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Biochemical and Biophysical Research Communications 301 (2003) 187–191
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Acetylation and level of mitochondrial transcription factor A in several organs of young and old rats

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Received 13 December 2002

Abstract

To gain further information on the role of mitochondrial transcription factor A (TFAM) in mitochondrial biogenesis, we studied the post-translational modifications of the protein in 6- and 28-month-old rat liver. Mass spectrometry and immunoblot analysis revealed that TFAM was acetylated at a single lysine residue and that the level of acetylation did not change with age. The measurement of the content of TFAM and of mitochondrial DNA (mtDNA) in several organs (cerebellum, heart, kidney, and liver) of young and old rats showed an age-related increase of mtDNA and TFAM in all the organs analyzed, except in heart. These data are discussed in the light of the multiple roles of TFAM in mitochondrial biogenesis and of the age-related change of the mitochondrial transcription.

Keywords: mtDNA; TFAM; Aging; Copy number; Acetylation

Several characteristics of mtDNA, such as "vertical" maternal transmission, high rate of mutation, poor capacity of repair, and the higher likelihood that mutations may be deleterious due to the lack of introns, suggested a pathogenic hypothesis of normal aging based on the cumulative effects of mtDNA mutations [1,2]. Indirect support for this hypothesis was provided by biochemical studies showing an age-related decline of respiratory rate in muscle mitochondria from subjects varying in age from 16 to 92 years [3]. Muller-Hocker [4] first observed that the number of COX-negative fibers increased with age in heart and muscle of normal individuals. Further reports confirmed this finding and showed also that the age-related decline in OXPHOS was associated with an increase in mtDNA damage in the form of base modifications, large scale deletions, and point mutations [5–7]. The replication and transcription of the mitochondrial genome depend exclusively on nuclear-gene products. One of these products, TFAM, plays a complex role in the regulation of both processes: it is required for mtDNA maintenance and together with two other factors, TFB1 and TFB2, stimulates mitochondrial transcription [8]. Moreover it has been shown that homozygous disruption of *TFAM* gene and tissue-specific *TFAM* knockout cause severe respiratory chain deficiency and increased apoptosis in mice embryos [9]. The content of TFAM and mtDNA increased during aging in human skeletal muscle; since this increase was associated with that of NRF-1 it is likely that it is the result of a compensatory response, which acts through nuclear-mitochondrial cross-talk [10].

Protein acetylation on the ε-amino groups of lysine is an important reversible modification that regulates gene expression. Although acetylation has been first described for histone proteins, site-specific acetylation of a growing list of non-histone protein, has been shown to play an important role in transcriptional regulation and cell proliferation [11,12]. In particular HMG1 and other HMG-box proteins are acetylated. This post-synthetic modification involves lysine at positions 2 and 11 in the N-terminal region of HMG1, located near the DNA-binding domain [13,14]. Binding affinity of acetylated protein is significantly higher to

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doi:10.1016/S0006-291X(02)03008-5

distorted DNA structures (due to UV light or cisplatin damage) and four-way junctions [15]. TFAM shares most of the characteristics of HMG proteins since it contains two HMG-box-like domains, causes bending and wrapping of mtDNA [16], binds to four-way DNA junctions [17], and is able to recognize cisplatin-damaged DNA [18] and oxidatively damaged DNA [19].

In the present work we have investigated whether TFAM was acetylated. Moreover, we have determined the relationship between mtDNA copy number and TFAM level during aging by measuring mtDNA and TFAM content in several rat organs (cerebellum, heart, kidney, and liver). The results of these studies show that TFAM is acetylated and that the extent of acetylation does not change appreciably during aging. Moreover we found a close association between the changes of TFAM and mtDNA level in aging.

Materials and methods

Animal samples. Male Wistar rats were maintained two per cage on a 12:12 h light–dark cycle at 25 °C and had access to standard laboratory chow and water ad libitum. All procedures were in accordance with the guiding principles in the care and use of laboratory animals of Bari University. Four rats for each of different ages (6 and 28 month) were killed and four of their own organs (cerebellum, heart, kidney, and liver) were quickly excised, immediately frozen in isopentane cooled by liquid nitrogen, and stored in liquid nitrogen until analysis.

Determination of mtDNA content in different rat organs. Total DNA was prepared from about 50–100 mg of frozen tissue as described by Reyes et al. [20] and suspended in 100 μl water. Five micrograms of total DNA was digested with *Pvu*II (MBI-Fermentas) and run on a 0.35% agarose gel (PFGE agarose, Celbio). The gel was blotted onto Hybond-N membrane (Amersham-Pharmacia) and simultaneously hybridized with a mitochondrial and a nuclear probe. The mitochondrial probe was a 482 bp fragment containing part of the ND2 gene subcloned in Blue Scribe vector. The nuclear probe was a 413 bp fragment containing part of the 18S rRNA gene [21] and subcloned in the TA vector (Clontech). Both probes were labelled by random priming (Random Prime DNA Labelling Kit, Invitrogen) and used in a 10:1 ratio of the nuclear DNA (nDNA) probe to the mtDNA probe. Blotting, prehybridization, and washings were carried out as described by Reyes et al. [20]. The filter was exposed to a X-ray film at −70 °C with an intensifying screen and the hybridization signals were quantified both by densitometry with LKB-Pharmacia (Uppsala Sweden) Ultrascan-XL laser densitometer, equipped with a GelScan-LX-Evaluation software, and by phosphorimaging using the program ImageQuant (Pharmacia). Both kind of analysis gave similar results.

Purification of rat TFAM. TFAM protein was purified from 6-, 18-, and 28-month-old rats as previously described [22]. Briefly, mitoplasts were prepared from rat liver mitochondria and lysed with Lubrol at a final concentration of 0.16 mg Lubrol per milligram of mitoplast proteins. The suspension was centrifuged at 130,000*g* and the supernatant, containing matrix proteins, was loaded onto a Heparin Sepharose CL-6B (Pharmacia) column. The protein fraction eluted at 0.5 M KCl was affinity purified using magnetic beads coated with the region of ND2 gene containing the TFAM-binding site (nucleotides 4252–4772 of rat mtDNA).

Mass spectrometry. Affinity purified TFAM was subjected to matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) using a VG TofSpec (Micromass, UK) equipped with a 337 nm nitrogen laser. The analysis was carried out by the Biopolymer Mass Spectrometry Core Facility, Weill Medical College of Cornell University, New York.

Western blotting. Total proteins were extracted by treating 100–200 mg of frozen rat tissues with 600 μl of 50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, and 10% glycerol. Proteins were quantified with the Bradford method (Bio-Rad) according to the supplier's instructions. Total proteins were separated in 12% SDS–polyacrylamide slab minigels and electroblotted onto PVDF membrane (Millipore or Amersham Bioscience). For acetylation analysis, samples of affinity purified TFAM were separated by SDS–PAGE and electroblotted. The membrane was subjected to incubation with primary antiserum, washings, incubation with secondary antibodies. and final washings according to Lezza et al. [10]. Primary antibodies were: rabbit anti-rat TFAM antiserum, a gift from Dr. H. Hinagaki (Department of Chemistry, National Industrial Research Institute of Nagoya, Japan), at 1:2500 dilution; mouse monoclonal anti-actin ascites (Oncogene Research) at 1:2000 dilution; and acetylated-lysine polyclonal antibody (Cell Signaling Technology) at 1:1000 dilution. Secondary antibody were goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) at 1:5000 dilution and horseradish peroxidase goat anti-mouse IgM (Oncogene Research) at 1:5000 dilution. Blots were visualized by chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham Bioscience) according to the supplier's instructions. Quantitative analysis of the blots was carried out by laser densitometry with LKB-Pharmacia (Uppsala Sweden) Ultrascan-XL equipped with a GelScan-LX-Evaluation software. Preliminary titration experiments allowed us to establish the protein extract range (2.5–25 μg), which gave a linear response of the signal. Different exposures of each blot, in the time range between 5 s and 5 min, were taken in order to ensure the linearity of the response for both assayed proteins.

Results

Acetylation of TFAM

In order to test whether TFAM was acetylated, the protein was purified from liver of 6-month old rat and subjected to MALDI-TOF mass spectrometry. As shown in Fig. 1A mass analysis, using bovine carbonic anhydrase as internal standard, showed a molecular species at m/z 23,721. Other internally calibrated spectra (not shown) resulted in molecular masses from 23,706 to 23,730 Da. The observed mass is significantly above the theoretical average molecular mass of 23,673, supporting the presence of a single acetylation (the mass of the acetyl-group is 42). The acetylation of TFAM was confirmed by western blot analysis. Purified TFAM was run on a 12% acrylamide SDS gel, electroblotted onto PVDF membrane, and probed with antibodies against acetylated lysine. Fig. 1B shows that the antibody recognizes TFAM (lanes 1 and 2) and acetylated BSA (lane 3), but does not react with non-acetylated BSA (lane 4).

To test whether the acetylation of TFAM changes during aging, we purified TFAM from 18- and 28-month old rats; then the same amount of protein was run on a SDS 12% gel and probed with acetylated-lysine antibody. Fig. 1C shows that the level of acetylated TFAM did not appreciably change during aging.

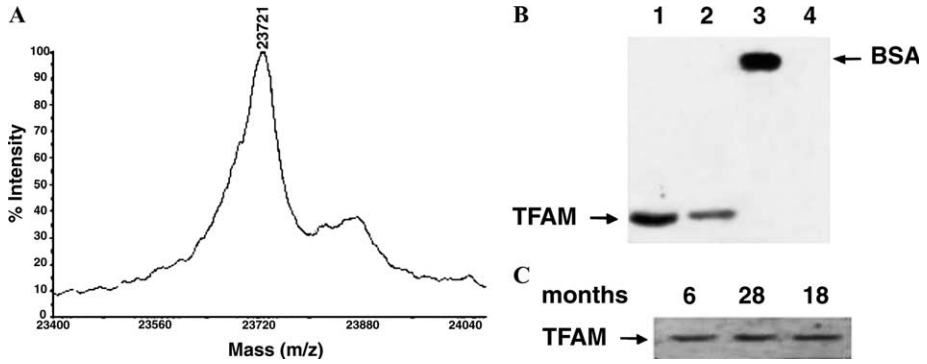

Fig. 1. TFAM acetylation. (A) Mass spectrometry of purified TFAM from 6-month-old rats. MALDI-TOF spectrum was obtained with Carbonic Anhydrase as internal standard spotted together with sample. The principal component resulted in a molecular mass of 23,721 Da. (B) Western blotting of affinity purified TFAM from 6-month-old rats. Lanes 1 and 2 contained 5 and 2 μl of affinity purified TFAM, respectively; lane 3 contained 5 ng of acetylated BSA (Promega); and lane 4 contained 100 ng of non-acetylated BSA. (C) Immunoblot analysis of TFAM purified from liver of 6-, 28-, and 18-month-old rats.

Content of mtDNA and TFAM in different organs of young and old rats

To analyze the variation of mtDNA copy number during rat aging, total DNA extracted from cerebellum, heart, kidney, and liver of 6- and 28-month-old rats was digested with *Pvu*II and blot hybridized with a mitochondrial and a nuclear probe. The mitochondrial probe detected a band of 16.5 kbp, corresponding to linearized mtDNA, whereas the nuclear probe detected a 12 kbp band, corresponding to a *Pvu*II fragment of the nuclear 18S rDNA gene. The ratio of the intensities of the two bands was used to estimate the relative amount of mtDNA in each subject. Fig. 2A shows a representative autoradiography from two animals of each age group, whereas Fig. 2B reports the quantitative data. The histograms show that aged animals have a higher content of mtDNA (from 1.5 to 2.4 times) compared to young individuals in cerebellum, kidney, and liver. In heart the mtDNA content did not change appreciably. In order to

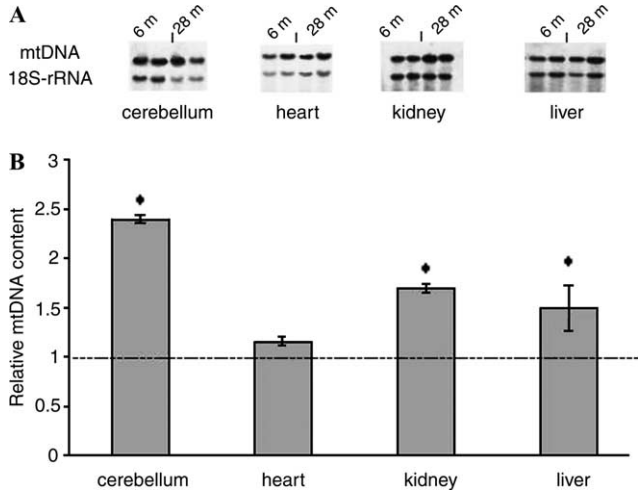

Fig. 2. Effects of aging on the mtDNA content in different rat organs. (A) Southern blot from two animals of each age group. Top and bottom bands show signals from mitochondrial and nuclear DNAs, respectively. (B) Quantitative estimate of the mtDNA copy number in different rat organs. The histograms show, for each organ, the mtDNA content (mtDNA/nDNA) of 28-month rats normalized to that of 6-month-old rats as indicated by the broken line. Bars represent the average ± SD of at least three experiments, each performed on independent DNA preparations from four young and four old animals. *, statistically significant result ($p < 0.05$) for young-old comparison using Student's t test for unpaired data.

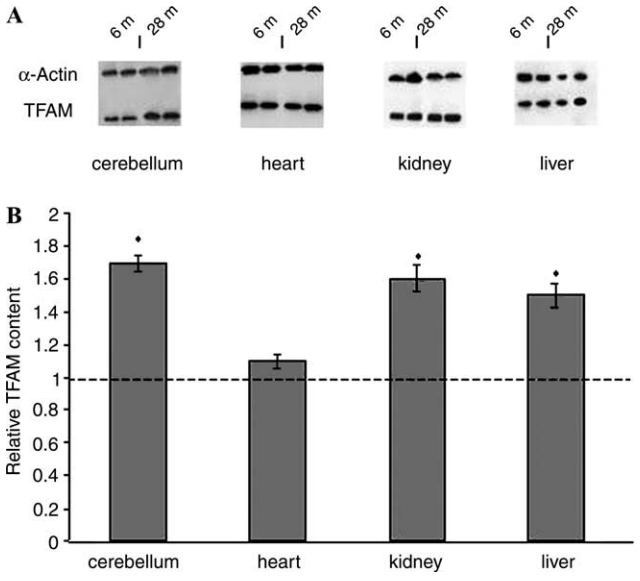

Fig. 3. Effects of aging on the TFAM protein level in different rat organs. (A) Western blot from two animals of each age group. Top and bottom bands show signals from the rat α-actin and TFAM, respectively. (B) Quantitative estimate of TFAM content in different rat organs. The data were obtained determining for each organ the TFAM/α-actin ratio of 28-month-old rats normalized to that of young rats as indicated by the broken line. Bars represent the average ± SD of values obtained from three experiments, each performed on independent protein preparation from four young and four old animals. *, statistically significant result ($p < 0.05$) for young-old comparison using Student's t test for unpaired data.

investigate the change of TFAM content during aging, total proteins from cerebellum, heart, kidney, and liver of 6- and 28-month-old rats were separated by SDS–PAGE, blotted onto PVDF membrane, and incubated with polyclonal antibodies against TFAM and α-actin. The result of a representative experiment is shown in Fig. 3A, whereas Fig. 3B reports the quantitative analysis of the data obtained normalizing vs actin the amount of TFAM. As shown in the histograms, all the organs of old animals, except heart, have a statistically significant higher content (from 1.5 to 1.7 times) of TFAM compared to the young ones.

Discussion

The results reported in this paper provide further information on qualitative and quantitative changes of TFAM during aging in different rat organs. Post-translational modification of a mitochondrial DNA-binding protein is reported here for the first time. TFAM is acetylated at a single lysine residue and the extent of acetylation does not change substantially with age. Protein acetylation is a regulatory mechanism for the expression of nuclear genes. In particular histone acetylation activates gene expression probably because the neutralization of part of histone positive charge weakens histone–DNA or nucleosome–nucleosome interactions [23]. This destabilizes the nucleosome structure and gives to other factors, such as the transcription complex, more access to a genetic locus. Since TFAM behaves as a histone-like protein its acetylation might have a role in regulating the expression of mitochondrial genes or in general the mtDNA maintenance. TFAM acetylation might regulate the binding of the protein to DNA, DNA conformational changes or interactions with other proteins of mtDNA transcription and replication apparatus. In addition, as it has been recently reported that the acetylation of p73 increases the activation of proapoptotic genes [24], and since mitochondria are involved in the apoptosis [9], the TFAM acetylation might be involved in the regulation of this process.

MtDNA and TFAM content in cerebellum, liver, and kidney showed a similar age-related increase from 1.5- to 2.4-fold. Such increases agree with similar data reported in human skeletal muscle, where Lezza et al. [10] and Pesce et al. [25] found that both TFAM and mtDNA content increased during aging. A close relationship between TFAM and mtDNA levels was found also in *TFAM* knockout mouse [26] and in pathologies with mtDNA depletion [27,28]. In cerebellum the increase of both TFAM and mtDNA content can compensate the substantial (about 60%) decrease of the transcriptional activity, measured as mtRNA/mtDNA molecules [29], so that the total number of mitochondrial transcript molecules remains unchanged. On the contrary in liver the increase of both TFAM and mtDNA is associated with an unchanged transcriptional activity, whereas in the heart the unchanged mtTFA and mtDNA levels are associated with a moderate decrease (about 30%) of mtRNA/mtDNA molecules [29]. Although the different behavior of these organs can be due to many factors, we can suggest some explanation. In cerebellum, which is a post-mitotic tissue, the increase of mtDNA level compensates the reduced transcriptional activity. For the heart it must be considered that it is a tissue with a unique incessant myocardial contractile activity and with a high efficiency of mitochondrial antioxidant defence systems. It is known that mitochondrial manganese-superoxide dismutase activity has large age-related increments in rat heart [30,31] and that a low level of mtDNA deletions is found in this organ [32]. These observations support the possibility that in heart mtDNA is less damaged than in other tissues and that therefore, it will be less need to increase mtDNA and TFAM levels. In liver, the increase of TFAM and mtDNA does not seem to depend on the change of the mitochondrial transcript level; this may be related to the active proliferation of liver cells even in old individuals. This relationship between the increase of mtDNA and TFAM in aging and the age-related changes of mtDNA transcription, suggests the existence of tissue-specific differences in the regulation of mitochondrial gene expression, although it must also be considered that TFAM has multiple roles in the mtDNA metabolism and that mtDNA transcription depends not only on TFAM but also on TFB1 and TFB2.

Acknowledgments

We thank Dr. H. Hinagaki (Department of Chemistry, National Industrial Research Institute of Nagoya, Japan) for the gift of rat TFAM antiserum. This work was supported by Progetto di Ateneo 'Regolazione della biogenesi mitocondriale mediata da proteine nucleari in diversi sistemi sperimentali,' by Sigma-Tau Research Program "Effetti dell'acetil-L-carnitina sulla replicazione ed espressione del DNA mitocondriale nel ratto," and by M.U.R.S.T. project Cluster C03 "Studio di geni di interesse biomedico e agroalimentare." M.M. Dinardo was supported by fellowship from ESF (P.O.P.'94-'99).

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