Genetic disruption of multiple α1,2-mannosidases generates mammalian cells producing recombinant proteins with high-mannose–type N-glycans

Recombinant therapeutic proteins are becoming very important pharmaceutical agents for treating intractable diseases. Most biopharmaceutical proteins are produced in mammalian cells because this ensures correct folding and glycosylation for protein stability and function. However, protein production in mammalian cells has several drawbacks, including heterogeneity of glycans attached to the produced protein. In this study, we established cell lines with high-mannose–type N-linked, low-complexity glycans. We first knocked out two genes encoding Golgi mannosidases (MAN1A1 and MAN1A2) in HEK293 cells. Single knockout (KO) cells did not exhibit changes in N-glycan structures, whereas double KO cells displayed increased high-mannose–type and decreased complex-type glycans. In our effort to eliminate the remaining complex-type glycans, we found that knocking out a gene encoding the endoplasmic reticulum mannosidase 1 (MAN1B1) in the double KO cells reduced most of the complex-type glycans. In triple KO (MAN1A1, MAN1A2, and MAN1B1) cells, Man9GlcNAc2 and Man8GlcNAc2 were the major N-glycan structures. Therefore, we expressed two lysosomal enzymes, α-galactosidase-A and lysosomal acid lipase, in the triple KO cells and found that the glycans on these enzymes were sensitive to endoglycosidase H treatment. The N-glycan structures on recombinant proteins expressed in triple KO cells were simplified and changed from complex types to high-mannose types at the protein level. Our results indicate that the triple KO HEK293 cells are suitable for producing recombinant proteins, including lysosomal enzymes with high-mannose–type N-glycans.

Biopharmaceuticals are drugs produced from organisms by biotechnology. Protein-based pharmaceuticals such as monoclonal antibodies have high specificity and high affinity against target molecules and few side effects compared with traditional small molecules. Recombinant therapeutic proteins are becoming very important pharmaceutical agents for treating intractable diseases, such as cancer and autoimmune diseases (1). Currently, most therapeutic proteins are produced in mammalian cells (2, 3) because these cell types improve the yields of correctly folded proteins and facilitate appropriate posttranslational modifications, which alternative protein-producing organisms do not always offer. Because most secreted recombinant proteins are modified with glycans, these proteins require proper glycosylation for stability and function and for avoiding immunogenicity (4–6).

Production of biopharmaceuticals in mammalian cells still has several drawbacks, including high cost and glycan heterogeneity. Heterogeneity of glycans attached to proteins is an important issue that needs to be solved to ensure protein quality and stability for use as pharmaceuticals. Some cytokines, including erythropoietin and granulocyte colony–stimulating factors, require sialylated complex-type glycans for their in vivo activity (7, 8). In addition, changing glycan structures on proteins can enhance their functional activity; for example, the absence of a core fucose on N-linked glycans of an antibody drastically enhances the antibody-dependent cellular cytotoxicity (9). Therefore, construction of mammalian cell lines that produce homogeneous glycoproteins is needed in the field of biopharmaceuticals.

Recombinant lysosomal enzymes are used as drugs for therapy of some lysosomal storage diseases (LSDs). Lyosomes contain many hydrolytic enzymes, such as nuclease, proteases, lipases, and glycosidases (10). These enzymes break down cel-

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3 The abbreviations used are: LSD, lysosomal storage disease; CHO, Chinese hamster ovary; Man, mannose; M6P, mannose 6-phosphate; HEK, human embryonic kidney; KO, knockout; D-KO, MAN1A1 and MAN1A2 double knockout; ER, endoplasmic reticulum; T-KO, MAN1A1, MAN1A2 and MAN1B1 triple knockout; PAM, protospacer adjacent motif; ConA, concanavalin A; sgRNA, single guide RNA; GLA, α-galactosidase-A; LIPA, lysosomal lipase; Endo-H, endoglycosidase H; PVDF, polyvinylidene difluoride; EGF, enhanced GFP; MW, methanol/deionized water; BMW, butanol/methanol/deionized water; DHB, 2,5-dihydroxybenzoic acid.

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lular metabolites in lysosomes. LSDs are inherited disorders caused by deficiency of specific lysosomal enzymes that metabolize substrates in lysosomes. Approximately 50 different kinds of LSDs are known (11, 12). Enzyme replacement therapy, which is normally performed by giving the patient an intravenous injection of recombinant lysosomal enzymes, is available for treatment of some LSDs. The N-glycans on lysosomal enzymes play critical roles in their delivery to the lysosome. The enzymes are incorporated into the cells through cation-independent M6P receptors or other receptors, such as Man receptors on the cell surface (12, 13). Therefore, enzymes require high-Man-type glycans containing M6P for their efficient incorporation into cells.

Imiglucerase (Cerezyme, Genzyme Corp.) is a Food and Drug Administration–approved recombinant acid β-glucosidase that has been produced using Chinese hamster ovary (CHO) cells and is used for type 1 Gaucher disease treatment (14). To that has been produced using Chinese hamster ovary (CHO) type should be suitable for therapeutic proteins with high-Man–type glycans without any treatment. The cells types. This is the first report that mammalian cells can produce recombinant enzymes produced in T-KO cells were high-Man N-glycan structures. The further decreased complex-type N-glycan structures. The enzymes incorporated into macrophages through Man receptors (17). Alternatively, inhibitors against α1,2-mannosidases, such as kifunensine and deoxymannojirimycin, can be used for the production of recombinant proteins having high-Man–type N-glycans with Man9GlcNAc2 structures (18). Kifunensine is used for the production of velaglucerase alfa (VPRIV, Shire), which treats type 1 Gaucher disease (19). Use of such deglycosylation enzymes or inhibitors is effective in the production of high-Man–type glycans; however, maintaining the quality and stability of the proteins is challenging. In CHO cells and human embryonic kidney (HEK) 293 cells, mutant cells defective in the Golgi-localized α1,2-mannosidase I (Fig. 1A). Most N-glycosylated proteins that are transported from the ER to the Golgi apparatus have Man9GlcNAc2 or Man8GlcNAc2 structures (Fig. 1B). At the Golgi apparatus, α1,2-linked Mans are trimmed by Golgi mannosidase I to yield the Man5GlcNAc2 structure (29, 30).

HEK293 cells knock out α1,2-mannosidases

Results

Knockout of the MAN1A1 or MAN1A2 gene did not change the glycan profiles

When glycoproteins are expressed in mammalian cells, heterogeneous glycan structures, which are generated in the glycosylation pathway at the ER and the Golgi, are found on the proteins (Fig. 1A). Such heterogeneity of glycans is an important issue in the production of therapeutic glycoproteins (23, 24). To simplify N-glycan structures, we chose HEK293 cells, which are widely used for basic cell biology and for applications such as viral particle production and expression of recombinant proteins (25–27). Because the cell line is a frequently used human-derived cell, precise genomic information, including chromosomal number and small nucleotide polymorphisms, has been well analyzed, and a significant body of available gene expression data has been accumulated (28). In addition, the methods for suspension culture and serum-free culture are established as for CHO cells (2). These advantages are valuable when establishing cell lines that produce recombinant proteins with simplified glycan structures.

To establish cells producing Man9 and Man8 containing high-Man–type glycans, we initially focused on Golgi-localized α-mannosidase I (Fig. 1A). Most N-glycosylated proteins that are transported from the ER to the Golgi apparatus have Man9GlcNAc2 or Man8GlcNAc2 structures (Fig. 1B). At the Golgi apparatus, α1,2-linked Mans are trimmed by Golgi mannosidase I to yield the Man5GlcNAc2 structure (29, 30).

In this study, we genetically engineered a glycosylation pathway and established cells that produce high-Man–type N-glycans with Man9GlcNAc2 and Man8GlcNAc2 structures. Two genes encoding the Golgi-located α-mannosidase I were disrupted in HEK293 cells. In the double knockout (D-KO), a decrease in the complex-type N-glycans was observed; however, the level of high-Man types increased. We further knocked out a gene encoding the ER α-mannosidase I in D-KO cells, and these triple KO (T-KO) cells further decreased complex-type N-glycans and simplified glycan structures. The N-glycans on the recombinant lysosomal enzymes produced in T-KO cells were high-Man types. This is the first report that mammalian cells can produce simple Man9GlcNAc2 and Man8GlcNAc2 structures of high-Man–type glycans without any treatment. The cells should be suitable for therapeutic proteins with high-Man–type N-glycans.
staining patterns of both PHA-L and ConA were similar to those observed in parental HEK293 wildtype cells (Fig. 3A), suggesting that MAN1A1 and MAN1A2 have overlapping functions.

Establishment of MAN1A1 and MAN1A2 double KO cells

We next established MAN1A1 and A2 D-KO cells. The MAN1A2 gene was knocked out using the A1-KO24 cell line as
HEK293 cells knock out α,1,2-mannosidases

In D-KO35 cells, a decrease in the level of complex-type glycans was observed, whereas staining of PHA-L4 still remained. Therefore, we postulated that other α,1,2-mannosidases are involved in the formation of complex-type N-glycans. MAN1A1 and MAN1A2 belong to glycoside hydrolase family 47 (GH47) in the carbohydrate active enzymes database (CAZy) (39). In mammals, there are five members (MAN1C1, MAN1B1, EDEM1, EDEM2, and EDEM3) other than MAN1A1 and MAN1A2 in the GH47 family (Fig. 1C) (40). MAN1C1 and MAN1B1 are another Golgi-α,1,2-mannosidase I and ER-mannosidase I, respectively (34, 41). EDEMs (EDEM1, 2, and 3) have α,1,2-mannosidase activities, which are required for Man trimming of misfolded glycoproteins in ER-associated degradation (42–45). We focused on the five genes (MAN1C1, MAN1B1, EDEM1, EDEM2, and EDEM3) to analyze the roles of complex glycan formation. D-KO35 cells were transfected with each gene KO target plasmid, and cells that transfected the plasmids expressing Cas9 and target single guide RNA (sgRNA) were sorted. After transfection, cells were cultured for more than 10 days, and the genomic DNAs were then isolated from the bulk population. Because two target sites in each gene were designed, the amplified fragments were shifted when the genes on the chromosomes were knocked out. We confirmed that a fraction of the target genes were knocked out in the bulk population (Fig. 3B). Using these cell populations, the surface glycan profiles were analyzed by PHA-L4 staining (Fig. 3C). The staining profiles for D-KO35 cells with plasmids for knockouts of either MAN1C1 or EDEM1, 2, or 3 showed no change in their staining compared with D-KO35 cells. However, the PHA-L4 staining was observed to have decreased in D-KO35 cells transfected with plasmids expressing sgRNA for MAN1B1 knockout compared with the parental cells, suggesting that this gene is actually involved in Man trimming to form complex-type N-glycan structures, at least in D-KO cells. We also knocked out the five genes in wildtype cells. Compared with wildtype cells, the staining profiles for cells with knockout of either MAN1C1 or EDEM1, 2, or 3 showed no change (Fig. S1). In some cells with knockout of MAN1B1, the PHA-L4 staining seemed to be decreased, whereas the majority of the cells had no change in staining. MAN1A1, A2, B1, and C1 have overlapping functions to process the same substrates. Therefore, the glycan patterns would not be changed and affected by the single knockout.

Establishment of a MAN1A1, A2, and B1 triple KO cell line

Because the bulk population of D-KO35 cells transfected with the MAN1B1-KO construct showed a decrease in PHA-L4 staining, we further analyzed this cell. From the D-KO35 cells

the parental strain. After transfection of the KO constructs, the clonal cell line was isolated. The genomic DNA sequences around the KO target regions were analyzed (Fig. 2). Compared with A2-KO37 cells, the D-KO35 cells showed three bands when the MAN1A2 target region was amplified, and the sequences were examined. The lower band arose from cleavage at two target sites and connected with the exposed ends. The middle band represented a 75-bp insertion from the plasmid sequence at the target 1 cleavage site and a 1-bp insertion at the target 2 cleavage site. The third band also represented 2-bp and 207-bp insertions at the target 1 and 2 cleavage site, respectively. Because both sequences cause frameshifts, the D-KO35 cell line has both MAN1A1 and MAN1A2 genes knocked out. The D-KO35 cells were stained with PHA-L4 and ConA. Compared with wildtype and single KO cells, surface staining of ConA increased, and PHA-L4 staining had significantly decreased in D-KO35 cells (Fig. 3A). The results indicate a change in the glycan profile compared with that of the wildtype cells. Here the level of complex-type N-glycans had decreased and the level of high-Man–type N-glycans had increased in D-KO cells.

Knockout of other genes responsible for α,1,2-mannosidases

Because the bulk population of D-KO35 cells transfected with the MAN1B1-KO construct showed a decrease in PHA-L4 staining, we further analyzed this cell. From the D-KO35 cells

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**Figure 2. Establishment of knocked out MAN1A1 and/or MAN1A2 cell lines.** A and B, genomic DNA was extracted from wildtype HEK293, MAN1A1-KO clone 24 (A1-KO24), MAN1A2-KO clone 37 (A2-KO37), and MAN1A1 and MAN1A2 double KO clone 35 (D-KO35) cells. The knockout region was amplified using primer sets for checking MAN1A1-KO (A) and MAN1A2-KO (B) and analyzed by agarose gel electrophoresis. The size of the designed DNA fragment for MAN1A1 is 431 bp for wildtype HEK293. If the KO of MAN1A1 is correctly deleted by the CRISPR/Cas9 system, then the size would be 358 bp (A). The size of the DNA fragment for MAN1A2 is 247 bp for the WT MAN1A2 (B). The knockout region was amplified, and the MAN1A2 genomic DNA sequences of target regions in WT, A1-KO24, A2-KO37, and D-KO35 cell lines. The coding amino acid sequences are shown under the nucleotide sequences. Red letters, target sequences of guide RNA; underlined letters, PAM sequence. The KO of MAN1A2 in D-KO35 cells had three variations.
with the MAN1B1 knockout, clonal cells were isolated and named T-KO. T-KO cells that contained a 48-bp deletion in the MAN1B1 coding sequence produced a MAN1B1 protein with a 16-amino acid deletion (Fig. 4, A and B). The staining of ConA was found to have increased compared with that of D-KO cells (Fig. 4, C and D). Significantly, PHA-L4 staining was highly reduced for the T-KO cells (Fig. 4, C and E).

We next analyzed the glycan structures in the D-KO and T-KO cells. Total cellular proteins were extracted from WT, D-KO, and T-KO cells. Sialic acids on glycans were amidated, followed by treatment with PNGase F to release N-glycans from proteins. The amidated N-glycans were analyzed by MALDI-TOF-MS. In the WT cells, at least 27 different types of glycan structures, including high-Man types, hybrid types, and complex types, were observed (Fig. 5A). Complex-type glycans consisted of biantennary and triantennary complex-type structures with or without sialic acids and/or fucose. On the other hand, in D-KO cells, a decrease in the variety of glycan structures was observed, with high-Man–type glycans present as major species and complex-type glycans still detected (Fig. 5B). Complex-type glycan structures were simplified to asialylated biantennary, disialylated biantennary, and trisialylated triantennary structures. The prominent peak was Man8GlcNAc2 in D-KO cells. In T-KO cells, the glycan structures were further simplified, and complex-type glycans were below the detection limit (Fig. 5C). All five glycan structures detected were high-Man types. The major peaks represented Man9GlcNAc2 and Man8GlcNAc2 structures in the profile. These results are consistent with the lectin profiles and indicate that the glycan profiles were simplified, with clear increases in high-Man types and
decreases in the level of complex-type glycans in D-KO and T-KO cells.

Expression of recombinant lysosomal enzymes and antibody proteins

A use for the mannosidase gene KO cells is the expression of recombinant proteins with high-Man–type glycans. In particular, lysosomal enzymes required for treating lysosomal storage diseases require high-Man types or phosphorylated high Man-type glycans for targeting to cells (46). We chose two lysosomal enzymes, α-galactosidase-A (GLA) and lysosomal acid lipase (LIPA). GLA hydrolyzes a glycosphingolipid Gb3 in lysosomes, and mutations in GLA cause Fabry disease (47). LIPA is an enzyme required for breakdown of lipids such as cholesterol esters and triacylglycerols in lysosomes, and deficiency of this enzyme leads to Wolman disease and cholesteryl ester storage
Both GLA and LIPA have four and six potential N-glycosylation sites, respectively. PNGase F cleaves all N-glycans from proteins expressed in mammalian cells, whereas Endo-H cannot digest complex type N-glycans attached to proteins (49). Such differences are useful for estimating glycan structures located on proteins. When the His6-FLAG tagged GLA (sHF-GLA) or LIPA (sHF-LIPA) was expressed in the wildtype cells, the glycans on proteins were digested completely by PNGase F but only partially cleaved by Endo-H (Fig. 6, A and B), suggesting that GLA and LIPA expressed in the medium possess complex-type N-glycans. In D-KO cells, fractions of the glycans on GLA and LIPA remained resistant to treatment with Endo-H. In contrast, the glycans on GLA and LIPA were almost completely digested by Endo-H in T-KO cells (Fig. 6), suggesting that the majority of the glycans are high-Man types.

To analyze the detailed glycan structures on the proteins, purified sHF-LIPA was electrophoresed by SDS-PAGE and blotted onto a PVDF membrane (Fig. 7A). The N-glycans released from the bands were captured by BlotGlyco beads, labeled, and analyzed by MALDI-MS (50). For LIPA prepared from wildtype cells, more than 30 different types of N-glycan structures were observed, which were mixtures of high-Man types, hybrid types, and complex types (Fig. 7B and Table S3). In particular, there are many fucosylated and sialylated structures. In contrast, the N-glycan structures on LIPA prepared from T-KO cells were simplified. The majority of the N-glycan structures were high-Man types, with some complex types giving rise to minor peaks. We further expressed EGFP-FLAG–tagged human IgG1 in wildtype and T-KO cells (Fig. 7C). In wildtype IgG1, several complex N-glycan structures consisting of fucosylated biantennary structures were detected (Fig. 7D and Table S3). In contrast, in IgG1 prepared from T-KO cells, the majority of glycan structures were changed to high-Man types. These data indicate that N-glycan structures on secretory proteins were simplified and changed from complex types to high-Man types at the protein level.

**Discussion**

The production of recombinant mammalian proteins is of significant interest because of their increasing use for biophar-
maceutical purposes. Homogenization of glycans is an important consideration in the production of recombinant proteins required for pharmaceutical use. In this study, we tried to overcome glycan heterogeneity in recombinant protein expression by producing proteins with only high-Man-type glycans. We first established multiple α,1,2-mannosidase gene KO cell lines that knocked out two Golgi mannosidase I genes, MAN1A1 and MAN1A2, and an ER mannosidase I gene, MAN1B1. The Golgi mannosidase single KO cells did not change the N-glycan structures. When we established D-KO (MAN1A1 and MAN1A2 double KO) cells, the presence of complex-type N-glycans decreased significantly, whereas high-Man types increased. To further decrease the remaining complex-type glycans, we checked the effects of other α,1,2-mannosidases belonging to GH47, including MAN1C1 and others in GH47, are involved in GH47, are involved in α1,2-Man trimming. This issue of identifying other glycan-trimming enzymes will be addressed in a future study to establish quadruple KO and penta KO cells by knocking out candidate genes using T-KO cells.

For enzyme replacement therapy of LSDs, cation–independent M6P receptors and mannose receptors recognize lysosomal enzymes for incorporation into cells. Therefore, lysosomal enzymes with high-Man–type structures are critical for efficient incorporation. For the production of pharmaceutical lysosomal enzymes, mannosidase inhibitors such as kifunensine are used (19). Treatment of cells with kifunensine leads to increases in the levels of proteins with high-Man–type N-glycans. However, use of chemicals increases the cost, and they would not be stable to maintain the required pharmaceutical quality level. Thus, T-KO cells provide an advantage because these cells do not require the use of chemicals to produce lysosomal enzymes. For the production of lysosomal enzymes, high contents of M6P residues on glycans would increase targeting of the proteins to cells. An increase of phosphorylated glycans represents a next step in the development of a cell line for production of these types of recombinant proteins in the future.

The cell lines established in this study are suitable for the production of lysosomal enzymes and the production of other proteins that require high-Man–type N-glycans. Conversion of native heterogeneous glycans to homogenous glycans of interests has been studied (56, 57). The method is mediated by
HEK293 cells knock out α1,2-mannosidases

chemo-enzymatic synthesis using the transglycosidase activity of endoglycosidases. In the first step of the system, native glycans must be removed from proteins by endoglycosidase activity, and these enzymes prefer high-Man–type glycans as substrates (58–60). Particularly, the existence of the α1,2-linked Man residues in the oligosaccharide is important for the activity.
HEK293 cells were cultured in Dulbecco’s Modified Eagle’s medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel). The appropriate antibiotic concentrations were used where necessary: puromycin (0.5 μg/ml) and streptomycin (100 units/ml)/penicillin (100 μg/ml).

To construct pME-pgpuro-shF-GLA and pME-pgpuro-shF-LIPA, DNA fragments coding mature GLA and mature LIPA were amplified by PCR and ligated into XhoI and NotI sites of the pME-puro vector containing an ER signal sequence of CD59 and a His6-FLAG sequence (62). To construct pHEK293Ultra-shF-GLA, DNA fragments coding shF-LIPA were amplified by PCR and ligated into Smal and SphI of pHEK293Ultra (Takara, Shiga, Japan). The DNA fragments coding HyHEL10 heavy chain were amplified by PCR and ligated into HindIII and NotI sites of pME-Neo2dH-mEGFP-F-CD59_CD59ss (63), generating pME-NeoH-ssEGFP-F-HyHEL10. A retroviral vector expressing human IgG1 CD59_CD59ss (63), generating pME-NeodH-ssEGFP-F-HyHEL10, was then constructed from pME-NeoH-ssEGFP-F-HyHEL10. The DNA fragments coding HyHEL10 light chain were amplified by PCR and ligated into a retroviral vector, pLIB2-pgkBSD, generating pLIB2-pgkBSD-HyHEL10-human-κ. pGP (Takara) and pLC-VSVG (64) were used for producing the retrovirus.

A mouse monoclonal anti-M2 FLAG antibody (Sigma) was used as the primary antibody. PHA-L4 conjugated with FITC (PHA-L4-FITC) and ConA conjugated with FITC (ConA-FITC) (1-oil Mills, Tokyo, Japan) were used for cell staining. Acetohydrazide, dithiothreitol, iodoacetamide, and N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide were purchased from Sigma.

Knockout of genes in HEK293 cells

The MAN1A1, MAN1A2, MANIB1, MANIC1, EDEM1, EDEM2, and EDEM3 genes were knocked out by the CRISPR/Cas9 system (35). The pX330-EGFP plasmid vector was digested with BbsI (65). The target sequences are listed in Table S1. The sequences were designed using the E-CRISP website (http://www.e-crisp.org/E-CRISP/) (66) and were ligated into digested pX330-EGFP.4 After transfection of HEK293 cells with knockout constructs, GFP-positive cells were sorted using an S3e cell sorter (Bio-Rad). Sorted cells were further cultured for more than 10 days and analyzed. From the bulk-sorted populations, clonal cell lines for MAN1A1-KO, MAN1A2-KO, MAN1A1 and A2 double-KO, and MAN1A1, A2, and B1 triple KO were obtained by limiting dilution. Knockout of genes was confirmed by PCR and sequencing. The primers used for the confirmation are listed in Table S2.

Flow cytometry

Cells (1 × 10^6) were harvested and washed with PBS. Cell suspensions were divided into three tubes, stained with/without 1 μg of ConA-FITC or 0.5 μg of PHA-L4-FITC in 50 μl of FACS solution (PBS, 1% BSA, and 0.1% NaN₃) for 15 min on ice, followed by washing three times with 150 μl of FACS solution to remove lectins. Stained cells were analyzed by Accuri C6 (BD Biosciences).

Mass spectrometric analysis of N-glycan structures from whole-cell lysates

Wildtype, D-KO, and T-KO HEK293 cells (10-cm dishes) were washed with 5 ml of PBS three times and lysed with 1 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, and protease inhibitor mixture (Sigma)) on ice for 30 min and then centrifuged to remove cell debris. Sialylated N-linked glycans were amidated by acetylation as described previously (67, 68). Briefly, 1.5 mg of protein material was placed in a size-exclusion spin filtration unit (Amicon Ultra-0.5 10 kDa, Millipore), denatured with 300 μl of 8 M urea, and then reduced and alkylated by 150 μl of 10 mM dithiothreitol and 150 μl of 10 mM iodoacetamide. The proteins were then washed three times with 150 μl of deionized water to desalt. Desalted proteins were dissolved in 100 μl of amiation buffer (1 M acetohydrazide, 20 μl of 1 M HCl, and 20 μl of 2 M N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide) and incubated at room temperature for ~4 h. The amidated proteins were washed three times with 150 μl of 40 mM NH₄HCO₃, resuspended in 300 μl of 40 mM NH₄HCO₃ containing 2 μl of PNGase F (1000 units, New England Biolabs, Ipswich, MA), and incubated overnight at 37 °C. The released N-glycans were collected by centrifugation and lyophilized. The N-glycans released from proteins were desalted by 100 μl of Sepharose CL4B (Sigma). Sepharose CL4B was prewashed with methanol/
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deionized water (MW, 1:1) and butanol/methanol/deionized water (BMW, 5:1:1). N-glycans were dissolved in 500 μl of BMW and loaded onto prewashed Sepharose CL4B. After shaking for ~45 min in an incubator, the mixture was washed three times with 1 ml of BMW and eluted with 1 ml of MW. N-glycans were collected by centrifugation and lyophilized.

N-glycans were analyzed by MALDI-TOF-MS (UltraflexXTreme, Bruker Daltonics, Bremen, Germany). Lyophilized N-linked glycans were dissolved in 10 μl of MW. One microliter of the sample mixtures was spotted onto an MTP AnchorChip sample target (Bruker Daltonics) and air-dried. One microliter of recrystallized glycans was also spotted onto the matrix (120 mM 2,5-dihydroxybenzoic acid (DHB, Sigma), 0.1 mM NaCl, and 20 mM N,N-dimethyl aniline (Sigma)). Measurements were taken in the positive ion mode, and m/z data were analyzed and annotated using the FlexAnalysis (Bruker Daltonics) and mMass (69) software programs.

Preparation of N-glycans from sHF-LIPA and EGFP-F-IgG, for mass spectrometry

To express sHF-LIPA, pHEK293Ultra-sHF-LIPA was transfected into wildtype and T-KO HEK293 cells (three 15-cm dishes). The medium was changed the next day, and cells were cultured for 3 days. Then 75 ml of culture medium was collected, and secreted sHF-LIPA was purified by 750 μl of nickel-nitrilotriacetic acid–agarose (Qiagen, Venlo, Netherlands) and eluted by elution buffer (250 mM imidazole (pH 7.4)). The eluted sHF-LIPA was further purified by 40 μl of anti-FLAG beads (Sigma). The proteins bound with anti-FLAG beads were eluted by 300 μl of FLAG peptide solution (500 μg/ml, Sigma).

To express EGFP-F-IgG, wildtype and T-KO HEK293 cells stably expressing EGFP-F-HyHEL10 (EGFP-F-IgG1) were infected with retroviral vectors, pLIB2-pgkHygssEGFP-F-HyHEL10 and pLIB2-pgkBSD-HyHEL10-human-κ. Cells (10 15-cm dishes) were cultured for 3 days, 250 ml of the culture medium was collected, and secreted EGFP-F-IgG1 was purified with 1 ml of protein-A Sefinose resin (Sangon Biotech, Shanghai, China), followed by 40 μl of anti-FLAG beads. The purified EGFP-F-IgG1 was confirmed by SDS-PAGE and Coomassie Brilliant Blue staining.

For RSV analyses, purified sHF-LIPA was electrophoresed by 10% SDS-PAGE and then blotted onto a PVDF membrane. The membrane was stained with Direct Blue-71 (Sigma), which gives no signals derived from the dye during MALDI-MS analysis. Stained sHF-LIPA spots were excised from the membrane and then transferred into a microtube. After the membrane pieces were wet with methanol, 30 μl of a solution containing 2 milliunits PNGase F (Takara) in 10 mM ammonium bicarbonate (pH 7.8) was added to the microtubes, which were incubated at 37 °C for 18 h. For RSV analyses of EGFP-F-IgG1, N-glycans were released from purified EGFP-F-IgG1 solution by PNGase F. The solutions in the microtubes were treated using the BlotGlyco glycan purification kit according to the manufacturer’s protocol (Sumitomo Bakelite Co., Kobe, Japan). Briefly, the released glycans in the solution were captured using BlotGlyco beads, followed by methyl esterification of sialic acids with 3-methyl-1-p-tolytriazine (Sigma). The captured glycans were then labeled and released with an aminoxy-functionalized peptide reagent (aoWR). Derivatized glycans were recovered from the resin by washing with 50 μl of distilled water. Finally, excess reagent was removed using a cleanup column provided in the kit. The obtained solution containing the glycan derivatives was analyzed via MALDI-TOF-MS.

MS spectra were acquired using a MALDI-TOF/TOF-MS (New ultraflexXtreme; Bruker Daltonics). Ions were generated by a pulsed 337-nm nitrogen laser and accelerated to 25 kV. All spectra were obtained using the reflectron mode with a delayed extraction of 200 ns. For sample preparation, 0.5 μl of DHB (10 mg/ml) in 30% ethanol was spotted onto a target plate (MTP 384 target plate ground steel, Bruker) and dried. Subsequently, an aliquot (0.5 μl) of the glycan solution was spotted onto the DHB crystal and dried.

Western blotting

Wildtype, D-KO, and T-KO cells transiently expressing His₆-FLAG–tagged GLA (sHF-GLA) or LIPA (sHF-LIPA) were cultured for 72 h, and then 1.5 ml of the medium was collected. After 20 μl of anti-DDDDK beads (MBL, Nagoya, Japan) were added to the medium, the tubes were rotated at 4 °C for 2 h. sHF-GLA and sHF-LIPA were eluted from the beads using 50 μl of DDDDK peptide (500 μg/ml, MBL). Eluted proteins (10 μl) were denatured by Laemmli sample buffer containing 100 mM dithiothreitol at 95 °C for 5 min and treated with Endo-H (1000 units, New England Biolabs) or PNGase F (500 units) for 3 h. Protein samples were subjected to SDS-PAGE and blotted onto a PVDF membrane. A mouse monoclonal anti-M2 FLAG antibody (5000-fold dilution) and horseradish peroxidase–conjugated goat anti-mouse IgG (5000-fold dilution, TransGen Biotech, Beijing, China) were used as the primary and secondary antibodies to detect the FLAG-tagged proteins, respectively.

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