



ELSEVIER

journal homepage: www.elsevier.com/locate/febsopenbio

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

Yuki Goto^a, Yuki Niwa^a, Takehiro Suzuki^b, Shiho Uematsu^a, Naoshi Dohmae^b, Siro Simizu^{a,*}

^aDepartment of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama 223-8522, Japan

^bGlobal Research Cluster, RIKEN, Wako 351-0198, Japan

ARTICLE INFO

Article history:

Received 25 April 2014

Revised 31 May 2014

Accepted 3 June 2014

Keywords:

Hyaluronidase1

N-glycosylation

Enzymatic activity

Secretion

ABSTRACT

Hyaluronidase1 (HYAL1) is a hydrolytic enzyme that degrades hyaluronic acid (HA) and has three predicted N-glycosylation sites at Asn⁹⁹, Asn²¹⁶, and Asn³⁵⁰. 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.

© 2014 The Authors. Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

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

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

* Corresponding author. Address: 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan. Tel./fax: +81 45 566 1778.

E-mail address: simizu@appplc.keio.ac.jp (S. Simizu).

<http://dx.doi.org/10.1016/j.fob.2014.06.001>

2211-5463/© 2014 The Authors. Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

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 β 1–4 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 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

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

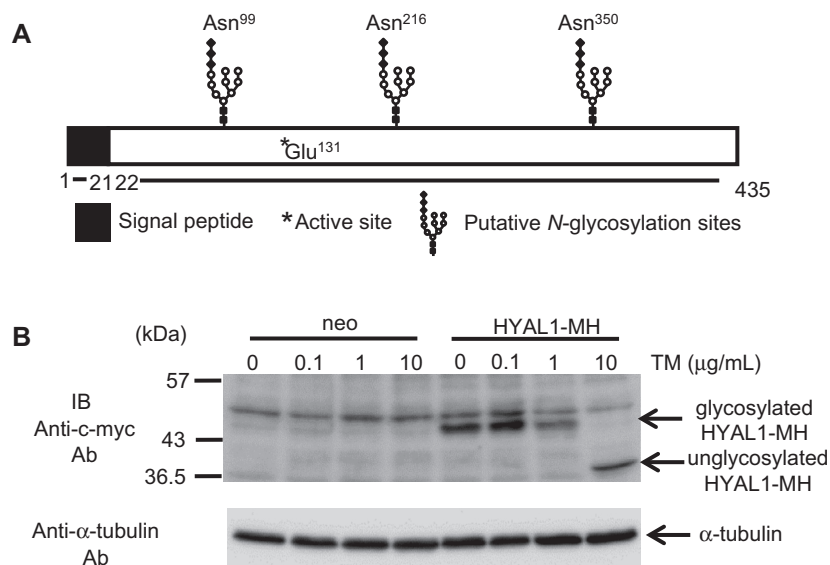


Fig. 1. N-glycosylation of HYAL1. (A) The locations of 3 putative N-glycosylation sites (Asn⁹⁹, Asn²¹⁶, and Asn³⁵⁰) are indicated by sugar chains. The black box denotes the signal peptide. The active site of HYAL1 (Glu¹³¹) is indicated by an asterisk. (B) Cells were treated with the indicated concentrations of TM for 24 h. Cells were lysed, and each cell lysate was electrophoresed and immunoblotted with the indicated antibodies.

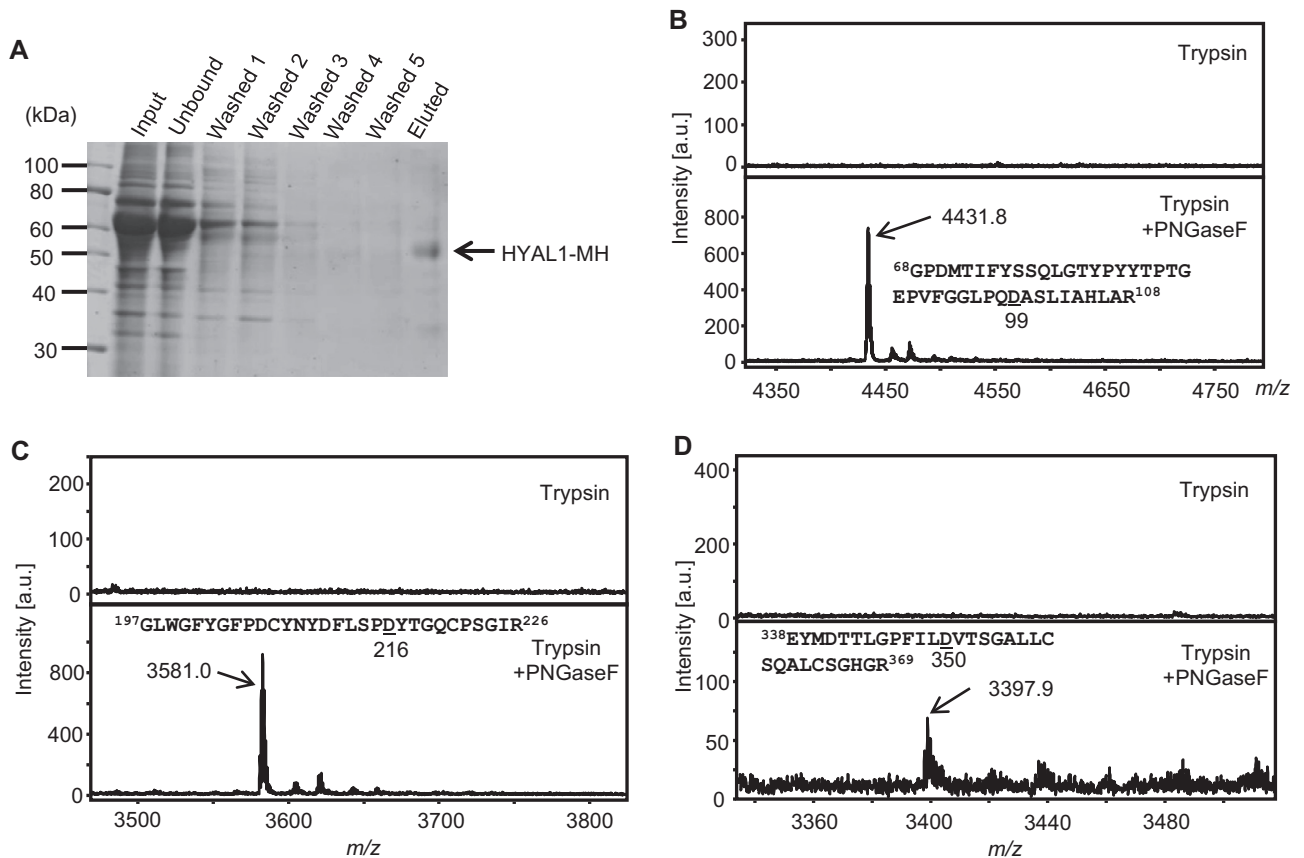


Fig. 2. N-glycosylation of HYAL1 at Asn⁹⁹, Asn²¹⁶, and Asn³⁵⁰ demonstrated by MALDI-TOF MS analysis. (A) Exponentially growing HT1080-HYAL1-MH cells were cultured in serum-free DMEM for 24 h before the conditioned medium was collected. The conditioned medium was concentrated by ultrafiltration membrane 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.

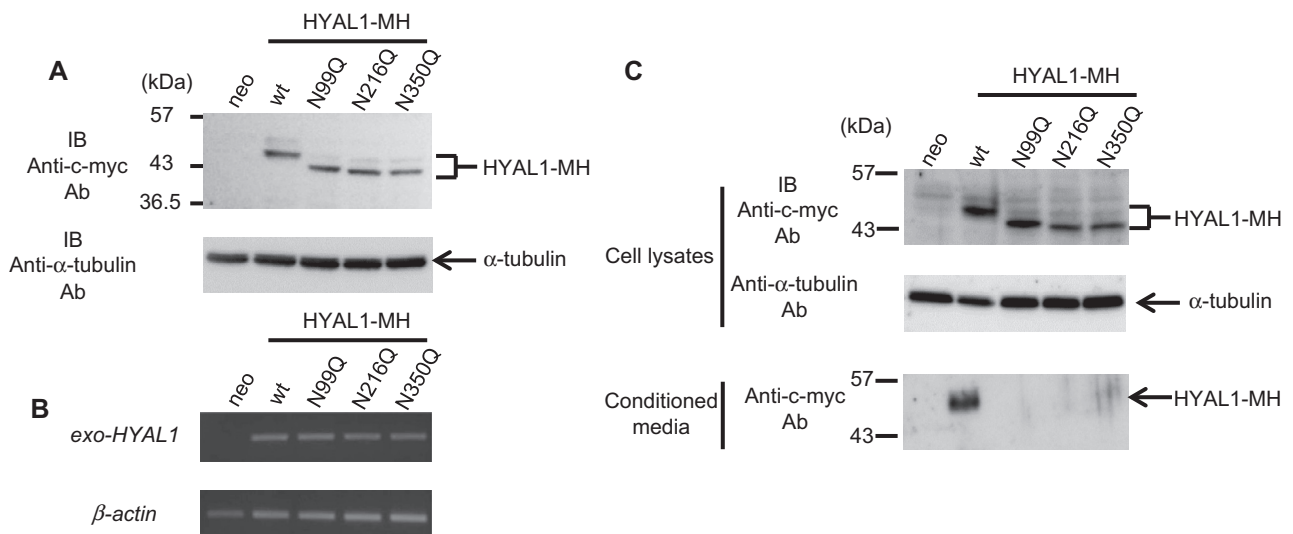


Fig. 3. N-glycosylation is essential for HYAL1 secretion. (A and B) Exponentially growing HT1080-neo (neo), HT1080-HYAL1-MH (wt), HT1080-HYAL1-MH/N99Q (N99Q), HT1080-HYAL1-MH/N216Q (N216Q), and HT1080-HYAL1-MH/N350Q (N350Q) cells were lysed, and each cell lysate was electrophoresed and immunoblotted 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.

Acknowledgments

This study was supported in part by grants from the programs Grants-in-Aid for Scientific Research (B) (nos. 23310163 and 24310167) and for JSPS Fellows (254256). Y.N. is a Research Fellow of the Japan Society for the Promotion of Science.

References

- [1] Dwek, R.A., Butters, T.D., Platt, F.M. and Zitzmann, N. (2002) Targeting glycosylation as a therapeutic approach. *Nat. Rev. Drug Discov.* 1, 65–75.
- [2] Ruddock, L.W. and Molinari, M. (2006) *N*-glycan processing in ER quality control. *J. Cell Sci.* 119, 4373–4380.
- [3] Lennarz, W.J. (1987) Protein glycosylation in the endoplasmic reticulum: current topological issues. *Biochemistry* 26, 7205–7210.
- [4] Lehrman, M.A. (2001) Oligosaccharide-based information in endoplasmic reticulum quality control and other biological systems. *J. Biol. Chem.* 276, 8623–8626.
- [5] Simizu, S., Ishida, K., Wierzbza, M.K. and Osada, H. (2004) Secretion of heparanase protein is regulated by glycosylation in human tumor cell lines. *J. Biol. Chem.* 279, 2697–2703.
- [6] Maeda, Y. and Kinoshita, T. (2008) Dolichol-phosphate mannosyl synthase: structure, function and regulation. *Biochim. Biophys. Acta* 1780, 861–868.
- [7] Parekh, R.B., Dwek, R.A., Sutton, B.J., Fernandes, D.L., Leung, A., Stanworth, D., Rademacher, T.W., Mizuochi, T., Taniguchi, T., Matsuta, K., Takeuchi, F., Nagano, Y., Miyamoto, T. and Kobata, A. (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 316, 452–457.
- [8] Dennis, J.W., Granovsky, M. and Warren, C.E. (1999) Glycoprotein glycosylation and cancer progression. *Biochim. Biophys. Acta* 1473, 21–34.
- [9] Hennet, T. (2012) Diseases of glycosylation beyond classical congenital disorders of glycosylation. *Biochim. Biophys. Acta* 1820, 1306–1317.
- [10] Overall, C.M. and Lopez-Otin, C. (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat. Rev. Cancer* 2, 657–672.
- [11] Chao, K.L., Muthukumar, L. and Herzberg, O. (2007) Structure of human hyaluronidase-1, a hyaluronan hydrolyzing enzyme involved in tumor growth and angiogenesis. *Biochemistry* 46, 6911–6920.
- [12] Tan, J.X., Wang, X.Y., Su, X.L., Li, H.Y., Shi, Y., Wang, L. and Ren, G.-S. (2011) Upregulation of HYAL1 expression in breast cancer promoted tumor cell proliferation, migration, invasion and angiogenesis. *PLoS One* 6, e22836.
- [13] Tan, J.X., Wang, X.Y., Li, H.Y., Su, X.L., Wang, L., Ran, L., Zheng, K. and Ren, G.S. (2011) HYAL1 overexpression is correlated with the malignant behavior of human breast cancer. *Int. J. Cancer* 128, 1303–1315.

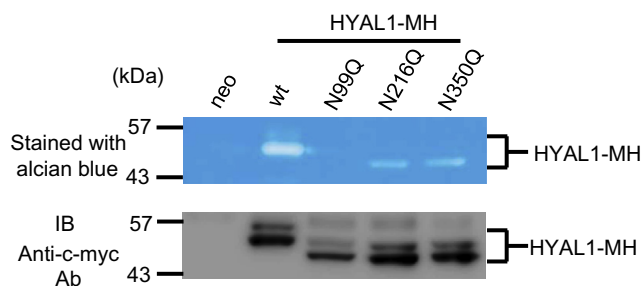


Fig. 4. *N*-glycosylation controls enzymatic activity of HYAL1. Exponentially growing neo, wt, N99Q, N216Q, and N350Q cells were lysed, and cell lysates were incubated with Ni-NTA agarose for 2 h. Ni-NTA-bound HYAL1 was washed and eluted with 500 mM imidazole. The amount of each protein was evaluated by western blotting with anti-c-myc antibody (lower). Equal amounts of samples were electrophoresed on an SDS polyacrylamide gel containing 0.2 mg/mL HA. The gel was incubated at 37 °C for 24 h after extraction of SDS and stained with alcian blue (upper). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

- [14] Ohno-Nakahara, M., Honda, K., Tanimoto, K., Tanaka, N., Doi, T., Suzuki, A., Yoneno, K., Nakatani, Y., Ueki, M., Ohno, S., Knudson, W., Knudson, C.B. and Tanne, K. (2004) Induction of CD44 and MMP expression by hyaluronidase treatment of articular chondrocytes. *J. Biochem.* 135, 567–575.
- [15] Imundo, L., Leduc, C.A., Guha, S., Brown, M., Perino, G., Gushulak, L., Triggs-Raine, B. and Chung, W.K. (2011) A complete deficiency of hyaluronoglucosaminidase 1 (HYAL1) presenting as familial juvenile idiopathic arthritis. *J. Inher. Metab. Dis.* 34, 1013–1022.
- [16] Triggs-Raine, B., Salo, T.J., Zhang, H., Wicklow, B.A. and Natowicz, M.R. (1999) Mutations in HYAL1, a member of a tandemly distributed multigene family encoding disparate hyaluronidase activities, cause a newly described lysosomal disorder, mucopolysaccharidosis IX. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6296–6300.
- [17] Fiszer-Szafarz, B., Litynska, A. and Zou, L. (2000) Human hyaluronidases: electrophoretic multiple forms in somatic tissues and body fluids. Evidence for conserved hyaluronidase potential N-glycosylation sites in different mammalian species. *J. Biochem. Biophys. Methods* 45, 103–116.
- [18] Zhang, L., Bharadwaj, A.G., Casper, A., Barkley, J., Barycki, J.J. and Simpson, M.A. (2009) Hyaluronidase activity of human Hyal1 requires active site acidic and tyrosine residues. *J. Biol. Chem.* 284, 9433–9442.
- [19] Goto, Y., Niwa, Y., Suzuki, T., Dohmae, N., Umezawa, K. and Simizu, S. (2014) C-mannosylation of human hyaluronidase 1: possible roles for secretion and enzymatic activity. *Int. J. Oncol.* 45, 344–350.
- [20] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77, 51–59.
- [21] Niwa, Y., Suzuki, T., Dohmae, N., Umezawa, K. and Simizu, S. (2012) Determination of cathepsin V activity and intracellular trafficking by N-glycosylation. *FEBS Lett.* 586, 3601–3607.
- [22] Yasukagawa, T., Niwa, Y., Simizu, S. and Umezawa, K. (2012) Suppression of cellular invasion by glybenclamide through inhibited secretion of platelet-derived growth factor in ovarian clear cell carcinoma ES-2 cells. *FEBS Lett.* 586, 1504–1509.
- [23] Guntenhöner, M.W., Pogrel, M.A. and Stern, R. (1992) A substrate-gel assay for hyaluronidase activity. *Matrix* 12, 388–396.
- [24] Wilkins, M.R., Gasteiger, E., Gooley, A.A., Herbert, B.R., Molloy, M.P., Binz, P.A., Ou, K., Sanchez, J.C., Bairoch, A., Williams, K.L. and Hochstrasser, D.F. (1999) High-throughput mass spectrometric discovery of protein post-translational modifications. *J. Mol. Biol.* 289, 645–657.
- [25] Liu, D., Pearlman, E., Diaconu, E., Guo, K., Mori, H., Haqqi, T., Markowitz, S., Willson, J. and Sy, M.S. (1996) Expression of hyaluronidase by tumor cells induces angiogenesis in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7832–7837.
- [26] Hiramatsu, N., Kasai, A., Hayakawa, K., Nagai, K., Kubota, T., Yao, J. and Kitamura, M. (2006) Secreted protein-based reporter systems for monitoring inflammatory events: critical interference by endoplasmic reticulum stress. *J. Immunol. Methods* 315, 202–207.
- [27] Lokeshwar, V.B., Lokeshwar, B.L., Pham, H.T. and Block, N.L. (1996) Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. *Cancer Res.* 56, 651–657.