Human NAT10 Is an ATP-dependent RNA Acetyltransferase Responsible for N4-Acetylcytidine Formation in 18 S Ribosomal RNA (rRNA)*

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Background: Post-transcriptional modifications of rRNAs play important roles in biogenesis and function of ribosome.

Results: NAT10 is an ATP-dependent RNA acetyltransferase responsible for N4-acetylcytidine formation of 18 S rRNA.

Conclusion: NAT10 and ac4C1842 are required for pre-18 S rRNA processing.

Significance: 40 S subunit formation is regulated by a single acetylation of 18 S rRNA, implying a regulatory mechanism for ribosome biogenesis by sensing the cellular energy budget.

Human N-acetyltransferase 10 (NAT10) is known to be a lysine acetyltransferase that targets microtubules and histones and plays an important role in cell division. NAT10 is highly expressed in malignant tumors, and is also a promising target for therapies against laminopathies and premature aging. Here we report that NAT10 is an ATP-dependent RNA acetyltransferase responsible for formation of N4-acetylcytidine (ac4C) at position 1842 in the terminal helix of mammalian 18 S rRNA. RNA-mediated knockdown of NAT10 resulted in growth retardation of human cells, and this was accompanied by high-level accumulation of the 30 S precursor of 18 S rRNA, suggesting that ac4C1842 formation catalyzed by NAT10 is involved in rRNA processing and ribosome biogenesis.

The ribosome is a large ribonucleoprotein complex that translates the genetic information carried by mRNA into protein sequence. The eukaryotic ribosome consists of the small 40 S subunit and the large 60 S subunit, each of which contains rRNAs and ribosomal proteins. In mammals, 47 S pre-rRNAs transcribed from the rDNA repeat region are processed into 18 S (see Fig. 1A), 5.8 S, and 28 S rRNAs through multistep processing events (see Fig. 2E) (1). More than 200 factors and small nucleolar RNAs are involved in ribosome assembly, and mutations in these assembly factors often result in pathological consequence (2, 3). During rRNA processing, rRNAs are modified post-transcriptionally; these modifications are involved in fine-tuning translational fidelity and/or modulating ribosome biogenesis (4). The most abundant rRNA modifications in mammals, 2′-O-methylation and pseudouridylation (Ψ), are introduced by box C/D and box H/ACA small nucleolar RNA-guided enzymes, respectively (5). In addition, 18 S and 28 S rRNAs contain several small nucleolar ribonucleoprotein-independent modifications (4), which are introduced by specific RNA-modifying enzymes. N4-Acetylcytidine (ac4C)2 (see Fig. 1A) is present in 18 S rRNA from rat, chicken, and Saccharomyces cerevisiae, although the exact positions of ac4C in these species remained unknown (6). In Dictostelium discoideum, ac4C is present at position 1844 in the 3′ terminal region of 18 S rRNA (7). Together, these observations indicate that ac4C is a highly conserved modification in eukaryotic 18 S rRNAs.

Very recently, we reported that ac4C is present at position 1773 in the 18 S rRNA of S. cerevisiae and found an essential gene, KRE33, which we renamed RRA1 (ribosomal RNA cytidine acetyltransferase 1) encoding an RNA acetyltransferase responsible for ac4C1773 formation (8). Here, we report that NAT10, a human homolog of Rra1p, is an ATP-dependent RNA acetyltransferase responsible for formation of ac4C at position 1842 in mammalian 18 S rRNA.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Growth Conditions**—HeLa and HEK293 cells were grown in DMEM supplemented with 10% FBS, at 37 °C under a humidified atmosphere containing 5% CO2.

**RNA Extraction and Preparation of 18 S rRNA**—Total RNA was extracted from cells using TriPure isolation reagent (Roche Diagnostics) and resolved by PAGE on 4% polyacrylamide gels containing 7 M urea. 18 S rRNA was excised from the gel and extracted from the gel slice by the “crush and soak” method in elution buffer (0.3 M NaOAc, 1 mM EDTA, 0.1% SDS).

**RNA Mass Spectrometry**—Highly sensitive analysis of RNA fragments by mass spectrometry was carried out essentially as described previously (9, 10). The isolated 18 S rRNA was digested with RNase T1, at 37 °C for 30 min in a 10-μl reaction mixture containing 10 mM ammonium acetate (pH 5.3) and 5 units/μl RNase T1, (Epitrend), followed by the addition of an equal volume of 0.1 M triethylamine acetate (pH 7.0). The digest was then subjected to capillary LC coupled with nano-electrospray ionization-mass spectrometry (ESI-MS) using a linear ion trap-orbitrap hybrid mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific).

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‡ The abbreviations used are: ac4C, N4-acetylcytidine; NAT10, N-acetyltransferase 10; CID, collision-induced dissociation; ESI, electrospray ionization; DIG, digoxigenin; ITS1, internal transcribed spacer 1; ETS, external transcribed spacer.
RNAi—Sequences of siRNAs used in this study are listed in Table 1. Two siRNAs targeting NAT10, siNAT10a and siNAT10c, were designed using the “siExplorer” algorithm (11). The sequence of siNAT10b was obtained from the literature (12). These siRNAs were chemically synthesized by FASMAC Co., Ltd. HeLa cells (2.5 × 10⁵ cells) or HEK293 cells (2.5 × 10⁵ cells) were transfected with siRNA (1 or 10 nM, respectively) using Lipofectamine RNAiMAX (Invitrogen). Cells were harvested 4 days after transfection. The efficiency of RNAi was estimated by measuring the steady-state level of NAT10 mRNA, normalized to the level of ACTB mRNA, on a LightCycler 480 real-time PCR system (Roche Diagnostics). Primer sets are listed in Table 1. Knockdown of NAT10 was confirmed by checking for RNAi knockdown by quantitative RT-PCR.

**Measuring Cell Proliferation**—HeLa cells (2.5 × 10⁵ cells) were transfected with siRNA (final concentration 1 nM) as described above. Cell proliferation in each well was evaluated daily using alamarBlue (BIOSOURCE International, Inc.) according to the manufacturer’s instructions. The absorbance of the wells was read at 570 nm and 600 nm with a SpectraMax 190 plate reader (Molecular Devices, Inc.). Cell proliferation was calculated from fraction of the reduced dye as percentage of reduced.

**Flow Cytometry**—HeLa cells were harvested 4 days after siRNA transfection, stained with annexin V and propidium iodide using the annexin V-FITC apoptosis kit (BioVision), and analyzed by flow cytometry (FACS, BD Biosciences).

**Northern Blotting**—Total RNA (1 μg) in loading solution (10 mM MOPS, 2.5 mM NaOAc (pH 5.0), 0.5 mM EDTA-NaOH (pH 8.0), 6.5% formaldehyde) was denatured at 65 °C for 10 min and left on ice. After the addition of 2 μl of dye solution (0.25% bromphenol blue, 0.25% xylene cyanol, 50% glycerol, and 1 mM EDTA-NaOH (pH 8.0)), total RNA was electrophoresed on a 1% agarose gel (10 × 10 cm, SeaKem GTG agarose, TaKaRa) containing 0.6 M formaldehyde in MOPS buffer (20 mM MOPS, 5 mM NaOAc (pH 5.0), 1 mM EDTA-NaOH (pH 8.0)) for 2.5 h at 100 V. The gel was stained with ethidium bromide in alkaline solution (50 mM NaOH and 1 μg/ml ethidium bromide), followed by two rounds of neutralization with 200 mM NaOAc (pH 5.0) for 20 min each. rRNAs in the gel were visualized using a FLA7000 imaging analyzer (Fuji-film). After equilibrating the gel twice with 10 × SSC for 10 min each, RNAs were transferred to a Hybond-N+ nylon membrane (GE healthcare) in 10 × SSC using a model 785 vacuum blotter (Bio-Rad).

Northern blotting was carried out using the DIG Northern starter kit (Roche Diagnostics). The RNA probes (~200-mers, complementary to 5’-ETS or ITS1) were transcribed by T7 RNA polymerase in the presence of digoxigenin-11-UTP. Primer sequences used to amplify the templates for the RNA probes are listed in Table 1. Hybridization was carried out at 68 °C overnight. The hybridized bands were visualized by CDP-Star chemiluminescence (Roche Diagnostics), using alkaline phoshatase-conjugated anti-digoxigenin, and detected on an LAS 4000 mini (GE healthcare).

**Sucrose Density Gradient Centrifugation and Ribosomal Subunit Profiling**—HeLa cells (1.6–2.4 × 10⁶ cells) were treated with siRNAs for luciferase (siLUC) and NAT10 (siNAT10c) as described under “RNAi.” The cells were harvested, washed with ice-cold PBS, and resuspended with 300 μl of lysis buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 15 mM EDTA, 0.5% Triton X-100 (v/v), 1 × cOmplete EDTA-free protease inhibitor mixture (Roche Diagnostics), 0.2 units/μl SUPERase-In RNase inhibitor (Life Technologies)), followed by passing a 25-gauge needle 20 times to prepare the whole-cell lysate. The lysate was gently rotated for 10 min at 4 °C and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was transferred to a new tube, recentrifuged at 10,000 × g for 10 min at 4 °C, and loaded onto a 10–40% (w/v) linear gradient of sucrose containing 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 15 mM EDTA, 1 mM DTT, followed by ultracentrifugation in SW41Ti rotor (Beckman...
Coulter) at 37,000 rpm for 3.5 h at 4 °C. The sucrose gradient was fractionated by a Piston gradient fractionator (BioComp), and ribosomal subunits were monitored by absorbance at 254 nm using a UV monitor (AC-5200, ATTO) and a digital recorder (AC-5150, ATTO).

Expression and Purification of Recombinant Nat10—The gene of mouse Nat10 was amplified by PCR using a cDNA clone from the Mammalian Gene Collection (Invitrogen) as a template, KOD-Plus-DNA polymerase (Toyobo), and a set of primers (Table 1). The PCR product was inserted into the SbfI and Ascl sites of a modified pPICZ vector (Invitrogen) to generate pPICZ-Nat10. The resultant vector was linearized with BstXI and then electroporated into Pichia pastoris strain SMD1168H. Electroporation was performed in a 0.2-cm cuvette in a Micro-Pulser (Bio-Rad) with a pulse voltage of 2 kV. Overproducing transformants harboring multiple copies of the Nat10 genes were selected with 500 µg/ml Zeocin on YPD plates and then cultured in buffered glycerol-complex (BMGY) medium at 30 °C for 24 h. Cells were collected by centrifugation at 4,500 × g for 15 min and then disrupted using a Multi-Beads Shocker (Yasui Kikai) in disruption buffer consisting of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF, and 5 mM 2-mercaptoethanol. Cell debris was removed by centrifugation at 40,000 × g for 30 min at 10 °C, and the supernatant was loaded onto a HisTrap HP column (GE Healthcare) pre-equilibrated with His binding buffer consisting of 20 mM HEPES-NaOH (pH 7.6), 300 mM NaCl, 10% glycerol, and 5 mM 2-mercaptoethanol. The recombinant Nat10 was eluted with a 0.3–2M gradient of imidazole in His binding buffer with heparin binding buffer consisting of 20 mM HEPES-NaOH (pH 7.6), 400 mM NaCl, 10% glycerol, and 5 mM 2-mercaptoethanol. After washing with His binding buffer containing 40 mM imidazole and 0.05% complete EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics), recombinant Nat10 with N-terminal hexahistidine tag was eluted with a 0.04–0.4 M gradient of imidazole in His binding buffer. Fractions containing Nat10 were loaded onto a HiLoad Heparin HP column (GE Healthcare) pre-equilibrated with heparin binding buffer consisting of 20 mM HEPES-NaOH (pH 7.6), 300 mM NaCl, 10% glycerol, and 5 mM 2-mercaptoethanol. The recombinant Nat10 was eluted with a 0.3–2 M gradient of NaCl in heparin binding buffer. The collected fractions were further purified on a HiLoad 26/60 Superdex 200 column (GE Healthcare) pre-equilibrated with size exclusion chromatography (SEC) buffer consisting of 20 mM HEPES-NaOH (pH 7.6), 300 mM NaCl, 10% glycerol, and 1 mM dithiothreitol. The purified Nat10 was stored at −80 °C until use.

**In Vitro Reconstitution of ac4C1842 Formation—**The DNA templates for in vitro transcription of RNA fragments carrying helix 45 of 18 S rRNA and its variants were constructed by PCR using synthetic DNAs (Table 1). The substrate RNA was transcribed by T7 RNA polymerase essentially as described (13). The transcripts were extracted from the reaction mixture with phenol-chloroform, passed through a NAP-5 column (GE healthcare) to remove free NTPs, and precipitated with ethanol. The transcript was further purified on a 10% PAGE gel containing 7 M urea.

**In vitro reconstitution of ac4C formation was carried out at 30 °C for 2 h in a reaction mixture (10 µl) consisting of 50 mM HEPES-KOH (pH 7.6), 150 mM KCl, 5 mM MgCl2, 1 mM DTT, 1 mM ATP, 1 mM acetyl-CoA, 1 µM substrate RNA, and 5.1 µM recombinant Nat10. After the reaction, the RNA fragment was extracted with phenol-chloroform, followed by ethanol precipitation. The ac4C formation was analyzed by mass spectrometry as described above.

**RESULTS AND DISCUSSION**

To determine the exact position of ac4C modification, we performed mass spectrometric analysis on 18 S rRNA from HEK293 cells. RNase T1-digested RNA fragments of 18 S rRNA were subjected to capillary LC coupled with nano-ESI-MS to detect RNA fragments containing modified nucleotides. We were able to assign most RNA fragments deduced from the human 18 S rRNA sequence with modified nucleotides. The doubly charged negative ion of the ac4C-containing hexamer was detected (Fig. 1B) and further analyzed by collision-induced dissociation (CID). This analysis revealed that the hexamer sequence was UUUUAc4CGp (Fig. 1C); thus, ac4C is present at position 1842 of the helix 45 in 18 S rRNA (Fig. 1A), which is equivalent to ac4C1844 in *D. discoideum* 18 S rRNA (7) and ac4C1773 in *S. cerevisiae* 18 S rRNA (8).

ac4C is a modified base frequently found in tRNAs and rRNA from all domains of life (14). In bacteria, ac4C is present at the wobble position of elongator tRNA^Met Met. We previously identified tRNA^Met cytidine acetyltransferase (TmcA) in *Escherichia coli*, which catalyzes ac4C formation in the presence of acetyl-CoA and ATP (15). TmcA has two catalytic domains, RNA helicase (ATPase) and N-acetyltransferase. According to the crystal structure of *E. coli* TmcA (16), the RNA helicase domain contains ADP (a hydrolysis product of ATP), whereas the N-acetyltransferase domain interacts with acetyl-CoA. Biochemical and genetic studies of *E. coli* TmcA revealed that both domains are required for ac4C formation in tRNA (15, 16). Orthologs of *tmcA* are widely distributed among archaea and euukaryotes. Based on the fact that ac4C is present in the 3′-terminal region of 18 S rRNAs in euukaryotes (6, 7), the euukaryotic homolog of TmcA was proposed to be the RNA acetyltransferase responsible for ac4C formation in 18 S rRNA (15). In fact, we recently reported that Rra1p (ribosomal RNA cytidine acetyltransferase 1), which is a yeast homolog of TmcA, catalyzes ac4C1773 formation using acetyl-CoA and ATP as substrates (8).

In humans and mouse, N-acetyltransferase 10 (NAT10 and Nat10, respectively) is an ortholog of bacterial TmcA and yeast Rra1p. Human NAT10 (also known as hALP) was initially identified as a transcriptional factor that interacts with the promoter region of hTERT (human telomerase reverse transcriptase) (17). Recombinant NAT10 (amino acids 164–834) lacking the N-terminal domain has the ability to acetylate calf thymus histones in vitro in the presence of acetyl-CoA (without ATP), indicating that NAT10 has a histone acetyltransferase activity that might promote hTERT transcription via decondensation of chromatin structure (17). In addition, it was reported that NAT10 is associated with U3 small nuclear RNA, and acetylates upstream binding factor to activate rRNA transcription (18). NAT10 is predominantly localized to the nucleolus during interphase, but to the mitotic midbody during telophase (19). Depletion of NAT10 causes defects in nuclear assembly and cytokinesis, and also reduces the level of acetylated α-tubulin, leading to G2/M arrest (19). NAT10 can also
acetylate porcine tubulin *in vitro* (20), although the acetylated residues have not yet been determined. These observations indicate that NAT10 plays an important role in cell division by facilitating reformation of the nucleolus and midbody in the late phase of mitosis.

To determine whether NAT10 is the RNA acetyltransferase responsible for ac^4^C formation in 18 S rRNA, we knocked down NAT10 in HeLa cells using three distinct siRNAs. We confirmed the absence of endogenous NAT10 in knockdown cells by Western blotting (Fig. 2A). As reported previously (19), NAT10 knockdown cells grew more slowly than control cells (siLUC) (Fig. 2B). Four days after the transfection, the NAT10 knockdown cells were harvested and stained with annexin V and propidium iodide, followed by flow cytometry. The fraction of apoptotic cells increased significantly upon NAT10 depletion (Fig. 2C), suggesting that the growth retardation of NAT10 knockdown cells can be partly explained by an elevated rate of apoptotic death. Next, we prepared 18 S rRNA from the knockdown cells and subjected it to capillary LC/ESI-MS analysis. Intriguingly, the level of the ac^4^C-containing hexamer fragment (UUUCac^4^CGp) was markedly reduced relative to the control fragment in NAT10 knockdown cells (Fig. 2D). This result
strongly suggested that NAT10 is responsible for ac\textsuperscript{4}C1842 formation in 18 S rRNA. In the case of \textit{S. cerevisiae}, RRA1 is an essential gene, and temporal depletion of Rra1p resulted in significant accumulation of the 23 S pre-rRNA, leading to complete loss of 18 S rRNA and 40 S subunit (8). We observed complete absence of ac\textsuperscript{4}C1773 in the accumulated 23 S pre-rRNA, but little reduction of ac\textsuperscript{4}C1773 in 18 S rRNA, which is likely to originate from a residual pool of 40 S subunit that accumulated in the cytoplasm before depletion of Rra1p (8). These results suggest that ac\textsuperscript{4}C1773 formation catalyzed by Rra1p plays an essential role in processing of the 23 S pre-rRNA to yield 18 S rRNA. Unlike the case of \textit{S. cerevisiae}, apparent reduction of ac\textsuperscript{4}C1842 in 18 S rRNA was observed in human cells upon NAT10 depletion. The ac\textsuperscript{4}C1842 formation mediated by NAT10 is not essential for pre-rRNA processing to yield 18 S rRNA.

Given that NAT10 localizes to the nucleolus and is involved in nucleolar assembly (19), it is likely that this protein plays a role in ribosome biogenesis. To examine whether 18 S rRNA processing is affected by NAT10 knockdown, we detected precursors of 18 S rRNAs by Northern blotting with three probes complementary to the 5'-ETS and ITS1 (Fig. 2E). Processing of the 47 S pre-rRNA was initiated with endonucleolytic cleavages at site 01 in the 5'-ETS and site 02 in the 3'-ETS to generate 45 S pre-rRNA, which was then processed into 30 S pre-rRNA by cleavage at site 2 in ITS1, splitting pathways for small subunit and large subunit maturation. 30 S pre-rRNA was further processed into 21 S pre-rRNA by removal of the 5'-ETS. The 45 S and 30 S pre-rRNAs were detected by the three probes, whereas 21 S pre-rRNA was detected only by probes b and c.

Further study will be necessary to reveal in molecular detail why this single acetylation mediated by NAT10 is involved in rRNA processing.

To detect an appropriate enzymatic activity of NAT10 to participate in ac\textsuperscript{4}C formation, we obtained recombinant Nat10 expressed in \textit{P. pastoris} and purified it to homogeneity. A 50-mer RNA fragment containing hexamers in the mass chromatograms were normalized to those of the control fragments.

As shown in Fig. 2H, the ac\textsuperscript{4}C-containing hexamer was clearly detected only in the presence of both ATP and acetyl-CoA. CID analysis revealed that the ac\textsuperscript{4}C in the hexamer was in position 1842.

A truncated recombinant NAT10 (amino acids 164–834) lacking the N-terminal RNA helicase domain has \textit{in vitro} lyssine acetyltransferase activity toward histones in the presence of acetyl-CoA; this activity does not require ATP (17). In this study, we clearly showed that a full-length Nat10 has RNA acetyltransferase ability that can catalyze formation of ac\textsuperscript{4}C1842 in 18 S rRNA in the presence of both acetyl-CoA and ATP; thus,
ac4C1842 formation requires both catalytic domains of NAT10. It remains to be determined whether full-length NAT10 has efficient and specific lysine acetyltransferase activity toward histones or other proteins.

Collectively, the results of this and previous studies indicate that NAT10 has multiple functions required for ribosome biogenesis, nucleolar architecture, cytokinesis, and mitosis. Moreover, NAT10 is highly expressed in malignant tumors (19, 21), and it is essential for growth of a subtype of epithelial ovarian cancer with poor prognosis (22). Recent work demonstrated that NAT10 is the molecular target of a chemical compound called “Remodelin,” which treats lamopathies, including premature aging syndromes, by correcting nuclear architecture (20). Functional studies of NAT10 as an rRNA acetyltransferase should facilitate development of novel strategies for treating cancer and premature aging.

Identification of NAT10 as an rRNA acetyltransferase provides a conceptual advance in RNA epigenetics (23). In S. cerevisiae, upon depletion of nuclear acetyl-CoA, we observed temporal accumulation of the 23 S pre-rRNA, indicating that Rra1p modulates 40 S biogenesis by sensing nuclear acetyl-CoA concentration (8). Further studies will be required in mammals to investigate the physiological significance of ac4C modification of 18 S rRNA in coordination with ribosome biogenesis with the cellular energy budget.

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