Cell lines for producing recombinant glycoproteins with di-antennary N-glycans, methods using the same, and recombinant glycoproteins

The present invention relates to a genetically modified cell line with reduced expression of GnTIVa/b and/or GnTV, a method for the production of glycoproteins having N-glycans with a reduced number of tri- and/or tetra-antennary N-glcyans using said cell line, and respective glycoproteins.

The development of therapeutic proteins has accelerated immensely over the past years. However, in particular for complexly glycosylated proteins, it can be difficult to obtain proteins with favorable glycosylation patterns. This often leads to suboptimal pharmacological properties such as *e.g.* reduced serum half-life or increased immunogenicity.

One difficulty in producing recombinant glycoproteins is the potential heterogeneity of the product due to variations in post-translational modifications, in particular of glyco-structures. This can lead to inconsistencies in product quality between different production batches.

Glycosylation is the most common post-translational modification. Nearly all biopharmaceuticals need to be correctly glycosylated in order to display the optimal therapeutic effect. In general, glycosylation refers to the covalent attachment of sugars to the protein surface, wherein the sugars are either linked to asparagine residues resulting in N-linked glycans (N-glycans) or serine or threonine residues resulting in O-linked glycans (O-glycans). The glycosylation pattern can be very diverse between different molecules as the attached glycans can be different in monosaccharide order, branching pattern, and length (micro-heterogeneity). Additionally, not all glycosylation sites are necessarily fully occupied (macro-heterogeneity).

Glycosylation influences *e.g.* the solubility of proteins, their resistance to proteolysis, and their binding behavior to other proteins or protein receptors such as *e.g.* the

ASGPR (asialoglycoprotein receptor) and, therefore, influences the half-life of the glycoprotein in the plasma.

For small size proteins under about 50 kDa, clearance occurs mainly via the kidney. Beside the size of a protein, also the protein surface charge has influence on renal clearance, as the filtration of highly charged proteins in the kidney is diminished. For large size proteins, clearance occurs mainly in the liver through specific and/or unspecific hepatic uptake. An example for a specific uptake mediator is the ASGPR, which binds specifically to non-sialylated N-linked glycoproteins with a terminal galactose. Due to the action of this receptor, glycoproteins with terminal sialic acids (N-acetylneuraminic acid; NeuAc) covering the adjacent carbohydrate, galactose, have an up to 100-fold increased half-life as compared to their non-sialylated counterparts with terminal galactose residues on their N-linked glycans. Other receptors bind specifically to mannose, N-acetyl-glucosamine, or fucose and clear glycoproteins with these terminal sugars from the system.

From this perspective, a native or nearly native N-glycosylation pattern including a high degree of terminal sialylation is crucial for therapeutic proteins since it determines the pharmacokinetic properties of the therapeutic protein. In addition, terminal sialic acids with the proper linkage on glycoproteins reduce the immunogenicity of glycoproteins.

The foremost strategy to obtain nearly native glycostructures and a high degree of sialylation is to produce the recombinant proteins in mammalian cell lines capable of linking mammalian-like glycostructures to proteins, *e.g.* CHO cells (Chinese hamster ovary cells). However, non-human mammalian cell lines often lack the enzyme needed to catalyze 2,6-linkage of sialic acids and, therefore, can only catalyze 2,3-linkage. Moreover, in addition to sialic acid, they often also link N-glycolylneuraminic acid (NeuGc) to glycans, a sugar not synthesized in humans and therefore immunogenic in humans when injected. Thus, a better strategy would be to generate therapeutic proteins from human cell lines like CAP cells (derived from human amniocytes) or HEK293 cells (derived from human embryo kidney cells).

However, sialylation of therapeutic proteins secreted from human cell lines during fermentation is often incomplete.

Accordingly, additional expression of sialyltransferases, *e.g.* β-galactoside α-2,6-sialyltransferase 1 (ST6Gal1) or β-galactoside α-2,3-sialyltransferase 4 (ST3Gal4), in mammalian cells could be advantageous to increase sialylation and consequently serum half-life of therapeutic proteins. Interestingly, human serum proteins are mostly 2,6-sialylated, and normally only the additional branches in tri- and tetra-antennary N-glycans are 2,3-sialylated. Reasons for this were revealed in *in vitro* studies with bovine ST6Gal1 enzyme. In particular, it could be shown that this sialyltransferase adds NeuAc very inefficiently to the third and fourth branch of N-glycans, in particular to the Galß1→4GlcNAcß1→6Manα1→6 branch (Figure 2). In line with these studies it was shown that overexpression of ST6Gal1 results in a lower degree of sialylation of complex N-glycans as compared to expression of ST3Gal4.

Reasons for this are that the third and fourth antennae of a tetra-antennary N-glycan are characterized by different glycosidic linkage and are sterically different. Therefore, they show differential substrate availability to ST3Gal4 and ST6Gal1 enzymes. Accordingly, the Galß1→4GlcNAcß1→2Manα1→3 and Galß1→4GlcNAcß1→2Manα1→6 arms are preferentially sialylated by 2,6-linked sialic acid while the Galß1→4GlcNAcß1→4Manα1→3 and Galß1→4GlcNAcß1→6Manα1→6 arms are preferred targets for 2,3-sialylation.

Therapeutic proteins secreted from mammalian cell lines during fermentation mostly display a higher degree of tri and tetra-antennary N-glycan structures as compared to their counterparts purified from human serum plasma. In line with the studies described above, additional overexpression of ST6Gal1 does not result in completely sialylated therapeutic proteins. Only overexpression of ST3Gal4 does, but in that case the sialylation linkage differs from the plasma-derived proteins. In order to recombinantly produce therapeutic proteins that are highly similar to their naturally occurring counterparts as they are found circulating in the human body, it

can thus be necessary to modify the antennarity of their N-linked glycans and optionally also the linkage type of the terminal sialic acids.

α1-Antitrypsin (AAT) is a protease inhibitor belonging to the serpin superfamily. AAT is a potent inhibitor of serine proteases, in particular neutrophil elastase. AAT is a 52 kDa glycoprotein carrying 3 N-glycosylation sites, namely Asn46, 83 and 247, which mainly carry di-antennary (Asn46, 83, and 247) and tri-antennary N-glycans (Asn83). AAT purified from human serum contains α-2,6-linked neuraminic acids at the di- and tri-antennary N-glycans, and some tri-antennary N-glycans contain additionally one single α-2,3-neuraminic acid per N-glycan. The amount of tri-antennary N-glycans in different human plasma derived AAT samples measured by HPLC averages 12% (+/- 0,4%), tetra-antennary N-glycans could not be detected.

Expression of human recombinant AAT in a broad range of mammalian cells has been shown to result in rhAAT (recombinant human AAT) with increased amounts of tri-antennary N-glycans, and even tetra-antennary N-glycan structures to an extent of 10% and more. This is in strong contrast to the human serum derived counterpart.

The same is true for other recombinant proteins expressed in mammalian cells, e.g. C1 Inhibitor (C1 Inh.). The protease inhibitor C1 esterase inhibitor (C1 Inh) belongs to the serpin superfamily. Its main function is the inhibition of the complement system to prevent spontaneous activation. The 500 amino acid protein is highly glycosylated with seven predicted N-glycans and eight predicted O-linked glycans.

Like rhAAT, rhC1 Inh from mammalian expression systems displays an increased amount of tri- and tetra-antennary N-glycan structures which is not seen in the serum derived counterpart.

In order to achieve full sialylation of all N-glycan branches in such proteins, additional expression of the ST3Gal4 sialyltransferase is necessary, as due to the low affinity to core ß1→4 and core ß1→6 branches of the tri- and tetra-antennary

N-glycans, expression of ST6Gal1 sialyltransferase is not efficient enough, resulting in recombinant proteins which differ from the plasma protein not only regarding their N-glycan antennarity but also regarding the sialylation linkage.

Therefore, the technical problem underlying the present invention is the provision of recombinant glycoproteins having homogenous post-translational modifications with primarily di-antennary N-glycans, as well as cell lines that are capable of recombinantly producing such proteins.

The solution to the above technical problem is achieved by the embodiments characterized in the claims.

In particular, in one aspect, the present disclosure relates to a cell line that is genetically modified to reduce the expression of at least one of GnTIVa/b and GnTV.

In another aspect, the present disclosure relates to a recombinant glycoprotein having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

In another aspect, the present disclosure relates to a method for the expression of a recombinant glycoprotein of interest in a cell, said glycoprotein having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, comprising the steps of providing a cell line as disclosed herein; expressing the glycoprotein of interest in said cell line; and isolating the glycoprotein from the cell or the cell culture supernatant.

GnTIVa and GnTIVb are glycosyltransferases that participate in the transfer of N-acetylglucosamine (GlcNAc) to the core mannose residues of N-linked glycans. They are isoenzymes that are often equally expressed. They catalyze the formation of the GlcNAc ß1-4 branch on the GlcNAc ß1-2 Man α 1-3 arm of the core structure

of N-linked glycans. GnTV catalyzes the addition of GlcNAc in ß 1-6 linkage to the α-linked mannose of di-antennary N-linked oligosaccharides. Both enzymes (GnTIVa/b and GnTV) are essential for the production of tetra-antennary N-linked sugar chains (Figure 1).

In this context, in cell lines wherein GnTIVa and GnTIVb are more or less equally expressed, preferably the expression of both is reduced. However, in cell lines that predominantly express one of GnTIVa and GnTIVb, such as CAP cells expressing predominantly GnTIVb, reduction of the expression of the predominantly expressed isoenzyme may be sufficient.

According to the present invention, the cell lines are genetically modified to reduce the expression of at least one of GnTIVa (α-1,3-mannosyl-glycoprotein 4-β-N-

acetylglucosaminyltransferase A; SEQ ID NO: 2), GnTIVb (α-1,3-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase B; SEQ ID NO: 3), and GnTV (α-1,6-mannosylglycoprotein 6-β-N-acetylglucosaminyltransferase A; SEQ ID NO: 1). In preferred embodiments, the expression of GnTIVa/b and GnTV is reduced.

In this context, the term "cell line that is genetically modified to reduce the expression of at least one of GnTIVa/b and GnTV" as used herein indicates that upon genetic modification, the individual cells of the cell line display lower expression levels and, thus, lower activities of the respective proteins than they did before the genetic modification.

The terms "reduced expression" or "reduced activity" as used herein refer to a reduction of protein expression or protein activity by at least 50%, preferably at least 60%, 70%, 75%, 80%, 82%, 84%, 86%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.25%, 98.5%, 98.75%, 99%, 99.25%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%.

Genetic modifications that allow the reduction of the expression of a given protein are not particularly limited and are known in the art. In a particular example, the cell line comprises an endogenous GnTIVa/b or GnTV gene, such as *e.g.* human cell lines. In such cases, the cells can be genetically modified by deletion or insertion of nucleotides into the genome of the cells in a position suitable to cause knock-out of said nucleic acid. This can be done by non-homologous end joining or homologous recombination using TALENS, Zn-finger proteins, CRISPR-CAS9, or other methods known in the art. Accordingly, in preferred embodiments, the expression of GnTIVa/b and/or GnTV is completely abolished, preferably by disruption of the respective genes. Reduced expression of a given protein can also be achieved by reduction of the levels of corresponding mRNA, *e.g.* the cells can be genetically modified in order to express interfering RNAs targeting the mRNA of GnTIVa/b and/or GnTV. Respective methods are not particularly limited and are known in the art.

In preferred embodiments, the cell line is selected from the group consisting of insect cell lines, avian cell lines, and mammalian cell lines, wherein mammalian cell lines, in particular human cell lines are particularly preferred. In specific embodiments, the cell line is derived from a cell line, selected from the group consisting of Muscovy Duck cells (AGE.CR®), African green monkey kidney epithelial cells (Vero), Madin Darby canine kidney cells (MDCK), baby hamster kidney cells (BHK), Chinese hamster ovary (CHO) cells, human hepatocarcinoma cell lines (HepG2, Huh7), human embryonic kidney 293 (HEK293) cells, human neuronal precursor cells (AGE1.HN®, NC5T11), human embryonic retinoblast cells (Per.C6), myeloma cell lines (HMCLs, MM.1, U266, RPMI8226), CML tumor cell lines (NM, NM-F9), hybrid HEK293 and lymphoma cell (HKB11), and human amniocytes-derived CAP cells. In this context, CAP cells are particularly preferred.

CAP cells are permanent amniocytic cell lines comprising a nucleic acid encoding the gene products of adenovirus, in particular adenovirus type 5 (Ad5), E1A and E1B regions. CAP cells are derived from primary human amniocytes that are transformed with a nucleic acid encoding Ad5 E1A and E1B.

Accordingly, in a preferred embodiment, the cell lines according to the present invention can be derived from human primary amniocytes comprising at least one nucleic acid encoding the gene products of the adenoviral E1 and pIX region, preferably E1 and pIX region of adenovirus type 5 (Ad5), more preferably from nucleotides 505 to 4079, in which preferably E1A is under the control of the murine phosphoglycerate kinase (pgk) promoter, while E1B and pIX expression is controlled from their natural promoters. The E1B downstream intron, splice acceptor and polyA signal are preferably replaced by corresponding motifs from SV40.

In preferred embodiments, the cell line is a suspension cell line, *i.e.*, a cell line that can be cultured in suspension culture, e.g. serum-free suspension culture. Respective cell lines are known in the art.

In preferred embodiments, the cell line is further genetically modified to overexpress β-galactoside α-2,6-sialyltransferase 1 (ST6Gal1) and/or α-2,3-sialyltransferase 4 (ST3Gal4).

In this context, the term "overexpressed" either refers to protein expression in cells that prior to genetic modification did not express the respective protein at all, or to an increase in endogenous expression of at least 1.2-fold, preferably at least 1.4-fold, 1.6-fold, 1.8-fold, 2-fold or more.

Methods for the expression/overexpression of a given protein in a cell are not particularly limited and are known in the art. Suitable genes encoding ST3Gal4 and suitable genes encoding ST6Gal1 are not particularly limited and include any genes from any origin that encode a protein having the respective activity, wherein mammalian, in particular human genes are particularly preferred.

In a further embodiment, the present disclosure relates to methods for the expression of recombinant glycoproteins of interest in a cell, said glycoproteins having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, said methods comprising the step of genetically modifying said cell to reduce the expression of GnTIVa/b and /or GnTV in said cell, and optionally to overexpress ST6Gal1 and/or ST3Gal4.

In particular embodiments, said methods comprise the steps of:

(a) providing a cell line according to the present invention;

(b) expressing the glycoprotein of interest in said cell line; and

(c) isolating the glycoprotein from the cell or the cell culture supernatant.

Means for recombinantly expressing glycoproteins in a given cell line, as well as for isolating said glycoproteins from the cell or the cell culture supernatant, are not particularly limited and are known in the art.

In these aspects, all of the definitions and preferred and/or specific embodiments described below for the recombinant glycoproteins of the present invention, and described above for the cell lines of the present invention apply in an analogous manner where applicable.

In a further embodiment, the present disclosure relates to a recombinant glycoprotein having N-glycans with a significantly increased proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

As used herein, the term "recombinant glycoprotein" indicates that the respective glycoproteins are biotechnologically produced in genetically modified or cells.

According to the present invention, the proportion of di-antennary N-glycans in the glycoproteins is preferably increased at least 1.2-fold, more preferably at least 1.4-fold, 1.6-fold, 1.8-fold, 2-fold or more, when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

In related preferred embodiments, the proportion of tetra-antennary N-glycans in the glycoproteins is significantly reduced when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, Preferably, said proportion is reduced at least 2-fold, more preferably at least 5-fold, at least 10-fold, at least 100-fold (corresponding to a reduction by at least 50%, more preferably at least 80%, at least 90%, at least 99%) or more, wherein any remaining N-glycans besides di-antennary N-glycans, if present at all, are tri-antennary N-glycans.

In preferred embodiments, at least 80% of the antennae of the N-glycans of the recombinant glycoproteins of the present invention are terminated by 2,6-linked sialic acid. More preferably, at least 85%, 90%, 92%, 93%, 94%, 95%, 95.5%, 96%,

96.5%, 97%, 97.5%, 98%, 98.25%, 98.5%, 98.75%, 99%, 99.25%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% of the N-glycans are terminated by 2,6-linked sialic acid.

Respective glycoproteins can be produced by recombinant expression in suitable organisms or cells, wherein concurrently with expression of the glycoproteins, and reduction of the expression of GnTIVa/b and/or GnTV, ST6Gal1 and/or ST3Gal4 are overexpressed, *e.g.* as indicated above. Preferably, the glycoproteins of the present invention are expressed in a cell line of the present invention as defined above.

The N-glycans of the recombinant glycoproteins of the present invention are characterized by a reduced micro-heterogeneity, *i.e.*, a reduced diversity of N-glycan structure and composition within a given glycoprotein preparation. Respective recombinant glycoproteins can be produced as described herein, *e.g.* by reducing the expression level and, this, activity of GnTIVa/b and/or GnTV, and optionally by additional overexpression of ST3Gal4 and/or ST6Gal1. Preferably, said glycoproteins are produced in a cell line according to the present invention as described herein.

Specific glycoproteins according to the present invention are not particularly limited, provided that said glycoproteins have N-glycans. Preferably, said glycoproteins are mammalian, more preferably human glycoproteins. Glycoproteins can be selected from the group consisting of growth factors, peptide hormones, cytokines, enzymes, antibodies, antibody fragments, blood clotting factors, and protease inhibitors. However, in a particular embodiment, said glycoproteins are not antibodies or antibody fragments, *i.e.*, the glycoproteins are selected from the group consisting of growth factors, peptide hormones, cytokines, enzymes, blood clotting factors, and protease inhibitors.

Preferably, the glycoprotein is selected from the group consisting of α1-antitrypsin (AAT), hepatocyte growth factor (HGF), erythropoietin (EPO), Factor VII (FVII),

Factor VIII (FVIII), Factor IX (FIX), von-Willebrand-Factor (vWF), and C1 esterase inhibitor (C1-inhibitor; C1 Inh), wherein C1 Inh is particularly preferred.

In a further embodiment, the present disclosure relates to a method for the generation of a cell line for the recombinant expression of glycoproteins according to the present invention, comprising steps of genetically modifying a cell line to reduce the expression of at least one of GnTIVa/b and GnTV, and optionally to overexpress ST6Gal1 and/or ST3Gal4.

In further embodiments, the present disclosure relates to methods for decreasing the micro-heterogeneity of N-glycans of recombinant glycoproteins produced in a cell, said method comprising the step of genetically modifying said cell to reduce the expression of GnTIVa/b and /or GnTV in said cell, and optionally to overexpress ST6Gal1 and/or ST3Gal4.

In particular embodiments, said methods comprise the steps of:

(a) providing a cell line according to the present invention;
(b) expressing the glycoprotein of interest in said cell line; and
(c) isolating the glycoprotein from the cell or the cell culture supernatant.

In these aspects, all of the definitions and preferred and/or specific embodiments described for the recombinant glycoproteins of the present invention and the cell lines of the present invention apply in an analogous manner where applicable.

As used herein, the term “about” is intended to be a modifier of ± 10%, preferably ± 5% of the specified value. As an example, the term “about 0.2” is intended to encompass the range of 0.18 to 0.22, preferably 0.19 to 0.21.

The terms “comprising/comprises”, “consisting of/consists of”, and “consisting essentially of/consists essentially of” are used herein in an interchangeable manner, *i.e.*, each of said terms can expressly be exchanged against one of the other two terms.

The figures show:

Figure 1:

Biosynthesis of tri- and tetra-antennary branched N-glycans. Tri- and tetra-antennary complex N-glycans are generated by the action of GnTIV and GnTV. These branches can be elongated further with e.g. galactose, N-acetylgalactosamine, sialic acid, and fucose.

Figure 2:

Branch specificity of ST6Gal1 activity. ST6Gal1 catalyzes sialylation of the primary branches of tri- and tetra-antennary N-glycans, whereas sialylation of the additional branches of tri- and tetra-antennary N-glycans is catalyzed by ST3Gal4.

Figure 3:

DSA and PHA-L lectin immunoblot analysis of N-glycans from human AAT purified from human serum (Prolastin) in comparison to rhAAT expressed in CAP wild-type cells.

Figure 4:

DSA lectin immunoblot of recombinant hAAT expressed in wildtype CAP cells (control) or CAP cells harboring a GnTIVb knock-out in the presence or absence of sialyltransferases. CAP wild-type cells or CAP cells harboring a GnTIVb knock-out (without (A) or with additional overexpression of sialyltransferases ST3Gal4 (B) or ST6Gal1 (C)) were modified to stably express rhAAT. rhAAT was purified from cell culture supernatants and was subject to lectin blot analysis. *Datura stramonium* (DSA) lectin detects core ß1→4 branched N-glycans synthesized by GnTIVb. Therefore, a diminished signal in the DSA blot means a decreased amount of N-glycans with ß1→4 branches, indicating a decreased or absent GnTIVb enzyme activity, resulting in N-glycan structures without a ß1→4 branch and therefore with only di- or tri-antennary structures. As loading control, the same samples were subjected to western blot analysis using a hAAT specific antibody.

Figure 5:

PHA-L lectin immunoblot of recombinant hAAT expressed in wildtype CAP cells or CAP cells harboring a GnTV knock-out in the presence or absence of sialyltransferases. CAP wild-type cells or CAP cells harboring a GnTV knock-out (with or without additional overexpression of ST6Gal1) were modified in order to stably express rhAAT. rhAAT was purified from cell culture supernatants and was subject to lectin blot analysis. *Phytohemagglutinin*-L (PHA-L) lectin detects core ß1→6 branched N-glycans synthesized by GnTV. Therefore, a diminished signal in the PHA-L blot is indicating a decreased or absent GnTV enzyme activity, resulting in N-glycan structures without a ß1→6 branch and therefore with only di- or tri-antennary structures. Binding of PHA-L lectin to the ß1→6 branched N-glycan is inhibited by sialic acid, therefore signal is decreased in the samples purified from cells additionally expressing ST6Gal1. Nevertheless, a diminished signal in the GnTV knock-out sample in comparison to the CAP wild-type sample can be observed. As loading control the same samples were subject to western blot analysis using an hAAT specific antibody.

Figure 6:

Summary of MALDI-TOF mass spectrum analysis of rhAAT N-glycans of protein expressed in CAP wild-type or in CAP ΔGnTIVb with or without additional overexpression of ST3Gal4 or ST6GAL1. Displayed are the sums of relative amounts of the hybrid, di-, tri- and tetra-antennary N-glycan structures of CAP ΔGnTIVb normalized to CAP wt. Glycan fragments generated by MALDI-TOF or negligible signal were not used for this analysis. Knock-out of GnTIVb results in a 3- to 15-fold decrease in the total amounts of tetra-antennary N-glycans. Residual amounts of tetra-antennary N-glycans could be due to the enzymatic activity of GnTIVa.

Figure 7:

DSA and PHA-L lectin immunoblot of recombinant hAAT expressed in wildtype CAP cells or CAP cells harboring a ΔGnTV or ΔGnTIVb/GnTV knock-out. CAP wild-type cells or CAP single cell clones harboring a GnTV, or GnTIVb/GnTV double knock-

out were modified in order to stably express rhAAT. rhAAT was purified from cell culture supernatants and was subject to lectin blot analysis. DSA lectin detects core ß1→4 branched N-glycans synthesized by GnTIVb. Therefore, a diminished signal in the DSA blot means a decreased amount of N-glycans with ß1→4 branches. PHA-L lectin detects core ß1→6 branched N-glycans synthesized by GnTV. Therefore, a diminished signal in the PHA-L blot means a decreased amount of N-glycans with ß1→6 branches. AAT purified from CAP single cell clones with the GnTIVb/GnTV double knock-out display only a minor signal in both lectin blot analysis in comparison to the wild type control indicating that almost all N-glycans are di-antennary.

Figure 8:

Comparison of rhAAT isoform patterns by IEF analysis. As the backbones of the different rhAAT are identical, changes in the IEF are most likely due to changes in the sialic acid content. rhAAT was expressed in wild-type, ΔGnTIVb, ΔGnTV, or ΔGnTIVb/GnTV CAP cells with additional expression of ST6Gal. AAT purified from knock-out cells shows a less acidic pI, indicating a decrease in the total amount of sialic acids per molecule. In addition, this indicates a decrease of potential NeuAc binding sites due to a shift from tri- and tetra-antennary structures to di-antennary N-glycan structures.

Figure 9:

ECL lectin immunoblot of recombinant hAAT expressed in wildtype CAP cells or CAP cells harboring a ΔGnTIVb/GnTV knock-out. *Erythrina crista-galli* (ECL) lectin detects ß1-4 linked terminal galactose on N-linked glycans. Due to the branch specificity of ST6Gal1 (Galß1→4GlcNAcß1→2Manα1→3 and Galß1→4GlcNAcß1→2Manα1→6), overexpression of ST6Gal1 results in complete sialylation of di-antennary N-glycans present on rhAAT expressed in ΔGnTIVb/GnTV cells, whereas rhAAT expressed in wild-type CAP cells with additional overexpression of ST6Gal1 still harbors non-sialylated N-glycan branches. Neuraminidase catalyzes the hydrolysis of N-acetyl-neuraminic acid residues from oligosaccharide, thus neuraminidase treated samples serve as positive control. As

loading control, the same samples were subjected to western blot analysis using an hAAT specific antibody.

Figure 10:

DSA and PHA-L lectin immunoblot of recombinant rhC1 Inh expressed in wildtype CAP cells or CAP cells harboring a ΔGnTIVb/GnTV double knock-out in comparison to plasma derived C1 Inhibitor (Berinert). CAP wild-type cells or CAP-ST6Gal1 single cell clones harboring a GnTIVb/GnTV double knock-out were modified in order to stably express rhC1 Inh. rhC1 Inh was purified from cell culture supernatants and was subjected to lectin blot analysis. Lectin *Datura stramonium* (DSA) lectin detects core ß1→4 branched N-glycans synthesized by GnTIVb. Therefore, a diminished signal in the DSA blot proves a reduced amount of N-glycans with ß1→4 branches. *Phytohemagglutinin-L (PHA-L)* lectin detects core ß1→6 branched N-glycans synthesized by GnTV. Therefore, a diminished signal in the PHA-L blot proves a decreased amount of N-glycans with ß1→6 branches. Plasma derived C1 Inh. as well as C1 Inh purified from CAP single cell clones with the GnTIVb/GnTV double knock-out display only a minor signal in both lectin blot analysis in comparison to the wild type control indicating that almost all N-glycans are di-antennary. The corresponding densitometrical analysis was normalized on the input using the C1-Inhibitor specific western blot as well as on the signal intensity of the CAP wild type.

The present invention will be further illustrated by the following examples without being limited thereto.

Examples

Material and Methods:

Cell lines

Cell lines used in the present application are indicated in Table 1 below.

Table 1: Stable cell lines used in the present invention.

Cell Line	Expression of GnTV/GnTIVb	Rec. Protein Expression	Overexpression of Sialyltransferases
CAP	wild-type	rhAAT	
CAP	ΔGnTIVb	rhAAT	
CAP	Δ-GnTV	rhAAT	
CAP	ΔΔGnTIVb/GnTV	rhAAT	
CAP	wild-type	rhAAT	ST6Gal1
CAP	ΔGnTIVb	rhAAT	ST6Gal1
CAP	Δ-GnTV	rhAAT	ST6Gal1
CAP	ΔΔGnTIVb/GnTV	rhAAT	ST6Gal1
CAP	wild-type	rhAAT	ST3Gal4
CAP	ΔGnTIVb	rhAAT	ST3Gal4
CAP	wild-type	rhC1 Inh	
CAP	ΔΔGnTIVb/GnTV	rhC1 Inh	ST6Gal1

Cell Culture and Fermentation

The permanent human amniocyte cell line CAP 1D5 and all their derivates with diminished expression of GnTIVa/b and/or GnTV were cultured in suspension, either in chemically defined, animal component free CAP-CDM medium (CEVEC Pharmaceuticals, Germany) supplemented with 6 mM stable glutamine (biochrom,

Germany), or in serum free PEM media (Life Technologies) supplemented with 4 mM stable glutamine (biochrom, Germany).

All cell lines were cultivated at 37 °C in shaker flasks (Corning, #431143, 125 mL (25 mL wv) or #431252, 3000 mL (1000 mL wv)) at 5% CO_2, and 185 rpm. During fermentation CAP cells were fed at d3, d5, and d7 with 10 % CAP-CDM feed solution (CEVEC Pharmaceuticals, Germany) and 4 mM stable glutamine (biochrom, Germany).

Cloning

For the generation of the CAP cell lines stably expressing ST3Gal4 and/or ST6Gal1, the cells were nucleofected with the corresponding nuclei acid constructs.

For designing the ST3Gal4 cDNA, sequence information of the precursor protein and mature protein was based of the database entry UniProt Q11206. For cloning, a ClaI restriction site and a Kozak sequence were added 5´ of the start codon of the human ST3Gal4 cDNA and an EcoRV restriction site was added 3´ of the stop codon to be inserted between the ClaI and EcoRV restriction sites in the pStbl-Neo-CMV-MCS(-) vector resulting in the expression plasmid pStbl-Neo-CMV-ST3Gal4. This vector contains a CMV promoter driving the expression of the gene of interest, followed by an SV40 intron for improved, splicing-mediated mRNA transport and a multiple cloning site for the insertion of the gene of interest. The selection marker is driven by the human ubiquitin (UbC) promoter. cDNA synthesis was performed at GeneArt (Germany, Life Technologies).

For designing the ST6Gal1 cDNA, sequence information of the precursor protein and mature protein was based of the database entry UniProt P15907. For cloning, a ClaI restriction site and a Kozak sequence were added 5´ of the start codon of the human ST6Gal1 cDNA and a EcoRV restriction site was added 3´ of the stop codon to be inserted between the ClaI and EcoRV restriction sites in the pStbl-Neo-CMV-

MCS(-) vector resulting in the expression plasmid pStbl-Neo-CMV-ST6Gal1. cDNA synthesis was performed at GeneArt (Germany, Life Technologies).

Nucleofection and Pool Generation

Nucleofection was performed using a Nucleofector (LONZA) with the appropriate Nucleofector Kit (KitV) according to the manufacturer's protocol. Briefly, during exponential growth phase of the culture 1 × 10^7 cells were harvested via centrifugation (150 g for 5 min) and resuspended in 100 µl complete nucleofector solution and mixed with a total of 5 µg plasmid. Nucleofection was performed using the X001 program (CAP cells). After the pulse, cells were recovered in 12 ml complete cell culture media in a 125 ml shaking flask. The cells were cultured as before at 37 °C, 5% CO_2, and 185 rpm. 72 to 96 h post-nucleofection cells were selected with 200 µg/ml neomycin in order to generate stable pools.

Purification of AAT from cell culture supernatants

Following harvest by tangential flow filtration and sterile filtration, the rhAAT containing cell culture supernatant was concentrated and diafiltrated against buffer for the first chromatography step. The diafiltrated solution was loaded on an AAT affinity chromatography column. Subsequently, rhAAT protein was eluted in the linear gradient with increasing $MgCl_2$ concentrations. The pooled fractions were concentrated and the buffer was exchanged against formulation buffer using desalting columns.

Purification of C1 Inh from cell culture supernatants

Following harvest by tangential flow filtration and sterile filtration, C1 Inh was purified from the cell culture supernatant by anion exchange chromatography column

followed by a phenyl hydrophobic interaction chromatography column, which was run in negative mode. The flow-through of this chromatography step was collected, concentrated and the buffer was exchanged against formulation buffer using desalting columns.

Lectin Immunoblotting

Lectins are proteins that bind specific carbohydrate structures. Biotin-coupled lectins can therefore be used to analyze N-linked glycans. DSA lectin detects core ß1→4 branched N-glycans synthesized by GnTIVb. PHA-L lectin detects core ß1→6 branched N-glycans synthesized by GnTV. Purified protein or cell culture supernatants with or without diminished expression of GnTIVa/b and/or GnTV and with or without co-expression of ST3Gal4 and/or ST6Gal1 were separated as described above and blotted onto Amersham Hybond ECL nitrocellulose membrane (GE healthcare). The membrane was blocked for 1 h at RT with PBSTB (phosphate-buffered saline, pH = 7.4, supplemented with 0.1% Tween 20 and 1 % BSA). Afterwards, the membrane was incubated with the lectin diluted 1:2000 in PBSTB. After washing the membrane with PBST (phosphate-buffered saline, pH = 7.4, supplemented with 0.1% Tween 20), the membrane was stained with streptavidin-coupled horseradish peroxidase for 1 h at RT (1:1000 diluted in PBSTB). The HRP signal was amplified using anti-streptavidin IgG and anti IgG-HRP. The proteins were detected using the Pierce ECL WB Substrate Kit via a chemiluminescence detector (INTAS).

IEF analysis

Isoelectric focusing (IEF) was performed in order to analyze the isoelectric point (pI) of rhAAT purified from CAP cells expressing rhAAT with or without diminished expression of GnTIVa/b and/or GnTV and with or without co-expression of ST3Gal4 and/or ST6Gal1. The degree of sialylation correlates with a given proteins acidity

and, therefore, with its pI. IEF analysis was done according to the manufacturers protocol (Invitrogen). Briefly, 5 µg of purified protein were loaded on pH3-7 gels and subjected to electrophoresis (1h 100 V, 1 h 200 V, 30 min 500 V). Proteins were stained with SimplyBlue SafeStain according to the manufacturers protocol (Invitrogen).

Example 1:

Cells of the human amniocyte cell line CAP 1D5 were stably transfected with a plasmid coding for rhAAT. After pool generation cells were subject to fed-batch in order to generate sufficient amounts of recombinant AAT.

To determine the degree of core ß1,6- and or core ß1,4-branched N-glycans of AAT expressed in wild-type CAP cells in comparison to plasma-derived AAT (Prolastin), DSA and PHA-L lectin blots were performed. PHA-L reacts specifically with core β1,6-branched products synthesized by GnTV and DSA detects core ß1→4 branched N-glycans synthesized by GnTIVb. Therefore, the increased signal in blots shown in Figure 3 indicates a higher degree of tri- and/or tetra-antennary N-glycans in the AAT derived from wild-type CAP cells in comparison to the plasma derived AAT.

Lectin blot analysis (Figure 3) reveals that as in other mammalian cell lines, expression of recombinant glycoproteins results in N-glycan structures with elevated amount of tri- and tetra-antennary structures compared to the corresponding plasma-derived human protein.

Example 2:

Cells of the human amniocyte cell line CAP 1D5 with either wild type expression or diminished expression of GnTIVb were stably transfected with a vector encoding

rhAAT. After pool generation, cell lines were subject to fed-batch in order to make sufficient amounts of recombinant AAT. rhAAT was purified from cell culture supernatants and was subject to lectin blot analysis. DSA lectin detects core ß1→4 branched N-glycans synthesized by GnTIVb. The reduction in DSA-signal shown in Figure 4A is due to a reduced or absent GnTIVb enzyme activity, resulting in N-glycan structures without a ß1→4 branch and therefore without tetra-antennary structures. As loading control same samples were subject to western blot analysis using a hAAT specific antibody (Figure 4).

These results were confirmed by MALDI-TOF analysis of N-glycans from AAT derived in CAP wildtype cells and CAP ΔGnTIVb (Figure 6), as knock-out of GnTIVb results in a 3- to 4-fold decrease in the total amounts of tetra-antennary N-glycans. Residual amount of tetra-antennary N-glycan structures are probably due to residual GnTIVb activity in the modified CAP cells or due to GnTIVa enzyme activity.

Example 3:

Cells of the human amniocyte cell line CAP 1D5 with either wild type expression or diminished expression of GnTV were stably transfected. After pool generation cell lines were subject to fed-batch in order to make sufficient amounts of recombinant AAT.

Purified recombinant AAT was then analyzed to determine the degree of core ß1,6-branched N-glycans in the AAT expressed in ΔGnTV vs. wild-type CAP cells. In order to do so, a PHA-L (phytohemagglutinin-L) lectin blot was performed. PHA-L reacts specifically with core β1,6-branched glycostructures synthesized by GnTV.

As shown in Figure 5 (lane 2 and 4) and Figure 7B a diminished signal in the PHA-L blot was detected, indicating a decreased or absent GnTV enzyme activity, decreased amount of ß1,6-branched products, and, therefore, reduced amount of tri- and/or tetra-antennary N-glycans.

Example 4:

In order to investigate if an absent enzyme activity of GnTIVb and GnTV results in only di-antennary N-glycans, the following experiment was performed. CAP cells with either wild type expression or reduced expression of GnTIVb and GnTV (ΔGnTIVb/GnTV cells) were nucleofected with a vector coding for rhAAT. After pool generation cell lines were subject to fed-batch in order to generate sufficient amounts of recombinant AAT. Subsequently, AAT was purified from the cell culture supernatant.

Next, the N-glycan structures from rhAAT expressed in wild-type or ΔGnTIVb/GnTV CAP cells were analyzed by DSA and PHA-L lectin blots
(Figure 7).

Interestingly, in the DSA as well as in the PHA-L lectin blots only a background signal was detectable for the rhAAT expressed in ΔGnTIVb/GnTV CAP, strongly suggesting that rhAAT expressed in these cells contains no or only minimal amounts of tri or tetra-antennary N-glycan structures.

Example 5:

CAP wild-type cells expressing rhAAT or cells with reduced expression of GnTIVb and/or GnTV expressing rhAAT were further transfected with an additional vector encoding for ST6Gal1 or ST3Gal4. After pool generation, generated cell lines were subject to fed-batch in order to generate sufficient amounts of recombinant AAT. Subsequently, AAT was purified from the cell culture supernatant of the following cell lines: CAP-AAT, CAP-AAT+ST6Gal1, CAP-AAT+ST3Gal4, ΔGnTIVb-AAT, ΔGnTIVb-AAT+ST6Gal1, ΔGnTIVb-AAT+ST3Gal4, ΔGnTV-AAT, ΔGnTV-AAT+ST6Gal1, ΔΔGnTIVb/V-AAT, and ΔΔGnTIVb/V-AAT+ST6Gal1.

Figures 4 and 7 show that additional expression of sialyltransferases like ST3Gal4 or ST6Gal1 has no influence on the diminished amount of ß1,6-branched N-glycans caused by the diminished expression of GnTIVb. In addition, Figures 5 and 7 show that additional expression of sialyltransferases has also no influence on the reduced amount of ß1,4-branched N-glycans caused by the diminished expression of GnTV. Of note, binding of PHA-L lectin to the ß1→6 branched N-glycan is inhibited by sialic acid, so that signal is decreased in the samples purified from cells additionally expressing ST6Gal1. Nevertheless, a diminished signal of the GnTV knock-out in comparison to the CAP wild-type cells can be observed.

As the backbones of the different rhAAT are identical, changes in the IEF indicate changes in the sialic acid content. rhAAT expressed in ΔGnTIVb, ΔGnTV, or ΔGnTIVb/GnTV CAP cells with additional expression of ST6Gal1 results in a modified rhAAT which slightly shifts towards a less acidic pI, in comparison to the rhAAT expressed in parental CAP cells overexpressing ST6Gal1. This indicates a decrease in the total amount of sialic acids per molecule. The observation is consistent with a decrease of potential NeuAc binding sites due to a shift from tri- and tetra-antennary structures to di-antennary N-glycan structures (Figure 8). This rational is supported by the ECL blot shown in Figure 9.

Example 6:

To explore if, like hAAT (see example 1), also rhC1 Inh from mammalian expression systems displays an increased amount of tri- and tetra-antennary N-glycan structures which is not seen in the serum derived counterpart, the degree of core ß1,6- and or core ß1,4-branched N-glycans of C1 Inh expressed in wild-type CAP cells in comparison to plasma-derived C1 Inh (Berinert) was determined by DSA (*Datura stramonium*) and PHA-L (Phytohemagglutinin-L) blots. PHA-L reacts specifically with core β1,6-branched antennae synthesized by GnTV and DSA detects core ß1→4 branched N-glycans synthesized by GnTIVb. Therefore, the signal ratio in blots shown in Figure 10 (lane1, CAP wild type, and lane 2, Berinert)

indicates a significantly higher degree of tri- and/or tetra-antennary N-glycans in the C1 Inh derived from wild-type CAP cells in comparison to the plasma derived C1 Inh.

Therefore, the lectin blot analysis in Figure 10 reveals that, like for rhAAT, expression of the recombinant glycoprotein C1 Inh in unmodified CAP cells results in N-glycan structures with elevated amounts of tri- and tetra-antennary structures compared to the corresponding plasma-derived human protein (Berinert).

In order to investigate if an absent enzyme activity of GnTIVb and GnTV results in only di-antennary N-glycans, not only in rhAAT but also in the complex glycoprotein C1 Inh, non-glycoengineered CAP cells with wild type expression of GnTIVb and GnTV and CAP-ST6Gal1 single cell clones with reduced expression of GnTIVb and GnTV (ΔGnTIVb/GnTV cells) were nucleofected with a vector coding for rhC1 Inh. After pool generation, cell lines were subjected to fed-batch in order to generate sufficient amounts of recombinant C1 Inh. Subsequently, C1 Inh was purified from the cell culture supernatant. Next, the N-glycan structures from rhC1 Inh expressed in wild-type or ΔGnTIVb/GnTV CAP-ST6Gal1 cells were analyzed by DSA and PHA-L lectin blots (Figure 10).

Interestingly, in the DSA as well as in the PHA-L lectin blots, only a background signal was detectable for the rhC1 Inh expressed in ΔGnTIVb/GnTV CAP-ST6Gal1 cells, strongly suggesting that rhC1 Inh expressed in these cells contains no or only minimal amounts of tri or tetra-antennary N-glycan structures.

The present invention discloses the following amino acid sequences.

SEQ ID NO: 1

Human GnTV

MALFTPWKLSSQKLGFFLVTFGFIWGMMLLHFTIQQRTQPESSSMLREQILDLSKRYIKA
LAEENRNVVDGPYAGVMTAYDLKKTLAVLLDNILQRIGKLESKVDNLVVNGTGTNSTNST

TAVPSLVALEKINVADIINGAQEKCVLPPMDGYPHCEGKIKWMKDMWRSDPCYADYGVDG
STCSFFIYLSEVENWCPHLPWRAKNPYEEADHNSLAEIRTDFNILYSMMKKHEEFRWMRL
RIRRMADAWIQAIKSLAEKQNLEKRKRKKVLVHLGLLTKESGFKIAETAFSGGPLGELVQ
WSDLITSLYLLGHDIRISASLAELKEIMKKVVGNRSGCPTVGDRIVELIYIDIVGLAQFK
KTLGPSWVHYQCMLRVLDSFGTEPEFNHANYAQSKGHKTPWGKWNLNPQQFYTMFPHTPD
NSFLGFVVEQHLNSSDIHHINEIKRQNQSLVYGKVDSFWKNKKIYLDIIHTYMEVHATVY
GSSTKNIPSYVKNHGILSGRDLQFLLRETKLFVGLGFPYEGPAPLEAIANGCAFLNPKFN
PPKSSKNTDFFIGKPTLRELTSQHPYAEVFIGRPHVWTVDLNNQEEVEDAVKAILNQKIE
PYMPYEFTCEGMLQRINAFIEKQDFCHGQVMWPPLSALQVKLAEPGQSCKQVCQESQLIC
EPSFFQHLNKDKDMLKYKVTCQSSELAKDILVPSFDPKNKHCVFQGDLLLFSCAGAHPRH
QRVCPCRDFIKGQVALCKDCL

SEQ ID NO: 2

Human GnTIVa

MRLRNGTVATALAFITSFLTLSWYTTWQNGKEKLIAYQREFLALKERLRIAEHRISQRSS
ELNTIVQQFKRVGAETNGSKDALNKFSDNTLKLLKELTSKKSLQVPSIYYHLPHLLKNEG
SLQPAVQIGNGRTGVSIVMGIPTVKREVKSYLIETLHSLIDNLYPEEKLDCVIVVFIGET
DIDYVHGVVANLEKEFSKEISSGLVEVISPPESYYPDLTNLKETFGDSKERVRWRTKQNL
DYCFLMMYAQEKGIYYIQLEDDIIVKQNYFNTIKNFALQLSSEEWMILEFSQLGFIGKMF
QAPDLTLIVEFIFMFYKEKPIDWLLDHILWVKVCNPEKDAKHCDRQKANLRIRFRPSLFQ
HVGLHSSLSGKIQKLTDKDYMKPLLLKIHVNPPAEVSTSLKVYQGHTLEKTYMGEDFFWA
ITPIAGDYILFKFDKPVNVESYLFHSGNQEHPGDILLNTTVEVLPFKSEGLEISKETKDK
RLEDGYFRIGKFENGVAEGMVDPSLNPISAFRLSVIQNSAVWAILNEIHIKKATN

SEQ ID NO: 3

Human GnTIVb

MRLRNGTFLTLLLFCLCAFLSLSWYAALSGQKGDVVDVYQREFLALRDRLHAAEQESLKR
SKELNLVLDEIKRAVSERQALRDGDGNRTWGRLTEDPRLKPWNGSHRHVLHLPTVFHHLP
HLLAKESSLQPAVRVGQGRTGVSVVMGIPSVRREVHSYLTDTLHSLISELSPQEKEDSVI
VVLIAETDSQYTSAVTENIKALFPTEIHSGLLEVISPSPHFYPDFSRLRESFGDPKERVR
WRTKQNLDYCFLMMYAQSKGIYYVQLEDDIVAKPNYLSTMKNFALQQPSEDWMILEFSQL
GFIGKMFKSLDLSLIVEFILMFYRDKPIDWLLDHILWVKVCNPEKDAKHCDRQKANLRIR
FKPSLFQHVGTHSSLAGKIQKLKDKDFGKQALRKEHVNPPAEVSTSLKTYQHFTLEKAYL

REDFFWAFTPAAGDFIRFRFFQPLRLERFFFRSGNIEHPEDKLFNTSVEVLPFDNPQSDK
EALQEGRTATLRYPRSPDGYLQIGSFYKGVAEGEVDPAFGPLEALRLSIQTDSPVWVILS
EIFLKKAD

Claims

1. A cell line that is genetically modified to reduce the expression of:
   (i) GnTIVa or GnTIVb, and
   (ii) GnTV.

2. The cell line according to claim 1, wherein the expression of all of GnTIVa/b and GnTV is reduced.

3. The cell line according to claim 1 or claim 2, wherein the expression of GnTIVa/b and/or GnTV is completely abolished.

4. The cell line according to any one of claims 1 to 3, wherein the cell line is further genetically modified to overexpress β-galactoside α-2,6-sialyltransferase 1 (ST6Gal1) and/or α-2,3-sialyltransferase 4 (ST3Gal4).

5. The cell line according to any one of claims 1 to 4, wherein the cell line is an insect cell line, an avian cell line, or a mammalian cell line, preferably a human cell line.

6. The cell line according to any one of claims 1 to 5, wherein the cell line is derived from a cell line, selected from the group consisting of Muscovy Duck cells (AGE.CR®), African green monkey kidney epithelial cells (Vero), Madin Darby canine kidney cells (MDCK), baby hamster kidney cells (BHK), Chinese hamster ovary (CHO) cells, human hepatocarcinoma cell lines (HepG2, Huh7), human embryonic kidney 293 (HEK293) cells, human neuronal precursor cells (AGE1.HN®, NC5T11), human *embryonic* retinoblast cells (Per.C6), myeloma cell lines (HMCLs, MM.1, U266, RPMI8226), CML tumor cell lines (NM, NM-F9), hybrid HEK293 and lymphoma cell (HKB11), and human amniocytes-derived CAP cells.

7. The cell line according to any one of claims 1 to 5, wherein the cell line is derived from human primary amniocytes comprising at least one nucleic acid encoding the gene products of the adenoviral E1 and pIX regions.

8. A recombinant glycoprotein having N-glycans with a 1.2-fold increase in the proportion of di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

9. The recombinant glycoprotein according to claim 8, wherein the proportion of tetra-antennary N-glycans is reduced by at least 80% when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV.

10. The recombinant glycoprotein according to claim 8 or claim 9, wherein at least 80% of the antennae of the N-glycans are terminated by 2,6-linked sialic acid.

11. The recombinant glycoprotein according to any one of claims 8 to 10, wherein the glycoprotein is selected from the group consisting of α1-antitrypsin (AAT), hepatocyte growth factor (HGF), Factor VII (FVII), Factor VIII (FVIII), Factor IX (FIX), von-Willebrand-Factor (vWF), and C1 esterase inhibitor (C1-inhibitor; C1 Inh).

12. The recombinant glycoprotein according to any one of claims 8 to 11, wherein the glycoprotein is a mammalian, preferably a human glycoprotein.

13. The recombinant glycoprotein according to any one of claims 8 to 12, wherein the glycoprotein has been expressed in a cell line according to any one of claims 1 to 7.

14. A method for the expression of a recombinant glycoprotein of interest in a cell, said glycoprotein having N-glycans with a significantly increased proportion of

di-antennary N-glycans when compared to the same recombinant glycoprotein expressed in a cell line with non-reduced expression of GnTIVa/b and/or GnTV, comprising the steps of:

(a) providing a cell line according to any one of claim 1 to 7;

(b) expressing the glycoprotein of interest in said cell line; and

(c) isolating the glycoprotein from the cell or the cell culture supernatant.

15. The method according to claim 14, wherein the recombinant glycoprotein is a recombinant glycoprotein according to any one of claims 8 to 13.