N-glycosylation is required for secretion and enzymatic activity of human hyaluronidase1

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1. Introduction

Protein glycosylation is important for protein functions, such as stability, folding, and secretion [1]. N-glycosylation is a well-known type of glycosylation in which N-glycans are directly attached to asparagine residue in proteins. This modification is typically observed within the sequence motif Asn-Xaa-Ser/Thr (Xaa represents any amino acids except Pro), and the asparagine residue may be N-glycosylated [2,3]. Protein glycosylation takes place enzymatically inside the lumen of the endoplasmic reticulum (ER) [4,5]. Dolichol-phosphate precursor, composed of pre-assembled oligosaccharide, is attached to certain Asn residues and is trimmed to various types of sugar chains. More than 30 enzymes, located in the cytosol, the ER, and the Golgi apparatus, are involved in N-glycosylation. They are required to generate, attach, and process the oligosaccharides; particularly, oligosaccharyltransferase plays an essential role in synthesis of N-glycans [4–6]. Because of the complicated structure and various patterns of N-glycans, the functions of N-glycosylation have not been clarified yet.

The aberrant condition of N-glycosylation is known to correlate with many diseases, such as cancer and rheumatoid arthritis [7,8]. Furthermore, in humans, defect in N-glycosylation induces disorders, which are often referred to as congenital disorders of glycosylation [9]. Therefore, further detailed investigations are required to clarify the roles of the modification.

Human hyaluronidase1 (HYAL1) is a member of the hyaluronidase family (HYALs), which hydrolyze the β1–4 linkage between N-acetylgalactosamine and glucuronic acid of hyaluronic acid (HA) polymers. Degradation of the extracellular matrix (ECM) by hydrolases, particularly matrix metalloproteinase 9, is known to associate with tumor cell growth, proliferation, and metastasis [10]. Similarly, upregulation of HYAL1 is reported to correlate with tumor cell proliferation, migration, invasion, and angiogenesis in many cancers [11–14]. In contrast, defects of HYAL1 are also known to correlate with juvenile idiopathic arthritis and mucopolysaccharidosis [15,16]. Thus, it is important to keep the optimal HYAL1 activity in the tissues.

HYAL1 has 3 possible N-glycosylation consensus sequences at Asn99, Asn216, and Asn350 [17]. A previous study predicted the presence of N-glycosylation at all asparagine residues by electron density map analysis [11]. However, there has been no direct evidence that demonstrates the presence of N-glycosylation within HYAL1 by mass spectrometry; therefore, we undertook matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis to examine whether HYAL1 is N-glycosylated. Furthermore, the importance of N-glycosylation was

Abbreviations: HYAL1, hyaluronidase1; HA, hyaluronic acid; ER, endoplasmic reticulum; ECM, extracellular matrix; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; CBB, Coomassie Brilliant Blue; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; SDS, sodium dodecyl sulfate

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demonstrated, particularly at Asn\(^{350}\), for enzymatic activity, evaluated by site-directed mutation (substitution of Asn to Ala) and microplate assay and kinetic analysis [18]. We evaluated the enzymatic activity of every N-glycosylation-defective mutant, changing asparagine to glutamine, by using in-gel digestion assay. In this report, we examined the presence of N-glycosylation and its functional significance for HYAL1. We could demonstrate that the 3 predicted asparagine residues were N-glycosylated and N-glycosylation played important roles in the secretion and enzymatic activity of HYAL1. Since HYAL1 is known to correlate with tumor malignancy, N-glycosylation of HYAL1 may be a new target of cancer therapeutics.

2. Materials and methods

2.1. Cell culture

Human fibrosarcoma HT1080 cells, purchased from Japanese Cancer Research Resources Bank, were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Nissui, Tokyo, Japan), supplemented with 10% (v/v) fetal bovine serum, 100 mg/L kanamycin, 100 units/mL penicillin G, 600 mg/L L-glutamine, and 2.25 g/L Na\(_2\)HCO\(_3\), at 37 °C in a humidified incubator with 5% CO\(_2\).

2.2. Construction of HYAL1 expression plasmids

Wild-type HYAL1-myc-his\(_6\) cDNA, which was subcloned into pcI-neo vector (Promega, Madison, WI), was constructed previously [19]. We substituted the Asn\(^{99}\), Asn\(^{216}\), and Asn\(^{350}\) residues in HYAL1 with Gln residues by PCR site-directed mutagenesis [20]. The sequences of primers used for the mutagenesis were as follows: N99Q: 5'-TGCCCGCCAAGCCACGGCTC-3' (forward) and 5'-CAGGCTGCTTGCTGGGGCC-3' (reverse), N216Q: 5'-CTAAGCCCCCAATACACCGG-3' (forward) and 5'-CCGGTGTAATGGGGCCTTAG-3' (reverse), and N350Q: 5'-TTCACTCTCACTTGCAGGATGAA-3' (forward) and 5'-GCACTGACGAGCAGAGTGGAGCCAG-3' (reverse).

2.3. Establishment of HYAL1-expressing cell lines

A permanent cell line expressing wild-type HYAL1 (HT1080-HYAL1-MH) was established previously [19]. Permanent cell lines expressing mutant HYAL1-myc-his\(_6\) were established by transfecting the vectors into HT1080 cells, followed by 400 μg/mL G418 (Roche Applied Sciences, Indianapolis, CA) selection. The cells that expressed high levels of myc-his\(_6\)-tagged HYAL1 (N99Q, N216Q, and N350Q) were designated HT1080-HYAL1-MH/N99Q, HT1080-HYAL1-MH/N216Q, and HT1080-HYAL1-MH/N350Q cells, respectively. The cells transfected with pcI-neo vector were designated HT1080-neo [19].

2.4. Western blotting

To perform western blotting, we carried out previously described methods with slight modifications [21,22]. Cells were lysed in a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C with sonication. The lysates were centrifuged at 14,000 rpm for 10 min, and the amount of protein was measured by staining with Coomassie Brilliant Blue (CBB) G-250 (Bio-Rad Laboratories, Hercules, CA). Loading buffer (350 mM Tris–HCl, pH 6.8, 30% glycerol, 6% SDS, 0.012% bromophenol blue, and 30% 2-mercaptoethanol) was added to each lysate, which was subsequently boiled for 3 min and electrophoresed on SDS–polyacrylamide gels. Proteins were transferred to PVDF membranes and immunoblotted with anti-c-myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-α-tubulin (Sigma) antibodies. Detection was performed with enhanced chemiluminescence reagent (Millipore Corporation, Billerica, MA).

2.5. Purification of recombinant protein from conditioned medium

To purify recombinant HYAL1 from the conditioned medium, HT1080-HYAL1-MH cells were cultured in serum-free DMEM for 24 h, and the conditioned medium was concentrated by ultrafiltration membrane and incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) for 2 h at 4 °C. The Ni-NTA agarose was washed 5 times with phosphate-buffered saline (PBS) and eluted with 500 mM imidazole. The obtained samples were electrophoresed on SDS–polyacrylamide gels and stained with CBB R-250. The purified proteins were used for mass spectrometry.

2.6. Mass spectrometry

Purified recombinant HYAL1 was subjected to SDS–polyacrylamide gel electrophoresis. After CBB staining, the bands were excised and destained. After reduction with DTT, they were alkylated with acrylamide and treated with 0.05 mg of sequencing-grade modified trypsin (Promega) at 37 °C for 12 h in 20 mM Tris–HCl, pH 8.0. The digests were applied to MALDI-TOF MS on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflector mode using α-cyano-4-hydroxycinnamic acid as the matrix.

2.7. Semi-quantitative RT-PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) as described in the manufacturer’s method, and solutions containing 1 μg of total RNAs were taken for the reverse-transcription reaction that was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA), as described previously [21,22]. The cDNA was used for PCR amplification with rTaq DNA polymerase (Takara Bio Inc., Shiga, Japan). The number of PCR cycles for each product was determined after confirmation of the efficacy of amplification and after having defined the linear exponential portion of the amplification. The sequences of the primers used in the semi-quantitative RT-PCR, the number of cycles, and the annealing temperatures were as follows: exogenous HYAL1, 5’-CAGCACAACAAACTTCTGG-3’ (forward) and 5’-GTATGTTGATGAGTCACTCTTCTGAGATGAG-3’ (reverse), 25 cycles, 55 °C and β-actin, 5’-CTCCGACAGAGATGGCCA-3’ (forward) and 5’-CCAGACGGACTGTGGTGGCC-3’ (reverse), 19 cycles, 58 °C. PCR products were electrophoresed, stained with ethidium bromide, and visualized with a UV illuminator.

2.8. Detection of secreted HYAL1

HT1080-neo, HT1080-HYAL1-MH, HT1080-HYAL1-MH/N99Q, HT1080-HYAL1-MH/N216Q, and HT1080-HYAL1-MH/N350Q cells were cultured in serum-free DMEM for 24 h, and the conditioned media and cell lysates were collected. Conditioned media were concentrated by Ni-NTA agarose for 2 h at 4 °C. The Ni-NTA agarose was washed 3 times with PBS and eluted with 300 mM imidazole. The cell lysates were prepared as described above. Loading buffer was added to both conditioned media and cell lysates, which were boiled for 3 min. Subsequently, the proteins were separated on SDS–polyacrylamide gels and analyzed by immunoblot with anti-c-myc and anti-α-tubulin antibodies.
2.9. Measurement of hyaluronidase activity

To measure HYAL1 activity by in-gel digestion assay, we carried out the experiment using previously reported methods [18,23]. HT1080-neo, HT1080-HYAL1-MH, HT1080-HYAL1-MH/N99Q, HT1080-HYAL1-MH/N216Q, and HT1080-HYAL1-MH/N350Q cells were lysed in binding buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole) and concentrated by using Ni-NTA agarose. Ni-NTA-bound proteins were eluted, and the samples were electrophoresed on an SDS–polyacrylamide gel containing rooster comb HA (0.2 mg/mL; Sigma) at 4°C. The gel was washed twice with SDS extraction buffer (50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 2.5% Triton X-100) for 1 h and incubated in assay buffer (50 mM sodium formate, pH 4.0, 150 mM NaCl) at 37°C for 24 h. After incubation, the gel was stained with 0.5% alcian blue (Sigma) containing 10% acetic acid and 20% ethanol solution for 2 h and destained with 25% methanol and 7.5% acetic acid solution. HYAL1 activity can be observed as a transparent band in the blue pigment background.

3. Results

3.1. HYAL1 was N-glycosylated

Human HYAL1 contains 3 possible N-glycosylation consensus sequences at the Asn99, Asn216, and Asn350 residues (Fig. 1A). Tunicamycin (TM) blocks the synthesis of all N-linked glycoproteins; therefore, treatment of cells with TM is predicted to cause N-glycosylated proteins to have a more rapidly migrating form compared with untreated cells. We treated HT1080-HYAL1-MH cells with TM, which were established previously [19], and as a result, 10 μg/mL TM treatment resulted in a size reduction of HYAL1-MH (Fig. 1B). These data suggested that HYAL1 was N-glycosylated.

3.2. HYAL1 is N-glycosylated at 3 predicted sites revealed by MALDI-TOF MS analysis

We undertook MALDI-TOF MS analysis to determine whether HYAL1 is N-glycosylated or not [24]. To obtain recombinant HYAL1 protein, we purified HYAL1 protein from conditioned medium of HT1080-HYAL1-MH cells by using Ni-NTA agarose (Fig. 2A). Purified HYAL1 was digested with trypsin and treated with or without PNGase F, which can cleave between the innermost GlcNAc and asparagine residues of N-linked glycoproteins, thereby converting N-glycosylated Asn to Asp. A resulting peptide treated with PNGase F was observed at the expected position (m/z 4431.8), at which Asn99 was replaced with Asp99, suggesting that HYAL1 is N-glycosylated at Asn99 (Fig. 2B). Similarly, MALDI TOF-MS analysis demonstrated that the peptides that were converted from Asn216 and Asn350 to Asp residues after treatment with PNGase F were observed at the expected position (m/z 3581.0 and 3397.9), respectively (Fig. 2C and D). These results indicated that HYAL1 was N-glycosylated at 3 predicted N-glycosylation consensus sequences: Asn99, Asn216, and Asn350.

3.3. N-glycosylation is essential for HYAL1 secretion

Since we demonstrated that HYAL1 was N-glycosylated at Asn99, Asn216, and Asn350, we tried to evaluate the roles of N-glycosylation in HYAL1 functions by using mutant forms of the protein, replacing asparagine residues with glutamine residues. Therefore, we established HT1080-HYAL1-MH/N99Q, HT1080-HYAL1-MH/N216Q, and HT1080-HYAL1-MH/N350Q cell lines. The electrophoretic migrations of the mutant HYAL1 cell lysates were slightly faster than that from HT1080-HYAL1-MH (Fig. 3A). Equal amounts of exogenous HYAL1 in the stable cell lines were confirmed by semi-quantitative RT-PCR (Fig. 3B). It suggests that inhibition of N-glycosylation by site-directed mutation induces size reduction.

Since the secretion of HYAL1 by tumor cells has been shown to correlate with tumor malignancy [25], we evaluated the effect of N-glycosylation on HYAL1 secretion by comparing wild-type HYAL1 with each N-glycosylation-defective HYAL1 mutant. As a result, secretion levels of N99Q, N216Q, and N350Q mutant HYAL1-MH were eliminated, although wild-type HYAL1 was secreted into conditioned medium (Fig. 3C). It suggested that a defect of in each N-glycosylation site within HYAL1 inhibited its secretion.

3.4. N-glycosylation is necessary for enzymatic activity of HYAL1

We examined the effects of N-glycosylation on HYAL1 enzymatic activity assessed by in-gel digestion assay [18,23]. We...
Fig. 2. N-glycosylation of HYAL1 at Asn99, Asn216, and Asn350 demonstrated by MALDI-TOF MS analysis. (A) Exponentially growing HT1080-HYAL1-MH cells were cultured in serum-free DMEM for 24 h before the conditioned medium was collected. The conditioned medium was concentrated by ultrafiltration membrane and incubated with Ni-NTA agarose for 2 h. The Ni-NTA agarose was washed 5 times and eluted with 500 mM imidazole. The obtained proteins were electrophoresed on an SDS–polyacrylamide gel and visualized by CBB staining. (B–D) Purified HYAL1 was treated with (lower) or without (upper) PNGase F and was subjected to an SDS-polyacrylamide gel. Samples were digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF MS. The fragments converting Asn99 (B), Asn216 (C), and Asn350 (D) with Asp residues by PNGase F had the expected masses of 4431.8, 3581.0, and 3397.9, respectively. Underlined “D”s indicate Asp residues converted from glycosylated Asn residues after treatment with PNGase F.

Fig. 3. N-glycosylation is essential for HYAL1 secretion. (A and B) Exponentially growing HT1080-neo (neo), HT1080-HYAL1-MH (wt), HT1080-HYAL1-MH/N99Q (N99Q), HT1080-HYAL1-MH/N216Q (N216Q), and HT1080-HYAL1-MH/N350Q (N350Q) cells were lysed, and each cell lysate was electrophoresed and immunoblotted with the indicated antibodies (A). Total RNAs were isolated from neo, wt, N99Q, N216Q, and N350Q cells, and semi-quantitative RT-PCR was performed (B). (C) Exponentially growing neo, wt, N99Q, N216Q, and N350Q cells were cultured in serum-free DMEM for 24 h before the conditioned media and cell lysates were collected. Conditioned media were incubated with Ni-NTA agarose. Ni-NTA-bound HYAL1 was washed and eluted with 300 mM imidazole. The eluted samples and cell lysates were electrophoresed and immunoblotted with the indicated antibodies.
purified wild-type and each mutant of HYAL1 from whole-cell lysates by using Ni-NTA agarose. The amount of obtained protein level was almost equivalent, confirmed by western blotting (Fig. 4, lower). In the same condition, we evaluated the enzymatic activity of each HYAL1 protein. Wild-type HYAL1 contained abundant enzymatic activity, but the N99Q mutant protein eliminated enzymatic activity (Fig. 4, upper). Moreover, the N216Q and N350Q HYAL1 mutants also decreased their enzymatic activities compared with wild-type HYAL1. These results demonstrated that N-glycosylation, especially Asn99, plays an essential role in HYAL1 enzymatic activity.

4. Discussion

In this report, we demonstrated that HYAL1 was N-glycosylated at Asn99, Asn216, and Asn350, revealed by mass spectrometry (Fig. 2). Protein N-glycosylation, the attachment of N-glycans to Asn, is known to change protein conformation and protein polarity, which is predicted to change many functions [1]. Our results showed that each N-glycosylation-defective mutant decreased its secretion and enzymatic activity (Figs. 3C and 4). It is probably due to conformational changes of HYAL1. Moreover, decrease of protein stability and/or defect of the recognition step of protein transport are possible reasons for the decrease of secretion. In fact, the protein level of each N-glycosylation-defective mutant HYAL1 in cells was also decreased compared with wild-type HYAL1 (Fig. 3C). Therefore, protein stability was decreased, and HYAL1 was probably degraded by ER-associated degradation, because the total amounts of HYAL1 protein—intracellular and secreted HYAL1—were decreased in each N-glycosylation-defective mutant HYAL1.

It has been reported that ER stress inhibited secretion of the proteins [26]. To address whether suppression of mutant form-HYAL1 secretion is due to the induction of ER stress, we examined the effect of ER stress inducer on the secretion. Treatment with DTT of the cells induced Bip expression, a well-known marker of ER stress induction, and secretion level of HYAL1 was reduced (data not shown). However, expression levels of Bip were not changed in mutant form-HYAL1 expressing cells (N99Q, N216Q and N350Q) compared with wild-type HYAL1 expressing cells and control cells (data not shown), indicating that mutant HYAL1 did not induce ER stress. These results suggested that suppression of mutant form-HYAL1 secretion was not due to the ER stress induction, and that defect of each three N-glycosylation might be changed the HYAL1 conformation not to be secreted. Taken together, our results revealed that N-glycosylation of HYAL1 affects its functions, such as enzymatic activity and secretion.

A previous study suggested the presence of N-glycosylation at 3 predicted asparagine residues, revealed by electron density map analysis [11]. On the other hand, we directly demonstrated that N-glycans were attached to Asn99, Asn216, and Asn350 revealed by mass spectrometry. Previous studies also suggested the importance of N-glycosylation for the enzymatic activity of HYAL1, revealed by microplate assay and kinetic analysis [18, 27]. However, these reports did not evaluate the effect of N-glycosylation at Asn99 and evaluated it by using a mutant that was changed from Asn to Ala. In contrast, we showed that N-glycosylation of HYAL1 at 3 Asn, especially Asn99, was important for enzymatic activity by using in-gel digestion assay (Fig. 4). Our results are consistent with a previous report and furthermore show novelty. Intracellular HYAL1 and secreted HYAL1 from tumor cells are known to correlate with tumor malignancy [25]. Thus, the secretion of every N-glycosylation-defective mutant HYAL1 was eliminated although wild-type HYAL1 was secreted (Fig. 3C), suggesting the significance of N-glycosylation for the behavior of malignant tumors. These results indicated that N-glycosylation is important for enzymatic activity of HYAL1 and its secretion. Therefore, inhibition of N-glycosylation will lead to regulation of malignant tumor behavior caused by overexpression of HYAL1.

In conclusion, we demonstrated that HYAL1 is N-glycosylated at 3 predicted N-glycosylation consensus sequences and revealed its roles in N-glycosylation for secretion and enzymatic activity of HYAL1. Since HYAL1 is known to promote tumor growth and metastasis, inhibition of HYAL1 N-glycosylation can be a new target for cancer therapeutics.

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