charged precursor ion at *m/z* of 535.3 corresponds to the acetylated peptide KacDSGPNPAR. C-terminal fragmentation "y" ions, at *m/z* 175.1 (y1), 229.0 (y2-NH_3), 326.2 (y3-NH_3), 457.0 (y4), 554.3 (y5), 698.4 (y7), 785.4 (y8), 900.5 (y9), and the internal fragmentation ions at *m/z* 157.0 (SP-28), 184.0 (NP-28), 281.1 (SPV-H_2O), and 347.1 (DSGS), are indicated and correspond to the peptide DSGSPNPAR whose isotopic mass is 170 units less than the precursor ion. This indicates the presence of an acetylated lysine at the N-terminus of the precursor ion and is confirmed by ions at *m/z* 413.1 (b4-NH_3), 489.1 (a5), and 808.3 (b8-NH_3). A signature immonium ion at *m/z* 126.1 is indicative of an acetylated lysine.

next to and within, respectively, a nuclear localization signal, and (iii) K371 which is located within the bHLH domain and directly contacts DNA in the Myc:Max:DNA ternary complex solved by X-ray crystallography [16]. The other acetylated lysine, K275, is located in a Myc region that has no known function at this time.

DNA-binding does not interfere with p300-mediated acetylation

To address whether acetylation of Myc:Max by p300 is influenced by pre-binding of the complex to DNA, recombinant Myc:Max was pre-incubated with an excess of a

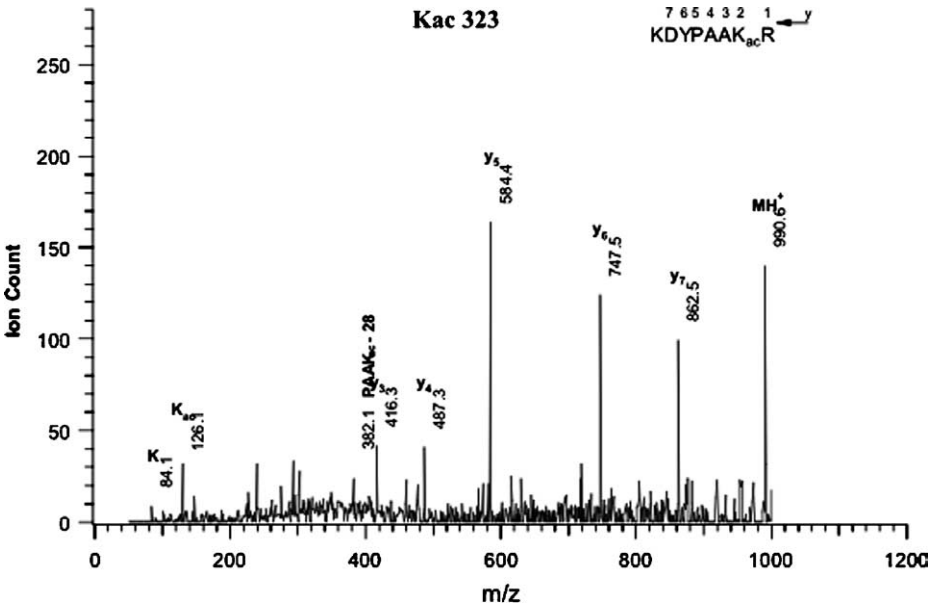


Fig. 5. The MS/MS spectrum of the double-charged precursor ion at m/z of 495.8 indicates single acetylation at K323 within the peptide KDYPAAKacR. The C-terminal fragmentation "y" ions, at m/z 416.3 (y_3), 487.3 (y_4), 584.4 (y_5), 747.5 (y_6), 862.5 (y_7), are all 42 Da heavier than the fragmentation ions produced from the peptide sequence DYPAA^{323}KR, indicating acetylation of lysine 323. Consistent with this, a signature ion at m/z 126.1 and an internal fragmentation ion at m/z 382.1 corresponding to the sequence PAAKac lacking a C=O group are also detected.

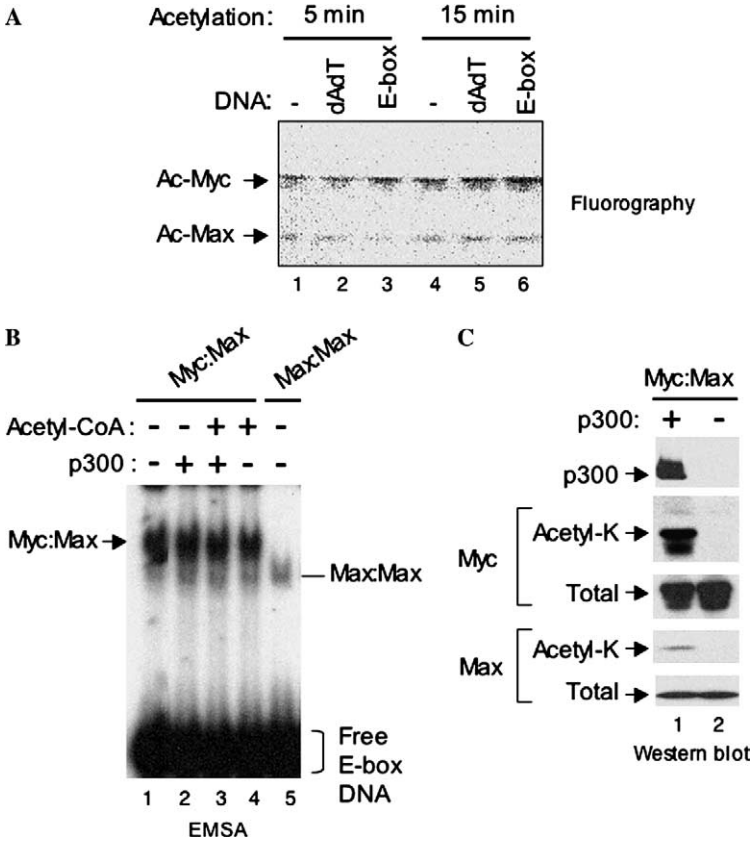


Fig. 6. DNA binding does not affect p300-mediated acetylation. (A) DNA-binding reactions were performed with Myc:Max (1 pmol) in the absence of DNA (−) and with either 150 ng poly(dA–dT):(dA–dT) or 150 ng E-box oligonucleotide, as indicated; p300 (15 ng) was then added and acetylation was performed in the presence of [^{3}H]acetyl-CoA for 5 min (lanes 1–3) and 15 min (lanes 4–6), and then analyzed by SDS–PAGE and fluorography. Acetylated Myc and Max proteins are indicated. (B) Myc:Max (1.6 pmol) was pre-acetylated in the absence and presence of p300 (50 ng) and acetyl-CoA (50 μM), as indicated, and 10% of each reaction was used in DNA-binding assays with a radiolabeled E-box oligonucleotide and analyzed by EMSA (lanes 1–4). Max:Max (0.04 pmol) was analyzed in parallel (lane 5). (C) The remaining 90% of the reactions used in lanes 3 and 4 of (B) were analyzed by Western blot (lanes 1 and 2, respectively) with an anti-acetyl-lysine (Acetyl-K) polyclonal antibody (Cell Signaling)."Total" indicates probing with antibodies to Myc (C-33, Santa Cruz Biotech) and Max (clone 2.6, BD Bioscience), as indicated.

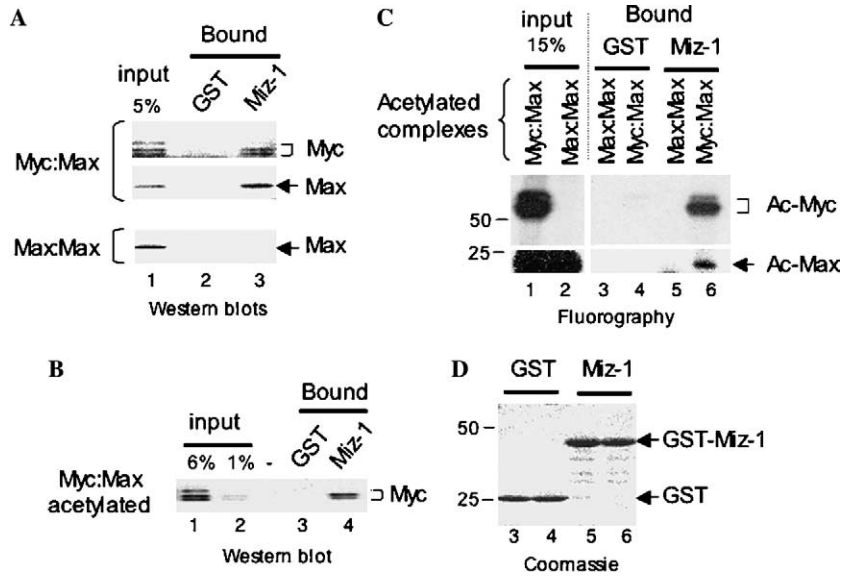


Fig. 7. Acetylated Myc:Max interacts with Miz-1 in vitro. (A) GST pull-down assay with GST (lane 2) and GST-Miz-1 (lane 3) resins and unacetylated Myc:Max (top panel) and Max:Max (bottom). Input (5%, lane 1) and resin-bound complexes (lanes 2 and 3) were analyzed by Western blot with specific antibodies. (B) GST pull-down assay as in (A) but the input was Myc:Max complex (2 μg) pre-acetylated with p300 resin (1 μg). Only Myc is shown. (C) GST pull-down as in (B) with [^{3}H]acetylated Myc:Max and Max:Max complexes and bound proteins were analyzed by fluorography. Acetylated Myc (Ac-Myc) and Max (Ac-Max) are indicated. (D) Coomassie-stained SDS–PAGE gel used for fluorography in (C) lanes 3–6. GST and GST-Miz-1 proteins are indicated.

consensus E-box oligonucleotide, which saturates the binding reaction under the conditions used (data not shown), and then acetylated in the presence of p300 and radio-labeled acetyl-CoA. As shown in Fig. 6A, pre-binding to the E-box DNA did not affect p300-mediated acetylation of either Myc or Max (compare lanes 1 and 2 with 3, and lanes 4 and 5 with 6). These observations suggest that Myc:Max could potentially be acetylated by p300 when bound to regulatory DNA sequences on specific target promoters. We also performed EMSA experiments to analyze the DNA-binding activity of recombinant Myc:Max complexes that were pre-acetylated by p300 in vitro (Fig. 6B). Western blot analyses with an antibody that specifically recognizes acetylated lysine residues verified that both Myc and Max were acetylated by p300 (Fig. 6C, lane 1). However, as shown in Fig. 6A, there was no detectable effect of acetylation on the E-box DNA binding activity of Myc:Max. While, it is possible that the DNA-binding lysine residue K371 is not a major substrate for acetylation under our in vitro conditions (and thus the role of its acetylation on DNA-binding cannot be addressed by this assay), this result is consistent with the above observation that DNA binding does not affect p300 acetylation and suggests that acetylation of Myc:Max at sites other than K371 may be compatible with DNA-binding.

Acetylated Myc:Max efficiently interacts with Miz-1

To further test whether Myc:Max acetylation might affect its promoter targeting functions independently of direct DNA-binding, we analyzed the ability of acetylated Myc:Max to interact with Miz-1, a gene-specific transcription factor that recruits the Myc:Max complex to certain promoters repressed by Myc (see Introduction). We analyzed the direct binding of Myc:Max to Miz-1 in vitro by the GST pull-down assay. As expected, recombinant Myc:Max, but not Max:Max, interacted with a GST-Miz-1 fusion protein (Fig. 7A, lane 3) and no interaction was observed with the GST control resin (lane 2). The specific interaction of Myc:Max with GST-Miz-1 was retained after acetylation of the Myc:Max complex with p300-HAT (Fig. 7B, lane 4). The binding of acetylated Myc:Max to GST-Miz-1 was verified more directly by using radio-labeled acetyl-Myc:Max obtained by p300-mediated acetylation in the presence of [^{3}H]acetyl-CoA. As shown in Fig. 7C, [^{3}H]acetyl-Myc:Max bound to GST-Miz-1 (lane 6) while, as expected, acetylated Max:Max did not (lane 5). About 5% of the acetylated Myc:Max bound to GST-Miz-1, which is similar to the binding efficiency of unacetylated Myc:Max under the same conditions (see Fig. 7A). These results suggest the possibility that p300-acetylated Myc:Max complexes may associate with Miz-1 on specific target promoters.

Concluding remarks

In this study, we have provided the first mapping of Myc residues that are direct substrates for acetylation by p300. We demonstrate that purified p300 directly acetylates Myc within a recombinant Myc:Max heterodimer at six lysine residues in vitro. These residues in human (and mouse) Myc are: K143(K144), K157(K158), K275, K317, K323,

and K371 (human and mouse coordinates differ only at the N-terminus). In cultured cells Myc is acetylated by overexpressed CBP [11] and mutagenesis studies indicate that maximal Myc acetylation induced by p300 in human cells requires four of the six lysine residues identified here—i.e., K143(144), K157(158), K317, and K323 (F. Faiola, X. Liu, and E. Martinez, unpublished). Thus, the acetylated residues identified here include the major acetylation sites targeted by p300/CBP in vivo. Interestingly, GCN5-induced acetylation of murine Myc in cultured cells involves K323 (a common target with p300) and K417, a GCN5-specific target [12]. This raises the interesting possibility that Myc acetylation by different HATs might occur at overlapping but also distinct residues and could influence Myc functions differently. Note however that it remains unclear whether K323 is indeed a direct substrate for GCN5 or whether acetylation at this site is an indirect effect of GCN5 overexpression in cultured cells.

A role for acetylation in modulation of Myc protein turnover has been reported [11,12]. Whether acetylation also regulates Myc transcription functions more directly remains unclear. We have shown that p300-acetylated Myc:Max complexes can interact with Miz-1 and that DNA-binding does not interfere with most Myc:Max acetylation by p300 in vitro. Since p300/CBP is recruited by Myc to target genes in mammalian cells ([11] and F. Faiola, X. Liu, and E. Martinez, unpublished), acetylation could potentially regulate specific events after promoter binding including transcription activation and/or repression by Myc. This is not incompatible with acetylation influencing Myc protein turnover. Indeed, ubiquitination and proteasomal degradation of Myc appear intimately linked to its transactivation activity [17,18]. Our identification of Myc acetylated residues should help address these questions and more generally the potential role of p300-mediated Myc acetylation during normal cell growth and division and during cellular transformation.

Acknowledgments

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