Improved Virus Neutralization by Plant-produced Anti-HIV Antibodies with a Homogeneous β1,4-Galactosylated N-Glycan Profile*[§]

Richard Strasser‡, Alexandra Castilho‡, Johannes Stadlmann‡, Renate Kunert‡, Heribert Quendler*,**, Pia Gattinger‡, Jakub Jez‡, Thomas Rademacher††, Friedrich Altmann, Lukas Mach‡, and Herta Steinkellner†††

From the ‡Department of Applied Genetics and Cell Biology, ‡‡Department of Chemistry, and ‡§Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, 1190 Vienna, Austria, ‡¶Polymun Scientific GmbH, 1190 Vienna, Austria, the **Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California 92037, and ‡‡‡Rheinisch-Westfälische Technische Hochschule, 52074 Aachen, Germany

It is well established that proper N-glycosylation significantly influences the efficacy of monoclonal antibodies (mAbs). However, the specific immunological relevance of individual mAb-associated N-glycan structures is currently largely unknown, because of the heterogeneous N-glycan profiles of mAbs when produced in mammalian cells. Here we report on the generation of a plant-based expression platform allowing the efficient production of mAbs with a homogeneous β1,4-galactosylated N-glycosylation structure, the major N-glycan species present on serum IgG. This was achieved by the expression of a highly active modified version of the human β1,4-galactosyltransferase in glycoengineered plants lacking plant-specific glycosylation. Moreover, we demonstrate that two anti-human immunodeficiency virus mAbs with full β1,4-galactosylated N-glycans display improved virus neutralization potency when compared with other glycoforms produced in plants and Chinese hamster ovary cells. These findings indicate that mAbs containing such homogeneous N-glycan structures should display improved *in vivo* activities. Our system, using expression of mAbs in tobacco plants engineered for post-translational protein processing, provides a new means of overcoming the two hurdles that limit the therapeutic use of anti-human immunodeficiency virus mAbs in global health initiatives, low biological potency and high production costs.

About 40 million people are estimated to be infected with HIV-1, and the HIV–1/AIDS epidemic continues to escalate, with the most devastating consequences seen in the most impoverished nations (1). Two strategies that have been pursued over the past 2 decades for stopping the AIDS pandemic/epidemic are the generation of vaccines to prevent HIV infection and the development of microbicides to prevent HIV transmission. Highly effective monoclonal antibodies (mAbs) are suitable to be used in both modalities. To date, only a handful of anti-HIV mAbs with neutralizing activities has been explored in more detail (2). In a recent clinical study, it has been demonstrated that a combination of three broadly neutralizing anti-HIV antibodies (2G12, 2F5, and 4E10) shows promise as AIDS treatment (3). However, despite effective *in vitro* neutralization activities, relatively modest *in vivo* effects were obtained, suggesting that the *in vivo* properties of these antibodies require further improvement (2). Noteworthy, these antibodies bind to HIV envelope proteins thus inhibiting viral entry into target cells (2, 4, 5). In addition to their potential use in therapeutic modalities, this renders them as promising candidates for microbicide development. However, high production costs using mammalian-cell technologies and insufficient efficacy of anti-HIV antibodies are remaining hurdles for their effective use. Among recent advances in generating antibodies with enhanced activities, glyco-engineering has been proven to be a powerful tool (6). It is well established that proper N-glycosylation significantly influences the efficacy of mAbs. Nevertheless, the specific immunological relevance of individual mAb-associated N-glycan structures is largely unknown, because of the heterogeneous N-glycan profiles of mAbs when produced in mammalian cells. A series of studies emphasize the critical role of IgG glycoforms lacking core α1,6-fucose for cell-mediated immunological activities (6). However, the immunological significance of N-glycans with terminal β1,4-galactose residues, the major N-glycan species present on serum IgG, has not yet been established.

During the last 2 decades, plants have been under intensive investigation to provide an alternative system for cost-effective, highly scalable, and safe production of recombinant proteins. This resulted in a significant enhancement of expression levels (up to 100-fold) and a reduction of production time (7, 8), which makes the system economically interesting. Another important achievement was the generation of plant glycosylation mutants, which allows a controlled human-type glycosylation of recombinant glycoproteins (9, 10). Recently, we have generated different glycoforms of anti-HIV mAb 2G12 in the

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‡ To whom correspondence should be addressed: Muthgasse 18, 1190 Vienna, Austria. Tel.: 43-1-36006-6700; Fax: 43-1-36006-6392; E-mail: herta.steinkellner@boku.ac.at.
§ The abbreviations used are: HIV–1, human immunodeficiency virus, type 1; HIV, human immunodeficiency virus; mAb, monoclonal antibody; CHO, Chinese hamster ovary cells; Gn, N-acetylglucosamine; ST, α2,6-sialyltransferase; GaIT, β1,4-galactosyltransferase; CTS, cytoplasmic–transmembrane–stem; HRP, horseradish peroxidase; RCA, *Ricinus communis* agglutinin; LC-ESI-MS, liquid chromatography–electrospray ionization–mass spectrometry; WT, wild type.

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tobacco-related plant species Nicotiana benthamiana (9). All of them were functionally active, and HIV neutralization potency was comparable with CHO-derived 2G12. This process involved the generation of a plant glycosylation mutant (ΔXT/FT), which was found to produce mAbs carrying homogeneous N-glycans with terminal N-acetylgalactosamine (Gn) residues (i.e. GnGn structures) lacking unwanted plant-specific β1,2-xylene and core α1,3-fucose residues. These glycans are devoid of any β1,4-linked galactose residues; thus in this study, we set out to glyco-engineer ΔXT/FT plants for quantitative β1,4-galactosylation. A highly active modified version of human β1,4-galactosyltransferase was used to transform ΔXT/FT and progeny screened for efficient protein β1,4-galactosylation. In total four glycoforms from the two anti-HIV mAbs 2G12 and 4E10 (plant- and CHO-derived) were generated and compared toward antigen binding and virus neutralization capacities.

EXPERIMENTAL PROCEDURES

Construction of Binary Constructs—For trans-Golgi targeting of human β1,4-galactosyltransferase (GalT), the N-terminal CTS domain was replaced with the CTS from the rat α2,6-sialyltransferase (ST, GenBank™ accession number M18769). For this, the catalytic domain of GaIT was first amplified from the vector pCDNA1- GT (11) with the primers tataCTTAGAcgccgtaccctgcgcgtct/tataGGATCCtcacgctgctgccatgtc and ligated to pPT2M (12). The CTS region of the ST was amplified from the pGAA482rST (13) with the primers tatATCTAGAtagctacattcaactggaatgtatGTTACCgccattctccgtcctgttgctccttgctc and subsequently ligated to the vector containing GaIT catalytic domain resulting in the vector ST-GalT. The generation of the binary vector used for transient expression of mAb 4E10 (pE40, Fig. 1) was generated according to 2G12 described by Schähs et al. (14).

Transformation and Screening of N. benthamiana—Leaf disk transformation of N. benthamiana using ST-GaIT was carried out by David M. Tricoli (Ralph M. Parsons Foundation Plant Transformation Facility, University of California, Davis). Putative transformed plantlets were selected on kanamycin-containing media, and genomic insertion of the plasmid was confirmed by PCR. Endogenous proteins were screened according to the presence of galactose by lectin blotting using Ricinus communis agglutinin (RCA) as described by Bakker et al. (15). ST-GaIT-transformed plants were crossed with ΔXT/FT, and the progeny thereof (GaIT⁺) was screened toward the presence of galactose (see above) and the absence of fucose and xylose by immunoblotting using rabbit anti-HRP antiserum (1:15,000; Sigma). For detection HRP-conjugated goat anti-rabbit IgG (1:100,000; Sigma) was used.

Agroinfiltration and Purification of 2G12 and 4E10—Agrobacterium strain GV3101:pMP90RK containing 2G12 and 4E10 binary vectors, respectively, was grown overnight (A600 1.0–1.5) in the presence of appropriate antibiotics, diluted to an A600 0.3, and infiltrated as described previously (9). 3 days post-infiltration of 2G12 and 4E10 were purified by protein A affinity as described recently (9).

4E10 and 2G12 Antigen Binding Assay—4E10 and 2G12 antigen binding specificity was carried out using HIV gp41-specific peptide (KKWNWFDETNWGGG) or gp60 as described recently (9, 14).

HIV Neutralization Assays—Neutralization assays were performed using either (i) AA-2 cells or (ii) TZM-bl target cells. (i) The AA-2 cell-based syncytium inhibition assay has been described previously (9). Briefly, 2-fold dilution series of antibodies were preincubated with virus at 10²–10⁵ 50% tissue culture infective dose (TCID₅₀)/ml for 1 h at 37 °C. CD4-positive human AA-2 cells were then added at a cell count of 4 × 10⁵ cells/ml and further incubated for 5 days. The read-out was performed according to the method of Reed and Muench (33), and the presence of at least one syncytium was scored positive. All assays were performed as duplicates. (ii) The single cycle TZM-bl neutralization assay has been described earlier (16). Pseudoviruses were generated by transfection of 293T cells by co-transfection of an env-mutated viral backbone and an env plasmid. 2-fold dilutions of antibody were preincubated with pseudoviruses for 1 h at 37 °C. TZM-bl cells that carry the luciferase reporter gene under control of the HIV Tat protein were then added at a cell count of 10⁵/ml. Two days after incubation, the cells were lysed, and luciferase activity was quantified by luminescence. Samples were run as duplicates.

N-Glycan Analysis of 4E10 and 2G12 by LC-ESI-MS—N-Glycan analyses of 4E10 and 2G12 were carried out by LC-ESI-MS of tryptic glycopeptides as described previously (9). Briefly, the heavy chain of SDS-PAGE-separated IgGs was excised from the gel, S-alkylated, digested with trypsin, and subsequently analyzed by LC-ESI-MS. Note during this procedure two glycopeptides are generated that differ in 482 Da. They are assigned as 1,4-Galactosylated mAb and amino acid N-glycans carrying xylose and core α1,3-fucose residues.

RESULTS

Generation of N. benthamiana Enabling Homogeneous IgG β1,4-Galactosylation—The rational design of the experiments is based on the observation that overexpression of mammalian β1,4-galactosyltransferase (GaIT) in tobacco causes the formation of largely incomplete processed N-glycan species (15, 18–20). We hypothesized improper sub-Golgi targeting of the mammalian enzyme allowing interference with endogenous N-glycan-processing enzymes. To overcome this shortcoming, a construct that targets GaIT to a late Golgi compartment, where the final steps of N-glycan processing occur, was designed. Thus, the N-terminal CTS region of GaIT that contains sub-Golgi targeting information was replaced by the ST-GaIT construct (Fig. 1) was used to transform N. benthamiana wild type. Transgenic lines were screened for the presence of galactosylated N-glycans using RCA blotting. A transgenic line that exhibits strong RCA staining was selected to transiently express mAbs (see below). The N-glycosylation profile of purified IgG was determined by ESI-time of flight/MS analysis and exhibited a quantitative portion of 1,4-linked galactose residues; thus in this study, we set to the presence of galactose by lectin blotting using Ricinus communis agglutinin (RCA) as described by Bakker et al. (15). ST-GaIT-transformed plants were crossed with ΔXT/FT, and the progeny thereof (GaIT⁺) was screened toward the presence of galactose (see above) and the absence of fucose and xylose by immunoblotting using rabbit anti-HRP antiserum (1:15,000; Sigma). For detection HRP-conjugated goat anti-rabbit IgG (1:100,000; Sigma) was used.

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N-Glycan Analysis of 4E10 and 2G12 by LC-ESI-MS—N-Glycan analyses of 4E10 and 2G12 were carried out by LC-ESI-MS of tryptic glycopeptides as described previously (9). Briefly, the heavy chain of SDS-PAGE-separated IgGs was excised from the gel, S-alkylated, digested with trypsin, and subsequently analyzed by LC-ESI-MS. Note during this procedure two glycopeptides are generated that differ in 482 Da. They are assigned as 1,4-Galactosylated mAb and amino acid N-glycans carrying xylose and core α1,3-fucose residues.

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(supplemental Fig. 1). Subsequently, the transgenic line was crossed with ΔXT/FT, and the progeny thereof was screened for the presence of galactose and the absence of plant-specific N-glycan residues (i.e. xylose and core α1,3-fucose) by lectin blots using RCA and immunoblotting using anti-xylose/fucose-specific antiserum (anti-HRP, Fig. 2). Plants selected in this manner (GalT⁺) were used to transiently express anti-HIV antibodies.

**Generation of 2G12 and 4E10 Glycoforms**—Anti-HIV mAbs 4E10 and 2G10 were transiently expressed in leaves of *N. benthamiana* WT, ΔXT/FT, and GalT⁺ using binary vectors that contain the respective cDNAs of heavy and light chains as described previously (Fig. 2) (9, 14). The N-glycosylation profile of purified IgGs were determined by ESI-time of flight/MS analysis and exhibited in each case the presence of a single major glycoforms as follows: GnGnXF (derived from WT), GnGn (ΔXT/FT), and fully galactosylated (AA) structures (GalT⁺). Most notably, galactosylation works very efficiently when ST-GalT is transiently co-expressed along with mAb constructs in ΔXT/FT. However, although AA represents the major glycoforms, to some extent incompletely processed and monoantennary galactosylated structures arise (Fig. 4). Notwithstanding this observation, galactosylated structures still represent about 80% of all glycoforms (Table 1). Our results demonstrate that the generation of fully β1,4-galactosylated mAbs is possible in glycoengineered plants as well as by transient expression of ST-GalT and emphasize the importance of sub-Golgi localization of glycosylation enzymes to obtain proper glycosylation.

**Functional Integrity of 2G12 and 4E10 Glycoforms**—To test the functional properties of the various 2G12 and 4E10 glycoforms, antigen-binding tests and HIV neutralization assays were carried out. Compared with CHO-derived 2G12 and 4E10, the antigen-binding capacity of plant-derived antibodies was 115–140% (Table 2) demonstrating that the binding was at least as efficient as that of the CHO-derived proteins. Finally, the ability of plant-derived mAbs to neutralize HIV-1 was examined using a syncytium inhibition assay (Table 2). Plant-derived GnGn and GnGnXF variants were found to be slightly better than CHO-derived 2G12 and 4E10. These results are in line with previously reported data where the virus neutralization potency of four different plant-derived 2G12 glycoforms was compared (GnGnXF, GnGnX, GnGnF, and GnGn, see Ref. 9). However, the neutralization activity of fully galactosylated (AA) variants of 2G12 and 4E10 was more than three times higher than that of other plant-derived glycoforms. To confirm these unexpected results, additional neutralization assays based on TZM-bl cells were performed using 2G12 glyco-forms and seven different HIV strains, including tier 1 and 2 strains (22). As in syncytium inhibition assays, AA glycoforms constantly exhibited a significantly increased potency to inhibit pseudovirus formation (Fig. 5). As the protein backbones of the respective mAb glycoforms are identical, our data indicate that these differences in function must be attributed to the altered glycosylation profile.

**DISCUSSION**

We report the generation of a plant-based expression platform that allows efficient expression of recombinant mAbs with quantitative β1,4-galactosylation (AA structures). This was achieved by addressing two pivotal factors that have not been considered previously: elimination of plant-specific N-glycosylation and targeting of GalT to a final stage of the glycosylation pathway. The presence of quantitative amounts of galactosylated structures in ST-GalT-transformed *N. benthamiana* wild type already indicated a highly active enzyme; however, the synthesis of major fractions of incompletely processed and mono-galactosylated structures indicated a severe interference with endogenous glycosylation enzymes (supplemental Fig. 1). This problem could be overcome by the use of ΔXT/FT that lacks plant-specific β1,2-xylo-
Homogeneous β1,4-Galactosylated mAb

WT

ΔXTFT

GalT+

CHO
Homogeneous β1,4-Galactosylated mAb

The presence of virtually exclusive and fully processed galactosylated structures on mAbs derived from GalT+ plants indicates that the rat-derived ST-CTS region contains an extremely efficient trans-Golgi targeting domain in plants. However, other CTS domains of plant glycosylation enzymes that act in the final stage of the pathway like β1,3-GaIT or α1,4-fucosyltransferase might be similarly effective (23, 24). Our results emphasize the importance of sub-Golgi localization of glycosylation enzymes to obtain proper glycosylation and indicate that spatial distribution is a driving force that directs N-glycan processing within the plant Golgi.

Here we show that fully galactosylated 2G12 and 4E10 neutralize HIV more efficiently in vitro compared with other glycoforms derived from plants and CHO cells. Notably, CHO-derived 2G12 as well as 4E10 carry some galactosylated structures (Fig. 3) (9, 17). However, most of them carry galactose moieties only on one branch (e.g. AGnF). Moreover, virtually all N-glycan species carry core α1,6-fucose residues, a glycan modification not produced in plants. Strategies have been developed to reduce/eliminate core α1,6-fucose in CHO cells (25, 26), and mAbs produced in such lines exhibit enhanced Fc-mediated effector functions. However, the lack of this N-glycan residue does not seem to improve virus neutralization activities as demonstrated in this study and previously (9). Our results indicate that AA structures favor the neutralization potency of antiviral mAbs. At the moment, the reason(s) for this unexpected observation are not clear; however, it is known from crystal and solution structural studies that even minor variations of terminal sugar residues affect antibody conformation, which may affect not only effector functions but also stability and in some cases antigen binding (27, 28). Another possible explanation for the observed increase in potency is that the glycosylation may cause the antibody to be adsorbed to the surface of the target cells used in our neutralization assays, thus pre-concentrating it at the spot of infection. However, additional intensive studies need to be carried out to elucidate the exact underlying mechanism(s) and to determine whether antibodies containing such a homogeneous galactosylated glycosylation display improved in vivo activities.

Expression of mAbs in wild type and glycoengineered N. benthamiana plants was achieved by agroinfiltration of binary vectors, allowing purification of functionally active mAbs 3 days post-inoculation. Notably, the entire procedure works equally well when the virus-based magnICON® expression system was used, resulting in yields of up to 500 μg of functional mAbs per kg of fresh leaf biomass (29). Additionally, a virtually unlimited number of different mAbs can be generated within 1 week post-DNA construct delivery, and the system is easily adaptable for the use of other expression vectors allowing high protein production (8). Interestingly, galactosylation works very efficiently when ST-GaIT is transiently expressed, indicating that time-consuming transformation procedures as needed for the generation of GalT+ plants may be dispensable. The occurrence of incompletely processed

syltransferase and core α1,3-fucosyltransferase activities. The presence of virtually exclusive and fully processed galactosylated structures on mAbs derived from GaIT plants indicates that the rat-derived ST-CTS region contains an extremely efficient trans-Golgi targeting domain in plants. However, other CTS domains of plant glycosylation enzymes that act in the

TABLE 1
Relative abundance of glycoforms (in %) on 4E10 derived from three different co-infiltration experiments with ST-GaIT in ΔXT/FT (numbers 1–3)

Data were deduced from MS analyses as illustrated in Fig. 3. Σ minor indicates a summary of all other minor glycoforms present on 4E10. A detailed description of CHO-4E10 glycan composition is given in Ref. 17.

| Glycoforms | Relative abundance |
|------------|-------------------|
|            | No. 1 | No. 2 | No. 3 | Mean |
| %          | %     | %     | %     |
| AA         | 37    | 54    | 46    | 46   |
| AGn        | 14    | 6     | 7     | 9    |
| AM         | 26    | 27    | 17    | 23   |
| GnM        | 19    | 13    | 24    | 18   |
| Σ minor    | 5     | 1     | 6     | 4    |

TABLE 2
Functional properties of 2G12 and 4E10 glycoforms

In vitro HIV-1 neutralization activity was determined by a syncytium inhibition assay using AA-2 cells as described previously (9). Laboratory strains SF2 and RF (both clade B) were used for neutralization tests of 4E10 and 2G12, respectively. 2G12 and 4E10 antigen (Ag)-binding tests were carried out by enzyme-linked immunosorbent assay using recombinant gp160- and a gp41-derived peptide (KKWNWFDETGWGGG) as antigens, respectively (4). The two virus neutralization experiments shown were performed using different batches of 2G12 and 4E10, respectively.

| Glycoforms | AG-binding | IC50 μg/ml | IC50 μg/ml | Average IC50 μg/ml |
|------------|------------|------------|------------|-------------------|
|            | %          | μg/ml      | μg/ml      | μg/ml             |
| 2G12       |            |            |            |                   |
| GnGnXF     | 117        | 3.71       | 1.30       | 2.50              |
| GnGn       | 115        | 4.29       | 1.90       | 3.30              |
| AA         | 138        | 0.93       | 0.26       | 0.59              |
| CHO        | 100        | 7.40       | 4.40       | 5.90              |
| 4E10       |            |            |            |                   |
| GnGnXF     | 120        | 0.55       | 0.70       | 0.62              |
| GnGn       | 125        | 0.83       | 0.83       | 0.83              |
| AA         | 140        | 0.15       | 0.22       | 0.18              |
| CHO        | 100        | 0.93       | 0.90       | 0.91              |

H. Steinkellner, unpublished results.
$N$-glycan structures may be due to differences in the expression levels of ST-GalT as a consequence of transient expression. This problem may be overcome by optimization and standardization of the transient expression system. (Note that incompletely processed and monoantennary galactosylated $N$-glycans were also observed in stable transformed GalT$^+$ plants. However, these transformants were omitted during the screening procedure.) These facts in combination with simple handling and maintenance conditions of (glycoengineered) plants provide a significant advantage over existing glyco-modified expression platforms, including yeast and mammalian cells. Notably, homogeneous $\beta$1,4-galactosylation of mAbs is not achieved by any state-of-the-art mammalian cell-based expression system.

There are over 20 anti-retroviral drugs available, and recent in vivo studies have raised the question of the requirements for an effective drug to slow down the HIV/AIDS epidemic. Evidence has accumulated that mAb-based HIV inactivation may be crucially dependent on a combination of humoral and cell-mediated immunity (2, 30). The rapid production of different variants of anti-HIV mAbs as described here will help to better understand the underlying neutralization mechanisms and will add to the antiviral strategies under development. Additionally, protein-based molecules highly specific for HIV hold great potential for microbicide development (31, 32), and monoclonal antibodies with improved activities as described in this work render them promising microbicide candidates. Our results highlight the potential of glycoengineered plants as a cost-effective expression platform for mAbs and other therapeutically relevant recombinant proteins with defined glycosylation profiles of great uniformity, thus providing a new means of overcoming the dual obstacles of higher activity and cost reduction in protein manufacturing.

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