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In-vitro acetylation of SARS-CoV and SARS-CoV-2 nucleocapsid proteins by human PCAF and GCN5

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ABSTRACT

Recently, the novel coronavirus (SARS-CoV-2), which has spread from China to the world, was declared a global public health emergency, which causes lethal respiratory infections. Acetylation of several proteins plays essential roles in various biological processes, such as viral infections. We reported that the nucleoproteins of influenza virus and Zaire Ebolavirus were acetylated, suggesting that these modifications contributed to the molecular events involved in viral replication. Similar to influenza virus and Ebolavirus, the coronavirus also contains single-stranded RNA, as its viral genome interacts with the nucleocapsid (N) proteins. In this study, we report that SARS-CoV and SARS-CoV-2 N proteins are strongly acetylated by human histone acetyltransferases, P300/CBP-associated factor (PCAF), and general control nonderepressible 5 (GCN5), but not by CREB-binding protein (CBP) *in vitro*. Liquid chromatography-mass spectrometry analyses identified 2 and 12 acetyl-lysine residues from SARS-CoV and SARS-CoV-2 N proteins, respectively. Particularly in the SARS-CoV-2 N proteins, the acetyl-lysine residues were localized in or close to several functional sites, such as the RNA interaction domains and the M-protein interacting site. These results suggest that acetylation of SARS-CoV-2 N proteins plays crucial roles in their functions.

1. Introduction

Coronaviruses (CoVs) are animal and human pathogens that can cause lethal zoonotic infections such as severe acute respiratory syndrome (SARS) [1]. We have experienced 2 outbreaks of SARS coronavirus (SARS-CoV) with zoonotic origins. The first outbreak of SARS-CoV emerged in Foshan, Guangdong province in southeastern China in 2002, and spread to other countries such as Hong Kong, Vietnam (Hanoi), Singapore, Canada (Toronto), and the USA. Since July 2003, no more infections were detected, and the SARS-CoV pandemic was declared over. However, other SARS-CoV-like viruses found in bats were shown to be capable of infecting human cells [2,3], suggesting that infections with other CoV could re-emerge. Concerningly, in December 2019, the second SARS-CoV-2 outbreak initially began in Wuhan, Hubei province, China, and has now affected more than 200 countries and was declared a global pandemic [4,5]. This new infectious disease developed by SARS-CoV-2 was formally named “coronavirus infectious disease (COVID)-19” by a consensus group of World Health Organization (WHO) experts. These highly pathogenic emerging coronaviruses have caused fatal pneumonia and have been recognized as new public health concerns in this century. To combat the threats of SARS-CoV and SARS-CoV-2, the molecular biological details of these viruses need to be understood.

The SARS-CoV and SARS-CoV-2 genomes, which are shared with betacoronaviruses, comprise a single-stranded positive sense RNA [6,7]. The nucleotide sequences of these vital genomes are 79% identical [8]. The 6 functional open reading frames (ORFs) were arranged in the order of replicase (ORF1a/1b), spike (S), envelope (E), membrane (M), and nucleocapsid (N). Amino acid sequences of 4 structural proteins (S, E, M, and N) of SARS-CoV and SARS-CoV-2 share more than 90% identity [8,9]. Of these structural proteins of both SARS-CoVs, the N proteins play essential roles in the packaging of viral genome RNA into the ribonucleocapsid by interacting with the viral genome and the M proteins and mediating viral

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assembly [10,11]. SARS-CoV and SARS-CoV-2 N proteins have been reported to undergo posttranslational modifications, such as phosphorylation [12–15], sumoylation [16] and ADP-ribosylation [17]. In addition, the N-terminal modification of the SARS-CoV N protein was theoretically indicated from the results of molecular weight resolution [18]. Previously, we reported that nucleoproteins (NPs) of influenza A virus and Zaire Ebolavirus, which functionally correspond to the SARS-CoV and SARS-CoV-2 N proteins, were acetylated in cells and *in vitro* by human histone acetyltransferases (HATs), P300/CBP-associated factor (PCAF), and general control nonderepressible 5 (GCN5), suggesting that these post-translational modifications play crucial roles in regulating viral RNA polymerase activity and viral particle budding [19,20]. However, there are no reports on the acetylation of lysine residues of SARS-CoV and SARS-CoV-2 N proteins.

In this study, we showed that SARS-CoV and SARS-CoV-2 N proteins were acetylated by PCAF and GCN5 *in vitro* as well as NPs of influenza A virus and Zaire Ebolavirus, and identified these acetylation sites by liquid chromatography-mass spectrometry (LC-MS/MS). These acetyl-lysine residues were localized in or close to several functional sites, such as the RNA interaction domains in both the N-terminal and C-terminal domains and the viral M-protein interacting site in the C-terminal domain of the SARS-CoV-2 N proteins, suggesting that acetylation of these proteins affects their functions.

2. Materials and methods

2.1. Acetylation assays using radioisotope-labeled acetyl-CoA

The experiments in this study were modified from those described in our previous reports [19–21]. Full-length recombinant SARS-CoV and SARS-CoV-2 N proteins with a histidine tag were purchased from Abcam (Product No., ab272108 and ab272107, respectively). Recombinant PCAF and GCN5 HAT domain proteins were synthesized in *E. coli* cells and purified as previously described [20,21]. Recombinant proteins of the catalytic domain of human CBP (Enzo Life Sciences) were purchased. The recombinant proteins (1 μg) of the SARS-CoV and SARS-CoV-2 N proteins were incubated with each HAT recombinant protein (1 μg), 7.4 kBq of acetyl-CoA (acetyl-1-^{14}C; Moravek Inc.) at 30 °C for 2 h in buffer containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM dithiothreitol, and 10 mM sodium butyrate. Reactions were separated on 10% SDS-PAGE gels, which were dried on filter paper. The imaging plates were exposed to the dried gels for several days. Signals were detected using a fluoroimage analyzer (FLA-2000; Fuji Film).

2.2. LC-MS/MS for identifying acetyl lysine residues

Digestion of each sample and quantitative proteomic analysis were performed as previously described [22]. Briefly, each sample was digested using the phase transfer surfactant (PTS) method, as previously reported [23,24]. Specific peptide data were detected by Sequential Window Acquisition of All Theoretical Fragment-ion Mass Spectra (SWATH-MS) on a TripleTOF 5600 instrument (SCIEX) interfaced with a DIONEX Ultimate 3000 RSLC nano system (Thermo Fisher Scientific). Peptides were identified by Protein Pilot 4.5 (SCIEX) with MS/MS data from Information-Dependent Acquisition (IDA). The peak of peptide acetylated lysine was analyzed using PeakView Software version 2.1 (SCIEX). The average peptide peak area of the samples in each group was compared between the 4 groups. Statistical analyses were performed using one-way analysis of variance (ANOVA).

2.3. In-silico simulation analysis of localization of acetylated lysine residues

Molecular modeling was performed using Molecular Operating Environment software (MOE; Chemical Computing Group). The X-ray crystallographic structure of the *N*- and *C*-terminal regions of the SARS-CoV-2 N proteins [Protein Data Bank (PDB) ID code: 6M3M and 6YUN, respectively] and C-terminal region of SARS-CoV N proteins (PDB ID code: 2CJR) were obtained from the PDB.

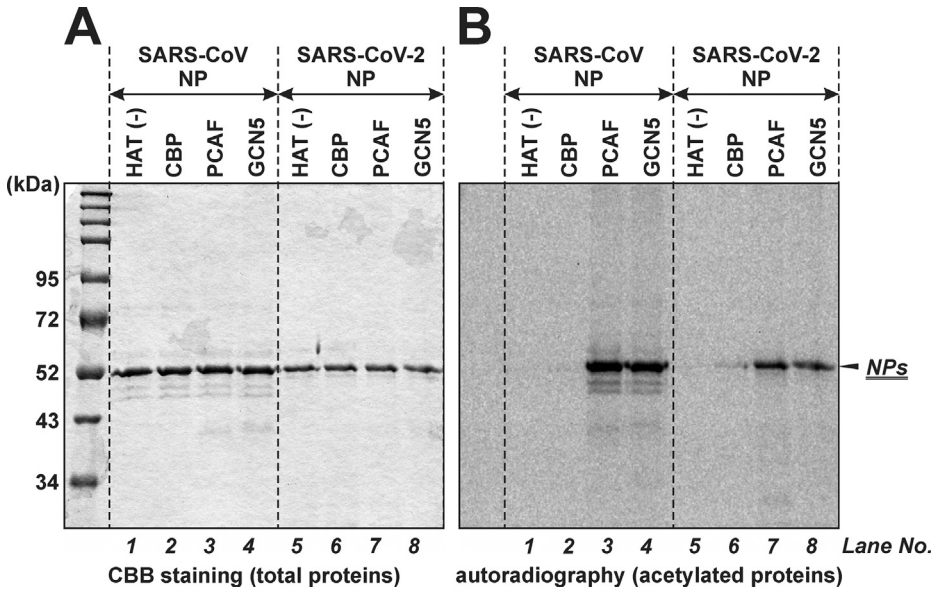


Fig. 1. Acetylation of the SARS-CoV and SARS-CoV-2 N proteins. (A) The full-length recombinant proteins (each 1 μg) of the SARS-CoV and SARS-CoV-2 N proteins were incubated with 3 different human HATs: CBP, PCAF, and GCN5. Treated N proteins were separated with SDS-PAGE gels and stained with CBB, showing that same amount of the recombinant N proteins were loaded in each sample. (B) Acetylation status was investigated by autoradiography for detecting β ray transferred to the N proteins. CBP weakly (lanes 2 and 6), and PCAF and GCN5 strongly acetylated SARS-CoV and SARS-CoV-2 N proteins (lanes 3, 4, 7 and 8).

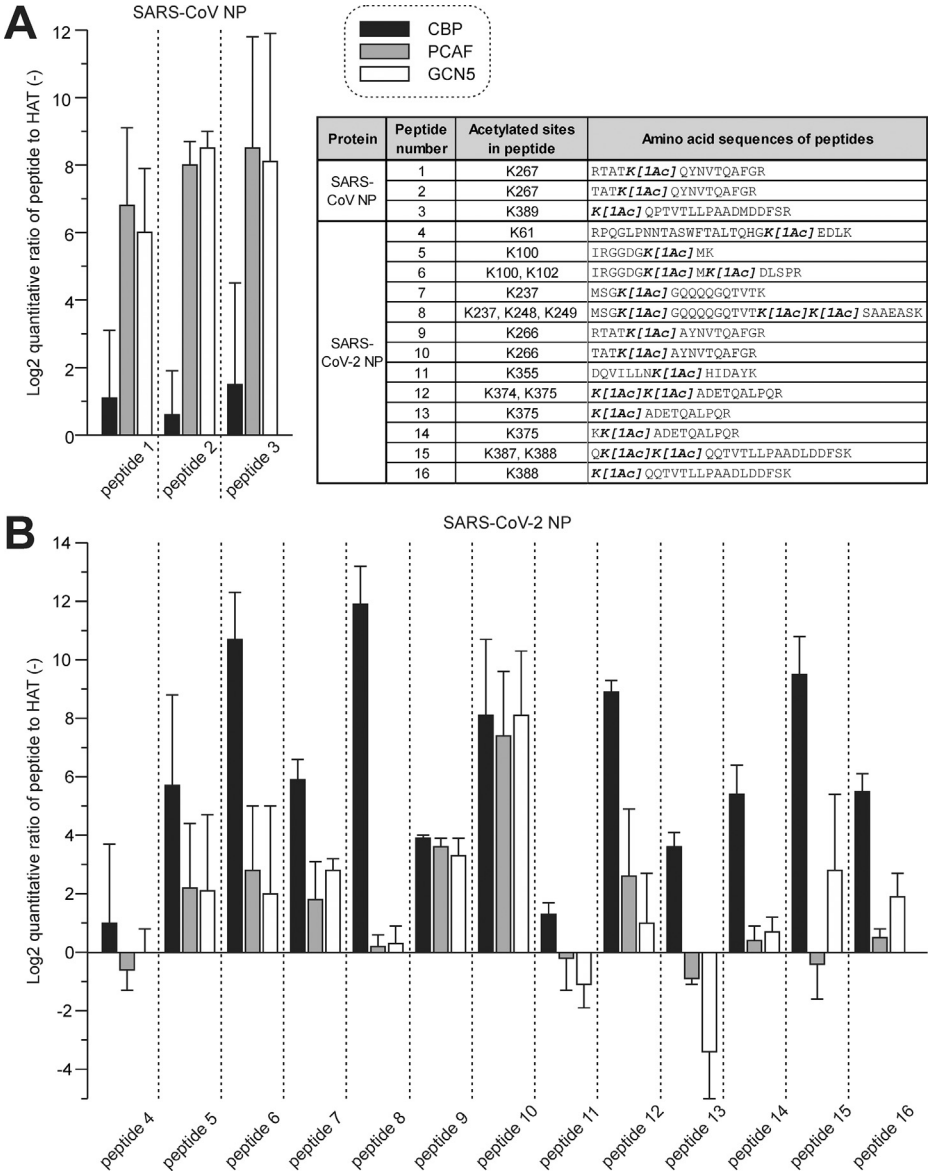
3. Results

3.1. Acetylation of the SARS-CoV and SARS-CoV-2 N proteins

First, we investigated the acetylation status of SARS-CoV and SARS-CoV-2 N proteins (Fig. 1). Full-length recombinant SARS-CoV and SARS-CoV-2 N proteins (each 1 μg) were incubated with the partial recombinant proteins containing HAT domains of human CBP, PCAF, and GCN5, and [^{14}C]-acetyl-CoA. Gel staining with Coomassie Brilliant Blue (CBB) showed that the same amount of recombinant N protein was loaded onto the gel (Fig. 1A). Acetylation status was investigated by detecting β rays released from [^{14}C] in the acetyl group, which was transferred from acetyl CoA to the N proteins (Fig. 1B). As a result, CBP faintly, and PCAF and GCN5 strongly acetylated the N proteins (Fig. 1B). In the SARS-CoV N protein lanes (3 and 4), double bands were detected between 43 and 52 kDa marker bands, with the SARS-CoV N protein putatively degraded, and acetylated. These results showed that the SARS-CoV and SARS-CoV-2 N proteins were acetylated by PCAF and GCN5 *in vitro*. As mentioned above, the NPs of influenza A virus and Zaire Ebolavirus were acetylated by PCAF and GCN5 [19,20]. These HATs, categorized in the N-acetyltransferase family, acetylate not only N-termini but also lysine residues of hundreds of non-histone proteins [25], suggesting that viral N proteins are preferably acetylated by these enzymes.

3.2. Identification of acetyl-lysine residues in the SARS-CoV and SARS-CoV-2 N proteins

We performed LC-MS/MS to identify acetyl-lysine residues in SARS-CoV and SARS-CoV-2 N proteins (Fig. 2). In the SARS-CoV N protein, 3 peptides containing acetyl-lysine residues were identified, and 2 acetyl-lysine residues (K267 and K389) were identified (Fig. 2A, S1A-C, and S2). The quantitative ratio of peptides digested



Protein	Peptide number	Acetylated sites in peptide	Amino acid sequences of peptides
SARS-CoV NP	1	K267	RTAT***K[1Ac]***QYNVTQAFGR
	2	K267	TAT***K[1Ac]***QYNVTQAFGR
	3	K389	***K[1Ac]***QPTVTLLPAADMDDFSR
SARS-CoV-2 NP	4	K61	RPQGLPNNTASWFTALTQHG***K[1Ac]***EDLK
	5	K100	IRGGDG***K[1Ac]***MK
	6	K100, K102	IRGGDG***K[1Ac]***M***K[1Ac]***DLSPR
	7	K237	MSG***K[1Ac]***GQQQQGQTVTK
	8	K237, K248, K249	MSG***K[1Ac]***GQQQQGQTVT***K[1Ac]K[1Ac]***SAAEASK
	9	K266	RTAT***K[1Ac]***AYNVTQAFGR
	10	K266	TAT***K[1Ac]***AYNVTQAFGR
	11	K355	DQVILLN***K[1Ac]***HIDAYK
	12	K374, K375	***K[1Ac]K[1Ac]***ADETQALPQR
	13	K375	***K[1Ac]***ADETQALPQR
	14	K375	K***K[1Ac]***ADETQALPQR
	15	K387, K388	Q***K[1Ac]K[1Ac]***QQTVTLLPAADLDDFSK
	16	K388	***K[1Ac]***QQTVTLLPAADLDDFSK

Fig. 2. Identification of acetyl-lysine residues in the SARS-CoV and SARS-CoV-2 N proteins. Amino acid sequences of peptides and contained putative acetyl-lysine residues are summarized in the list of this figure. (A) Quantitative ratio of 3 peptides of the SARS-CoV N protein, that contained acetyl-lysine residues, detected by LC-MS/MS. These peptides may be more acetylated by PCAF and GCN5 than CBP. (B) Quantitative ratio of 13 peptides of the SARS-CoV-2 N proteins. Eleven peptides, and except for peptides 9 and 10, may be acetylated by CBP more strongly than PCAF and GCN5.

from the N protein incubated with CBP, PCAF, or GCN5 suggested that PCAF and GCN5 more strongly acetylated K267 and K389 than CBP (Fig. 2A, S1A-C, and S2). These results were consistent with those of the biochemical experiments shown in Fig. 1. In the SARS-CoV-2 N protein, 13 peptides containing acetyl-lysine residues were identified, and 12 acetyl-lysine residues (K61, K100, K102, K237, K248, K249, K266, K355, K374, K375, K387, and K388) were identified (Fig. 2B, S1D-P, and S2). Of the 13 peptides, 11 peptides except for peptides 9 and 10, may be acetylated by CBP more strongly than PCAF and GCN5. These results were not consistent with those of the biochemical experiments shown in Fig. 1, suggesting that some modified peptides were failed to be detected by LC-MS analyses or peptide identification. Acetylation levels of peptides 9 and 10 were similarly acetylated by 3 different HATs (Fig. 2B). These peptides contained the same acetyl-lysine residue K266, suggesting that K266 is a strong target of acetylation catalyzed by many kinds of HATs.

3.3. Comparison of acetylation sites between SARS-CoV and SARS-CoV-2 N proteins

The conservation of acetylation sites between SARS-CoV and SARS-CoV-2 N proteins was investigated by comparing these primary structures. Alignment of amino acid sequences between the SARS-CoV (GenBank Accession No.: AAR87518.1) and SARS-CoV-2 (GenBank Accession No.: YP_009724397.2) N proteins used in this experiment are shown in Fig. 3. These amino acid sequences were 89.2% identical. This alignment found 2 pairs of identical acetyl-lysine residues (267th of SARS-CoV and 266th of SARS-CoV-2, and 389th of SARS-CoV and 388th of SARS-CoV-2) in both N proteins. Lysine residues in the SARS-CoV N protein corresponding with other 10 acetyl-lysine residues (K61, K100, K102, K237, K248, K249, K355, K374, K375, and K387) in the SARS-CoV-2 N protein were not acetylated. However, there were differences in amino acid sequences around the acetylation targets, such as (1) E64 and R66 in SARS-CoV N protein, D63 and K65 in SARS-CoV-2 N protein, (2) E104 in SARS-CoV N protein and D105 in SARS-CoV-2 N protein, and (3) T377 in SARS-CoV N protein and A376 in SARS-CoV-2 N protein. These differences may affect the affinity between enzymes (HATs) and substrates (N proteins).

3.4. Localizations of acetylation targets in tertiary structures of SARS-CoV and SARS-CoV-2 N proteins

The N proteins of SARS-CoV and SARS-CoV-2 consist of N- and C-terminal domains separated by a serine-rich linker domain [26,27]. The N- and C-terminal domains are known to possess an RNA-binding domain and an oligomerization domain, respectively [28–31]. Partial tertiary structures of the *N*- and *C*-terminal domains of SARS-CoV and SARS-CoV-2 N proteins have been reported [31–33]. Based on this structural knowledge, we investigated the tertiary positions of acetyl-lysine residues in silico using molecular simulation of the X-ray crystallographic structure of the SARS-CoV and SARS-CoV-2 N proteins (Fig. 4).

In the SARS-CoV-2 N protein N-terminal domain (PDB ID: 6M3M) [34], 2 acetylation targets, K100 and K102, were contained in the "basic finger," which forms the finger-like structure by the long basic β-hairpin (β2′ and β3′) comprised mostly of basic amino acid residues (Fig. 4A) [31]. Together with the other acetylation site, K61, these 3 acetylated lysine residues were localized along the RNA interaction surface (Fig. 4A) [31]. This surface is positively charged, and this electrical property is an important factor for interaction

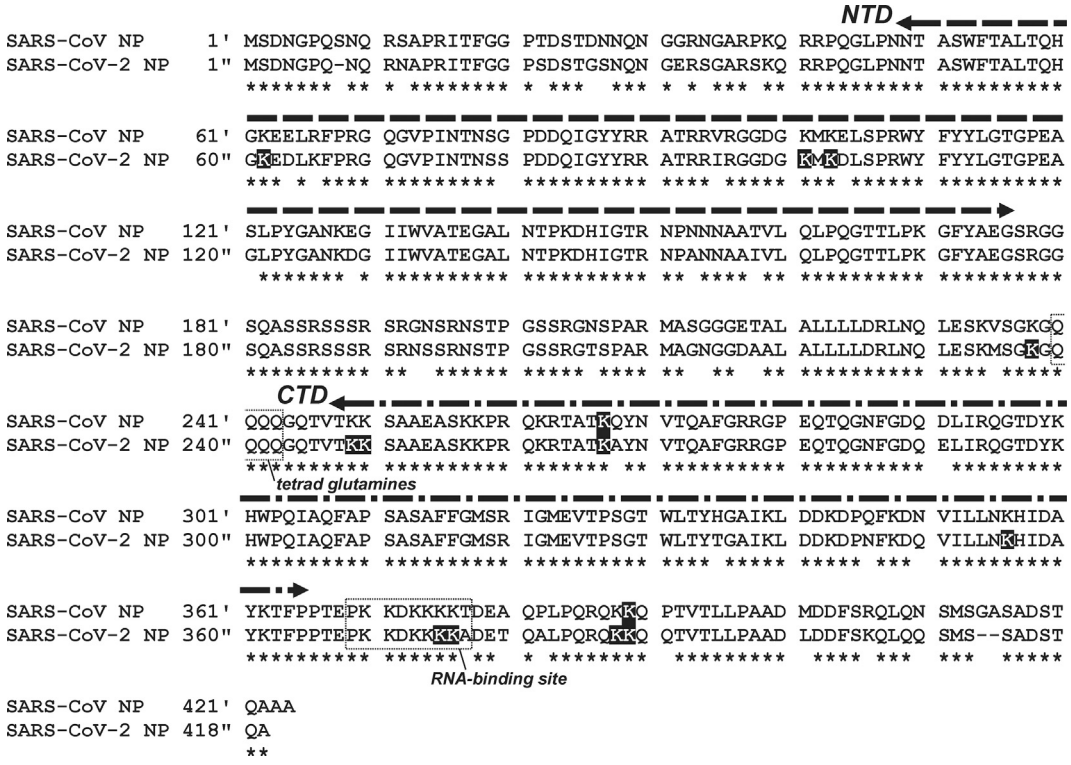


Fig. 3. Alignment of amino acid sequences between the SARS-CoV and SARS-CoV-2 N proteins. GenBank Accession Numbers of the SARS-CoV and SARS-CoV-2 N proteins were AAR87518.1 and YP_009724397.2, respectively. Putative acetyl-lysine residues identified by LC-MS/MS are shown with filled boxes. Asterisks indicate identical amino acid residues between 2 sequences. This alignment found 2 pairs of identical acetyl-lysine residues (267th of SARS-CoV and 266th of SARS-CoV-2, and 389th of SARS-CoV and 288th of SARS-CoV-2) in both N proteins. NTD: N-terminal domain (dotted line), CTD: C-terminal domain (dash-dot line). The tetrad glutamines, essential for interaction with the M proteins, and the RNA-binding site in CTD are highlighted with dotted lines. Acetylated lysines were identified nearly close to these domains, suggesting that the acetylation affects these functions.

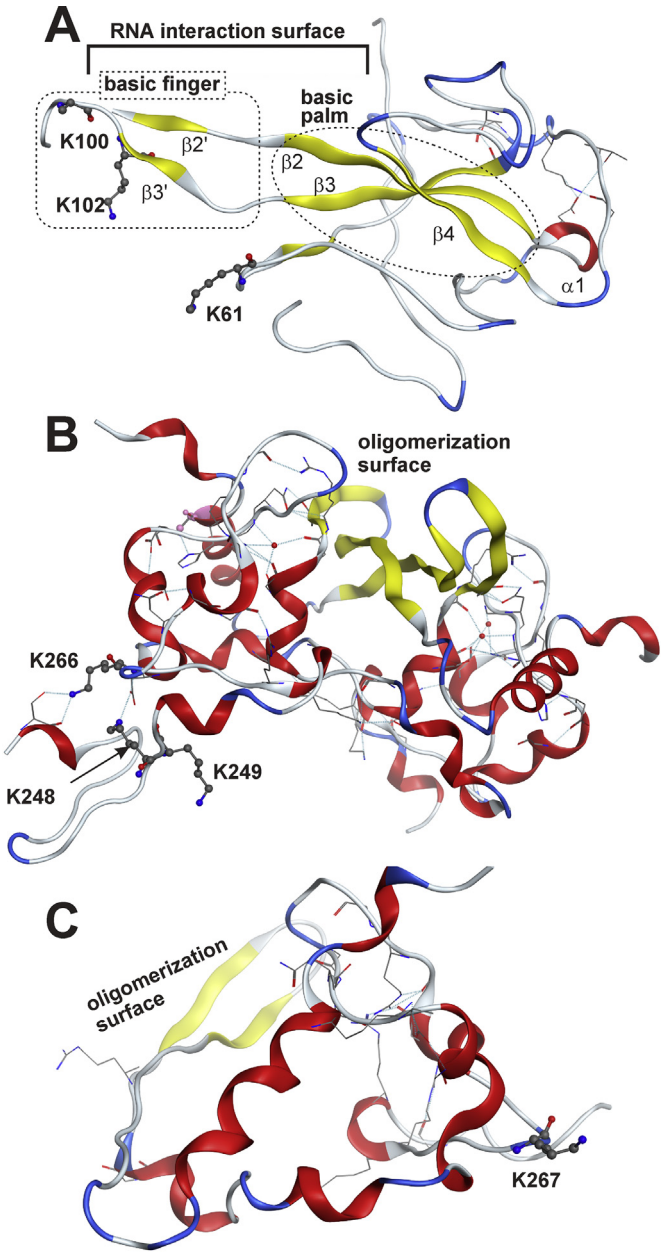


Fig. 4. Localizations of acetyl-lysine residues in SARS-CoV and SARS-CoV-2 N proteins. (A) Ribbon diagram of the SARS-CoV-2 N protein *N*-terminal domain containing the RNA binding surface (PDB ID: 6M3M) [33]. The RNA interaction surface is constructed with the "basic finger" and the "basic palm" [30]. Two acetylation targets, K100 and K102, were contained in the long basic β-hairpin (β2′ and β3′) constructing the basic finger. These lysine residues and K61 were localized in the RNA interaction surface. (B) Ribbon diagram of the dimerized SARS-CoV-2 N protein *C*-terminal domain containing the oligomerization surface (6YUN) [32]. Three acetylation targets, K248, K249 and K266, were localized in the opposite side of the oligomerization surface. (C) Ribbon diagram of SARS-CoV N protein *C*-terminal domain containing the oligomerization surface (2CJR) [31]. Also, in the SARS-CoV-2 N protein *C*-terminal region, an acetylation target, K267, was localized in the opposite side of the oligomerization surface. Yellow and red plains show β-sheets and α-helixes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

affinity with RNA [35]. Interestingly, a computational mutagenesis approach suggested that the substitution of K61A reduced binding affinity with RNA [36], suggesting that the cancelation of the positive charge of a lysine residue by replacing with an alanine residue is a reason for decreasing the affinity. K61 acetylation shown in this study is expected to have the same effects as substitution with an alanine residue. Therefore, K61 acetylation may be a key factor in reducing the interaction affinity between N proteins and RNA. Conversely, substitution of K102A increased binding affinity with RNA [36]. The molecular mechanisms of this phenomenon are expected with a more detailed investigation.

The localization of acetylated lysine residues in the SARS-CoV-2 and SARS-CoV N protein *C*-terminal region was also visualized in silico. In the diagram of the dimerized SARS-CoV-2 N protein *C*-terminal region containing the oligomerization domain (PDB ID: 6YUN, Fig. 4B) [33], acetylated lysines, K248, K249, and K266, were localized on the opposite surface of the oligomerization surface. K267 was also localized in a similar position in the SARS-CoV N protein C-terminal region (PDB ID: 2CJR, Fig. 4C) [32]. The acetylation of these lysine residues may affect the interaction with other proteins, rather than the oligomerization of the N proteins.

4. Discussion

Here, we found that SARS-CoV and SARS-CoV-2 N proteins were acetylated by human PCAF and GCN5 *in vitro*. LC-MS/MS analyses succeeded in identifying the acetylated lysine residues, and acetylation of the SARS-CoV and SARS-CoV-2 N proteins was suggested to play important roles in the modification of N protein functions. In particular, acetylation of K61 may regulate the binding affinity between the N proteins and viral RNA.

Several acetyl-lysines in the SARS-CoV and SARS-CoV-2 N proteins were unfortunately out of the tertiary structural data, and their positions were not clear. We speculated the functions of acetylation at (1) K237, K248, and K249, and (2) K374 and K375 of the SARS-CoV-2 N proteins.

(1) K237, K248, and K249 of the SARS-CoV-2 N proteins: The tertiary positions of K248 and K249 were clearly investigated (Fig. 4B). However, the tetrad glutamines, which are functional sites located close to K248 and K249 (Fig. 3), were out of the tertiary structural data, and we discussed the relationship between the 2 acetyl-lysines and the tetrad glutamines. A previous report showed that the tetrad glutamines (240th to 243rd in SARS-CoV N protein, Fig. 3) play essential roles in interacting with the viral M proteins [37]. These 4 serial glutamine residues were also conserved in the SARS-CoV-2 N protein (239th to 242nd, Fig. 3). In the SARS-CoV-2 N protein, K237 and K248–K249, closely localizing upstream and downstream sides of the tetrad glutamines, respectively, were acetylated (peptide 8 in Fig. 2). Therefore, acetylation of K237, K248, and K249 may regulate the interaction affinity between the N and M proteins. The tertiary position of K237 has not yet been investigated.

(2) K374 and K375 of the SARS-CoV-2 N proteins: A previous report showed that the SARS-CoV N protein C-terminus can also associate with nucleic acids, and residues 363rd to 382nd are responsible interaction partners [38]. In particular, the 6 lysines (369-PKKDKKKKT-377 and 368-PKKDKKKKA-376 in the SARS-CoV and the SARS-CoV-2 N proteins, respectively; Fig. 3) were thought to be responsible for its RNA binding [38]. In the present study, we found that 2 acetylated lysine residues, K374 and K375, were present in this RNA-binding site (Figs. 2 and 3). In addition to the N-terminal domain, acetylation of the SARS-CoV-2 N protein may prominently affect its affinity with RNA. We have reported the identification of many kinds of proteins interacting with SARS-CoV and SARS-CoV-2 N proteins [39–41], and acetylation of these proteins is expected to regulate interactions.

Previous reports showed that the SARS-CoV N protein underwent other posttranslational modifications, such as sumoylation, which was implied to be controlled by the human SUMO-conjugating enzyme (hUbc9) [16,42]. The target of this modification was K62, which corresponded to K61 of the SARS-CoV-2 N protein and was acetylated by PCAF and GCN5 (Fig. 3). Both acetylation and sumoylation occurring at the same lysine residues have been reported in the tumor suppressors *HIC1* and p53 [43,44]. Sumoylation of K62 of the SARS-CoV N protein drastically promoted its homo-oligomerization and played a role in the N protein-mediated interference of host cell division [16]. Therefore, acetylation of this lysine residue in the SARS-CoV-2 N protein may function as a switch to launch homo-oligomerization of N proteins.

In conclusion, we suggest that acetylation of SARS-CoV and SARS-CoV-2 N proteins plays a crucial role in modifying N protein functions. Further analyses using viral and cellular preparations are required to investigate the biological significance of acetylation of the SARS-CoV and SARS-CoV-2 N proteins and to understand their relationships with viral replication.

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Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.173.

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