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Regulating Cell Surface Glycosylation by Small Molecule Control of Enzyme Localization

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Summary

Cell surface carbohydrates mediate interactions between the cell and its environment. Glycosyltransferases responsible for synthesis of cell surface oligosaccharides are therefore essential administrators of cellular communication. These enzymes often comprise large families. Redundancy of related family members and embryonic lethality both complicate genetic methods for deconvoluting functions of glycosyltransferases. We report a chemical method in which the activity of an individual glycosyltransferase is controlled by a small molecule. The approach exploits the requirement of Golgi localization, a common feature of glycosyltransferase superfamily members. In our approach, the glycosyltransferase is separated into two domains, one that determines localization and one responsible for catalysis. Control of enzyme activity is achieved using a small molecule to regulate association of the two domains. We used this method to regulate production of sialyl Lewis x by α1,3-fucosyltransferase VII in living cells.

Introduction

Compartmentalization within eukaryotic cells allows for the formation of microenvironments containing distinct sets of enzymes and substrates; consequently, enzymes only utilize substrates that they can access [1, 2]. Nowhere is this more true than in the secretory pathway [3–6]. The spatial distribution of glycosyltransferases and sulfotransferases within the Golgi cisternae forms an assembly line for glycoprotein and glycolipid biosynthesis. Newly synthesized proteins destined for secretion or for the plasma membrane traverse the individual compartments of the secretory pathway before arriving at the cell surface. Along the way, proteins become decorated with complicated carbohydrates in an ordered and regulated fashion. Changes in localization of individual Golgi-resident enzymes can result in profound changes in glycosylation, either by denying enzymes access to their normal substrates or by allowing them access to uncharacteristic substrates. The observation that localization is a critical determinant of Golgi-resident enzyme activity suggests regulation of localization as a possible mechanism for controlling that activity.

Many of the Golgi-resident enzymes responsible for glycosylation exhibit similar primary structural arrangements of functional elements (Figure 1A). Most of these enzymes are type II transmembrane proteins, with a short amino terminus extending into the cytosol, a hydrophobic transmembrane region spanning the Golgi membrane, and a large carboxy-terminal catalytic domain positioned in the lumen of the Golgi [4]. The transmembrane region and the catalytic domain are separated by the so-called stem region, a region of variable length and sequence. For many, although certainly not all, Golgi-resident enzymes, the regions controlling catalysis and localization appear to be modular and separable at the level of amino acid sequence [3, 4]. The amino-terminal residues comprising the cytoplasmic, transmembrane, and stem (CTS) regions of the enzyme are often both necessary and sufficient for localization [7, 8]. For catalysis, only those amino acids of the C-terminal catalytic domain (CAT) are required [9]. By taking advantage of the modular nature of Golgi-resident enzymes, we designed a system in which a small molecule can be used to control the activity of a glycosyltransferase catalytic domain.

We reasoned that a protein that contained only the localization domain of a glycosyltransferase would be retained at the enzyme's normal location within the Golgi and have access to the enzyme's usual substrates, but lack the ability to catalyze the reaction. Conversely, a protein that contained only the catalytic domain would maintain the ability to catalyze the enzyme's reaction, but would not be retained at a location where it had access to the appropriate substrates. Conditions that caused these two proteins to associate would result in the retention of the catalytic domain at the site of the localization domain and would reconstitute the enzyme's normal activity. To control association of the two protein domains, we took advantage of the rapamycin-mediated heterodimerization [10] of FKBP [11] and FRB [12]. This ternary interaction has been used widely to mediate the dimerization of protein fragments [13–15] and provides a mechanism for small molecule control of the association of localization and catalytic domains (Figure 1B). Herein, we demonstrate the strategy as applied to the glycosyltransferase human α1,3-fucosyltransferase VII (FucT7) [16, 17].

Results and Discussion

Engineering FucT7 for Small Molecule Control

FucT7 and another fucosyltransferase with similar activity, FucT4, collaborate in fucosylation of the glycans that mediate leukocyte-endothelial cell interactions in the lymph node and at sites of inflammation [18, 19]. FucT7 installs fucose in an α-1,3 linkage to GlcNAc in the substrate sialyl LacNAc, generating sialyl Lewis x (sLe^x) and related structures [20]. FucT7 accepts only sialylated structures [16, 17]; in order to access the appropriate substrates, FucT7 must localize to the Golgi in the same compartment or a compartment subsequent to that where sialylation takes place. We relied on previous work

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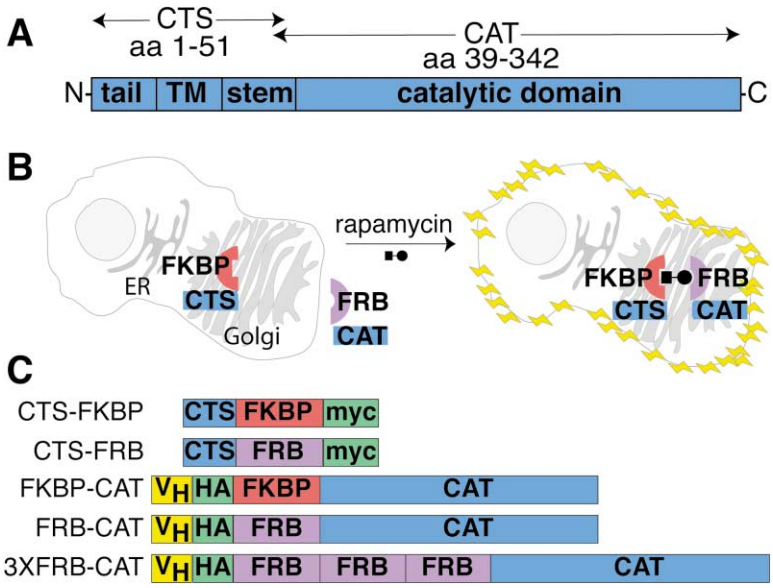

Figure 1. Design of the Conditional Glycosylation System for Small Molecule Control of Golgi Enzyme Activity

(A) Anatomy of a type II membrane-resident glycosyltransferase. The localization (CTS) domain is comprised of the cytoplasmic tail (C), the transmembrane region (T), and the lumenal stem region (S). The catalytic (CAT) domain is responsible for performing the glycosyltransfer reaction. Residue numbers correspond to human FucT7.

(B) Mechanism of small molecule control of glycosyltransferase activity. In the absence of rapamycin, the catalytic domain is secreted to the exterior of the cell, and little of its product is observed on the cell surface. When rapamycin is added, the catalytic domain is retained in the Golgi, and the enzyme's normal activity is reconstituted.

(C) Fusion proteins are composed of either FKBP or FRB linked to either the localization (CTS) or catalytic (CAT) domain of FucT7. V_H, the secretion signal derived from the murine Ig κ-chain; HA, hemagglutinin peptide epitope tag; myc, *myc* peptide epitope tag.

to identify the portion of FucT7 necessary and sufficient for localization [21] and, similarly, the region that comprised a functional catalytic domain [22]. We designed plasmids encoding the localization and catalytic domains of FucT7 fused to FKBP and FRB (Figure 1C). For localization plasmids, FKBP or FRB was appended C terminal to amino acids 1–51 of FucT7 (CTS, Figure 1A), generating the plasmids CTS-FKBP and CTS-FRB. For the catalytic domain plasmids, FKBP or FRB was appended N terminal to amino acids 39–342 of FucT7 (CAT, Figure 1A). These plasmids were designated FKBP-CAT and FRB-CAT. The catalytic domain plasmids also contained an N-terminal secretion tag derived from the mouse immunoglobulin heavy chain (V_H). The addition of the secretion tag was necessary for the chimeric protein to enter the endoplasmic reticulum. Once in the secretory pathway, the ER signal peptide is cleaved, and the protein, lacking any localization domain, is secreted by default to the exterior of the cell. All plasmids also encoded epitope tags, either myc or HA, for antibody recognition.

Plasmids encoding these chimeric proteins, as well as one encoding wild-type FucT7, were transfected into CHO cells. Two days after transfection, cell surfaces were stained using biotin-conjugated HECA-452 followed by staining with streptavidin conjugated to the fluorescent dye Tricolor. HECA-452 recognizes sLe^x and related epitopes; these are absent on wild-type CHO cells due to a lack of α1,3-fucosyltransferase activity [23]. Binding of HECA-452 has an absolute requirement for fucosylation. Flow cytometry analysis showed that cells transfected with full-length FucT7 displayed high levels of sLe^x, as evidenced by their high mean fluorescence, while mock-transfected cells lack sLe^x. When transfected with either of the localization plasmids CTS-FRB or CTS-FKBP, cells displayed only background levels of staining with HECA-452, similar to that observed on wild-type CHO cells (Figure 2A, open bars). Transfection with either catalytic domain plasmid, FRB-CAT or FKBP-CAT, produced cell surface sLe^x slightly above background levels but significantly below that observed for full-length FucT7. This very low level of activity may result from catalytic domains passing through the Golgi as they are secreted. Simultaneous transfection with a localization domain plasmid and a catalytic domain plasmid resulted in sLe^x levels similar to those observed for a catalytic domain alone.

To test the effect of rapamycin on cells expressing FucT7 chimeras, cells were split one day after transfection into two populations. One population was incubated with normal media and one with media containing 200 nM rapamycin. After 18 hr of rapamycin exposure, we measured cell surface sLe^x levels and compared the mean fluorescence of rapamycin-treated cells with the mean fluorescence of the corresponding untreated cells. The filled bars in Figure 2A show the mean fluorescence observed for cells incubated with rapamycin and subsequently stained for sLe^x expression. Cells transfected with CTS-FRB and FKBP-CAT or with CTS-FKBP and FRB-CAT were expected to respond to rapamycin. The populations incubated with rapamycin displayed an increase in sLe^x expression when compared to the population incubated in the absence of rapamycin (compare open and filled bars in Figure 2A). We attribute the increase in sLe^x production to rapamycin-induced dimerization of FRB and FKBP, which thereby retains the CAT protein at the site of the CTS protein, a location where the catalytic domain can encounter appropriate substrates. Rapamycin treatment had no effect on sLe^x production for mock-transfected cells or cells transfected with the full-length FucT7 gene. Similarly, cells transfected with only a localization domain plasmid or only a catalytic domain plasmid showed no rapamycin-mediated increase in sLe^x expression. Importantly, cells transfected with CTS-FKBP and FKBP-CAT or with CTS-FRB and FRB-CAT did not respond to rapamycin, consistent with fact that rapamycin does not induce homodimerization of FKBP or FRB.

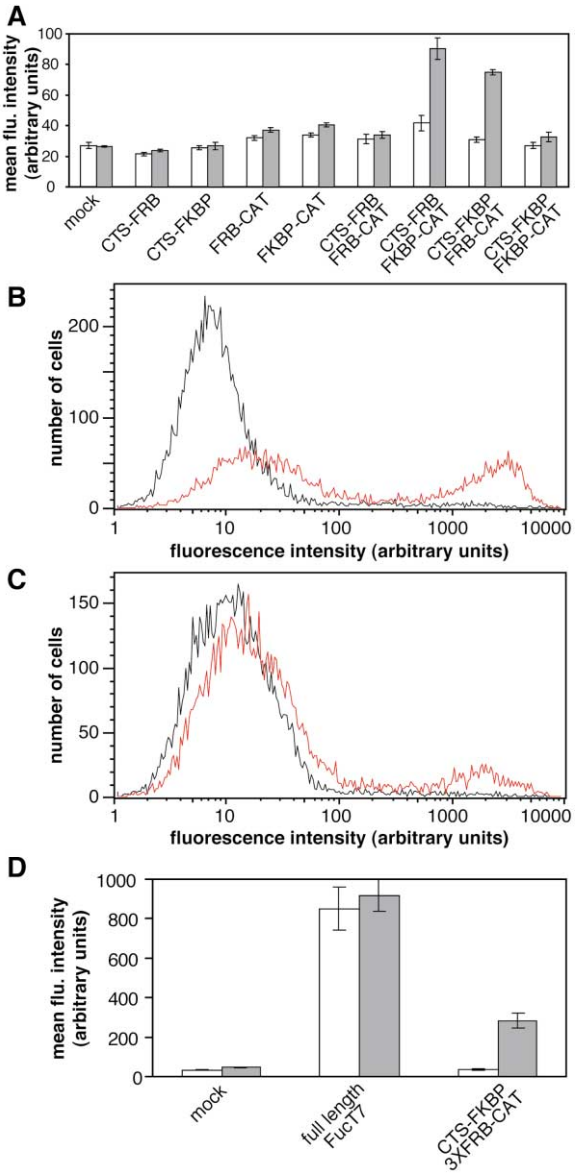

Figure 2. Rapamycin Increases Cell Surface Expression of sLe^x

(A) Cells were transfected with the indicated plasmids, then treated with 0 (open bars) or 200 nM (filled bars) rapamycin. The mean fluorescence of all live cells was quantified by flow cytometry.

(B–D) Comparison of the activity of the wild-type FucT7 gene and the small molecule-controlled system. (B) shows a histogram presentation of flow cytometry data. Fluorescence intensity measures sLe^x expression on mock transfected cells (black line) and cells transiently transfected with FucT7 (red line). (C) shows a histogram presentation of flow cytometry data. Fluorescence intensity measures sLe^x expression on cells transfected with CTS-FKBP and 3XFRB-CAT and treated with 0 (black line) or 200 nM rapamycin (red line). (D) shows a quantitative comparison of the data shown in (B) and (C). Cells were transfected with the indicated plasmids, then treated with 0 (open bars) or 200 nM (filled bars) rapamycin. The mean fluorescence is the average fluorescence of all live cells.

Multiple Copies of FRB Improve the Rapamycin Response

The results of previous studies that used the rapamycin-mediated interaction between FRB and FKBP to regulate transcription or signaling pathways suggested that multiple FRB or FKBP domains might enhance the rapamycin response [24, 25]. We explored a similar strategy to increase the rapamycin-dependent FucT7 activity in our system, with the aim of achieving rapamycin-induced activity comparable to that of full-length FucT7. A series of plasmids was produced in which the FRB or FKBP domains were repeated two or three times (3XFRB-CAT in Figure 1C is an example). These plasmids were transfected pairwise into CHO cells and assayed for rapamycin-induced changes in expression of sLe^x. For all cognate plasmid pairs, cells incubated in the presence of rapamycin displayed sLe^x expression greater than or equal to equivalent cells incubated without rapamycin (data not shown). The strongest rapamycin response was observed for cells transfected with plasmids encoding two or three copies of FRB linked to the catalytic domain. The plasmid pair CTS-FKBP and 3XFRB-CAT showed the largest increase in sLe^x expression in response to rapamycin and was used for subsequent experiments.

Comparison of Rapamycin-Inducible and Wild-Type FucT7 Activities

Our goal was to develop a system in which the presence or absence of a small molecule yielded a change in cell surface glycosylation equivalent to that observed in the presence or absence of the glycosyltransferase gene. To assess the ability of our conditional glycosylation system to achieve this objective, we quantified the rapamycin-induced sLe^x expression of cells transfected with CTS-FKBP and 3XFRB-CAT and compared it to the change in sLe^x expression that resulted from transfecting cells with full-length FucT7. The histogram in Figure 2B shows the difference in sLe^x expression between CHO cells that were mock transfected and those that were transfected with FucT7. This can be compared to the histogram in Figure 2C that shows the sLe^x expression for cells transfected with CTS-FKBP and 3XFRB-CAT and treated with 0 or 200 nM rapamycin. On a qualitative level, the conditional glycosylation system succeeds, yielding very low levels of sLe^x in the absence of rapamycin and, upon addition of rapamycin, producing a population of cells expressing high levels of sLe^x, comparable to levels produced by cells expressing FucT7. These data are quantified in Figure 2D, which shows that the mean fluorescence associated with rapamycin-induced sLe^x expression is about a third of that observed by introduction of the FucT7 gene. The inability of our system to quantitatively reproduce wild-type FucT7 activity may result from the transient transfection conditions, in which some cells are likely to receive nonoptimal ratios of the two (CTS-FKBP and 3XFRB-CAT) plasmids.

FucT7 Activity Depends on the FRB-Rapamycin-FKBP Interaction

We measured how enhanced expression of sLe^x depends on rapamycin concentration. The mean fluorescence of rapamycin-treated cells is plotted versus rapa-

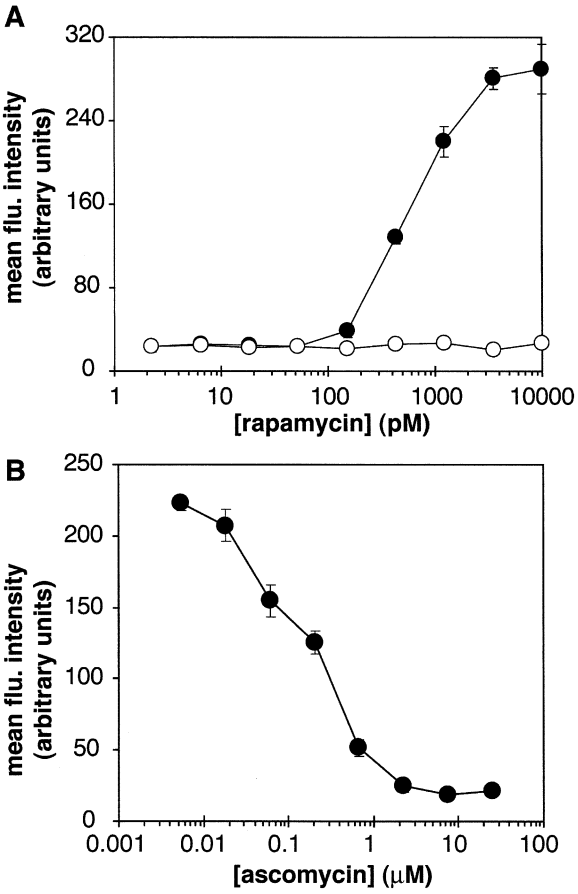

Figure 3. Rapamycin Dimerizes FKBP and FRB

(A) Cells were transfected with CTS-FKBP and 3XFRB-CAT (closed circles) or with CTS-FRB and 3XFRB-CAT (open circles). They were treated with various rapamycin concentrations, then stained for sLe^x expression. The mean fluorescence of rapamycin-treated cells is plotted versus rapamycin concentration.

(B) CTS-FKBP/3XFRB-CAT-transfected cells were incubated with 1 nM rapamycin and the indicated concentration of ascomycin, then stained for cell surface sLe^x. The mean fluorescence of the ascomycin- and rapamycin-treated cells is plotted versus ascomycin concentration.

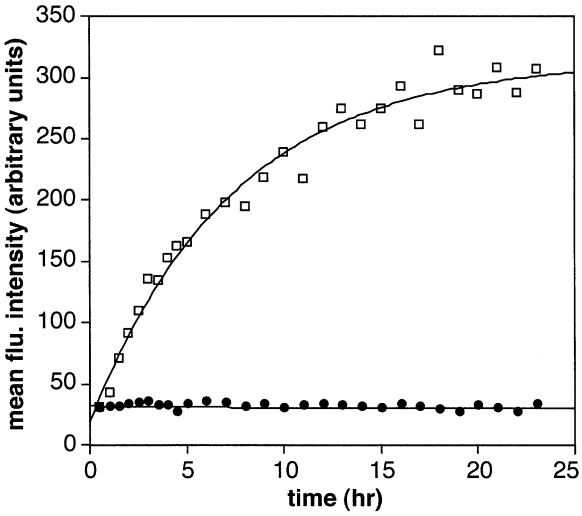

Figure 4. Time Course of the Rapamycin Response

CTS-FKBP/3XFRB-CAT-transfected cells were stained for sLe^x expression after being treated with 0 (closed circles) or 200 nM (open squares) rapamycin for the indicated period of time. The solid lines show the best fit of each data set to a single exponential.

mycin concentration in Figure 3A. For cells expressing CTS-FKBP and 3XFRB-CAT, the mean fluorescence was dependent on the rapamycin concentration, with half-maximal stimulation occurring at about 600 pM rapamycin. As expected, at no concentration of rapamycin did cells transfected with CTS-FRB and 3XFRB-CAT show detectable expression of sLe^x. Similarly, cells transfected with CTS-FKBP and FRB-CAT showed an increase in sLe^x production that was dependent on rapamycin concentration, while cells transfected with CTS-FRB and FRB-CAT did not show increased sLe^x production at any rapamycin concentration (data not shown).

To provide further support that the observed changes in expression of sLe^x were mediated by dimerization of FRB and FKBP, we tested the ability of the small molecule ascomycin to abrogate the rapamycin-induced increase in FucT7 activity. Ascomycin competes with rapamycin for the same binding pocket of FKBP but does not mediate the interaction of FKBP and FRB [26]. Cells were transfected with CTS-FKBP and 3XFRB-CAT, then treated with 1 nM rapamycin and various concentrations of ascomycin. These cells were probed for sLe^x expression, and the mean fluorescence was plotted versus ascomycin concentration (Figure 3B). Ascomycin showed concentration-dependent competition, with half-maximal signal occurring at about 200 nM. Ascomycin was also able to compete away the rapamycin-dependent fluorescence change observed for CTS-FKBP/FRB-CAT transfected cells (data not shown).

We performed a time course experiment to assess the ability of rapamycin treatment to provide temporal control over FucT7 activity. Cells transfected with CTS-FKBP and 3XFRB-CAT were treated with media containing 0 nM or 200 nM rapamycin for various times and then analyzed by flow cytometry for sLe^x expression. A measurable increase in sLe^x expression was observed for cells that had been exposed to rapamycin for time periods as short as 1 hr (Figure 4). Subsequent to a short (about 0.5 hr) lag time, the rate of increase in sLe^x expression was fit well by a single exponential with half-maximal signal observed at about 5 hr. Cells that were not exposed to rapamycin displayed no time-dependent change in sLe^x expression.

Localization of the Catalytic Domain Is Controlled by Rapamycin

We took advantage of the HA epitope tag encoded in the catalytic domain plasmids to confirm that the rapamycin-induced change in FucT7 activity is due to a change in the physical location of the catalytic domain. Cells were transfected with CTS-FKBP and 3XFRB-CAT, then split into two populations, one of which was treated with rapamycin. Cells were stained with HECA-452 to quantify sLe^x expression, then fixed, permeabilized, and stained intracellularly with a FITC-conjugated anti-HA antibody. Cells treated with rapamycin showed higher

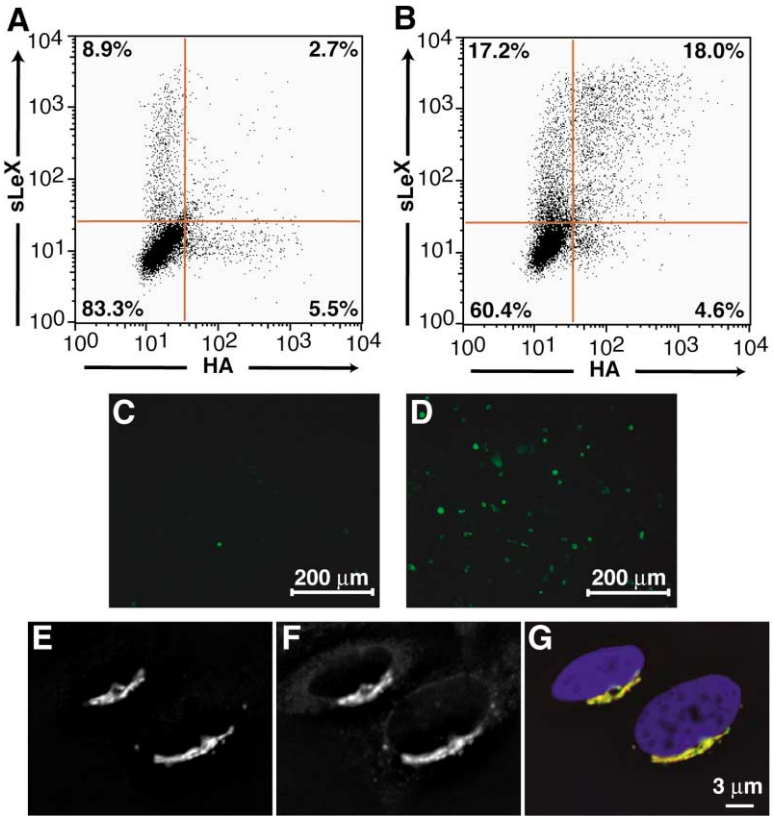

Figure 5. Rapamycin Causes the HA-Tagged 3XFRB-CAT to Be Retained Intracellularly

(A and B) Flow cytometry plots showing anti-sLe^x staining versus anti-HA staining for cells transfected with CTS-FKBP and 3XFRB-CAT, then treated with 0 (A) or 200 nM (B) rapamycin. Numbers inside the plots indicate the percent of cells found in each quadrant.

(C and D) Fluorescence microscopy of cells transfected with CTS-FKBP and 3XFRB-CAT, then treated with 0 (C) or 1 μM (D) rapamycin. Cells were probed with mouse anti-HA followed by FITC-labeled goat anti-mouse antibody.

(E–G) Cells treated with 1 μM rapamycin and probed with antibodies against HA and mannosidase II. The panels show single sections of a deconvolved data set. (E), the signal from mannosidase II staining shown in monochrome; (F), the signal from HA staining shown in monochrome; (G), three-color overlay with mannosidase II staining shown in green, HA staining in red, and nuclear DAPI staining shown in blue. Overlap between mannosidase II and HA staining appears yellow in this image.

levels of anti-HA antibody staining (compare Figures 5A and 5B), suggesting that more catalytic domain is retained in these cells. Furthermore, there was a substantial increase in the fraction of cells that were positive for both sLe^x and the HA tag, indicating that increased expression of sLe^x structures is correlated with an increase in intracellular catalytic domain levels.

The rapamycin-inducible production of sLe^x indicates that the small molecule causes the catalytic domain to accumulate in the appropriate cellular compartment, where sialylated substrates are present. This suggests that the catalytic domain is accumulating in FucT7's normal subcellular location, the Golgi. We used immunofluorescence microscopy to verify the localization of the catalytic domain. Cells were transfected with CTS-FKBP and 3XFRB-CAT, then split, and half were treated with rapamycin. Both rapamycin-treated and -untreated cells were incubated with cycloheximide directly prior to fixing in order to clear the secretory pathway of catalytic domain transiently present during trafficking to the exterior of the cell. Cells were permeabilized and probed with anti-HA antibody, then visualized by fluorescence microscopy. Due to the transient transfection conditions, not all cells produce the catalytic domain protein, and those that do produce it vary in expression levels. Nonetheless, clear differences were apparent between those cells treated with rapamycin and those untreated (compare Figure 5C and 5D). The immunofluorescence experiments show that more catalytic domain is present in rapamycin-treated cells. Moreover, for cells treated with rapamycin, the majority of the HA staining overlaps with staining for a medial Golgi marker protein, mannosidase II (Figures 5E–5G).

In summary, we present a system in which the activity of a Golgi-resident enzyme has been engineered for regulation by a small molecule. Due to the similar domain architecture of most Golgi-resident enzymes, we expect the conditional glycosylation approach to be readily extended to the control of other glycosyltransferases and sulfotransferases. In the case described here, rapamycin regulates production of sLe^x, a critical mediator of cell-cell communication. Furthermore, since the level of sLe^x expression depends on rapamycin concentration, this system has the potential to allow for production of cells bearing controlled levels of cell surface glycans of interest. Such cells could be utilized in biological assays to probe the relationship between glycan expression levels and function. For example, cells bearing various levels of sLe^x could be tested in a cell rolling assay, which provides a model of leukocyte adhesion to endothelial cells at sites of inflammation, to determine the critical density of sLe^x necesssary for recruitment of cells under conditions of shear stress.

In addition, the small molecule method provides temporal control over glycan production. It is notable that, in this system, control is at the level of protein activity rather than of RNA or protein production. For this reason, we expect the conditional glycosylation system to present a kinetic advantage over systems where small molecule control of gene activity is achieved by regulation of transcription (such as tetracycline [27, 28], ecdysone [29], mifepristone [30], rapamycin [25], dexamethasone

[31], streptogramin [32], or 3-oxo-C_8-HSL [33]-inducible systems) or translation (such as Hoechst dye 33258 [34], rapamycin [35], or tetracycline [36]-controlled systems). Although we have not yet compared our system to any of these others, we expect that deferring control of glycosylation to a late step in the biosynthetic pathway will allow for a relatively rapid response to rapamycin. Furthermore, in our system, turning "off" the activity of the glycosyltransferase requires withdrawal of rapamycin and disassembly of the FKBP-rapamycin-FRB complex; unlike other systems, degradation of the target protein may not be necessary.

Traditionally, small molecule control of enzymes is pursued through active-site inhibitors. However, Golgi-resident enzymes often comprise large families in which members recognize polar, charged substrates and have similar active sites; therefore, cell permeability and specificity of inhibitors are difficult to achieve. Since different members of these families seem to have distinct developmental roles [37, 38], both specific and temporal control of enzyme activity will be necessary to deconvolute their activities. If a "knocked-out" glycosyltransferase gene were replaced with rapamycin-inducible chimeras analogous to the ones described here, conditional glycosylation might find application in resolving the in vivo function of individual members of Golgi-resident enzyme families. If this strategy is successfully applied in vivo, an added benefit could be circumvention of the problem of embryonic lethality associated with some glycosyltransferase knockouts. We envision a system in which administration of a small molecule activates essential glycosyltransferases during early developmental stages, and the targeted enzymes are later inactivated by withdrawal of the drug. In this way, one could study glycosyltransferase knockouts at previously inaccessible developmental stages. While the implementation of this system in vivo to bypass embryonic lethality is currently only a theoretical possibility, the successful use of rapamycin and its analogs to control transcription in animal experiments [25, 39–41] suggests that this vision may be achievable. In vivo experiments may be facilitated by the use of rapamycin analogs that bind to mutant versions of FKBP and/or FRB and not to the endogenous proteins [42–44]; orthogonal small molecule-protein pairs should allow for small molecule control of glycosyltransferase activity without causing the immunosuppressive effects that are associated with rapamycin use.

Significance

We present a general strategy for controlling the cellular activity of glycosyltransferases using small molecules. Glycoconjugates, the products of glycosyltransferases, mediate cell-cell interactions fundamental to organ development, cell trafficking, learning and memory, and the immune response; glycosyltransferases are therefore key regulators of complex biological processes. They comprise a superfamily numbering around 250 in humans, and there is considerable biochemical redundancy within subfamilies (such as fucosyltansferases, sialyltransferases, etc.). In some cases, gene knockouts have been instructive in determining the biological targets and functions of individual enzymes, but lethality and compensation by upregulated relatives have limited this approach.

Chemical tools can overcome limitations associated with genetic methods [45, 46]. Small molecule "switches" offer temporal control and reversibility and can be applied to cells and organisms for which the corresponding gene knockout would be lethal. For glycobiologists, chemical approaches have amplified importance, since polysaccharides are not primary gene products and are therefore difficult to perturb using conventional genetic methods. Yet, there is a relative dearth of chemical tools with which to modulate the synthesis of glycans. A general strategy for controlling activities of specific glycosyltransferases with small molecules could accelerate efforts to understand the functions of glycans.

Our strategy exploits the one commonality shared by many members of the glycosyltransferase family: their requirement of Golgi localization for cellular activity. We applied the "chemical inducer of dimerization" strategy [13–15] to control the association of a glycosyltransferase catalytic domain with its Golgi localization domain and thereby regulate the enzyme's activity. Because the approach exploits the common domain structure of the glycosyltransferase family rather than a specific feature of a particular enzyme's active site, it can be generalized readily to other family members.

Experimental Procedures

Reagents

DNA primers were purchased from Sigma-Genosys. The plasmids pcDNA3.1-Zeo and pCMV/myc/ER were purchased from Invitrogen. Restriction enzymes and T4 DNA ligase were from New England BioLabs. Calf intestinal alkaline phosphatase (CIAP) was from Amersham. Pfu polymerase from Stratagene was used for all PCRs. DNA sequencing was performed by Davis Sequencing, LLC (Davis, CA). Rapamycin and ascomycin were purchased from Calbiochem. Rapamycin was dissolved in ethanol to yield a 1 mM stock concentration and stored at −20°C, and ascomycin was dissolved in dimethyl sulfoxide to yield a 1 mM stock concentration and stored at −20°C.

Plasmid Construction

Localization Domain Plasmids

The CTS region of human FucT 7 [16, 17] (GenBank accession number Q11130) was amplified from the plasmid pcDNA3.1(−)FucT 7 (a gift from Steven Rosen, UCSF) by PCR using primers CTS_5′ (5′-TGGCTAGCGTTTAAACGGGCCC-3′) and CTS_3′ (5′-TCTCTAGA GACAAGGATGGTGATCG-3′), then digested with XbaI to yield a fragment coding for the first 51 amino acids of FucT 7. This fragment was ligated into the plasmid pcDNA3.1-Zeo, which had been digested with Nhe I and Xba I and treated with CIAP. The identity of the product, pcDNA3.1-CTS, was verified by sequencing.

A PCR product encoding FKBP-myc was produced using DNA encoding human FKBP12 [11] (GenBank accession number 1613455A) as a template (a gift from David Austin and Fei Liu, Yale University) and FKBP_5′ (5′-ACTCTAGAATCCTCTGGCATGAGAT GTGG-3′) and FKBPmycstop_3′ (5′-TCACTAGTCTACAGATCCTCT TCTGAGATGAGTTTTGTTCTTCCAGTTTTAGAAGCTCC-3′) as primers. The PCR product was cloned into the plasmid pCR4Blunt-TOPO using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) to generate TOPO-FKBP-myc, whose identity was confirmed by sequencing. The insert, containing amino acids 2–108 of FKBP12 followed by the myc epitope tag and a stop codon, was excised with XbaI and SpeI. FKBP-myc was ligated into plasmid pcDNA3.1-CTS that had

been digested with XbaI and treated with CIAP, yielding the plasmid CTS-FKBP. The orientation of the insert was confirmed by restriction digest. Thus, the chimeric gene encoded by CTS-FKBP consists of amino acids 1–51 of FucT7, followed by amino acids Ser, Arg (a vestige of the cloning process), followed by amino acids 2–108 of FKBP, and concluding with the myc epitope tag (EQKLISEEDL).

FRB-myc was produced by PCR using the Marathon human fetal brain cDNA library (catalog #1702-1, Clontech) as a template and FRB_5′ (5′-ACTCTAGAATCCTCTGGCATGAGATGTGG-3′) and FRBmycstop_3′ (5′-AGACTAGTCTACAGATCCTCTTCTGAGATGAG TTTTTGTTCCTTTGAGATTCGTCGGAAC-3′) as primers. The PCR product was cloned into the plasmid pCR4Blunt-TOPO using the Zero Blunt TOPO PCR Cloning Kit to generate TOPO-FRB-myc, whose identity was confirmed by sequencing. A point mutation of FRB, T2098L, that allows binding to rapamycin analogs not discussed here was introduced into this plasmid using the Quikchange method (Stratagene). Primers used were T2098L_top (5′-GGAATGT CAAGGACCTCCTCCAAGCCTGGGACCTC-3′) and T2098L_bottom (5′-GAGGTCCCAGGCTTGGAGGAGGTCCTTGACATTCC-3′). The FRB insert, containing amino acids 2021–2113 of FRAP [12] (GenBank accession number AAA58486) followed by the myc epitope tag and a stop codon, was excised with XbaI and SpeI. FRB-myc was ligated into plasmid pcDNA3.1-CTS that had been digested with XbaI and treated with CIAP, yielding the plasmid CTS-FRB. The orientation of the insert was confirmed by restriction digest. Thus, the chimeric gene encoded by CTS-FRB consists of amino acids 1–51 of FucT7, followed by amino acids Ser, Arg (a vestige of the cloning process), followed by amino acids 2021–2113 of human FRAP (including the T2098L mutation), and concluding with the myc epitope tag.

Catalytic Domain Plasmids

The plasmid pCMV/myc/ER was digested with NdeI and XhoI to yield a fragment that contains the murine V_H chain signal peptide. This fragment was ligated into pcDNA3.1-Zeo that had been digested with NdeI and XhoI and treated with CIAP. The resulting plasmid was designated pcDNA3.1-Vh. The catalytic domain of FucT7 (amino acids 39–342) was PCR amplified from pcDNA3.1 (−)FucT7 using the primers CAT_5′ (5′-AGTCTAGAGGTACCCCGG CACCCC-3′) and CAT_3′ (5′-ATACTAGTTCAGGCCTGAAACCAACC-3′). The PCR product was cloned into the plasmid pCR4Blunt-TOPO using the Zero Blunt TOPO PCR Cloning Kit to generate TOPO-CAT, whose identity was confirmed by sequencing. The insert was excised with XbaI and SpeI, then ligated into pcDNA3.1-Vh that had been digested with XbaI and CIAP treated. The orientation of the insert was confirmed by restriction digest. The product was termed pcDNA3.1-Vh-CAT.

The inserts encoding HA-FRB and HA-FKBP were produced in a fashion analogous to the production of FRB-myc and FKBP-myc (see above). In this case, the primers used were HAFRB_5′ (5′-ACTCTAGATATCCGTACGACGTACCAGACTACGCAATCCTCTGGC ATGAGATGTGG-3′) and FRB_3′ (5′-AGACTAGTCTTTGAGATTCGT CGGAAC-3′) and HAFKBP5′ (5′-ACTCTAGATATCCGTACGACGT ACCAGACTACGCAGGAGTGCAGGTGGAAACC-3′) and FKBP_3′ (5′-TCACTAGTTTCCAGTTTTAGAAGCTCC-3′). The inserts were cloned into the plasmid pCR4Blunt-TOPO, sequenced, and excised using the restriction enzymes XbaI and SpeI. The plasmid pcDNA3.1-Vh-CAT was digested with XbaI and treated with CIAP. The linearized plasmid was ligated with the insert HA-FRB to generate the plasmid FRB-CAT or ligated with the insert HA-FKBP to generate the plasmid FKBP-CAT. The orientations of the inserts were confirmed by restriction digest. Hence, the chimeric gene encoded by the plasmid FRB-CAT consists of the murine V_H chain signal peptide, followed by the HA epitope tag (YPYDVPDYA), followed by amino acids 2021–2113 of human FRAP (including the T2098L mutation), amino acids Ser, Arg (resulting from the cloning process), and concluding with amino acids 39–342 of human FucT7. Similarly, the gene encoded by FKBP-CAT is composed of the murine V_H chain signal peptide, followed by the HA epitope tag, amino acids 2–108 of FKBP, amino acids Ser, Arg (resulting from the cloning process), and ending with amino acids 39–342 of human FucT7.

Production of the Plasmid 3XFRB-CAT

A series of plasmids were produced containing multiple repeats of FRB or FKBP. The construction of 3XFRB-CAT is described here; other plasmids were produced in an analogous manner. The DNA encoding FRB was amplified by PCR using the primers FRB_5′ and FRB_3′ (sequences shown above). The PCR product was cloned into the plasmid pCR4Blunt-TOPO, sequenced, and excised using the restriction enzymes XbaI and SpeI. The FRB insert was ligated into pcDNA-Vh-CAT that had been digested with XbaI and CIAP treated. The resulting plasmid, 1XFRB-CAT, was digested with XbaI, CIAP treated, and ligated with the FRB insert. The plasmid resulting from this ligation, 2XFRB-CAT, was digested with XbaI, CIAP treated, and ligated with the HA-FRB insert (described above). The final product, the plasmid 3XFRB-CAT, encodes the murine V_H chain signal peptide, followed by the HA epitope tag, followed by three copies of the FRB domain (amino acids 2021–2113 of human FRAP; the first copy includes the T2098L mutation) each followed with amino acids Ser, Arg (resulting from the cloning process), and concluding with amino acids 39–342 of human FucT7. The orientations of all inserts were confirmed by restriction digests.

Flow Cytometry Experiments

Chinese hamster ovary (CHO-K1) cells were cultured in F12 nutrient mixture (Ham) with L-glutamine (Gibco) containing 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cell cultures were maintained in a water saturated, 5% CO_2 atmosphere at 37°C.

One day prior to transfection, CHO cells were seeded at a density of 5×10^5 cells per well of a 6-well plate (10 cm^2 surface area/well). For transfection, LipofectAMINE PLUS Reagent (Gibco–BRL) was used according to the manufacturer's directions, using unsupplemented OptiMEM I (Gibco) as media during the transfection. One day after transfection, cells were removed from the plate by trypsinization, counted, and reseeded at a density of 1×10^5 cells per well of a 12-well plate (4 cm^2 surface area/well). Cells were incubated for 18 hr in media containing the indicated concentrations of ascomycin and/or rapamycin. Three wells were prepared for each ascomycin/rapamycin concentration to allow the data to be collected in triplicate.

To remove the cells from the plate for flow cytometry analysis, they were washed twice with Dulbecco's phosphate buffered Saline (1× PBS, Gibco), then incubated with 1× PBS containing 1 mM EDTA (0.2 ml/well) for 20 min at 37°C. Cells were maintained at 4°C for all subsequent steps. The cells were then washed twice with FACS buffer (1× PBS, 1 mg/ml BSA, 0.2% NaN_3). Each sample was incubated in 50 μl of FACS buffer containing 2 μl of biotinylated HECA-452 (BD Pharmingen) for 1 hr. The cells were washed twice with FACS buffer, then stained with the secondary reagent, 1 μl of tricolor-streptavidin conjugate (Caltag Laboratories) in 50 μl of FACS buffer, for 0.5 hr. Following two more washes with FACS buffer, the cells were resuspended in 150 μl of FACS buffer, and the fluorescence of at least 10,000 live cells per sample was measured on the FL3 channel of a FACSCalibur flow cytometer (BD Biosciences). The mean fluorescence intensity was quantified for all live cells.

To assess the time course of sLe^x expression, 4.5×10^6 cells were transfected with CTS-FKBP and 3XFRB-CAT. One day after transfection, cells were seeded into 60 wells (1×10^5 cells per well) of 12-well plates. At 0.5 or 1 hr intervals, the media on two wells was replaced: one well with plain media and one with media containing 200 nM rapamycin. After 25 hr, the cells were removed from the plates and probed for sLe^x expression, as above.

For intracellular staining of the HA-tagged catalytic domain, the Cytofix/Cytoperm Kit (BD Biosciences) was used. Cells were first stained for sLe^x expression using biotinylated HECA-452 and tricolor-streptavidin, as above. Cells were then fixed by incubating for 20 min at 4°C in 200 μl of BD Cytofix/CytoPerm solution per well. Cells were washed twice with BD Perm/Wash buffer, then stained with 2 μl of FITC-labeled anti-HA (Covance Research Products, Denver, PA) in 50 μl of BD Perm/Wash buffer for 30 min at 4°C. After two washes with BD Perm/Wash buffer, cells were resuspended in 400 μl of FACS Buffer. Fluorescence of at least 10,000 live cells per sample was measured on the FL1 and FL3 channels of the flow cytometer.

Immunofluorescence Microscopy

Cells were transfected with CTS-FKBP and 3XFRB-CAT, as above. One day after transfection, they were seeded (1×10^5 cells per well) into two wells of a Labtek II Chamber slide (Nalge Nunc Incorpo-

rated). One well was incubated with plain media and one with media containing 1 μM rapamycin. After 2 days, 10 μg/ml cycloheximide (Sigma-Aldrich) was added to both wells for 3 hr. The cells were washed two times with 1× PBS, then fixed with 3% paraformaldehyde in PBS for 20 min. Cells were washed three times with 1× PBS, then blocked 15 min with 1× PBS containing 2% BSA. After permeabilizing for 5 min with 0.1% Triton X-100 in PBS containing 2% BSA, cells were stained overnight with the primary antibody, mouse anti-HA (1-1000 dilution, Covance Research Products), in 1× PBS containing 2% BSA. Cells were washed three times with 1× PBS containing 0.1% Triton X-100. They were stained for 1 hr with the secondary antibody, FITC-conjugated goat anti-mouse (1-1000 dilution, Caltag Laboratories), in 1× PBS with 2% BSA. Cells were washed three times with 1× PBS containing 0.1% Triton X-100, three times with 1× PBS, and three times with water. They were mounted in Vectashield media containing DAPI (Vector Laboratories, Inc.) and photographed using a inverted stage microscope equipped with a FITC filter set (Chroma) and a Spot camera.

For colocalization experiments, samples were prepared as above with the following modifications. To minimize sulfation and consequent masking of the HA epitope, sodium chlorate (50 mM) was included during the rapamycin incubation. The primary antibody staining utilized mouse anti-HA (3-1000 dilution, Covance Research Products) and a polyclonal rabbit serum against mannosidase II (1-1000 dilution, a gift from Dr. Marilyn Farquhar). During the secondary antibody staining, a Alexa488-conjugated goat anti-rabbit antibody (1-1000 dilution, Molecular Probes) and a Alexa546-conjugated goat anti-mouse antibody (1-1000 dilution, Molecular Probes) were used. A Leica DMIRB/E inverted microscope equipped with a xenon arc lamp using a 63× lens was employed for imaging. Image stacks containing 20–25 sections spaced 0.3 μm apart were acquired using a Quantix CCD camera (Roper Scientific). An 8600 Sedat quad filter set with single band excitation and emission filters (Chroma) was used, allowing the imaging of DAPI, Alexa488, and Alexa546 without any concern for register among the different channels. Slidebook software (Intelligent Imaging Solutions) was used to control the microscope and the camera. The image stacks were digitally deconvolved using the nearest neighbor algorithm of Slidebook.

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