Structural and Mechanistic Insights into Lunatic Fringe from a Kinetic Analysis of Enzyme Mutants*3

The Notch receptor is critical for proper development where it orchestrates numerous cell fate decisions. The Fringe family of β1,3-N-acetylgalactosaminyltransferases are regulators of this pathway. Fringe enzymes add N-acetylgalactosamine to O-linked fucose on the epidermal growth factor repeats of Notch. Here we have analyzed the reaction catalyzed by Lunatic Fringe (Lfng) in detail. A mutagenesis strategy for Lfng was guided by a multiple sequence alignment of Fringe proteins and solutions from docking an epidermal growth factor-like O-fucose acceptor substrate onto a homology model of Lfng. We targeted three main areas as follows: residues that could help resolve where the fucose binds, residues in two conserved loops not observed in the published structure of Manic Fringe, and residues predicted to be involved in UDP-N-acetylgalactosamine (UDP-GlcNAc) donor specificity. We utilized a kinetic analysis of mutant enzyme activity toward the small molecule acceptor substrate 4-nitrophenyl-α-L-fucopyranoside to judge their effect on Lfng activity. Our results support the positioning of O-fucose in a specific orientation to the catalytic residue. We also found evidence that one loop closes off the active site coincident with, or subsequent to, substrate binding. We propose a mechanism whereby the ordering of this short loop may alter the conformation of the catalytic aspartate. Finally, we identify several residues near the UDP-GlcNAc-binding site, which are specifically permissive toward UDP-GlcNAc utilization.

Defects in Notch signaling have been implicated in numerous human diseases, including multiple sclerosis (1), several forms of cancer (2–4), cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy (5), and spondylocostal dysostosis (SCD)3 (6–8). The transmembrane Notch signaling receptor is activated by members of the DSL (Delta, Serrate, Lag2) family of ligands (9, 10). In the endoplasmic reticulum, O-linked fucose glycans are added to the epidermal growth factor-like (EGF) repeats of the Notch extracellular domain by protein O-fucosyltransferase 1 (11–13). These O-fucose monosaccharides can be elongated in the Golgi apparatus by three highly conserved β1,3-N-acetylgalactosaminyltransferases of the Fringe family (Lunatic (Lfng), Manic (Mfng), and Radical Fringe (Rfng) in mammals) (14–16). The formation of this GlcNAC-β1,3-Fuc-α1, O-serine/threonine disaccharide is necessary and sufficient for subsequent elongation to a tetrasaccharide (15, 19), although elongation past the disaccharide in Drosophila is not yet clear (20, 21). Elongation of O-fucose by Fringe is known to potentiate Notch signaling from Delta ligands and inhibit signaling from Serrate ligands (22). Delta ligands are termed Delta-like (Delta-like1, -2, and -4) in mammals, and the homologs of Serrate are known as Jagged (Jagged1 and -2) in mammals. The effects of Fringe on Drosophila Notch can be recapitulated in Notch ligand in vitro binding assays using purified components, suggesting that the elongation of O-fucose by Fringe alters the binding of Notch to its ligands (21). Although Fringe also appears to alter Notch-ligand interactions in mammals, the effects of elongation of the glycan past the O-fucose monosaccharide is more complicated and appears to be cell type-, receptor-, and ligand-dependent (for a recent review see Ref. 23).

The Fringe enzymes catalyze the transfer of GlcNAc from the donor substrate UDP-α-GlcNAc to the acceptor fucose, forming the GlcNAc-β1,3-Fuc disaccharide (14–16). They belong to the GT-A-fold of inverting glycosyltransferases, which includes N-acetylgalactosaminyltransferase 1 and β1,4-galactosyltransferase 1 (17, 18). The mechanism is presumed to proceed through the abstraction of a proton from the acceptor substrate by a catalytic base (Asp or Glu) in the active site. This creates a nucleophile that attacks the anomeric carbon of the nucleotide-sugar donor, inverting its configuration from α (on the nucleotide sugar) to β (in the product) (24, 25). The enzyme then releases the acceptor substrate modified with a disaccharide and UDP. The Mfng structure (26) leaves little doubt as to the identity of the catalytic residue, which in all likelihood is aspartate 289 in mouse Lfng (we will use numbering for mouse Lunatic Fringe throughout, unless otherwise stated). The structure of Mfng with UDP-GlcNAc soaked into the crystals (26) showed density only for the UDP portion of the nucleotide-sugar donor and no density for two loops flanking either side of the active site. The presence of flexible loops that become ordered upon substrate binding is a common observation with...
glycosyltransferases in the GT-A fold family (18, 25). Density for the entire donor was observed in the structure of rabbit N-acetylglucosaminyltransferase I (27). In this case, ordering of a previously disordered loop upon UDP-GlcNAc binding may have contributed to increased stability of the donor. In the case of bovine β1,4-galactosyltransferase I, a section of flexible random coil from the apo-structure was observed to change its conformation to α-helical upon donor substrate binding (28). Both loops in Lfng are highly conserved, and we have mutated a number of residues in each to test the hypothesis that they interact with the substrates. The mutagenesis strategy was also guided by docking of an EGF-O-fucose acceptor substrate into the active site of the Lfng model as well as comparison of the Lfng model with a homology model of the β1,3-glucosyltransferase (β3GlcT) that modifies O-fucose on thrombospondin type 1 repeats (29, 30). The β3GlcT is predicted to be a GT-A fold enzyme related to the Fringe family (17, 18, 29).

**EXPERIMENTAL PROCEDURES**

*Homology Models of Lfng and β3GlcT—Lfng was aligned with Mfng using ClustalW (31). This alignment and the Mfng structure (PDB 2J0B) were used as input to the automodel script for the program Modeler 9 version 4 to produce five homology models of the Lfng catalytic domain (32). The best model as judged by Modeler’s objective function, and a Ramachandran analysis was chosen. This model was then superimposed on the Mfng structure using the Superpose program (33). The experimentally determined positions of 14 amino acid side chains of identical residues in the active site from the Mfng structure were substituted for the Modeler-produced sidechain orientations because of the fact that they were derived experimentally.

The mouse β3GlcT sequence was submitted to the ESyPred3D server (which uses Modeler) (34) along with the Mfng structure (PDB 2J0B) as template. This homology model showed significant differences near the donor-substrate-binding site that we surveyed by mutation.

Although we did not include UDP-GlcNAc in our subsequent docking experiments, we did want a UDP-GlcNAc model to allow us to analyze how well our docked solutions might fit with the donor substrate bound. We produced a model of UDP-GlcNAc in the active site of the Lfng homology model by superimposing the β-phosphate of the ud1 ideal coordinate model of UDP-GlcNAc from the HIC-Up data base (35) onto the β-phosphate of the UDP bound in the Mfng structure.

*Docking of EGF-O-Fucose—*The EGF-O-fucose model was prepared as described previously (16). We used the program HEX4.5 (36) to dock the EGF-O-fucose onto the complete Lfng model. The crystal structure of the Factor IX EGF repeat showed dual side-chain conformations for a number of residues. We first docked the structure containing both conformations of all side chains, and we determined that three amino acid side chains (a phenylalanine, tryptophan, and lysine) were likely to be oriented toward the Lfng protein when docked. We prepared eight separate PDB files for all combinations of the three amino acid side-chain conformations and docked each one separately. Any result that positioned the fucose near the catalytic aspartate was saved. We saved the first 6–20 solutions (lowest energy) for each of the eight docked EGF-O-fucose ligands resulting in 80 solutions, which were then loaded into the molecular visualization software PyMOL (37). It should be noted that the HEX program clusters similar solutions based on a user set root mean square deviation cutoff. In our first round of manual culling, we accepted only the first (lowest energy) solution for any given HEX cluster, and we ignored subsequent solutions that belonged to the same cluster. Thus when we describe clustering of solutions under “Results,” we are not referring to the original clusters of solutions produced by the HEX program. We further manually culled inappropriate solutions where the fucose and aspartate 289 were too far apart using PyMOL resulting in a final total of 73 solutions. These solutions fall into two main classes, which are shown in Fig. 2.

*Preparation of Lfng Enzyme Mutants—*Mutations were introduced using mutant primer pairs and PCR (Pfu Turbo polymerase, Stratagene) with pSecTag2CLfng (16) as template. This construct encodes Lfng with C-terminal Myc and His6 tags. Eight μg of endo-free maxi-prep DNA of each construct was transfected into a 10-cm dish of ~50% confluent HEK293T cells using 40 μl of GenePorter reagent (Gene Therapy Systems). The transfected cells were grown for 3 days in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 9% bovine calf serum (Hyclone) and 0.9% penicillin and streptomycin (Invitrogen). One tablet of complete Mini EDTA-free protease inhibitor mixture (Roche Applied Science) was dissolved in 10 ml of MilliQ H2O. Ten ml of media was harvested into 1 ml of the protease inhibitor on ice. The media were centrifuged at ~4500 × g for 10 min to remove cell debris and decanted into 275 μl of 1 m Tris-HCl (Fisher), pH 8.0, on ice. Ni2+-nitrilotriacetic acid beads (Qiagen) were equilibrated in wash 2 (150 mM NaCl (Fisher), 10 mM Tris-HCl, pH 7.8) and added to the media (120 μl of 50% slurry per 10 ml of media). The beads were placed on a rotator at 4 °C for 1 h and then poured into a small disposable column in a cold room. The beads were washed with 10 bed volumes of 500 mM NaCl, 10 mM Tris-HCl, pH 7.8, 10 bed volumes of wash 2, three times with 1 bed volume of 50 mM imidazole (Sigma), 150 mM NaCl, 10 mM Tris-HCl, pH 7.8, and then eluted five times with 1 bed volume of 250 mM imidazole, 150 mM NaCl, 10 mM Tris-HCl, pH 7.8. The elutions were consolidated, and glycerol (J. T. Baker Inc.) was added to 20% by volume. A sample was removed and mixed with 5× SDS gel loading buffer for immunoblot quantification, and the remainder was aliquoted and immediately frozen at −80 °C.

*Enzyme Quantification—*Lfng-MycHis6 protein levels were quantified by immunoblot of 10% SDS-PAGE compared with adjacent lanes containing 400-, 200-, 100-, 50-, 25-, and 12.5-ng samples of purified Lfng-MycHis6, standard purified and quantified as described previously (16). The gel was then blotted onto nitrocellulose (Bio-Rad) and blocked at 4 °C overnight in 5% nonfat dried milk in 150 mM NaCl, 10 mM phosphate buffer, pH 7.4 (PBS). The blot was incubated at room temperature with gentle rocking with a 1:500 dilution of a His probe rabbit polyclonal IgG antibody (H15, Santa Cruz Biotechnology) in PBS plus 0.1% Tween 20 (Sigma) (PBS/Tween) for 45 min. The blot was rinsed and washed in PBS/Tween followed by incubation in a 1:10,000 dilution of the Alexa-Fluor 660 goat anti-rabbit IgG
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(H+L) antibody (Molecular Probes) in PBS/Tween for 1 h in the dark, after which the blot was rinsed and washed as before. The blot was then exposed using the Odyssey infrared imaging system (LI-COR) using the 700-nm channel, 169-μm resolution setting, set to medium quality, and intensity 5.0. The bands were quantified using the Odyssey software with a reciprocal fit to the standard curve.

Enzyme Assays—Lfng assays were adapted from previous assays (16). The assays were performed for 30 min at 37 °C, in 50 mM HEPES, pH 6.8, 10 mM MnCl₂, 400 μM UDP-GlcNAc (0.5 μCi of UDP-[6-3H]GlcNAc (60 Ci/mmol, American Radiolabeled Chemicals)) in 50-μl final volume. The pNP-fucose substrate was prepared in DMSO to allow higher concentrations to be used. The final concentration of DMSO in the reaction was 30% (v/v). Alkaline phosphatase (3.5 units, Roche Applied Science) was added to each reaction to degrade UDP. Finally, 200 ng of Lfng enzyme in 20% (v/v) glycerol was added to initiate the assay. The reactions were stopped and loaded directly onto C18 cartridges (Agilent) as described (16). The samples were eluted from the C18 columns three times with 500 μl of 80% methanol. The eluted samples were then vortexed with 5 ml of Scintiverse LS6500 scintillation counter for 1 min per sample. The pNP-fucose saturation curves were performed with concentrations of pNP-fucose between 5 and 100 mM and 400 μM UDP-GlcNAc (0.5 μCi of UDP-[6-3H]GlcNAc (60 Ci/mmol, American Radiolabeled Chemicals)). The UDP-GlcNAc saturation curve was measured with UDP-GlcNAc concentrations of 1, 2, 5, 10, 20, 100, 250, and 500 μM with a pNP-fucose concentration of 100 μM. UDP inhibition curves were performed with UDP-GlcNAc concentrations of 5, 20, 100, 250, and 500 μM with UDP concentrations of 0, 2, 5, and 10 μM and pNP-fucose concentrations of 5 and 100 mM. UMP inhibition curves were performed with UDP-GlcNAc concentrations of 5, 20, 100, 250, and 500 μM, UMP concentrations of 0, 20, 50, and 100 μM, and a pNP-fucose concentration of 100 μM. UDP-Glc utilization assays were performed with 100 μM UDP-Glc (0.5 μCi of UDP-[6-3H]Glc (60 Ci/mmol, American Radiolabeled Chemicals)) and 100 μM pNP-fucose. The Vₘₐₓ values for each enzyme variant were normalized using a standard source of Lfng with saturating pNP-fucose in triplicate.

Fitting of Enzyme Curves—The efficiency of 3H counting was determined by preparation of a calibration curve from known amounts of 3H. The equation of this curve was used to determine the disintegrations/min, which were converted into moles based on the specific activity of the radiolabel. Velocities in pmol/liter/s and substrate concentrations in picomoles were fit to Michaelis-Menten curves using the program EnzFitter (Biosoft®). UDP and UMP inhibition data were fit to various inhibition curves using EnzFitter (Biosoft®). All fits used the Marquardt-Levenberg algorithm (39, 40).

UDP-hexanolamine-Agarose Binding Assay—Between 400 ng and 1 μg of enzyme was incubated 30 min on ice with an excess of UDP-hexanolamine-agarose (11 μmol/ml, a generous gift of Dr. Gerald Hart, Johns Hopkins University School of Medicine) in 50 mM HEPES, pH 6.8, 10 mM MnCl₂, with or without 100 mM EDTA. The beads were equilibrated in 50 mM HEPES, pH 6.8, 10 mM MnCl₂ prior to use. Subsequent to the incubation on ice, the beads were pelleted; the supernatant was removed and the bead volume brought back to an equivalent of the supernatant, and samples were removed and mixed with 5× SDS gel loading buffer. In the case of some mutants, we could not produce 1 μg of enzyme in a small enough volume. For this reason, the amount of each sample loaded on the gel was normalized to the lowest concentration sample so that in each case an equivalent amount of total enzyme was present. The electrophoresis and immunoblotting were performed as described above under “Enzyme Quantification.”

RESULTS

Generation of an Lfng Homology Model—Elimination of Lfng causes a significant somitogenesis phenotype when knocked out in mice (41, 42), whereas Rfng has no known phenotype (43), and an Mfng knock-out has not yet been published. In addition, a mutation in human Lfng has recently been suggested to result in the human genetic disorder SCD (7). Finally, previous work has shown that Mfng and Rfng show significantly lower activity toward EGF-O-fucose in vitro than Lfng (16). For these reasons, we decided to focus our efforts on understanding the catalysis and substrate recognition of mouse Lfng. Mouse Lfng has a stretch of 285 residues with 49.8% identity and 71.6% similarity to Mfng, and these enzymes are 52.5% identical and 72.2% similar over the stretch of sequence for which density was observed in the Mfng structure (supplemental Fig. S1). This high degree of identity made it possible for us to create a homology model for Lfng on the basis of the Mfng structure (Fig. 1).

The Lfng homology model superimposes on the Mfng structure with a root mean square deviation over the backbone of 0.48 Å. Comparison of the Mfng structure and Lfng homology...
model shows nearly identical Ramachandran statistics. The Mfng structure has all residues in most-favored (91.7%) or additionally allowed regions (8.3%). The Lfng model has 94.4% in most-favored 4.7% in additionally allowed regions and 0.9% (2 residues) in generously allowed regions. These two residues are very near the C terminus, and we do not consider them significant.

Docking FIX EGF-O-Fucose onto the Lfng Homology Model—To gain an understanding of how Lfng interacts with fucose on an EGF repeat, we docked a previously described (16) model of Factor IX EGF bearing O-fucose (EGF-O-fucose) onto the Lfng model using the HEX program (36). This program docks the ligand based primarily on shape complementarity. After manually culling the lowest energy solutions of any docked EGF repeats where the fucose was not in proximity to the active site, we retained 73 solutions. The fucose of docked EGF repeats was observed to cluster in roughly two groups, on opposite sides of the catalytic aspartate 289 (Fig. 2, C and D). Interestingly, the docking solutions reveal a cavity for the donor substrate UDP-GlcNAc between the enzyme and docked EGF-O-fucose, adding weight to the reliability of these models (Fig. 2, C and D). We achieved identical docking results using the Mfng structure and our Lfng homology model.

Kinetic Characterization of the Wild-type Lfng Enzyme—Initially we performed a thorough kinetic analysis of wild-type Lfng using the conditions described above (Table 1). We chose to use the small molecule, pNP-fucose, as acceptor substrate for the following reasons. First, the EGF-O-fucose acceptor would undoubtedly allow many protein-protein contacts with the enzyme that would mask the effect of mutants on the \( K_m \) value for the fucose. Second, pNP-fucose is readily available, whereas EGF-O-fucose is hard to produce in large amounts and, as we discuss later, cannot be produced in quantities capable of saturating the enzyme. We also evaluated inhibition of Lfng by UDP and UMP (Table 1). Many published glycosyltransferase structures with donor substrates present show density for the nucleotide portion of the donor, but no density for the sugar in the absence of acceptor substrate (44–46). The Mfng structure was no exception, suggesting that in the absence of acceptor substrate, Mfng interacts mainly with the nucleotide portion of the donor (26). Not surprisingly then, glycosyltransferases are strongly inhibited by the nucleotide product generated after glycan transfer. In the Golgi, a nucleoside diphosphatase rapidly degrades UDP to UMP to eliminate this inhibition (47). We characterized the inhibition of Lfng by UDP and UMP and found both to be competitive inhibitors of the enzyme, with a \( K_i \) of 11.04 \( \mu \)M for UDP and \( K_i \) of 96.35 \( \mu \)M for UMP (Table 1). Thus, we added alkaline phosphatase to our standard assays to degrade UDP.

Analysis of Lfng Mutants—We used the docked EGF-O-fucose solutions and sequence alignment data to inform our choice of Lfng mutants. We aligned all sequences annotated as Fringe or Fringe-like enzymes in the NCBI protein data base excluding any plant sequences (supplemental Fig. S2). Although proteins annotated as Fringe-like proteins may not be Fringe enzymes, we found that inclusion of the more distantly related Fringe-like sequences made several residues stand out as extremely conserved. The most obvious example is the DXD motif (\(^{190}\)DDD\(^{201}\)) in mouse Lfng, supplemental Figs. S1 and S2), which is conserved across many glycosyltransferases (48). Mutating this well characterized motif is known to abrogate enzymatic activity in a number of glycosyltransferases, including both Drosophila Fng (15) and mouse Mfng (19). However, serine 228 and serine 312 are two other residues in the vicinity of the active site that also showed this very high level of conser-
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Table 1

Kinetic data for wild-type and mutant lunatic fringe

All $K_m$ and $V_{max}$ values reported here were produced from the appropriate fit using EnzFitter (Biosoft®). The $V_{max}$ values reported here are per mg of enzyme. All assays were performed with 110 mM enzyme in a 50-μl volume. All errors reported are standard error values from the corresponding fit produced with EnzFitter (Biosoft®). Due to variation in Lfng activity from day to day, all $V_{max}$ values were normalized to a standard Lfng assay using a standard Lfng source. The percent wild-type (WT) activity values for the donor specificity mutants were produced by dividing mutant enzyme activity utilizing UDP-GlcNAc or UDP-Glc by the wild-type Lfng activity with the appropriate donor.

| Wild type | UMP inhibition | UDP inhibition |
|-----------|---------------|---------------|
| $V_{max}$ (μmol/liter/s) | UDP GlcNAc | pNP-fucose |
| $K_m$ (μM) | 43.93 ± 0.31 | 0.08 |
| $K_m$ (μM) | 37.94 ± 2.47 | 10.91 ± 0.65 |
| $K_i$ (μM) | 11.04 ± 2.50 | |
| Mutants that did not express | | |
| R173A | D289N | D289S | C290A | S312Q |
| Catalytically inactive active site mutants | | |
| S228L | S228Y | F251S | T253A | D289E |
| Active site mutants to short loop side of aspartate 289 | | |
| G254A | D288A |
| Active site mutants to long loop side of aspartate 289 | | |
| S228A | S228T | F251Y | C290S |
| Short loop mutants | | |
| S168A | S168V | H171A | H171D | A175V | L176A |
| UDP-GlcNAc saturation curves for short loop mutants | | |
| S168A | S168V | H171A | H171D | A175V |
| Long loop mutants | | |
| L229Q | I233A | A235Y | E237A |
| Donor specificity mutants | | |
| S312T | H313A | L314R | G334H | H313A + G334H |

$^a$ We were unable to saturate the enzyme with an EGF-O-fucose acceptor substrate despite reaching a maximum concentration in excess of 190 μM in the assay. We estimate the $K_m$ for EGF-O-fucose to be approximately 2 mM from a Hanes-Woolf plot of the available data (data not shown). This is unexpectedly high, given that the EGF-O-fucose is a much better substrate for the enzyme than the pNP-fucose (16). However, the extrapolated $K_m$ for the enzyme with these data would be approximately 2 s⁻¹, which is considerably better than the value of 0.08 that we see with pNP-fucose. We had previously published a specific activity for Lfng with 5 μM Factor IX EGF-O-fucose of 16 nmol/min/mg (16). In this study we found a similar value of 13.5 nmol/min/mg. We see activity of approximately 309 nmol/min/mg at approximately 190 μM EGF-O-fucose compared to approximately 132 nmol/min/mg when saturated with pNP-fucose.

$^b$ In some cases activity was too low to measure below saturating pNP-fucose concentrations, and a Michaelis-Menten curve could not be produced. In these cases we report the velocity at 100 μM pNP-fucose rather than $V_{max}$, and no value for $K_m$, which could not be determined.

viation. Fig. 3 shows the sequence conservation mapped onto the Lfng model surface in the vicinity of the active site.

Our mutagenesis strategy focused on four areas. First, we focused on conserved residues in the two disordered loops flanking the active site for which there was no electron density in the Mfng structure (26). Second, residues in the vicinity of the two clusters of fucose on either side of the putative catalytic aspartate 289 were mutated. Third, we mutated residues in the vicinity of the UDP-GlcNAc-binding site. We chose residues that might confer specificity for this bulkier nucleotide sugar donor compared with UDP-Glc, the substrate for a related, fucose-specific β1,3-glucosyltransferase (β3GlcT) (29, 30).

Finally, we also produced a mutant associated with the human genetic disorder SCID (7). All together we made 35 mutants encompassing 22 separate residues (Table 1 and Figs. 6 and 7). Five of these (R173A, D289N, D289S, C290A, and S312Q) failed to produce assayable protein, three of which could not be visualized in an immunoblot of cell lysates or media (supplemental Fig. S3).

Catalytically Inactive Mutants—Of the mutants that produced protein that could be purified and assayed, eight (L176A, S228L, S228Y, F251S, T253A, D288A, D289E, and S312T) are essentially catalytically inactive with the UDP-GlcNAc donor, as measured in our assay (Table 1). The S312T mutant is unique.
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FIGURE 4. The position of the short loop relative to the catalytic aspartate. Lfng (gray schematic) with Cys-175, Ser-177, Cys-178, Asp-288, Asp-289, and Cys-290 in black sticks with all-bonds coloring. UDP-GlcNAc is shown in all bonds representation with white carbon atoms. The Mn²⁺ ion is shown as a green sphere. The short loop is drawn with a dashed line.

among this group in that despite a lack of Fringe β1,3GlcNAc transferase enzyme activity, this mutant retains some residual activity with a UDP-glucose (UDP-Glc) donor (discussed below). Interestingly, the inactive mutants include the D288A variant, next to the putative catalytic aspartate 289. Although some residual catalytic activity remains, it is measurable only at saturating acceptor substrate concentrations and is barely above background. The Mfng structure (26) and Lfng model show that aspartate 288 forms a hydrogen bond with serine 177 at the base of the short loop (Fig. 4). Both of these residues are highly conserved in fringes (either serine or cysteine at position 177, and aspartate at position 288 for all fringes, supplemental Fig. S2). At a minimum, it appears that the aspartate 288–serine 177 hydrogen bond is critical for catalysis. Alternatively, if the short loop becomes ordered during or subsequent to substrate binding, the position of serine 177 may be altered, which could in turn affect aspartate 288 and the backbone conformation of the putative catalytic aspartate 289. Thus, ordering of the short loop may move aspartate 289 into a catalytically optimal configuration (Fig. 4). We were unable to express either the D289S or D289N mutants, despite robust expression and secretion of mutants affecting the adjacent residues on either side of this aspartate, as illustrated by the D288A and C290S mutants (Table 1). The D289E variant was the only mutant of the catalytic aspartate that produced secreted protein that we could purify and assay. This mutant was catalytically inactive, consistent with the prediction that this is the catalytic aspartate (Table 1).

Effects of Mutations in the Loops and Residues Flanking Aspartate 289—Jinek et al. (26) referred to the region between the long loop and the catalytic site as a putative fucose binding pocket. This is approximately the area encompassed by Thr-253, Ser-228, and Phe-251 in Fig. 3. Although disordered, the long loop is adjacent to one of the fucose clusters we observed in our docking solutions (Fig. 2D), whereas the short loop is adjacent to the other (Fig. 2C). When we tabulate $V_{\text{max}}$ and $K_m$ values for our loop mutants (Table 1), we find a striking pattern where most of the $K_m$ defects localize to mutants in or near the long loop and the putative fucose binding pocket, whereas the more significant $V_{\text{max}}$ defects localize to the short loop region near the alternative fucose cluster we observed in our docking experiment (Fig. 5). This suggests that the fucose is indeed localized near the putative fucose-binding pocket on the long loop side of the active site (as shown in Fig. 2D), and not on the short loop side. The $V_{\text{max}}$ defects indicate that the short loop closes one side of the active site pocket and does not make significant contributions to the affinity of the acceptor for the active site. These short loop mutants do not show substantial $K_m$ defects for UDP-GlcNAc (Table 1) suggesting minimal contact is made with the donor substrate.

Four mutants showed a large $K_m$ defect and little or no $V_{\text{max}}$ defect (S228A, S228T, F251Y, and C290S, see Table 1). Perhaps the most striking defect occurred with the C290S mutant (Fig. 6A). This free cysteine is a direct neighbor of the catalytic aspartate 289. Despite the conservative nature of the mutation, the large $K_m$ defect strongly suggests that the fucose binds near the long loop and that the sulfur of Cys-290 makes an important
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FIGURE 5. Clustering of \( V_{\text{max}} \) and \( K_m \) defects to either side of the catalytic aspartate. Lfng is shown in white surface representation, UDP-GlcNAc in black sticks and all-bonds coloring, mutants with \( V_{\text{max}} \) defects in yellow, and those with \( K_m \) defects in orange, and the catalytic aspartate in red. The loops are drawn with a dashed line.

contact with the acceptor. Phenylalanine 251 when mutated to tyrosine (Fig. 6B) exhibits a striking \( K_m \) defect, whereas the serine mutant of this residue is catalytically inactive (Table 1). This phenylalanine forms part of the floor of the active site toward the long loop side of aspartate 289, and it would appear from these results to provide needed hydrophobic bulk at the base of the long loop (Fig. 3). The \( K_m \) defect from the tyrosine mutation implies a steric clash with the pNP-fucose reinforcing the conclusion that the substrate is on this side of the catalytic pocket. Mutating the highly conserved residue serine 228 to leucine or tyrosine produced inactive enzyme (Table 1 and Fig. 6, C and D). Making the less dramatic mutant S228A produced a significant \( K_m \) defect (Table 1) suggesting that the hydroxyl of the serine makes contact with the pNP-fucose substrate, further reinforcing the probability that the acceptor is positioned in this area of the active site (Fig. 3). The S228T mutant produced a mixed \( K_m \) and \( V_{\text{max}} \) defect (Table 1) suggesting that any additional bulk in this position is poorly tolerated. This is not a surprise considering the broad conservation of serine at this position in Fringe and Fringe-like enzymes (supplemental Fig. S2).

Flanking aspartate 289, threonine 253 forms part of the floor of the active site, near the edge of the donor-substrate binding site (Fig. 3). Mutation of threonine 253 to alanine (Fig. 6E), a relatively conservative mutation, produces inactive enzyme (Table 1). Because there are no obvious hydrogen bonds involving the hydroxyl of the threonine, this suggests that the bulk of the threonine is important for the conformation of the pocket. Glycine 254 is positioned near the mouth of a small cavity on the short loop side of the catalytic aspartate 289 (Fig. 3). The mutation of glycine 254 to alanine produces a \( V_{\text{max}} \) defect presumably through steric effects on the positioning of aspartate 289 (Table 1 and Fig. 6F).

The possibility existed that the mutants exhibiting \( V_{\text{max}} \) defects were in fact unstable, which would decrease the overall catalytic efficiency of a given aliquot of enzyme. We eliminated this possibility by testing their ability to bind to UDP-hexanolamine-agarose and observed behavior identical to wild-type enzyme (supplemental Fig. S4). This indicates that these mutations are affecting catalysis, and the \( V_{\text{max}} \) deficits are not because of misfold catalytically inactive contaminants in the enzyme aliquots.

Effects of Mutants on the Utilization of the EGF-O-Fucose Acceptor—We were unable to generate sufficient amounts of the EGF-O-fucose acceptor substrate to saturate the enzyme. Because of this, only the linear portion of the Michaelis-Menten curve was accessible in our assays. Because of the limited amount of this substrate available, we decided to measure the effect of the mutants on Lfng utilization of EGF-O-fucose by looking for a change in the slope of the linear portion of the Michaelis-Menten curve. Because the \( V_{\text{max}} \) and \( K_m \) defects clustered with the pNP-fucose acceptor substrate, we looked for evidence of clustering with the EGF-O-fucose as well. The \( K_m \) cluster of mutants (supplemental Fig. S5A) showed very little difference from wild-type Lfng in the linear portion of the Michaelis-Menten curve. This suggests that our initial prediction that \( K_m \) effects for the fucose would be masked by contacts between the enzyme and the EGF portion of EGF-O-fucose is reasonable. The \( V_{\text{max}} \) cluster of mutants showed obvious changes in slope compared with wild-type Lfng (supplemental Fig. S5B). Although these results are consistent with a \( V_{\text{max}} \) defect, we cannot rule out an effect on \( K_m \) because the enzyme is not saturated with substrate. Nonetheless, these data suggest that we are drawing reasonable conclusions about general acceptor substrate behavior because the two groups of mutants continue to distinctly cluster with the larger EGF-O-fucose substrate. These results also suggest that use of the small molecule acceptor allows us to observe mechanistic details of the interaction between the enzyme and the fucose that would not be evident in a kinetic analysis with the larger EGF-O-fucose substrate.

Specificity for UDP-GlcNAc Versus UDP-Glc—Recently, a Fringe homologue was identified that transfers glucose from UDP-\( \alpha \)-glucose to O-fucose on TSRs forming a Glc-\( \beta \)1,3-Fuc-\( \alpha \)-O-Ser/Thr disaccharide (29, 30). Interestingly, both enzymes can utilize pNP-fucose as an acceptor substrate (49). Although the degree of sequence homology between Mfnf and the \( \beta 3 \)GlcT is somewhat low (20.7% identity, see supplemental Fig. S6), we were able to create a homology model of the \( \beta 3 \)GlcT threaded onto the Mfnf structure (PDB 2J0B) using the ESyPred3D server (34). We compared the models of Lfng and the \( \beta 3 \)GlcT to help ascertain which residues might contribute to the ability of these enzymes to discriminate between UDP-GlcNAc and UDP-Glc. Comparisons of these two structures are shown in Fig. 7. Fig. 7A shows four side chains from the \( \beta 3 \)GlcT homology model that protrude from the Lfng surface, and may affect the shape of the UDP-sugar binding pocket. Fig.
7B shows these side chains from Lfng and β3GlcT overlapped, whereas Fig. 7C shows the wild-type residues, and Fig. 7D shows those residues mutated to the β3GlcT sequence. Both the Lfng and β3GlcT models show a histidine chelating the Mn²⁺ ion, although in Lfng this is histidine 313 (alanine in β3GlcT), whereas in the β3GlcT it is histidine 334 (glycine in Lfng) (Fig. 7, B, C and D). Additionally, the side chain of leucine 314 in Lfng corresponds to an arginine in β3GlcT (Fig. 7B), whereas the highly conserved serine 312 in Lfng is in the same position as a glutamine in β3GlcT (Fig. 7, B, C and D).

To examine whether any of these residues affected the ability of Lfng to utilize UDP-glucose versus UDP-GlcNAc, we generated a series of mutants converting the corresponding residues to those of the β3GlcT. Wild-type Lfng transfers glucose from UDP-glucose to pNP-fucose at ~10-fold reduced catalytic efficiency when compared with transfer of GlcNAc from UDP-GlcNAc. Thus, we measured mutant catalytic efficiency toward each donor separately as a percentage of the wild-type activity with that donor. We took the activity for the mutants utilizing a specific donor substrate, and we divided by the activity of wild-type Lfng with that donor to compute the percentage of wild-type activity retained. The H313A mutant is all but dead when analyzed for UDP-GlcNAc transfer, although it retains 75% of the wild-type glucose transfer activity (Table 1 and Fig. 8). Thus, the loss of this histidine is considerably more permissive toward UDP-Glc utilization in a productive complex, and it seems to prevent UDP-GlcNAc utilization. Replacement of glycine 334 with histidine (Fig. 7, A–D) does not seem to have a dramatic effect on catalytic efficiency with either donor (Fig. 8). However, this highly nonconservative mutation is strikingly permissive to the utilization of either of the donor nucleotide sugars tested, with roughly 78% of activity retained with UDP-Glc and 94% of activity with UDP-GlcNAc (Fig. 8). Not surprisingly, the H313A/G334H double mutant is all but dead when utilizing UDP-GlcNAc, which is likely due mainly to the H313A mutation. In contrast there is significant (34%) retention of UDP-Glc utilization with this double mutant (Table 1 and Fig. 7D). Leucine 314 is pointing toward the GlcNAc of the donor substrate in Lfng (Fig. 7, A–C), and mutation of this residue to arginine disrupts GlcNAc transfer considerably but is highly permissive toward Glc transfer, exhibiting an even higher than wild-type catalytic efficiency when utilizing the UDP-Glc donor (Fig. 8). Jinek et al. (26) mutated this leucine in Mfng to phenylalanine and saw no effect on their in vivo ligand binding assay. This suggests that although Leu-314 is a conserved residue in the enzyme, the introduction of side-chain bulk by phenylalanine can be toler-
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FIGURE 7. Lfng donor specificity mutants. A, Lfng is displayed as white surface, and β3GlcT residues from the ESyPred3D model are displayed as black sticks with all-bonds coloring; UDP-GlcNAc is displayed as brown sticks with all-bonds coloring, and the Mn²⁺ is displayed as a green sphere. This panel shows S228Y, S312Q, H313A, L314R, and G334H. The red arrow shows the approximate viewpoint of the following panels. B, nucleotide-sugar binding pocket showing all mutants plus the DDD motif chelating the manganese. Lfng is displayed as white sticks with all-bonds coloring. Mutated residues are displayed as black sticks with all-bonds coloring. C, wild-type Lfng residues (white sticks) from B. D, β3GlcT model residues (black sticks) from B. The numbering of residues in all panels refers to the corresponding mouse Lunatic fringe residues.

FIGURE 8. Relative activity of Lfng mutants with UDP-GlcNAc and UDP-Glc donor substrates. The percentage of wild-type Lfng activity with each donor is shown for several mutants. The white bars indicate UDP-GlcNAc utilization, and the shaded bars indicate UDP-Glc utilization. The error bars show the standard deviation of the measurements. These data are tabulated in Table 1 under the heading Donor specificity.

DISCUSSION

Until now, no kinetic characterization of Fringe enzymes with saturating concentrations of an acceptor substrate has been published. More importantly, the ability to saturate has allowed us to utilize assays to assess the relative effect of various mutations in the Lfng active site. Our data support fucose binding in a specific region of the Lfng active site. We conclude that the fucose is situated on the long loop side of aspartate 289 near the putative
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fucose binding pocket proposed by Jinek et al. (26). The fact that mutations in the short loop result in \( V_{\text{max}} \) but not \( K_m \) defects led us to the proposal that the short loop becomes ordered coincident with, or subsequent to, acceptor substrate binding. We propose that ordering of the short loop may affect the hydrogen bond between serine 177 and aspartate 288 and as such could affect the conformation of the catalytic aspartate 289. Furthermore, we conclude that several residues in the vicinity of the bound UDP-GlcNAc are specifically permissive toward utilization of this larger donor compared with UDP-Glc.

All our data (Table 1 and Fig. 5) indicate that the fucose cluster on the long loop side of the catalytic aspartate represents the correct site of interaction (Fig. 2D). Furthermore, the authors of the MfnG structure refer to this area as the putative fucose-binding pocket based on a comparison with human glucuronyltransferase I (26, 51). They also modeled the UDP-GlcNAc into the active site based on the rabbit GlcNAc-transferase I structure (27), with the GlcNAc folded over to expose the anomeric carbon toward the long loop side of the enzyme. This fits well with our data suggesting the fucose is positioned on this side of the active site. The dramatic \( K_m \) defect from mutating cysteine 290 to serine is highly suggestive that the sulfur of the cysteine makes contact with the acceptor substrate, and that the acceptor substrate is on the long loop side of the active site. An alternative explanation would be that the cysteine makes a critical contact with the long loop as it becomes ordered and, as such, affects acceptor substrate binding indirectly, a possible albeit not very likely explanation. The conservative nature of the mutation and the dramatic effect it has on \( K_m \) can most easily be explained by the decreased size of the oxygen of the serine relative to the sulfur of the cysteine. If this were affecting the ordering of the long loop, one might expect a far smaller \( K_m \) defect as additional contacts between the ordered loop and the enzyme-substrate complex would contribute to the overall stability of the complex. Indeed, we see more modest \( K_m \) defects when we introduce nonconservative mutations for identically conserved residues in the long loop such as with I233A, A235Y, and E237A. It seems unlikely a single contact, altered in such a conservative manner as the C290S mutation, could so dramatically affect the large protein mass of the loop, whereas a dramatic effect from a single contact with the small molecule acceptor is easily conceivable.

It is possible that the N-acetyl group could be oriented up and out of the pocket, rather than in the position shown in Fig. 7. There appears to be room for such an arrangement in our docked solutions (Fig. 2, C and D). Although there is no reason this could not be the case, it seems more likely that the N-acetyl group would be probed by the binding pocket to discern the identity of the donor. With the N-acetyl moiety of the GlcNAc oriented toward Leu-314, we can see an explanation for the severely decreased utilization of UDP-GlcNAc by the L314R mutant (Fig. 7, A, B, and D), whereas UDP-GlC utilization remains at or above wild-type levels (Fig. 8). Another possibility is that following activation there is a rearrangement allowing the activated fucose to access the anomeric carbon of the GlcNAc sugar.

The \( V_{\text{max}} \) defects associated with the short loop mutants could be attributed to an altered local environment in the catalytic pocket if the loop is unable to close off the active site from the solvent. However, our docked solutions show little access to solvent on this side of the pocket (Fig. 2A). Another possibility is that the closing of the loop upon substrate binding alters the positioning of the catalytic aspartate. The A175V mutant is at the base of the loop closest to the catalytic aspartate. This residue, along with Leu-176, appears to anchor one side of the loop, at a point that is behind the catalytic aspartate, and movement of the anchor point, or disruption of the anchoring residues by mutation, could propagate to the backbone of the catalytic residue through the serine 177–aspartate 288 hydrogen bond (Fig. 4). Although the Richardson laboratory has found that altering the backbone for most residues may change the frequency of the preferred side-chain rotamer, but not its position (52), the situation is different for aspartate and asparagine, where altering the backbone also changes the side-chain position (52). The mutation of aspartate 288 to alanine produces enzyme that is almost completely inactive. Whereas some residual activity with saturating pNP-fucose concentrations can be measured, it is barely above background, clearly indicating that this mutant produces a most dramatic \( V_{\text{max}} \) deficit. It is hard to imagine that an alanine at this position could produce such a dramatic effect without larger structural implications. This suggests at the very least that the hydrogen bond between aspartate 288 and serine 177 is critical for enzymatic activity. Aspartate 288 is at the beginning of an \( \alpha \)-helix, and it is reasonable to suggest from the effects of the D288A mutation that the active site terminus of this helix is less stable in the absence of the Ser–177 to Asp–288 hydrogen bond. We suggest that perturbation of this hydrogen bond by an altered short loop conformation could propagate a conformational change to the neighboring aspartate 289 residue. The question remains whether the hydrogen bond is critical for the proper orientation of aspartate 289 in a static fashion, or whether a conformational change occurs upon substrate binding. Our data are suggestive of the latter interpretation.

Interestingly, Correia et al. (50) in a genetic screen of Fringe in Drosophila found a lethal phenotype when the residue equivalent to leucine 176 at the base of one of the short loop termini was mutated to phenylalanine, suggesting that disruption of this loop can have similar effects in vivo. The presence of an aspartate or glutamate in the Asp–288 position is conserved across a large number of glycosyltransferases (50). There is an aspartate involved in a hydrogen bond at a helix terminus next to an aspartate facing the active site in at least two other enzyme structures (27, 38). Additionally, mutation of the corresponding residue in peptidoglycan glycosyltransferase (38) results in severely defective enzyme, mirroring our result with this residue.

Mutations of residues surrounding the donor substrate suggested by the \( \beta3GlcT \) homology model were effective at curtailing or eliminating utilization of UDP-GlcNAc as a donor while showing markedly less or no effect on the ability of the enzyme to utilize UDP-Glc. Leucine 314 appears to be permissive toward UDP-GlcNAc utilization in Fringe enzymes, because an arginine in this position severely curtails utilization of the larger donor, whereas activity with the smaller UDP-Glc donor is higher than wild type (Table 1 and Fig. 7, A, B, and D). Interestingly, this leucine is conserved in all of the sequences we
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included in the alignment, except for the Fringe-like protein from the sea squirt *Ciona intestinalis*, which has an arginine at this position (supplemental Fig. S2). This sea squint protein also contains a threonine at the position of histidine 313 (supplemental Fig. S2), which suggests, considering what we have learned about histidine 313 and leucine 314 in Lfng, that this Fringe-like protein is not a fringe enzyme. Finally, S312T, although catalytically dead toward utilization of UDP-GlcNAc (Table 1 and Fig. 8), retains some activity (18%) with the UDP-Glc donor-substrate. Correia et al. (50) reported a lethal phenotype for the corresponding mutant in *Drosophila* Fringe. Because elongation past the disaccharide does not appear to be essential for Fringe function in *Drosophila* (21), one wonders if overexpression of this particular mutant could at least partially rescue the lethal phenotype caused by the same mutation through addition of Glc rather than GlcNAc.

We have observed that Lfng migrates as a doublet during SDS-PAGE (supplemental Figs. S3 and S4) and that peptide-N-glycosidase F digestion of N-linked glycans eliminates this doublet (data not shown). Mutation of serine 168 to alanine or valine in the sequence NCS eliminates this doublet and causes a shift on the gel, suggesting that asparagine 166 carries an N-linked glycan (supplemental Fig. S4). This coincides with asparagine 109 in mouse Mfng which was mutated to glutamine by the authors of the Mfng structure to eliminate presumed N-linked glycosylation at this site (26). Unlike Mfng, mouse Lfng does not contain a second N-linked glycosylation site in the long loop. In mouse Mfng, asparagine 185 in the sequence NRT is likely glycosylated, whereas the corresponding position in mouse Lfng is histidine 242 (supplemental Fig. S1). Human Lfng however retains the asparagine, but the sequence is NRV, which should not be glycosylated. Because Mfng exhibits considerably lower catalytic efficiency toward EGF-O-fucose in *vitro* in comparison with Lfng (16), it is possible that this glycan contributes, at least in part, to this lower efficiency by modulating the interaction of the long loop with the acceptor substrate.

The kinetic data from the Lfng mutants suggests that the fucose is most likely positioned near what has previously been referred to as the putative fucose binding pocket (26). The *V*<sub>max</sub> and *K*<sub>m</sub> values of the short loop mutants, coupled with a lack of *K*<sub>m</sub> deficit on that side of the active site, suggest the possibility that this loop changes conformation subsequent to substrate binding, affecting the hydrogen bond between serine 177 and asparagine 288. The altered conformation could then affect the catalytic residue to achieve maximum catalytic efficiency. This type of mechanism could be significant for a number of glycosyltransferases besides the Fringe enzymes.

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