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ヒトゲノム・再生医療等研究【ヒトゲノム遺伝子治療研究】研究事業

糖鎖シグナルの異常による肺気腫の発生機構の解明と治療戦略

平成１８年度　総括研究報告書

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総括研究報告書

糖鎖シグナルの異常による肺気腫の発生機構の解明と治療戦略

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研究要旨：本研究事業は肺気腫の病態とその成因を糖鎖異常によるシグナル伝達経路の変化を通して解析することにより、肺気腫に対する治療戦略を提供することを目的として開始された。TGF-β受容体に対するコアフコース修飾の欠損が肺気腫様病変を引き起こすFut8ノックアウトマウスをモデル動物として用い、受容体シグナル経路におけるコアフコース修飾の影響の解析、肺気腫の第一原因である喫煙とFut8活性の相関関係をモデルマウスでの喫煙実験ならびに培養細胞系において解析し、さらにはヒト肺気腫患者におけるFut8活性の変化を検討することの３点から本年度の研究を開始した。

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Ａ．研究目的

肺気腫を代表疾患とする慢性閉塞性肺疾患（COPD）は全世界での罹患者が６億人、死亡者が年間200万人、日本での推定患者数は530万人以上、そのうち年間13,000人が死亡しており、2020年までには先進国における死亡原因の第３位になることが予想される主要疾患である。COPDの社会的影響は、患者に対する医療費のみならず労働力人口の減少ならびに労働生産性の減弱にもつながる。またCOPDを引き起こす主要な原因の一つとしてあげられる喫煙がもたらす社会的な損失は、年間５兆６千億円と試算されている。一方、現在の治療方針は対症療法に限られており、有効な特異的治療法はごく限られたものとなっている。

これらの状況から、肺気腫の治癒をめざすのみならず、病態の進行を遅らせ社会活動能力を保つことのできる治療法の開発が急務となっている。

我々は、糖鎖の機能解析の研究において、Ｎ－型糖鎖にコアフコースが付加（α１、６フコシル化）されると、そのターゲットタンパク質の機能が変化すること、またコアフコシル化を触媒するFut8（α1,6 fucosyltransferase）の遺伝子欠損マウス（ノックアウトマウス）は、著明な成長障害と肺気腫様病変を示すことを明らかにしてきた。この肺気腫様病変は、TGF-β受容体に対するコアフコースの付加がなされないため、TGF-β受容体の下流シグナルが減弱し、抑制系に働くSmadのリン酸化が十分に起こらなくなることから細胞外マトリックスの分解を主につかさどるマトリックスメタロプロテアーゼ（MMP）の遺伝子発現が高まり、肺胞壁の合成と分解のバランスが崩れることにより肺気腫様の病変が引き起こされることを明らかにした。この研究により、糖鎖異常が肺気腫の原因になる可能性を示したことから、このFut8ノックアウトマウスを肺気腫モデルマウスとして用いることにより、肺気腫の病因ならびに病態の解明に糖鎖生物学・グライコームをもちいることを本研究の目的とした。

これまで肺気腫に対する特異的な治療方法の開発には多くの試みがなされてきたが、依然として有効な治療手段の提供には結びついてはいない。病因・病態に関する有効な知見を今後得るためには、これまでとは異なった分野・方向性による解析の進展とそこから得られた知見をもととした研究開発体制が必要になろうと考えられる。

そこで、本研究事業では糖鎖によるシグナル伝達経路の制御について考察を深めるとともに、喫煙等のこれまで知られてきた肺気腫の原因となる物質と糖鎖構造の変化の相関関係、さらにはヒト肺気腫患者における糖鎖変化に関する検討を加え、基礎研究から臨床検体まで含めた複合的な検討を平行して行うことを目的とする。

Ｂ．研究方法

３つの研究課題から検討を開始する。

1. 糖鎖によるTGF-β受容体修飾と機能制御のメカニズムの基礎的解析
2. タバコ等によるFut8修飾および制御の有無を培養細胞と動物において検討
3. ヒト肺気腫患者より採取された気管支肺胞洗浄液におけるFut8活性の測定

これらは、受容体を介した細胞内シグナル伝達の異常から細胞外マトリックスの破壊へと至る肺胞

破壊カスケードの解明とその制御方法の検討、肺気腫の発症・増悪に関する既知因子と糖鎖異常の相互関連性についての検討、ヒトにおける糖鎖異常と肺気腫との関連性の検討へとつながり、細胞レベルから動物、さらにはヒトへと各ステップに対する検討から、総合的な検討を効率よく進めていくことを目的としている。

1. 糖鎖によるTGF-β受容体修飾と機能制御のメカニズムの基礎的解析：TGF-β受容体に結合しているN-型糖鎖に対しα1,6-フコシル化が通常は起きているが、このコアフコース修飾が起こらないとTGF-βリガンドと受容体結合が弱いため、受容体刺激に反応しておこるSmad2のリン酸化が減弱する。Smad2は核内における負の転写因子として働いているため、MMP(マトリックスプロテアーゼ)の遺伝子発現が促進され、酵素活性も上昇する。この細胞外マトリクス破壊系が合成系を上回り、結果として肺胞壁の破壊が起こり肺気腫様の変化が生じるものと考えられている。薬物等によるこの肺胞破壊カスケードの制御を目指す場合、どの段階に作用する薬物を選択するかは重要な因子となるため、これら各段階における分子生物学的なメカニズムの解析が重要な基礎データとなる。すなわち、TGF-β受容体1,2とリガンドであるTGF-βの結合に果たす糖鎖修飾の影響、受容体複合体形成における糖鎖修飾の影響、タンパク質の挙動変化を観察することを試みる。これら培養細胞系を用いた実験で、糖鎖変化、特にFut8活性の有無による影響を直接的に評価するために、Fut8ノックアウトマウスから得られた線維芽細胞において受容体の挙動ならびに機能変化を調べ、その結果を野生型マウスから得られた線維芽細胞での結果と比較することによりコアフコース依存的な機能変化を見いだす。

2. タバコ等によるFut8による修飾および制御の有無を培養細胞と動物において検討：肺気腫発症の第一原因である喫煙が肺気腫の発症・進展に及ぼす分子メカニズムは明らかではない。喫煙により糖鎖構造に変化が起きることが新たな発症原因となる可能性を探るため、培養細胞ならびにマウスに対する喫煙実験を行ない相互作用が複合的に作用するかどうかを検討する。野生型マウスとFut8遺伝子改変マウスに対してそれぞれ喫煙暴露し、一定期間終了後の肺胞の肺気腫様変化（肺胞径）を測定し、Fut8活性と喫煙暴露、さらには喫煙によるFut8活性の変化を検討する。Fut8のホモ型ノックアウトマウスは生後間もなく死亡してしまうため、安定して喫煙実験に使用することができないが、ヘテロ型のノックアウトマウスは、野生型とほぼ同じ生存を示し、さらにFut8活性が野生型に比較して約半分に減弱していることが知られている。このヘテロ型ノックアウトマウスを用いて喫煙実験を行なう。一般にマウスに対する喫煙実験は雌マウスに対して行なうことが常法となっているため、本実験系でも雌マウスに対する喫煙実験を行なう。

タバコ煙中には様々な有害物質が含まれていることが知られている。これらの喫煙由来物質のどのようなものがFut8活性に影響を与えることがあるのか、さらには肺胞破壊カスケードに対して影響を与えているのかを検討するため、培養細胞系を用いた検討も行う。タバコ煙中にはニコチン・タールを代表とした化学物質に加え、特に反応性ならびに毒性の高いことが知られているアクロレイン、さらには窒素酸化物（NOx）等が含まれている。これらの物質によるFut8活性等への影響も幅広く検討する。

3. ヒト肺気腫患者より採取された気管支肺胞洗浄液におけるFut8活性の測定：Fut8ノックアウトマウスで認められた肺気腫様病変であったが、ヒトの肺気腫においては逆にFut8の活性に変化が認められないかを検討することを目的とする。特に、気管支肺胞洗浄液より集められた細胞におけるFut8活性を測定することにより、直接的な肺気腫とFut8活性の変化について検討することを目標とする。

（倫理面への配慮）

ヒトの気管支肺胞洗浄に関する研究については、研究対象者に対する人権擁護上の配慮、研究方法による不利益、危険性の排除や説明を行い、理解と同意（インフォームドコンセント）を取得することを前提に、北海道大学「医の倫理委員会」の審査を受けた。

また、動物実験に関しては「厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針」ならびに「動物実験の適正な実施に向けたガイドライン」を尊守して作成された実験計画により実施中である。

C．研究結果

1. 糖鎖によるTGF-β受容体修飾と機能制御のメカニズムの基礎的解析：TGF-β受容体2型全長を発現ベクターへクローニングした。さらに、C末端側に単量体型蛍光タンパク質を融合させ、細胞内における受容体局在を実時間測定できるようにするとともに、N末端側にはペプチドタグを付加することにより、細胞膜表面における受容体を生化学的に認識することができる様にした。さらに、TGF-β受容体1型に対しても同様のプラスミドを作製し、受容体複合体形成についての解析も行な

える様にしているところである。また、肺胞破壊カスケードに関連している受容体以外の因子、TGF-β、MMP等に関してもクローニングを終了し、発現系の構築を行なっている。今後、糖転移酵素・細胞外マトリクスに存在する糖鎖・その結合タンパク質等に関しても解析用プラスミドを構築し、今後の解析に役立てていく予定である。また、解析母体となるマウス線維芽細胞についても、Fut8および野生型マウスから確立した不死化線維芽細胞ならびに初代培養系線維芽細胞の両系を樹立した。一般的にこれらの細胞系はプラスミドを用いた発現系に利用することが容易ではないが、一時的な発現系で５０％以上の効率で目的遺伝子を発現させる系の構築に成功した。以上の状況から、今後発現系プラスミド構築等が終了すれば、早期に解析に取りかかることができるものと考えている。

2. タバコ等によるFut8修飾および制御の有無を培養細胞と動物において検討：Fut8を過剰発現させることなく内在性の活性が十分に高く、細胞本来のFut8活性をそのまま測定に用いることのできる細胞を選択した。この結果、ヒト胎児由来腎上皮細胞であるHEK293細胞が適していることがわかった。正常組織由来の細胞であり、本解析に適当であると考えられた。また、十分な内在性の活性をもつため培養ディッシュ（６cm径）１枚より収集された細胞が１回の測定に十分量であることがわかった。Fut8測定には蛍光標識したアスパラギン結合型asialo-, agalacto-biantennary sugar chainをacceptor substrateとして用い、HPLCにてフコシル化基質の生成量を測定した。現在、アクロレイン・一酸化窒素・ニトロシル化合物・窒素酸化物・タバコ抽出物等の物質を異なった酸素濃度条件下にて作用させ、そのFut8活性に与える影響の評価を継続している。今後、転写・合成レベルでの制御も含めて解析を進めていく予定である。マウスを用いた喫煙実験も開始した。喫煙実験には、世界的な実験用標準タバコとなっているアメリカケンタッキー大学タバコ健康研究所製の研究用タバコを用い、世界的な標準化基準に適合する様にした。野生型マウスを用いた肺気腫モデルの作製には６ヶ月間の連続した喫煙暴露が必要である。また、これらの実験を行なうためには、雌のマウスのみが用いられてきているため、本研究においても雌マウスを対象動物として喫煙実験を行なった。Fut8ヘテロ型ノックアウトマウスと野生型マウスの双方を同週令にて繁殖させ、実験に用いるが、分娩後の約半数しか本実験に用いることができないため、やや大掛かりな繁殖計画を立てなければならなかった。現在、短期間（１ヶ月）と長期間（３ヶ月）暴露の系を平行して行なっており、暴露期間終了後に肺を摘出し、右肺を肺胞径測定用、左肺をFut8活性測定用など生化学的解析用に採取し、測定を行なっていく。これらの実験により、肺気腫形成におけるFut8活性とタバコの影響が相互作用をもたらすかについて検討を進めていく。さらに、このFut8ノックアウトマウスが作成が容易な肺気腫モデルマウスとして治療薬の評価等に使用可能かどうかについても同様に検討を加えていく。

3. ヒト肺気腫患者より採取された気管支肺胞洗浄液におけるFut8活性の測定：ヒト肺気腫患者にFut8活性変化に基づく発症メカニズムを適応できるのかについて解析することを目的として、気管支肺胞洗浄液より得られた細胞のFut8活性を測定することをめざす。気管支肺胞洗浄液から得られる細胞数は極めて限られており貴重であることから、Fut8活性測定に関しても技術的にも十分に検討してから患者由来のサンプルの測定を開始することとした。現在は、健常ボランティアから得られた気管支肺胞洗浄液中から得られた細胞を用いることにより、安定してFut8活性を測定する系をほぼ確立した。今後、症例数の蓄積ならびに年齢・喫煙との関連性等を考慮したデータ解析を行なっていく予定である。

D. 考察

第二次募集により開始されたため、実質的には平成１８年１１月からプロジェクトが開始されたため、各研究課題が準備段階にある。

１：培養細胞系を用いたTGF-β受容体機能を解析する技術を応用し、Fut8ノックアウトマウス由来の線維芽細胞内におけるTGF-β受容体機能を観察することを目的とする実験を進めた。受容体発現系に用いる各種プラスミドの構築ならびにそのアッセイ系の確立をおこなっている。TGF-β受容体・リガンド・受容体複合体形成・細胞内局在・Smadリン酸化能・MMP活性化能についてそれぞれ検討を行うことにより、肺胞破壊カスケードのいずれの部分を制御することが肺気腫様病態の制御に有効であるか、今後の肺気腫に対する治療戦略を考慮するために有用な知見を与えることが期待される。

２：喫煙がFut8活性に与える影響の評価と、肺気腫様病変の発現に及ぼす喫煙とFut8活性の相乗効果について動物モデルならびに培養細胞系の双方から検討を開始した。動物モデルでは通常６ヶ月間の喫煙暴露が必要なため、現在長期（３ヶ月）ならびに短期間（１ヶ月）モデルに対する喫煙暴露実験を行なっている。喫煙とFut8活性の相互作用に関する検討が深まるのみならず。Fut8ヘテロ型ノックアウトマウスを肺気腫治療薬の効果判定

に有用に用いることができるかどうかをについての知見を与えることとなる。また、細胞を用いた各種タバコ関連物質のFut8活性に与える影響の検討から、喫煙により肺気腫が形成される原因物質の推定が行なえる可能性を持つ。
３：ヒト肺気腫患者由来の気管支肺胞洗浄液（BALF）から取得された細胞を用い、Fut8活性と病態との関連性を調べるためのサンプル収集を倫理委員会の承認のもと開始した。肺気腫患者からの気管支肺胞洗浄液の採取は大変難しく症例数を揃えることが難しいことも予想しておかなければならない。そのため、気管支肺胞洗浄液のみならず血液・手術標本などのヒト由来サンプルにおけるFut8活性の測定についても検討していくこととし、より幅広い検討が行える様に努力する必要があろう。

E．結論
１：線維芽細胞における発現系の構築を終え、アッセイに用いるプラスミド構築・アッセイ系の確立を行なっている。
２：Fut8ヘテロノックアウトマウスに対する喫煙暴露実験を実施中である。培養細胞系におけるFut8活性に影響を与える物質ならびに条件の検索をおこなっている。
３：気管支肺胞洗浄液より回収された細胞におけるFut8活性の測定法を確立した。今後、臨床検体を用いた検討を継続していく。

F．健康危険情報
特になし。

G．研究発表
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Crystal Structure of Mammalian 1,6-Fucosyltransferase, FUT8

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ABSTRACT

FUT8, mammalian α1,6-fucosyltransferase, catalyses the transfer of a fucose residue from a donor substrate, GDP-β-L-fucose, to the reducing terminal GlcNAc of the core structure of an asparagine-linked oligosaccharide. α1,6-Fucosylation, also referred to as a core fucosylation, plays an essential role in various pathophysiological events. Our group reported that FUT8 null mice show severe growth retardation and emphysema-like lung-destruction as a result of the dysfunction of EGF and TGF-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 catalysis, thus playing a key role in the fucose-transferring reaction.

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, various microbial and virus infections (Staudacher *et al.*, 1999; Becker *et al.*, 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 *et al.*, 2003). Asparagine-linked oligosaccharides (*N*-glycans) of glycoproteins are ubiquitously α1,6-fucosylated (Miyoshi *et al.*, 1997; Miyoshi E, Noda K, Yamaguchi Y, *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 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 E, Noda K, Ko JH, *et al.*, 1999). Moreover, the core fucosylation of *N*-glycans in human IgG1 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 EGF, TGF-β receptors (Wang *et al.*, 2005; Wang *et al.*,

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.

FUT8 catalyzes the transfer of a fucose residue from the donor substrate, GDP-β-L-fucose, to the innermost GlcNAc residue in *N*-glycans via α1,6-linkage with inversion of the anomeric center of the transferred fucose (Fig. 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 *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; (I) 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 (II) 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 (Paschinger *et al.*, 2005; Staudacher *et al.*, 1998). 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; Martinez-Duncker *et al.*, 2003; Takahashi *et al.*, 2000; 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 basis 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 Fig. 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 fifteen strands and sixteen helices (Figs. 2 and 3). Several distinct features are observed in the structure of FUT8 (Fig. 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, 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, 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 *et al.*, 1993). As summarized in table 2, 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), *E. coli* ADP-heptose lps

heptosyltransferase II (PDB No., 1PSW), which is belong 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. The SH3 domain (residues 502-562) of FUT8 has relatively high similarities to that of many proteins, and will be described in detail below. 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 (Fig. 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 less than 5, were ADP-heptose lipopolysaccharide heptosyltransferase II (PDB No., 1PSW), and Gtfb, β-glucosyltransferase (Mulichak *et al.*, 2001), trehalose-6-phosphate synthase (Gibson, R.P. *et al.*, 2002), and sialyltransferase from *Pasteurella*

multocida (Ni, L. *et al.*, 2006) (Table 2). 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 (Fig. 4). At the same time, the catalytic region of FUT8 was compared to 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 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; 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; Ramakrishnan *et al.*, 2004; Ramasamy *et al.*, 2003). 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 form and/or donor substrate-bound form (Unligil *et al.*, 2000; Gastinel *et al.*, 2001; Patenaude *et al.*, 2002; Pedersen *et al.*, 2000; Kakuda *et al.*, 2004; Pedersen *et al.*, 2003; Mulichak *et al.*, 2001). In the case of FUT8, the region consisting of residues 368-372 is disordered probably due to its flexibility (Figs. 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 (Figs. 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; Martinez-Duncker *et al.*, 2003; Takahashi *et al.*, 2000; 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 (Fig.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 (Fig. 5A). As shown by the structural analysis of FUT8 in this study (Fig. 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 motif I, II and III of Fig. 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 SDS-PAGE and FUT8 enzyme activity assay (Figs. 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 (Fig.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 known as the inactive mutant (Takahashi *et al.*, 2000) (Fig. 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 motif I and III of Fig. 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 three 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 (Fig. 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 (Zechel and Withers, 2001; White and Rose, 1997; Tarbouriech *et al.*, 2001; Ünligil and Rini, 2000). 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 within the flexible loop of FUT8, were found to be essential for enzyme activity (Fig. 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 (Fig. 5A and B). Cys-266 of human FUT8 is predominantly conserved except in *C.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 (Fig. 5A). Cys-465 and 472 are conserved in vertebrates, insect and nematode, but not in asidian (Fig. 5A). Because it has been reported that human FUT8 is sensitive to reducing condition (Kaminska *et al.*, 1998; Yanagidani *et al.*, 1997; 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 and 2003). The structural analysis confirms that the SH3 domain is actually folded at the C-terminus of FUT8 (Figs.2 and 6). By searching the DALI database, some other proteins were identified from the database as having similarities with the FUT8 structure, and it was found that these proteins contain an 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, 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 2. As shown in Fig. 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. Since 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; Fritz *et al.*, 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.