Lunatic Fringe, Manic Fringe, and Radical Fringe Recognize Similar Specificity Determinants in O-Fucosylated Epidermal Growth Factor-like Repeats*

Received for publication, August 30, 2005, and in revised form, October 11, 2005 Published, JBC Papers in Press, October 12, 2005, DOI 10.1074/jbc.M509552200

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Notch signaling is a component of a wide variety of developmental processes in many organisms. Notch activity can be modulated by O-fucosylation (mediated by protein O-fucosyltransferase-1) and Fringe, a β1,3-N-acetylgalcosaminyltransferase that modifies O-fucose in the context of epidermal growth factor-like (EGF) repeats. Fringe was initially described in Drosophila, and three mammalian homologues have been identified, Manic fringe, Lunatic fringe, and Radical fringe. Here for the first time we have demonstrated that, similar to Manic and Lunatic, Radical fringe is also a fucose-specific β1,3-N-acetylgalcosaminyltransferase. The fact that three Fringe homologues exist in mammals raises the question of whether and how these enzymes differ. Although Notch contains numerous EGF repeats that are predicted to be modified by O-fucose, previous studies in our laboratory have demonstrated that not all O-fucosylated EGF repeats of Notch are further modified by Fringe, suggesting that the Fringe enzymes can differentiate between them. In this work, we have sought to identify specificity determinants for the recognition of an individual O-fucosylated EGF repeat by the Fringe enzymes. We have also sought to determine differences in the biochemical behavior of the Fringes with regard to their in vitro enzymatic activities. Using both in vitro and in vivo experiments, we have found two amino acids that appear to be important for the recognition of an O-fucosylated EGF repeat by all three mammalian Fringes. These amino acids provide an initial step toward defining sequences that will allow us to predict which O-fucosylated EGF repeats are modified by the Fringes.

The Notch protein is a transmembrane receptor involved in various cell fate decisions. It was initially characterized in Drosophila and has subsequently been found in all known metazoa (1). Notch becomes activated upon binding to its ligands located on apposing cells. These ligands, known collectively as the DSL (for Delta-Serrate-Lag2) family, fall into two classes, Serrate/Jagged and Delta. A large portion of the extracellular domain of Notch is composed of epidermal growth factor-like (EGF) repeats, and many of these EGF repeats contain consensus sites for modification by O-fucose (a process mediated by the protein O-fucosyltransferase-1 (O-FucT-1) (2, 3). Some of these O-fucosylated EGF repeats can be further modified by the actions of Fringe, a β1,3-GlcNAc transferase (4–6). The modification of Notch by Fringe plays an important role in the modulation of Notch signaling in various contexts (7, 8).

Fringe was initially described in Drosophila as a gene that modulates dorsal-ventral cell interactions in the developing wing (9). Further studies reveal that Fringe functions specifically by modulating the response of Notch to its ligands, potentiating Delta signaling while inhibiting Serrate signaling (10–12). Three mammalian homologues to Drosophila Fringe (Dfng) have been identified, Manic fringe (Mfng), Lunatic fringe (Lfng), and Radical fringe (Rfng). Lfng and Rfng are known to play important roles in developmental processes in vertebrates. For example, disruption of Lfng expression in chick or mouse embryos severely disrupts the formation of somitic borders (13–15). In mice lacking Lfng, there is increased perinatal death due to rib malformation. Mice that survive into adulthood display multiple defects related to somitogenesis (malformation of the vertebral column and ribs, fusion of the neural arches, and shortened tails). Rfng also functions in chick wing development (16, 17). In contrast, Rfng knock-outs in mice exhibit no obvious phenotype, and Lfng/Rfng double knock-outs in mice display phenotypes similar to those observed in Lfng knock-outs (15). Data regarding knock-out of Mfng in mice is not currently available.

Characterization of Dfng, Lfng, and Mfng has revealed that these enzymes are fucose-specific β1,3-GlcNAc transferases (4, 5). No data on the enzymatic activity of Rfng has yet been reported. Dfng, Lfng, and Mfng all require manganese for activity, as is the case for most other glycosyltransferases, and utilize uridine diphosphate N-acetylgalcosamine (UDP-GlcNAc) as a donor substrate (4). Little else is known about their specificity. The question of whether and how these enzymes differ is important for a complete understanding of how they modulate the Notch-signaling pathway. Furthermore, although Notch contains numerous EGF repeats that are modified by O-fucose, not all are subsequently modified by one of the Fringes (i.e. Fringes show a preference for some fucosylated EGF repeats over others) (6). The information necessary for the Fringes to differentiate between O-fucosylated EGF repeats appears to be encoded in the EGF repeats themselves (6). Nonetheless, the question of how the Fringe enzymes differentiate between O-fucosylated EGF repeats remains. Here we have begun to examine the specificity determinants for the recognition of individual O-fucosylated EGF repeats by the Fringe enzymes. We have also demonstrated that Rfng is indeed a fucose-specific β1,3-N-GlcNAc transferase (similar to Lfng and Mfng (4)), and we have sought to determine the differences in

*This work was supported in part by National Institutes of Health Grant GM 61126. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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3 The abbreviations used are: EGF, epidermal growth factor-like; Bnz-fuc, benzyl α-O-fucose; Mfng, Manic fringe; Lfng, Lunatic fringe; Rfng, Radical fringe; Dfng, Drosophila fringe; O-FucT-1, protein O-fucosyltransferase-1; CHO, Chinese hamster ovary; HPAEC, high pH anion exchange chromatography; Ni-NTA, nickel-nitrilotriacetic acid; MS/MS, mass spectrometry/mass spectrometry; HPLC, high pressure liquid chromatography.
the biochemical behavior of Lfng, Rfng, and Mfng with regard to their in vitro enzymatic activities.

EXPERIMENTAL PROCEDURES

Preparation of Constructs—Amino acids 93–129 of human factor IX and amino acids 106–147 of human factor VII were cloned into pSecTag2 (Invitrogen) using the HindIII and XhoI restriction sites. pSecTag2 encodes an IgG signal sequence for secretion and C-terminal Myc and hexahistidine tags. Point mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene) with wild-type factor VII and factor IX pSecTag2 constructs as templates. Constructs for expression of the mouse Fringe enzymes were produced by amplification from constructs containing the full-length sequence of each enzyme (generously provided by Dr. Tom Vogt (18)). In the case of Lfng and Rfng, sequences corresponding to the predicted lumenal domain (as described in Ref. 18) were amplified by PCR and subcloned into pSecTag2 (Invitrogen). The mature peptide corresponds to amino acids (as described in Ref. 18) were amplified by PCR and subcloned into pSecTag2 constructs as templates. Constructs for expression of the mouse Fringe enzymes were produced by amplification from constructs containing the full-length sequence of each enzyme (generously provided by Dr. Tom Vogt (18)). In the case of Lfng and Rfng, sequences corresponding to the predicted lumenal domain cloned into pSecTag2 failed. The primers used for amplification of Lfng were: forward, 5'-GATACTATAAAAGGTTTACGAGATTGATACAGGGGCCG-3' and reverse, 3'-TATATATCTCGAGGCGGCCGTGCACCAGGACA-5'. The products were digested with HindIII and XhoI and subcloned into pSecTag2 (restriction sites on primers are underlined). Primers used for amplification of Rfng were: forward, 5'-GATACTATAAAAGGTTTACGAGATTGATACAGGGGCCG-3' and reverse, 3'-AAGGCATATCCCTTGAGAACGTCCCTCACCT-5'. The products were digested with HindIII and EcoRV and subcloned into pSecTag2. In the case of Mfng, the full-length sequence was used (including the endogenous signal sequence/transmembrane domain, amino acids 1–31), as attempts to express the protein using the predicted lumenal domain cloned into pSecTag2 failed. The primers used for extension were: forward, 5'-TTTTAAAATAGGCTTATGCGACTGCCAGCCTTTT-3' and reverse, 3'-GAGGGCCCTCTAGAGGGGCCGTGCACCAGGACA-5'. The products were digested with HindIII and XbaI and subcloned into pCDNA4 (Invitrogen); this plasmid also encodes C-terminal Myc and hexahistidine tags. All constructs were confirmed by sequencing. For bacterial expression of EGF repeats, pET20b (Novagen) constructs encoding the first EGF repeat from human factor IX (amino acids 93–129) and factor IX bearing a double mutation were created using the constructs described above. Sequences were amplified from these constructs and subcloned into pET20b vector using BamHI and XhoI restriction sites in-frame with the pelB signal sequence for bacterial expression. The pET20b constructs were generated by the Stony Brook University Cloning Center and confirmed by sequencing. A pET20b construct encoding mouse Notch1 EGF 26 was similarly created using a previously reported pET20b construct of Notch1 EGF 26 (6).

Cell Lines—The Lec1 Chinese hamster ovary (CHO) cell line (19) was obtained from the American Type Culture Collection (Manassas, VA). Lec1-CHO cells were developed by the laboratory of Dr. Pamela Stanley (Albert Einstein College of Medicine). Lec1-CHO cells were grown in α-MEM/10% calf serum for 48 h. Proteins were purified from medium using Ni-NTA-agarose (Qiagen), and O-glycans were released by alkali-induced β elimination and analyzed by gel filtration chromatography as previously described (4, 20).

Purification of Fringe Enzyme—Constructs encoding each of the mouse Fringe enzymes were transfected into Lec1-CHO cells using Geneporter reagent (Gene Therapy Systems) according to the manufacturer’s recommendations. Stable transfectants were selected by the addition of 680 µg/ml hygromycin B (Calbiochem) (Lfng and Rfng) or 250 µg/ml zeocin (Invitrogen) (Mfng) and identified by dilution cloning. The expression levels of different clones were determined by immunoblot of the medium using an anti-hexahistidine antibody (Santa Cruz Biotechnology), and clones expressing the highest level of protein were identified. Cells were grown to confluency in 100-mm dishes with α-MEM supplemented with 10% calf serum. Subsequently, the medium was removed, and the cells were washed once with 5 ml of 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl (TBS) and then switched to 10 ml of OptiMEM (Invitrogen) for ~60 h. The medium was collected and clarified by centrifugation and then incubated with Ni-NTA-agarose (Qiagen) for 1 h at 4°C with rotation. Approximately 60 µl of Ni-NTA-agarose were used/ml of medium. The slurry was poured into a 0.8 × 4-cm Bio-Rad disposable column, and the Ni-NTA-agarose was then washed sequentially with 10 volumes of 0.5 M NaCl in 10 mM Tris-HCl (pH 7.4), followed by 10 volumes of TBS and three 1-ml aliquots of 25 mM imidazole in TBS. Protein was eluted with 5 1-ml aliquots of 50 mM imidazole in TBS. Aliquots of elutions were analyzed by 10% SDS-PAGE followed by Coomassie Blue staining and immunoblot with mouse anti-hexahistidine antibody (Santa Cruz Biotechnology). The identity of enzymes was confirmed by proteomic analysis. Briefly, protein samples were reduced in SDS sample buffer (lacking reducing agents) using 10 mM Tris (2-carboxyethyl)phosphine hydrochloride (Pierce) for 5 min at 95 °C and then carboxymidomethylated with 30 mM iodoacetamide for 30 min at room temperature in the dark prior to loading onto an SDS-polyacrylamide gel. The band corresponding to Lfng, Rfng, or Mfng was identified after staining the gel with Gel Stain (Pierce), cutting it out, and subjecting it to in-gel digestion with modified trypsin (Promega), essentially as described previously (21). The resulting peptides were extracted from the gel pieces and desalted on a C18 ZipTip microcolumn (Millipore). The peptides were then fractionated by reversed phase liquid chromatography electrospray ionization mass spectrometry using a Zorbax C8 capillary column (0.3 × 150 mm diameter) (Agilent) with a 60-min linear gradient from 0 to 95% Buffer B (Buffer A = 0.1% formic acid; Buffer B = 95% CH3CN, 0.1% formic acid) at 5 µl/min. The column effluent was sprayed into an XCT ion trap mass spectrometer (Agilent). MS/MS of the two major ions in each scan was performed automatically to identify peptides. The data were analyzed using data analysis software (Agilent) combined with the on-line Mascot and Global Protein Machine data base-searching programs. The activity of the enzymes was confirmed using the assays described below. The enzymes were quantified by Coomassie Blue staining on a 10% SDS gel and comparison of the density of bands with those of bovine serum albumin standards using NIH Image program.

Fringe Assays—Fringe assays were carried out essentially as described previously (4). Briefly, reactions were carried out in a volume of 50 µl containing 10 mM MnCl2, 50 mM HEPES (pH 6.8), 0.5 µCi UDP-[3H]GlcNAc as the donor substrate and 10 mM benzyl-α-O-fucose (generously provided by Dr. Khusi Matta, Roswell Park Cancer Institute) as the acceptor substrate. For UDP-GlcNAc saturation curves and activity curves employing EGF 26-O-fucose as the acceptor substrate, unlabeled UDP-GlcNAc was added to the reactions to bring the total concentration of UDP-GlcNAc to above Km. Reactions were carried out for 30 min at 37 °C unless otherwise indicated. Reactions were stopped by
the addition of 900 μl of 30 mM EDTA (pH 8.0). Reaction mixtures were loaded onto C18 cartridges (100 mg, Agilent). The cartridges were washed with 8 ml of water and eluted in 1.5 ml of 80% methanol. The samples were then subject to liquid scintillation counting. For determination of optimal pH of Fringe reactions, 50 mM Tris acetate was used as the buffer in reactions and adjusted to the appropriate pH.

Preparation of EGF Repeats—The first EGF repeat of factor IX, the factor IX double mutant, and EGF repeat 26 were produced in Escherichia coli BL21 (DE3) (the first EGF repeat from factor VII was a kind gift of Dr. Yang Wang (described in Ref. 22)). Transformation and growth of E. coli BL21 (DE3) was carried out as previously described (23). The bacteria were pelleted and then resuspended in 50 mM Tris (pH 8.0) and sonicated 10 times for 20 s each on ice. The suspension was clarified by centrifugation and incubated with Ni-NTA-agarose for 1 h at 4 °C with rotation. The slurry was poured into a 0.8 × 4-cm Bio-Rad
disposable column, and the Ni-NTA-agarose was washed twice with 10 ml of 10 mM imidazole in 50 mM Tris (pH 8.0). Protein was eluted from beads with eight 1-ml washes of 250 mM imidazole in 50 mM Tris (pH 8.0). EGF repeats were further purified by reversed phase HPLC on a 250 × 4.6-mm Dynamax C18 column (Rainin) as previously described (23). Purified EGF repeats were then dried in a SpeedVac (Savant) to near dryness. In vitro O-fucosylation of EGF repeats was carried out by resuspending the EGF repeat in 1 ml of 5 mM imidazole (pH 7.4), 5 mM MnCl₂, and 1 mM GDP-L-fucose (Sigma). Purified human His₆-O-FucT-1 (~40 microunits, purified as described for Fringe above) (24) was added to the reaction mixture, and the reaction was allowed to proceed overnight at 37 °C. The O-fucosylated EGF repeat was then purified by reversed phase HPLC as described above using a 90-min 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid. To confirm fucosylation of the EGF repeats, samples of HPLC fractions containing the EGF repeats were dried in the SpeedVac (Savant) and resuspended in 20% acetonitrile and 0.1% formic acid. The resuspended sample was centrifuged to pellet insoluble material and directly infused into the XCT ion trap mass spectrometer (Agilent) at a rate of 5 μl/min. The MS/MS peaks for MS/MS were chosen manually, and the data were analyzed using Agilent data analysis software. To quantify the amount of the fucosylated EGF repeat, carbohydrate compositional analysis was performed (25, 26). Briefly, a sample of each O-fucosylated EGF repeat was hydrolyzed in 2 M trifluoroacetic acid for 2 h at 100 °C. The samples were dried in a SpeedVac (Savant) and resuspended in water. The samples were then analyzed by high pH anion exchange chromatography (HPAEC) on a Dionex DX300 using a PA-1 column. The amount of fucose was determined by comparing the signal from the hydrolyzed EGF repeats with that obtained by injecting known amounts of L-fucose.

Other Methods—Analysis of the Rfneg product was performed essentially as described previously (2, 3), except that the HPAEC analysis was performed using an isocratic gradient at 26 mM NaOH.

RESULTS

Specificity of Fringe for Individual EGF Repeats Is Encoded by Distinct Amino Acids of EGF Repeats—Many of the EGF repeats of Notch1 contain O-fucose sites; however, not all are subsequently modified by Fringe (6). Furthermore, previous studies have demonstrated that the specificity of Fringe for an EGF repeat is encoded by the amino acid sequence of a given EGF repeat. More specifically, the endogenous Fringes found in Lec1-CHO cells (Rfneg and Lfneg) have been shown to modify O-fucose on EGF repeat 26 (but not EGF repeat 24) of mouse Notch1 (6). Another important example of Fringe specificity is provided by comparison of the O-fucose saccharides on human clotting factors VII and IX, both of which are modified with O-fucose from factor IX were in the form of tetrasaccharide (indicating Fringe recognition) (Fig. 3A).

Site mutants were then made to interchange the non-conservative amino acids between the factor IX and factor VII EGF repeats (Fig. 1). Analysis of the point mutations introduced into factor IX (Fig. 2D) revealed that substitution of the glutamic acid for isoleucine at position 24 (E24I) and the proline at position 28 for leucine (P28L) each independently led to a reduction in the elongation of O-fucose, indicating that these residues are important for Fringe recognition. None of the other mutations had a major effect on tetrasaccharide formation on factor IX. When both the E24I and P28L mutations were simultaneously introduced into factor IX, there was significant reduction in tetrasaccharide and an increase in monosaccharide. This change in tetrasaccharide...
ride/monosaccharide ratio was greater than that observed for either mutation alone, indicating an additive effect of the mutations. In the case of factor VII (Fig. 2E), there was no change in the relative amounts of tetrasaccharide and monosaccharide observed in any of the factor VII site mutants. However, when glutamic acid and proline were introduced into positions 24 and 28 of factor VII, respectively, a slight increase in tetrasaccharide, and a slight decrease in monosaccharide, was observed. Thus, the glutamic acid at position 24 and the proline at position 28 of factor IX appear necessary but not sufficient for Fringe recognition of O-fucose on these EGF repeats.

In Vitro Analysis of Lfng, Rfng, and Mfng Activity—The cell-based analysis of factor VII and factor IX elongation shown in Fig. 2 is constrained by the fact that several parameters (e.g. relative expression levels of the transfected EGF repeats or of the endogenous Fringes, transport rates through the secretory pathway) are difficult to control. Therefore, in vitro assays using purified components (enzymes and EGF repeats) are necessary to verify that the in vivo results are actually measuring specificity. As a first step toward this analysis, Lec1-CHO cells were stably transfected with constructs encoding secreted forms of the predicted lumenal domains of the three mouse Fringe enzymes (Fig. 3A). Cells expressing high levels of protein were identified by dilution cloning and immunoblot analysis. Protein was purified from medium using Ni²⁺-NTA chromatography and evaluated by Coomassie Blue staining of SDS-PAGE (Fig. 3B). The identity of each protein was con-
Activity of each of the Fringe enzymes (Lfng, Rfng, and Mfng, Fig. 3, A–C). All three Fringe enzymes showed a 10-fold higher activity compared with each other. The Km values for UDP-GlcNAc of Lfng is nearly 10-fold lower than that of Rfng and Mfng.

We next sought to determine whether the Fringe enzymes differ in their utilization of the donor substrate. Each Fringe enzyme was incubated with increasing amounts of UDP-GlcNAc using saturating Bnz-fuc as an acceptor substrate (Fig. 5, A–C). Lineweaver-Burke analysis of these curves indicated that Lfng and Rfng display similar Km values for UDP-GlcNAc, whereas that for Mfng is somewhat higher. Interestingly, Vmax and catalytic efficiency (Vmax/Km) of Lfng was nearly 10-fold higher than either Rfng or Mfng. Similar differences in catalytic efficiency were observed with multiple independent preparations of enzyme, suggesting this difference is not due simply to differences between preparations.

We next characterized the Fringe enzymes with respect to acceptor substrates. To generate fucosylated EGF repeats in sufficient quantities for use as acceptor substrates in in vitro assays, EGF repeats (EGF 26 from mouse Notch1 and EGF1 from factors VII and IX) were expressed in bacteria and purified as described under “Experimental Procedures.” Each EGF repeat was fucosylated in vitro using purified FucT-1. FucT-1 will only modify properly folded EGF repeats (30), and activity was only observed with properly folded EGF repeats. The extent of fucosylation was used as a measure of whether the bacterially expressed enzyme has shown that Fringe is inhibited by EDTA (4, 29). Several different metal ions were tested in enzyme assays to determine which metal ion is preferred by each of the Fringe enzymes. All three Fringe enzymes showed strong preference for manganese (Fig. 4, G–I), although they could also use cobalt. Lfng also showed some activity with magnesium and calcium as metal ions. Both Lfng and Mfng showed some activity with magnesium, whereas Rfng did not. The significance of these differences is not clear. None of the enzymes were active in the presence of zinc or EDTA. To determine the optimal pH for the enzymatic activity of each Fringe, assays were carried out in a pH range of 5.0–9.0 (Fig. 4, J–L). Surprisingly, Mfng and Lfng displayed the greatest activity at pH 5.0, with decreased activity at higher pH values. Rfng, however, showed enzymatic activity over a broad range, from pH 5.0 to 8.0.

Because both bands reacted with the anti-hexahistidine antibody, the difference in migration is believed to be the result of an unknown post-translational modification. As such, both bands were used in the densitometry analysis and quantification of the enzymes.

To optimize the conditions for in vitro assays using Lfng, Rfng, and Mfng, various properties of these enzymes were characterized. The enzymes were first characterized with regard to the time dependence of GlcNAc transferase activity. Each enzyme was incubated with benzyl-α-O-fucose (Bnz-fuc, a synthetic O-fucose acceptor substrate) for varying amounts of time (Fig. 4, A–C). All three Fringe enzymes showed a linear increase in product formation with respect to time. These data indicate that Rfng is a fucose-specific GlcNAc transferase, similar to Lfng and Mfng. The Fringe enzymes were next compared with regard to temperature. All three enzymes showed the strongest enzymatic activity at 37 °C compared with activity at 4 and 23 °C (Fig. 4, D–F). Glycosyltransferases often require divalent cations for activity, and prior studies have shown that Fringe is inhibited by EDTA (4, 29). Several different metal ions were tested in enzyme assays to determine which metal ion is preferred by each of the Fringe enzymes. All three Fringe enzymes showed strong preference for manganese (Fig. 4, G–I), although they could also use cobalt. Lfng also showed some activity with magnesium and calcium as metal ions. Both Lfng and Mfng showed some activity with magnesium, whereas Rfng did not. The significance of these differences is not clear. None of the enzymes were active in the presence of zinc or EDTA. To determine the optimal pH for the enzymatic activity of each Fringe, assays were carried out in a pH range of 5.0–9.0 (Fig. 4, J–L). Surprisingly, Mfng and Lfng displayed the greatest activity at pH 5.0, with decreased activity at higher pH values. Rfng, however, showed enzymatic activity over a broad range, from pH 5.0 to 8.0.

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repeats were in the fucosylated form, although small amounts (<25%) of unfucosylated EGF repeat could be detected in the factor VII and IX preparations (Fig. 6). Mixing experiments showed that Fringe assays containing up to 50% unfucosylated EGF repeat did not significantly affect results (data not shown). The amount of fucose in each preparation was quantified by acid hydrolysis followed by HPAEC analysis (see “Experimental Procedures”). Because small amounts of unfucosylated EGF repeats were present in the preparations, all quantification was based on moles of fucose added to assays.

We have been unable to saturate activity with any acceptor substrate (e.g. Bnz-fuc or fucosylated EGF repeats). Thus, we cannot determine kinetic constants for acceptors. Nonetheless, all three Fringes were able to use fucosylated EGF repeat 26 of mouse Notch1 as acceptor substrate (Fig. 5, D–F). Consistent with what was seen above using Bnz-fuc as substrate, Lfng was significantly more effective at modifying EGF26-O-fucosylated EGF repeat than either Rfng or Mfng.

Because Rfng has never before been shown to have fucose-specific β1,3-GlcNAc transferase activity, we performed product analysis of the EGF 26-O-fucose modified by Rfng to demonstrate that it forms the expected linkage. The modified EGF 26-O-fucose was purified by reversed phase HPLC to demonstrate that the [3H]GlcNAc was covalently associated with the fucosylated EGF repeat (Fig. 7A). The purified product was then subjected to alkaline-induced β elimination to release the O-fucose saccharides from the protein. Analysis of the released saccharides by gel filtration chromatography on a Superdex peptide column revealed that the radiolabel migrated at the size predicted for a GlcNAc-fucitol disaccharide (Fig. 7B). The linkage between the GlcNAc and fucitol was confirmed to be β1,3 by analyzing the disaccharide on HPAEC with the appropriate standards (Fig. 7C). Thus, similar to Lfng and Mfng, Rfng is indeed a fucose-specific β1,3-GlcNAc transferase.

To verify the cell-based findings regarding factor VII and factor IX discussed above, fucosylated EGF repeat 1 from factor VII, factor IX, and factor IX bearing both the E24I and P28L mutation were compared as acceptor substrates for the Fringes (Fig. 8). This analysis revealed that factor IX EGF-O-fucose was a better substrate for each of the three fringe enzymes than factor VII EGF-O-fucose, consistent with the data shown in Fig. 2, B and C. Furthermore, introduction of the E24I and P28L mutations into factor IX EGF-O-fucose made this EGF repeat a much poorer substrate for each of the three Fringes, although the effects of the mutations were most profound on Mfng. This supports the in vivo findings described above (Fig. 2D) and, again, indicates the importance of residues Glu-24 and Pro-28 for the recognition of a fucosylated EGF repeat by each of the mammalian Fringe enzymes. In addition, these data indicate that both Lfng and Rfng modify factor IX EGF-O-fucose to a similar extent. This is in contrast to EGF26-O-fucose from mouse Notch1, which Lfng modified more efficiently than Rfng or Mfng (TABLE ONE). Thus additional sequences other than Glu-24 and Pro-28 must be present in EGF 26 from mouse Notch1 to account for the difference in activity with Lfng and Rfng.

**DISCUSSION**

Here we have examined differences in the biochemical behavior of the three mammalian Fringe enzymes Mfng, Lfng, and Rfng. We demonstrated that Rfng, like Mfng and Lfng, is a fucose-specific β1,3-GlcNAc transferase. We found no significant difference between the three enzymes in terms of the effects of time, temperature, and metal ion requirement in their enzymatic activities. All of the enzymes had surprisingly broad pH distributions, even retaining activity at pH 5. The main difference between the enzymes appeared to be their catalytic efficiency, with Lfng displaying significantly higher activity than either Mfng or Rfng for most acceptors (TABLE ONE). Specific analysis of
EGF repeats from human clotting factor VII and IX identified glutamic acid residue 24 and proline residue 28 (within an EGF repeat) as important recognition determinants for all three of the Fringes. The fact that the Fringe enzymes appear to recognize similar specificity determinants in fucosylated EGF repeats is consistent with earlier genetic studies that show all three mammalian fringes are capable of complementing Fringe mutants in Drosophila (18). Nonetheless, the fact that the introduction of these amino acids into the EGF repeat from factor VII did not result in modification by Fringe suggests additional recognition determinants must exist.

Comparison of the specificity of the three Fringes with respect to all acceptor substrates tested in this study supports the concept that Lfng is generally more active than either Rfng or Mfng and that determinants other than residues 24 and 28 are important (TABLE ONE). Lfng shows ~10-fold higher activity with Bnz-fuc than either Rfng or Mfng, although all three enzymes work more efficiently with fucose attached to any of the EGF repeats examined. Lfng shows a dramatic preference for EGF26-O-fucose over all other substrates tested. Rfng also preferred EGF26-O-fucose over other substrates, whereas Mfng showed a preference for factor IX EGF-O-fucose. These results show that, although residues 24 and 28 influence substrate recognition, there are differences in substrate preference for each of the Fringes.

Structural studies of the first EGF repeat of factor IX showed that glutamic acid residue 24 and proline residue 28 are in close proximity to the serine residue that bears the O-fucose modification (Fig. 9). This
indicates that these residues, along with the O-fucose, may form part of a binding epitope necessary for Fringe elongation of O-fucose in the context of an EGF repeat. Alternatively, it is possible that the corresponding residues found on the factor VII EGF repeat 1 (Ile-24, Leu-28) may prevent Fringe binding. Thus, structural data together with the biochemical data presented in this work argues for the importance of glutamic acid 24 and proline 28 in the recognition of a fucosylated EGF repeat by Fringe.

Identification of residues that increase modification by Fringe may help in making predictions as to which O-fucosylated EGF repeats are modified. The fact that EGF 26 from mouse Notch1 is modified by Lfng and/or Rfng (6) but has a threonine in position 24 and a proline in position 28 (see Fig. 1) suggests that a threonine may be acceptable in position 24. The fact that EGF 24 from mouse Notch1, which is not modified by Lfng and/or Rfng (6) and has a hydrophobic residue at position 24 (and no proline at 28), is consistent with this idea (Fig. 1). Several other sites of Fringe modification have been mapped over the past several years (6). None of these EGF repeats contains both a glutamic acid (or threonine) and proline in positions 24 and 28, respectively, consistent with the importance of these residues for Fringe recognition. Interestingly, EGF 3 contains a lysine at position 24 and a proline at position 28, suggesting that the lysine at 24 may prevent Fringe recognition in this context. This is complicated by the observation that O-fucose on EGF 4 from mouse Notch3 is elongated in Lec1-CHO cells (31). EGF 4 from Notch3 contains an arginine at position 24 and a proline at position 28. These conflicting observations indicate that accurate predictions cannot be made when a positively charged amino acid (lysine or arginine) is present at position 24. Thus, additional residues must also be involved in recognition by the Fringes, as was also indicated by the fact that mutation of residues at positions 24 and 28 in factor VII were not sufficient to cause it to be modified (Fig. 2).

Although additional residues are obviously important, the presence of glutamic acid or threonine at position 24 together with a proline at position 28 appears to be a strong indicator that an O-fucosylated EGF repeat will be modified by one of the Fringes. Although EGF repeat 26 is the only other site specifically mapped containing these residues, other EGF repeats within mouse Notch1 fit these criteria (EGF repeats 18 and 32). Consistent with these predictions, mapping studies have shown that each of these EGF repeats occurs within a region of mouse Notch1 known to be modified by one of the Fringes (6). However, it is important to note that this does not mean that O-fucosylated EGF repeats without these residues cannot be substrates for Fringe. A good example of this is EGF repeat 12 of Notch1, which does not contain glutamic acid/threonine or proline in positions 24 and 28. This EGF repeat is modified by Mfng in CHO cells (6) and the endogenous Fringe enzymes found in Cos-7 cells (32). However, both of these studies indicate that EGF repeat 12 is a poorer substrate than EGF repeat 26, which does contain the threonine and proline residues. Thus, the presence of these residues provides an indication of how effective a substrate an O-fucosylated EGF repeat is for Fringe modification.

Previous studies on Fringe specificity involving Notch2 indicated that Lfng and Mfng modify different fragments of Notch2 (33). These data show that certain fragments are only modified by Lfng, whereas others can be modified by either Lfng or Mfng. This observation is consistent with our data, as it indicates that both of these enzymes are capable of modifying the same EGF repeats but that Lfng is capable of modifying more fragments than Mfng because of the fact that Lfng is a more efficient enzyme than Mfng. Presumably, if higher levels of Mfng were expressed in the cells, Mfng would be able to modify all of the EGF repeats modified by Lfng.

Based on the finding that the mammalian Fringe enzymes differ in their kinetic efficiency, it is plausible that Notch would undergo different degrees of modification depending on which Fringe orthologue was present. Thus, we would predict that expression of the same level of different Fringes would modify Notch signaling somewhat differently. Recent work by Yang et al. (34), in which an NIH-3T3 cell-based binding assay was used to determine the effects of each of the mammalian Fringes on modulation of Delta/Jagged-mediated Notch signaling, demonstrates this. Although both Lfng and Mfng were found to potentiate Delta signaling while inhibiting Jagged signaling, they did so to different extents; Lfng was a much stronger potentiator of Delta signaling than was Mfng. In contrast, Rfng was found to potentiate both Delta and Jagged signaling. The fact that Rfng has a different effect than Lfng and Mfng in NIH-3T3 cells cannot be explained by our data and suggests that cell-specific factors may influence Fringe specificity. Analysis of the specific sites on Notch1 modified in the NIH-3T3 cell system will need to be determined to resolve this conflict.

The expression patterns of Mfng, Rfng, and Lfng have been characterized in both mice and zebra fish (18, 35, 36). These studies have revealed that different Fringes are expressed at different times and in distinct locations in developing embryos (although there are overlaps in expression). For example, in mouse embryos, Lfng and Mfng are both
expressed in the hindbrain, whereas only Lfng is expressed in presomitic mesoderm (18). This would again seem to indicate that each of the Fringe enzymes regulates Notch signaling in distinct contexts. Indeed, this notion is substantiated by the fact that mice mutant for Lfng result in defects in somite formation (14), indicating that Rfng and Mfng are not capable of compensating for the loss of Lfng activity. Thus, the individual activity of Lfng, by virtue of its expression pattern or inherent abilities to modify Notch relative to Mfng and Rfng, has a unique function in the modulation of Notch signaling. It is possible that Mfng and Rfng have somewhat redundant functions. This would explain the lack of an obvious phenotype in Rfng mutant mice. No data regarding Mfng knock-out mice have yet been reported. Thus, in vivo functions for Rfng and Mfng in mice are still unknown.

The fact that we can nearly eliminate Fringe recognition of an EGF repeat while retaining O-fucosylation suggests that it is now possible to differentiate the effects of the O-fucose monosaccharide versus tetrasaccharide at an individual site. For example, EGF repeat 26 is a known substrate for Fringe. In previous work, we have demonstrated that mutation of the O-fucose site in this EGF repeat results in hyperactivation of the Notch receptor (32), but it is unclear whether this effect is due to the mono- or tetrasaccharide form of O-fucose. Selective mutation of threonine 24 and proline 28 residues in EGF 26 should reduce O-fucose at this site without compromising O-fucosylation, thus allowing study of the Fringe modification at this site. Such studies are in progress.

Acknowledgments—We thank Dr. Khushi Matta (Roswell Park Cancer Institute) for providing benzyl-fucose and disaccharide standards, Dr. Tom Vogt (Merck) for providing mouse Fringe constructs, Drs. Todd Miller and Ken Irvine for helpful discussions, and members of the Haltiwanger laboratory for critically reading the manuscript.

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