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Eukaryotic expression, purification, crystallization and preliminary X-ray analysis of murine Manic Fringe

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Fringe proteins are Golgi-resident β1,3-*N*-acetylglucosaminyltransferases that regulate development in metazoa through glycosylation of the Notch receptor and its ligands. The catalytic domain of murine Manic Fringe was expressed in the baculovirus/insect-cell system as a secreted protein. Mass-spectrometric analysis of the purified protein indicated the presence of two N-linked glycans. Abolishing the glycosylation sites by site-directed mutagenesis was necessary in order to obtain orthorhombic crystals that diffracted to 1.8 Å resolution. For phasing, a highly redundant data set was collected using a crystal soaked with halide salts.

1. Introduction

Notch-mediated signalling is a conserved intercellular communication pathway that regulates cell-fate decisions in metazoan development (Artavanis-Tsakonas *et al.*, 1999). The extracellular domain of the Notch receptor consists of 36 tandem epidermal growth factor (EGF) motifs that mediate interactions between Notch and its protein ligands, Delta and Serrate. In *Drosophila*, the protein Fringe acts as a modulator of Notch–ligand interactions, enhancing the activation of Notch by Delta, while inhibiting Notch activation by Serrate (Panin *et al.*, 1997). This ligand-specificity switch elicited by Fringe is employed in establishing tissue boundaries in the developing fly embryo (Irvine, 1999). Three Fringe homologues, Manic Fringe, Lunatic Fringe and Radical Fringe, have been identified in vertebrate genomes and shown to regulate Notch signalling during development (Irvine & Rauskolb, 2001).

Fringe and its vertebrate homologues are fucose-specific β1,3-*N*-acetylglucosaminyltransferases that attach *N*-acetylglucosamine (GlcNAc) to *O*-fucose glycans present on a specific subset of Notch EGF motifs (Moloney *et al.*, 2000; Bruckner *et al.*, 2000). The Fringe glycosyltransferases are ~40 kDa type II membrane proteins that localize to the Golgi compartment by means of an N-terminal signal peptide. The enzymes use UDP-GlcNAc as the donor substrate and require Mn^{2+} for activity (Moloney *et al.*, 2000; Bruckner *et al.*, 2000).

Glycosyltransferases are a diverse group of enzymes belonging to over 80 families that share little sequence homology with each other (Coutinho *et al.*, 2003). Fringe glycosyltransferases belong to a family for which no structural information is currently available. Here, we report insect-cell expression, crystallization and X-ray diffraction analysis of the catalytic domain of murine Manic Fringe (MFNG), a 32 kDa protein of 277 amino acids.

2. Methods

2.1. Molecular cloning

A customized baculovirus transfer vector (termed pKHis3C) was generated as follows. A DNA sequence encoding a hexhistidine tag, a rhinovirus 3C protease cleavage sequence and a portion of the multiple cloning site was PCR-amplified from the bacterial expression vector pETM-14 (G. Stier, EMBL, unpublished work) using a forward primer 5′-ATAAGAATGCGGCCGCATCACCATCACCA-

TCACTCCGCGGG-3′ and a reverse primer 5′-ACGCGTCGACG-GAGCTCGAATTCGG-3′. The PCR product was digested with *Not*I and *Sal*I and ligated downstream of the sequence encoding a secretion signal and a FLAG tag in pK503-9 (Keinanen *et al.*, 1998), a derivative of the pFastBac1 baculovirus transfer vector (Invitrogen). The DNA sequence encoding the catalytic domain of murine Manic Fringe (residues 45–321; numbering based on SWISS-PROT entry O09008) was amplified by PCR from a murine Manic Fringe cDNA clone (RZPD German Resource Center for Genomic Research, Heidelberg, Germany) using a forward primer 5′-GTGATCAT-GAACCC-
AGGACCCCTGGAC-3′ and a reverse primer 5′-CGCGGATCCTT-AGGGCGCTGCCAGCAGCGG-3′. The PCR product was digested with *Bsp*HI and *Bam*HI and ligated into the pKHis3C vector cut with *Nco*I and *Bam*HI, yielding pKHis3C-MFNG. Recombinant baculovirus was generated in Sf9 cells using the Bac-to-Bac system (Invitrogen) according to the manufacturer's protocol.

2.2. Protein expression and purification

Sf9 cells were grown in suspension culture in ExCell 420 serum-free medium (JRH Bioscience). For large-scale protein expression, 5 l of Sf9 cell culture (at 1.5×10^6 cells per millilitre) were infected with recombinant virus (multiplicity of infection of ∼2). Conditioned medium was harvested 60 h post-infection and filtered sequentially using a 0.22 µm filter and a 300 kDa cutoff Prep/Scale tangential flow filtration cartridge (Millipore). In order to reduce the handling volume and to render the medium compatible with chromatographic matrices, the medium was concentrated to a volume of 250 ml using a 10 kDa cutoff Prep/Scale tangential flow ultrafiltration cartridge (Millipore), diluted to 2 l with 20 m*M* MES–NaOH pH 6.0, 50 m*M* NaCl and re-concentrated. The concentrate was applied in batches to 12 ml of High S Macro Prep cation-exchange resin (BioRad). The resin was packed into a column and washed and bound protein was eluted with a linear gradient of 20 m*M* Tris–HCl pH 8.0, 1 *M* NaCl. Peak fractions were directly loaded onto a 10 ml His-Select Co^{2+}-affinity column (Sigma) and the protein was eluted with a linear imidazole gradient (0–100 m*M*). Rhinovirus 3C protease was used to remove the N-terminal affinity tags while the protein was dialyzed against 20 m*M* MES–NaOH pH 6.5, 100 m*M* NaCl, 10%(*w*/*v*) sucrose overnight. The protein was further purified by gel-filtration chromatography on a Superdex 75 column (Pharmacia) in 20 m*M* MES–NaOH pH 6.5, 100 m*M* NaCl and concentrated to 10 mg ml^{-1}.

2.3. N-Glycan analysis

Enzymatic deglycosylation assays with Endoglycosidase H and Peptide N-glycosidase F (New England Biolabs) were performed according to the supplier's instructions using 1 µg purified MFNG and 500 U of each endoglycosidase and incubating the reaction at 310 K for 3 h. Reactions were analyzed by electrophoresis on a 15% SDS–polyacrylamide gel. ESI–TOF mass spectrometry of native and deglycosylated MFNG was performed at the Proteomics Core Facility, EMBL, Heidelberg.

2.4. Site-directed mutagenesis

Residues Asn109 and Asn185 in MFNG were mutated to glutamine using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the mutations was verified by DNA sequencing. The mutant protein MFNG-N109Q/N185Q was expressed and purified as for wild-type MFNG.

2.5. Crystallization

All crystallization experiments employed the sitting-drop vapour-diffusion method and were performed at 291 K. Initial screening against 288 crystallization conditions (Classic and PEGs Screens from Nextal and Index Screen from Hampton Research) was performed in 96-well trays (Greiner Bio-One) using a Mosquito nanovolume crystallization robot (Molecular Dimensions). 0.1 µl MFNG protein solution (10 mg ml^{-1} in 20 m*M* MES–NaOH pH 6.5, 100 m*M* NaCl) was mixed with 0.1 µl precipitant solution and equilibrated against 75 µl reservoir solution. Bar-shaped crystals of MFNG-N109Q/

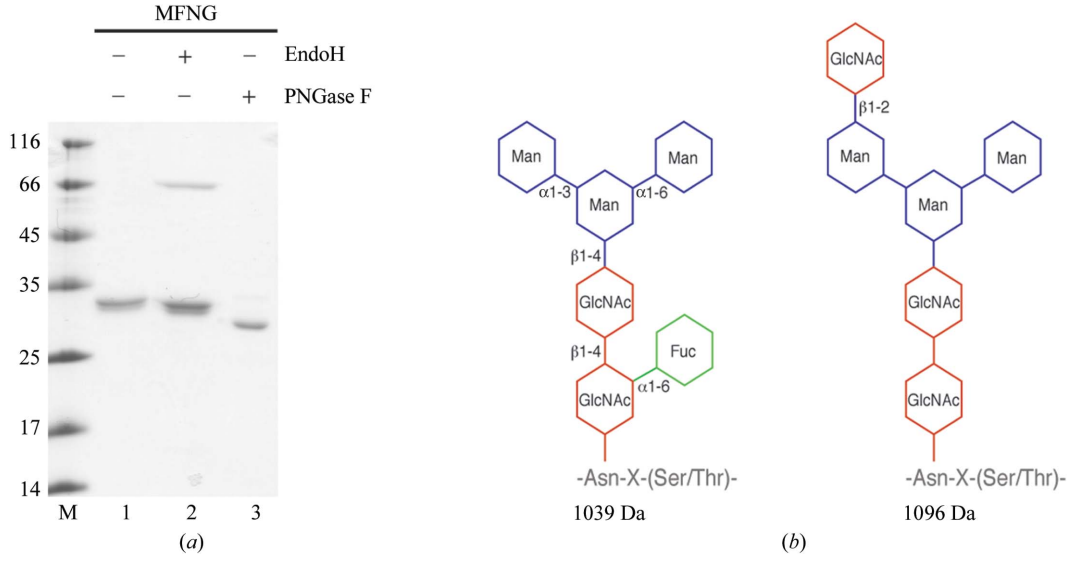

Figure 1
N-Glycan analysis of MFNG. (*a*) MFNG was digested with Endoglycosidase H (Endo H, lane 2) or Peptide N-glycosidase F (PNGase F, lane 3). The extent of deglycosylation was monitored by electrophoresis on a 15% SDS–polyacrylamide gel. A shift in electophoretic mobility is observed with PNGase F, indicating that MFNG is N-glycosylated. The absence of a shift upon treatment with Endo H indicates that the N-glycans are not of a high-mannose type. Lane M, molecular-weight markers (kDa). (*b*) Schematic structures of the N-glycans found in native MFNG, as deduced by mass-spectrometric analysis of native and deglycosylated proteins. GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; Fuc, L-fucose. The molecular weight of each glycan chain and the type of each glycosidic linkage are indicated.

N185Q were obtained in 200 m*M* K_2SO_4, 20%(*w*/*v*) PEG 3350. For data collection, crystals were grown in standard 24-well trays (Hampton Research) by mixing 1 µl protein solution with 1 µl precipitant solution containing 200 m*M* K_2SO_4 and 16–26%(*w*/*v*) PEG 3350. The crystals grew to maximum dimensions of 20 × 40 × 150 µm within a week.

2.6. X-ray diffraction

For native data collection, crystals were soaked in 20 m*M* MES pH 6.5, 200 m*M* K_2SO_4, 20%(*w*/*v*) PEG 3350 and 18%(*v*/*v*) glycerol for 10 s prior to flash-cooling in liquid N_2. Native diffraction data were collected at 100 K at beamline ID14-3 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) equipped with a MAR 165 CCD detector. Single-wavelength anomalous dispersion (SAD) data were collected at a wavelength of 1.725 Å at beamline BM-14 of the ESRF (equipped with a MAR 225 CCD detector) using a crystal soaked in the cryoprotectant solution supplemented with 0.5 *M* NaI for 30 s. The crystal was mounted on a swinging-arc goniometer head. A total of 1440 diffraction images were recorded with 1° oscillation per image. The crystal was reoriented after each 360° of rotation to further boost the redundancy of the data. All data were integrated, scaled and merged with *XDS* (Kabsch, 1993).

3. Results and discussion

Initial attempts to express soluble Fringe proteins in *Escherichia coli* were unsuccessful (data not shown). The presence of strictly conserved cysteine residues in the Fringe protein family suggested that the proteins might contain disulfide bonds, prompting us to employ a eukaryotic expression system in order to express Fringe in a secreted form. A truncated construct of MFNG lacking the transmembrane domain and the linker was expressed in the insect-cell/baculovirus system using a customized baculovirus transfer vector. The protein was purified by a combination of ion-exchange, affinity and size-exclusion chromatographic steps to give a final yield of approximately 1 mg of protein per litre of culture. Crystallization trials with this protein preparation failed to give crystals suitable for X-ray diffraction analysis.

SDS–PAGE analysis of purified MFNG revealed that the protein migrated in two closely spaced bands, indicating heterogeneity in the protein preparation. Mass-spectrometric analysis of the purified protein identified two species with molecular masses of 33 757.6 and 33 636.9 Da (data not shown), suggesting the presence of N-linked glycans in the MFNG protein. Treating MFNG with peptide N-glycosidase F (PNGase F) resulted in a significant shift in electrophoretic mobility in SDS–PAGE, confirming that the protein was N-glycosylated (Fig. 1*a*). The mass spectrum of PNGase F-treated MFNG revealed a single peak with a molecular mass of 31 560.9 Da. In insect cell lines, the final product of the N-glycosylation pathway is a hexasaccharide of 1039 Da (Manneberg *et al.*, 1994). Comparison of the molecular masses of native and deglycosylated MFNG suggested that the 33 636.9 Da species in the native preparation carried two such N-linked glycans, while the 33 757.6 Da species carried two 1098 Da glycans that are likely to represent an intermediate in the insect N-glycan pathway (Fig. 1*b*). The observation of two N-glycans attached to MFNG is consistent with the presence of two predicted N*X*S/T N-glycosylation sites in the catalytic domain of Manic Fringe at asparagine residues Asn109 and Asn185.

The chemical heterogeneity and conformational flexibility of glycans are generally considered to be major obstacles to the crystallization of extracellular proteins (Wyss & Wagner, 1996; Kwong *et al.*, 1999). In order to improve the crystallization potential of MFNG, we attempted large-scale enzymatic deglycosylation of MFNG with either PNGase F or Endoglycosidase F3. However, this resulted in precipitation of the MFNG protein at relatively dilute protein concentrations in both cases. This could be a consequence of the insolubility of the deglycosylated protein at the pH required for deglycosylation or, in the case of PNGase F, the introduction of two negative charges to the protein. In another attempt to obtain diffracting crystals of MFNG, the N-glycosylation sites at Asn109 and Asn185 were abolished by asparagine-to-glutamine mutations. The resulting double mutant MFNG-N109Q/N185Q was expressed and purified in soluble form as for the wild-type protein, yielding approximately 0.15 mg of pure protein per litre of cell culture. Owing to the relatively low yield, screening for crystallization conditions was carried out using nanovolume robotics (using approximately 35 µl of the protein solution at 10 mg ml^{-1} to screen against 288 crystallization conditions). Single crystals of MFNG-N109Q/N185Q were obtained in 0.2 *M* K_2SO_4, 20% PEG 3350 (Fig. 2). The crystals were of sufficient size and quality to permit X-ray diffraction analysis without major optimization of the crystal-growth condition. Crystals of MFNG-N109Q/N185Q diffracted to a maximum resolution of 1.8 Å at synchrotron X-ray sources. A complete data set was collected and revealed that the crystals belonged to space group $P2_12_12$ (Table 1). The unit-cell parameters (a = 162.6, b = 41.4, c = 38.8 Å) are consistent with the presence of one molecule in the asymmetric unit,

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Data set	Native	Iodide SAD
X-ray source	ESRF ID14-EH3	ESRF BM-14
Wavelength (Å)	0.931	1.725
Space group	$P2_12_12$	$P2_12_12$
Unit-cell parameters		
a (Å)	162.6	163.0
b (Å)	41.4	41.4
c (Å)	38.8	38.9
Resolution (Å)	81.4–1.8 (1.9–1.8)	100–2.5 (2.6–2.5)
Total reflections	109385	530689
Unique reflections	25066	17520
Completeness (%)	99.5 (97.9)	99.5 (98.1)
Multiplicity	4.4 (3.6)	30.1 (29.8)
$\langle I/\sigma(I)\rangle$	13.2 (2.9)	34.7 (9.6)
R_{sym}† (%)	6.5 (44.1)	10.3 (47.2)

† $R_{sym} = \sum_{\mathbf{h}}\sum_i |I_{i\mathbf{h}} - \langle I_{\mathbf{h}}\rangle| / \sum_{\mathbf{h}}\sum_i \langle I_{\mathbf{h}}\rangle$, where $I_{i\mathbf{h}}$ is the ith observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations I of reflection **h**.

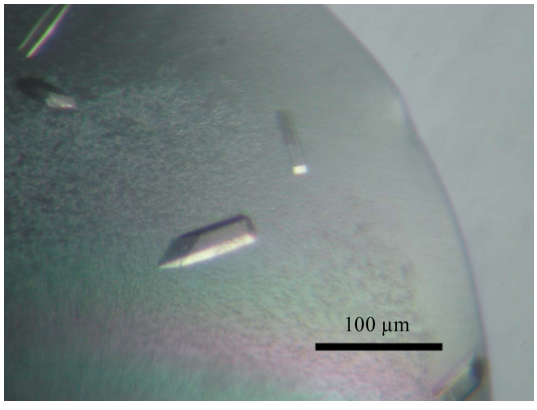

Figure 2
Crystals of MFNG-N109Q/N185Q. The crystals grew in 0.2 *M* K_2SO_4, 20%(*w*/*v*) PEG 3350 within one week.

giving a Matthews coefficient of 2.08 Å^3 Da^{-1} and a solvent content of 40.8%. Given the limited supply of MFNG-N109Q/N185Q crystals and the need for experimental phases, we chose single-wavelength anomalous dispersion experiments using crystals soaked in halide salts (Dauter *et al.*, 2001). A highly redundant data set was collected on a crystal soaked in sodium iodide (Table 1). Structure determination is currently in progress.

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