Characterization of nucleolin K88 acetylation defines a new pool of nucleolin colocalizing with pre-mRNA splicing factors

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A B S T R A C T

Nucleolin is a multifunctional protein that carries several post-translational modifications. We characterized nucleolin acetylation and developed antibodies specific to nucleolin K88 acetylation. Using this antibody we show that nucleolin is acetylated in vivo and is not localized in the nucleoli, but instead is distributed throughout the nucleoplasm. Immunofluorescence studies indicate that acetylated nucleolin is co-localized with the splicing factor SC35 and partially with Y12. Acetylated nucleolin is expressed in all tested proliferating cell types. Our findings show that acetylation defines a new pool of nucleolin which support a role for nucleolin in the regulation of mRNA maturation and transcription by RNA polymerase II.

1. Introduction

Nucleolin was first identified as one of the major nucleolar phosphoproteins [1]. Because of its predominant nucleolar localization, the different functions of nucleolin in ribosome biogenesis have been extensively studied [2]. In particular, nucleolin is involved in the first processing step of pre-rRNA maturation in vitro [3] and it interacts with numerous ribosomal proteins [4]. Nucleolin–rRNA interaction studies suggest that nucleolin could be involved in the co-transcriptional folding of pre-rRNA which is necessary for the correct maturation of pre-rRNA [5]. Indeed, in nucleolin knockout DT40 cells [6], the processing of 45S pre-rRNA is moderately affected. However, the most prominent effect of nucleolin knockout or silencing, is on the processing of 4S pre-rRNA which remains unaffected. However, the most prominent effect of nucleolin knockout or silencing, is on the processing of 4S pre-rRNA which remains unaffected. However, the most prominent effect of nucleolin knockout or silencing, is on the processing of 4S pre-rRNA which remains unaffected. However, the most prominent effect of nucleolin knockout or silencing, is on the processing of 4S pre-rRNA which remains unaffected. Nucleolin is also involved in DNA repair, mRNAs metabolism, internalization of growth factors and viral ligands and virus replication [5].

These multiple functions can be achieved via numerous protein–protein interactions, and probably also thanks to the numerous post-translational modifications (PTM) whose functions are still largely unexplored.

The best characterized PTM of nucleolin is undoubtedly the phosphorylation of the N-terminal domain [11,12], which has been involved in the regulation of transcription [13,14] and nucleic acid interaction [15]. The C-terminal domain of nucleolin is the site of Nε,Nε-dimethylarginine [16] that can modulate the interaction of nucleolin with nucleic acids [17]. Nucleolin is also glycosylated in human U397 cells [18].

Acetylation is another PTM that has been extensively studied. p300/CBP, the GNAT family of histone acetyltransferase (HAT) acetylates a large number of non-histone proteins [19]. Acetylation analysis by mass spectrometry identified acetylated residues on nucleolin [20].

In this study, we identified several acetylated lysines in the N-terminal domain of nucleolin and we developed an antibody specific to nucleolin acetylated lysine 88 (NCL-K88ac). Using this antibody, we demonstrated that a fraction of nucleolin could be acetylated in vivo. Interestingly, we found that NCL-K88ac is not localized within the nucleolus, but rather in nucleoplasmic speckles that colocalized with splicing factors SC35. This speckle...
localization was found in HeLa cells and in stimulated Peripheral Blood Mononuclear Cells (PBMC).

2. Materials and methods

2.1. In vitro acetylation assay

In vitro acetylation assays were performed using 2 μg of proteins, incubated at 30 °C for 30 min in a 30 μl acetylation buffer (50 mM Tris–HCl (pH 8.0), 10% (vol/vol) glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA (pH 8.0), 10 mM sodium butyrate) and 0.5 μl of 3.3 Ci/mmol [3H]acetyl-CoA and analyzed as described in the legend of Supplementary Fig. 1. For the mass acetylation reaction, 2 μg of baculovirus-expressed nucleolin and 100 ng full-length p300 were incubated in the presence of 50 μM acetyl-CoA at 37 °C for 1.5 h. To achieve efficient acetylation, p300 and acetyl-CoA were added at every 30-min interval.

2.2. Generation of polyclonal antibodies specific for acetylated nucleolin and characterization of the in vivo status of nucleolin acetylation

Based on the identified acetylation sites by mass spectrometry, four different peptides having acetylated lysine residues (AcK) were designed (see sequences in Supplementary Fig. 2). Each acetylated peptide was used for immunization of a rabbit (Covalab, Lyon). The specificity of the four resulting sera was first checked by an ELISA with the acetylated and non-acetylated peptides. The serum obtained from the immunization with AcNcl1 peptide showed the highest specificity, and was chosen to perform the experiments described in this manuscript. For some experiments (as indicated in the text) we used the purified IgG of AcNcl1 that showed the highest specificity, and was chosen to perform the immunoprecipitation experiments using nucleolin antibodies were done as described previously [10].

2.3. Cell culture, siRNA transfection and immunofluorescence studies

HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco) at 37 °C in 5% CO2 incubator. HeLa cells (6 × 10^6) were transfected twice with siRNA Ncl2 and Ncl4 (Eurogentec) using Lipofectamine 2000 (Invitrogen) as described previously [8]. As a siRNA control we used Stealth high GC siRNA (Invitrogen). Proteins were harvested at 96 h after the first transfection, and analysed by Western blot.

2.4. Antibodies

The following antibodies were used: A rabbit polyclonal antibody against human nucleolin no 5567 developed by our laboratory and used previously [21], a rabbit polyclonal antibody against human acetylated nucleolin (AcNcl1) (Covalab, Lyon; this study), anti-acetyl-Histone H3 (Lys14) (17-305,Upstate) anti-SC35 (and as AcNcl1 peptide) with the AcNcl1 serum, indicating that this antibody (raised against the AcNcl1 peptide) was further studied in this manuscript. The specificity of AcNcl1 serum was then characterized by ELISA with the AcNcl1 modified and unmodified peptide used for immunization (Supplementary Fig. 2B). These data clearly indicate that the AcNcl1 serum is specifically directed against the acetyl modification of the peptide. In addition, cross reactivity of AcNcl1 serum with AcNcl2, AcNcl3 and AcNcl4 modified peptides was also studied (Supplementary Fig. 2C) and the reactivity toward acetylated histones was determined by Western blot (Supplementary Fig. 2D). These experiments show that AcNcl1 serum present a good specificity only for the AcNcl1 peptide. Since AcNcl1 peptide contains 2 acetylated Lysines (K79 and K88), ELISA tests were performed with two new peptides carrying only one of the acetylated Lysine (Supplementary Fig. 3). Remarkably, only the peptide AcNcl1_Pep1 carrying acetylated K88 reacted efficiently (and as AcNcl1 peptide) with the AcNcl1 serum, indicating that this serum contains antibodies specific to this nucleolin K88 acetylated lysine (NCL-K88ac).

Then, the specificity of this AcNcl1 antibody was characterized by Western blot analysis using full-length nucleolin purified from baculovirus that was subjected or not to an in vitro acetylation reaction with p300 (Fig. 2C). AcNcl1 antibody reacts only very weakly with baculovirus purified nucleolin (lane 1) whereas a strong signal is detected after the acetylation reaction (lane 2). In addition, with a HeLa whole cell extract, AcNcl1 serum detects only one weak protein bands at the size of nucleolin (Fig. 2D). These experiments suggest that this AcNcl1 serum is specific of nucleolin acetylation and that the level of nucleolin acetylation is probably low in the cell.

To further demonstrate that AcNcl1 antibody is specific to nucleolin, we performed a series of immuno-precipitation (IP) experiments from HeLa cells with AcNcl1 serum and with another previously well characterized polyclonal nucleolin antibody [21] (Fig. 2E). After an IP with AcNcl1 serum, nucleolin Ab gives a strong signal (lane 5, second row); and inversely after IP with nucleolin of nucleolin purification from HeLa cells [21]. (Fig. 1A, B). Western blot on this extract with global anti-acetyl Lysine antibody (Ab21623, Abcam) clearly detects a faint band corresponding to nucleolin size (Fig. 1C) suggesting that a fraction of nucleolin protein could be acetylated in vivo. To confirm this data, and identify the lysine acetyltransferase(s) (KAT) that are able to post-translationally modify nucleolin, we carried out an in vitro acetylation assay using different KATs and [3H]acetyl-CoA. The normalized KATs (Supplementary Fig. 1) were tested for their ability to acetylate nucleolin in vitro (Fig. 1D). Interestingly, it was found that only p300 and PCAF were able to acetylate nucleolin in vitro (Fig. 1D, lanes 4 and 6).

3. Results

3.1. Nucleolin is acetylated both in vivo and in vitro

With the aim to determine if nucleolin could be acetylated in vivo, we prepared a nuclear S2 extract which is the first step
Ab, AcNc1 is able to recognize the nucleolin immunoprecipitated protein (lane 4, first row). As previously shown, AcNc1 serum gives only a weak signal with total "input" proteins, suggesting again that the level of NCL-K88ac is low in cells. Altogether, these experiments show that this AcNc1 serum is specific to nucleolin acetylation and that nucleolin is indeed acetylated in vivo.

3.3. NCL-K88ac localizes in the nucleoplasm and is colocalized with different nuclear markers

We used our AcNc1 Ab to determine the cellular localization of NCL-K88ac by immunofluorescence (Fig. 3). Interestingly, AcNc1 Ab did not label the nucleoli structures as typical nucleolin antibodies do, but instead detected nucleoplasmic speckles (Fig. 3A). The nucleolin polyclonal antibody which strongly labels the nucleoli also gives some faint signal in the nucleoplasm that colocalize with the signal of AcNc1 Ab (Fig. 3A) which confirm the Western blot analysis (Fig. 2E) showing that the polyclonal Ab recognize also the pool of acetylated nucleolin. The signal specificity was demonstrated by treating the cells with nucleolin siRNA (Supplementary Fig. 4). Inhibition of deacetylases with a sodium butyrate (NaBu) treatment (Fig. 3B–E) leads to a twofold increase of the signal observed by Western blot (Fig. 3B and C) and immunofluorescence (Fig. 3D and E) with the AcNc1 Ab whereas the signal obtained with the nucleolin polyclonal Ab is not changed (Fig. 3B). Since global nucleolin Ab detects mainly nucleolin in the nucleoli and associated with the coding region of rDNA chromatin using ChIP-seq [10] we performed a ChIP-seq analysis of NCL-K88ac interaction with rDNA (Supplementary Fig. 5). In contrast with the interaction of nucleolin with rDNA, NCL-K88ac does not seem significantly bound to rDNA chromatin. Altogether, these data confirm that AcNc1 Ab is specific of nucleolin acetylation and show that NCL-K88ac is excluded from the nucleolus and predominantly localized in the nucleoplasm.

To identify the nucleoplasmic structures labeled with AcNc1 Ab, we studied the distribution of the AcNc1 antibody and different nuclear markers (Fig. 4) that are also known to give a speckle-like distribution such as the splicing factor SC35 and the snRNP marker Y12. The amount of colocalization was quantified by NIH Imagej software and is depicted as Pearson’s coefficient (Fig. 4, bottom right insert of each panel, and 4D) where a number near +1 suggests perfect correlation between 2 biomolecules, and a number near 0 indicates no correlation. Strikingly, the signal obtained with AcNc1 Ab is almost completely co-localized with SC35 (Fig. 4A) and partially with Y-12 (Fig. 4B). In contrast, AcNc1 Ab did not co-localize at all with coilin (Fig. 4C). In agreement with the co-localization of NCL-K88ac and SC35, immunoprecipitation with an anti-SC35 antibody is able to pull down NCL-K88ac as detected by AcNc1 Ab (Supplementary Fig. 6), suggesting that NCL-K88ac and SC35 are indeed present in the same cellular structures.

3.4. Acetylation of nucleolin in different cell lines

The level of NCL-K88ac was checked in different cells (Fig. 5A). Acetylated nucleolin as detected by AcNc1 seems absent from resting PBMC (lane 1) but is present in stimulated cells (lane 2). Interestingly, the level of expression of NCL-K88ac follows the level of expression of nucleolin in the different cell lines as the ratio of the signal AcNc1/Ncl is very similar in all tested cell lines (Fig. 5B). Like
in HeLa cells (Fig. 3A), acetylated nucleolin detected by AcNcl1 Ab is perfectly co-localized with the splicing factor SC35 in stimulated PBMC (Fig. 5C).

4. Discussion

In this report, we show that nucleolin is acetylated in vivo. We identified several acetylated lysines, which are present exclusively within the first 150 N-terminal residues of nucleolin. Other acetylated residues may however exist as the purpose of this work was not to have an exhaustive analysis of nucleolin PTM. Also, it is not known if all these modifications are present simultaneously in the same molecules. NCL-K88ac represent a distinct pool of protein with a distinct cellular localization: NCL-K88ac is apparently absent from the nucleolar structures, but is present in speckle structures in the nucleoplasm (Figs. 3 and 4).

The absence of NCL-K88ac from the nucleoli suggests that it is not involved in ribosome biogenesis and, in particular, in the regulation of transcription by RNAPI. ChIP-Seq with a polyclonal antibody show that nucleolin is associated with the coding region of rDNA, similar to the distribution of UBF and RNAPII subunit RPA116 [10,22]. However, ChIP-Seq with AcNcl1 does not detect any significant binding of NCL-K88ac on rDNA chromatin (Supplementary Fig. 5). This finding is in agreement with the
absence of NCL-K88ac in nucleolar structure as observed by immunofluorescence.

If NCL-K88ac is not involved in RNAPI regulation, then what could be its function? Interestingly, it was found that nucleolin in the presence of PCAF enhanced IRF-2-dependent H4 promoter activity [23]. Recruitment of nucleolin to acetylated IRF-2 is required for this promoter activity. However, since we show in this manuscript that nucleolin is also a substrate of PCAF, it would be interesting to reevaluate the role of nucleolin acetylation in this transcriptional regulation. NPM1 that share many functional similarities with nucleolin [24] is also acetylated by p300 [25]. Acetylated-NPM1 is predominantly localized in the nucleoplasm [26] and has an enhanced ability to activate transcription from chromatin templates. However, we were unable to see any colocalization for NCL-K88ac with transcriptionally active RNA polymerase II or any activation of transcription on chromatin template in vitro (data not shown).

It is remarkable that NCL-K88ac distribution in the nucleoplasm is co-localized with the nuclear domains enriched in splicing factor SC35 and with the snRNP marker Y12 (Fig. 4). These nuclear speckles (also called interchromatin granule clusters, IGC) are very dynamic structures [27] and are also enriched in many other mRNA splicing factors, RNA polymerase II subunits and diverse transcription factors [28] but, transcription and pre-mRNA splicing do not seem to take place within these structures [29]. The association of NCL-K88ac with these speckles suggests two possible functions for NCL-K88ac. First, it was shown that the
Fig. 4. Immunofluorescence of NCL-K88ac and different nuclear markers. (A) Immunofluorescence staining of AcNcl1 (green) with nuclear markers SC35 (red), (B) Y-12 (red), (C) Coilin (red) in formaldehyde fixed HeLa cells. DNA staining (blue) was counterstained with DAPI. Scale bar, 5 μm. Lower panels of each section (A, B, C) represent the enlargements represented by the squares in the corresponding upper images (scale bar 1 μm). The lower right insert of each panel correspond to the cytofluorogram which was used to determine the Pearson’s coefficient (D) Histogram showing Pearson’s coefficient for 15 individual cells analyzed in 3D for SC35 and AcNcl, Y-12 and AcNcl, coilin and AcNcl. (E) Histogram showing Menders’ coefficient for 15 individual cells analyzed in 3D for SC35 and AcNcl, Y-12 and AcNcl, coilin and AcNcl.
serine-2-phosphorylated form of the RNA polymerase II large subunit, which is involved in transcription elongation was co-localized with nuclear speckles [30]. As nucleolin has been involved in the transcription of several Pol II genes and in transcription elongation [5] it is also possible that the presence of NCL-K88ac in the speckles participates to the formation of transcription elongation complexes for the nearby genes.

The second possibility is that nucleolin participates in the splicing of some mRNAs. There are many examples of interactions of nucleolin with different mRNAs with different effects on mRNA stability and translation. Recently, a pull down assay followed by microarray analysis identified several hundred of potential nucleolin mRNA targets [31]. Although the nucleolin interactome is not known, several reports describe the interaction of nucleolin with proteins involved in pre-mRNA splicing in particular, different hnRNPs [32]. Our results also show that SC35 complexes contain NCL-K88ac. Recently, RNP complexes formed on a specific HIV pre-mRNA splicing site (SLS2-A7 RNA transcripts) in HeLa cell nuclear extracts identified hnRNP A1, nucleolin, hnRNP H and hnRNP K that directly interact with SLS2-A7 RNA [33]. Nucleolin binds to a cluster of successive canonical nucleolin recognition element (NRE motifs) [21] in SLS2-A7 RNA. The authors showed a strong effect of hnRNP K on HIV-1 alternative splicing, but they have not tested the effect of the interaction of nucleolin with HIV-1 mRNA. This opens the possibility that the interaction of nucleolin with this RNA affects its alternative splicing.

Fig. 5. Acetylation of nucleolin in different cell lines. (A) Cell lysates were prepared from different cell lines as indicated on the figure (lanes 1–9) and immunoblotted with AcNcl1, nucleolin and β-actin antibodies. (B) Quantification of Western blot results showing the ratio of NCL-K88ac versus total nucleolin. (C) Immunofluorescence staining of nucleolin in stimulated PBMC cells using AcNcl1 (green) and SC35 antibodies (red). DNA was stained with DAPI (blue). Scale bar: 5 µm.
The characterization and functional significance of nucleolin post-translational modifications are still unexplored. In this work, we provide evidence that nucleolin is acetylated in vivo and this modification drastically changes its cellular localization. The presence of NCL-K88ac in nuclear speckles suggests that this nucleolin pool may be involved in pre-mRNA synthesis or metabolism. The specific antibodies developed against NCL-K88ac herein, should be useful to explore this novel nucleolin function.

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References