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

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ABSTRACT

Hyaluronidase1 (HYAL1) is a hydrolytic enzyme that degrades hyaluronic acid (HA) and has three predicted *N*-glycosylation sites at Asn^{99}, Asn^{216}, and Asn^{350}. In this report, we show the functional significance of *N*-glycosylation on HYAL1 functions. Using mass spectrometry, we demonstrated that HYAL1 was *N*-glycosylated at the three asparagine residues. *N*-glycosylation of HYAL1 is important for secretion of HYAL1, as demonstrated by site-directed mutation. Moreover, a defect of *N*-glycosylation attenuated the enzymatic activity of HYAL1. Thus, HYAL1 is *N*-glycosylated at the three asparagine residues, and its secretion and enzymatic activity are regulated by *N*-glycosylation.

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*-acetylglucosamine 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 Asn^{99}, Asn^{216}, and Asn^{350} [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 $NaHCO_3$, 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 by using overlap extension technique [20]. The sequences of primers used for the mutagenesis were as follows: N99Q; 5′-TGCCCCA GCAAGCCAGCCTG-3′ (forward) and 5′-CAGGCTGGCTTGCTGGGGC A-3′ (reverse), N216Q; 5′-CTAAGCCCCCAATACACCGG-3′ (forward) and 5′-CCGGTGTATTGGGGGCTTAG-3′ (reverse), and N350Q; 5′-TTCATCCTGCAAGTGACCAG-3′ (forward) and 5′-CTGGTCACTTGC AGGATGAA-3′ (reverse).

2.3. Establishment of HYAL1-overexpressing 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, IN) selection. The clone 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′-CACGACAAACCACTTTCTGC-3′ (forward) and 5′-GTGATGGTGATGATGCAGATCCTCTTCTGAGATGAG-3′ (reverse), 25 cycles, 55 °C and β-actin, 5′-CTTCGAGCACGAG ATGGCCA-3′ (forward) and 5′-CCAGACAGCACTGTGTTGGC-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.