Glycan modulation and sulfoengineering of anti–HIV-1 monoclonal antibody PG9 in plants

Andreas Loos[a], Johannes S. Gach[b], Thomas Hackl[a], Daniel Maresch[c], Theresa Henkel[a], Andreas Porodko[a], Duc Bui-Minh[a], Wolfgang Sommeregger[d], Gordana Wozniak-Knopp[d], Donald N. Forthal[b], Friedrich Altmann[c], Herta Steinkellner[a,1], and Lukas Mach[a,1]

[a]Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, A-1190 Vienna, Austria; [b]Division of Infectious Diseases, University of California, Irvine, CA 92697; [c]Department of Chemistry, University of Natural Resources and Life Sciences, A-1190 Vienna, Austria; and [d]Department of Biotechnology, University of Natural Resources and Life Sciences, A-1190 Vienna, Austria

Edited by C. Thomas Caskey, Baylor College of Medicine, Houston, TX, and approved August 7, 2015 (received for review May 8, 2015)

Broadly neutralizing anti–HIV-1 monoclonal antibodies, such as PG9, and its derivative RSH hold great promise in AIDS therapy and prevention. An important feature related to the exceptional efficacy of PG9 and RSH is the presence of sulfated tyrosine residues in their antigen-binding regions. To maximize antibody functionalities, we have now produced glycan-optimized, fucose-free versions of PG9 and RSH in *Nicotiana benthamiana*. Both antibodies were efficiently sulfated in planta on coexpression of an engineered human tyrosylprotein sulfotransferase, resulting in antigen-binding and virus neutralization activities equivalent to PG9 synthesized by mammalian cells (^{CHO}PG9). Based on the controlled production of both sulfated and nonsulfated variants in plants, we could unequivocally prove that tyrosine sulfation is critical for the potency of PG9 and RSH. Moreover, the fucose-free antibodies generated in *N. benthamiana* are capable of inducing antibody-dependent cellular cytotoxicity, an activity not observed for ^{CHO}PG9. Thus, tailoring of the antigen-binding site combined with glycan modulation and sulfoengineering yielded plant-produced anti–HIV-1 antibodies with effector functions superior to PG9 made in CHO cells.

antibody | biopharmaceutical | glycosylation | plant | sulfation

Monoclonal antibodies (mAbs) offer great promise for AIDS treatment (1). In particular, the recent discovery of broadly neutralizing anti–HIV-1 mAbs (bNAbs) with extraordinary potency as exemplified by the antibodies PG9, PG16 (2), or those of the PGT series (3) creates hope for effective therapy by passive antibody transfer. PG9 and its close relative PG16 neutralize ∼80% of HIV-1 isolates across all clades (2, 4). The recognized epitopes are within the hypervariable and heavily glycosylated V1/V2 loops of the viral envelope glycoprotein gp120 and preferentially displayed in its trimeric state (2). Both mAbs use their unusually long complementarity-determining region (CDR) H3 domains (4–6) to penetrate the glycan shield of the virus and make contact with the underlying protein backbone (7). In addition, PG9 and PG16 recognize two highly conserved gp120 *N*-glycans attached to Asn^{160} and $Asn^{156/173}$, which flank the peptide epitope (7–9). Remarkably, the glycan-binding properties of the two antibodies could be combined by modification of the PG9 light chain with $R^{L94}SH^{L95A}$ as found in PG16. This PG9 variant (here termed RSH) has a superior neutralization capacity and broader coverage of HIV-1 isolates than either wild-type PG9 or PG16, which makes it an excellent choice for additional drug development studies (7).

Proper *N*-glycosylation is important for aspects of mAb functionality, because the oligosaccharides attached to Asn^{297} of the crystallizable fragment (Fc) are known to strongly affect binding to cellular Fc receptors and thus, in vivo functionalities (10). In particular, core α1,6-fucosylation, typically found on mAbs produced in mammalian cell lines, has been shown to hinder antibody-dependent, cell-mediated cytotoxicity (ADCC) and antibody-dependent, cell-mediated virus inactivation (11), key effector functions in the context of anti–HIV-1 immune responses (12–14). Hence, considerable efforts have been undertaken to establish mAb production systems generating human-type *N*-glycans lacking this modification. Plants, particularly *Nicotiana benthamiana*, are well-suited for glycan engineering processes. The advantages of plant-based expression platforms include a high extent of glycan homogeneity, the flexibility with which glycosylation can be modulated, high production speed, and ease of large-scale production (15). The superior efficiency of glycocengineered mAbs produced in plants has recently been highlighted by ZMapp, an mAb mixture developed for the treatment of Ebola patients (16). Similarly, improved effector potency has been observed for plant-made anti–HIV-1 bNAb 2G12 (17), rendering glycoengineered plants an interesting production system for mAbs.

Another posttranslational modification, namely tyrosine sulfation of selected residues in the CDR H3 region of PG9 and PG16, has recently been shown to be critical for high-affinity interactions with their antigen (4, 6). In humans, tyrosine sulfation is carried out by two closely related type II transmembrane proteins: tyrosylprotein sulfotransferase 1 (TPST1) and TPST2 (reviewed in refs. 18 and 19). Although plants contain TPSTs, these proteins are phylogenetically unrelated to the human enzymes and could, therefore, exhibit different enzymatic properties (18, 20). Previous attempts to produce bioactive PG9 and PG16 in *N. benthamiana* have failed, possibly because of deficient tyrosine sulfation (21). Hence, it is currently uncertain whether plant-based expression

Significance

The broadly neutralizing anti–HIV-1 monoclonal antibody (mAb) PG9 requires multiple posttranslational modifications to exhibit its full biological activity, including proper *N*-glycosylation and tyrosine sulfation. We now describe a technology that permits the controlled synthesis of these modifications in *Nicotiana benthamiana*. This technology allowed us to show that sulfated PG9 neutralizes HIV-1 with much higher potency than unsulfated antibody. We also found that glycooptimized mAb versions made in plants are superior to PG9 produced in mammalian cells with respect to mediating antibody-dependent cellular cytotoxicity, an important mAb effector function. Because effector functions are of key importance for antibody-mediated immune control of HIV-1 infections, our results can instruct the development of improved immunotherapeutics for the treatment of AIDS patients.

Author contributions: A.L., H.S., and L.M. designed research; A.L., J.S.G., T. Hackl, D.M., T. Henkel, A.P., D.B.-M., W.S., and G.W.-K. performed research; A.L., J.S.G., D.M., D.N.F., F.A., H.S., and L.M. analyzed data; and A.L., H.S., and L.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence may be addressed. Email: herta.steinkellner@boku.ac.at or lukas.mach@boku.ac.at.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1509090112/-/DCSupplemental.

Table 1. Tyrosine sulfation of plant-produced PG9 and RSH

Sulfates	^{CHO}PG9	$^{\Delta XF}$PG9	$^{\Delta XF}$PG9$_{Sulf}$	$^{\Delta XF}$PG9$_{SulfSia}$	$^{\Delta XF}$RSH	$^{\Delta XF}$RSH$_{Sulf}$	$^{\Delta XF}$RSH$_{SulfSia}$
0	19	>98	49	43	>98	53	49
1	60	<1	33	34	<1	31	34
2	21	<1	18	23	<1	17	17
1 or 2	82	<2	51	57	<2	47	51

Relative amounts of unsulfated, singly sulfated, and doubly sulfated PG9 and RSH when coexpressed with p^{RST}TPST1 in plants. The sulfation status of ^{CHO}PG9 is shown for comparison.

platforms are naturally capable of sulfating tyrosine residues in recombinant proteins.

Here, we aimed to maximize the potency of bNAbs against HIV-1 using a plant-based expression system. For this goal, PG9 and RSH were expressed in a xylosyltransferase (XT)- and fucosyltransferase (FT)-deficient *N. benthamiana* mutant (ΔXT/FT) supporting the synthesis of glycan-optimized, fucose-free mAbs (15). Whereas tyrosine sulfation of PG9 by endogenous plant enzymes was barely detectable, this additional posttranslational modification was efficiently introduced by coexpression of human TPST1 (hsTPST1) modified with a plant Golgi-targeting sequence. When sulfated, plant-derived PG9 had essentially the same antigen-binding and virus neutralization properties as its counterpart produced in CHO cells. Importantly, ADCC activity was displayed by fucose-free, plant-produced mAbs but not by CHO-derived PG9. Furthermore, the controlled production of both sulfated and unmodified PG9 in the same expression system enabled us to establish the impact of tyrosine sulfation on the functionality of this important bNAb.

Results

Coexpression of TPST1 Enables the in Planta Production of Sulfated PG9. We expressed PG9 and its derivative RSH in ΔXT/FT *N. benthamiana* plants that have been glycoengineered to remove the plant-typical *N*-glycan residues β1,2-xylose and core α1,3-fucose ($^{\Delta XF}$PG9 and $^{\Delta XF}$RSH) (22, 23). RSH differs from PG9 by three amino acids in the light chain (PG9: T^{94}RR^{95A}; RSH: R^{94}SH^{95A}). MS analysis of CDR H3 peptides did not provide evidence for sulfation of PG9 and RSH by endogenous plant enzymes, whereas a high degree (82%) of CHO-derived PG9 (^{CHO}PG9) was sulfated (Table 1). Although a functional TPST has been described in *Arabidopsis thaliana* (20), we could not retrieve a TPST candidate from the *N. benthamiana* draft genome (24). Because previous reports showed that inefficient tyrosine sulfation of PG9 by HEK293 cells can be rescued by TPST overexpression (6), PG9 was coexpressed with hsTPST1 in ΔXT/FT plants. To mediate proper targeting to sub-Golgi compartments, three constructs carrying different cytoplasmic tail, transmembrane domain, and stem (CTS) regions were tested. Expression of hsTPST1 combined with its native CTS region (p^{Full}hsTPST1) led to 15–20% sulfated $^{\Delta XF}$PG9 (Table S1). Interestingly, replacement of the CTS region with the corresponding domain of glycosylation enzymes known to be targeted to the medial/trans region of the plant Golgi (p^{Fut11}hsTPST1 and p^{RST}hsTPST1) led to the production of $^{\Delta XF}$PG9$_{Sulf}$ and $^{\Delta XF}$RSH$_{Sulf}$ with substantially higher levels of sulfation, almost reaching the extent of tyrosine sulfation observed for ^{CHO}PG9. Using p^{RST}hsTPST1, up to 57% of plant-produced mAbs were monosulfated or disulfated (Table 1 and Tables S1 and S2).

Tyrosine Sulfation of PG9 Produced in Plants and CHO Cells Occurs at the Same Positions. Sulfation of PG9 at specific tyrosine residues is believed to be important for high-affinity antigen binding. Thus, we set out to establish which tyrosines are sulfated in hsTPST1-expressing plants. The tryptic CDR H3 peptide used for analyzing the PG9 sulfation status by liquid chromatography-electrospray ionization (LC-ESI) MS (N^{100C}GYNYYDFYDGYYNYHYMDVWGK^{105}) contains several tyrosine residues that are potential TPST targets. Additional digestion by AspN cleaved the peptide into three parts. In ^{CHO}PG9 as well as sulfated PG9 and

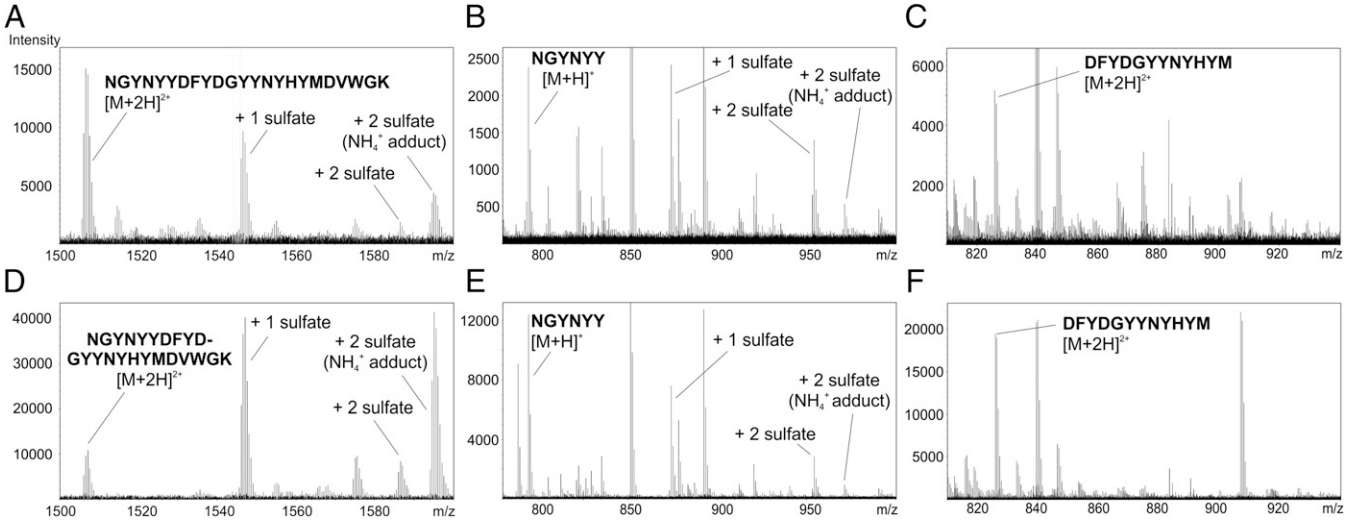

Fig. 1. $^{\Delta XF}$PG9$_{Sulf}$ and ^{CHO}PG9 are singly and doubly sulfated in the region N^{100C}GYNYY^{100H}. The sulfation sites of (*A*) $^{\Delta XF}$PG9$_{Sulf}$ and (*D*) ^{CHO}PG9 were mapped by liquid chromatography-electrospray ionization MS to the tryptic PG9 peptide N^{100C}GYNYYDFYDGYYNYHYMDVWGK^{105}. Additional digestion with AspN revealed nonsulfated, singly sulfated, and doubly sulfated variants of the peptide N^{100C}GYNYY^{100H} [(*B*) $^{\Delta XF}$PG9$_{Sulf}$ and (*E*) ^{CHO}PG9]. No sulfated residues were found on the other tyrosine-containing AspN fragment: D^{100I}FYDGYYNYHYM^{100T} [(*C*) $^{\Delta XF}$PG9 and (*F*) ^{CHO}PG9].

Table 2. *N*-glycosylation of PG9 and RSH expressed in plants and CHO cells

Glycans	$^{\Delta XF}$PG9	$^{\Delta XF}$PG9$_{Sulf}$	$^{\Delta XF}$PG9$_{SulfSia}$	$^{\Delta XF}$RSH	$^{\Delta XF}$RSH$_{Sulf}$	$^{\Delta XF}$RSH$_{SulfSia}$	^{CHO}PG9
Gn	50	56	19	44	60	20	79
Gal	1	1	30	2	1	39	21
Sia	0	0	6	0	0	13	0
Man5-9	50	43	45	55	39	28	1
Fuc	1	2	3	1	1	5	96

N-glycan acronyms are based on the ProGlycAn nomenclature (www.proglycan.com). Fuc, sum of core-fucosylated *N*-glycans; Gn/Gal/Sia, sum of complex *N*-glycans with terminal GlcNAc/galactose/sialic acid residues; Man5-9, sum of oligomannosidic *N*-glycans with five to nine mannose residues.

RSH produced in plants, only one of three fragments was found to be singly or doubly sulfated (N^{100C}GYNYY^{100H}), whereas the other two (D^{100I}FYDGYYNYHYM^{100T} and D^{101}WGK^{105}) were not modified (Fig. 1). This finding indicates that the sulfate groups are attached to Y^{100E}, Y^{100G}, and/or Y^{100H} independent of the expression platform used for PG9 production. It has been shown previously by X-ray crystallography that Y^{100G} and Y^{100H} of PG9 produced in mammalian cells can be sulfated (6). Hence, it is likely that hsTPST1 also modifies the same tyrosine residues in planta.

PG9 Carries Human-Type *N*-Glycans When Expressed in Glycoengineered Plants. Because *N*-glycans are known to modulate binding to cellular Fc receptors (10), several glycoforms of PG9 and RSH were produced. MS *N*-glycan analysis of $^{\Delta XF}$PG9, $^{\Delta XF}$PG9$_{Sulf}$, $^{\Delta XF}$RSH, and $^{\Delta XF}$RSH$_{Sulf}$ revealed the presence of a predominant complex *N*-glycan species, G0 (GnGn) (Table 2 and Table S2). This glycoform accounted for roughly 45–50% of all *N*-glycan species. On coexpression of PG9 and RSH with mammalian genes necessary for terminal galactosylation and sialylation in planta (25) resulting in the synthesis of $^{\Delta XF}$PG9$_{SulfSia}$ and $^{\Delta XF}$RSH$_{SulfSia}$, the *N*-glycosylation profiles shifted to 30–40% galactosylated and 6–12% sialylated oligosaccharides, with G0 reduced to 15–20% (Table 2). Importantly, core α1,3-fucose and β1,2-xylose residues were hardly detectable in plant-produced mAbs (below 5%). By contrast, ^{CHO}PG9 carried mainly α1,6-fucosylated *N*-glycans (more than 95%), with G0F^6 (GnGnF^6) being the most prevalent structure (70%). Roughly 20% of ^{CHO}PG9 was galactosylated, and less than 1% was sialylated (Table 2).

Antigen Binding by $^{\Delta XF}$PG9 Is Enhanced by Tyrosine Sulfation. All six PG9 variants produced in plants ($^{\Delta XF}$PG9, $^{\Delta XF}$PG9$_{Sulf}$, $^{\Delta XF}$PG9$_{SulfSia}$, $^{\Delta XF}$RSH, $^{\Delta XF}$RSH$_{Sulf}$, and $^{\Delta XF}$RSH$_{SulfSia}$) could be purified from leaf extracts in good yields. When analyzed by SDS/PAGE under reducing conditions, the heavy and light chains of the plant-made mAbs showed the expected migration pattern, with the light chains displaying higher electrophoretic mobilities than those of ^{CHO}PG9 (Fig. S1). This difference is due to the removal of a functionally unnecessary *N*-glycosylation site in the light chain of the latter antibody (4), which could accelerate its clearance from the circulation (21). Under nonreducing conditions, plant- and CHO-derived PG9 and RSH variants yielded single bands migrating at the same position (Fig. S1).

PG9 has been described to bind with high affinity to trimeric envelope glycoproteins of a wide variety of HIV-1 isolates, and also to gp120 monomers of selected HIV-1 strains, including ZM109 (2, 6). To investigate the antigen-binding properties of plant-derived PG9 and RSH variants compared with ^{CHO}PG9, we expressed gp120^{ZM109} containing a C-terminal hexahistidine tag in FreeStyle 293 (FS293) cells and purified it to apparent homogeneity. SDS/PAGE revealed a diffuse band as expected for a heavily glycosylated protein (Fig. S2). *N*-glycosylation of two gp120 asparagines (Asn^{160} and Asn^{173}) has been shown to be important for PG9 binding (2, 6). Glycosylation analysis by MS revealed mainly Man5 structures on either of these *N*-glycosylation sites (Fig. S3 and Table S3). Importantly, PG9 is known to prefer such *N*-glycans on Asn^{160}, while tolerating them on Asn^{173}. Only minor amounts of other *N*-glycans were detected on either site, showing that FS293-derived gp120^{ZM109} meets the prerequisites for a high-affinity PG9 ligand.

Binding of the different PG9 and RSH variants to monomeric gp120^{ZM109} and trimeric gp140$^{BG505.SOSIP.664}$ (26) was tested by ELISA (Table 3). Although unsulfated $^{\Delta XF}$PG9 and $^{\Delta XF}$RSH had substantially lower affinity to either antigen than ^{CHO}PG9, sulfation increased their affinities 10–16 times for trimeric gp140$^{BG505.SOSIP.664}$ and 2–5 times for monomeric gp120^{ZM109}. Thus, sulfated PG9 produced in plants displays a similar affinity to its antigens as ^{CHO}PG9. RSH showed up to threefold better binding than PG9 to either gp120 or gp140. The different glycoforms of each mAb showed comparable EC_{50} values.

The avidity of the antigen–antibody interaction was also determined by biolayer interferometry (Table 4). $^{\Delta XF}$PG9$_{Sulf}$, $^{\Delta XF}$RSH$_{Sulf}$, and ^{CHO}PG9 showed roughly the same affinity for gp120^{ZM109} (K_d values of 525, 605, and 756 nM, respectively), whereas unsulfated $^{\Delta XF}$RSH exhibited a roughly fourfold lower affinity (K_d value of 2,510 nM). The affinity of unsulfated PG9 for gp120^{ZM109} was too low to be accurately determined under the experimental conditions used (K_d > 3 μM). Overall, these results confirm that tyrosine sulfation increases the affinity of both antibodies.

Increased Virus Neutralization by Sulfated PG9 and RSH Variants. Neutralization efficiencies of the antibodies were tested on a panel of HIV-1 clade B and clade C pseudoviruses (Table 5 and Table S4). The viruses were chosen based on previously published data regarding their susceptibility to PG9 and RSH produced in mammalian cells (2, 6, 7) ranging from well-neutralized to resistant isolates. As expected, a number of pseudoviruses was not neutralized under the tested conditions (JRFL, ZM214M, PVO, and TRO.11), whereas neutralization of others was intermediate (ADA, YU2, and MN), good (DU156.12, DU422.1, and ZM109), or very efficient (JRCSF and CAP45). Interestingly,

Table 3. Sulfation enhances antigen binding of plant-derived PG9 and RSH

Antibody	EC_{50} (ng/mL) gp120	gp140
^{CHO}PG9	89 ± 2	290 ± 85
$^{\Delta XF}$PG9	421 ± 42	4,870 ± 1105
$^{\Delta XF}$PG9$_{Sulf}$	92 ± 9	490 ± 170
$^{\Delta XF}$PG9$_{SulfSia}$	101 ± 7	310 ± 30
$^{\Delta XF}$RSH	179 ± 49	2,230 ± 115
$^{\Delta XF}$RSH$_{Sulf}$	82 ± 19	180 ± 5
$^{\Delta XF}$RSH$_{SulfSia}$	73 ± 13	180 ± 25

Binding of PG9 and RSH to immobilized monomeric gp120^{ZM109} or trimeric gp140$^{BG505.SOSIP.664}$ was measured by ELISA. Data are presented as means ± SEM of two or three independent experiments.

APPLIED BIOLOGICAL SCIENCES

Table 4. Affinities of PG9 and RSH for gp120^{ZM109}

Antibody	K_d (nM)
^{CHO}PG9	756 ± 163
$^{\Delta XF}$PG9$_{Sulf}$	525 ± 75
$^{\Delta XF}$RSH$_{Sulf}$	605 ± 138
$^{\Delta XF}$RSH	2,510 ± 39
$^{\Delta XF}$PG9	>3,000

Biolayer interferometry data are presented as means ± SEM of two ($^{\Delta XF}$RSH) or four to six individual determinations. The binding of $^{\Delta XF}$PG9 to gp120^{ZM109} was too weak for accurate determination of K_d under the experimental conditions used.

PG9 variants displayed pronounced differences with respect to their neutralization efficiencies. In accordance with the results of the antigen-binding assays, tyrosine sulfation strongly enhanced neutralization of highly sensitive isolates (50-fold and more; e.g., JRCSF, DU156.12, ZM109, CAP45, and DU422.1), whereas only a modest improvement was observed for more resistant strains (up to 1.5-fold; ADA, YU2, and MN). These data provide unprecedented quantitative evidence for the pivotal role of CDR H3 sulfotyrosines in effective HIV-1 neutralization by PG9 as previously proposed based on the tertiary structure of the PG9/gp120 complex (6). In general, the varying sensitivities of the tested HIV-1 strains to PG9 and RSH were in good agreement with the presence or absence of PG9-interacting residues in their gp120 V2 sequences (Fig. S4). The increase in neutralization efficiency observed for RSH is in good agreement with results obtained in antigen-binding assays. Notably, glycoengineering of plant-derived PG9 and RSH did not affect virus neutralization.

Plant-Derived PG9 and RSH Are Capable of Mediating Antibody-Dependent Cellular Cytotoxicity. Finally, the capability of PG9 and RSH to elicit ADCC was evaluated. This effector function is suggested to have an important role in the control of HIV-1 viral load and infection (12–14), although its significance in virus clearance in vivo remains controversial, because not all bNAbs against HIV-1 mediate ADCC (27). The latter also applies to ^{CHO}PG9, which did not support ADCC activity of human peripheral blood mononuclear cells against target cells inoculated with a PG9-sensitive strain of HIV-1 (JRCSF). In contrast, plant-derived PG9 and RSH induced a potent ADCC response in a concentration-dependent manner (Fig. 2). This difference can be explained by the absence of core fucose residues in the *N*-glycans of the plant-produced mAbs, which confers an up to 100-fold increase in ADCC potency (11).

Discussion

In this study, we aimed to optimize the potency of anti–HIV-1 bNAb PG9 by combining CDR engineering with the modulation of different posttranslational modifications (i.e., glycosylation and tyrosine sulfation). Sulfation of specific tyrosine residues of PG9 and its CDR-engineered variant RSH is considered important for high-affinity binding to antigens. Although some plants have a TPST and can sulfate phytohormones (20), PG9 expressed in *N. benthamiana* did not contain detectable amounts of sulfotyrosines, indicating that mammalian-type sulfation does not occur naturally in *N. benthamiana* leaves. Overexpression of the hsTPSTs hsTPST1 and hsTPST2 (18) increased sulfation of recombinantly produced proteins in mammalian cells (6, 28). However, expression of full-length hsTPST1 in *N. benthamiana* did not yield efficient levels of PG9 tyrosine sulfation. We and others have shown previously that engineering of posttranslational modifications in plants by overexpression of human enzymes necessitates targeting of the respective enzyme to its correct subcellular location (29). Indeed, on replacing the authentic CTS sequence of hsTPST1 with a plant CTS region known to target proteins to late Golgi compartments, a marked increase in sulfation efficiency was observed. This improvement suggests that the native CTS of hsTPST1 is not capable of mediating the efficient delivery of the enzyme to its proper intracellular destination in plant cells.

The crystal structure of PG9 in complex with its antigen has revealed that Y^{100G} and Y^{100H} of the PG9 heavy chain can be sulfated (4, 6). The functional relevance of this posttranslational modification has been largely inferred from structural studies and a comprehensive mutational assessment of the antibody's CDR H3 region. Replacement of Y^{100G} and Y^{100H} with alanine or phenylalanine, respectively, resulted in substantially weaker antigen binding and HIV-1 neutralization. However, the functionality of PG9 was also reduced by mutagenesis of other CDR H3 tyrosines (4). We could map the sulfation sites of ^{CHO}PG9 and plant-produced PG9/RSH by MS to a short CDR H3 peptide containing three tyrosine residues (Y^{100E}, Y^{100G}, and Y^{100H}). Sulfation of plant-produced PG9 and RSH enhanced antigen binding and virus neutralization, indicating that, in plants as well, Y^{100G} and Y^{100H} are the sulfated residues. Although the impact of tyrosine sulfation on neutralization efficiency was previously only assessed for singly and doubly sulfated PG9 (4), we now compared sulfated and unsulfated PG9/RSH and observed a far more pronounced difference in antiviral potency. Taken together, these results show that singly sulfated PG9 binds and neutralizes HIV-1 better than nonsulfated antibody and that the doubly sulfated mAb displays even further enhanced binding. Interestingly, the effect of tyrosine sulfation on antigen binding was more pronounced for trimeric gp140 than for monomeric gp120.

Table 5. Neutralization efficiencies of PG9 and RSH against a panel of pseudoviruses

Strain	^{CHO}PG9	$^{\Delta XF}$PG9	$^{\Delta XF}$PG9$_{Sulf}$	$^{\Delta XF}$PG9$_{SulfSia}$	$^{\Delta XF}$RSH	$^{\Delta XF}$RSH$_{Sulf}$	$^{\Delta XF}$RSH$_{SulfSia}$
JRFL	>50	>50	>50	>50	>50	>50	>50
PVO	>50	>50	>50	>50	>50	>50	>50
TRO.11	>50	>50	>50	>50	>50	>50	>50
ZM214M	>50	>50	>50	>50	>50	>50	>50
YU2	>50	>50	>50	>50	42.47	34.75	26.08
MN	>50	>50	>50	>50	43.70	30.77	36.14
ADA	42.81	>50	37.36	40.17	26.15	19.58	20.06
DU422.1	10.93	>50	14.28	6.60	>50	9.72	8.45
ZM109F	0.78	>50	1.66	1.31	>50	1.46	1.38
DU156.12	0.35	>50	1.20	0.90	33.85	0.56	0.56
CAP45	<0.02	0.65	0.03	<0.02	0.25	<0.02	<0.02
JRCSF	<0.02	0.19	<0.02	0.03	0.06	<0.02	<0.02

IC_{50} values (in micrograms per milliliter) are color-coded (green, <1 μg/mL; orange, 10–50 μg/mL; red, >50 μg/mL; yellow, 1–10 μg/mL).

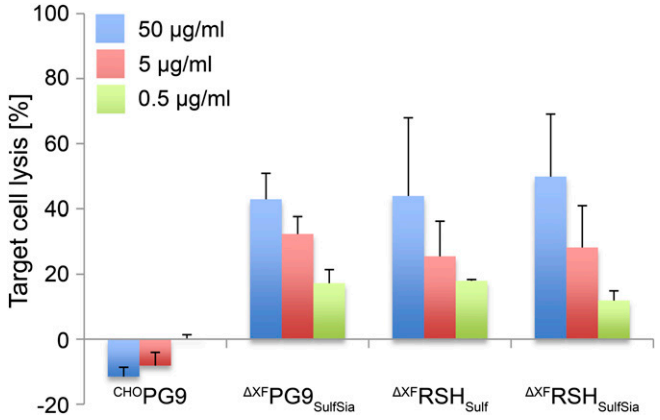

Fig. 2. Plant-derived PG9 and RSH variants are capable of inducing ADCC. ADCC assays were performed in the presence of the indicated PG9 or RSH concentrations. Data were corrected for the extent of nonspecific cell lysis observed in the presence of a control antibody and expressed as means ± SEM of three independent experiments (each performed with effector cells isolated from a different donor).

A dependence on sulfated tyrosines was also observed for other anti–HIV-1 mAbs binding to the V1/V2 loops or the coreceptor binding site (30). This finding indicates that tyrosine sulfation is a critical posttranslational modification common to many of these antibodies (5, 30). It is of note that tyrosine sulfation also affects the antigenicity of gp120 itself. It has been reported that gp120 from $CD4^+$ T cell-produced virions is more extensively sulfated than cell line-produced gp120 (28) and that V2 needs to be sulfated to stabilize V3. Thus, efficient tyrosine sulfation of gp120 vaccine candidates might be crucial for eliciting a sustained antibody response. Other biotherapeutic candidates also depend on sulfation for optimal activity (31, 32). Hence, the procedures developed for human-type protein sulfation in *N. benthamiana* further widen the scope of this powerful expression system.

We also intended to improve the in vivo efficacies of PG9 and RSH by means of glycoengineering. In IgGs, elimination of core fucose from the conserved Fc glycans strongly increases Fc-mediated effector functions, like ADCC or antibody-dependent, cell-mediated virus inactivation (11, 17, 33). These effector functions seem to play an important role in HIV-1 control by the immune system (12–14). For this reason, we produced mAbs decorated with fucose-free, human-type *N*-glycans (22). Such structures are considered "glycan-optimized," and they are the basis for next generation antibodies in cancer therapies (34) and other indications (16). As expected, PG9 and RSH produced in ΔXT/FT plants contained, in all cases, less than 5% fucosylated glycans. By contrast, ^{CHO}PG9 was highly fucosylated (>95%), which is typical for CHO-produced mAbs. The elimination of core fucose residues clearly improves the potential of the plant-derived antibodies to mediate ADCC. However, it should be noted that the significance of ADCC in HIV-1 clearance by bNAbs in vivo is still a controversial issue. Although a non-fucosylated version of the anti–HIV-1 mAb b12 was more effective in ;inducing the ADCC-dependent killing of virus-infected cells in vitro, it did not improve protection against vaginal challenge with a hybrid simian–human immunodeficiency virus in macaques (35). Nevertheless, it is now well-established that Fc effector functions are of pivotal importance for the therapeutic activity of bNAbs against HIV-1 (36, 37).

It cannot be excluded that even the low residual levels of nonmammalian glycans (less than 5%) present on plant-derived PG9 and RSH could lead to adverse immune reactions on regular treatment of humans with therapeutically relevant doses of these bNAbs (10–30 mg/kg) (38). However, biweekly infusions of Gaucher disease patients with up to 2 mg/kg human glucocerebrosidase produced in carrot cells have proven clinically safe over a period of 9–12 mo, with none of the treated individuals developing neutralizing antibodies to the recombinant enzyme (39, 40). Noteworthy, the nonmammalian glycan content of plant-made glucocerebrosidase (41, 42) is per mass unit about 100 times higher than that of PG9 and RSH produced in ΔXT/FT *N. benthamiana* plants. This observation suggests that human recipients could tolerate even long-term therapy with these bNAbs. Notwithstanding, efforts should be undertaken to completely eliminate undesirable *N*-glycan species from *N. benthamiana* and other plant-based expression platforms (for instance, by means of genome editing) (43).

In addition to core fucosylation, it has been also reported that Fc sialylation can modulate the biological activities of antibodies (44, 45). Although this modification has been shown to impede the binding of IgG to FcγRII and FcγRIII (44, 46), recent structural evidence suggests that sialylated Fc glycans could tighten the interaction with FcγRI (47). Because it has not been tested before whether sialylation of bNAbs influences their effector functions, we have produced terminally sialylated PG9 and RSH variants in *N. benthamiana* by coexpression of genes from the mammalian galactosylation and sialylation pathway (25). Plant-produced PG9 and RSH were 30–40% galactosylated and 6–12% sialylated, similar to human serum IgG (48) and clearly higher than for ^{CHO}PG9. Interestingly, none of the tested functionalities of PG9 and RSH (antigen binding, virus neutralization, and ADCC) were affected by the extent of galactosylation and sialylation. However, an in vivo contribution of these *N*-glycan moieties to serum half-life or other pharmacokinetically important properties cannot be ruled out.

Materials and Methods

Experimental procedures are detailed in *SI Materials and Methods*. These outlines include cloning of expression constructs, recombinant protein purification and biochemical characterization, glycosylation and sulfation analysis by MS, ELISA, and biolayer interferometry. The protocols used for virus neutralization and ADCC assays are also provided.

ACKNOWLEDGMENTS. We thank Michaela Bogner (Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences) for technical support, Dietmar Katinger (Polymun Scientific) for PG9 produced in CHO cells, and John P. Moore and Albert Cupo (Weill-Cornell Medical College) for BG505 SOSIP.664 gp140. MagnIcon vectors were provided by Viktor Klimyuk (Icon Genetics). This work was supported by Austrian Research Promotion Agency Laura Bassi Centres of Expertise Grant 822757 and Austrian Science Fund Grants L575-B13 and W1224-B09.

1. Klein F, et al. (2012) HIV therapy by a combination of broadly neutralizing antibodies in humanized mice. *Nature* 492(7427):118–122.
2. Walker LM, et al.; Protocol G Principal Investigators (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* 326(5950):285–289.
3. Walker LM, et al.; Protocol G Principal Investigators (2011) Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 477(7365): 466–470.
4. Pejchal R, et al. (2010) Structure and function of broadly reactive antibody PG16 reveal an H3 subdomain that mediates potent neutralization of HIV-1. *Proc Natl Acad Sci USA* 107(25):11483–11488.
5. Pancera M, et al. (2010) Crystal structure of PG16 and chimeric dissection with somatically related PG9: Structure-function analysis of two quaternary-specific antibodies that effectively neutralize HIV-1. *J Virol* 84(16):8098–8110.
6. McLellan JS, et al. (2011) Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. *Nature* 480(7377):336–343.
7. Pancera M, et al. (2013) Structural basis for diverse N-glycan recognition by HIV-1-neutralizing V1-V2-directed antibody PG16. *Nat Struct Mol Biol* 20(7):804–813.
8. Amin MN, et al. (2013) Synthetic glycopeptides reveal the glycan specificity of HIV-neutralizing antibodies. *Nat Chem Biol* 9(8):521–526.
9. Julien JP, et al. (2013) Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9. *Proc Natl Acad Sci USA* 110(11):4351–4356.

10. Jefferis R (2012) Isotype and glycoform selection for antibody therapeutics. *Arch Biochem Biophys* 526(2):159–166.
11. Shields RL, et al. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* 277(30):26733–26740.
12. Lewis GK (2013) Qualitative and quantitative variables that affect the potency of Fc-mediated effector function in vitro and in vivo: Considerations for passive immunization using non-neutralizing antibodies. *Curr HIV Res* 11(5):354–364.
13. Holl V, Peressin M, Moog C (2009) Antibody-mediated Fcγ receptor-based mechanisms of HIV inhibition: Recent findings and new vaccination strategies. *Viruses* 1(3): 1265–1294.
14. Baum LL, et al. (1996) HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. *J Immunol* 157(5):2168–2173.
15. Strasser R, Altmann F, Steinkellner H (2014) Controlled glycosylation of plant-produced recombinant proteins. *Curr Opin Biotechnol* 30:95–100.
16. Qiu X, et al. (2014) Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature* 514(7520):47–53.
17. Forthal DN, et al. (2010) Fc-glycosylation influences Fcγ receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. *J Immunol* 185(11):6876–6882.
18. Moore KL (2009) Protein tyrosine sulfation: A critical posttranslation modification in plants and animals. *Proc Natl Acad Sci USA* 106(35):14741–14742.
19. Stone MJ, Chuang S, Hou X, Shoham M, Zhu JZ (2009) Tyrosine sulfation: An increasingly recognised post-translational modification of secreted proteins. *N Biotechnol* 25(5):299–317.
20. Komori R, Amano Y, Ogawa-Ohnishi M, Matsubayashi Y (2009) Identification of tyrosylprotein sulfotransferase in Arabidopsis. *Proc Natl Acad Sci USA* 106(35):15067–15072.
21. Rosenberg Y, et al. (2015) Pharmacokinetics and immunogenicity of broadly neutralizing HIV monoclonal antibodies in macaques. *PLoS One* 10(3):e0120451.
22. Strasser R, et al. (2008) Generation of glyco-engineered Nicotiana benthamiana for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnol J* 6(4):392–402.
23. Niemer M, et al. (2014) The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: Down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms. *Biotechnol J* 9(4):493–500.
24. Bombarely A, et al. (2012) A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. *Mol Plant Microbe Interact* 25(12):1523–1530.
25. Castilho A, et al. (2010) In planta protein sialylation through overexpression of the respective mammalian pathway. *J Biol Chem* 285(21):15923–15930.
26. Sanders RW, et al. (2013) A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog* 9(9):e1003618.
27. Pollara J, et al. (2013) Epitope specificity of human immunodeficiency virus-1 antibody dependent cellular cytotoxicity [ADCC] responses. *Curr HIV Res* 11(5):378–387.
28. Cimbro R, et al. (2014) Tyrosine sulfation in the second variable loop (V2) of HIV-1 gp120 stabilizes V2-V3 interaction and modulates neutralization sensitivity. *Proc Natl Acad Sci USA* 111(8):3152–3157.
29. Loos A, Steinkellner H (2014) Plant glyco-biotechnology on the way to synthetic biology. *Front Plant Sci* 5:523.
30. Huang CC, et al. (2004) Structural basis of tyrosine sulfation and VH-gene usage in antibodies that recognize the HIV type 1 coreceptor-binding site on gp120. *Proc Natl Acad Sci USA* 101(9):2706–2711.
31. Niehrs C, Huttner WB, Carvallo D, Degryse E (1990) Conversion of recombinant hirudin to the natural form by in vitro tyrosine sulfation. Differential substrate specificities of leech and bovine tyrosylprotein sulfotransferases. *J Biol Chem* 265(16): 9314–9318.
32. Leyte A, et al. (1991) Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. *J Biol Chem* 266(2):740–746.
33. Zeitlin L, et al. (2011) Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. *Proc Natl Acad Sci USA* 108(51): 20690–20694.
34. Reichert JM, Dhimolea E (2012) The future of antibodies as cancer drugs. *Drug Discov Today* 17(17-18):954–963.
35. Moldt B, et al. (2012) A nonfucosylated variant of the anti-HIV-1 monoclonal antibody b12 has enhanced FcγRIIIa-mediated antiviral activity in vitro but does not improve protection against mucosal SHIV challenge in macaques. *J Virol* 86(11):6189–6196.
36. Hessell AJ, et al. (2007) Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* 449(7158):101–104.
37. Bournazos S, et al. (2014) Broadly neutralizing anti-HIV-1 antibodies require Fc effector functions for in vivo activity. *Cell* 158(6):1243–1253.
38. Caskey M, et al. (2015) Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 522(7557):487–491.
39. Zimran A, et al. (2011) Pivotal trial with plant cell-expressed recombinant glucocerebrosidase, taliglucerase alfa, a novel enzyme replacement therapy for Gaucher disease. *Blood* 118(22):5767–5773.
40. Zimran A, et al. (2015) Safety and efficacy of two dose levels of taliglucerase alfa in pediatric patients with Gaucher disease. *Blood Cells Mol Dis* 54(1):9–16.
41. Shaaltiel Y, et al. (2007) Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. *Plant Biotechnol J* 5(5):579–590.
42. Tekoah Y, et al. (2013) Glycosylation and functionality of recombinant β-glucocerebrosidase from various production systems. *Biosci Rep* 33(5):771–781.
43. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31(8):691–693.
44. Kaneko Y, Nimmerjahn F, Ravetch JV (2006) Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313(5787):670–673.
45. Anthony RM, et al. (2008) Recapitulation of IVIG anti-inflammatory activity with a recombinant IgG Fc. *Science* 320(5874):373–376.
46. Anthony RM, Wermeling F, Karlsson MC, Ravetch JV (2008) Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc Natl Acad Sci USA* 105(50):19571–19578.
47. Lu J, et al. (2015) Structure of FcγRI in complex with Fc reveals the importance of glycan recognition for high-affinity IgG binding. *Proc Natl Acad Sci USA* 112(3):833–838.
48. Stadlmann J, Pabst M, Kolarich D, Kunert R, Altmann F (2008) Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. *Proteomics* 8(14):2858–2871.
49. Ouyang Yb, Lane WS, Moore KL (1998) Tyrosylprotein sulfotransferase: Purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. *Proc Natl Acad Sci USA* 95(6): 2896–2901.
50. Strasser R, et al. (2005) Molecular basis of N-acetylglucosaminyltransferase I deficiency in Arabidopsis thaliana plants lacking complex N-glycans. *Biochem J* 387(Pt 2):385–391.
51. Schähs P, et al. (2008) Cellular repressor of E1A-stimulated genes is a bona fide lysosomal protein which undergoes proteolytic maturation during its biosynthesis. *Exp Cell Res* 314(16):3036–3047.
52. Strasser R, et al. (2007) A unique beta1,3-galactosyltransferase is indispensable for the biosynthesis of N-glycans containing Lewis a structures in Arabidopsis thaliana. *Plant Cell* 19(7):2278–2292.
53. Pabst M, Chang M, Stadlmann J, Altmann F (2012) Glycan profiles of the 27 N-glycosylation sites of the HIV envelope protein CN54gp140. *Biol Chem* 393(8):719–730.
54. Gach JS, et al. (2013) A human antibody to the CD4 binding site of gp120 capable of highly potent but sporadic cross clade neutralization of primary HIV-1. *PLoS One* 8(8): e72054.
55. Gach JS, et al. (2014) HIV-1 specific antibody titers and neutralization among chronically infected patients on long-term suppressive antiretroviral therapy (ART): A cross-sectional study. *PLoS One* 9(1):e85371.