

A novel baculovirus vector for the production of nonfucosylated recombinant glycoproteins in insect cells

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Glycosylation is an important attribute of baculovirus-insect cell expression systems, but some insect cell lines produce core α 1,3-fucosylated *N*-glycans, which are highly immunogenic and render recombinant glycoproteins unsuitable for human use. To address this problem, we exploited a bacterial enzyme, guanosine-5'-diphospho (GDP)-4-dehydro-6-deoxy-D-mannose reductase (Rmd), which consumes the GDP-L-fucose precursor. We expected this enzyme to block glycoprotein fucosylation by blocking the production of GDP-L-fucose, the donor substrate required for this process. Initially, we engineered two different insect cell lines to constitutively express Rmd and isolated subclones with fucosylation-negative phenotypes. However, we found the fucosylation-negative phenotypes induced by Rmd expression were unstable, indicating that this host cell engineering approach is ineffective in insect systems. Thus, we constructed a baculovirus vector designed to express Rmd immediately after infection and facilitate the insertion of genes encoding any glycoprotein of interest for expression later after infection. We used this vector to produce a daughter encoding rituximab and found, in contrast to an Rmd-negative control, that insect cells infected with this virus produced a nonfucosylated form of this therapeutic antibody. These results indicate that our Rmd⁺ baculoviral vector can be used to solve the immunogenic core α 1,3-fucosylation problem associated with the baculovirus-insect cell system. In conjunction with existing glycoengineered insect cell lines, this vector extends the utility of the baculovirus-insect cell system to include therapeutic glycoprotein production. This new vector also extends the utility of the baculovirus-insect cell system to include the production of recombinant antibodies with enhanced effector functions, due to its ability to block core α 1,6-fucosylation.

Keywords: baculovirus / fucosylation / glycoengineering / glycoprotein production / insect cells

Introduction

In view of bioinformatic analyses suggesting that well over half of all human proteins are glycosylated, the ability to support glycosylation is an increasingly significant attribute of recombinant protein production systems, such as the baculovirus-insect cell system (reviewed by Jarvis 2009; Usami et al. 2010). The native human protein glycosylation process often results in the addition of terminally sialylated *N*-glycans that dramatically influence key properties, such as the *in vivo* half-lives, of glycoproteins in the human body (Ngantung et al. 2006; reviewed by Varki and Gagneux 2012). Most investigators know that the baculovirus-insect cell expression system supports recombinant protein glycosylation, but it is also important to recognize that this system cannot produce human-type, terminally sialylated *N*-glycans (Marchal et al. 2001; Hillar and Jarvis 2010). This limitation has been addressed by glycoengineering baculovirus vectors and/or insect cell lines to encode and express mammalian glycogenes that extend endogenous insect cell *N*-glycan processing capabilities (Jarvis and Finn 1996; Wagner et al. 1996; Hollister et al. 1998, 2002; Ailor et al. 2000; Hollister and Jarvis 2001; Jarvis et al. 2001; Seo et al. 2001; Aumiller et al. 2003, 2012; Chang et al. 2003; Tomiya et al. 2003; Yun et al. 2005; Hill et al. 2006; Okada et al. 2010; Geisler and Jarvis 2012; Palmberger et al. 2012; Mabashi-Asazuma et al. 2013). These efforts ultimately yielded new baculovirus-insect systems that can produce recombinant glycoproteins with human-type, terminally sialylated *N*-glycans. This was an important step towards the broader goal of extending the utility of the baculovirus-insect cell system to include therapeutic glycoprotein production, which is not currently considered to be a legitimate application of this system. In order to finally achieve this goal, however, it also will be necessary to eliminate core α 1,3-fucosylation of recombinant glycoproteins in certain insect cell lines commonly used as hosts for baculovirus vectors, as this modification generates a highly immunogenic carbohydrate epitope (reviewed by Fötisch and Vieths 2001; Altmann 2007).

There are two distinct types of *N*-glycan core fucosylation involving the addition of either α 1,6- or α 1,3-linked fucose residues to the core *N*-acetylglucosamine. Humans encode a single core fucosyltransferase (FUT8), which can only add α 1,6-linked fucose residues, but many insects encode two core fucosyltransferases, FucT6 and FucTA, which can add α 1,6- or α 1,3-linked fucose residues, respectively, to the core *N*-acetylglucosamine (Figure 1A; Fabini et al. 2001; Paschinger et al. 2005; Rendić et al. 2007). Because humans have no FucTA counterpart, we

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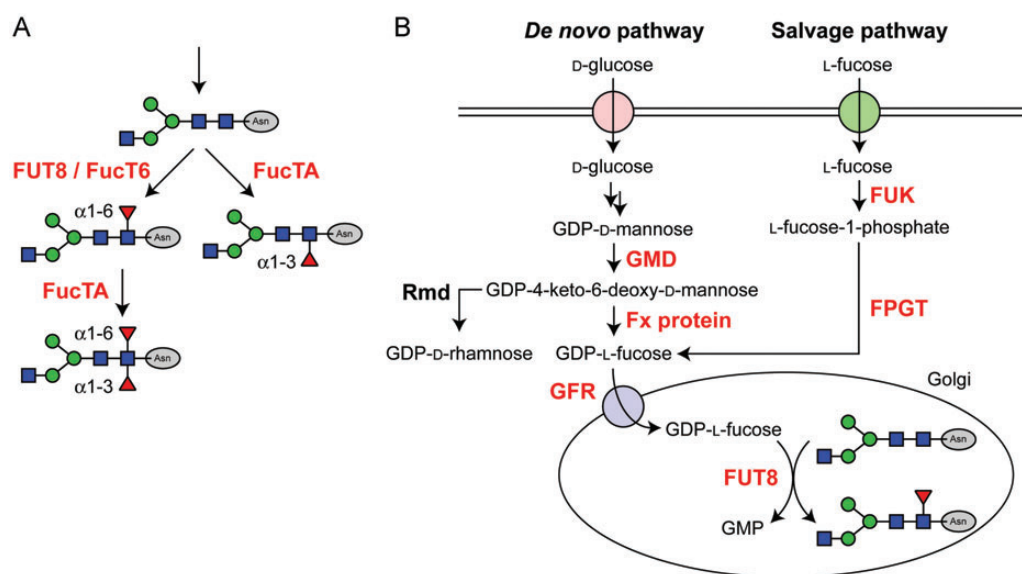


Fig. 1. Pathways of *N*-glycan fucosylation (A) and GDP-L-fucose biosynthesis (B). In theory, GDP-L-fucose biosynthesis in eukaryotic cells can be blocked by expression of bacterial Rmd, which converts GDP-4-keto-6-deoxy-D-mannose to GDP-D-rhamnose. Abbreviations: FUT8, human core α 1,6-fucosyltransferase 8; FucT6, insect core α 1,6-fucosyltransferase; FucTA, insect core α 1,3-fucosyltransferase; GMD, GDP-D-mannose 4,6-dehydratase; Fx protein, GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/4-reductase; GFR, Golgi GDP-L-fucose transporter; Rmd, GDP-4-dehydro-6-deoxy-D-mannose reductase; FUK, fucokinase; FPGT, fucose-1-phosphate guanylyltransferase.

cannot produce core α 1,3-fucosylated *N*-glycoproteins and, as a result, the core α 1,3-fucosylated sugar epitope is immunogenic for many people. In fact, the presence of this epitope on bee venom glycoproteins accounts for the life-threatening allergic responses, such as anaphylactic shock, induced by bee stings in some humans (King et al. 1976; Prenner et al. 1992; Kubelka et al. 1995; reviewed by Altmann 2007). In addition, pre-existing human antibodies against the immunogenic sugar epitope can produce false positives in diagnostic tests that use α 1,3-fucosylated recombinant glycoproteins produced in the baculovirus-insect cell system (Hancock et al. 2008; Seismann et al. 2010).

Among the insect cell lines commonly used as hosts for baculovirus-mediated recombinant protein production, BTI-Tn-5B1-4 (Wickham et al. 1992; commercialized as High FiveTM by Life Technologies, Inc., Carlsbad, CA), which is derived from *Trichoplusia ni*, produces high levels of the core α 1,3-fucosylated *N*-glycan epitope, whereas Sf9 (Summers and Smith 1987), derived from *Spodoptera frugiperda*, and BmN (Maeda 1989), derived from *Bombyx mori*, do not (Rudd et al. 2000; Hancock et al. 2008; Seismann et al. 2010; Blank et al. 2011; Palmberger et al. 2011). Importantly, the capacity to produce immunogenic, core α 1,3-fucosylated *N*-glycans trumps the opportunity to exploit the potentially higher recombinant glycoprotein production capacity of insect cell lines derived from *Trichoplusia ni*, such as High FiveTM (Davis et al. 1992; Krammer et al. 2010).

While core α 1,3-fucosylation is a relatively host-specific modification, core α 1,6-fucosylation is a common feature of recombinant glycoproteins produced by all insect cell lines used as hosts for baculovirus vectors. As noted above, core α 1,6-fucosylation also occurs in humans and, therefore, does not produce an immunogenic sugar epitope. Nevertheless, this form of core fucosylation is also biotechnologically significant because it inhibits the

effector functions of certain types of therapeutic antibodies (Shields et al. 2002; Shinkawa et al. 2003; reviewed by Satoh et al. 2006; Jefferis 2009), which comprise a large and growing share of the human biologics market (reviewed by Elvin et al. 2013). The inhibition of antibody effector functions by core fucosylation stimulated efforts to block pathways responsible for this modification and enable the production of nonfucosylated antibodies in mammalian cell expression systems (Figure 1B). Various approaches included repressing or eliminating FUT8 gene expression (Yamane-Ohnuki et al. 2004; Imai-Nishiya et al. 2007; Malphettes et al. 2010), overexpressing upstream processing enzymes to produce *N*-glycan structures that are not recognized as acceptor substrates (Ferrara et al. 2006; Zhong et al. 2012), and blocking production of GDP-L-fucose, which is required as the donor substrate for fucosylation by FUT8 (Imai-Nishiya et al. 2007; Kanda et al. 2007; von Horsten et al. 2010). To date, however, there have been no reported efforts to block recombinant glycoprotein fucosylation in the baculovirus-insect cell system, despite the arguably more serious problem of immunogenicity associated with core α 1,3-fucosylation in this system.

Thus, the basic purpose of this study was to develop a new baculovirus-insect cell system that can produce nonfucosylated recombinant glycoproteins. Our initial approach was to engineer insect cell lines to constitutively express a *Pseudomonas aeruginosa* gene encoding GDP-4-dehydro-6-deoxy-D-mannose reductase (Rmd), which consumes the immediate precursor to GDP-L-fucose (Figure 1B), and was previously used to block core α 1,6-fucosylation in Chinese hamster ovary (CHO) cells (von Horsten et al. 2010). Interestingly, while this approach appeared to be temporarily effective, we discovered that it cannot be used successfully in the baculovirus-insect cell system because the fucosylation-negative phenotype induced by constitutive Rmd

expression in insect cell lines is unstable. Thus, we constructed a novel baculovirus vector designed to express Rmd immediately after infection and to facilitate downstream isolation of daughter vectors capable of expressing any recombinant glycoprotein of interest later in infection. We subsequently isolated a daughter encoding a therapeutic anti-CD20-immunoglobulin G (IgG), rituximab, and demonstrated that our new vector can be successfully used to produce nonfucosylated recombinant glycoproteins. By eliminating core α 1,3-fucosylation, the new baculovirus vector described in this study solves the significant problem of immunogenic recombinant glycoprotein production associated with the baculovirus-insect cell system. In conjunction with glycoengineered insect cell lines, this new vector extends the utility of the baculovirus-insect cell system as a legitimate tool for the production of therapeutic glycoproteins. Finally, by eliminating core α 1,6-fucosylation, this new vector also extends the utility of the baculovirus-insect cell system to include the production of recombinant antibodies with enhanced effector functions.

Results

Analysis of core α 1,3-fucosylation in three insect cell lines

As mentioned above, High Five™ cells, derived from *Trichoplusia ni*, but not Sf9 cells, derived from *Spodoptera frugiperda*, produce core α 1,3-fucosylated glycoproteins (Rudd et al. 2000; Seismann et al. 2010; Blank et al. 2011; Palmberger et al. 2011). Another *Trichoplusia ni* cell line used as a host for baculovirus expression vectors is Tni PRO™ (Kwon et al. 2009; Bourhis et al. 2010; Bongiovanni et al. 2012; He et al. 2013; Merchant et al. 2013), but its capacity for core α 1,3-fucosylation has not been reported. Thus, we analyzed intracellular extracts of uninfected Tni PRO™ cells by western blotting with anti-horseradish peroxidase (HRP), which detects core α 1,3-linked fucosylation, using extracts from Sf9 and High Five™ cells as negative and positive controls. Coomassie brilliant blue staining showed that approximately equal amounts of protein were loaded in each case (Figure 2A). The anti-HRP antibody did not detectably react with the Sf9 lysates,

but reacted with several glycoproteins in the High Five™ lysates, as expected (Figure 2B). In addition, this antibody reacted with several glycoproteins in the Tni PRO™ lysates (Figure 2B), indicating that Tni PRO™ cells produce the immunogenic core α 1,3-fucosylated sugar epitope at levels roughly comparable to High Five™ cells. These results show that it will be necessary to block core α 1,3-fucosylation in both of these cell lines before we can exploit their potentially higher capacity for recombinant glycoprotein production (Davis et al. 1992; Krammer et al. 2010).

Glycoengineering insect cells to block glycoprotein fucosylation

Our plan to block glycoprotein fucosylation in insect cell lines focused on blocking the biosynthesis of GDP-L-fucose, which is the donor substrate required for this process. This was a particularly attractive approach in our system because insects appeared to be the only multicellular organisms lacking two enzymes, fucokinase (FUK) and fucose-1-phosphate guanylyltransferase (FPGT), required for the GDP-L-fucose salvage pathway in other organisms (Figure 1B). We drew this conclusion from a previous study indicating there are no FUK and FPGT orthologs in the *Drosophila melanogaster* genome, which was the only insect genome sequenced at that time (Rhombert et al. 2006). However, because we now have more information from silkworm, honeybee and mosquito genome sequencing projects, among others, we also searched the National Center for Biotechnology Information database using mammalian FUK and/or FPGT genes as queries. We identified putative orthologs in some invertebrates, including arthropods and nematodes, but none in any insects (Supplementary data, Figure S1A and B). In contrast, using genes required for de novo GDP-L-fucose synthesis as queries, we found putative orthologs in a wide variety of insects, as expected (Supplementary data, Figure S1C and D). Although we could not exclude the possibility that insects have an unknown salvage pathway, these results strengthened the idea that we

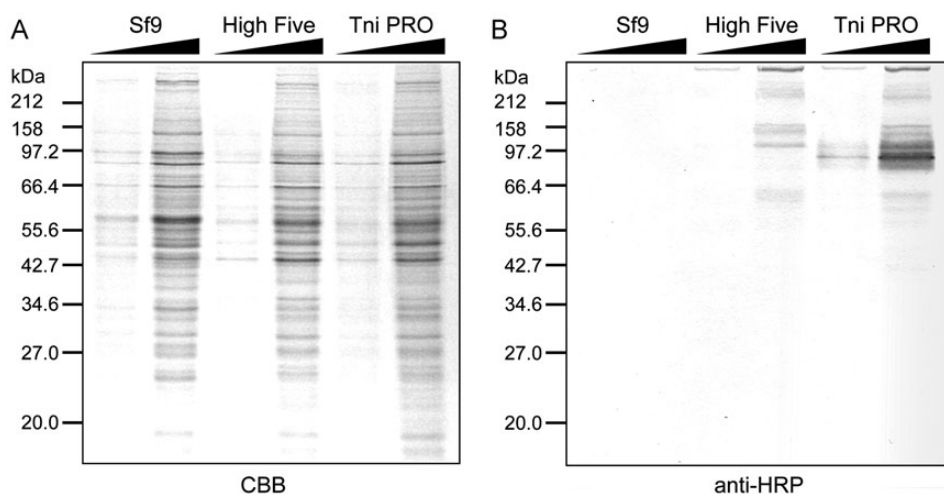


Fig. 2. Core α 1,3-fucosylation of endogenous insect cell glycoproteins. Total proteins in Sf9, High Five™ or Tni PRO™ cell lysates were resolved by SDS-PAGE in 12% acrylamide gels and stained with Coomassie Brilliant Blue (A) or transferred to a PVDF membrane and analyzed by western blotting with primary anti-HRP rabbit IgG and secondary α -rabbit IgG conjugated to alkaline phosphatase (B).

could effectively block GDP-L-fucose biosynthesis by blocking the de novo biosynthetic pathway, alone, in insect cell lines.

In principle, we might have achieved this goal by inactivating any of the genes encoding enzymes involved in this pathway, including GDP-D-mannose 4,6-dehydratase (GMD), Fx, GDP-L-fucose transporter (GFR) or FUT8 (Figure 1B). However, there are no reported examples of targeted gene knockouts in any lepidopteran insect cell line and this approach is technically complicated by the fact that neither the *Spodoptera frugiperda* nor the *Trichoplusia ni* genomes have been sequenced. On the other hand, we have reported many examples of foreign gene knock-ins using both Sf9 (Hollister et al. 1998, 2002; Hollister and Jarvis 2001; Aumiller et al. 2003, 2012; Geisler and Jarvis 2012; Mabashi-Asazuma et al. 2013) and High Five™ (Breitbach and Jarvis 2001) cells, as part of our broader effort to glycoengineer the baculovirus-insect cell system. Thus, we pursued an analogous glycoengineering strategy that involved transforming Sf9 and High Five™ cells with a constitutively expressible *Pseudomonas aeruginosa* Rmd gene. This gene encodes an enzyme that consumes the immediate precursor to GDP-L-fucose to produce GDP-D-rhamnose, which we believed would be a dead-end product in insect cells (Figure 1B; Rocchetta et al. 1998). Thus, we expected Rmd to block the production of GDP-L-fucose and glycoprotein fucosylation because GDP-L-fucose is required as the donor substrate for this process.

We constructed an expression plasmid encoding Rmd under the transcriptional control of the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) immediate early 1 (*ie1*) promoter, as described in the Materials and methods section. We then co-transfected Sf9 and High Five™ cells with a mixture of this plasmid and an antibiotic-resistance marker and selected polyclonal transformed Sf9 and High Five™ cell subpopulations, which were designated SfRMD and TnRMD, respectively. We assayed both polyclonal transformed cell populations for cell surface fucosylation by staining with *Aleuria aurantia* lectin (AAL), as described in the Materials and methods section. The results showed that 100% of the parental Sf9 and High Five™ cells were stained, while only 23% of the SfRMD and 33% of the TnRMD cells were stained with AAL (Figure 3). These results demonstrated that Rmd

overexpression significantly reduced glycoprotein fucosylation in insect cells. We subsequently isolated single cell clones from the polyclonal transformed SfRMD and TnRMD populations, identified several that exhibited no detectable AAL cell surface staining, and amplified one of each, designated SfRMD 2B2 and TnRMD 6A6. We then infected Sf9, SfRMD 2B2, High Five™ and TnRMD 6A6 cells with a baculovirus vector encoding a 6X HIS-tagged Fc domain of mouse IgG2a (mIgG2a-Fc) under the control of the baculovirus *p6.9* promoter, which is activated during the late phase of infection (Passarelli and Guarino 2007), and affinity-purified the mIgG2a-Fc from the extracellular fractions, as described in the Materials and methods section. Samples were then treated with peptide-N⁴-(N-acetyl-β-glucosaminyl)asparagine amidase (PNGase)-F or reaction buffer alone, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane and probed with AAL to detect fucose or with anti-mouse IgG to detect the protein (Supplementary data, Figure S2). The results of this analysis showed that the mIgG2a-Fc preparations produced by the parental cell lines were AAL-reactive and either completely (Sf9) or partially (High Five™) sensitive to PNGase-F, indicating they were core α1,6- (Sf9) or α1,6- and α1,3 (High Five™)-fucosylated, respectively. In contrast, neither of the mIgG2a-Fc preparations from SfRMD 2B2 or TnRMD 6A6 cells was AAL-reactive, indicating that RMD effectively blocked glycoprotein fucosylation in both cell types. However, we were surprised to find that both of these prototype SfRMD and TnRMD clones recovered strong AAL reactivity after 38 and 35 passages in culture, respectively, indicating that the fucosylation-negative phenotype is unstable in these cells (data not shown). We obtained this same result with other SfRMD and TnRMD clones, which was extremely surprising, because this knock-in approach had been used previously to isolate a stable, fucosylation deficient CHO cell derivative (von Horsten et al. 2010) and because we had previously demonstrated that a transgenic insect cell line transformed with six different transgenes was stable for at least 300 passages in culture (Aumiller et al. 2012). Although we found that they still expressed the Rmd gene at the transcriptional level, we did not expend any further effort to determine how the SfRMD and TnRMD cells recovered their original fucosylation-positive

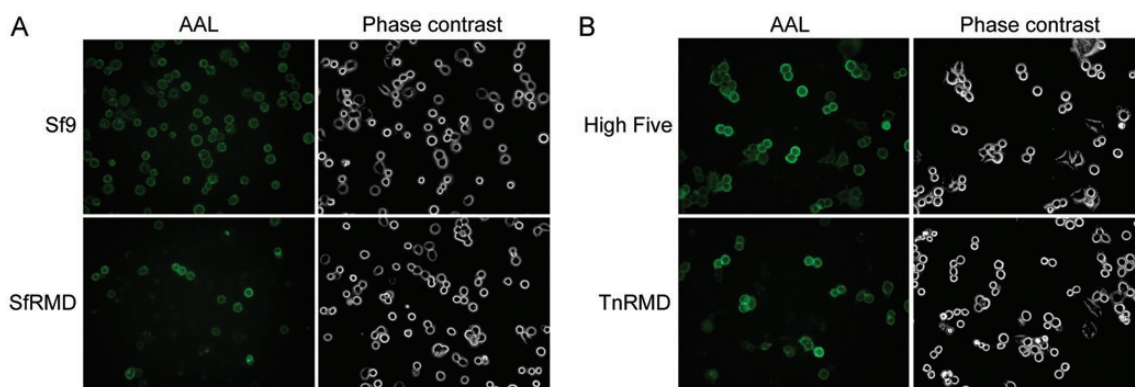


Fig. 3. Cell surface fucosylation. Sf9 and polyclonal SfRMD (A) or High Five™ and polyclonal TnRMD (B) cells were seeded into culture plates, and then stained with AAL, as described in the Materials and methods section. The cell surface staining patterns are shown alongside corresponding phase contrast micrographs, as indicated.

phenotypes. Rather, after finding our insect cell glycoengineering approach was unsuccessful, we began to develop a new approach for blocking glycoprotein fucosylation in the baculovirus-insect cell system.

Assessing a vector-based glycoengineering strategy for blocking glycoprotein fucosylation

In view of the surprising phenotypic instability of SfRMD and TnRMD cells, we abandoned our efforts to glycoengineer the host cell component and focused our attention on the baculoviral vector component of the baculovirus-insect cell system. The baculovirus-insect cell system is a transient expression system in which baculovirus-infected insect cells express the glycoprotein of interest for a period of ~2 days, beginning about a day after infection, when the gene encoding the glycoprotein of interest begins to be expressed during the late or very late phase of infection. Baculovirus-infected cells are typically harvested by ~60–72 h postinfection because the host cells die and lyse at later times of infection. Given the transient nature of this expression system, we realized that the phenotypic instability observed with long-term constitutive expression of Rmd in the host cells could be overcome by engineering the viral vector to express this enzyme. However, we also realized we would have to design the new vector to express Rmd early in infection, in order to reduce endogenous GDP-L-fucose to low enough levels to block fucosylation before the glycoprotein of interest began to be expressed during a later phase of infection.

To assess our ability to meet these requirements, we isolated a recombinant baculovirus designated AcP(+)IE1-RMD, which encodes Rmd under the control of the AcMNPV *ie1* promoter and was expected to express the Rmd gene immediately after baculovirus infection (Guarino and Summers 1986). We then used this virus to examine the impact of immediate early Rmd expression on fucosylation of mIgG2a-Fc. This was accomplished by co-infecting Sf9 or Tni PRO™ cells with equal doses of AcP(+)IE1-RMD and Acp6.9-mIgG2a-Fc, or with Acp6.9-mIgG2a-Fc alone as a control, and then affinity-purifying the mIgG2a-Fc from the extracellular fractions for

analysis, as described in the Materials and methods section. Samples of the purified mIgG2a-Fc were then treated with PNGase-F or reaction buffer alone, resolved by SDS-PAGE, transferred to a PVDF membrane and probed with AAL to detect fucose, anti-HRP to detect core α 1,3-linked fucose or anti-mouse IgG to detect the protein. The results showed that the control mIgG2a-Fc preparation produced by Sf9 cells infected with Acp6.9-mIgG2a-Fc alone were AAL-reactive (Figure 4, top panel), indicating it was fucosylated. In addition, PNGase-F pretreatment eliminated its AAL reactivity (Figure 4, top panel), indicating that it was exclusively core α 1,6-fucosylated, because PNGase-F does not remove core α 1,3-fucosylated *N*-glycans (Tretter et al. 1991). This interpretation was supported by the fact that anti-HRP, which recognizes the immunogenic sugar epitope produced by core α 1,3-fucosylation, did not react with this mIgG2a-Fc preparation (Figure 4, middle panel). In contrast, the mIgG2a-Fc preparation from Sf9 cells co-infected with Acp6.9-mIgG2a-Fc and AcP(+)IE1-RMD was not AAL-reactive (Figure 4, upper panel), indicating it was not fucosylated and, therefore, that baculovirus-mediated Rmd expression blocked core α 1,6-fucosylation in Sf9 cells. The mIgG2a-Fc from Tni PRO™ cells infected with Acp6.9-mIgG2a-Fc alone reacted not only with AAL (Figure 4, top panel), but also with anti-HRP (Figure 4, middle panel), whether or not it was pretreated with PNGase-F, indicating that it was core α 1,3-fucosylated. This interpretation was supported by the fact that PNGase-F did not detectably alter the electrophoretic mobility of this mIgG2a-Fc preparation (Figure 4, bottom panel). In contrast, the mIgG2a-Fc from Tni PRO™ cells co-infected with Acp6.9-mIgG2a-Fc and AcP(+)IE1-RMD had no detectable AAL (Figure 4, top panel) or anti-HRP (Figure 4, middle panel) reactivity and was sensitive to PNGase-F (Figure 4, bottom panel). These results indicated that baculovirus-mediated Rmd expression blocked both core α 1,6- and core α 1,3-fucosylation in Tni PRO™ cells.

To more directly assess the impact of AcP(+)IE1-RMD co-infection on core fucosylation of mIgG2a-Fc, we enzymatically released, permethylated and analyzed the *N*-glycans from each mIgG2a-Fc preparation in Figure 4 by matrix assisted laser

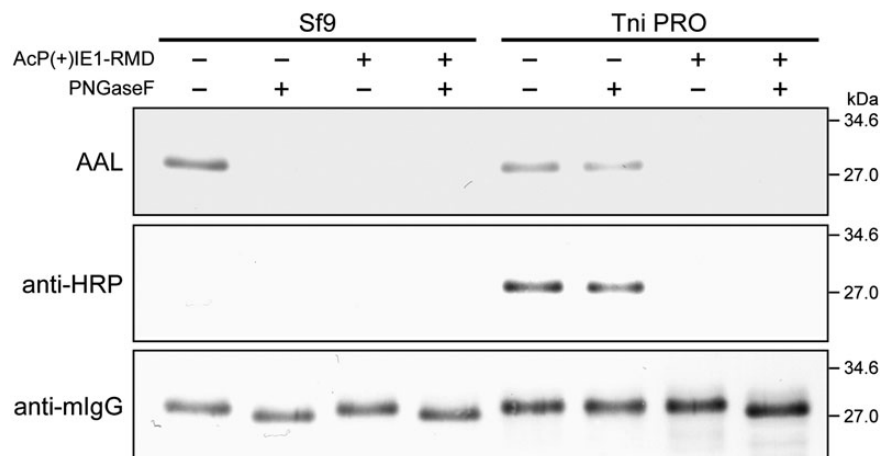


Fig. 4. Impact of AcP(+)IE1-RMD co-infection on mIgG2a-Fc fucosylation. Sf9 or Tni PRO™ cells were infected with Acp6.9-mIgG2a-Fc, a recombinant baculovirus encoding mIgG2a-Fc, or with equal doses of Acp6.9-mIgG2a-Fc and AcP(+)IE1-RMD, a recombinant baculovirus encoding Rmd. The cell-free media were harvested at 48-h postinfection and used to affinity purify each mIgG2a-Fc preparation for lectin blotting analysis with AAL (specific for fucose) or western blotting analysis with anti-HRP (specific for core α 1,3 fucose) or anti-mouse IgG with (+) or without (-) PNGase-F pretreatment, as indicated.

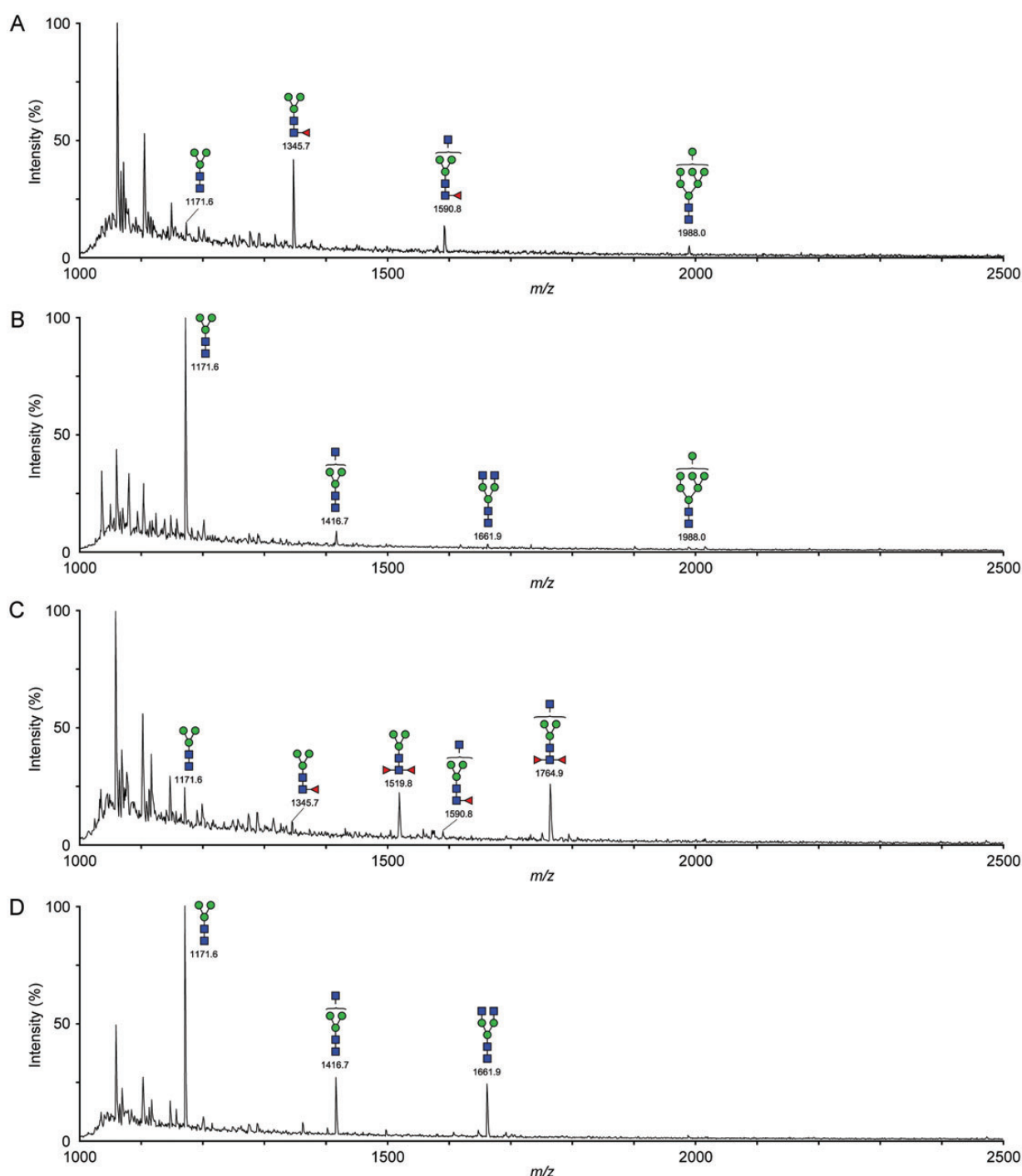


Fig. 5. *N*-glycan profiling of various mIgG2a-Fc preparations by MALDI-TOF MS. mIgG2a-Fc preparations were produced and purified from Sf9 and Tni PROTM cells infected with Acp6.9-mIgG2a-Fc alone or Acp6.9-mIgG2a-Fc and Acp(+)-IE1-RMD, as described in the legend to Figure 4. PNGaseAr was used to remove the *N*-glycans, which were then recovered, permethylated, and analyzed by MALDI-TOF MS, as described in the Materials and methods section. mIgG2a-Fc from Sf9 cells infected with Acp6.9-mIgG2a-Fc alone (A), Sf9 cells co-infected with Acp6.9-mIgG2a-Fc and Acp(+)-IE1-RMD (B), Tni PROTM cells infected with Acp6.9-mIgG2a-Fc alone (C) or Tni PROTM cells co-infected with Acp6.9-mIgG2a-Fc and Acp(+)-IE1-RMD (D). All molecular ions were detected as $[M + Na]^+$, assigned and annotated using the standard cartoon symbolic representations.

desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), as described in the Materials and methods section. The results indicated that the major *N*-glycans isolated from the mIgG2a-Fc produced by Sf9 cells infected with Acp6.9-

mIgG2a-Fc alone were mono-fucosylated (m/z 1345.7 and 1590.8; Figure 5A). Based on the results shown in Figure 4, these are most likely core α 1,6-fucosylated *N*-glycans. In contrast, none of the peaks assigned as fucosylated *N*-glycans were detected in

the mIgG2a-Fc produced by Sf9 cells co-infected with Acp6.9-mIgG2a-Fc and Acp(+)-IE1-RMD (Figure 5B). The major *N*-glycans isolated from the mIgG2a-Fc produced by Tni PROTM cells infected with Acp6.9-mIgG2a-Fc alone included mono-, but predominantly di-fucosylated structures (Figure 5C). While their masses cannot directly reveal the nature of the linkages between the fucose and core *N*-acetylglucosamine residues in these *N*-glycans, it is most reasonable to conclude that the di-fucosylated *N*-glycans have both α 1,6- and α 1,3-linked fucose residues. Again, none of the peaks assigned as mono- or di-fucosylated *N*-glycans were detected in the mIgG2a-Fc produced by Tni PROTM cells co-infected with Acp6.9-mIgG2a-Fc and Acp(+)-IE1-RMD (Figure 5D). Together, the AAL blotting, anti-HRP antibody blotting, and MALDI-TOF MS data clearly demonstrate that baculovirus-mediated expression of Rmd at an early stage of infection can effectively block core α 1,6- and α 1,3-fucosylation of recombinant glycoproteins expressed later in infection in the baculovirus-insect cell system.

A novel baculovirus vector designed to produce nonfucosylated recombinant glycoproteins

While co-infection with multiple baculoviruses is an approach that can be used to co-express two or more different recombinant proteins in insect cells, balanced co-infections can be difficult to achieve, infection with multiple baculoviruses can dramatically reduce recombinant glycoprotein yields, and optimal co-infection conditions must be established for each experiment (Sokolenko et al. 2012). We chose to circumvent these issues by isolating a novel baculovirus vector designed not only to express Rmd immediately after infection, but also to facilitate downstream insertion of genes encoding any glycoprotein of interest for expression at a later time of infection. The experimental design used to isolate this new baculovirus vector, designated AcRMD, included using the *ie1*-Rmd gene to replace portions of the viral *chiA* and *v-cath* genes, which encode a chitinase purported to interfere with secretory pathway function (Hawtin et al. 1997; Kaba et al. 2004;

Hitchman et al. 2010) and a cathepsin-like protease that can degrade recombinant proteins of interest (Slack et al. 1995; Kaba et al. 2004; Hitchman et al. 2010), respectively. The complete details of the strategy used to isolate this new vector are given in the Materials and methods section and genetic maps of the parental viral genome, transfer plasmid and final baculoviral vector are shown in Figure 6.

AcRMD produces nonfucosylated recombinant glycoproteins

To examine the capabilities of the new baculovirus vector described in the preceding section, we isolated two independent daughter recombinants encoding rituximab, an anti-CD20-IgG, with the light and heavy chain genes placed under the independent transcriptional control of dual back-to-back promoters from either the AcMNPV *p6.9* or *polyhedrin* genes (see genetic maps in Supplementary data, Figure S3). We chose rituximab as the model glycoprotein for this work because it is a biotechnologically relevant therapeutic antibody that is core α 1,6-fucosylated in mammalian cells, has enhanced effector function in the absence of fucosylation, and has been used to treat rheumatoid arthritis and non-Hodgkin's lymphoma in human patients (Li et al. 2013; reviewed by Lim et al. 2010). We isolated AcRMD daughters encoding anti-CD20-IgG under the control of either the *p6.9* or the *polyhedrin* promoters because the latter is activated during the very late phase of infection and is widely used to drive recombinant gene expression in baculoviral vectors, whereas the former is activated somewhat earlier, during the late phase of infection, and can sometimes provide higher efficiencies of protein secretion (Sridhar et al. 1993; Rankl et al. 1994; Bozon et al. 1995; Kost et al. 1997; Toth et al. 2011). We then infected Sf9 cells with these viruses, designated Ac- α CD20-IgG, Acp6.9- α CD20-IgG, AcRMD- α CD20-IgG or AcRMDp6.9- α CD20-IgG, and measured the relative amounts of anti-CD20-IgG produced and secreted by viruses encoding the heavy and light chains under the control of the two different promoters. The results showed that all four baculovirus vectors induced production and

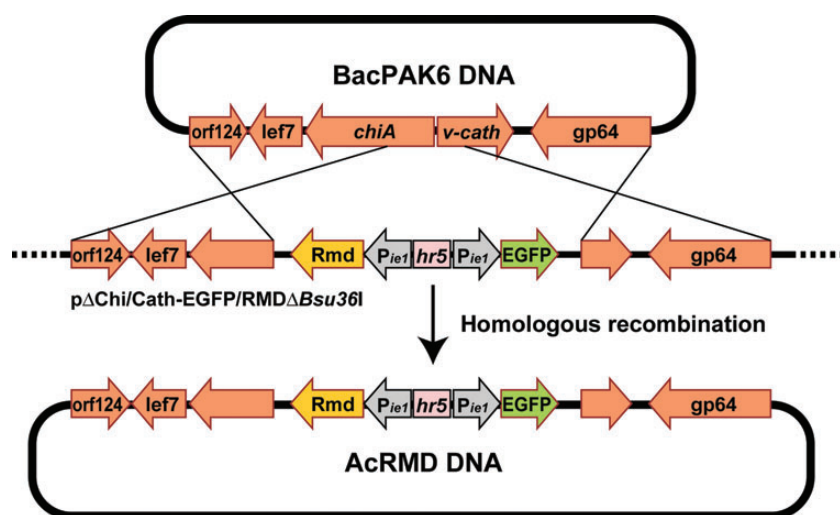


Fig. 6. Genetic maps of parental baculoviral vector, baculovirus transfer plasmid and AcRMD. The new baculovirus vector designated AcRMD was isolated by replacing the 5' regions of the AcMNPV *chiA* and *v-cath* genes in BacPAK6 (Kitts and Possee 1993) baculoviral DNA with an expression cassette encoding EGFP and Rmd under the control of dual, back-to-back *ie1* promoters separated by the AcMNPV *hr5* enhancer, as described in the Materials and methods section.

secretion of the anti-CD20-IgG heavy and light chains (Supplementary data, Figure S4). The results also showed that cells infected with the baculovirus vectors encoding anti-CD20-IgG under the control of the late *p6.9* promoters secreted higher levels of heavy and light chains than those infected with the baculovirus encoding this product under the control of the very late *polyhedrin* promoters. Together with the lower levels of heavy and light chains observed in the intracellular fraction, these results indicated that the *p6.9*-based vectors provided higher efficiencies of anti-CD20-IgG secretion than the *polyhedrin*-based vectors (Supplementary data, Figure S4). Based on these results, we used Acp6.9- α CD20-IgG and AcRMDp6.9- α CD20-IgG for the remainder of the experiments described in this study.

Acp6.9- α CD20-IgG or AcRMDp6.9- α CD20-IgG were used to infect Sf9 or Tni PROTM cells and the anti-CD20-IgG secreted by all four virus-cell combinations was affinity purified, resolved by SDS-PAGE under reducing or nonreducing conditions with commercial human IgG as a control, and then stained with Coomassie Brilliant Blue. The results showed that each anti-CD20-IgG preparation analyzed under reducing conditions contained approximately equal proportions of the heavy and light chains, indicating that each virus-cell combination produced and secreted properly assembled forms of anti-CD20-IgG (Supplementary data, Figure S5A). This was confirmed by SDS-PAGE analysis under nonreducing conditions, which revealed

that each anti-CD20-IgG preparation migrated with relative electrophoretic mobilities that were consistent with their calculated molecular weights (~ 165 kDa) and comparable to the human IgG control (Supplementary data, Figure S5B). Further analysis showed that the anti-CD20-IgG heavy chain produced by Acp6.9- α CD20-IgG-infected Sf9 cells was PNGase-F-sensitive and AAL-reactive, indicating that it had core $\alpha 1,6$ -fucosylated *N*-glycans (Figure 7). In contrast, the anti-CD20-IgG heavy chain produced by AcRMDp6.9-

α CD20-IgG-infected Sf9 cells was PNGase-F-sensitive, but not AAL-reactive, indicating that it had nonfucosylated *N*-glycans (Figure 7). The anti-CD20-IgG heavy chain produced by Acp6.9- α CD20-IgG-infected Tni PROTM cells was PNGase-F-resistant and AAL-reactive, presumably because its *N*-glycans were $\alpha 1,3$ -fucosylated in these cells (Figure 7). In contrast, the anti-CD20-IgG heavy chain produced by AcRMDp6.9- α CD20-IgG-infected Tni PROTM cells was PNGase-F-sensitive and did not react with AAL (Figure 7). Together, these results strongly suggested that early expression of Rmd by the vectors derived from the AcRMD parent blocked core fucosylation of anti-CD20-IgG in the baculovirus-insect cell system.

To confirm and extend these results using a more direct approach, we enzymatically released, permethylated and used MALDI-TOF MS to analyze the *N*-glycans from anti-CD20-IgG preparations purified from various virus-cell combinations, as described in the Materials and methods section. The *N*-glycan

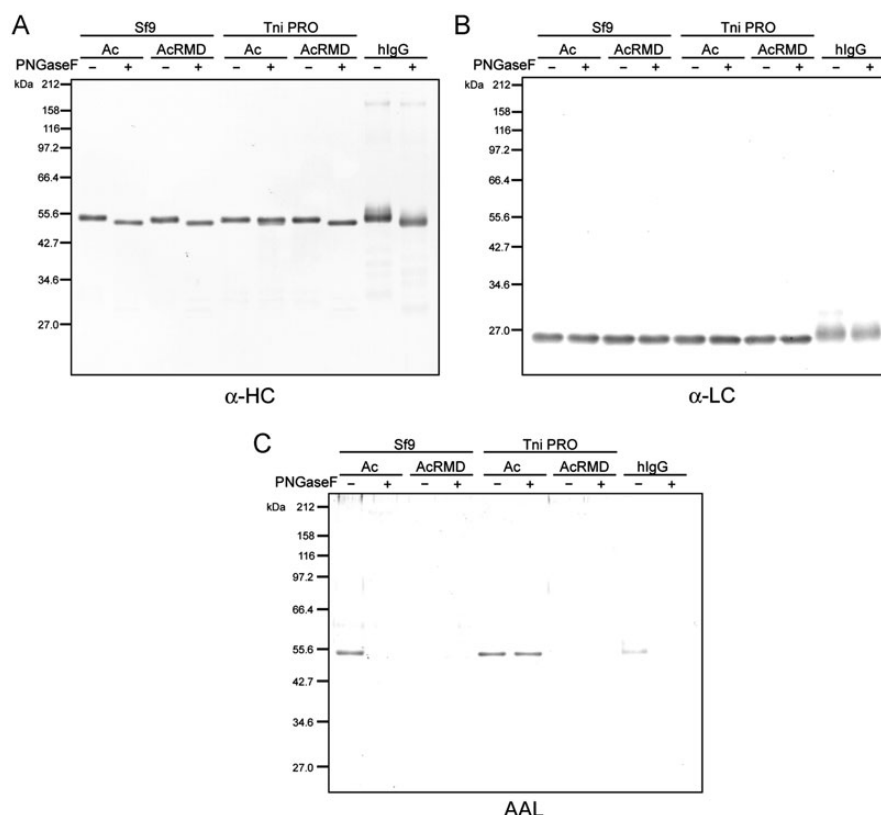


Fig. 7. Fucosylation of an anti-CD20-IgG (rituximab) produced using conventional or AcRMD baculovirus vectors. Sf9 and Tni PROTM cells were infected with either Acp6.9- α CD20-IgG or AcRMDp6.9- α CD20-IgG, and the cell-free media were harvested and used to affinity purify anti-CD20-IgG at 48-h postinfection. The results of western blotting assays with anti-human IgG Fc (A, α -HC) or anti-human IgG κ chain (B, α -LC) and lectin blotting assays with AAL (C) with (+) or without (-) PNGase-F pretreatment are shown.

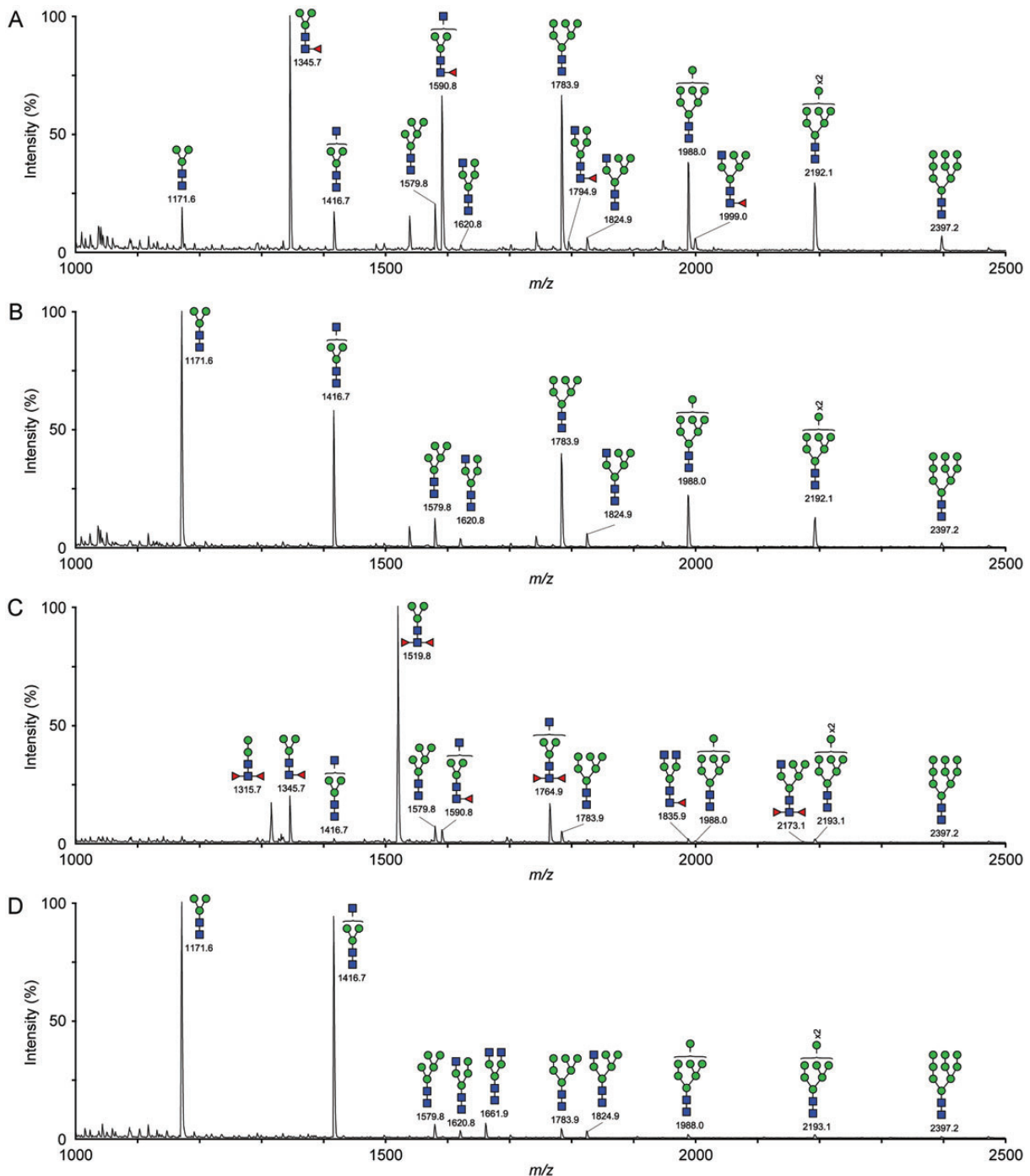


Fig. 8. *N*-glycan profiling of various anti-CD20-IgG preparations by MALDI-TOF MS. Anti-CD20-IgG was isolated from Sf9 or Tni PRO[™] cells infected with Acp6.9- α CD20-IgG or AcRMDp6.9- α CD20-IgG, as described in the legend to Figure 7. The *N*-glycans were removed by PNGaseAr treatment, recovered, permethylated and analyzed by MALDI-TOF MS, as described in the Materials and methods section. Anti-CD20-IgG from Sf9 cells infected with Acp6.9- α CD20-IgG (A), Sf9 cells infected with AcRMDp6.9- α CD20-IgG (B), Tni PRO[™] cells infected with Acp6.9- α CD20-IgG (C) or Tni PRO[™] cells infected with AcRMDp6.9- α CD20-IgG (D). All molecular ions were detected as $[M + Na]^+$, assigned and annotated using the standard cartoon symbolic representations.

profiles observed with the anti-CD20-IgG from Acp6.9- α CD20-IgG-infected Sf9 cells included four peaks assigned as mono-fucosylated *N*-glycans (m/z 1345.7, 1590.8, 1794.9 and 1999.0; Figure 8A), which are presumably core α 1,6-fucosylated, based on the PNGase-F-sensitivity of the heavy chain (Figure 7). In

contrast, none of these mono-fucosylated peaks were detected among the *N*-glycans isolated from the anti-CD20-IgG produced by AcRMDp6.9- α CD20-IgG-infected Sf9 cells (Figure 8B). In addition, the loss of the fucosylated *N*-glycan peaks was accompanied by increased *N*-glycan peaks corresponding to their

nonfucosylated counterparts, especially those with m/z values of 1171.6 and 1416.7 (Figure 8A and B). The N -glycan profiles observed with the anti-CD20-IgG from Acp6.9- α CD20-IgG-infected Tni PROTM cells included seven peaks assigned as fucosylated N -glycans, three of which were mono-fucosylated (m/z 1345.7, 1590.8 and 1835.9) and four of which were di-fucosylated (m/z 1315.7, 1519.8, 1764.9 and 2173.1; Figure 8C). The di-fucosylated N -glycans represented $\sim 65.5\%$ of total (Figure 8C), reflecting the high levels of core $\alpha 1,3$ -fucosylation observed in Tni PROTM and High FiveTM cells (Figure 2). In contrast, no fucosylated N -glycans were detected in the profiles obtained with the anti-CD20-IgG produced by AcRMDp6.9- α CD20-IgG-infected Tni PROTM cells (Figure 8D) and, again, the loss of fucosylated N -glycan peaks was accompanied by increases in the N -glycan peaks corresponding to their nonfucosylated counterparts (Figure 8C and D; m/z 1171.6 and 1416.7). It is tempting to speculate that the large increase in the m/z 1416.7 peak, which represents an N -glycan with a single N -acetylglucosamine residue on its nonreducing end, reflects the relative inability of *spodoptera frugiperda* fused lobes (Geisler et al. 2008) to remove this sugar from nonfucosylated substrates. Regardless, the mass spectrometric analysis of the N -glycans isolated from anti-CD20-IgG produced by the Rmd-negative or Rmd-positive baculovirus vectors clearly demonstrated that the new vector designed to express Rmd early in infection can block recombinant glycoprotein fucosylation in the baculovirus-insect cell system.

Discussion

Core $\alpha 1,3$ -fucosylation generates an immunogenic sugar epitope that has significantly hindered development and utilization of the baculovirus-insect cell system for the production of recombinant glycoproteins for therapeutic drug and diagnostic applications in human medicine. In addition, core $\alpha 1,6$ -fucosylation of certain types of recombinant antibodies in this system and others represses their effector functions. Thus, the basic purpose of this study was to develop new tools that could be used to produce nonfucosylated recombinant glycoproteins, including antibodies, in the baculovirus-insect cell system.

It is well established that High FiveTM, which is a widely used insect cell line derived from *Trichoplusia ni*, produces high levels of immunogenic core $\alpha 1,3$ -fucosylated N -glycans. In the first part of this study, we showed that Tni PROTM cells, also derived from *Trichoplusia ni*, produce high levels of immunogenic core $\alpha 1,3$ -fucosylated N -glycans, as well. This finding is relevant because High FiveTM and Tni PROTM cells can potentially produce recombinant glycoproteins at higher levels than other insect cell lines (Davis et al. 1992; Krammer et al. 2010). Tni PROTM cells have the additional advantage of being easier to culture in suspension and, unlike High FiveTM cells (Dee et al. 1997; Taticek et al. 1997; Savary et al. 1999), are directly transferrable without adaptation from serum containing to serum-free ESF 921 medium (unpublished observations). In the course of this study, we found that a mouse IgG2a-Fc domain and a therapeutic anti-CD20-IgG were both core $\alpha 1,6$ -fucosylated when produced in Sf9 cells and core $\alpha 1,6$ - and core $\alpha 1,3$ -fucosylated when produced in High FiveTM or Tni PROTM cells. While these results were not

surprising in view of previous literature, they were important because they clearly justified an effort to block recombinant glycoprotein fucosylation in the baculovirus-insect cell system.

To accomplish this goal, we focused on a bacterial enzyme, Rmd, which consumes the direct precursor to GDP-L-fucose and was expected to block recombinant glycoprotein fucosylation in insect cell lines. In fact, previous work had shown that core $\alpha 1,6$ -fucosylation could be blocked in CHO cells genetically transformed to overexpress this enzyme (von Horsten et al. 2010). Thus, our analogous initial approach was to transform Sf9 and High FiveTM cells to constitutively express Rmd under the control of the AcMNPV *ie1* promoter. We successfully isolated Sf9 and High FiveTM cell subclones that initially had fucosylation-negative phenotypes and were able to produce a nonfucosylated recombinant glycoprotein. However, this phenotype was unstable, as both insect cell lines reverted to fucosylation-positive phenotypes after a relatively small number of passages in culture. This surprising result revealed that the cell engineering approach used to block core $\alpha 1,6$ -fucosylation in CHO cells could not be used successfully to block core $\alpha 1,6$ - and/or $\alpha 1,3$ -fucosylation in insect cell systems. Thus, we subsequently focused on a new approach that involved glycoengineering the baculovirus vector, rather than the host.

We assessed the efficacy of our proposed vector engineering approach by co-infecting Sf9 and Tni PROTM cells with separate baculovirus vectors encoding Rmd under the control of the *ie1* promoter or mIgG2a-Fc under the control of the *polyhedrin* promoter. Analysis of the resulting N -glycosylation patterns showed that Rmd pre-expression could block core fucosylation of mIgG2a-Fc produced later in infection. This encouraged us to proceed with our plan to isolate a new baculovirus vector designed not only to express Rmd immediately after infection, but also to enable quick and efficient isolation of daughter vectors capable of expressing any recombinant glycoprotein of interest at later times after infection. After isolating and characterizing AcRMD, the parent baculovirus vector, we used it to isolate a daughter encoding the heavy and light chains of rituximab, an anti-CD20-IgG, under the control of dual, back-to-back *p6.9* promoters. We chose the late *p6.9*, rather than the very late *polyhedrin* promoter to drive expression of this biotechnologically relevant recombinant glycoprotein because we found that it provided a higher efficiency of IgG secretion. Finally, we showed that this novel baculovirus vector could be used to produce recombinant anti-CD20-IgG with no detectable core $\alpha 1,6$ - or $\alpha 1,3$ -fucosylation. This conclusion was based on results obtained from several different methods of N -glycan analysis, including endoglycosidase treatments, lectin blotting assays and MALDI-TOF MS profiling. It is important to note that our MALDI-TOF MS profiling results indicating there were no detectable fucosylated N -glycans on the recombinant anti-CD20-IgG produced by the Rmd-positive baculovirus vector were obtained using N -glycans isolated with a highly active form of PNGase-A (PNGaseA_R; New England Biolabs), which effectively removes core $\alpha 1,3$ -fucosylated structures, and with glycan detection levels in the picomolar range. It also is important to note that our MALDI-TOF MS results revealed no detectable N -glycans containing any deoxyhexose, which supported the idea that GDP-rhamnose is a dead-end product that cannot be utilized for N -glycan modification in the baculovirus-insect cell system.

There are clear advantages to engineering the baculovirus vector, rather than host cell lines to block recombinant glycoprotein fucosylation in the baculovirus-insect cell system. One is that any investigator familiar with the system can use the new AcRMD vector in conjunction with an established linearized viral DNA (Kitts and Possee 1993) approach for homologs recombination with conventional, familiar, even pre-existing baculovirus transfer plasmids to efficiently isolate daughter baculovirus vectors encoding their favorite recombinant glycoprotein. Another is that the resulting daughter vectors can be used to produce nonfucosylated forms of that product in standard, familiar, commercially available insect cell lines, such as Sf9, High Five™, and Tni PRO™, even if the investigators favorite cell line normally produces high levels of α 1,3-fucosylated *N*-glycans. This eliminates the need to maintain specialized cell lines transformed to block recombinant glycoprotein fucosylation, which might require different growth media and/or conditions and have different growth properties, all of which complicate routine cell culture operations. Overall, it is highly advantageous to be able to produce nonfucosylated recombinant glycoproteins by simply replacing the conventional recombinant baculovirus used for standardized production runs with its AcRMD counterpart, without having to alter or re-optimize existing protocols. Most importantly, engineering the virus reduces the likelihood of genetic instability because low passage virus stocks can be produced, checked and stored in a biologically inert state. In contrast, cell lines transformed to constitutively express Rmd must be maintained in culture and, therefore, subjected to constant selective pressure, which can drive loss of the fucosylation-negative phenotype, as observed in this study. Mammalian cell expression systems engineered to produce nonfucosylated recombinant glycoproteins, such as FG1, CHO SM 3G1, CHO FUT8^{-/-}, RMD-CHO and CHO-DUKX (Imai-Nishiyama et al. 2007; Kanda et al. 2007; Malphettes et al. 2010; von Horsten et al. 2010; Zhong et al. 2012), are also subject to the potential problem of long-term instability. In addition, mammalian cells have a salvage pathway that can produce GDP-L-fucose using exogenous fucose, which is a common contaminant of many cell culture components. This salvage pathway can rescue recombinant glycoprotein fucosylation in all mammalian cell lines engineered to eliminate this modification except the FUT8 knockout line. This is unlikely to be a problem in the baculovirus-insect cell system because insects do not appear to encode the enzymes involved in the salvage pathways for GDP-L-fucose biosynthesis.

Arguably, the most important feature of the novel baculovirus vector described in this study is its ability to eliminate the immunogenic sugar epitope resulting from core α 1,3-fucosylation of recombinant glycoproteins. This will enable investigators to exploit the potentially higher productivity of insect cell lines derived from *Trichoplusia ni* for recombinant glycoprotein manufacturing (Davis et al. 1992; Krammer et al. 2010). It also will expand the utility of the baculovirus-insect cell system to include the production of recombinant glycoproteins for human clinical applications, including therapeutics and diagnostics. Historically, the production of recombinant glycoproteins for therapeutic use in humans has not been a legitimate application of the baculovirus-insect cell system. This study shifts this paradigm because the new baculovirus vector described herein can

block core α 1,3-fucosylation in insect cell lines glycoengineered to produce humanized, terminally sialylated *N*-glycans. The combination of these emerging tools constitutes a novel baculovirus-insect cell platform that can be used to manufacture safe and efficacious glycoproteins for human therapy.

Another important feature of the novel baculovirus vector described in this study is its ability to block core α 1,6-fucosylation, which is a common modification of a conserved *N*-glycan on the Fc domain that represses the effector functions of certain types of therapeutic antibodies. As noted above, core α 1,6-fucosylation has been blocked in other expression systems in efforts to produce therapeutic antibodies with enhanced effector functions and, therefore, higher efficacy at lower doses (reviewed by Yamane-Ohnuki and Satoh 2009). Sf9 and Tni PRO™ cells infected with the AcRMD daughter vector encoding anti-CD20-IgG produced a nonfucosylated form of this antibody, which is expected to have enhanced effector function, based on a significant body of previous literature (Shields et al. 2002; Shinkawa et al. 2003; Li et al. 2013; reviewed by Lim et al. 2010; Owen and Stewart 2012). Moreover, while many recombinant antibodies have been produced in the baculovirus-insect cell system (reviewed by Cérutti and Golay 2012), most were expressed using dual, back-to-back, very late *polyhedrin* and *p10* promoters to express the heavy and light chains and the heavy chain product. In this study, we also found that expression of the heavy and light chains under the control of dual, back-to-back, late *p6.9* promoters separated by the second intron from the *Drosophila* white gene provided a higher secretion efficiency than the analogous arrangement of very late *polyhedrin* promoters (Supplementary data, Figure S4). This result is consistent with those obtained in similar studies on the relationship between the timing of promoter activation and the efficiency of recombinant glycoprotein processing (Sridhar et al. 1993; Rankl et al. 1994; Bozon et al. 1995; Kost et al. 1997; Toth et al. 2011).

Materials and methods

Plasmid constructions

The *Pseudomonas aeruginosa* Rmd (GenBank: AAG08839.1) coding sequence was optimized for *Spodoptera frugiperda*, synthesized by GeneArt® (Life Technologies, Gaithersburg, MD), and cloned into pMK-T to produce a plasmid designated pKan-RMD.

pIE1-RMD, a plasmid designed to express Rmd under the control of the AcMNPV *ie1* promoter, was constructed by subcloning the *Bam*HI-*Not*I fragment of pKan-RMD into the *Bam*HI and *Not*I sites of pIE1TV4 (Jarvis et al. 1996).

pAcP(+)*IE1*-RMD, a baculovirus transfer plasmid, was constructed by subcloning the *Bam*HI-*Not*I fragment of pIE1-RMD into the *Bgl*II and *Not*I sites of pAcP(+)*IE1*TV3 (Jarvis et al. 1996).

pDIE1-EGFP/RMD Δ Bsu36I, a plasmid designed to express both EGFP and Rmd under the control of dual, back-to-back AcMNPV *ie1* promoters, was constructed in four sequential steps. First, the bovine growth hormone (BGH) polyadenylation (poly A) signal was amplified by polymerase chain reaction (PCR) with pcDNA3.1 (Life Technologies) as the template and PmeI-BGHpolyA-Fw and SpeI-BGHpolyA-Rv as the primers. The resulting amplicon was digested with *Pme*I and

SpeI and cloned into the corresponding sites of pIE1TV4 to produce a plasmid designated pIE1-BR. Secondly, the EGFP coding sequence was amplified by PCR with pIE1-EGFP as the template and BamHI/KpnI-EGFP-Fw and NruI-EGFPstop-Rv as the primers. The resulting amplicon was digested with BamHI and NruI and subcloned into the BamHI and PmeI sites of pIE1-BR to produce a plasmid designated pIE1-EGFP-BGH. Thirdly, the *Bsu36I* site of Rmd was deleted by overlapping PCR mutagenesis. This involved amplifying the 5'-region of Rmd with NruI-RMD-Fw and RMD-BsuDel-Rv as the primers and the 3'-region of Rmd with RMD-BsuDel-Fw and ApaI-RMD-Rv as the primers, with pIE1-RMD as the template in both cases. The resulting 5'- and 3'-region Rmd amplicons were mixed and used as templates for an overlapping PCR with NruI-RMD-Fw and ApaI-RMD-Rv as the primers. The resulting RMDΔ*Bsu36I* amplicon was subcloned into pGEM-T (Promega, Madison, WI) to produce pGEMT-RMDΔ*Bsu36I*. Finally, the EGFP-BGH poly A signal and RMDΔ*Bsu36I* coding sequence were assembled by sequentially subcloning the *KpnI-HindIII* fragment of pIE1-EGFP-BGH and the *NruI-ApaI* fragment of pGEMT-RMDΔ*Bsu36I* into the corresponding sites of pDIE1-TOPO.3 (Shi et al. 2007) to produce the plasmid designated pDIE1-EGFP/RMDΔ*Bsu36I*.

To construct a baculovirus transfer vector targeting the *chiA/v-cath* locus, we produced three PCR amplicons comprised of AcMNPV orf124/lef7, *chiA* or *v-cath*/gp64 sequences and independently subcloned each one into pGEM-T. After verifying their sequences, these three DNA fragments were assembled by sequentially subcloning the *PmeI-ApaI* fragment of *chiA* and then the *ApaI* fragment of *v-cath*/gp64 into the corresponding sites of pGEMT-orf124/lef7 to produce a new plasmid designated pChi/CathTV6. Finally, we blunt-ended the *HindIII-BamHI* fragment of pDIE1-EGFP/RMDΔ*Bsu36I* with T4 DNA polymerase (New England Biolabs, Beverly, MA) and inserted the resulting DNA fragment into the T4 DNA polymerase blunt-ended *HindIII* and *EcoRI* sites of pChi/CathTV6. This transfer plasmid, which was designated pΔChi/Cath-EGFP/RMDΔ*Bsu36I*, was used to create the new baculovirus vector in which the viral *chiA* and *v-cath* genes were replaced by genes encoding Rmd and EGFP, each under the control of the AcMNPV *ie1* promoter.

pVL1393-polh-αCD20-IgG and pVL1393-p6.9-αCD20-IgG, which are baculovirus transfer plasmids used to isolate baculovirus expression vectors encoding anti-CD20-IgG, were constructed in four sequential steps. First, the BGH poly A signal was PCR amplified with pIE1-BR as the template and EcoRI-BGHpolyA-Fw and EcoRV-BGHpolyA-Rv as the primers. The resulting amplicon was digested with EcoRI and EcoRV and then subcloned into the corresponding sites of pVL1393 (Summers and Smith 1987) to produce pVL1393-BGH. Secondly, the *NotI-SbfI* fragment of pGEMT-αCD20HC, which encodes the heavy chain, and the *NotI-EcoRI* fragment of pGEMT-αCD20LC, which encodes the light chain of anti-CD20-IgG were sequentially subcloned into the *NotI-PstI* and *NotI-EcoRI* sites of pVL1393-BGH to produce pVL1393-αCD20-IgG-no promoter. Thirdly, two copies of the AcMNPV *polyhedrin* or *p6.9* promoter were assembled in back-to-back orientation, with the second intron of the *Drosophila melanogaster white* gene as an intervening spacer, to create plasmids designated either pGEMT-ppol-wi-ppol or pGEMT-p6.9-wi-p6.9, respectively. The former was constructed by

PCR-amplifying two copies of the *polyhedrin* promoter with pAcGT N-term 8xHis pPol (Toth et al. 2011) as the template and either *NheI*-ppol-Fw and *NotI*-ppol-Rv or *XhoI*-ppol-Fw and *SphI*/PmeI-ppol-Rv as the primers. The resulting amplicons were digested with *NheI* and *NotI* or *XhoI* and *SphI*, respectively, and sequentially subcloned into the corresponding sites of pGEM-WIZ (Bao and Cagan 2006). Similarly, pGEMT-p6.9-wi-p6.9 was constructed by PCR-amplifying two copies of the *p6.9* promoter with pAcp6.9GT N-term 8xHis pPol (Toth et al. 2011) as the template and either *NheI*-p6.9-Fw and *NotI*-p6.9-Rv or *XhoI*-p6.9-Fw and *SphI*/PmeI-p6.9-Rv as the primers. The resulting amplicons were digested with *NheI* and *NotI* or *XhoI* and *SphI*, respectively, and sequentially subcloned into the corresponding sites of pGEM-WIZ. Finally, each back-to-back promoter cassette was independently subcloned into the anti-CD20-IgG baculovirus transfer plasmid described above. pVL1393-polh-αCD20-IgG was constructed by subcloning the *PmeI-NotI* fragment of pGEMT-ppol-wi-ppol and pVL1393-p6.9-αCD20-IgG was constructed by subcloning the *PmeI-NotI* fragment of pGEMT-p6.9-wi-p6.9 into the corresponding sites of pVL1393-αCD20-IgG_no promoter, respectively (Supplementary data, Figure S3).

Phusion® *Taq* DNA polymerase (New England Biolabs) and a Biometra TProfessional Standard Thermocycler (Göttingen, Germany) were used for all PCRs and all primer sequences are given in Supplementary data, Table S1.

Cells and viruses

Sf9 and Tni PRO™ (Expression Systems, Woodland, CA) cells were routinely maintained as shake-flask cultures in ESF 921 medium (Expression Systems) at 28°C. High Five™ cells (Life Technologies) were maintained as adherent cultures in Trichoplusia ni medium-Fred Hink (TNM-FH) medium containing 10% (v/v) fetal bovine serum (Atlanta Biologics, Atlanta, GA).

SfRMD is a transgenic Sf9 cell derivative that was produced for this study using a modification of an established procedure (Harrison and Jarvis 2007). Briefly, Sf9 cells were co-transfected with a mixture of pIE1-Neo (Jarvis et al. 1990) and pIE1-RMD using a modified calcium phosphate method (Summers and Smith 1987). The transfected cells were allowed to recover for 1 day, treated with 1 mg of G418 (Life Technologies)/mL of TNM-FH medium containing 10% (v/v) fetal bovine serum (Atlanta Biologics) for 1 week, and G418-resistant clones were isolated by limiting dilution (Hollister and Jarvis 2001). After stepwise amplification into larger cultures, individual clones were assayed by cell surface staining with a fucose-specific lectin, as described below in the Materials and methods section. Unstained clones were designated SfRMD, characterized in more detail, and used for downstream experiments, as described below in the Materials and methods section.

TnRMD is a transgenic High Five™ cell derivative that was designed to constitutively express the Rmd gene and produced for this study as for SfRMD.

The baculovirus expression vector designated AcmIgG2a-Fc has been described previously (Geisler and Jarvis 2012).

The baculovirus expression vector designated AcP(+)IE1-RMD was isolated by co-transfecting Sf9 cells with a mixture of pAcP

(+)IE1-RMD and BacPAK6 (Kitts and Possee 1993) baculoviral DNA after pre-linearizing the latter by digestion with *Bsu36I*. Viral progeny were harvested, clones were resolved by plaque assay in the presence of X-Gal (Sigma-Aldrich; St. Louis, MO), and an isolated clone with a white plaque phenotype was amplified and titered in Sf9 cells.

Baculovirus expression vectors designated Ac- α CD20-IgG and Acp6.9- α CD20-IgG were isolated by co-transfecting Sf9 cells with mixtures of either pVL1393-polh- α CD20-IgG or pVL1393-p6.9- α CD20-IgG and AcGT baculoviral DNA (Toth et al. 2011) after pre-linearizing the latter by digestion with *Bsu36I* (Supplementary data, Figure S3). The transfected cells were cultured for 5 days in growth medium containing 100 μ M ganciclovir (Life Technologies) and then viral progeny were harvested, clones were resolved by plaque assay, and an isolated clone with a white plaque phenotype was amplified and titered in Sf9 cells.

The baculovirus expression vector designated AcRMD was isolated by co-transfecting Sf9 cells with a mixture of p Δ Chi/Cath-EGFP/RMD Δ *Bsu36I* and BacPAK6 (Kitts and Possee 1993) baculoviral DNA. This recombination strategy was custom-designed to replace the AcMNPV *chiA* and *v-cath* coding sequences with Rmd and EGFP coding sequences placed under the control of dual AcMNPV *ie1* promoters separated by the AcMNPV *hr5* enhancer. Viral progeny were harvested, clones were resolved by plaque assay, and fluorescent plaques were identified using an Axiovert 25 microscope equipped with HBO 50 epi-fluorescence unit (Carl Zeiss, Oberkochen, Germany). Several plaques were picked, screened by PCR, as described below in the Materials and methods section, and those that included viral progeny with the Rmd and EGFP genes in the correct genomic location were used for a second round of plaque purification. The PCR screen was repeated, promising clones were used for a third round of plaque purification, and well-isolated clones with fluorescent plaque phenotypes were picked and amplified in Sf9 cells. A Wizard genomic DNA purification kit (Promega) was used to extract total DNA from Sf9 cells infected with each of those viral clones and used as the templates for final PCR analyses, which were the same as those performed for the prior screening steps. In each case, we performed PCRs with two different primer pairs, orf124-Fw and RMD-Rv or EGFP-Fw and gp64-Rv to determine if the final AcRMD isolates had the EGFP/RMD Δ *Bsu36I* expression cassette in the correct genomic location, that is, in place of the viral *chiA* and *v-cath* genes. In addition, we used a third primer pair, *chi*-Fw and *cath*-Rv, to directly confirm the absence of those viral genes and show that the final AcRMD isolates were not detectably contaminated with parental BacPAK6 vector DNA. Three clones were positive with the first two primer pairs and negative with the third and one clone was designated AcRMD, further amplified in Sf9 cells, and used for the remainder of this study.

Baculovirus expression vectors designated AcRMD- α CD20-IgG and AcRMDp6.9- α CD20-IgG were isolated by co-transfecting Sf9 cells with a mixture of pVL1393-polh- α CD20-IgG or pVL1393-p6.9- α CD20-IgG and AcRMD baculoviral DNA pre-linearized by digestion with *Bsu36I*. Viral progeny were harvested, clones were resolved by plaque assay, and an isolated clone with a white plaque phenotype was amplified and titered in Sf9 cells.

Cell surface lectin staining

Sf9, SfRMD, High Five™ or TnRMD cells were stained with a mixture of biotinylated AAL (Vector Labs, Burlingame, CA) and fluorescein-conjugated streptavidin (Vector Labs) in lectin staining buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.5, 50 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM MnCl₂) for 10 min at room temperature. The cells were then washed twice with lectin staining buffer and imaged using an Olympus FSX 100 fluorescence microscope (Tokyo, Japan).

Recombinant protein expression and purification

For small scale recombinant protein expression experiments, Sf9 cells were seeded at a density of 2×10^6 cells/well into 6-well plates and infected with Ac- α CD20-IgG, Acp6.9- α CD20-IgG, AcRMD- α CD20-IgG or AcRMDp6.9- α CD20-IgG at a multiplicity of ~ 2 plaque forming units/cell. The virus was allowed to adsorb for 1 h and then the infected cells were washed once with 1 mL of ESF 921 and cultured in 1 mL of fresh ESF 921 at 28°C to 48 h postinfection. At that time, the cell-free media were prepared by low speed centrifugation and used as the extracellular fractions for downstream analysis. The cell pellets were washed once with phosphate buffered saline, lysed with 1 mL of extraction buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 0.1 mM ethylenediaminetetraacetic acid, 0.5% Nonidet P-40), clarified by centrifugation at 13,000 rpm for 10 min, and the supernatants were used as the intracellular fractions for downstream analysis.

For larger scale recombinant protein expression and purification, Sf9, Tni PRO™ or High Five™ cells were seeded into 50 mL shake-flask cultures at a density of 2×10^6 cells/mL in ESF 921 and then infected with Acp6.9- α CD20-IgG, AcRMDp6.9- α CD20-IgG, AcmlgG2a-Fc or a mixture of AcmlgG2a-Fc and AcP(+)IE1-RMD using a multiplicity of ~ 2 plaque forming units/cell for each virus. After a 1 h adsorption period, the infected cells were gently pelleted, resuspended in 50 mL of fresh ESF 921 supplemented with antibiotics (1.25 μ g/mL amphotericin B and 25 μ g/mL gentamicin), returned to the shake flasks, and incubated at 28°C to 48 h postinfection. Cells and debris were pelleted by centrifugation at $1000 \times g$ for 10 min at 4°C, the supernatants were harvested, and budded baculovirus progeny were removed by centrifugation at $70,000 \times g$ for 30 min at 4°C. One Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics, Indianapolis, IN) was dissolved in each final supernatant and then each was transferred into a 12–14,000 molecular weight cut-off membrane (Spectrum Labs, Rancho-Dominguez, CA) and dialyzed against 0.1 M NaCl for 6 h. Each anti-CD20-IgG or mlgG2a-Fc preparation was subsequently dialyzed against 0.15 M or 0.5 M NaCl, respectively, and then each was purified by was affinity-purified Protein A agarose (GenScript, Piscataway, NJ) or ProBond nickel (Life Technologies) affinity chromatography, respectively, according to the manufacturer's instructions. Eluted fractions containing the purified proteins were pooled and desalted on PD10 columns (GE Healthcare) equilibrated with phosphate buffered saline.

SDS-PAGE, western blotting and lectin blotting analyses

Several different types of samples were isolated from Sf9, Tni PRO™ and/or High Five™ cells and used for SDS-PAGE,

western blotting and/or lectin blotting analysis. These included intracellular fractions from uninfected cells, extracellular fractions from cells infected with various recombinant baculoviruses, and anti-CD20-IgG or mIgG2a-Fc purified from cells infected with various recombinant baculoviruses. In some cases, the samples were pretreated with PNGase-F in reaction buffer (New England Biolabs) or PNGase-F reaction buffer alone according to the manufacturer's instructions, as described in the figure legends. All samples were boiled in Laemmli sample buffer and then proteins were resolved by SDS-PAGE on 12% polyacrylamide gels and either stained with Coomassie Brilliant Blue, destained and imaged or electrophoretically transferred to Immobilon-P membranes (Millipore). The latter were blocked for 1 h at room temperature with Tris-buffered saline (150 mM NaCl in 50 mM Tris-HCl, pH 7.5) containing either 5% bovine serum albumin (w/v; Sigma-Aldrich) and 0.5% (v/v) Tween 20 (Sigma-Aldrich) or 1% Tween 20 for western or lectin blotting assays, respectively. After blocking, western blotting assays were completed using alkaline phosphatase-conjugated goat anti-human IgG κ chain (Sigma-Aldrich) to detect the anti-CD20-IgG light chain, alkaline phosphatase-conjugated goat anti-human IgG Fc (Sigma-Aldrich) to detect the anti-CD20-IgG heavy chain, alkaline phosphatase-conjugated goat anti-mouse IgG to detect mIgG2a-Fc or rabbit anti-HRP IgG (Gentaur, Brussels, Belgium) as the primary and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) as the secondary antibody to detect core α 1,3-linked fucose. The lectin blotting assays were completed using alkaline phosphatase-conjugated AAL (Vector Laboratories) and the probes used for all western and lectin blotting assays were detected using a standard chromogenic assay for alkaline phosphatase activity (Blake et al. 1984).

Mass spectrometry

N-glycans were enzymatically released from various purified mIgG2a-Fc and anti-CD20-IgG preparations by exhaustive digestion with PNGaseAr (New England Biolabs). The spent reactions were applied to preconditioned C18 SepPak cartridges (Waters Corp., Milford, MA) and the flow-through and a 5% (v/v) aqueous acetic acid wash were pooled, evaporated and permethylated (Dell et al. 1994). The permethylated *N*-glycan derivatives were extracted into chloroform, pooled with several aqueous washes, re-evaporated, and then resuspended in acetonitrile, mixed 1:1 with 2,5-dihydroxybenzoic acid matrix (10 mg/mL in 50% aqueous acetonitrile), and samples were spotted onto the MALDI-TOF target plate. Data acquisition was performed manually on a Model 4700 Proteomics Analyzer equipped with an Nd:YAG laser (Applied Biosystems, Framingham, MA) and 1000 shots were accumulated in the reflectron positive ion mode.

Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

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Conflict of interest

D.L.J. is the founder of a new biotech company, GlycoBac, LLC that will focus on the glycoengineered insect cell platform as a tool for recombinant glycoprotein production and serves Expression Systems, LLC, as a paid consultant. The other authors have no potential conflict of interest.

Abbreviations

AAL, *Aleuria aurantia* lectin; AcMNPV, *Autographa californica* multicapsid nucleopolyhedrovirus; BGH, bovine growth hormone; CHO, Chinese hamster ovary; EGFP, FPGT, fucose-1-phosphate guanylyltransferase; FUK, fucokinase; FUT8, fucosyltransferase; Fx protein, GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase; GFR, Golgi GDP-L-fucose transporter; GMD, GDP-D-mannose 4,6-dehydratase; HRP, horseradish peroxidase; *Ie1*, immediate early 1; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; mIgG2a-Fc, Fc domain of mouse IgG2a; PCR, polymerase chain reaction; PNGase-F, peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase; Rmd, GDP-4-dehydro-6-deoxy-D-mannose reductase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Table S1. Primers used for this study.

Primer name	Sequence (5' to 3')
PmeI-BGHpolyA-Fw	gtttaaagcctcgactgtgccttctagttg
SpeI-BGHpolyA-Rv	actagttccccagcatgcctgctatt
BamHI/KpnI-EGFP-Fw	aatggatccggtaccaccatggtagcaagggcg
NruI-EGFPstop-Rv	ggcacttcgcgattactgtacagctcgtccatgcc
NruI-RMD-Fw	ttatctcgcgaacctgactcaacgcttggtcg
RMD-BsuDel-Rv	atcacggaatgcctcgggtacgtatg
RMD-BsuDel-Fw	gctgggtcaaatacgtacccgaggc
ApaI-RMD-Rv	tggggccttactcctcttaacacgagattccca
EcoRI-BGHpolyA-Fw	attgtgaattcgctcgactgtgccttctagttgc
EcoRV-BGHpolyA-Rv	taattgatatctccccagcatgcctgctattg
NheI-ppol-Fw	gccgcgctagcatcatggagataattaaatgataaccatctcgaaataa
NotI-ppol-Rv	aatttgccggccgcagcgcccgatgggtgggacg
XhoI-ppol-Fw	gggcctcgagatcatggagataattaaatgataaccatctcgaaataa
SphI/PmeI-ppol-Rv	atttgcattgcgtttaaacagcgcccgatgggtgggacg
NheI-p6.9-Fw	gccgcgctagcaaattccgttttgcgacgatg
NotI-p6.9-Rv	aatttgccggccgcgtttaaattgtgtaatttatgtagctgtaattttacc
XhoI-p6.9-Fw	gggcctcgagaaattccgttttgcgacgatg
SphI/PmeI-p6.9-Rv	atttgcattgcgtttaaacgtttaaattgtgtaatttatgtagctgtaattttacc
orf124-Fw	atttgattttaatcaatcgaaccgtgcac
RMD-Rv	gattgggaatctcgtgtagagaggagtaa
EGFP-Fw	atcttctcaaggacgacggcaac
gp64-Rv	agcaagatggtaagcgctattgttttatatgtgc
chi-Fw	agatgggtatgaaaccatacaacaagtgtg
cath-Rv	cgctaccataatctttgttgatcgatg

Supplementary Figure Legends

Fig. S1. Phylogenetic trees of genes involved in GDP-L-fucose biosynthesis. Phylogenetic analysis of FUK (A), FPGT (B), GMD (C), and Fx protein (D) genes was performed using the Maximum Likelihood method with MEGA5 software (Tamura et al. 2011). The bars indicate the number of substitutions per site. Abbreviations: Hs, *Homo sapiens*; Mm, *Mus musculus*; Bt, *Bos taurus*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; Dp, *Daphnia pulex*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Dm, *Drosophila melanogaster*; Aa, *Aedes aegypti*; Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*; Bm, *Bombyx mori*

Fig. S2. Fucosylation of mIgG2a-Fc produced in pIE1-RMD-transformed insect cell lines. Sf9, SfRMD 2B2, High Five™, and TnRMD 6A6 cells were infected with Acp6.9-mIgG2a-Fc, a recombinant baculovirus encoding mIgG2a-Fc. The cell-free media were harvested at 48 hours post-infection and used to affinity purify each mIgG2a-Fc preparation for lectin blotting analysis with AAL (specific for fucose) or western blotting analysis with anti-mouse IgG with (+) or without (-) PNGase-F pre-treatment, as indicated.

Fig. S3. Genetic maps of recombinant baculoviruses encoding anti-CD20-IgG. The recombinant baculoviruses designated Ac- α CD20-IgG and Acp6.9- α CD20-IgG were isolated by using homologous recombination to replace the *lacZ* sequence in the parental baculovirus, AcGT (Toth et al. 2011), with expression cassettes encoding the anti-CD20-IgG heavy and light chains under the control of dual, back-to-back *polyhedrin* or *p6.9* promoters separated by the second intron of the *Drosophila melanogaster white* gene, respectively. Similarly, the recombinant baculoviruses designated AcRMD- α CD20-IgG and AcRMDp6.9- α CD20-IgG were isolated by using homologous recombination to

replace the *lacZ* sequence of the parental baculovirus, AcRMD (this study), with the same expression cassettes used to isolate Ac- α CD20-IgG and Acp6.9- α CD20-IgG.

Fig. S4. Expression and secretion of anti-CD20-IgG under the control of late or very late baculoviral promoters. Sf9 cells were infected with AcMNPV, Ac-CD20-IgG, Acp6.9- α CD20-IgG, AcRMD- α CD20-IgG, or AcRMDp6.9- α CD20-IgG. At 48 hours post-infection, the cell-free culture media were collected as the extracellular fraction, the cells were lysed, and the clarified supernatants were collected as the intracellular fraction. Proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-human IgG Fc-specific (α -HC) or anti-human IgG κ chain-specific antibodies (α -LC).

Fig. S5. Analysis of purified anti-CD20-IgG preparations from Sf9 and Tni PROTM. The anti-CD20-IgG preparations produced by Sf9 or Tni PROTM cells infected with Acp6.9- α CD20-IgG or AcRMDp6.9- α CD20-IgG were affinity purified using protein A, as described in Materials and methods, resolved by SDS-PAGE under reducing (A) or non-reducing (B) conditions, and stained with Coomassie Brilliant Blue.

