Max is acetylated by p300 at several nuclear localization residues

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Max is a ubiquitous transcription factor with a bHLHZip [basic HLH (helix–loop–helix) leucine zipper] DNA-binding/dimerization domain and the central component of the Myc/Max/Mad transcription factor network that controls cell growth, proliferation, differentiation and apoptotic cell death in metazoans. Max is the obligatory DNA-binding and dimerization partner for all the bHLHZip regulators of the Myc/Max/Mad network, including the Myc family of oncoproteins and the Mad family of Myc antagonists, which recognize E-box DNA elements in the regulatory regions of target genes. Max lacks a transcription regulatory domain and is the only member of the network that efficiently homodimerizes. Binding of Max homodimers to E-box elements suppresses the transcription regulatory functions of its network partners and of other non-network E-box-binding regulators. In contrast with its highly regulated partners, Max is a constitutively expressed and phosphorylated protein. Phosphorylation is, however, the only Max post-translational modification identi-

fied so far. In the present study, we have analysed Max post-translational modifications by MS. We have found that Max is acetylated at several lysine residues (Lys-57, Lys-144 and Lys-145) in mammalian cells. Max acetylation is stimulated by inhibitors of histone deacetylases and by overexpression of the p300 co-activator/HAT (histone acetyltransferase). The p300 HAT also directly acetylates Max *in vitro* at these three residues. Interestingly, the three Max residues acetylated *in vivo* and *in vitro* by p300 are important for Max nuclear localization and Max-mediated suppression of Myc transactivation. These results uncover novel post-translational modifications of Max and suggest the potential regulation of specific Max complexes by p300 and reversible acetylation.

Key words: histone acetyltransferase, Max, MS, Myc, nuclear localization, p300.

INTRODUCTION

Max is a DNA-binding transcription factor and the central and obligatory partner of a network of transcription regulators that control cell proliferation, differentiation, apoptotic cell death and cell transformation. This network includes the family of Myc oncoproteins (c-Myc, N-Myc, L-Myc) that stimulate cell division and growth and are deregulated in many types of cancer, and several Myc antagonists (i.e. Mad1, Mxi1/Mad2, Mad3, Mad4, Mnt/Rox and Mga) that inhibit cell proliferation and/or function during cell differentiation. While Max is a stable and ubiquitous protein that is constitutively expressed, the biosynthesis and stability of Myc and most of its network antagonists are highly regulated [1]. Max and all the other members of the Myc/Max/Mad network have a highly related bHLHZip [basic HLH (helix–loop–helix) leucine zipper] DNA-binding and dimerization domain and recognize common E-box DNA elements characterized by the core consensus sequence CACGTG. Although Max can bind DNA as a homodimer, Myc, Mad and Mnt proteins do not efficiently homodimerize, but bind E-box DNA elements only as heterodimers with Max [2–12]. Thus Max is essential for the transcription functions of the other regulators of the network. In addition, Max complexes can also impinge on the functions of other bHLHZip proteins [such as USF (upstream stimulatory factor)], which recognize the same E-box sequences but do not dimerize with members of the Myc/Max/Mad network [1,13–17].

A model for transcription activation by Myc–Max heterodimers involves the recruitment by Myc of several HATs (histone acetyltransferases)-containing co-activator complexes and Myc-induced hyperacetylation of chromatin within the regulatory regions of target genes [18–24]. Consistent with this, the Myc antagonists Mad and Mnt proteins inhibit transcription of target genes through conserved repression domains, which recruit mSin3 (mammalian Sin3) co-repressor complexes containing HDACs (histone deacetylases) [10,25–29]. In contrast, Max does not have a transcription regulatory domain and has generally been viewed as an obligatory but passive partner for the other factors of the network [1,4,6,30]. Max overexpression, however, has been shown to inhibit both Myc-dependent transcription activation and Myc/Ras-dependent cellular transformation, possibly via competition of Max homodimers (or Mnt–Max or Mad–Max heterodimers) with Myc–Max complexes for binding to E-box elements on target genes [4,5,30–36]. These inhibitory effects of Max have been shown to require a functional bHLHZip domain [35,37] and a 26-amino-acid region at the extreme C-terminus of Max that is important for efficient DNA binding by Max homodimers [37,38] and includes a nuclear localization signal and protein kinase CK2 phosphorylation sites [6,31,33,37].

Post-translational modifications play an important role in regulating the output of the Myc/Max/Mad network. For instance, Myc is a target for several post-translational modifications, which interplay to regulate its stability and transactivating functions and are often controlled by specific signalling pathways [22,38–46]. In

Abbreviations used: acetyl-K, acetyl-lysine; HLH, helix–loop–helix; bHLHZip, basic HLH leucine zipper; DAPI, 4',6-diamidino-2-phenylindole; EMSA, electrophoretic mobility-shift assay; ESI, electrospray ionization; GCN5, positive general control of transcription-5; HA, haemagglutinin; HAT, histone acetyltransferase; HDAC, histone deacetylase; HEK-293 cell, human embryonic kidney cell; hTERT, human telomerase reverse transcriptase; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MS/MS, tandem MS; Q-TOF, quadrupole–time-of-flight; TSA, trichostatin A.

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contrast, Max appears to be constitutively phosphorylated *in vivo*, most likely by protein kinase CK2, within an N-terminal region preceding its bHLHZip domain and within a C-terminal acidic region required for Max suppression of Myc functions [33,47–50]. Max phosphorylation by protein kinase CK2 at its N-terminal region *in vitro* has been shown to inhibit DNA binding by Max homodimers, but not by Myc–Max or Mad–Max heterodimers [7,48], and both N-terminal and C-terminal protein kinase CK2 sites inhibit Max's ability to interfere with Myc functions *in vivo* [33,50]. Interestingly, contrary to the traditional view of Max being unregulated, de-phosphorylation of Max during Fas-induced apoptosis stimulates cleavage of Max N-terminal region by caspase 5 and enhances DNA binding by cleaved Max homodimers [38].

We have reported recently that Max can be specifically acetylated by p300, but not by GCN5 (general control of amino acid synthesis-5) or TIP60 (HIV-1 Tat interacting protein, 60 kDa) HATs, *in vitro* [46]. In the present study, we demonstrate that Max is acetylated in mammalian cells at three lysine residues, Lys-57, Lys-144 and Lys-145 (co-ordinates in Max p21 isoform) that are also the major direct targets for p300-mediated acetylation *in vitro*. Interestingly, we show that these three residues are important for the nuclear localization of Max and its ability to suppress Myc-activated transcription. These results identify novel Max post-translational modifications and suggest the possibility that the sub-cellular localization and transcription activities of specific Max complexes may be regulated by p300-mediated Max acetylation.

MATERIALS AND METHODS

Plasmids

The bacterial expression vectors for His₆-tagged human Max short isoform (pET-His-Max) [51], His₆-tagged human GCN5 short form (pRSETA-His-GCN5-S) [52] and FLAG-tagged human p300 HAT domain (pET28c-FLAG-p300[1195–1810]) [53] and the mammalian expression vectors for FLAG-tagged mouse c-Myc (pCbs-FLAG-cMyc) [54], untagged human Max short isoform (pSP271-Max) [30] and FLAG-tagged human Max short isoform (pCbs-FLAG-Max p21) [46] were described previously. The mammalian expression vector pCMV β -p300-C/HA was obtained from Dr Richard Goodman (Vollum Institute, Oregon Health and Science University, Portland, OR, U.S.A.). The hTERT (human telomerase reverse transcriptase) luciferase reporter vector p2xEB was a gift from Dr J. Carl Barrett (National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.). The mammalian expression vector for HA (haemagglutinin)-tagged mouse c-Myc (pCbs-HA-cMyc) was generated by inserting between the BamHI and HindIII sites of pCbs a DNA fragment generated by PCR of pCbs-FLAG-cMyc with the forward primer 5'-CCTGC-CGGATCCACCATGGGCTACCCCTTATGACGTGCCCGATT-ACGCTCCCGGGATGCCCTGAACGTGAACCTTCACCAA-CAGG-3' (containing the HA tag) and the reverse primer 5'-CT-GCCCAAGCTTATGCACAGAGTTTCGAAGCTGTTTCGAG-TTTGTGTTTCAACTGTTC-3', and digested with BamHI and HindIII. To construct the mammalian expression vector for HA-tagged human Max p22 (pCbs-HA-Max p22), the XmaI site in Max cDNA from pCbs-FLAG-Max p21 plasmid was destroyed by site-directed mutagenesis with the QuikChange[®] Site-Directed Mutagenesis kit (Stratagene) and the primers 5'-GGAGAGAAG-GCATCCCGTGCCCAAATCCTAGAC-3' and 5'-GTCTAGG-ATTTGGGCACGGGATGCCTTCTCTCC-3'. Then, the additional nine codons specific of Max p22 were added by site-directed mutagenesis with the primers 5'-CATCGAGGTGGAGAG-

CGACGAAGAGCAACCCAGGTTTCAATCTGCGGCTGAC-AAACGGGCTCATC-3' and 5'-GATGAGCCCGTTTGTCAG-CCGCAGATTGAAACCTCGGTTGCTCTTCGTCGCTCTCC-ACCTCGATG-3' to obtain pCbs-FLAG-Max p22. The Max p22 fragment was excised from pCbs-FLAG-Max p22 by digestion with XmaI and HindIII, and ligated into the pCbs-HA vector (recovered from pCbs-HA-cMyc digested with XmaI and HindIII) to obtain the final pCbs-HA-Max p22 vector. The plasmids pET-His-Max and pCbs-FLAG-Max were used to create the bacterial and mammalian expression vectors respectively for Max K → R and K → Q mutants with the QuikChange[®] Site-Directed Mutagenesis kit. All plasmid constructs were verified by DNA sequencing.

Antibodies and Western blot

For Western blot analyses, proteins were resolved by SDS/PAGE and transferred on to nitrocellulose membranes and detected with specific antibodies: acetylated-lysine (Cell Signaling Technology), FLAG M2 (Sigma), Max C-17 and Myc N262 (Santa Cruz Biotechnology), by using the ECL[®] (enhanced chemiluminescence) kit (Amersham Biosciences) according to the manufacturer's instructions. When indicated, membranes were stripped for 60 min at 55 °C in stripping solution [62.5 mM Tris/HCl, pH 6.7, 2% (w/v) SDS and 100 mM 2-mercaptoethanol], and then washed and blocked with 6% (w/v) non-fat dry milk in TBST (10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20), and re-probed with a different primary antibody.

Recombinant protein expression and purification

Human His₆-tagged Max (wild-type and mutants) were expressed in bacteria BL21(DE3)pLysS (Stratagene) and purified from the soluble fraction as previously described [55]. For the experiment of Figure 4(A), His₆-tagged Max wild-type and the triple mutant Max K57R/K144R/K145R were further purified (after elution from the Ni²⁺-nitrilotriacetate-agarose) by batch binding to heparin-Sepharose (Amersham Biosciences) for 3 h at 4 °C (under constant rotation), followed by successive washes with BC-100 [20 mM Tris/HCl, pH 7.9 at 4 °C, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 10 mM 2-mercaptoethanol and 0.2 mM PMSF], BC-350 (containing 350 mM KCl) and BC-500. Max proteins were eluted with BC-1000, dialysed to BC-100, and stored at –80 °C. FLAG-p300 HAT domain was expressed in BL21-CodonPlus(DE3)-RP bacteria (Stratagene) and purified from the soluble fraction by batch binding to FLAG M2 affinity resin (Sigma) for 9–12 h at 4 °C. The resin was then extensively washed with, successively, bacteria lysis buffer (20 mM Hepes, pH 7.9 at 4 °C, 500 mM NaCl, 10% glycerol, 0.1% Igepal CA-630, 2 mM 2-mercaptoethanol and 1 mM PMSF), BC-500 and BC-100 (as above but with 2 mM 2-mercaptoethanol). FLAG-p300 was eluted with 0.4 mg/ml FLAG peptide in BC-100 for 30 min at 22 °C.

In vitro acetylation assays

Acetylation reactions were performed by incubating 100–400 ng of Max proteins (or 2 μ g of a mixture of calf thymus histones H1, H2A, H2B, H3 and H4; Roche) with 10–40 ng of recombinant p300-HAT and 3–5 μ M [³H]acetyl-CoA (27.5 Ci/mmol, 1.0 mCi/ml, Sigma) in 50 mM Tris/HCl (pH 8.0), 14% glycerol, 70 mM KCl, 0.1 mg/ml BSA, 0.09% Igepal CA-630, 8 mM 2-mercaptoethanol, 10 mM sodium butyrate and 0.3 mM PMSF for 1 h at 30 °C. Reactions were stopped by adding SDS-sample buffer, and proteins were resolved by SDS/PAGE and stained with

Coomassie Brilliant Blue. For fluorography, stained SDS/polyacrylamide gels were further soaked in 'Amplify' solution (Amersham Biosciences) for 30 min, quickly rinsed with water, dried under vacuum, and exposed to X-ray films with an intensifying screen for at least 15 h at -80°C . Unlabelled acetylation reactions were performed similarly (but with unlabelled acetyl-CoA) and acetylated proteins were detected by Western blot with the acetyl-K (acetyl-lysine) antibody, as described above.

MS analyses

In vitro acetylation of recombinant Max (wild-type, 8 μg) was performed essentially as described above but with unlabelled acetyl-CoA (100 μM) and FLAG-p300 HAT domain (2 μg) immobilized on the anti-FLAG M2 resin (alternatively 2 μg of Max and 0.5 μg of full-length p300 [46] were used with similar results, except that the use of p300 full-length further led to identification of acetylated Lys-31). Approximately 1 μg of acetylated Max was digested with 10 ng of trypsin in 25 mM NH_4HCO_3 for 9 h at 37°C , and the peptides were purified using a ZipTip (Millipore) and subjected to MS. Alternatively, after the acetylation reaction, proteins were resolved by SDS/PAGE and stained with Colloidal Blue staining kit (Invitrogen). Max protein bands were cut, destained and digested with trypsin. Tryptic peptides were first analysed by MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS. Monoisotopic masses of all peptides were measured by MALDI using a Voyager DE-STR Biospectrometry Workstation (Applied Biosystems) with delayed extraction operated in the reflectron mode. LC (liquid chromatography)-MS/MS (tandem MS) analyses were performed with an electrospray Q-TOF (quadrupole-time-of-flight) mass spectrometer (QTOF Ultima-Global; Micromass) coupled online with a capillary HPLC (Agilent 1100; Hewlett-Packard). An Agilent 0.5 mm $\times$ 150 mm ZORBAX SB-C18 column (5 μm particle diameter, 80 Å pore size; 1 Å = 0.1 nm) with mobile phase of A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) was used with a linear gradient of 2–65 % of mobile phase B over 65 min at a flow rate of 6 $\mu\text{l}/\text{min}$. The flow was directly introduced into the electrospray source of the mass spectrometer. Q-TOF experiments were run at a capillary voltage of 3.0 V and a cone voltage of 77 V. The source block and desolvation gas (N_2) temperatures were 90 and 120°C respectively. CAD (collision-activated dissociation) experiments were performed in a hexapole collision cell with Ar (69 kPa) as the collision gas and collision energy was optimized at 1.9 eV. The collision energy was ramped over the m/z range from 20 to 40 V using a pre-optimized collision-energy file. The quadrupole mass filter before the TOF analyser was set with LM (low mass) and HM (high mass) resolution of 10 (arbitrary units), which is approximately equivalent to a 2.0-Da mass window for transmission of precursor ions.

For MS analyses of endogenous Max in HeLa cells, 11 confluent 10-cm plates of cells were incubated at 37°C under 5 % CO_2 with HDAC inhibitors [10 mM sodium butyrate, 10 mM nicotinamide and 2 μM TSA (trichostatin A)] for 4 h and with the proteasome inhibitor MG-132 (the proteasome inhibitor carboxy-benzoyl-L-leucyl-L-leucyl-leucinal; 20 μM) for 1.5 h just before lysing the cells with Lysis Buffer (50 mM Hepes, pH 7.9 at 4°C , 500 mM NaCl, 0.1 % Igepal CA-630, 0.2 mM EDTA and 0.2 mM PMSF) supplemented with 10 mM sodium butyrate, 10 mM nicotinamide, 2 μM TSA and 20 μM MG-132. Endogenous Max was immunoprecipitated from the pre-cleared cell lysates by incubation with the anti-Max C-17 antibody (105 μg of IgG) for 9 h at 4°C . Protein A-Sepharose CL-4B (GE/Pharmacia) resin (220 μl) was then added and the lysates were rotated for 14 h at

4°C . Immunoprecipitates were washed three times with Lysis Buffer and once with 1 % sodium deoxycholate in Lysis Buffer, and eluted in SDS/PAGE loading buffer. Max proteins were resolved by SDS/PAGE and stained with Colloidal Blue. Max protein bands were cut, destained and digested with trypsin or V8 protease (staphylococcal V8 protease; Roche Diagnostics) overnight in the NH_4HCO_3 buffer. The final proteolytic peptide samples were prepared and analysed by nano-LC/ESI (electrospray ionization)/MS/MS essentially as previously described [46], with the exception that the Q-TOF Premier MS/MS system and the nanoAcquity ultra-performance LC (nanoUPLC; Waters Corp.) were used in the present study. For the experiment in Figure 2(C), HEK-293 cells (human embryonic kidney cells) were transfected three times with pCbs-FLAG-Myc (3 μg), pCbs-HA-Max p22 (0.5 μg) and pCMV β -p300-C/HA (4.5 μg) at 4-h intervals and cells were cultured for an additional 40 h. The cells were incubated with HDAC inhibitors (as above) for 12 h before cell lysis (see above). Cell lysates from four confluent 10-cm plates were immunoprecipitated with FLAG M2-agarose and analysed by MS as above.

Cell culture, transfection, co-immunoprecipitation, luciferase assays and EMSA (electrophoretic mobility-shift assay)

HeLa, COS-7, HEK-293 and U2OS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum at 37°C with 5 % CO_2 .

For transfection and co-immunoprecipitation experiments, the cells were seeded on to 10-cm plates to reach 40–80 % confluency the next day at the time of transfection. Transfections were performed with LipofectamineTM 2000 (Invitrogen) and the indicated mammalian expression vectors and/or corresponding empty vectors to maintain total DNA constant. For COS-7 cells (Figure 2A), 12 h after transfection the medium was replaced with complete medium containing or lacking HDAC inhibitors (10 mM sodium butyrate, 10 mM nicotinamide and 2 μM TSA) and the cells were lysed 12 h later with ice-cold Hypotonic Lysis Buffer (50 mM Hepes, pH 7.9 at 4°C , 20 mM NaCl, 0.1 % Igepal CA-630, 0.2 mM EDTA, 0.2 mM PMSF and 2 mM 2-mercaptoethanol). Cell extracts were adjusted to 250 mM NaCl and 20 % glycerol (IP-250 buffer) and lysates equivalent to one 10-cm plate were immunoprecipitated with 10 μl of FLAG M2-agarose resin (Sigma) for 8 h at 4°C , under constant rotation. The resin was washed 3 times with 1 ml of IP-250 buffer, and resin-bound proteins were analysed by Western blot. For HEK-293 cells (Figure 2B), the medium was replaced 24 h after transfection with complete medium containing HDAC inhibitors (as above) and the cells were lysed 8 h later with Lysis Buffer containing 10 mM sodium butyrate and 1.4 mM 2-mercaptoethanol. Anti-FLAG immunoprecipitations were performed as above. For U2OS cells (Figure 6) the cells were lysed 48 h after transfection with Lysis Buffer containing 2 mM 2-mercaptoethanol. Lysates were adjusted to 179 mM NaCl and 6 % glycerol, and immunoprecipitated with the anti-FLAG M2-agarose resin as above.

For luciferase assays, U2OS cells were transfected in 6-well plates with LipofectamineTM reagent (Invitrogen) and with 100 ng of p2xEB, 100 ng of pCMV- β -galactosidase, 25 ng of pCbs-FLAG-Myc and the indicated amounts of pCbs-FLAG-Max expression vectors or the empty pCbs vector to keep total DNA constant. Expression of the pCMV- β -galactosidase was not affected by co-transfection of the expression vectors for Myc or Max (results not shown). The relative luciferase activities were normalized to β -galactosidase activities and were plotted as percentage of the activity in extracts of cells transfected with the empty pCbs vector. The means and standard deviation obtained

Figure 1 Endogenous Max in human HeLa cells is acetylated at Lys-144 and Lys-145

Whole cell extracts were prepared from HeLa cells treated for 4 h with HDAC inhibitors and endogenous Max proteins were immunoprecipitated with the anti-Max C-17 antibody, resolved by SDS/PAGE, in-gel digested with V8 protease, and subjected to MS/MS (LC-MS/MS) analysis. Shown is the MS/MS spectrum of fragmentation ions (y and y^{++}) derived from a triple-charged precursor ion of the indicated m/z value and corresponding to the Max peptide sequence shown containing a single acetylated lysine residue at position Lys-145 or alternatively at Lys-144, as indicated by the two y_5 ions, K145-ac and K144-ac respectively (see also text). The errors for the precursor and fragmentation ions are indicated in the box.

from at least three independent experiments each performed in duplicate are shown in Figure 5(A).

For EMSA, U2OS cells were transfected in 6-cm plates with LipofectamineTM 2000 and 3 μ g of pCbs-FLAG-Max (wild-type or mutant) or the empty pCbs vector. After 24 h expression cell extracts were prepared in 200 μ l of Lysis Buffer (see above) and diluted 1:4 in buffer BC-133/BSA (20 mM Tris/HCl, pH 7.9, 133 mM KCl, 20 % glycerol, 0.2 mM EDTA, 0.2 mM PMSF, 10 mM 2-mercaptoethanol and 0.5 mg/ml BSA). DNA binding was performed in 10 μ l final volume with 1 μ l of diluted extract, 3 ng of ³²P-labelled E-box DNA oligonucleotide (5'-GCTC-AGGGACCACTGGTTCGGGGATC-3'), 500 ng of poly(dA-dT) · (dA-dT) and 7.5 μ l of BC-133/BSA for 30 min at 22 °C. Where indicated the extract in 7.5 μ l of BC-133/BSA was pre-incubated for 15 min at 22 °C with 1 μ l of anti-FLAG M2 antibody (Sigma) diluted 1:10 with 0.2 mg/ml BSA in BC-100. Protein-DNA complexes were resolved by native 6%-(w/v)-PAGE and visualized by autoradiography of the dried gels [55].

Indirect immunofluorescence

U2OS cells in 6-well plates containing three microscope coverslips were transfected with 1.5 μ g of pCbs-FLAG-Max p21. After 30 h the cells were washed with PBS and fixed with 100 % methanol and permeabilized with 0.2 % Triton X-100 in PBS. The cells were incubated with 5 % (v/v) goat serum and 0.1 % Triton X-100 (in PBS) for 30 min at room temperature (22 °C) and then incubated with anti-FLAG M2 antibody (Sigma) diluted 1:400 in PBS containing 1 % goat serum and 0.1 % Triton X-100 at 4 °C overnight. The cells were washed three times briefly with 0.1 % Triton X-100 in PBS and then incubated for 1 h at room temperature with Alexa Fluor[®] 488-conjugated goat anti-mouse IgG antibodies (Molecular Probes) diluted 1:400 in PBS containing 1 % goat serum and 0.1 % Triton X-100. After three washes with 0.1 % Triton X-100 in PBS, the cells were stained with DAPI (4',6-diamidino-2-phenylindole), and the coverslips were mounted on to microscope slips and analysed by fluorescence microscopy and digital imaging.

Figure 2 Max acetylation in mammalian cells is stimulated by HDAC inhibitors and p300 overexpression

(A) FLAG-Myc from transfected COS-7 cells treated (+) or mock treated (–) with a cocktail of HDAC inhibitors (HDAC inh.) was immunoprecipitated with the FLAG antibody (IP: FLAG) and analysed by Western blot with FLAG (FLAG-Myc), Max (Max total), and acetyl-K (Max-Ac) antibodies, as indicated. Different regions of the same nitrocellulose blot are shown. The two bottom panels represent the same nitrocellulose filter probed first with acetyl-K antibodies (Max-Ac), stripped and then re-probed with Max-specific antibodies (Max total). The position of pre-stained molecular-mass markers (in kDa) is indicated. The asterisk (*) points to a non-specific band. (B) p300 acetylates endogenous Max in HEK-293 cells. Extracts of cells transfected with expression vectors for FLAG-Myc and/or p300 (as indicated by + and –) and treated with HDAC inhibitors were immunoprecipitated with the FLAG antibody and analysed by Western blot. The same filter was probed first with acetyl-K antibodies (top) and then re-probed with Max-specific antibodies (bottom). (C) p300 acetylates Max at Lys-57 in HEK-293 cells. Cells transfected with expression vectors for FLAG-Myc, HA-Max and p300 were treated with HDAC inhibitors for 12 h before whole cell extract preparation and immunoprecipitation with the FLAG antibody. The HA-Max protein band in an SDS/PAGE was in-gel digested with trypsin, and tryptic peptides were analysed by LC-MS/MS. The MS/MS spectrum of fragmentation ions (y) is shown for the Max peptide LDKAcATEYIQYMR acetylated at Lys-57 (K57ac).

RESULTS

Acetylation of Max in mammalian cells

We have shown recently that Max is acetylated *in vitro* specifically by p300 [46], suggesting that Max might be acetylated *in vivo*.

To determine whether Max is acetylated in human cells and to identify the potential acetylated residues, endogenous Max in HeLa cells was immunoprecipitated with a specific Max antibody, resolved by SDS/PAGE and analysed by MS/MS (LC-MS/MS). Figure 1 shows the MS/MS spectrum of two acetylated

forms of a Max peptide containing one single acetylated lysine residue at positions 144 (Lys-144) and 145 (Lys-145) respectively. This was confirmed by database searching using ‘all entries’ against the entire NCBI_{nr} (National Center for Biotechnology Information non-redundant) protein database and by comparing the experimental spectrum with spectra derived from a synthetic peptide containing an acetyl group at either Lys-144 or Lys-145 (results not shown). To determine whether Max is also acetylated in other mammalian cell lines and to test whether acetylated Max can interact with Myc, endogenous Myc-associated proteins in extracts of COS-7 cells transfected with a FLAG-tagged Myc expression vector were immunoprecipitated with the anti-FLAG M2 resin and, after extensive washes, bound proteins were analysed by Western blot with an acetyl-K antibody that specifically recognizes Max acetylated *in vitro* by p300, but not unacetylated Max (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/403/bj4030397add.htm>). As shown in Figure 2(A), a FLAG–Myc-associated protein of approx. 20 kDa that co-migrated with endogenous Max was recognized by the acetyl-K antibody most strongly after treatment of the cells with a cocktail of HDAC inhibitors (lane 1 versus lane 2), suggesting that endogenous Max is acetylated in COS-7 cells and stably interacts with Myc. Acetylation of Max in association with Myc in COS-7 cells was verified by MS analyses of HA-tagged Max co-immunoprecipitated with FLAG–Myc, which identified a Max peptide with a mass indicative of acetylation at either Lys-144 or Lys-145 (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/403/bj4030397add.htm>).

We further analysed p300-dependent acetylation of Max in HEK-293 cells transfected with expression vectors for FLAG–Myc and p300. FLAG–Myc and stably associated endogenous proteins were immunoprecipitated with the FLAG antibody and analysed by Western blot as above. A Myc-associated protein that co-migrated with endogenous human Max was found preferentially acetylated in cells that were transfected with p300 (Max-Ac, lane 3 versus lane 4, Figure 2B). To verify that Max is indeed acetylated by p300, FLAG–Myc in extracts of HEK-293 cells co-transfected with HA–Max and p300 was immunoprecipitated with the FLAG antibody and Max proteins were resolved by SDS/PAGE and analysed by LC-MS/MS. As shown in Figure 2(C), a tryptic Max peptide was found acetylated at residue Lys-57.

Taken together, these results demonstrate that in mammalian cells (i) Max is acetylated at three lysine residues (i.e. Lys-57, Lys-144 and Lys-145), (ii) HDAC inhibitors and p300 induce acetylation of Max, and (iii) acetylated Max stably interacts with Myc.

Max lysine residues Lys-31, Lys-57, Lys-144 and Lys-145 are direct substrates for p300 *in vitro*

To identify Max lysine residues that are direct substrates for acetylation by p300, purified recombinant Max and p300 were used in acetylation reactions *in vitro* (Supplementary Figure S1 and [46]) and acetylated Max was analysed by MS (MALDI- and ESI-MS and LC-MS/MS). A total of 29 Max peptides were identified by MALDI–TOF (M) and ESI-Q-TOF (E), which covered 88 % of Max residues (Figure 3, top). ESI-Q-TOF identified several acetylated peptides with masses indicative of acetylation at residues Lys-144 and Lys-145 (peptides 1–3), including a peptide (peptide 4) that was acetylated at Lys-31 and verified by LC-MS/MS. MALDI–TOF analyses identified 25 different peptides, of which only two had masses suggestive of an internal acetylated residue (peptides 9 and 24). Consistent with this, LC-MS/MS identified acetylation of Lys-144 (peptide 9) and

Peptide	Mass	Position	Sequence
1	586.4 (E)	144/147	KacKLR and/or KKacLR
2	628.4 (E)	144/147	KacKacLR
3	876.4 (E)	145/151	KacLRMEAS
4	1397.7 (E)	28/38	DHIKacDSFHSLR ----- (MS/MS)
5	824.5 (M)	26/31	RRDHIK
6	861.4 (M)	32/38	DSFHSLR
7	947.5 (M)	17/24	AHHNALER
8	978.5 (M)	144/151	KKLRMoxEAS
9	1020.5 (M)	144/151	KacKLRMoxEAS ----- (MS/MS)
10	1059.5 (M)	39/48	DSVPSLQGEK
11	1075.6 (M)	17/25	AHHNALERK
12	1174.6 (M)	58/66	ATEYIQYMR
13	1181.6 (M)	82/91	Pyro-QNALLEQQVR
14	1190.5 (M)	58/66	ATEYIQYMoXR
15	1198.5 (M)	82/91	QNALLEQQVR
16	1354.8 (M)	81/91	RQNALLEQQVR
17	1510.4 (M)	27/38	RDHIKDSFHSLR
18	1619.4 (M)	69/81	NHTHQDIDDLKR
19	1747.9 (M)	68/81	KNHTHQDIDDLKR
20	1768.8 (M)	-19/-4	GSSHHHHHHSSGLVPR
21	1810.8 (M)	-19/-4	Ac-GSSHHHHHHSSGLVPR
22	1842.9 (M)	52/66	AQILDKATEYIQYMR
23	1858.9 (M)	52/66	AQILDKATEYIQYMoXR
24	1884.9 (M)	52/66	AQILDKacATEYIQYMR ----- (MS/MS)
25	2119.9 (M)	-3/16	GSHMSDNDIEVESDADKR
26	2135.9 (M)	-3/16	GSHMoxSDNDIEVESDADKR
27	2289.1 (M)	98/118	SSAQLQTNYPSSDNSLYTNAK
28	2395.2 (M)	28/48	DHIKDSFHSLRDSVPSLQGEK
29	2543.1 (M)	119/143	GSTISAFDGGSDSSSESEPEEQSR

Figure 3 Identification of Max residues acetylated by p300 *in vitro* by MS

Upper panel: list of Max tryptic peptides with their measured mass, position and predicted sequence, as determined by ESI-Q-TOF (E) and MALDI–TOF (M) MS. Sequences further verified by LC-MS/MS are indicated (MS/MS). Ac- indicates N-terminal acetylation; Kac, acetyl-K; Mox, oxidized methionine; Pyro-Q, pyroglutamine. The position of peptides refers to amino acid co-ordinates in recombinant His₆-tagged Max p21 short isoform (see bottom sequence). Lower panel: sequence of His₆–Max p21. The basic, Helix1 (H1)–Loop–Helix2 (H2) and leucine zipper (Zip) are indicated above the amino acid sequence. The His₆-tag sequence is in italics (*Tag*) with negative numbering. Filled arrowheads indicate the lysine residues that are acetylated in mammalian cells and *in vitro* by p300 (Lys-57, Lys-144 and Lys-145) and the open arrowhead points to a lysine residue weakly acetylated *in vitro* by p300 and not detectably acetylated in cells (Lys-31).

Lys-57 (peptide 24). Thus p300 directly acetylates Max at Lys-31 and Lys-57 within, respectively, the H1 and H2 α-helices of the HLH domain and the doublet Lys-144/Lys-145 near the C-terminus (Figure 3, bottom, arrowheads). Except for Lys-31, the other three p300-targeted residues correspond to sites also found acetylated in mammalian cells (see above).

To determine whether the Max lysine residues Lys-57, Lys-144 and Lys-145 correspond to the major direct target sites for p300, all three residues were changed to arginine residues by site-directed mutagenesis, and the resulting Max triple mutant K57R/K144R/K145R protein was then tested in acetylation

Figure 4 Analysis of p300-acetylated Max residues by site-directed mutagenesis *in vitro*

(A) Recombinant Max wild-type (WT) and triple K → R mutant K57R/K144R/K145R ('Max K[57,144,145]R') were acetylated *in vitro* with p300 HAT (40 ng) in the presence of [³H]acetyl-CoA and analysed by SDS/PAGE and fluorography of the Coomassie-stained gel. Max protein amounts used: —: no protein; +: 100 ng; ++: 200 ng; +++: 400 ng. The upper panel is a fluorogram of the SDS/PAGE showing the acetylated products (p300-Ac and Max-Ac) and the lower panel shows total Max proteins stained with Coomassie on the same SDS/PAGE. (B) Acetylation assays (as above) in the presence (+) or absence (–) of p300 (10 ng). Reaction in lane 1 contained no Max protein. Equivalent amounts (400 ng) of Max WT, Max single mutants K57R ('[57]'), K144R ('[144]') and K145R ('[145]') and Max double mutants K57R/K144R ('[57,144]') and K57R/K145R ('[57,145]') were used, as indicated. The bottom panel shows the Coomassie-stained Max proteins in the dried gel used for fluorography.

assays *in vitro* in the presence of p300, as above. While the wild-type Max protein was efficiently acetylated (Figure 4A, lanes 2–4), the triple mutant Max K57R/K144R/K145R was not detectably acetylated by p300 (lane 5). Note that overexposure of the gel did not reveal any acetylation signal (results not shown), indicating that these three lysine residues are the major sites acetylated by p300 and thus that Lys-31 is only very weakly acetylated. To address further which of these three lysine residues is the preferred substrate for p300, acetylation assays were performed with Max mutants having only one or two lysine residues changed to arginine. Max single mutants K57R and K145R and the double mutant K57R/K145R were all acetylated by p300 (Figure 4B, lanes 4, 6 and 8). In contrast, acetylation of the Max mutants K144R and K57R/K144R was very weak (lanes 10 and 12). Therefore we conclude that p300 directly and preferentially acetylates Max at three lysine residues, Lys-57, Lys-144 and Lys-145, which are also acetylated in human cells (Figure 3, bottom, filled arrowheads), and that Lys-144 appears to be the preferred acetylation site for p300, while acetylation of Lys-31 is very inefficient *in vitro*. This is consistent with the fact that we did not detect acetylation of Lys-31 in mammalian cells.

Figure 5 Analysis of Max wild-type and K → Q mutants in suppression of Myc transactivation

(A) U2OS cells were transfected with the hTERT promoter-luciferase (p2xEB) reporter, the internal control pCMV-β-galactosidase, an expression vector for FLAG-Myc (F-Myc, 25 ng) and increasing amounts (20–80 ng) of expression vectors for FLAG-Max (F-Max) wild-type (WT) and mutants, as indicated. Relative luciferase (Luc) activities (normalized to β-galactosidase) are presented in percentage (%) of the activity of the hTERT-Luc reporter in the absence of co-transfected Myc and Max (lane 1, 100%). (B) The expression levels of FLAG-Myc and FLAG-Max wild-type (wt) and mutant proteins in whole cell extracts of transfected U2OS cells were analysed by Western blot with a Myc antibody (upper panel, FLAG-Myc) and the FLAG antibody (lower panel, FLAG-Max). K[57]Q, K57Q; K[144,145]Q, K144Q/K145Q; K[57,144,145]Q, K57Q/K144Q/K145Q.

Function of lysine residues Lys-57, Lys-144 and Lys-145 in Max nuclear localization

To address the function of the three major acetylated Max residues in human cells, Max expression vectors encoding single (Lys-57), double (Lys-144 and Lys-145) and triple (Lys-57, Lys-144 and Lys-145) amino acid substitutions were generated by site-directed mutagenesis. These lysine residues were changed to either arginine (K → R triple mutant) to keep the positive charge or glutamine (K → Q mutants) to mimic an acetylated lysine side chain. We first analysed the ability of Max K → Q mutants to suppress Myc-dependent activation of an hTERT promoter-luciferase reporter gene in transiently transfected human U2OS cells. The hTERT promoter contains two E-box elements and is

Figure 6 Dimerization of Max wild-type and K→Q mutant proteins in human cells

(A) U2OS cells were transfected with expression vectors for FLAG-tagged (FLAG-Max) wild-type (WT) or the indicated mutants and untagged Max (Max, +) or its empty vector (–). FLAG-Max WT and mutants were immunoprecipitated with the FLAG antibody and analysed by Western blot (WB) with the Max C-17 antibody. Lanes 1–8 are the whole cell extracts (input) and lanes 9–10 are the corresponding immunoprecipitates (IP: FLAG). (B) Same as in (A), except that FLAG-Max WT and mutants were co-transfected with the HA-Myc expression vector. HA-Myc protein in whole cell extracts (lanes 1–6) or after co-immunoprecipitation with FLAG-Max (lanes 7–12) was detected by Western blot with a Myc (N262) antibody (top panel). K[57]Q, K[57]Q, K[144,145]Q, K[144Q/K145Q; K[57,144,145]Q, K[57Q/K144Q/K145Q; K[57,144,145]R, K[57R/K144R/K145R].

a well-established direct target for regulation by Myc/Max/Mad proteins (reviewed in [16,46]). As expected, in the absence of overexpressed Max, Myc activated the hTERT promoter approx. 3-fold (Figure 5A, lane 2) and this was dependent on the two E-box elements (results not shown, and see [46]). This suggests that Max is not limiting for Myc transactivation in U2OS cells. As previously reported in other systems (see the Introduction section), overexpression of Max wild-type strongly inhibited Myc transactivation in a dose-dependent manner (lanes 3, 7 and 11). Similarly, Max K57Q and Max K144Q/K145Q mutants having one and two lysine residues changed to glutamine respectively repressed Myc transactivation as efficiently as Max wild-type (lanes 4, 5, 8, 9, 12 and 13). In contrast, the triple mutant Max K57Q/K144Q/K145Q had a significantly reduced ability to repress Myc transactivation (lanes 6, 10 and 14). This was not due to differences in the level of expression of the different Max or Myc proteins, as verified by Western blot (Figure 5B). Furthermore, Max triple K→R mutant (i.e. Max K57R/K144R/K145R) repressed transcription as efficiently as Max wild-type (results not shown). Thus neutralization of the positively charged side chains at these three positions affects the ability of overexpressed Max to inhibit Myc transactivation in human cells.

The defect of Max K57Q/K144Q/K145Q was not merely the result of a complete inactivation of the Max protein, since co-

Figure 7 DNA binding analysis of Max wild-type and mutant proteins

(A) Extracts of U2OS cells transfected with expression vectors for FLAG-Max wild-type (WT) and the indicated mutants or the corresponding empty vector (–, lanes 1 and 7) were used in DNA binding reactions with a consensus E-box DNA probe and analysed by EMSA (lanes 1–6). Extracts were pre-incubated with the FLAG M2 antibody in lanes 7–12. An arrow indicates the position of DNA-bound Max homodimers, and an arrowhead labelled with an asterisk (*) points to the super-shifted antibody-FLAG-Max-DNA complexes. F is the 'free' E-box DNA probe. (B) The extracts used in (A) were analysed by Western blot (WB) with the FLAG M2 antibody. K[57]Q, K57Q; K[144,145]Q, K144Q/K145Q; K[57,144,145]Q, K57Q/K144Q/K145Q; K[57,144,145]R, K57R/K144R/K145R.

immunoprecipitation experiments indicated that all Max mutants dimerized with Max wild-type (Figure 6A) and with Myc (Figure 6B) in U2OS cells. The latter is also consistent with the fact that acetylated Max was found stably associated with Myc in co-immunoprecipitation experiments (see above). To address possible effects at the level of DNA binding, we analysed the E-box-binding activities of the different Max homodimers (Figure 7A). FLAG-tagged Max wild-type and mutant proteins were overexpressed in U2OS cells by transient transfection, and whole cell extracts containing comparable amounts of Max wild-type and mutant proteins, as determined by Western blot (Figure 7B), were used in EMSA with a consensus E-box-containing DNA probe. Under the conditions used, the extracts of empty-vector-transfected cells did not contain any detectable E-box-binding activity (Figure 7A, lane 1). As expected, a specific protein-DNA complex was observed with extracts containing FLAG-Max wild-type (lane 2), which was specifically super-shifted by the monoclonal anti-FLAG M2 antibody (lane 8,

Figure 8 Replacement of Max lysine residues Lys-57, Lys-144 and Lys-145 with glutamine but not arginine relocates Max to the cytoplasm of human cells

FLAG-tagged Max wild-type (wt) and the indicated FLAG-Max K → Q and K → R mutant proteins were expressed in U2OS cells by transient transfection and visualized with the FLAG antibody by indirect immunofluorescence (top row, α-FLAG, green). The middle row shows the DAPI-stained nuclei (blue) and the bottom row (Merge) shows the merged images of α-FLAG (green) and DAPI (blue) staining. The α-FLAG cell staining in each of the fields is representative of more than 80% of the cell population. K[57]Q, K57Q; K[144,145]Q, K144Q/K145Q; K[57,144,145]Q, K57Q/K144Q/K145Q; K[57,144,145]R, K57R/K144R/K145R.

asterisk), but not by a FLAG peptide-blocked antibody or by an unrelated monoclonal antibody (results not shown). All the FLAG-Max mutant proteins were able to bind to the E-box DNA probe (lanes 3–6), although a small but reproducible decrease in the amount of protein–DNA complexes formed was observed with the mutant Max proteins having specifically the K57Q substitution, i.e. Max K57Q and Max K57Q/K144Q/K145Q (lanes 3 and 5 compared with lanes 2, 4 and 6). This effect was more pronounced after pre-incubation of the extracts with the anti-FLAG antibody, which resulted in reduced amounts of super-shifted complexes containing specifically FLAG-Max K57Q and K57Q/K144Q/K145Q (lanes 9 and 11 compared with lanes 8, 10 and 12, asterisk). How the K57Q substitution affects the formation of Max–DNA complexes in this *in vitro* assay is unclear; however, this effect cannot by itself explain the reduced repressive function of selectively Max K57Q/K144Q/K145Q but not Max K57Q in the reporter gene assay described above.

Since Lys-144 and Lys-145 are located within a C-terminal basic motif (PQSRKKLR) previously reported to have nuclear localization activity [6,33,37], we analysed the subcellular localization of FLAG-tagged Max wild-type and the above K → Q and K → R mutants in transfected U2OS cells by indirect immunofluorescence with the FLAG monoclonal antibody (Figure 8). As expected, FLAG-Max wild-type was localized exclusively to the nucleus. The FLAG-Max K57Q was also exclusively nuclear. In accord with previous observations indicating that this C-terminal basic motif is not absolutely essential for Max nuclear localization [6,33,37], FLAG-Max K144Q/K145Q was also predominantly nuclear. Interestingly, however, FLAG-Max K57Q/K144Q/K145Q was mostly localized to the cytoplasm, while the triple mutant FLAG-Max K57R/K144R/K145R re-

mained exclusively nuclear. Thus we conclude that the positively charged side chains of Lys-57 within H2 and of Lys-144 and/or Lys-145 within the C-terminal basic sequence PQSRKKLR are essential for the nuclear localization of Max. The predominantly cytoplasmic localization of selectively Max K57Q/K144Q/K145Q is consistent with the reduced repressive function of only this mutant in the Myc transactivation/reporter gene assay.

DISCUSSION

We have shown recently that Max is acetylated *in vitro* by p300 but not by GCN5 or TIP60 HATs, while both p300 and GCN5 acetylate Myc [46]. In the present study, we provide direct evidence that Max is acetylated in mammalian cells by endogenous HATs and by overexpressed p300, and that Max acetylation is stimulated upon inhibition of cellular HDACs. This suggests that Max acetylation *in vivo* might be reversible and dynamic and that cellular HDACs could directly deacetylate Max. However, indirect effects of cellular HDAC inhibition on Max acetylation are possible and are not excluded by our experiments. We have identified three Max lysine residues (Lys-57, Lys-144 and Lys-145) that are acetylated in mammalian cells and are also direct substrates for p300 *in vitro*. Interestingly, replacement of Lys-57, Lys-144 and Lys-145 with glutamine efficiently relocates Max to the cytoplasm in transfected human cells. In contrast, substitution of neither Lys-57 nor the Lys-144/Lys-145 doublet alone (Figure 8) affects Max nuclear localization. This is in accord with the previous observation that deletion of the C-terminal basic nuclear localization sequence PQSRKKLR (which includes Lys-144 and Lys-145) does not prevent Max nuclear localization

[6,33,37] and suggests an essential co-operative role of these three lysine residues in the control of Max subcellular distribution. Accordingly, Max's ability to suppress Myc transactivation (presumably as a Max homodimer) is only affected by the substitution of all three residues. The fact that replacement of these three lysine residues with arginine does not affect Max nuclear localization (or inhibition of Myc transactivation) further underscores the importance of a positively charged side chain at these positions. While these three lysine residues are clearly important for Max nuclear localization, our results do not exclude the possible essential contribution of additional residues, as suggested by previous studies [37]. This would also be consistent with the fact that deletion of the basic DNA-binding region of Max moderately affects Max nuclear localization [56]. Similarly, since Lys-31 was found weakly acetylated by p300 *in vitro*, we cannot formally exclude the possible acetylation of additional Max lysine residues *in vivo* (that might not have been detected by our methods) and their potential involvement in Max nuclear localization.

The mechanism by which Lys-57 and the Lys-144/Lys-145 doublet co-operate to influence the subcellular localization of Max is unknown. As mentioned above, Lys-144 and Lys-145 are within an established, but 'weak'/dispensable, C-terminal nuclear localization signal in Max, while Lys-57 is part of the H2 α -helix within the HLH dimerization domain. However, the Lys-57 side chain is not part of the dimerizing interface of the HLH domain, but is exposed to the solvent in Max–Max and Myc–Max complexes [57–59]. Consistent with this, the replacement of Lys-57 with glutamine did not detectably influence Max dimerization or its interaction with Myc, and there were no differences in the *in vitro* DNA binding activities of the Max mutants that would explain the defect of selectively Max K57Q/K144Q/K145Q in nuclear localization and in repression of Myc transactivation. Thus the Lys-57 residue (and the Lys-144/145 doublet) might mediate interactions of Max with other factors that control its subcellular localization.

The above observations suggest the possibility that reversible acetylation might regulate the nuclear–cytoplasmic shuttling of specific Max complexes. However, the role of Max acetylation, the specific Max complexes affected, and the physiological conditions that induce acetylation (or prevent deacetylation) of specific Max complexes remain to be established. For instance, Max is essential for Mad4 to localize to the nucleus [56], and Myc is acetylated by p300 (and GCN5) at residues located within a nuclear localization signal [44–46]. In addition, Max could be differentially acetylated in different complexes, due for instance to the selective association of HDACs with Mad and Mnt proteins [10,25–29] or to the recruitment of p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein] HATs by Myc [43,46]. These possibilities and the potential interplay between Max acetylation and phosphorylation warrant further investigation. Given the different post-translational modifications already identified, Max is likely to represent an additional regulatory switch for signalling pathways that control the output of the Myc/Max/Mad network.

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