N-α-acetyltransferase 10 protein is a negative regulator of 28S proteasome through interaction with PA28β

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ABSTRACT

N-α-acetyltransferase 10 protein (Naa10p) regulates various pathways associated with cancer cell proliferation, metastasis, apoptosis and autophagy. However, its role in protein quality control is unknown. Here, we report that Naa10p is physically associated with proteasome activator 28β (PA28β). Naa10p also interacts with PA28α in a PA28β-dependent manner. Naa10p negatively regulates PA28β-dependent chymotrypsin-like proteasome activity in cancer cells and in a cell-free system reconstituted with purified proteins, which is not related to 26S proteasome. Acetyltransferase activity of Naa10p is not required for its effect on chymotrypsin-like proteasome activity. Therefore, our data reveal that Naa10p suppresses 28S proteasome activity through interaction with PA28β.

Structured summary of protein interactions:
Naa10p physically interacts with PA28α and PA28β by anti bait coimmunoprecipitation (View Interaction: 1, 2)

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1. Introduction

N-α-acetyltransferase 10 protein (Naa10p, also known as ARD1) is the catalytic subunit of N-acetyltransferase A (NATa), one of major N-terminal acetyltransferase complexes in eukaryotes [1]. Naa10p catalyzes N-α-acetylation of nascent polypeptides emerging from ribosomes [2–4], lysine ε-acetylation of mature proteins [5], lysine autoacetylation [6], and acts as a transcriptional co-factor [7]. By synergizing with diverse signaling proteins, Naa10p has been implicated in controlling cell proliferation [5,6], apoptosis [8,9], metastasis [10], autophagy [11], hypoxic signaling [12], and neuronal development [13,14]. Recently, overexpression and prognostic value of Naa10p had been validated in several types of cancer [7,10,15,16] and more than one potential mechanism was proposed to explain the association between Naa10p and carcinogenesis [5–7,10–12,17], despite that the precise role of Naa10p may differ in distinct cancer types [18].

Proteasomes play a fundamental role in protein turnover and therefore are essential for different cellular functions, including tumor development and response to anti-cancer agents [19–21]. It is also responsible for the proteolytic maturation of diverse polypeptide precursors and for the degradation of cell regulators, whose destruction is necessary for progression through such cellular events as cell cycle (e.g. β-catenin and cyclins) [22], cell death (e.g. p53 and NFκB) [23], cell division, differentiation, and adaptation to environmental stresses [24]. Generally, most of these functions are conducted by 26S proteasome, which is formed by a 20S proteasome core plus one or two 19S proteasome activator [19].

The proteasome activator is essential for the access of target proteins to 20S core. Besides the well-studied 19S activator, other forms of proteasome activator were also characterized. 11S activator (proteasome activator 28, a heteroheptamer consisting of PA28α and PA28β), for example, can only stimulate the hydrolysis of model peptide substrates but not proteins in an ATP-independent manner [25,26]. It was reported to activate proteasomes to generate the antigenic peptides presented by MHC class I molecules, owning to the ATP-independent peptide processing ability.
However, the concrete function of 11S activator of 28S proteasome remains obscure.

In this present study, PA28β was revealed as a novel binding protein of Naa10p and the consequential biological effect was investigated, which offers a new insight into the role of Naa10p.

2. Materials and methods

2.1. Antibodies and reagents

Anti-Naa10p monoclonal antibodies (14D4 for Western blot analysis, 4D10 and 10C12 for immunoprecipitation assay) were characterized in our previous studies[15]. Anti-PA28β and anti-PA28α antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-V5 tag antibody was characterized in our previous studies[15]. Anti-PA28β and anti-PA28α antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-V5 tag antibody was purchased from Abcam (Cambridge, MA). Rabbit anti-Myc tag (AB103) and anti-GST tag (AB101) were from TianGen Biotech (Beijing, China). Rabbit anti-Myc tag (AB103) and anti-GST tag (AB101) were from TianGen Biotech (Beijing, China). Recombinant human PA28α/β complex was purchased from Sigma–Aldrich (St. Louis, MO). Recombinant human 20S proteasome was purchased from Enzo (France). 26S Proteasome Fraction was purchased from GeneTex (San Antonio, TX). GST-Naa10p and GST protein were expressed using pGEX-5X3 vector by Escherichia coli strain JM109 and purified. Proteasome-Glo™ Chymotrypsin-Like Assay Kit was purchased from Promega (Madison, WI).

2.2. Cell culture and transfection

Colon cancer cell line RKO, lung cancer cell lines H1299 and PG were purchased from American Type Culture Collection (ATCC) and maintained in standard culture conditions. Stable shNaa10p-silencing cell lines generated with RKO cells and H1299 cells were described in our previous papers[8,15,16]. Plasmids encoding V5-tagged wild-type Naa10p and mutant (R82A) Naa10p were kindly provided by Dr. Li-Jung Juan and Dr. Shih-Huan Peng (Genomics Research Center, Academia Sinica, Taipei, Taiwan). Targeted sequence for short hairpin RNA (shRNA)-induced silencing of Naa10p was 5'-CCCUGCACCUCUAUUCCAA-3', using psilencer2.1-U6/neo plasmid. Control shRNA sequence was 5'-UUCUCCGAAGUGUCACGU-3', PA28α-specific siRNA sequence was 5'-AAGCCAAAGGT-GATGTTGT-3', PA28β-specific siRNA sequence was 5'-CGUCAA GACAAAAUGGAA-3'. Control siRNA sequence was 5'-UUCUCC-GAAGUGUCACGU-3'. Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

2.3. Cell-based proteasome activity assay

RKO cells were cultured in 96-well plate at a density of 1 × 10^4 cells per well with 100 µl RPMI 1640 medium (HyClone, Logan, UT), supplemented with 10% fetal calf serum (Invitrogen). 12 h later, the proteasome activity was determined using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit according to the manufacturer's protocol. The luminescent intensity was measured by Lmax II luminometer (Agilent, Santa Clara, CA).

2.4. In vitro proteasome activity assay

Human 20S proteasomes (0.5 µg) or 26S proteasome fraction (0.25 µg) were assayed in a reaction buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM Mg^2+ *, 1 mM DTT. 28S activator (0.75 µg), GST-Naa10p (0.4 µg), and GST (0.2 µg) protein were added selectively according to groups for proteasome assays. Reaction was performed at 30 °C for 2 min, then the proteasome activity was detected using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit. Fluorescence was measured every 2 min for 1 h.

2.5. Homology modeling of Naa10p

The tertiary structure of Naa10p was built by homology modeling with Drug Discovery studioTM 2.5 software package (Accelrys Inc., San Diego, CA). The 3D model of Naa10p was generated by MODELLER developed by Sali et al. [28], which performs automated protein homology modeling and loop modeling for Naa10p. Then the quality of 3D model of Naa10p was evaluated by a series

Fig. 1. Ligand peptide library screening suggested Naa10p interacts with PA28β. (A) Phage ELISA was used to screen the phage clones that specifically bound with Naa10p. Microtiter wells were coated with 5 µg/ml GST-Naa10p or with GST as negative control, then blocked with 5% skim milk. Phage clones were added to the wells. Bound phages were detected by incubation with HRP-conjugated anti-M13 antibody. The values shown are the mean OD492 of triplicate samples. S.D. are indicated by error bars. (B) GST Pull-down assay of Naa10p and X12 peptide interaction. GST, GST-X12 peptide and human recombinant His-Naa10p were purified from E. coli. (C) Top scoring conformation of the docked complex of Naa10p-X12. X12 is represented by stick and ball model and the surface of Naa10p are shown by green color. (D) A Sequence homology between X12 peptide and PA28β.
of tests for internal consistency and reliability including Raman- 
chandran Plot and Profile 3D.

### 2.6. Molecular dynamic simulation and in silicon interaction study

3D structure of X12 was simulated by CHARMM in DS modeling which provides powerful mechanics and dynamics protocols for studying the energetic and motion of molecules [29]. Accelrys CHARMM forcefield was used throughout the simulation. The obtained optimized structure of X12 was used for the following docking.

Docked construction and interaction energies were obtained using the docking program ZDOCK [30]. The free energies were calculated based on shape complementarity as type of correlation using a default grid spacing of 2.0 Å. The highest-ranked 2000 decoys were clustered based on structural similarity among the predicted docking forms. The features of generated clusters were analyzed to estimate the biological relevance of Naa10p and X12 interactions. 10 top-scored docked conformations were retained for further optimization using docking program RDOCK. The binding interfaces of refined docked complexes were analyzed.

### 2.7. Immunoprecipitation, Western blot, and GST pull-down assay

Immunoprecipitation and Western blot were performed as previously reported [8]. To generate the GST-X12 fusion protein, the sense and the antisense oligonucleotide fragments encoding the protein directly interacted with His-Naa10p (Fig. 1B).

To further investigate the dynamic characteristics of this interaction, we conducted protein docking analysis of Naa10p and X12 peptide. Since the sequence identity of 2X7B to Naa10p is 37% (Fig. S1A), the crystal structure of 2X7B (available from PDB) was used as the template for homology modeling of Naa10p (ribbon representation of the 3D model of Naa10p was shown in Fig. S1B). Ramanchandran Plot and ZDOCK were performed successively, to get top scoring conformations, which show the best interaction tendency with the lowest free energy of the docked complex (detail description and parameters seen in Supplementary information). Docked conformations were retained for further optimization using docking program RDOCK and the best scored conformation of refined docked complex of Naa10p-X12 was shown in Fig. 1C. Then identification of binding interface was performed to get the best scored conformations using a grid spacing of 2.0 Å, and residues of X12 dedicated to hydrogen bonds were shown in Fig. S1C, indicating the key residues of X12 involved in the relevant interactions are Asp2, Leu3, Asp5, and Tyr7. Binding interface of Naa10p includes Arg82, Arg83, Leu84, Gly85, Leu86, Gin88, Asp92, and Tyr122, which could also be critical for the X12–Naa10p interaction.

### 2.8. Statistical analysis

Results are expressed as mean ± standard error (represented as error bars) from at least three experiments. P values were calculated by performing the two-tailed Student’s t-test and P < 0.05 was considered statistically significant.

### 3. Results

#### 3.1. Ligand peptide library screening suggested Naa10p–PA28β interaction

To gain an insight into the biological role of Naa10p, phage-displayed peptide library was applied to find the motif sequence of Naa10p-interacting proteins with GST-Naa10p as bait (detail method shown in Supplementary information). Five positive clones significantly higher than the background in ELISA assay were picked up after three rounds of panning (Fig. 1A), and all the five clones had an identical sequence, (NH2)-WDLWDFYAEAV, named X12. To verify the interaction between Naa10p with X12, GST pull-down assay was performed with recombinant proteins. The data suggested that the GST-X12 fusion protein directly interacted with His-Naa10p (Fig. 1B).

To further investigate the dynamic characteristics of this interaction, we conducted protein docking analysis of Naa10p and X12 peptide. Since the sequence identity of 2X7B to Naa10p is 37% (Fig. S1A), the crystal structure of 2X7B (available from PDB) was used as the template for homology modeling of Naa10p (ribbon representation of the 3D model of Naa10p was shown in Fig. S1B). Ramanchandran Plot and ZDOCK were performed successively, to get top scoring conformations, which show the best interaction tendency with the lowest free energy of the docked complex (detail description and parameters seen in Supplementary information). Docked conformations were retained for further optimization using docking program RDOCK and the best scored conformation of refined docked complex of Naa10p-X12 was shown in Fig. 1C. Then identification of binding interface was performed to get the best scored conformations using a grid spacing of 2.0 Å, and residues of X12 dedicated to hydrogen bonds were shown in Fig. S1C, indicating the key residues of X12 involved in the relevant interactions are Asp2, Leu3, Asp5, and Tyr7. Binding interface of Naa10p includes Arg82, Arg83, Leu84, Gly85, Leu86, Gin88, Asp92, and Tyr122, which could also be critical for the X12–Naa10p interaction.

#### 3.2. Naa10p interacts with PA28β–PA28α complex

To investigate the potential interaction between Naa10p and PA28β, we performed co-immunoprecipitation assays. Our results indicated that endogenous Naa10p was able to associate with Myc-tagged PA28β overexpressed in RKO cells (Fig. 2A). GST pull-down assay with recombinant GST-Naa10p and lysate from cells expressing Myc-PA28β also verified an association between Naa10p and PA28β (Fig. 2B). Moreover, we found that bacterially expressed recombinant His-tagged PA28β was able to bind with purified GST-Naa10p protein (Fig. 2C), indicating Naa10p interacts with PA28β directly.

In agreement with these data, immunoprecipitation was used to detect the interaction between endogenous Naa10p and PA28β in both RKO cells (Fig. 2D) and PG cells (Fig. S1D). We found that endogenous Naa10p associates with PA28β as well as PA28α (Fig. 2D and Fig. S1D). Although PA28β always complexes with PA28α and they are 48% conserved at protein level [19,31], we predicted that PA28α–Naa10p interaction may be indirect because PA28α has no DLRAFYAE motif (Fig. S1E). To verify this notion, PA28β was knocked down by siRNA in RKO cells. Silencing of PA28β had no effect on the protein level of Naa10p or PA28α, but PA28β could no longer be precipitated by anti-Naa10p antibody (Fig. 2E). However, PA28β–Naa10p interaction was not affected by PA28α silencing (Fig. 2F). These results indicated that the interaction between Naa10p and PA28β is direct and the binding of PA28α with Naa10p is PA28β-dependent.

#### 3.3. Naa10p inhibits PA28-activated chymotrypsin-like proteasome activity in vivo and in vitro

Since PA28β and PA28α form a heterohexamer to stimulate the hydrolysis of model peptide substrates in 20S core of the 28S proteasome [19], it is tempting to speculate that Naa10p may affect the activity of 28S proteasome through its interaction with PA28β. To verify this hypothesis, RKO cells stably transfected with shNaa10p were used to detect chymotrypsin-like proteasome activity, a typical PA28-activated proteasome function. As shown in Fig. 3A, the activity was up-regulated in Naa10p-silenced RKO cells. Similar results were observed in Naa10p-silenced H1299 cells
Fig. 2. Naa10p directly interacts with PA28β independent of PA28α. (A) The interaction between endogenous Naa10p and exogenous Myc-tagged PA28β in RKO cells. Coimmunoprecipitation assays was performed with anti-Naa10p antibody to pull-down proteins from lysate of RKO cells overexpressing Myc-PA28β. LC, IgG light chain. HC, IgG heavy chain. (B) GST pull-down assays using recombinant GST-Naa10p. RKO cell lysate was used as the source of exogenous Myc-PA28β. GST served as negative control. (C) GST pull-down assays, using recombinant GST-Naa10p and His-PA28β. (D) The interaction between endogenous Naa10p, PA28β and PA28α. Anti-Naa10p antibody was applied to precipitate proteins from lysate of RKO cells. (E) The interaction between endogenous Naa10p and PA28α upon PA28β silencing. (F) The interaction between endogenous Naa10p and PA28β upon PA28α silencing. Anti-Naa10p antibody was applied to precipitate proteins from lysate of RKO cells. IgG served as a negative control.
Fig. 3. Naa10p inhibits PA28-activated chymotrypsin-like proteasome activity in vivo and in vitro. (A, B and C) Naa10p inhibits PA28-activated chymotrypsin-like proteasome activity in vivo. RKO (A) and H1299 (B) cells were stably transfected with shNaa10p, and RKO (C) cells were transiently transfected with pcDNA3.1-V5-Naa10p and pcDNA3.1 (control). Upper panels showed Western blot analysis of Naa10p protein levels, β-Actin served as loading control. Lower panels displayed chymotrypsin-like proteasome activity of these cells in duplicates. *P* values were determined by Student’s *t*-test. (D) RKO cells were co-transfected with pcDNA3.1-V5-Naa10p and pCMV-Myc-PA28β, plus their respective control vectors. Protein levels of V5-Naa10p, Myc-PA28β and β-Actin were detected by Western blot. The chymotrypsin-like proteasome activity of each group was measured in triplicate. *P* values were determined by Student’s *t*-test. (E) Naa10p inhibits PA28-activated chymotrypsin-like proteasome activity in vitro. A cell-free system consisting of purified proteins was established to validate effect of Naa10p on 28S proteasomes. Each group was tested in triplicates. Different groups were discriminated by color. (F) Naa10p has no effect on chymotrypsin-like proteasome activity of 26S proteasomes.
In contrast, RKO cells overexpressing V5-tagged Naa10p exhibited lowered chymotrypsin-like proteasome activity compared with the control cells (Fig. 3C). Therefore, Naa10p functions as a negative regulator of 28S proteasome activity in cells. Protein levels of PA28α and PA28β were also evaluated to rule out the possibility that Naa10p may influence proteasome activity by altering the expression of PA28 components (Fig. 3A–C). Moreover, we co-transfected RKO cells with PA28β and Naa10p (Fig. 3D). The data demonstrated that exogenous PA28β significantly upregulated chymotrypsin-like proteasome activity, which was diminished by co-transfection of Naa10p (Fig. 3D), suggesting that PA28β and Naa10p regulated chymotrypsin-like proteasome activity simultaneously, not mutually exclusively. Next, we established a cell-free system consisting of purified proteins to further validate the influence of Naa10p on 28S proteasome activity. In agreement with the previous findings [31], 28S activator upregulated chymotrypsin-like proteasome activity of 20S proteasome core, and this activation was interfered by GST-Naa10p protein (Fig. 3E). However, GST-Naa10p protein has no effect on 20S proteasome core in the absence of 28S activator (Fig. 3E), indicating the effect of Naa10p on chymotrypsin-like proteasome activity was PA28-dependent. Additionally, we found Naa10p had no effect on chymotrypsin-like proteasome activity of 26S proteasome in the same in vitro system (Fig. 3F).

3.4. Acetyltransferase activity of Naa10p is not necessary for inhibiting chymotrypsin-like proteasome activity

To investigate the potential contribution of Naa10p’s acetyltransferase activity in regulating chymotrypsin-like proteasome activity, we used a V5-tagged mutant Naa10p construct (R82A, namely Ala was substituted for Arg82), which had lost its ability to bind with acetyl-CoA, thus exhibiting defective acetyltransferase activity as characterized by previous studies [6,7]. RKO cells transfected with V5-tagged wild-type Naa10p and Naa10p-R82A inhibited chymotrypsin-like proteasome activity to a similar extent (Fig. 4A). Same conclusion was obtained by using cell-free system using immunoprecipitated wild-type Naa10p and Naa10p-R82A (Fig. 4B). Thus, Naa10p negatively regulates PA28-activated chymotrypsin-like proteasome activity in an acetylation-independent manner.

4. Discussion

Chymotrypsin-like proteasome activity is a representative function of 20S core proteasomes, which can be activated by PA28α/β complex [19,26]. It was reported that 20S proteasome’s catalytic subunit could be inactivated by NatA in yeast [22], but association between proteasome and N-α-acetyltransferase is largely elusive. Our study presents a potential regulatory mechanism between PA28 complex and acetyltransferase Naa10p in human cells. We revealed that Naa10p interacted with both exogenous and endogenous PA28β. The result that PA28β can also interact with Naa10p strongly argues that PA28 complex may interact with Naa10p as a whole, since α subunits and β subunits never being found alone in cells [19]. However, probably due to lacking of DLRFYAE motif, PA28α’s interaction with Naa10p is mediated by PA28β.

Our data also demonstrated that Naa10p negatively regulates chymotrypsin-like proteasome activity in cells. Consistent with these, in a cell-free system consisting of purified 20S core protea-
some and 28S activator complexes, GST-Naa10p could also compromise the speed of substrate peptides degradation. However, Naa10p alone had no effect on chymotrypsin-like proteasome activity of 20S core proteasome in the absence of PA28 complex. Together, PA28β-dependent inhibition of Naa10p on chymotrypsin-like proteasome activity partially explains the biological relevance of the interaction between Naa10p and PA28β.

Although Naa10p is an acetyltransferase, there are indications that it also mediates non-catalytic functions [7,10]. In this study, we used enzyme-dead mutant Naa10p to check the contribution that it also mediates non-catalytic functions [7,10]. In this study, we used enzyme-dead mutant Naa10p to check the contribution that it also mediates non-catalytic functions [7,10]. In this study, we used enzyme-dead mutant Naa10p to check the contribution that it also mediates non-catalytic functions [7,10]. In this study, we used enzyme-dead mutant Naa10p to check the contribution that it also mediates non-catalytic functions [7,10].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.jfebslet.2013.04.016](http://dx.doi.org/10.1016/j.jfebslet.2013.04.016).

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