Directing Flux in Glycan Biosynthetic Pathways with a Small Molecule Switch

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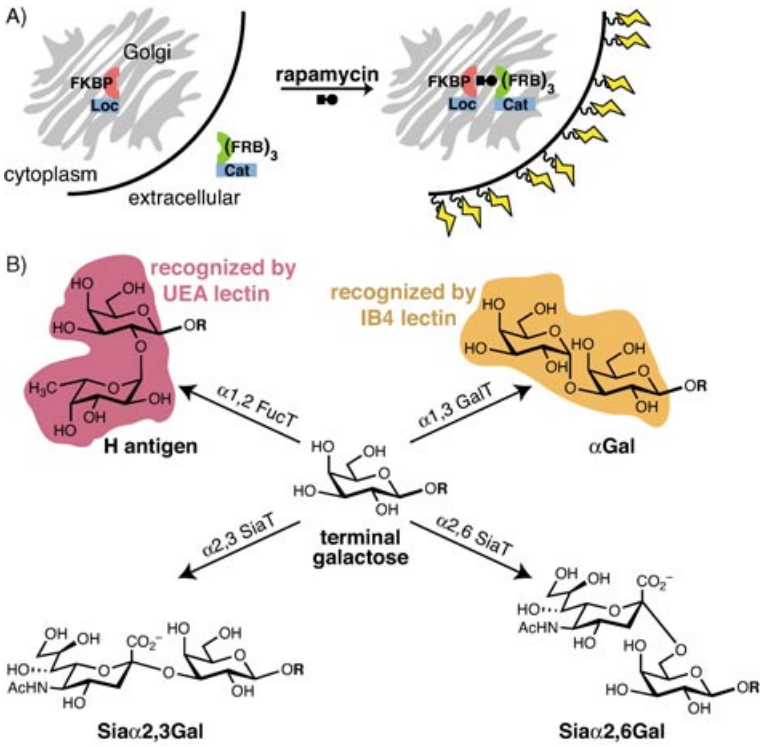
The diverse array of complex glycans displayed on the surface of mammalian cells is synthesized by the coordinate action of Golgi-resident glycosyltransferases. Deciphering glycosyltransferases' relative contributions in oligosaccharide biosynthesis is complicated by their functional redundancy and the embryonic lethality of gene knock-outs. While the in vitro activities and specificities of certain glycosyltransferases have been delineated, cellular activity of these enzymes is often more limited. Localization of glycosyltransferases to particular subcompartments of the Golgi complex is one mechanism for their restricted cellular activity.

Taking advantage of the critical role of Golgi localization, we previously reported a general method for small-molecule control of glycosyltransferase activity in cells.[1] This method (Scheme 1 A) does not demand the synthesis of bioavailable active-site inhibitors; rather, it takes advantage of the modularity of glycosyltransferases' localization (Loc) and catalytic (Cat) domains.[2, 3] The two domains are expressed as separate polypeptides, each fused to a rapamycin-binding protein, either FKBP or FRB. In the absence of rapamycin, the catalytic domain fails to localize to the Golgi and, consequently, is unable to access its normal substrates. Therefore, no product is observed. The addition of rapamycin induces heterodimerization of the localization and catalytic domains, reconstituting the enzyme and restoring activity. In these experiments, we use the T2098L mutant of FRB, which is susceptible to degradation in the absence of rapamycin.[4] The activity increase that we observe with addition of rapamycin may be due in part to rapamycin-induced stabilization of FRB-containing constructs.



Scheme 1. *Directing biosynthetic traffic by small-molecule activation of FUT1. A) Schematic representation of the method by which rapamycin activates FUT1, which results in cell-surface expression of FUT1's product. B) Terminal β-linked galactose is a substrate for four different families of glycosyltransferases: α-1,2-fucosyltransferases, α-1,3-galactosyltransferases, α-2,3-sialyltransferases, and α-2,6-sialyltransferases. The relative activities and localization of these enzymes determines the biosynthetic flux toward possible final glycans. H antigen (Fucα1,2Gal) can be detected with* Ulex europaeus *lectin 1 (UEA) while αGal (Galα1,3Gal) is recognized by isolectin B_4 from* Bandeiraea simplicifolia *(IB4).*

Here we apply the small-molecule method to direct flux through the biosynthetic pathways that determine the ultimate fate of glycans terminating in β-linked galactose (Scheme 1 B). Fucosyltransferase 1[5] (FUT1) catalyzes the transfer of fucose to the 2-position of terminal β-linked galactose; this generates Fucα1,2Gal, also known as H antigen. Terminal β-linked galactose is also a substrate for a variety of other Golgi-resident glycosyltransferases, including α-1,3-galactosyltransferases, α-2,3-sialyltransferases, and α-2,6-sialyltransferases.[6] Modification of galactose by any of these other enzymes precludes the production of H antigen. In any particular glycan, the observed modification of galactose will depend on the set of glycosyltransferases expressed in that cell type and their localization within the Golgi to sites proximal to enzymes responsible for generating terminal galactose substrate. Thus, modulation of FUT1 activity effectively directs glycan biosynthetic traffic toward, or away from, production of H antigen.

Competition between FUT1 and α-1,3-galactosyltransferase (GGTA1) for Galβ1,4GlcNAc (LacNAc) is of particular interest in the area of xenotransplantation. The action of GGTA1 on galactose generates a structure (Galα1,3Gal) known as αGal

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(Scheme 1B). This structure is present in porcine and other nonprimate mammals, but absent in humans due to their lack of a functional α-1,3-galactosyltransferase.[7] As a result, pig organs transplanted into humans are subject to attack by an anti-αGal immune response.[8,9] Expression of FUT1 in porcine tissue has been postulated as a mechanism for circumventing this problem: FUT1 can usurp the terminal galactose-containing substrate and convert it to H antigen, a normal blood-group antigen found in almost all humans.[10,11]

In this report, we converted FUT1 into two constructs that encode a rapamycin-inducible pair of proteins. The small-molecule-controlled FUT1 engaged in the same competition with GGTA1 as wild-type FUT1 does. We also show that heterologous localization domains can be used to retain the FUT1 catalytic domain within the Golgi, where it has the potential to be active on various pools of terminal galactose.

To identify the localization and catalytic domains of human FUT1, we performed a sequence alignment of eleven α-1,2-fucosyltransferases from various species (See Figure S1 in the Supporting Information). These enzymes are highly homologous in their C-terminal catalytic domains but diverge in the N-terminal regions. Based on similarities among these enzymes, we chose to define amino acids 1–66 of FUT1 as its localization domain and amino acids 58–365 as the catalytic domain. In previous experiments with another fucosyltransferase,[1] we found that attachment of one copy of FKBP to the localization domain and three copies of FRB to the catalytic domain yielded the most rapamycin-responsive proteins. We prepared plasmids that encode analogous constructs—Loc-FKBP and 3XFRB-Cat—based on FUT1. (We also prepared the 1XFRB-Cat plasmid based on FUT1, but found it to be less effective. See Figure S2 in the Supporting Information.)

Wild-type Chinese hamster ovary (CHO) cells, which lack endogenous α-1,2-fucosyltransferase activity, were transfected with plasmids that encode the FUT1 domains. The resulting production of cell-surface H antigen was probed by staining with a fluorescently labeled lectin that recognizes H antigen (TRITC-UEA) and quantified by flow-cytometry analysis. Cells transfected with Loc-FKBP and 3XFRB-Cat showed low levels of H antigen expression in the absence of rapamycin (Figure 1A). Addition of rapamycin to these cells caused H antigen expression to increase to the level observed for cells transfected with a plasmid that encodes the full-length FUT1 gene. As expected, rapamycin control of FUT1 activity required the localization domain; cells transfected with 3XFRB-Cat alone did not show a rapamycin-induced increase in H antigen expression. Unlike results observed in our previous experiments with FUT7, here we observed some background H antigen expression when catalytic domains were expressed in the absence of rapamycin. It is possible that the FUT1 catalytic domain retains some Golgi-targeting information and that use of a truncated version of this domain would decrease the background activity.

To test whether rapamycin-inducible FUT1 was also effective in cells where GGTA1 is present to compete for the same substrate, we produced CHO cells that stably express GGTA1.[12] While wild-type CHO cells lack GGTA1 activity, the GGTA1(+)CHO cells we generated constitutively express high levels of

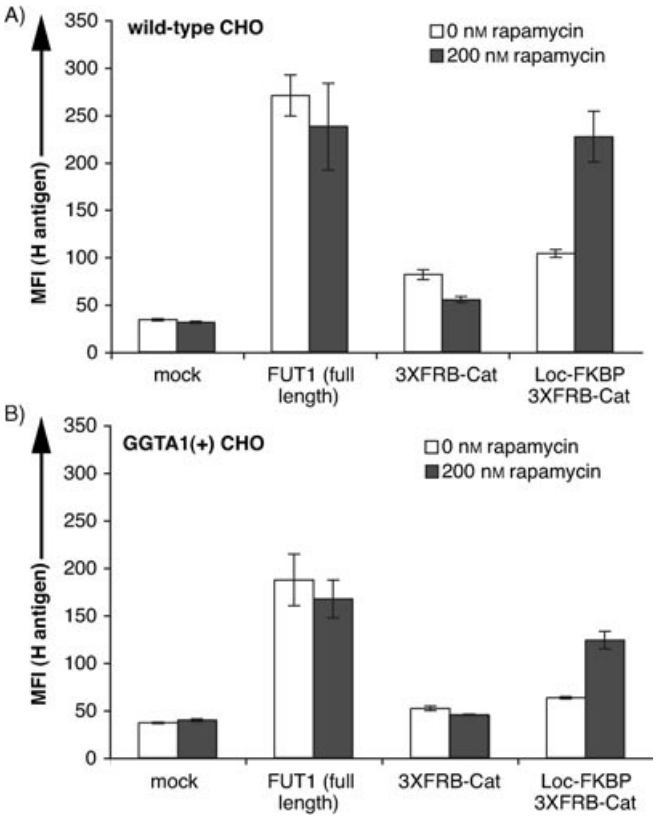

Figure 1. *FUT1 activity can be controlled with rapamycin. A) wild-type CHO or B) GGTA1(+)CHO were transfected with the indicated FUT1-derived plasmids, treated with 0 or 200 nM rapamycin, then probed for H antigen expression. The mean fluorescence intensity (MFI) of all live cells is plotted. Error bars indicate the standard deviation of three measurements.*

cell-surface αGal (not shown). When GGTA1(+)CHO cells were transfected with Loc-FKBP and 3XFRB-Cat, they showed rapamycin-inducible cell-surface H antigen expression (Figure 1B). Rapamycin treatment of GGTA1(+)CHO cells transfected with only 3XFRB-Cat did not increase H antigen production.

Consistent with the observations of others,[10,11] we found that transfection of GGTA1(+)CHO cells with the full-length FUT1 gene both increased H antigen expression and also decreased αGal production. A flow cytometry experiment was performed in which GGTA1(+)CHO cells were transfected with FUT1 and then stained with a fluorescently labeled lectin that recognizes αGal (FITC-IB4; Scheme 1B). When compared to mock transfected cells, FUT1-transfected cells produced lower levels of αGal (Figure 2A). GGTA1(+)CHO were also transfected with Loc-FKBP and 3XFRB-Cat; for these cells αGal expression levels depended on rapamycin treatment. As expected, rapamycin-treated cells expressed lower levels of αGal than their untreated counterparts (Figure 2A). This result indicates that rapamycin-inducible FUT1 functions analogously to the full-length FUT1 from which it is derived, similarly engaging in competition with GGTA1 for substrate. The small molecule can be used to divert their common substrate toward H antigen production and away from αGal.

We used immunofluorescence microscopy to determine the localization of the 3XFRB-Cat protein in cells treated with rapa-

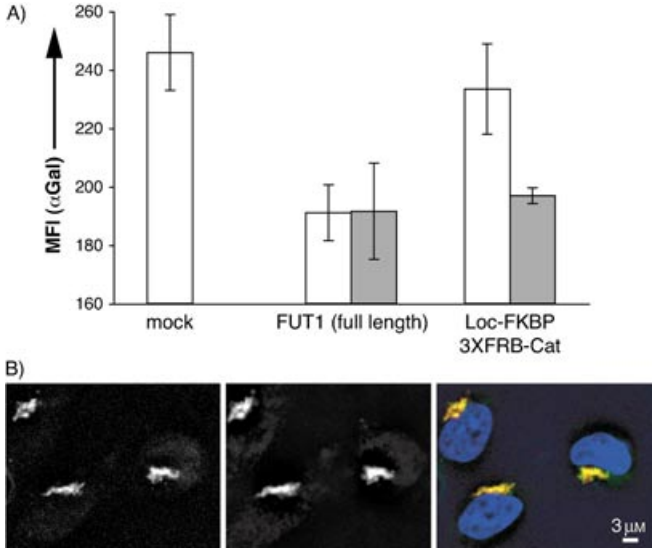

Figure 2. *A) H antigen production competes with αGal production. GGTA1(+) CHO cells were transfected with the indicated plasmids and treated with 0 (open bars) or 200 nM (filled bars) rapamycin, then probed for αGal expression, which was quantified by flow cytometry. The mean fluorescence intensity (MFI) of all live cells is plotted. Error bars indicate the standard deviation of three measurements. B) Rapamycin causes HA-tagged 3XFRB-Cat to be retained in the Golgi complex. Cells were transfected with Loc-FKBP and 3XFRB-Cat, treated with 200 nM rapamycin, and probed with antibodies against HA and giantin. The panels show single sections of a deconvolved data set. Left, the signal from HA staining shown in monochrome; center, the signal from giantin staining shown in monochrome; right, three-color overlay with HA staining shown in green, giantin staining in red, and nuclear DAPI staining shown in blue. Overlap between giantin and HA staining appears yellow in this image.*

mycin (Figure 2B). Cells were transfected with Loc-FKBP and 3XFRB-Cat plasmids, treated with rapamycin, then permeabilized and stained with two antibodies: one against the HA tag encoded in the 3XFRB-Cat construct and one against a resident marker of the *medial* Golgi, giantin. The overlap in the two staining patterns indicates that the catalytic domain resides in the Golgi in these cells.

Separating glycosyltransferases into modular localization and catalytic genes allows for their facile recombination. We took advantage of this feature of our system and tested the ability of a variety of localization domains to reconstitute FUT1 activity when combined with the FUT1 3XFRB-Cat chimera. We used localization domain-FKBP chimeras based on two other glycosyltransferases, β-1,2-*N*-acetylglucosaminyltransferase (GnTloc-FKBP)[13,14] and α-1,3-fucosyltransferase 7 (FUT7 loc-FKBP),[1] and two sulfotransferases, GlcNAc-6-sulfotransferase 1 (GlcNAc6ST1 loc-FKBP)[15] and GlcNAc-6-sulfotransferase 2 (GlcNAc6ST2 loc-FKBP).[15] Each of these localization domain constructs, as well as the one derived from FUT1 (FUT1 loc-FKBP), was transfected into wild-type or GGTA1(+) CHO cells along with the FUT1-derived 3XFRB-Cat. Previous studies suggest that FUT1 localizes in the *medial* Golgi;[16] terminal galactose available at this location is likely to be in the form of poly-*N*-acetyllactosamine (–Galβ1,4GlcNAc–).[17] Other localization domains that would be expected to localize similarly within the Golgi are GnTloc-FKBP[13,14] and GlcNAc6ST2 loc-FKBP,[15] while the localization domains FUT7 loc-FKBP[16] and GlcNAc6ST1 loc-FKBP[15] are expected to be targeted to late Golgi compartments.

All five localization domains examined were able to support rapamycin-inducible FUT1 activity both in wild-type (Figure 3A) and in GGTA1(+)CHO cells (Figure 3B). In all cases, transfected cells displayed lower levels of H antigen expression in the absence of rapamycin, and increased H antigen production with addition of rapamycin. Although we observed small differences in activity among the reconstituted enzymes, all were within threefold of that produced by the full-length FUT1 enzyme. The differences that we observed might result from different localization domains' relative proximity to the sites at which terminal galactose is synthesized. Alternatively, localization domains might vary in their expression levels, stability, or geometric constraints, all of which could affect their ability to reconstitute FUT1 activity. (Interestingly, we observed large dif-

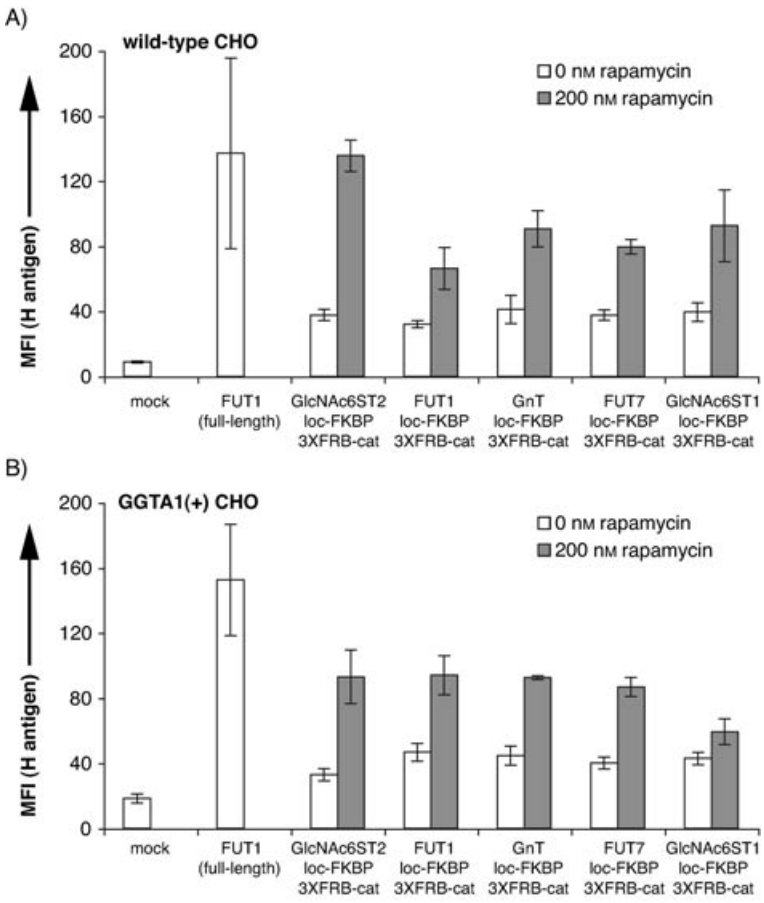

Figure 3. *Various localization domains support reconstituted FUT1 activity. A) Wild-type or B) GGTA1(+) CHO cells were transfected with the indicated plasmids, treated with 0 nM (open bars) or 200 nM (filled bars) rapamycin, then probed for H antigen expression. The mean fluorescence intensity (MFI) of all live cells is plotted. Error bars indicate the standard deviation of three measurements.*

ferences in localization domain expression levels, which do not seem to correlate with the levels of H antigen production. See Supporting Information, Figure S3.)

In summary, we have shown that FUT1 can be engineered for control by a small molecule. Small-molecule control of glycosyltransferase activity allows glycan flux to be effectively redirected from one biosynthetic pathway (production of αGal) to another (production of H antigen). Furthermore, the modularity of this system allows for easy recombination of various localization and catalytic domains.

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