FEBS Letters 588 (2014) 111–116

FEBS Letters

journal homepage: www.FEBSLetters.org

N-terminal signal sequence is required for cellular trafficking and hyaluronan-depolymerization of KIAA1199

Hiroyuki Yoshida *, Aya Nagaoka, Sachiko Nakamura, Megumi Tobiishi, Yoshinori Sugiyama, Shintaro Inoue

Innovative Beauty Science Laboratory, Kanebo Cosmetics Inc., 3-28, 5-chome, Kotobuki-cho, Odawara-shi, Kanagawa 250-0002, Japan

ARTICLE INFO

Article history:
Received 4 November 2013
Accepted 10 November 2013
Available online 20 November 2013

Edited by Veli-Pekka Lehto

Keywords:
KIAA1199
Hyaluronan
Catabolism
Signal sequence
N-Glycosylation
Post-translational modification

ABSTRACT

Recently, we disclosed that KIAA1199-mediated hyaluronan (HA) depolymerization requires an acidic cellular microenvironment (e.g. clathrin-coated vesicles or early endosomes), but no information about the structural basis underlying the cellular targeting and functional modification of KIAA1199 was available. Here, we show that the cleavage of N-terminal 30 amino acids occurs in functionally matured KIAA1199, and the deletion of the N-terminal portion results in altered intracellular trafficking of the molecule and loss of cellular HA depolymerization. These results suggest that the N-terminal portion of KIAA1199 functions as a cleavable signal sequence required for proper KIAA1199 translocation and KIAA1199-mediated HA depolymerization.

1. Introduction

Hyaluronan (HA) is a high molecular weight, non-sulfated glycosaminoglycan component of the extracellular matrix present in many tissues, such as skin, cartilage, and other connective tissues, providing structural and functional integrity to the organs. HA is rapidly depolymerized under physiological conditions from extra-large native molecules to intermediate-size fragments in the extracellular milieu [1]. HA degradation is enhanced under certain pathological conditions, and its lower-molecular-weight products are commonly detected in diseases such as arthritis and cancers [2,3]. Two hyaluronidases HYAL1 and HYAL2 and cell surface HA receptor CD44 were reported to play key roles in HA degradation [4,5]. However, we have recently shown that KIAA1199 is essential for endogenous HA degradation in human skin fibroblasts and that transfection of *KIAA1199* cDNA into cells confers on them the ability to catabolize HA via the clathrin-coated pit pathway [6]. In addition, KIAA1199 was expressed by dermal fibroblasts in normal skin and over-expressed by synovial fibroblasts and tissues from arthritic joints [6]. All these data suggest that KIAA1199 plays a key role in HA catabolism under certain physiological and pathological conditions.

KIAA1199 contains a predicted 4083 bp ORF encoding a protein with a molecular weight of 153 kDa (1361 amino acids) (Fig. 1A) [7], which is composed of four PbH1 domains, consisting of parallel β-helix repeats, believed to be involved in polysaccharide hydrolysis; one G8 domain, containing eight conserved glycine residues and consisting of five β-strand pairs and one α-helix, expected to take part in extracellular ligand binding; two GG domains, consisting of seven β-strands and two α-helices, of no predicted function; and seven predicted *N*-glycosylation sites [8–10]. And, the N-terminal portion of 30 amino acids is predicted to be a cleavable signal sequence for ER (endoplasmic reticulum) targeting [7]. Our previous study has demonstrated that two mutations at the Arg^{187} residue (R187C and R187H) located in one of the GG domains result in loss of HA depolymerization [6]. All these data suggest that the GG, G8 and PbH1 domains are responsible for KIAA1199-mediated binding and depolymerization of HA. We have also shown that KIAA1199 is localized close to the clathrin heavy chain in some vesicles in the periphery of KIAA1199-expressing cells, and thus an acidic, dynamic and energy-coupling microenvironment may be crucial for KIAA1199-mediated HA depolymerization [6]. It has also been shown that KIAA1199 is secreted to

Abbreviations: HA, hyaluronan; HYAL, hyaluronidase; ORF, open reading frame; ER, endoplasmic reticulum; HEK, human embryonic kidney; FBS, fetal bovine serum; FA, fluoresceinamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; CPC, cetylpyridinium chloride; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% (vol/vol) Tween 20; DMEM, Dulbecco's Modified Eagle's Medium; Endo H, endo-β-*N*-acetylglucosaminidase H; DTT, dithiothreitol; PDI, protein disulfide isomerase

* Corresponding author. Fax: +81 465343037.
E-mail address: yoshida.hiroyuki@kanebocos.co.jp (H. Yoshida).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.11.017

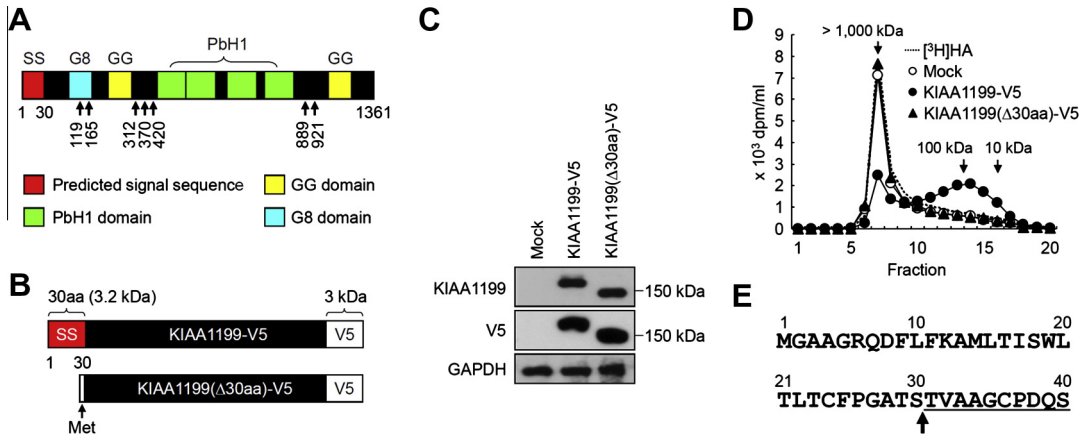

Fig. 1. HA depolymerization by HEK293 cells expressing KIAA1199-V5 and KIAA1199(Δ30aa)-V5, and N-terminal amino acid sequence of functionally expressed KIAA1199. (A) The schematic representation of the structure of full-length KIAA1199. Functional domains of KIAA1199 are indicated as follows: SS, predicted N-terminal signal sequence; PbH1, PbH1 domain; GG, GG domain; G8, G8 domain. The predicted *N*-glycosylation sites are shown by arrows. Numbers indicate the positions of the residues relative to the N-terminus of the full length KIAA1199. (B) A schematic diagram showing constructs of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 used for transfection. V5, C-terminal V5-epitope tag. Molecular weights of SS and V5 tag are about 3.2 kDa and 3 kDa, respectively. (C) HEK293 cells were transiently transfected with either an empty vector (Mock) or a vector containing *KIAA1199-V5* or *KIAA1199(Δ30aa)-V5* cDNA. The expression of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 proteins was assessed by immunoblotting using anti-KIAA1199 and V5 monoclonal antibodies. GAPDH, a loading control. (D) Mock, KIAA1199-V5 and KIAA1199(Δ30aa)-V5 transfectants were incubated with [^{3}H]HA for 24 h, and HA depolymerization was then examined by chromatography on a Sepharose CL-2B column. Dotted lines indicate [^{3}H]HA without incubation. (E) N-terminal amino acid sequence of KIAA1199. The signal sequence cleavage site is shown by an arrow, and the actual N-terminal protein sequence obtained is underlined.

culture media from KIAA1199-overexpressing SW480 cells [10]. However, the structural basis underlying the cellular targeting and functional modification of KIAA1199 to exert cellular HA depolymerization and secretion to extracellular space remains elusive. In the current study, we investigated post-translational maturation of KIAA1199 and its possible roles in the functional expression of the molecule in HA depolymerization, and demonstrated that the N-terminal 30 amino acid portion of KIAA1199 is the signal sequence essential for proper translocation of KIAA1199 and cellular HA depolymerizing activity.

2. Materials and methods

2.1. Preparations of plasmids and transfectants

Expression vectors for KIAA1199 with a C-terminal V5-epitope (KIAA1199-V5) and its N-terminal truncated form (KIAA1199(-Δ30aa)-V5) were constructed by inserting the full length *KIAA1199* cDNAs and the N-terminal 30 amino acid-deleted cDNAs for which the codon of residues of Met^1 to Ser^{30} was replaced by an ATG start codon into the expression vector pcDNA3.1/V5-His (Invitrogen) according to the manufacture's protocol, respectively. The authenticity of the cDNAs was verified by sequencing using Applied Biosystems 3730xl DNA Analyzer (Life Technologies). Transient transfection was done using Lipofectamine LTX (Invitrogen), and transfectants were used for the experiments at 48 h after transfection.

2.2. Antibodies

A rat monoclonal antibody against KIAA1199 was previously developed using a peptide of CA^{762}RYSPHQDADPLKPRE^{777}, which corresponds to the amino acid residues Ala^{762} to Glu^{777} of KIAA1199 (GenBank accession number NM_018689) [6]. The mouse anti-V5 antibody was from Invitrogen. The rabbit anti-PDI and anti-GAPDH antibodies were from Santa Cruz Biotechnology. The mouse anti-GM130 antibody was from BD Biosciences. The rabbit anti-TGN46 antibody was from Abcam.

2.3. Determination of signal peptide cleavage site of KIAA1199

To prepare cell homogenate supernatants, HEK293 cells expressing KIAA1199-V5 were lysed in 50 mM Tris–HCl buffer, pH 8.0 containing 1% Nonidet P-40 and a cocktail of proteinase inhibitors (Roche Diagnostics), and cleared by centrifugation. Conditioned media were also collected from the cells, and concentrated 20-fold with Amicon Ultra-10 kDa NMWL (Millipore). KIAA1199-V5 in the homogenate supernatants and the conditioned medium concentrates were immunoprecipitated using Dynal magnetic beads protein G (Invitrogen) covered with monoclonal antibody against KIAA1199. The beads were washed and then incubated with NuPAGE LDS sample-loading buffer (Invitrogen) for 10 min at 70 °C. The samples were electrophoresed on NuPAGE 4–12% Bis–Tris gels (Invitrogen) under reducing conditions, and proteins were transferred onto polyvinylidene difluoride membrane and stained with Coomassie Brilliant Blue. The bands corresponding to KIAA1199 were excised from the membrane for peptide sequencing. N-terminal amino acids up to the tenth were determined by standard Edman degradations using a Procise 494 HT Protein Sequencing System (Applied Biosystems).

2.4. Deglycosylation of KIAA1199 with N-glycanase or Endo H

HEK293 cells expressing KIAA1199-V5 or KIAA1199(Δ30aa)-V5, and Detroit 551 skin fibroblasts were homogenized in 50 mM phosphate buffer, pH 7.0, containing a cocktail of proteinase inhibitors (Roche Diagnostics) and cleared by centrifugation. Conditioned media were collected from the KIAA1199-V5 transfectants, and concentrated 5-fold with Amicon Ultra-10 kDa NMWL (Millipore). For *N*-glycanase digestion, the homogenate supernatants and the conditioned medium concentrates were boiled in the presence of 0.1% SDS and 50 mM β-mercaptoethanol for 5 min, and then incubated with 0.1 U/ml *N*-glycanase (Glyko) for 20 h at 37 °C according to the manufacturer's protocol. In the case of Endo H, samples were boiled in the presence of 0.5% SDS and 40 mM DTT for 10 min, and then incubated with 25 000 U/ml Endo H (New England BioLabs) according to the manufacturer's

protocol. The samples were electrophoresed on NuPAGE 6% Tris–glycine gels (Invitrogen) and immunoblotted with anti-KIAA1199 monoclonal antibody.

2.5. Immunofluorescence microscopy of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 in HEK293 cells

For double immunostaining of KIAA1199 or V5-epitope and Golgi markers (GM130 and TGN46) or ER marker (PDI), HEK293 cells expressing KIAA1199-V5, KIAA1199(Δ30aa)-V5 were grown on glass multi-chamber slides (BD Biosciences) to 70–80% confluence, and fixed with 4% (wt/vol) paraformaldehyde in PBS. After washing in PBS containing 0.05% (vol/vol) Tween 20 (PBS-T), the cells were reacted with anti-KIAA1199 monoclonal antibody conjugated to Alexa-Fluor 488 and mouse anti-GM130 antibody conjugated to Alexa-Fluor 555. The samples were mounted in VECTASHIELD (Vector). As for controls, samples were reacted with non-immune IgG conjugated to Alexa-Fluors. These samples were observed using Zeiss LSM 510 confocal microscope (Carl Zeiss). Signal intensity line-scan analysis and co-localization analysis were performed with Zeiss Efficient Navigation software (Carl Zeiss).

2.6. Pulse-chase labeling and immunoprecipitation

HEK293 cells expressing KIAA1199-V5 were preincubated with Met/Cys-free Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) for 1 h, and then pulsed for 30 min with medium containing [^{35}S]Met (PerkinElmer), then chased for 2, 4, 8 or 24 h with complete DMEM (Sigma). The treated cells were lysed in 50 mM Tris–HCl buffer, pH 8.0 containing 1% Nonidet P-40 and a cocktail of proteinase inhibitors (Roche Diagnostics), and cleared by centrifugation. Conditioned media were also collected from the cells, and concentrated 10-fold. KIAA1199-V5 in the homogenate supernatants and conditioned medium concentrates was immunoprecipitated with monoclonal antibody against KIAA1199 as described above. The beads were washed and then incubated with Tris–glycine SDS sample buffer (Invitrogen) for 10 min at 70 °C. The samples were analyzed using NuPAGE 6% Tris–glycine gels (Invitrogen) and autoradiography with the Typhoon Imager (GE Healthcare).

3. Results and discussion

3.1. Cleavage of N-terminal portion of 30 amino acids occurs in functionally mature KIAA1199 in HEK293 cells transfected with the full-length KIAA1199 cDNA

To assess the possible role of N-terminal 30 amino acids of KIAA1199 in cellular HA depolymerization, HEK293 cells were transfected with expression vectors encoding the full-length KIAA1199 with a C-terminal V5-epitope tag (KIAA1199-V5) and its N-terminally truncated form whose residues of Met^{1} to Ser^{30} have been replaced by Met before Thr^{31} (KIAA1199(Δ30aa)-V5), respectively (Fig. 1B). KIAA1199-V5 was detected with an apparent molecular weight of about ~160 kDa by immunoblotting using anti-KIAA1199 monoclonal antibody, and estimated to be larger than KIAA1199(Δ30aa)-V5 by ~10 kDa (Fig. 1C). The similar results were obtained using an antibody against the V5-epitope (Fig. 1C), indicating that there is no C-terminal processing. We then examined HA depolymerization by HEK293 cells expressing KIAA1199-V5 or KIAA1199(Δ30aa)-V5, and found that KIAA1199-V5 transfectants degrade exogenous high-molecular-weight [^{3}H]-labeled HA ([^{3}H]HA) to intermediate size fragments in a similar manner to normal human skin fibroblasts Detroit 551 cells [6], whereas mock and KIAA1199(Δ30aa)-V5 transfectants showed negligible activity (Fig. 1D), suggesting that the expression of full-length KIAA1199 containing the N-terminal 30 amino acids is essential for HA depolymerization. In order to verify the prediction that the signal sequence of KIAA1199 is cleaved at the Ser^{30} and Thr^{31} bond [7], we performed the N-terminal sequence analysis of the purified KIAA1199-V5 protein from cell lysates of KIAA1199-V5 transfectants by immunoprecipitation with anti-KIAA1199 monoclonal antibody, and identified it as T^{31}VAAGCPDQS (Fig. 1E), indicating that recombinant KIAA1199-V5 is indeed cleaved between the Ser^{30} and Thr^{31} bond. Since pre-processed KIAA1199-V5 was not observed, N-terminal processing seems to occur promptly after translation. The size difference between the two forms is related to differences in *N*-glycosylation as described in the next section.

3.2. KIAA1199 is N-glycosylated, and N-terminal 30 amino acids of KIAA1199 are necessary for proper KIAA1199 translocation

To examine the possibility that mature forms of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 are glycosylated, both proteins were treated with *N*-glycanase or Endo H to remove *N*-linked oligosaccharides. After deglycosylation, KIAA1199-V5 protein reduced in apparent size and located at the position close to KIAA1199(-Δ30aa)-V5 by immunoblotting using anti-KIAA1199 antibody (Fig. 2A). Similar results were observed with endogenous KIAA1199 in Detroit 551 skin fibroblasts (Fig. 2A). On the other hand, treatments with *N*-glycanase or Endo H did not affect the size of KIAA1199(Δ30aa)-V5 (Fig. 2A). After concurrent *O*-glycosidase and neuraminidase treatment, there was no further reduction in the molecular weights of the *N*-deglycosylated KIAA1199-V5, KIAA1199(Δ30aa)-V5 or endogenous KIAA1199 (data not shown). Thus, it seems likely that KIAA1199 is *N*-glycosylated, and the KIAA1199-V5 protein could be an *N*-glycosylated form of KIAA1199(Δ30aa)-V5. Our earlier study showed that KIAA1199-mediated HA depolymerization involves a clathrin-coated pit endocytic pathway, and KIAA1199 is localized in vesicles in peripheral regions of KIAA1199-expressing cells [6]. The observation that KIAA1199(Δ30aa)-V5 lacked *N*-glycanase and Endo H-sensitive *N*-glycosylation, an ER/early Golgi transportational indicator, raised the question of whether this construct failed to translocate to ER/Golgi, leading to a defect in the subsequent transport to the vesicles in the cell periphery. To address this question, we examined the subcellular distribution of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 in HEK293 cells. By immunocytochemistry using anti-KIAA1199 antibody, we found that KIAA1199-V5 is concentrated at a perinuclear region together with the staining in some vesicles in the peripheral region (Fig. 2B). Since this perinuclear distribution pattern of KIAA1199-V5 seemed consistent with distribution in the Golgi apparatus, we performed double immunostaining of KIAA1199-V5 and GM130 or TGN46 (markers for the Golgi apparatus), and demonstrated the co-localization, and a line-scan analysis showed fluorescence intensities of KIAA1199-V5 and GM130 or TGN46 rising and falling in parallel (Fig. 2B, *KIAA1199-V5 column in top and middle panels*). In contrast, KIAA1199(Δ30aa)-V5 was localized diffusely throughout the cytoplasm without localization in the Golgi area, and a line-scan analysis revealed that fluorescence intensities of KIAA1199(Δ30aa)-V5 rise where there are reductions in GM130 or TGN46 intensities (Fig. 2B, *KIAA1199(Δ30aa)-V5 column in top and middle panels*). A co-localization analysis of KIAA1199-V5 or KIAA1199(Δ30aa)-V5 with GM130 revealed mild co-localization (35.53% ± 6.34) between KIAA1199-V5 and GM130, but negligible co-localization (<1%) between KIAA1199(Δ30aa)-V5 and GM130 (Supplementary Fig. 1A). Similar distribution patterns and line-scan profiles of KIAA1199-V5 or KIAA1199(Δ30aa)-V5 and GM130 were obtained with anti-V5 and anti-GM130 antibodies (Fig. 2B, *bottom panels*). In addition,

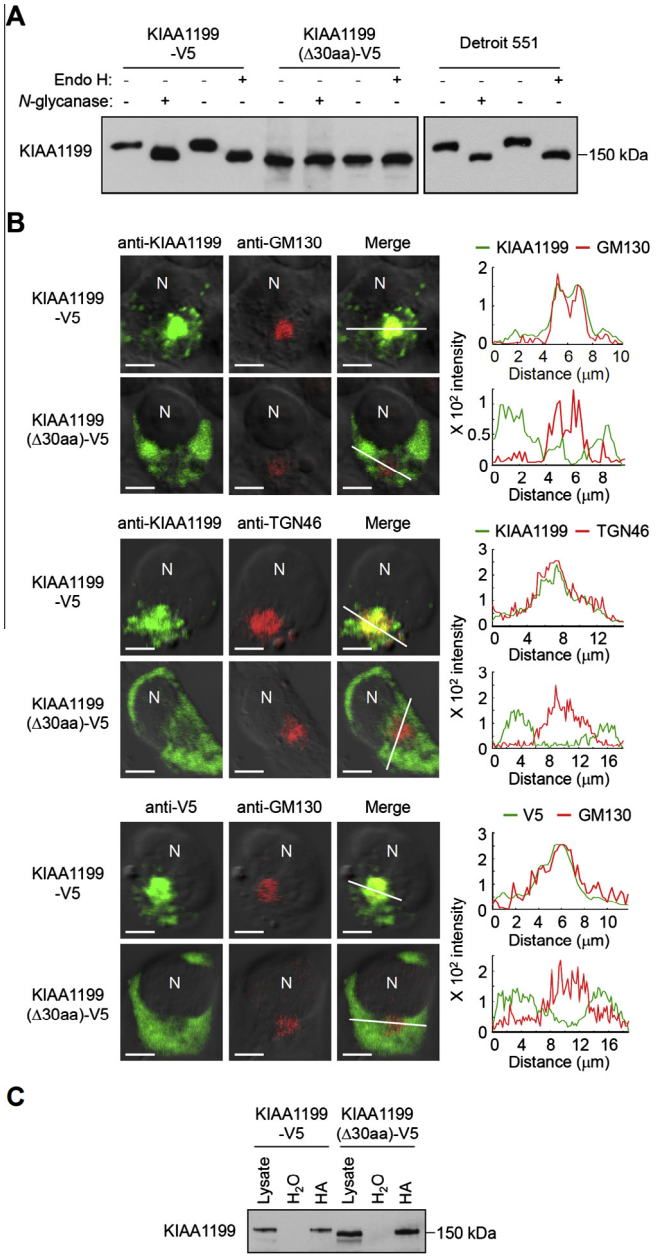

Fig. 2. Deglycosylation, cellular localization and HA-binding capability of KIAA1199-V5 and KIAA1199(Δ30aa)-V5. (A) Deglycosylation of KIAA1199. The lysates of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 transfectants, and Detroit 551 skin fibroblasts were treated with *N*-glycanase or Endo H, and then subjected to immunoblotting with anti-KIAA1199 antibody. (B) Double immunostaining of KIAA1199-V5 or KIAA1199(Δ30aa)-V5 (*green*) and Golgi markers (*red*) with anti-KIAA1199 or anti-V5 antibodies and anti-GM130 or anti-TGN46 antibodies in HEK293 cells expressing KIAA1199-V5 or KIAA1199(Δ30aa)-V5. Line-scans were taken from the merge column and showed profiles of fluorescence intensity against line distance. N: nucleus; Scale bar: 5 μm. (C) Binding of KIAA1199-V5 and KIAA1199(Δ30aa)-V5 to HA. The lysates of HEK293 cells expressing KIAA1199-V5 or KIAA1199(Δ30aa)-V5 were incubated with H_2O (negative control) or HA. The samples were precipitated with CPC and subjected to immunoblotting with anti-KIAA1199 antibody.

we performed double immunostaining using anti-KIAA1199 and PDI antibodies (ER marker), and showed that merged images of some of the KIAA1199-V5 and PDI were observed in the perinuclear region in KIAA1199-V5 transfectants, but were lacking in KIAA1199(Δ30aa)-V5 transfectants (Supplementary Fig. 1B). No fluorescence was observed by non-immune IgG (data not shown). These results suggest that due to the lack of N-terminal 30 amino acids, KIAA1199(Δ30aa)-V5 abolishes the translocation to ER during protein translation and/or results in a folding defect that is recognized by the quality control system of the ER, which in turn results in a defect in a subsequent transportation to the Golgi apparatus and an accumulation of the protein in the cytoplasm. In order to assess the possible role of *N*-glycosylation in KIAA1199 function, we examined the HA-binding activity of KIAA1199-V5 and KIAA1199(Δ30aa)-V5. When KIAA1199-V5 and KIAA1199 (Δ30aa)-V5 proteins isolated from the corresponding transfectants were incubated with HA and then precipitated with cetylpyridinium chloride (CPC), both proteins were co-precipitated with HA (Fig. 2C), indicating that both possess the capability to bind to HA, and therefore suggesting that *N*-linked oligosaccharides are unnecessary for HA binding.

3.3. Mature form of KIAA1199 is secreted extracellularly

As previously reported with SW480 cells [10], we also detected KIAA1199 protein in conditioned media from KIAA1199-V5 transfectants (Fig. 3A) and Detroit 551 skin fibroblasts (Fig. 3B) by immunoblotting using anti-KIAA1199 or V5 antibodies, but it was absent in conditioned media from mock and KIAA1199(-Δ30aa)-V5 transfectants (Fig. 3A), and KIAA1199 siRNA-treated skin fibroblasts (Fig. 3B). The treatment of KIAA1199-V5 transfectants with 0–100 μg/ml cycloheximide showed that KIAA1199 proteins in cell lysates and conditioned media decrease dose-dependently in parallel (Fig. 3C), and pulse-chase experiment using KIAA1199-V5 transfectants demonstrated that KIAA1199 is not accumulated intracellularly but observed extracellularly, suggesting that most of KIAA1199 seems to be efficiently secreted into the conditioned medium (Fig. 3C and D). The secreted form of KIAA1199-V5 in conditioned media was sensitive to *N*-glycanase and Endo H (Fig. 3E) and its N-terminal sequence was identified as TVAAGCPDQS (Fig. 3F), which are consistent with the results with KIAA1199-V5 in cell lysate (Fig. 1E and 3E). We also found that the secreted form of KIAA1199-V5 retains HA-binding capability (Fig. 3G). However, [^{3}H]HA was not depolymerized when it was incubated with conditioned media from KIAA1199-V5 transfectants or Detroit 551 skin fibroblasts at a pH range between 4.0 and 7.0 (data not shown).

The present study is the first to investigate the functional modification of KIAA1199 molecule. By determining the N-terminal sequence and the *N*-glycanase treatment, we showed that functionally mature KIAA1199 is indeed cleaved at the Ser^{30} and Thr^{31} bond and *N*-glycosylated as previously predicted [7,10]. On the other hand, deletion of the N-terminal 30 amino acids of KIAA1199 resulted in altered intracellular trafficking and a loss of *N*-glycosylation and HA depolymerization without affecting HA-binding capability. All these findings provide the evidence that the N-terminal portion of the pre-processed KIAA1199 is a cleavable signal sequence essential in mediating the proper translocation and the functional expression of KIAA1199 in HA depolymerization; targeting the molecule to ER and the subsequent transport to vesicles in cell periphery via Golgi apparatus, which are crucial for KIAA1199-mediated HA depolymerization. Alternatively, we could not exclude the possibility that *N*-linked oligosaccharides are directly involved in the degrading activity of HA, since KIAA1199-mediated HA depolymerizing activity was lost after KIAA1199 protein was isolated from cells, probably because the fine conditions for HA degradation could not be reconstituted in cell lysates [6]. In addition, since we showed that secreted KIAA1199 has the mature form, and deletion of the N-terminal 30 amino acids of KIAA1199 resulted in a defect in secretion, KIAA1199 may be secreted from the cells after targeting to ER and post-translational modification. KIAA1199 was previously identified in exosomes released from LIM1215 colorectal carcinoma cells [11], but we found that KIAA1199 secreted from

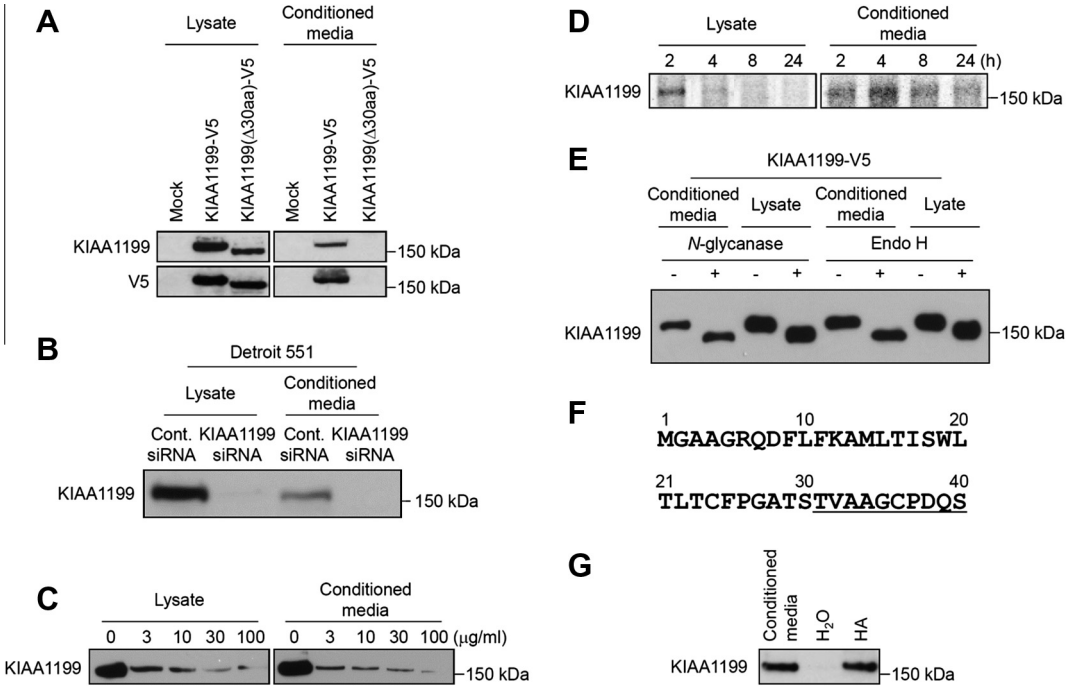

Fig. 3. Deglycosylation, N-terminal amino acid sequence, and HA-binding capability of KIAA1199 secreted extracellularly. (A) HEK293 cells were transiently transfected with an empty vector (Mock) or a vector containing *KIAA1199-V5* or *KIAA1199(Δ30aa)-V5* cDNA. KIAA1199-V5 and KIAA1199(Δ30aa)-V5 proteins in the cell lysates and 5-fold concentrated conditioned media were assessed by immunoblotting using anti-KIAA1199 or anti-V5 monoclonal antibodies. (B) KIAA1199 protein in the cell lysates and 5-fold concentrated conditioned media from Detroit 551 skin fibroblasts treated with control non-silencing siRNA or targeting siRNA for KIAA1199 were assessed by immunoblotting using anti-KIAA1199 antibody. (C) Effects of cycloheximide on KIAA1199 secretion. KIAA1199-V5 transfectants were incubated for 24 h in the absence or presence of 3, 10, 30, 100 μg/ml cycloheximide. KIAA1199 protein in the cell lysates and 10-fold concentrated conditioned media were assessed by immunoblotting using anti-KIAA1199 antibody. (D) Pulse-chase experiment in KIAA1199-V5 transfectants. Cells were starved for 1 h in Met/Cys free media and then pulse labeled with [^{35}S]Met for 30 min, followed by 2, 4, 8, or 24-h chase. Cell lysates and 10-fold concentrated conditioned media were subjected to anti-KIAA1199 immunoprecipitation and NuPAGE 6% Tris–glycine gels and detected by autoradiography. (E) Deglycosylation of KIAA1199-V5. The lysate and 5-fold concentrated conditioned media of KIAA1199-V5 transfectants were treated with *N*-glycanase or Endo H and then subjected to immunoblotting with anti-KIAA1199 antibody. (F) N-terminal amino acid sequence of KIAA1199-V5 secreted into conditioned media. The actual N-terminal protein sequence obtained is underlined. (G) Binding of secreted KIAA1199-V5 to HA. Conditioned media of KIAA1199-V5 transfectants were incubated with H_2O (negative control) or HA. The samples were precipitated with CPC and subjected to immunoblotting with anti-KIAA1199 antibody.

KIAA1199-V5 transfectants and Detroit 551 skin fibroblasts was not precipitated after ultracentrifugation at 100000×*g* for 1 h (data not shown), suggesting that secretion of KIAA1199 from these cells is unlikely to involve exosome-dependent pathway. Importantly, the observations in which secreted mature-form KIAA1199 showed no HA degrading activity support the idea that KIAA1199-mediated HA depolymerization occurs through rapid vesicle endocytosis [6]. On the other hand, the finding that secreted KIAA1199 had an HA-binding capability may reflect the need for a specific condition and/or interaction with other molecules to show the activity extracellularly, or another potential role of the protein apart from HA degradation. However, further detailed studies are necessary to better understand the roles of secreted KIAA1199. Because of the biological and therapeutic significance of KIAA1199 in HA catabolism in healthy skin [6], a major determinant organ for total body HA turnover [1], and in the KIAA1199-linked diseases such as arthritis, cancers, hearing loss, and Werner syndrome [6,7,12,13], further study to clarify the molecular mechanism underlying post-translational maturation and spatial regulation for the functional expression of KIAA1199, as well as its expressional regulation at the transcription level, would be of importance.

Acknowledgement

We thank Dr. Yasunori Okada of Keio University for reviewing the manuscript.

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.febslet.2013.11.017.

References

[1] Pandey, M.S., Harris, E.N., Weigel, J.A., et al. (2008) The cytoplasmic domain of the hyaluronan receptor for endocytosis (HARE) contains multiple endocytic motifs targeting coated pit-mediated internalization. J. Biol. Chem. 283, 21453–21461.
[2] Ghosh, P. (1994) The role of hyaluronic acid (hyaluronan) in health and disease: interactions with cells, cartilage and components of synovial fluid. Clin. Exp. Rheumatol. 12, 75–82.
[3] Sugahara, K.N., Murai, T., Nishinakamura, H., et al. (2003) Hyaluronan oligosaccharides induce CD44 cleavage and promote cell migration in CD44-expressing tumor cells. J. Biol. Chem. 278, 32259–32265.
[4] Harada, H. and Takahashi, M. (2007) CD44-dependent intracellular and extracellular catabolism of hyaluronic acid by hyaluronidase-1 and -2. J. Biol. Chem. 282, 5597–5607.
[5] Csoka, A.B., Frost, G.I. and Stern, R. (2001) The six hyaluronidase-like genes in the human and mouse genomes. Matrix Biol. 20, 499–508.
[6] Yoshida, H., Nagaoka, A., Kusaka-Kikushima, A., et al. (2013) KIAA1199, a deafness gene of unknown function, is a new hyaluronan binding protein involved in hyaluronan depolymerization. Proc. Natl. Acad. Sci. USA 110, 5612–5617.
[7] Abe, S., Usami, S. and Nakamura, Y. (2003) Mutations in the gene encoding KIAA1199 protein, an inner-ear protein expressed in Deiters' cells and the fibrocytes, as the cause of nonsyndromic hearing loss. J. Hum. Genet. 48, 564–570.
[8] Guo, J., Cheng, H., Zhao, S., et al. (2006) GG: a domain involved in phage LTF apparatus and implicated in human MEB and non-syndromic hearing loss diseases. FEBS Lett. 580, 581–584.

[9] He, Q.Y., Liu, X.H., Li, Q., et al. (2006) G8: a novel domain associated with polycystic kidney disease and non-syndromic hearing loss. Bioinformatics 22, 2189–2191.
[10] Birkenkamp-Demtroder, K., Maghnouj, A., Mansilla, F., et al. (2011) Repression of KIAA1199 attenuates Wnt-signalling and decreases the proliferation of colon cancer cells. Br. J. Cancer 105, 552–561.
[11] Mathivanan, S., Lim, J.W., Tauro, B.J., et al. (2010) Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature. Mol. Cell. Proteomics 9, 197–208.
[12] Michishita, E., Garces, G., Barrett, J.C., et al. (2006) Upregulation of the KIAA1199 gene is associated with cellular mortality. Cancer Lett. 239, 71–77.
[13] Matsuzaki, S., Tanaka, F., Mimori, K., et al. (2009) Clinicopathologic significance of KIAA1199 overexpression in human gastric cancer. Ann. Surg. Oncol. 16, 2042–2051.