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Crystal structure of mammalian α1,6-fucosyltransferase, FUT8

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Mammalian α1,6-fucosyltransferase (FUT8) catalyses the transfer of a fucose residue from a donor substrate, guanosine 5′-diphosphate-β-L-fucose to the reducing terminal *N*-acetylglucosamine (GlcNAc) of the core structure of an asparagine-linked oligosaccharide. α1,6-Fucosylation, also referred to as core fucosylation, plays an essential role in various pathophysiological events. Our group reported that FUT8 null mice showed severe growth retardation and emphysema-like lung-destruction as a result of the dysfunction of epidermal growth factor and transforming growth factor-β receptors. To elucidate the molecular basis of FUT8 with respect to pathophysiology, the crystal structure of human FUT8 was determined at 2.6 Å resolution. The overall structure of FUT8 was found to consist of three domains: an N-terminal coiled-coil domain, a catalytic domain, and a C-terminal SH3 domain. The catalytic region appears to be similar to GT-B glycosyltransferases rather than GT-A. The C-terminal part of the catalytic domain of FUT8 includes a Rossmann fold with three regions that are conserved in α1,6-, α1,2-, and protein *O*-fucosyltransferases. The SH3 domain of FUT8 is similar to other SH3 domain-containing proteins, although the significance of this domain remains to be elucidated. The present findings of FUT8 suggest that the conserved residues in the three conserved regions participate in the Rossmann fold and act as the donor binding site, or in catalysis, thus playing key roles in the fucose-transferring reaction.

Key words: fucosyltransferase/core fucosylation/*N*-glycan/crystal structure/glycosyltransferase

Introduction

The fucosylation of glycoconjugates in mammalian organisms is related to a wide variety of biological processes, including cell adhesion, blood antigens, and some severe diseases including cancer metastasis, congenital disorders of glycosylation, and various microbial and virus infections (Staudacher et al. 1999; Becker and Lowe 2003). Fucosylation via α1,2-, α1,3-, α1,4-, α1,6-linkages, and protein *O*-fucosylation are accomplished by the action of specific individual fucosyltransferases (Staudacher et al. 1999; Oriol and Mollicone 2002; Miyoshi and Taniguchi 2002; Becker and Lowe 2003). Asparagine-linked oligosaccharides (*N*-glycans) of glycoproteins are ubiquitously α1,6-fucosylated (Miyoshi et al. 1997; Miyoshi, Noda, Yamaguchi et al. 1999). α1,6-Fucosylation, also known as core fucosylation, is frequently observed in *N*-glycans of α-fetoprotein, a well-known tumor marker for hepatocellular carcinoma but not for chronic liver disease (Taketa et al. 1993; Noda et al. 1998). To elucidate the biological functions associated with core fucosylation, overexpression experiments with mammalian α1,6-fucosyltransferase (FUT8), which is an eukaryotic α1,6-fucosyltransferase, and a key enzyme in core fucosylation synthesis, were performed. The findings showed that experimental cancer metastasis is suppressed by the core fucosylation of α5β1 integrin (Miyoshi, Noda, Ko et al. 1999). Moreover, the core fucosylation of *N*-glycans in human immunoglobulin (Ig) G1 was found to regulate antibody-dependent cellular cytotoxicity (ADCC) (Shields et al. 2002; Shinkawa et al. 2003). The lack of core fucose on the IgG1 molecule results in an enhancement in ADCC activity of up to 100-fold, suggesting that this core fucose-deficient IgG1 would be useful in terms of antibody therapy in cancer treatment. In addition, it has been directly verified that α1,6-fucosylation regulates the function of immunoglobulin by modifying its physicochemical characteristics (Okazaki et al. 2004). Very recently, our group reported that disruption of the *FUT8* gene in mice leads to phenotypes of growth retardation, lung emphysema, and death during postnatal development (Wang et al. 2005). These severe phenotypes were found to be mainly due to the lack of core fucosylation of epidermal growth factor, transforming growth factor-β receptors (Wang et al. 2005, 2006; Taniguchi et al. 2006), and other molecules (Lee et al. 2006; Li et al. 2006; Zhao et al. 2006). These studies strongly suggest that FUT8 and its enzymatic product, a core fucose play pivotal roles in a variety of physiological and pathophysiological events.

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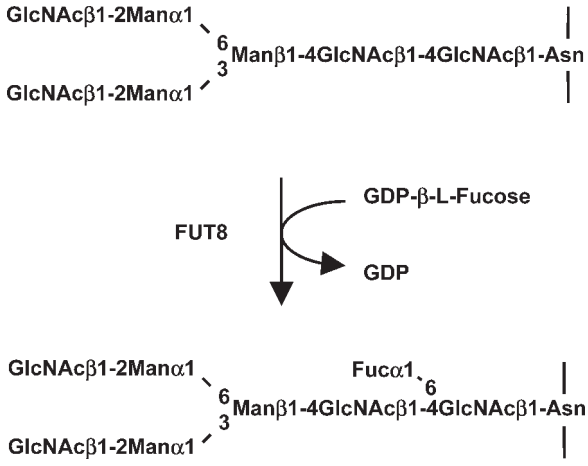

Fig. 1. Reaction catalyzed by FUT8. FUT8 transfers a fucose residue from GDP-β-L-fucose to the innermost GlcNAc of an Asn-linked oligosaccharide.

FUT8 catalyzes the transfer of a fucose residue from the donor substrate, GDP-β-L-fucose, to the innermost *N*-Acetylglucosamine (GlcNAc) residue in *N*-glycans via α1,6-linkage with inversion of the anomeric center of the transferred fucose (Figure 1) (Wilson et al. 1976). FUT8 is known to be a typical type II membrane protein and is localized in the Golgi apparatus, along with many other glycosyltransferases (Uozumi, Yanagidani et al. 1996), and is also known to be abundant especially in brain tissue (Nakakita et al. 1999). FUT8 has been extensively studied with respect to its substrate specificity (Longmore and Schachter 1982; Voynow et al. 1991; Kaminska et al. 1998; Paschinger et al. 2005; Ihara et al. 2006). As indicated in earlier studies, FUT8 requires at least two structural features in oligosaccharide acceptors: (a) β1,2-GlcNAc residue linked to the α1,3-mannose arm in the tri-mannose core structure of *N*-glycans (Longmore and Schachter 1982; Voynow et al. 1991; Kaminska et al. 1998; Paschinger et al. 2005) and (b) β-linkage between the reducing terminal GlcNAc and the asparagine residue in the *N*-glycosylation consensus sequence (Voynow et al. 1991). In addition, the reaction of FUT8 with the substrate is prevented by the presence of the bisecting GlcNAc, which is produced by the action of β1,4-*N*-acetylglucosaminylytransferase (GnT-III) (Longmore and Schachter 1982), and also by α1,3-fucose at the reducing terminal GlcNAc residue, as observed in insects (Staudacher and Marz 1998; Paschinger et al. 2005). Regarding donor substrate specificity, on the other hand, our recent study indicated that FUT8 strongly recognizes the base portion and diphosphoryl group of GDP, a part of the donor substrate (Ihara et al. 2006). As reported previously (Breton et al. 1998; Oriol et al. 1999; Takahashi et al. 2000; Martinez-Duncker et al. 2003; Okajima et al. 2005), three small regions that are highly conserved among α1,2-, α1,6-, and protein *O*-fucosyltransferases appear to participate in the possible binding site for GDP-β-L-fucose. Furthermore, site-directed mutagenesis studies indicated that two arginine residues in one of the conserved regions, Arg-365 and 366 in human FUT8 play important roles in donor binding (Takahashi et al. 2000). In kinetic analyses, it was found that the reaction of FUT8 follows a rapid equilibrium random mechanism (Ihara et al. 2006). However, the catalytic mechanism for this enzyme is not known in detail, and the molecular and chemical bases for the catalysis remains to be investigated.

In the present study, we solved the overall structure of human FUT8 at 2.6 Å resolution, in order to more understand the molecular basis for the action of FUT8. The results showed that FUT8 is comprised of three domains, an N-terminal coiled-coil domain, a catalytic domain, and a C-terminal SH3 domain. The C-terminal part of the catalytic domain of FUT8 includes a Rossmann fold with three conserved regions in α1,6-, α1,2-, and protein *O*-fucosyltransferases. Furthermore, site-directed mutagenesis experiment showed that several residues, which are all highly conserved in the three fucosyltransferases in this fold are essential for the enzyme activity of FUT8.

Results and discussion

Overall structure of FUT8

The recombinant human FUT8 used in the structural analysis was designed to express a soluble form by the truncating transmembrane and stem regions, as described previously (Ihara et al. 2006). The expressed protein corresponds to residues 68–575 and contained some additional amino acids. We used this recombinant protein for the crystallographic analysis, and succeeded in solving its structure at 2.6 Å resolution. As illustrated in Figure 2, the residues of Leu-108 to Glu-572 are modeled, however, the N-terminus (residues 68–107), C-terminus (residues 573–575), and the residues 368–372 are disordered in this structure. The overall structure of FUT8 is comprised of 15 strands and 16 helices (Figures 2 and 3). Several distinct features are observed in the structure of FUT8 (Figure 2). (a) At the N-terminus of FUT8, two long antiparallel α-helices (residues 109–173, α1 and α2 helices) form a coiled-coil structure. (b) The putative catalytic domain is comprised of two structures, an open sheet α/β structure and a Rossmann fold which is frequently found in nucleotide binding proteins including glycosyltransferases. The former structure contains five helices and three β-strands (residues 203–297, α4 and 3H1–3 helices, and β1-3 strands), and is at the N-terminal side of the catalytic domain of FUT8. The latter Rossmann fold contains five helices and five β-strands (residues 359–492, α8–11 and 3H5 helices, and β5–9 strands), and is at C-terminal side of the catalytic domain of FUT8. These two structures are linked via three helices, the α5, α6, and α7 helices. (c) The SH3 domain, which has been reported in various types of cytosolic proteins, is located at the C-terminus of FUT8.

In order to compare the fold of FUT8 with known structures, each part of the FUT8 structure was applied to database searching (DALI server) (Holm and Sander 1993). As summarized in Table I, the matching proteins are human lysine-specific demethylase-1 (Stavropoulos et al. 2006) to the coiled-coil structure of FUT8 (residues 108–174), ATP-binding hypothetical protein (Zarembinski et al. 1998) to the catalytic region of FUT8 (residues 201–500), *Escherichia coli* ADP-heptose lps heptosyltransferase II (PDB No., 1PSW), which belongs to the GT-B glycosyltransferase group, to the Rossmann fold (residues 348–500), *E. coli* carbamoyl phosphate synthetase (Thoden et al. 1999) to the open sheet α/β structure (residues 201–300) of the N-terminal region of the catalytic domain.

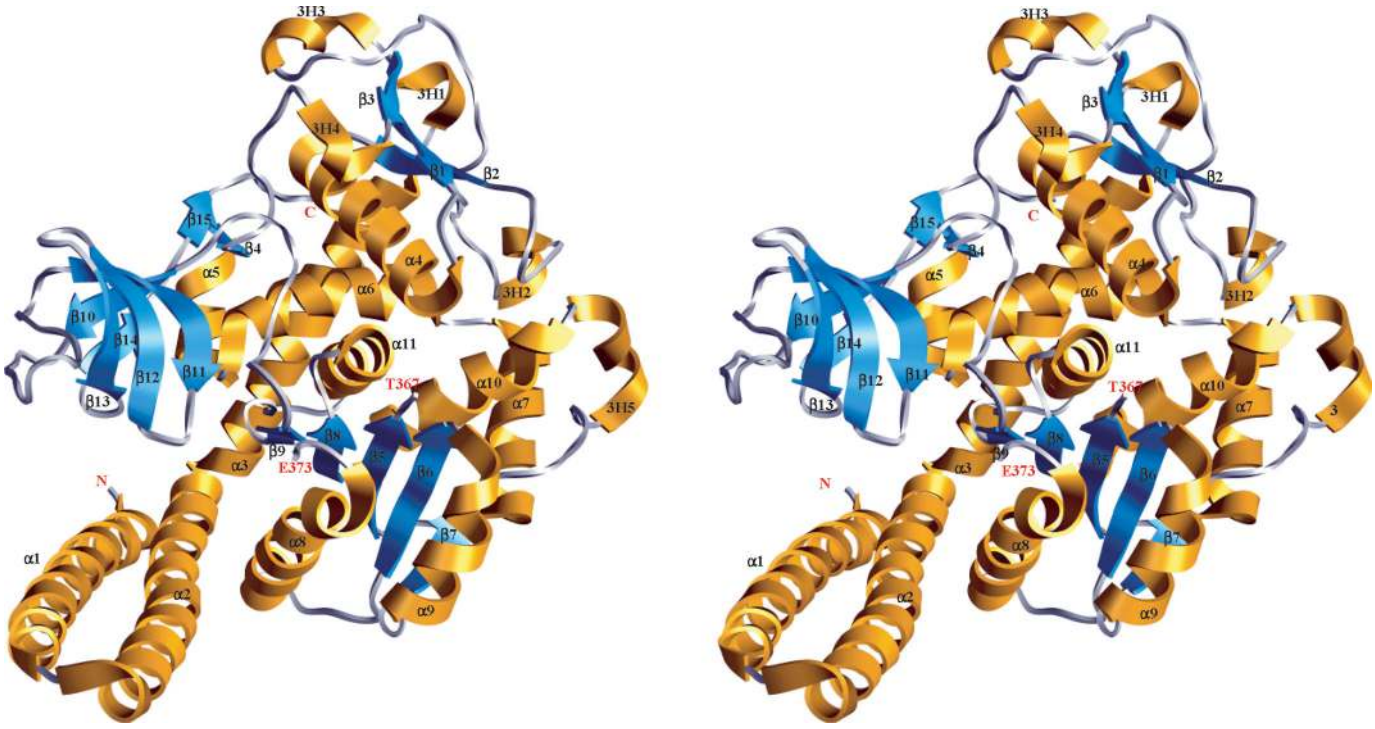

Fig. 2. Overall structure of human FUT8. Stereo view of the overall structure of the catalytic region of FUT8 (PDB No. 2DE0) is indicated by a ribbon diagram. The secondary structures are highlighted, and helices and β-strands are shown in orange and blue, respectively. Thr-367 and Glu-373 colored in red means that the residues, Asp-368 to Thr-372 are disordered. All figures in this study were produced using the program, UCSF Chimera (Pettersen et al. 2004).

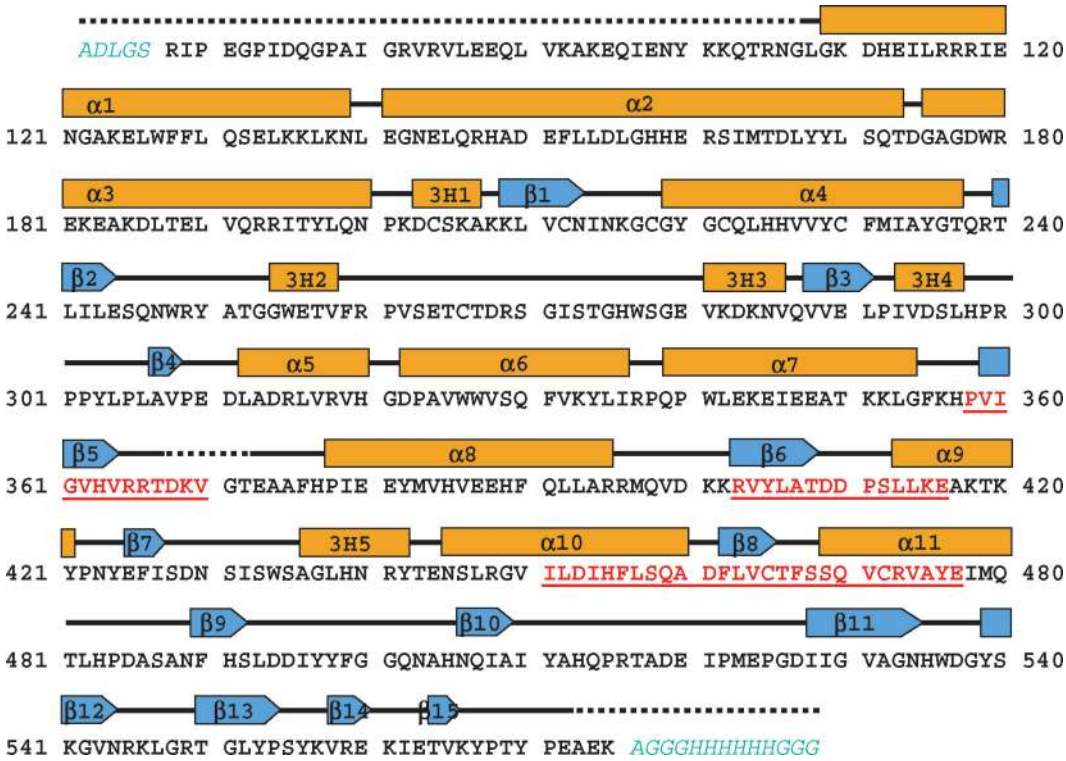

Fig. 3. The amino acid sequence and secondary structure of human FUT8. Amino acid residues 68–575 that were examined by structural analysis are shown. Residues 358–370, 403–416, and 451–477, underlined and in red, indicate the three conserved regions among the α1,2-, α1,6-, and protein *O*-fucosyltransferases, as shown in Figure 5A. As illustrated in the diagram of the secondary structures above the sequence, the cylinder and arrow denote the helix and β-strand, respectively. Some residues indicated in light blue and italic are additional residues for recombinant expression at the N- and C-terminus. The dashed line denotes regions that are disordered in the structural analysis (residues 68–117, 368–372, and 573–575). Helices, α1 − 11, are α-helices and 3H1–4 mean 3_{10}-helices.

Table I. Structural similarities of FUT8 to other proteins

Applied query of FUT8 (residues)	Protein	PDB	*Z* score	Root mean square deviation
Coiled-coil region (residues 108–174)	Human lysine-specific demethylase-1	2H94	8.3	2.0
Open sheet α/β structure (residues 201–300)	*E. coli* carbamoyl phosphate synthetase	1C3O	3.6	3.3
Rossmann fold (residues 348–500)	ADP-heptose lipopolysaccharide heptosyltransferase II	1PSW	6.1	3.0
Catalytic region (residues 201–500)	ATP-binding hypothetical protein	1MJH	6.0	3.0
	Gtfb, β-glucosyltransferase	1IIR	5.2	3.3
	Trehalose-6-phosphate synthase	1GZ5	5.1	3.9
	Sialyltransferase from *Pasteurella multocida*	2EX0	5.0	3.3
SH3 domain (residues 502–562)	Yeast actin binding protein	1JO8	11.0	1.3
	Neutrophil cytosol factor 4	1W6X	11.0	1.2
	c-Crk, oncogene protein	1CKA	10.3	1.4
	p56 Lck, the Src family kinase	1LCK	11.6	1.1

The SH3 domain (residues 502–562) of FUT8 has relatively high similarities to that of many proteins, and will be described in detail in the section *Src homology 3 domain of FUT8*. These results indicate that some parts of FUT8, the coiled-coil structure, the Rossmann fold, and the SH3 domain have structural similarities to some proteins, however, the overall structure of FUT8 has low similarities to any currently known proteins.

Comparison to other known glycosyltransferase structures

The structures of several glycosyltransferases have been solved by crystallographic analysis to date, and are classified into two structural superfamilies, GT-A and GT-B (Couinho et al. 2003; Qasba et al. 2005). GT-A enzymes have a specific motif, the DXD or EXD motif, which is required for metal ion and donor substrate interaction. The folds of these enzymes contain a Rossmann fold of two tightly associated domains at the N-terminal. The mixed β-sheets, which functions as the acceptor-binding domain are also located at the C-terminal (Couinho et al. 2003; Qasba et al. 2005). On the other hand, GT-B enzymes have folds consisting of two similar Rossmann folds (Couinho et al. 2003; Qasba et al. 2005). It is known that the Rossmann fold in many proteins is a nucleotide or nucleotide-sugar binding domain. In fact, several glycosyltransferases, whose structures have been solved, were shown to bind the nucleotide sugar to the Rossmann fold regardless of whether a metal ion is required for activity (Qasba et al. 2005).

Our structural study reveals that the structure of FUT8 contains one Rossmann fold, similar to GT-A enzymes (Figure 2). However, the results of database searching retrieved GT-B enzymes from the database when the catalytic region (residues 201–500) of FUT8 was used as a search query. Retrieved GT-B enzymes with a *Z* score of not <5, were ADP-heptose lipopolysaccharide heptosyltransferase II (PDB No., 1PSW) and Gtfb, β-glucosyltransferase (Mulichak et al. 2001), trehalose-6-phosphate synthase (Gibson et al. 2002), and sialyltransferase from *Pasteurella multocida* (Ni et al. 2006) (Table II). These enzymes were found to be similar to only the Rossmann fold part of FUT8. However, the overall shape of the catalytic region of FUT8 seems to be like that of GT-B enzymes (Figure 4). At the same time, the catalytic region of FUT8 was compared with some GT-A glycosyltransferases, CstII, sialyltransferase from *Campylobacter jejuni* (Chiu et al. 2004), yeast α1,2-mannosyltransferase (Lovsanov et al. 2004), mannosylglycerate synthase from *Rhodothermus marinus* (Flint et al. 2005), and leukocyte type core2 β1,6-*N*-acetylglucosaminyltransferase (Pak et al. 2006). Leukocyte type core2 β1,6-*N*-acetylglucosaminyltransferase belongs to the GT-A enzyme family, and is a metal-independent glycosyltransferase without a DXD motif (Pak et al. 2006). Yeast α1,2-mannosyltransferase and mannosylglycerate synthase from *Rhodothermus marinus*, which are also GT-A enzymes, are known to utilize a GDP–sugar as the donor substrate for enzyme reaction like FUT8. CstII is classified as a GT-A enzyme, because this enzyme contains a single Rossmann fold. However, the connectivity of secondary structure and the lack of a DXD motif in this enzyme are different from typical GT-A enzymes (Chiu et al. 2004). The catalytic region of FUT8 was observed to have no or little similarity to these GT-A enzymes (mannosylglycerate synthase, *Z* score 2.7; CstII, 1.2; α1,2-mannosyltransferase, 1.6; and core2 β1,6-*N*-acetylglucosaminyltransferase, 2.0). These results support that the catalytic region of FUT8 is likely to be closer to GT-B than GT-A enzymes, although the structure of the N-terminal part of the catalytic region of FUT8 is not to be a typical Rossmann fold. In addition, FUT8 does not contain a DXD motif and is fully active without a metal ion. These properties of FUT8 are also similar to those of GT-B than GT-A enzymes.

Putative catalytic region of FUT8

For glycosyltransferases like nucleotide-binding proteins, it has been reported that the flexible loop which is essential for their enzymatic reactions is located in close proximity to the nucleotide-sugar binding site (Qasba et al. 2005). This flexible loop was determined in the structures of β1,4-galactosyltransferase I, which is one of the most extensively investigated glycosyltransferases by structural analyses involving the donor substrate-complexed form, as well as the unliganded form (Gastinel et al. 1999; Ramasamy et al. 2003; Ramakrishnan et al. 2004). By contrast, in several enzymes, for example, blood group A- and B-transferases, β1,3-glucuronyltransferases, and β-glucosyltransferase, the flexible loop could not be determined in the structure of the ligand-free

Table II. Data collection and refinement statistics for the structure of FUT8

Data set	Native2	Pt derivative
Data collection		
Space group	$P6_522$	$P6_522$
Wavelength (Å)	0.9	0.9
Unit cell		
a (Å)	90.0	90.6
b (Å)	90.0	90.6
c (Å)	380.7	380.6
Resolution	50–2.6 (2.69–2.60)	50–3.0 (3.11–3.00)
Number of unique reflections	28706	19663
Completeness (%)	97.7 (80.2)	99.9 (99.8)
R_{merge} (%)[b]	5.7 (39.5)	9.2 (68.6)
$I/\sigma(I)$	16.1 (1.4)	11.39 (1.34)
Phase determination		
R_{Cullis} (acentric/centric, isomorphous)[c]	0.86/0.85	
R_{Cullis} (anomalous)[c]	0.98	
Phasing power (acentric/centric)[d]	0.81/0.78	
Number of heavy atom sites		1
Mean overall figure of merit (after DM[e])	0.83	
Refinement		
Resolution (Å)	50–2.61	
R_{work} (%)[f]	22.0	
R_{free} (%)[g]	28.3	
Root mean square deviation		
Bond length (Å)	0.017	
Bond angle (°)	1.75	
Ramachandran plot		
Most favored (%)	88.1	
Additional allowed (%)	11.4	
Generously allowed (%)	0.5	

[a]The number in parentheses represents statistics in the highest resolution shell.
[b]$R_{merge} = \Sigma|I - <I>|/\Sigma <I>$, where I is the observed intensity, and $<I>$ is the averaging intensity of multiple symmetry-related observations of that reflection.
[c]$R_{Cullis} = \Sigma\varepsilon/\Sigma|F_{PH}-F_P|$, where ε = phase-integrated lack of closure.
[d]Phasing Power = $<|F_{Hcalc}|/\varepsilon,>$, where ε = phase-integrated lack of closure.
[e]DM means density modification performed with SOLOMON and DM implemented in SHARP.
[f]$R_{work} = \Sigma||F_{obs}|$ − $|F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors for data used for refinement, respectively.
[g]$R_{free} = \Sigma||F_{obs}|$− $|F_{calc}||/\Sigma|F_{obs}|$ for 5% of the data not used at any stage of the structural refinement.

form and/or donor substrate-bound form (Pedersen et al. 2000; Unligil et al. 2000; Gastinel et al. 2001; Mulichak et al. 2001; Patenaude et al. 2002; Pedersen et al. 2003; Kakuda et al. 2004). In the case of FUT8, the region consisting of residues 368–372 is disordered probably due to its flexibility (Figures 2 and 3), and it seems likely that the region corresponds to a flexible loop. This flexible loop is located in the Rossmann fold of the FUT8 structure, as has been found in other glycosyltransferases (Figures 2 and 3), and thus it is likely that this loop plays an important role in the catalytic mechanism of FUT8.

It has been previously reported that three short regions are highly conserved in the amino acid sequences of FUT8, α1,2-fucosyltransferase related to H-antigen synthesis, NodZ, which is a bacterial α1,6-fucosyltransferase and modifies the Nod factor related to plant root nodulation, and protein *O*-fucosyltransferase, which is involved in Notch signaling (Breton et al. 1998; Oriol et al. 1999; Takahashi et al. 2000; Martinez-Duncker et al. 2003; Okajima et al. 2005). As indicated in our previous study involving site-directed mutagenesis and kinetic analysis, two arginine residues (Arg-365 and 366 of human FUT8) in one of the conserved regions play important roles in the binding of GDP-β-L-fucose (Takahashi et al. 2000). Recently, another group also reported similar roles for the equivalent arginine residues in the *O*-fucosyltransferase (Okajima et al. 2005). Our current study of the structure of FUT8 shows that three conserved regions are located adjacently to one another and are located within the Rossmann fold of FUT8 (Figure 3). These results strongly suggest that the three conserved regions and the flexible loop of FUT8 are functional in fucose-transferring reactions.

Site-directed mutagenesis

Structural analyses of the complex forms with the substrates are desired to better elucidate the catalytic mechanism of the α1,6-fucose transfer reaction. Despite much effort, such an analysis has not yet been successful for FUT8. Therefore, site-directed mutagenesis experiment was performed to determine roles for amino acid residues in or around Rossmann fold which is presumably the active site of FUT8. Eight amino acid residues, Asp-368, Lys-369, Glu-373, Tyr-382, Asp-409, Asp-410, Asp-453, and Ser-469 of human FUT8 were selected to be mutagenized because, in addition to their location, these residues are perfectly conserved among various species, vertebrates, insect, nematode, and ascidian (Figure 5A). As shown by the structural analysis of FUT8 in this study (Figure 5B), these selected residues were found to be located in the proximity of Arg-365 which is known to be the essential residue for its activity, as reported previously (Takahashi et al. 2000). These residues are also highly conserved in the motif conserved among other fucosyltransferases, α1,2-, and protein *O*-fucosyltransferase (Martinez-Duncker et al. 2003), as shown in motifs I, II, and III of Figure 5A, and those requirements expected from the alignment are consistent with the suggestion by the present structural analysis.

The mutant enzymes, in which the residues to be examined are replaced by alanine, were expressed in COS-1 cells, and were investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and FUT8 enzyme activity assay (Figure 5C and D). As indicated by a western blot analysis, the expression levels of these mutants were similar to that of the wild-type enzyme (Figure 5C). In the activity assay, however, it was found that D368A, K369A, E373A, Y382A, D409A, D453A, and S469A mutants were inactive because the level of enzyme activities in the transfected COS-1 cells were as low as that of the parental COS-1 cell, vector-transfected cell and the R365A mutant, which is

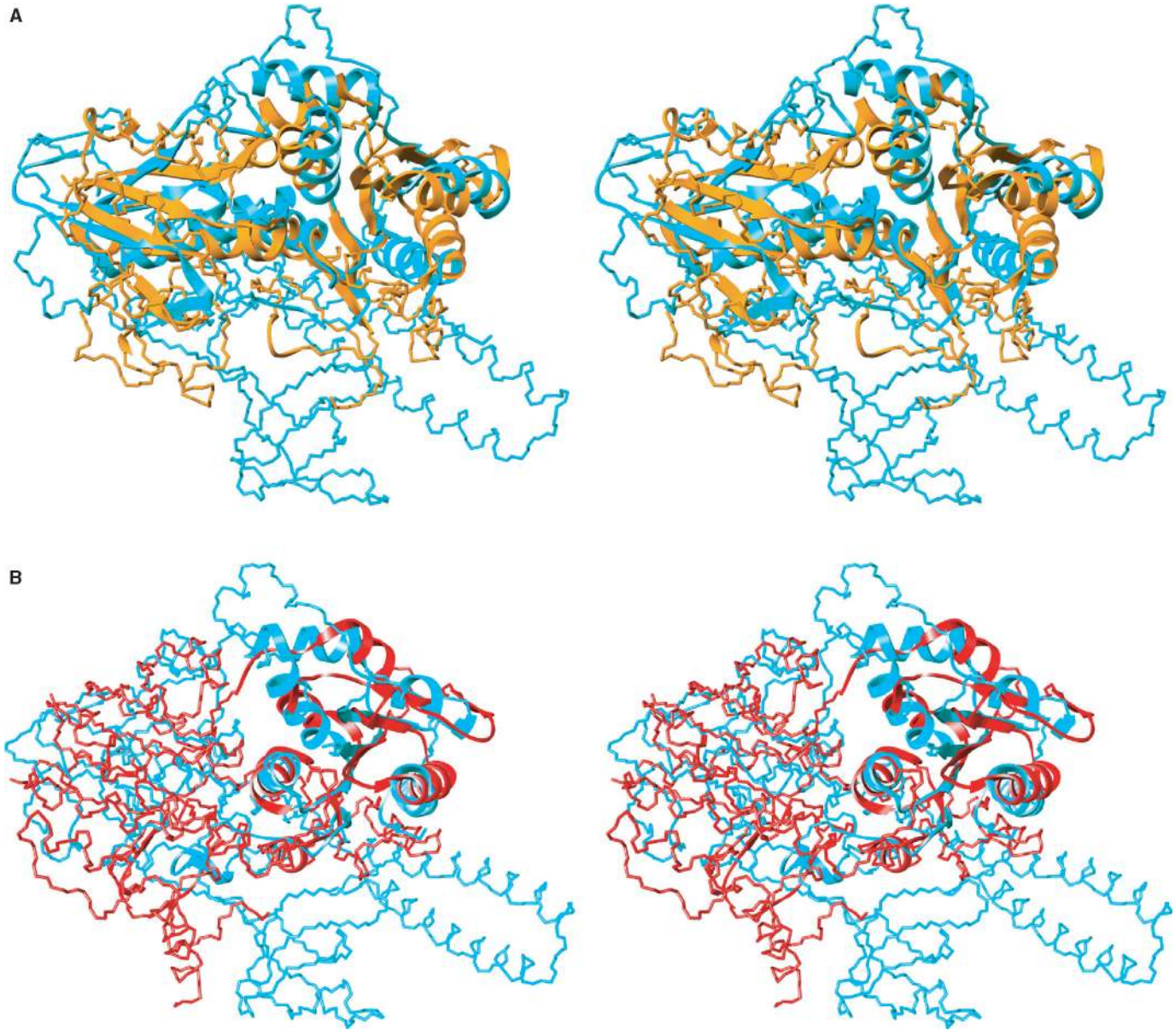

Fig. 4. Overlapping of GT-B glycosyltransferase on FUT8.Two GT-B glycosyltransferases, in which the Rossmann fold is similar to that of FUT8, were overlapped on FUT8. The main-chains of the enzymes are indicated. Regions with similarities are shown as a ribbon model. ADP-heptose lps heptosyltransferase II (**A**, PDB No. 1PSW) and Gtfb, β-glucosyltransferase (**B**, PDB No. 1IIR) are indicated in orange and red, respectively. FUT8 is indicated in blue.

known as the inactive mutant (Takahashi et al. 2000) (Figure 5D). The D410A mutant was found to be fully active, similar to the wild-type enzyme, suggesting that Asp-410 is not required for enzyme activity despite its location. It appears that Asp-368, Lys-369, Glu-373, Tyr-382, Asp-409, Asp-453, and Ser-469 play essential roles in the activity of FUT8 and are involved in the catalytic mechanism.

Asp-453 of human FUT8 as well as Arg-365 appears to be perfectly conserved among α1,2-, α1,6-, and *O*-fucosyltransferases (Martinez-Duncker et al. 2003), as shown in motifs I and III of Figure 5A. Consistent with the requirement of Asp-453 in FUT8, the equivalent Asp residue of NodZ is also essential for enzymatic activity (Chazalet et al. 2001). These results strongly suggest that Asp-453 and its equivalents serve a common function in α1,2-, α1,6-, and *O*-fucosyltransferases, possibly as a critical catalytic residue. Another essential aspartic residue, Asp-409 is located near Arg-365 and arranged face-to-face with Asp-453 (Figure 5B and D), and thus it seems more likely that a pair of Asp-409 and Asp-453 play a critical role as a general acid–base catalyst in the catalytic mechanism, as expected in inverting glycosidases and glycosyltransferases (White and Rose 1997; Unligil and Rini 2000; Tarbouriech et al. 2001; Zechel and Withers 2001). Such pairs of the residues have not yet been observed in the crystal structures of inverting glycosyltransferases.

On the other hand, Asp-368 and Lys-369, the locations of which were not determined but would be expected to be

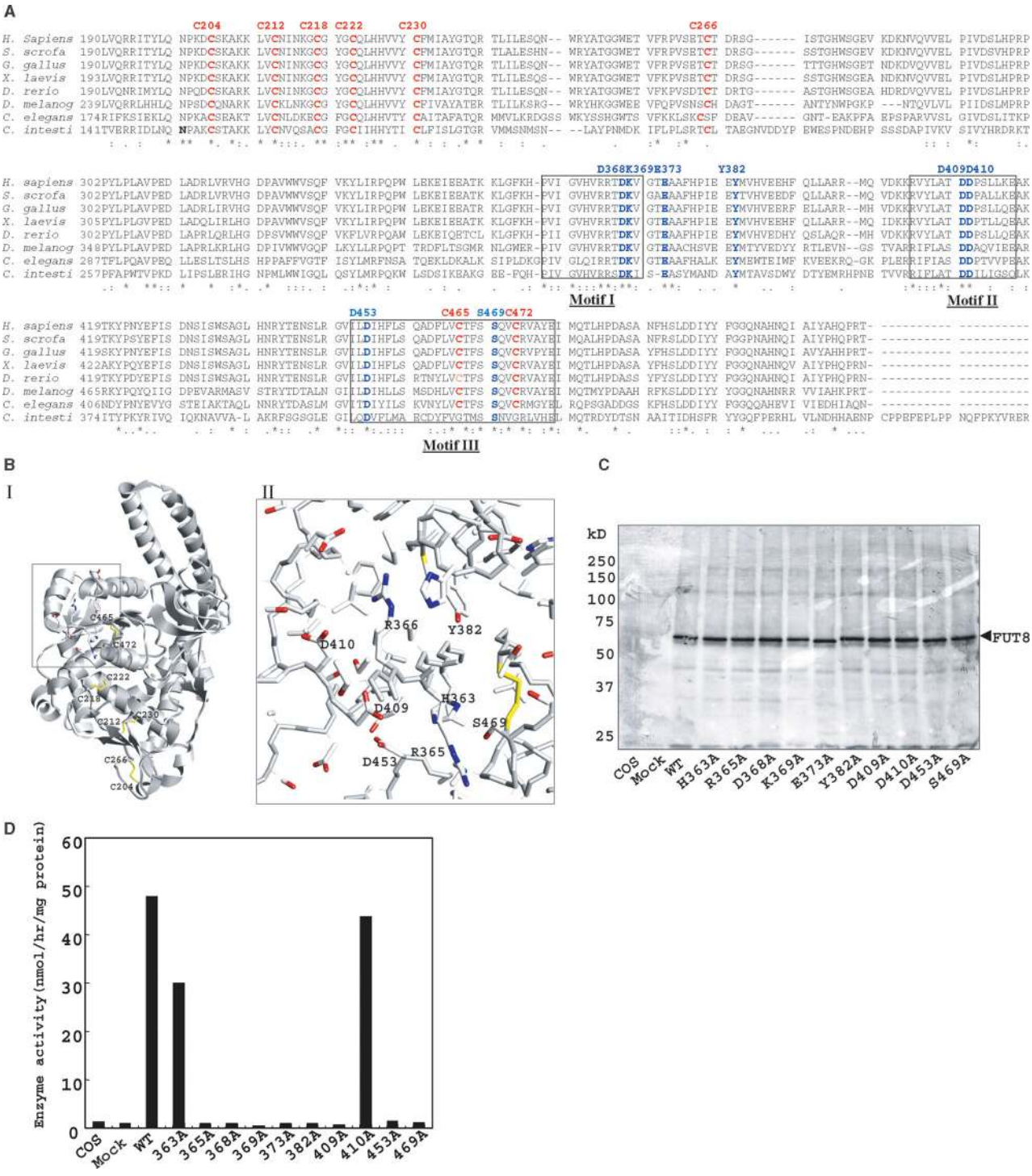

Fig. 5. Site-directed mutagenesis of the conserved amino acid residues of FUT8. (**A**) An alignment analysis of the catalytic regions of FUT8 from several species was carried out using CLUSTAL W. The amino acid residues which are conserved in all species are highlighted in bold. The conserved cysteine residues are indicated in red, and mutated residues are indicated in blue. The motif I (residues 358–370), motif II (residues 403–416), and motif III (451–477), which are conserved in three fucosyltransferases, α1,2-, α1,6-, and protein O-fucosyltransferases, are indicated in a box. GenBank accession numbers for FUT8 are: *Homo sapiens*, BAA19764; *Mus musculus*, NP_058589; *Sus scrofa*, BAA13157; *Gallus gallus*, CAH25853; *Xenopus laevis*, AAH79978; Danio *rerio*, CAH03675; *Drosophila melanogaster*, AAF48079; *C. elegans*, AAN84870; and *Ciona intestinalis*, CAD561622. (**B**) (I) The overall structure is shown as a ribbon model. Locations of the mutated residues near Arg-365 of FUT8 are indicated in a box. Side chains of the mutated residues are shown by stick. Conserved disulfide bonds are indicated in yellow. (II) The region around Arg-365 in box of Figure 5B-I is closed up. His-363, Arg-365, and Arg-366 were examined, as reported previously (Takahashi et al. 2000). The σ_A weighted $2F_o-F_c$ map contoured at 1 is also indicated. (**C**) The wild-type and mutant enzymes were transiently expressed in COS-1 cells. Cell lysates were separated on 8% SDS-gels and analyzed by immunoblot using anti-(FUT8) IgG. COS and mock indicate nontransfected and vector-transfected COS-1 cells, respectively. H363A and R365A are the controlled mutants. (**D**) Enzyme activities of FUT8 and its mutants were assayed using Asn-linked oligosaccharide acceptor labeled with *N*-[2-(2-pyridylamino)ethyl]-succinamic acid 5-norbornene-2,3-dicarboxyimide ester, as described in *Materials and methods*.

within the flexible loop of FUT8, were found to be essential for enzyme activity (Figure 5D). These results suggest that the flexible loop of FUT8, containing Asp-368 and Lys-369, plays an important role in the function of the enzyme.

Conserved disulphide bonds of FUT8 in vertebrate and invertebrate

Human FUT8 contains eight cysteine residues (Cys-204, 212, 218, 222, 230, 266, 465, and 472) (Yanagidani et al. 1997). This study identified all combinations of cysteines that form four disulfide bonds (Cys204–266, 212–230, 218–222, and 465–472 of human FUT8). Five cysteine residues (Cys-204, 212, 218, 222, and 230) of human FUT8 are perfectly conserved among vertebrates, insect, nematode, and ascidian (Figure 5A and B). Cys-266 of human FUT8 is predominantly conserved except in *Chlostridium elegans*, although in the *C. elegans* enzyme, the amino acid residue equivalent to Cys-266 of human FUT8 is replaced by serine (Ser-252 in *C. elegans*), the neighboring Cys-251 in *C. elegans* appears to be the substitute for the conserved cysteine residue (Figure 5A). Cys-465 and 472 are conserved in vertebrates, insect, and nematode, but not in asidian (Figure 5A). Because it has been reported that human FUT8 is sensitive to reducing condition (Yanagidani et al. 1997; Kaminska et al. 1998; Kaminska et al. 2003), some of the four disulfide bonds of FUT8 which are strongly conserved may play important roles in the correct folding of the protein and/or its stability rather than for the enzymatic function of catalysis.

Src homology 3 domain of FUT8

Early studies reported that the amino acid sequence of FUT8 is similar to some proteins that contain an SH3 domain (Javaud et al. 2000, 2003). The structural analysis confirms that the SH3 domain is actually folded at the C-terminus of FUT8 (Figures 2 and 6). By searching the DALI database, some other proteins were identified from the database as

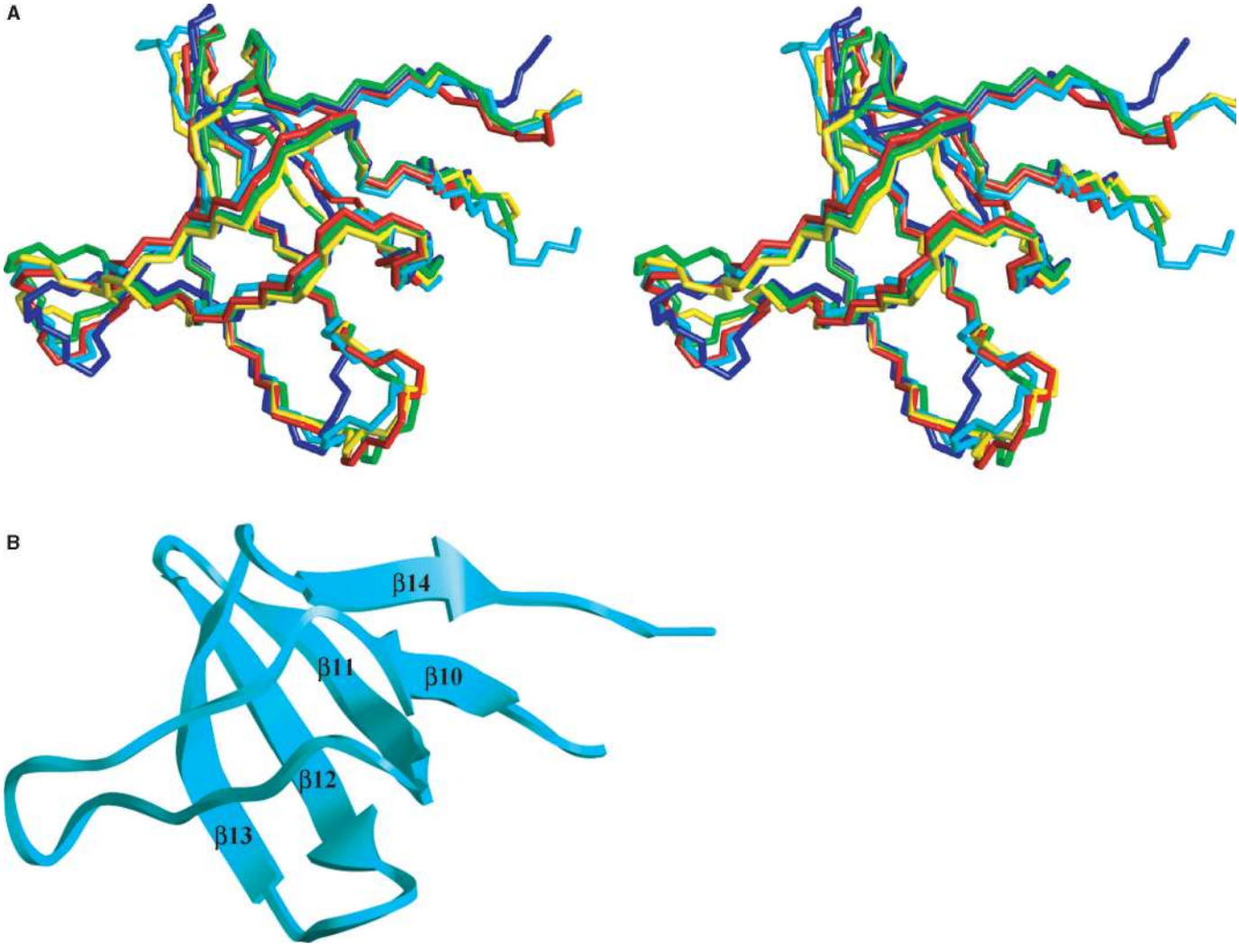

Fig. 6. Superimposition of SH3 domain of FUT8 onto various proteins containing SH3 domain. (**A**) SH3 domain of FUT8 at C-terminal was superimposed onto various proteins in a stereo view. Residues 502–562 comprise the SH3 domain of FUT8. The main chain of the SH3 domain of FUT8 is colored in cyan in this figure. Other SH3 domains were indicated in red, Abp1, the yeast actin binding protein (PDB No. 1JO8); blue, SH3 domain of neutrophil cytosol factor 4, the $p40^{phox}$, component of NADPH oxidase, (PDB No. 1W6X); green, the SH3 domain of c-Crk, a viral oncogene product (PDB No. 1CKA); and yellow, p56lck(Lck), a T-lymphocyte-specific member of the Src family of nonreceptor tyrosine kinases (PDB No. 1LCK). (**B**) A ribbon model of the SH3 domain of FUT8 is indicated.

having similarities with the FUT8 structure, and it was found that these proteins contain a SH3 domain. In particular, four proteins, yeast actin binding protein (Fazi et al. 2002), neutrophil cytosol factor 4 (Massenet et al. 2005), c-Crk, oncogene protein (Wu et al. 1995), p56 Lck, and the Src family kinase (Eck et al. 1994) were all found to have considerable similarities to the SH3 domain (residues 502–562) of FUT8, as summarized in Table I. As shown in Figure 6A, the SH3 domain of FUT8 contains a fold that is quite homologous to these proteins, regardless of the low homologies of the amino acid sequences. Because these proteins are known to interact and form a complex with a specific proline-rich peptide via the SH3 domain (Fazi et al. 2002; Massenet et al. 2005), the SH3 domain of FUT8 may be able to interact with a proline-rich peptide. SH3 domain-containing proteins are typically localized in the cytosol and mediate numerous signal-transducing pathways via critical protein–protein interactions. On the other hand, FUT8 is a type II membrane protein that is localized in the Golgi apparatus, and its catalytic domain and C-terminal SH3 domain are located in the lumen. It is not clear whether the luminal SH3 domain of FUT8 is functional in glycosyl-transfer, for example, through the selection of acceptor proteins, like the lectin domain of polypeptide α-*N*-acetylgalactosaminyltransferases (Fritz et al. 2004, 2006; Kubota et al. 2006), and the regulation of its enzymatic activity, or whether it has any other functions including localization, subunit formation or unknown functions. To elucidate the function of the SH3 domain on FUT8, further functional studies are needed and are currently in progress.

Conclusion

The overall structure of FUT8, human α1,6-fucosyltransferase, was solved and the findings indicate that the enzyme appears to have a catalytic region similar to GT-B glycosyltransferases rather than GT-A. In addition, it was found that FUT8 contains an SH3 domain at the C-terminus, which is quite similar to the SH3 domains found in other proteins but is unique in glycosyltransferase proteins. Although it would be expected that the SH3 domain of FUT8 may function to associate with substrate glycoproteins or unknown regulatory proteins, the definite significance of this domain remains to be elucidated. Consistent with earlier studies involving homology analyses and mutagenesis, the present structural and enzymatic studies of FUT8 also suggest that conserved residues in the three conserved motifs participate in the Rossmann fold and are involved in donor binding or in catalysis, thus playing a key role in the fucose-transferring reaction.

Materials and methods

Preparation of recombinant protein

FUT8 was expressed in soluble form, with a C-terminal polyhistidine tag, and purified by Ni^{2+} chelating affinity chromatography as described previously (Ihara et al. 2006). The protein was expressed using a baculovirus/insect cell expression system, as described previously (Ihara et al. 2006). Solutions of the purified proteins, at a concentration of 10 mg/mL, in 50 mM Tris–HCl buffer at pH 8.3, were used for crystallization. Protein concentrations were determined using a BCA Kit (Pierce, IL), with bovine serum albumin (BSA) as a standard.

Crystallization

Crystallization of the recombinant FUT8 was performed at 27 °C using the hanging drop vapor diffusion method. The drop contained protein at a concentration of 2.5–6.0 mg/mL in 50 mM HEPES-Na, 0.75 M lithium sulfate monohydrate, pH 7.5, and the reservoir solution contained 100 mM HEPES-Na, 1.5 M lithium sulfate monohydrate, pH 7.5. To prepare heavy atom derivatives of protein crystals, the crystals were soaked for 5 h in the presence of 10 mM potassium tetrachloroplatinate(II) in the same buffer that was used in the reservoir solution. The crystals were then transferred to a cryoprotectant solution containing 20% glycerol in the reservoir solution to prevent the formation of ice under the cryogenic environment.

X-ray data collection and structure determination

Crystals were flash-frozen in cryogenic nitrogen gas at 100 K, and data sets were then collected at the same temperature. Diffraction data for native and Pt derivative were collected using an imaging plate detector at the beamline BL44XU at SPring-8. The obtained data were indexed, integrated, and scaled using DENZO/SCALEPACK (Otwinowski et al. 1997). There were two non-isomorphous crystals, native1 and native2. The calculated R_{iso} between them was 38.5% (100–2.8 Å). The FUT8 crystal used in this study belongs to the $P6_522$ space group with one molecule per asymmetric unit. The unit cell dimensions for the native1 FUT8 crystal using structure determination are: $a = 90.46$ Å, $b = 90.46$ Å, and $c = 381.9$Å. Data collection and processing statistics for the data sets are summarized in Table II. Phases were determined by a single isomorphous replacement with anomalous scattering (SIRAS) using the data sets for native1 and Pt derivative. One clear platinum position was found on Harker sections from both the calculated difference and the anomalous Patterson map. The determined platinum position was refined using the program SHARP (de La Fortelle and Bricogne 1997). Density modification with solvent flattening was performed with the program SOLOMON (Abrahams and Leslie 1996) implemented in SHARP. The resultant experimental map using native1 data was traced and the model building was performed with the program O (Jones et al. 1991). The final model was built using native2 data and refined with program REFMAC5 (Murshudov et al. 1997) and ARP/wARP (Lamzin et al. 2001) in CCP4 suite (Collaborative Computational Project Number 4, 1994). The Ramachandran plot was also defined by PROCHECK (Lakowsky et al. 1993). Figures were produced using the UCSF Chimera program (Pettersen et al. 2004).

Cell culture

COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5 g/L glucose under a humidified atmosphere of 95% air and 5% CO_2.

Construction of expression plasmids

For transient expression in COS-1 cells, a cDNA encoding human FUT8 (Yanagidani et al. 1997) was subcloned into

the *Eco*RI sites of an expression vector, pCXNII (Niwa et al. 1991). In this vector, FUT8 was expressed under the control of the β-actin promoter and the CMV enhancer.

Site-directed mutagenesis

Site-directed mutagenesis experiments were carried out using the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA), as described previously (Ihara et al. 2004). The primers used in this study were 5′- GTCAGACGCACAGCG AAAGTGGGAACAG-3′ and 5′-CTGTTCCCACTTTCGCTG TGCGTCTGAC-3′ for replacement of Asp-368 (designated as D368A), 5′-GACGCACAGACGCAGTGGGAACAGAAG C-3′ and 5′- GCTTCTGTTCCCACTGCGTCTGTGCGTC-3′ for replacement of Lys-369 (K369A), and 5′- AAAGTGGG AACAGCAGCTGCCTTCCAT-3′ and 5′-ATGGAAGGCAG CTGCTGTTCCACTTT-3′ for replacement of Glu-373 (E373A), 5′-CCCATTGAAGAGGCAATGGTGCATGTT-3′ and 5′-AACATGCACCATTGCCTCTTCAATGGG-3′ for replacement of Tyr-382 (Y382A), 5′-GTATTTGGCCACAG CGGACCCTTCTTTATTAAAGG-3′ and 5′-CCTTTAAT AAAGAAGGGTCCGCTGTGGCCAAATAC-3′ for replacement of Asp-409 (D409A), 5′-GTATTGGCCACAGATGC GCCTTCTTTATTAAAGG-3′ and 5′-CCTTTAATAAAGAA GGCGCATCTGTGGCCAAATAC-3′ for replacement of A sp-410 (D410A), 5′-GGAGTGATCCTGGCGATACATTTTC TCTC-3′ and 5′-

GAGAGAAAATGTATCGCCAGGATCACTCC-3′ for replacement of Asp-453 (D453A), and 5′-TGTACTTTTT CAGCACAGGTCTGTCGA-3′ and 5′-TCGACAGACCTGT GCTGAAAAAGTACA-3′ for replacement of Ser-469 (S469A). The resulting mutations were verified by using BigDye® Terminator v3.1 Cycle Sequencing Kit and a DNA sequencer, ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA).

DNA transfection

Expression plasmids were transfected into cells by electroporation (Chu et al. 1987) using a Gene Pulser (Bio-Rad, CA), as described previously (Ihara et al. 2002). In a typical experiment, the cells were washed with HEPES-buffered saline and resuspended in the same solution. Plasmids (30 μg) were added to the cell suspension, followed by electrification. For the transient expression in Huh-6 cells, transfected cells were harvested after an appropriate growth period. The expression of FUT8 was verified by immunoblot analysis and enzyme activity assay for FUT8.

Electrophoresis and immunoblot analysis

SDS–PAGE was carried out on 8% gels, according to Laemmli (1970). Immunoblot analyses were performed as described previously (Ihara et al. 2002). The separated proteins were transferred onto a nitrocellulose membrane (PROTORAN, Schleicher & Schuell Inc., NH), and the resulting blot was blocked with 5% skim milk and 0.5% BSA in phosphate-buffered saline (PBS) containing 0.05% Tween-20. The resulting membrane was incubated with the 15C6 antibody against FUT8. The 15C6 antibody is a monoclonal antibody against human and porcine FUT8, and was obtained from Fujirebio Inc. (Tokyo, Japan). After washing with PBS containing 0.05% Tween-20, the membrane was reacted with a horseradish peroxidase -conjugated rabbit anti-(mouse IgG) Ig from Promega (Madison, WI). The immuno-reactive protein bands were visualized by HRP reaction using 4-chloro-1-naphtol and 3,3′-diaminobenzidine tetrahydrochloride (Pirece, IL) as substrates.

FUT8 activity assay

α1,6-Fucosyltransferase activity was assayed using a fluorescence-labeled sugar chain substrate, as described previously (Uozumi, Teshima et al. 1996; Ihara et al. 2006). Cell lysates were incubated at 37 °C with 10 μM of the acceptor substrate and 0.5 mM GDP-fucose as a donor in 0.1 M MES-NaOH, 1% Triton X-100 (pH 7.0). The reactions were terminated by boiling after an appropriate reaction time, and the reaction mixtures were centrifuged at 15 000*g* in a microcentrifuge for 10 min. The resulting supernatants were injected to a reversed phase HPLC equipped with TSKgel, ODS 80TM (4.6 × 150 mm) (Tosoh, Tokyo, Japan). The product and substrate were separated isocratically with 20 mM ammonium acetate buffer (pH 4.0) containing 0.15% *n*-butanol. The fluorescence of the column elute was detected with fluorescence detector (2475 Multi λ Fluorescence Detector, waters, MA) at excitation and emission wavelengths of 315 and 380 nm, respectively.

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Conflict of interest statement

None declared.

Abbreviations

FUT8, mammalian α1,6-fucosyltransferase; GDP, guanosine 5′-diphosphate; GlcNAc, N-Acetylglucosamine; Ig, immunoglobulin; *N*-glycan, asparagines-linked oligosaccharide; PBS, phosphate-buffered saline

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