(macrophage colony stimulating factor, M-CSF)類を示すサブステムである。M-CSFは，単球，マクロファージの前駆細胞に特異的に作用し，その分化，増殖を促進してコロニー形成を誘導する作用を持つ。ヒトM-CSFは149個または214個のアミノ酸残基からなる同一のサブユニット2分子で構成される，分子量約45,000と約84,000の2種類の糖タンパク質が知られている。

ステム「-mostim」を持つINNは以下のものがある。

Cilmostim

Lanimostim

Mirimostim(ミリモスチム)

これらのうち，Mirimostim(ミリモスチム)は，ヒト尿より精製したM-CSFで，214個のアミノ酸残基からなるタンパク質のホモ2量体で構成される糖タンパク質(分子量：約84,000)であり，日本で承認され顆粒球減少症治療薬として使用されている。

(4)「-plestim」：インターロイキン-3類

「-plestim」は，インターロイキン-3(interleukin-3, IL-3)類を示すサブステムである。IL-3は多能性コロニー刺激因子(multi-CSF)とも呼ばれていたもので，顆粒球，マクロファージ，マスト細胞，赤血球，好酸球，巨核球系と多様な造血系細胞の分化，増殖を促進する作用を有する。IL-3はインターロイキンに分類されているが，ステムはインターロイキンのステム「-kin」ではなく，コロニー刺激因子のステム「-stim」が用いられている。ヒトIL-3は133個のアミノ酸残基からなり，4個の*N*-結合型糖鎖を有する糖タンパク質である。

ステム「-plestim」を持つINNには以下のものがある。

Muplestim(ムプレスチム)

Daniplestim

Muplestim(ムプレスチム)は，遺伝子組換えヒトIL-3で，JANに登録されているが，未承認である。Daniplestimは，IL-3の14番目から125番目のアミノ酸残基のうち，27個のアミノ酸残基を改変したIL-3誘導体で，IL-3よりも強力なIL-3受容体アゴニストとして開発中の医薬品である。

(5)「-distim」：2種類のコロニー刺激因子の融合タンパク質

「-distim」は，2種類の異なるコロニー刺激因子の融合タンパク質を示すサブステムである。INNでは以下の2種類が登録されている。

Leridistim

Milodistim

Leridistimは，IL-3誘導体とG-CSF誘導体との融合タンパク質，Milodistimは，GM-CSF誘導体とIL-3誘導体との融合タンパク質である。

(6)その他の「-stim」類

INNにはその他の「-stim」として，以下のものが登録されている。

Ancestim(アンセスチム)

Garnocestim

Pegacaristim

Ancestim(アンセスチム)は，造血幹細胞の増殖に重要な分子であるヒト幹細胞因子(stem cell factor, hSCF)の可溶性(分泌型)タンパク質を遺伝子組換えで製造したもので，hCSFの1-165番目のアミノ酸残基のN末端にメチオニン残基が付加したタンパク質の2量体からなる。JANに登録され，再生不良性貧血治療薬として開発が進められていたが，臨床開発は中止されている。

Garnocestimは，白血球遊走活性を有するCXCケモカインのひとつであるGROβ／マクロファージ炎症性タンパク質(macrophage inflammatory protein, MIP)2αの5-73番目のアミノ酸残基に相当するペプチドである。

Pegacaristimは，血小板産生を促進するヒトトロンボポエチン(thrombopoetin, TPO)の活性領域(recombinant human megakaryocyte growth and development factor, rhMGDF)にPEGを結合した修飾タンパク質で，血小板減少症治療薬として開発中である。

ステム30 「-kin」：サイトカイン／インターロイキン類

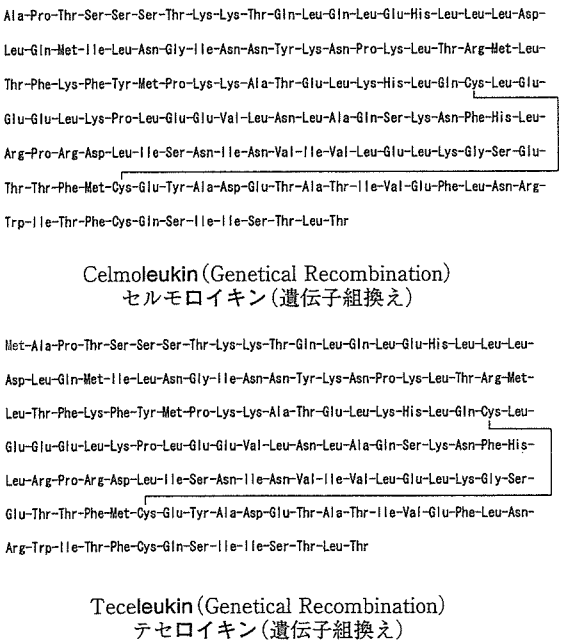
「-kin」は，サイトカインの中の一群の分子種であるインターロイキン(interleukin)類に共通するステムである。インターロイキンはリンパ球や単球，マクロファージなどの免疫担当細胞が産生放出する(糖)タンパク質性の生物活性物質の総称で，細胞表面に存在する受容体を介して細胞の活性化，分化，増殖，細胞間相互作用などに関与する。インターロイキンはタンパク質として同定された順にインターロイキン(IL)の後に番号を付けて呼ばれている。インターロイキンのステムの「-kin」もイ

ンターロイキンの種類ごとにサブステムが与えられている。インターロイキンおよびインターロイキンに関連する医薬品のステムは表２に示した。

(1)「-leukin」：インターロイキン-２類

「-leukin」はインターロイキン-２(interleukin-2, IL-2)類を示すサブステムである。インターロイキン類の中で，日本で医薬品として実用化されているのはIL-2のみである。IL-2はT細胞増殖因子と呼ばれていたもので，T細胞より産生され，T細胞の増殖と分化を促進するほか，ナチュラルキラー細胞の活性化，B細胞の増殖など多様な作用を示す。ヒトIL-2はアミノ酸133個からなる糖タンパク質である。

ステム「-leukin」を持つINNは７品目が登録されている(表２)。これらのうち，Celmo**leukin**(セルモ**ロイキン**)，Tece**leukin**(テセ**ロイキン**)は新たに日局に収載された医薬品である(**図２**)。これらはいずれもヒトIL-2のcDNAを導入した大腸菌で製造されるタンパク質で，Celmo**leukin**(セルモ**ロイキン**)は天然のIL-2と同じ133個のアミノ酸残基から，また，Tece**leukin**(テセ**ロイキン**)はN末端にメチオニン１残基が付加した134個のアミノ酸残基からなるタンパク質である。いずれも天然のIL-2とは異なり糖鎖は付加していない。腎がん，血管肉腫の治療薬として使用されている。



Celmo**leukin** (Genetical Recombination)
セルモ**ロイキン**(遺伝子組換え)

Tece**leukin** (Genetical Recombination)
テセ**ロイキン**(遺伝子組換え)

図２　インターロイキン-２を示すステム「-leukin」を持つ医薬品

また，Alde**sleukin**，Deni**leukin** Diftitoxは海外で承認されている医薬品である。Alde**sleukin**はIL-2の2-133番目のアミノ酸残基のうち，125番目のシステインをセリンに置換したIL-2誘導体で，適応症は腎がん，悪性黒色腫である。Deni**leukin** DiftitoxはIL-2とジフテリア毒素との融合タンパク質で，IL-2受容体を介して標的細胞に取り込まれ，ジフテリア毒素により細胞死を誘導する。IL-2受容体α鎖(CD25)を発現している皮膚T細胞リンパ腫の治療薬として使用されている。

(2)その他の「-kin」類

IL-2以外のインターロイキン類はまだほとんど実用化されていない。しかし，インターロイキンの機能解明が進み，インターロイキンを利用したり，インターロイキンの機能を阻害する医薬品の開発が進められており，海外ではすでに承認されている医薬品もある。

①「-elvekin」：インターロイキン-11

「-elvekin」は，インターロイキン-11(IL-11)を示すサブステムである。IL-11は骨髄間質細胞や繊維芽細胞から産生される178個のアミノ酸残基からなる分子量23,000のタンパク質で，造血前駆細胞や間質細胞に作用し，巨核球の増殖と成熟，脂肪細胞分化の抑制などの作用を持つ。Opre**lvekin**(オプ**レルベキン**)は遺伝子組換えで製造されたIL-11の2-178番目のアミノ酸残基に相当するタンパク質である。血小板増殖因子として開発が進められ，米国では血小板減少症治療薬として承認されているが，日本では承認申請が取り下げられている。

②「-nakinra」：インターロイキン-1受容体アンタゴニスト

「-nakinra」はインターロイキン-1受容体アンタゴニスト(interleukin-1 receptor antagonist, IL-1RA)を示すサブステムで，IL-1のステム「-nakin」と受容体アンタゴニスト(receptor antagonist)に由来する。IL-1RAは単球系細胞で産生分泌される分子量23,000～25,000の糖タンパク質で，IL-1受容体に結合し，IL-1がIL-1受容体に結合するのを競合阻害する生理的アンタゴニストである。IL-1は炎症性サイトカインで，慢性関節リウマチなどの炎症性疾患にも深く関与している。A**nakinra**は遺伝子組換えで製造されたN末端にメチオニン1残基が結合したIL-1受容体アンタゴニストで，欧米では関節リウマチ治療薬として承認されている医薬品である。しか

し日本での臨床開発は進んでいない。

ステム31 「interferon」：インターフェロン類

インターフェロン(interferon, IFN)はウイルス感染などの刺激を受けた細胞が産生，分泌する分子量約20,000の一群の生理活性糖タンパク質で，サイトカインの１種である。ウイルス増殖抑制作用のほかに細胞増殖抑制作用，抗腫瘍作用，免疫調節作用等の生物活性を持つ。主な分子種として産生細胞や構造の異なるIFN-α，IFN-β，IFN-γの３種類がある。

医薬品としてのインターフェロン類にはステムはなく，学術用語と同じ「**interferon**」がINNとしても用いられている。**Interferon**(**インターフェロン**)がINNになったのは非常に古く1962年のことで，「動物細胞とウイルスの相互作用により産生される(糖)タンパク質で，動物細胞にウイルス感染に対する抵抗力を持つようにする物質」と定義された。1980年代になり，IFN-α，IFN-β，IFN-γやこれらのバリアント(アミノ酸変異体)が遺伝子組換えで製造されるようになり，名称や定義の変更が検討された。INN委員会は「Alfaferon」，「Betaferon」，「Gammaferon」等の名称を検討したが，これらはすでに製品名として登録されており変更はできなかった。そこでINNでは，

Interferon Alfa(インターフェロン アルファ)

Interferon Beta(インターフェロン ベータ)

Interferon Gamma(インターフェロン ガンマ)

とα，β，γのアルファベット綴りを略さずに記載し，２語式で表す方法が採用された。前にも説明したように，INNには糖タンパク質の糖鎖の異なるものに対して名称の後にアルファ，ベータとつけて２語式に表して区別するというルールがある。しかし，インターフェロンでは，アミノ酸配列の異なるインターフェロンの分類を示すために生化学名に使われているアルファ，ベータ，ガンマをINNでもそのまま例外的に使用している。さらに必要に応じて数字やアルファベットを付加したり，混合物の場合にはコードを付加することにより，遺伝子の違いやアミノ酸の違いを区別するというルールが採用されている。

(1)「Interferon Alfa」：インターフェロン アルファ類

ヒトIFN-αは，白血球インターフェロンとして知られていたもので，ウイルス感染した白血球で産生分泌される糖タンパク質ファミリーである。塩基配列の異なる14種類以上のIFN-α遺伝子群から発現されるサブタイプが存在する。アミノ酸残基165-172個からなり，*N*-結合型糖鎖を持つものが多い。

INNではヒトIFN-α遺伝子のサブタイプはハイフンの後に数字を付けて，**Interferon** Alfa-2(**インターフェロン** アルファ-2)のように表す。Interferon Alfa-2には23番目と34番目のアミノ酸残基の異なるバリアントがあり，これらは数字の後にアルファベットをつけて区別する(Alfa-2a, Alfa-2b, Alfa-2c)(**図３**)。また，混合物の場合は**Interferon** Alfa-n1, **Interferon** Alfa-n2などと表す。

現在，日本では以下の７品目が承認されている。

Interferon Alfa(NAMALWA)(**インターフェロン** アルファ(NAMALWA))

Interferon Alfa(BALL-1)(**インターフェロン** アルファ(BALL-1))

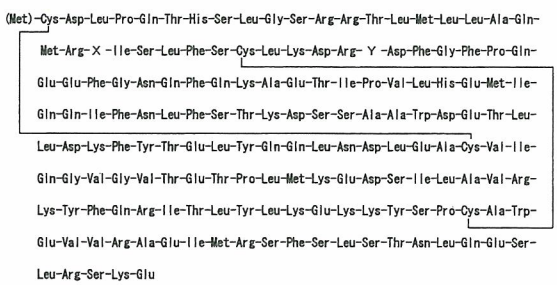
Interferon Alfa-2a(Genetical Recombination)(**インターフェロン** アルファ-2a(遺伝子組換え))

Interferon Alfa-2b(Genetical Recombination)(**インターフェロン** アルファ-2b(遺伝子組換え))

Interferon Alfacon-1(Genetical Recombination)(**インターフェロン** アルファコン-1(遺伝子組換え))

Peg**interferon** Alfa-2a(Genetical Recombination)(ペグ**インターフェロン** アルファ-2a(遺伝子組換え))

Peg**interferon** Alfa-2b(Genetical Recombination)(ペグ**インターフェロン** アルファ-2b(遺伝子組換え))



	各位置のアミノ酸残基	
	23(X)	34(Y)
Alfa-2a	Lys	His
Alfa-2b	Arg	His
Alfa-2c	Arg	Arg

図３　Interferon Alfa-2(インターフェロン アルファ-2)のアミノ酸配列

これらの医薬品のうち，Interferon Alfa(インターフェロン アルファ)は，INNでは１種類であるが，細胞培養技術を用いて製造したIFN-αは用いる細胞によりサブタイプの組成が異なるため，JANでは，INNの後に用いた細胞の名称を括弧書きで記載して区別しているため２品目となる。これらの医薬品は慢性C型肝炎の治療薬として用いられているほか，慢性B型肝炎，腎がん，慢性骨髄性白血病，多発性骨髄腫の治療薬としても用いられる。

Interferon Alfa(NAMALWA)(インターフェロン アルファ(NAMALWA))は，ヒトリンパ芽球NAMALWA細胞をセンダイウイルスで誘発することにより産生される分子量17,000～30,000の糖タンパク質で，サブタイプの混合物であり，日局収載候補品目となっている。

Interferon Alfa(BALL-1)(インターフェロン アルファ(BALL-1))は，ヒトリンパ芽球BALL-1細胞をセンダイウイルスで誘発することにより産生される分子量13,000～21,000の糖タンパク質で，IFN-α2，α7およびα8のサブタイプから構成される。

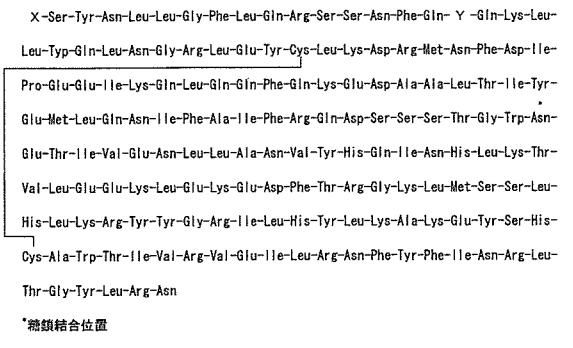
Interferon Alfa-2a(インターフェロン アルファ-2a)，Interferon Alfa-2b(インターフェロン アルファ-2b)は，それぞれ対応する遺伝子を導入した組換え体で産生される165個のアミノ酸残基からなるタンパク質である。

Interferon Alfacon-1(インターフェロン アルファコン-1)は，ヒトIFN-αの12種類のサブタイプのアミノ酸配列において，各位置の出現頻度が最も高いアミノ酸残基をコードするように人工的に設計した遺伝子の発現により組換え体で産生される，一部N末端にメチオニン残基が付加している166個のアミノ酸残基からなるタンパク質で，インターフェロン アルファよりも高い生物活性を示す。

Peginterferon Alfa-2a(ペグインターフェロン アルファ-2a)，Peginterferon Alfa-2b(ペグインターフェロン アルファ-2b)は，それぞれInterferon Alfa-2a(インターフェロン アルファ-2a)，Interferon Alfa-2b (インターフェロン アルファ-2b)をPEG化したもので，血中半減期が延長され，投与回数を減らすことが可能な医薬品である。

(2)「Interferon Beta」：インターフェロン ベータ類

ヒトIFN-βは，繊維芽細胞インターフェロンとして知られていたもので，ウイルスや２本鎖RNAの刺激により繊維芽細胞で産生される166個のアミノ酸残基からなるN-結合型糖鎖を持つ糖タンパク質である。IFN-β遺伝子はIFN-αと異なり１種類である。



	各位置のアミノ酸残基 1(X)	17(Y)	糖鎖結合
Beta-1a	Met	Cys	Asn80
Beta-1b	-	Ser	-

図４　Interferon Beta(インターフェロン ベータ)のアミノ酸配列

INNではIFN-βのサブタイプはハイフンの後に数字を付けて，Interferon Beta-1(インターフェロン ベータ-1)と表す。Interferon Beta-1では，１番目と17番目のアミノ酸残基および糖鎖結合の有無が異なるものがあり，これらは数字の後のアルファベットで区別する(Beta-1a, Beta-1b)(図４)。また，混合物の場合はInterferon Beta-n1, Interferon Beta-n2などと表す。

現在，日本で承認されているのは以下の３品目である。

Interferon Beta(インターフェロン ベータ)

Interferon Beta-1a(Genetical Recombination)(インターフェロン ベータ-1a(遺伝子組換え))

Interferon Beta-1b(Genetical Recombination)(インターフェロン ベータ-1b(遺伝子組換え))

Interferon Beta(インターフェロン ベータ)は，ヒト繊維芽細胞に誘発剤を作用させて産生した166個のアミノ酸残基からなる糖タンパク質であり，悪性黒色腫，膠芽腫，髄芽腫，星細胞腫，慢性B型肝炎，慢性C型肝炎などの治療薬として使われている。

Interferon Beta-1a(インターフェロン ベータ-1a)は，CHO細胞を用いて遺伝子組換えにより製造した166個のアミノ酸残基からなる糖タンパク質で，天然型IFN-βと同じアミノ酸配列でN-結合型糖鎖を持つ。一方，Interferon Beta-1b(インターフェロン ベータ-1b)は，17番目のシステインをセリンに置換し，分子内ジスルフィド結合が正しく架橋されるようにしたもので，大腸菌

で製造した165個のアミノ酸からなるタンパク質である。これらはともに，多発性硬化症の治療薬として使用されている。

(3)「Interferon Gamma」：インターフェロン ガンマ類

ヒトIFN-γは，免疫インターフェロンとして知られていたもので，マイトジェンや特異抗原刺激によりT細胞で産生される，146個のアミノ酸残基からなるN-結合型糖鎖を持つ糖タンパク質で，IFN-γ遺伝子は1種類である。IFN-αとIFN-βは構造上の類似性が高く受容体も共通しているが，IFN-γとIFN-α，IFN-βに類似性はなく，αとβはⅠ型，γはⅡ型インターフェロンに分類される。

INNではIFN-γのサブタイプはハイフンの後に数字を付けて，**Interferon** Gamma-1(**インターフェロン** ガンマ-1)と表す。Interferon Gamma-1ではN末端，C末端のアミノ酸配列の異なるものを数字の後のアルファベットで区別し，Gamma-1a，Gamma-1b，Gamma-1cが定義されている(**図5**)。また，混合物の場合は**Interferon** Gamma-n1, **Interferon** Gamma-n2などと表す。

現在，日本では以下の2品目が承認されており，腎がん，菌状息肉症，慢性肉芽腫，成人T細胞白血病の治療薬として用いられている。

Interferon Gamma-1a(Genetical Recombination)(**インターフェロン** ガンマ-1a(遺伝子組換え))

Interferon Gamma-n1(**インターフェロン** ガンマ-n1)

Interferon Gamma-1a(**インターフェロン** ガンマ-1a)は，対応する遺伝子を導入した組換え体で産生されるアミノ酸146個からなるタンパク質である。また，**Interferon** Gamma-n1(**インターフェロン** ガンマ-n1)は，ヒトミエロモノサイト細胞株HBL-38をリポポリサッカライドで刺激して産生される，126，127，128，129および138個のアミノ酸残基からなる分子量約15,000～26,000の糖タンパク質の混合物である。

X-Gln-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr-Phe-Asn-Ala-Gly-His-Ser-
Asp-Val-Ala-Asp-Asn-Gly-Thr-Leu-Phe-Leu-Gly-Ile-Leu-Lys-Asn-Trp-Lys-Glu-Glu-Ser-
Asp-Arg-Lys-Ile-Met-Gln-Ser-Gln-Ile-Val-Ser-Phe-Tyr-Phe-Lys-Leu-Phe-Lys-Asn-Phe-
Lys-Asp-Asp-Gln-Ser-Ile-Gln-Lys-Ser-Val-Glu-Thr-Ile-Lys-Glu-Asp-Met-Asn-Val-Lys-
Phe-Phe-Asn-Ser-Asn-Lys-Lys-Lys-Arg-Asp-Asp-Phe-Glu-Lys-Leu-Thr-Asn-Tyr-Ser-Val-
Thr-Asp-Leu-Asn-Val-Gln-Arg-Lys-Ala-Ile-His-Glu-Leu-Ile-Gln-Val-Met-Ala-Glu-Leu-
Ser-Pro-Ala-Ala-Lys-Thr-Gly-Lys-Arg-Lys-Arg-Ser-Gln-Met-Leu-Phe-Arg-Gly-Arg-Y

	末端アミノ酸配列		糖鎖結合
	X	Y	
Gamma-1a	Cys-Tyr-Cys	Arg-Ala-Ser-Gln	-
Gamma-1b	Met	-	-
Gamma-1c	Met	Arg-Ala-Ser-Gln	-

図5 Interferon Gamma(インターフェロン ガンマ)のアミノ酸配列

ステム32 「-poetin」：エリスロポエチン類

「**-poetin**」は，エリスロポエチン(erythropoetin，EPO)型の血液因子に共通のステムである。EPOは，赤血球前駆細胞に作用して赤血球への分化と増殖を促す造血因子で，主として腎臓から分泌される。天然のヒトEPOは，165個のアミノ酸残基からなる分子量約30,000の糖タンパク質で，Asn24，38，および83にN-結合型糖鎖，またSer126にO-結合型糖鎖が結合している。糖鎖の非還元末端に結合しているシアル酸数が多いものほど血中半減期が長く，高い生物活性を示す。

ステム「**-poetin**」を持ち，現在，日本で承認されている医薬品には以下の2品目がある(**図6**)。

Epoetin Alfa(Genetical Recombination)(**エポエチン** アルファ(遺伝子組換え))

Epoetin Beta(Genetical Recombination)(**エポエチン** ベータ(遺伝子組換え))

これらの医薬品は主に透析施行中の腎性貧血治療薬として用いられているほか，未熟児貧血にも用いられる。今後，日局への収載が予定されている医薬品である。

Epoetin Alfa(**エポエチン** アルファ)は，ヒトEPOをコードするゲノムDNAを導入したCHO細胞で産生され

Ala-Pro-Pro-Arg-Leu-Ile-Cys-Asp-Ser-Arg-Val-Leu-Glu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-
Glu-Ala-Glu-*Asn-Ile-Thr-Thr-Gly-Cys-Ala-Glu-His-Cys-Ser-Leu-*Asn-Glu-Asn-Ile-Thr-
Val-Pro-Asp-Thr-Lys-Val-Asn-Phe-Tyr-Ala-Trp-Lys-Arg-Met-Glu-Val-Gly-Gln-Gln-Ala-
Val-Glu-Val-Trp-Gln-Gly-Leu-Ala-Leu-Leu-Ser-Glu-Ala-Val-Leu-Arg-Gly-Gln-Ala-Leu-
Leu-Val-*Asn-Ser-Ser-Gln-Pro-Trp-Glu-Pro-Leu-Gln-Leu-His-Val-Asp-Lys-Ala-Val-Ser-
Gly-Leu-Arg-Ser-Leu-Thr-Thr-Leu-Leu-Arg-Ala-Leu-Gly-Ala-Gln-Lys-Glu-Ala-Ile-Ser-
Pro-Pro-Asp-Ala-Ala-**Ser-Ala-Ala-Pro-Leu-Arg-Thr-Ile-Thr-Ala-Asp-Thr-Phe-Arg-Lys-
Leu-Phe-Arg-Val-Tyr-Ser-Asn-Phe-Leu-Arg-Gly-Lys-Leu-Lys-Leu-Tyr-Thr-Gly-Glu-Ala-
Cys-Arg-Thr-Gly-Asp

Epoetin(Genetical Recombination)
エポエチン(遺伝子組換え)

図6 Epoetin(エポエチン)のアミノ酸配列
*N-結合型糖鎖結合位置，**O-結合型糖鎖結合位置

る165個のアミノ酸残基からなる糖タンパク質で，グリコフォームαからなる。一方，Epoetin Beta(**エポエチン** ベータ)は，ヒトEPOをコードするcDNAを導入したCHO細胞で産生される165個のアミノ酸残基からなる糖タンパク質で，グリコフォームβからなる。すなわち，この2つの医薬品のアミノ酸配列は同一であるが，結合している糖鎖の分布は異なっている。なお，グリコフォームとは，タンパク質部分の一次構造が同一で，糖鎖部分のみが異なるサブユニットのことである。

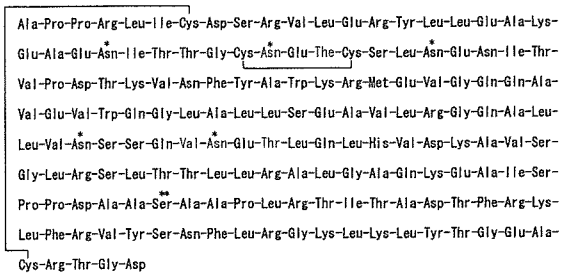
その他，ステム「**-poetin**」を持ちJANに登録されている医薬品として以下のものがある。

Epoetin Epsilon(Genetical Recombination)(**エポエチン** イプシロン(遺伝子組換え))

Darbepoetin Alfa(Genetical Recombination)(ダルベポエチン アルファ(遺伝子組換え))

Epoetin Epsilon(**エポエチン** イプシロン)は，ヒトEPOをコードする遺伝子を導入したBHK細胞で産生される165個のアミノ酸残基からなる糖タンパク質で，グリコフォームεからなる。Darbepoetin Alfa(ダルベポエチン アルファ)は，ヒトEPOの5カ所のアミノ酸残基を置換することによって新たに2本の*N*-結合型糖鎖を導入し，分子あたりのシアル酸数を最大14から22へと増加させた改変型糖タンパク質で(**図7**)，ヒトEPOよりも血中半減期が長い。欧米ではすでに貧血治療薬として承認されている。図7には天然型と異なるアミノ酸残基を赤文字で示した。

その他，INNにはステム「**-poetin**」を持つ以下のものが登録されている。いずれもヒトEPOと同じアミノ酸配列を有し，異なるグリコフォームからなる糖タンパク質である。



Darbepoetin(Genetical Recombination)
ダルベポエチン(遺伝子組換え)

図7　Darbpoetin(ダルベポエチン)のアミノ酸配列
N*-結合型糖鎖結合位置，*O*-結合型糖鎖結合位置

Epoetin Gamma

Epoetin Delta

Epoetin Zeta

Epoetin Theta

Epoetin Iota

Epoetin Omega

おわりに

今回は，生物薬品のステムとして，サイトカイン類に属するコロニー刺激因子類のステム「**-stim**」，インターロイキン類のステム「**-kin**」，インターフェロン類「**interferon**」，およびエリスロポエチン類のステム「**-poetin**」を紹介した。次回以降も順次，生物薬品のステムを紹介していきたい。

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宮田直樹：
本連載第1回(本誌2006年8月号)を参照。

参考文献

1) INTERNATIONAL NONPROPRIETARY NAMES(INN) FOR BIOLOGICAL AND BIOTECHNOLOGICAL SUBSTANCES(A REVIEW), INN Working Document 05.179, 15/06/2006 http://www.who.int/medicines/services/inn/BioRevforweb.pdf
2) pINNおよびrINNのリストhttp://www.who.int/druginformation/general/innlists.shtml
3) 日本医薬品一般名称データベースhttp://moldb.nihs.go.jp/jan/Default.htm
4) 医療用医薬品の添付文書情報(医薬品医療機器総合機構) http://www.info.pmda.go.jp/info/pi_index.html
5) 医薬品一般名称辞典 JAN1996,(財)日本公定書協会(1996)
6) FDA Drug information-Product approval information-http://www.fda.gov/cder/drug/default.htm
7) 生化学辞典，第2版，東京化学同人(1991)
8) 免疫学辞典，第1版，大沢利昭・小山次郎・奥田研爾・矢田純一【編】，東京化学同人(1993)
9) 分子細胞生物学辞典，第1版，村松正實編集代表，東京化学同人(1997)
10) 日経バイオ年鑑2006，バイオセンター編集，日経バイオテク・日経バイオビジネス(2006)
11) 薬学用語解説，日本薬学会http://www.pharm.or.jp/dictionary/
12) 宮田直樹，中野達也，川崎ナナ，内田恵理子，瀧明子，長谷川式子，山本美智子：平成15年度「日本薬局方の試験法に関する研究」研究報告－日本薬局方収載医薬品などの名称，構造式，化学名の国際調和に関する研究(第3報)－，医薬品研究，**35**(12)，627-637(2004)

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Deletion of Core Fucosylation on α3β1 Integrin Down-regulates Its Functions*

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The core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues. Recently α1,6-fucosylation has been further reported to be very crucial by the study of α1,6-fucosyltransferase (*Fut8*)-knock-out mice, which shows the phenotype of emphysema-like changes in the lung and severe growth retardation. In this study, we extensively investigated the effect of core fucosylation on α3β1 integrin and found for the first time that *Fut8* makes an important contribution to the functions of this integrin. The role of core fucosylation in α3β1 integrin-mediated events has been studied by using $Fut8^{+/+}$ and $Fut8^{-/-}$ embryonic fibroblasts, respectively. We found that the core fucosylation of α3β1 integrin, the major receptor for laminin 5, was abundant in $Fut8^{+/+}$ cells but was totally abolished in $Fut8^{-/-}$ cells, which was associated with the deficient migration mediated by α3β1 integrin in $Fut8^{-/-}$ cells. Moreover integrin-mediated cell signaling was reduced in $Fut8^{-/-}$ cells. The reintroduction of *Fut8* potentially restored laminin 5-induced migration and intracellular signaling. Collectively, these results suggested that core fucosylation is essential for the functions of α3β1 integrin.

α1,6-Fucosyltransferase (*Fut8*) catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of the hybrid and complex types of *N*-linked oligosaccharides on the glycoproteins (Fig. 1) (1). Core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues and altered under pathological conditions, such as hepatocellular carcinoma and liver cirrhosis (2, 3). A high expression of *Fut8* was observed in 33.3% of papillary carcinoma, and the incidence was directly linked to tumor size and lymph node metastasis, thus *Fut8* expression may be a key factor in the progression of thyroid papillary carcinomas (4). It has also been reported that the deletion of the core fucose from the IgG1 molecule enhances antibody-dependent cellular cytotoxicity activity by up to 50- to 100-fold. This indicates that the core fucose is an important sugar chain in terms of antibody-dependent cellular cytotoxicity activity (5). Recently, the physiological functions of the core fucose have been further investigated by our group using analysis of core fucose-deficient mice (6). The $Fut8^{-/-}$ mice showed severe growth retardation, and 70% died within 3 days after birth. The surviving mice suffered from emphysema-like changes in the lung that appear to be due to the lack of core fucosylation of transforming growth factor-β1 receptor, which consequently resulted in a marked dysregulation of transforming growth factor-β1 receptor activation and signaling. We also found that the loss of core fucosylation resulted in the down-regulation of EGF[3] receptor-mediated signaling pathway (7). These results together suggest that core fucose performs the important physiological functions through modification of some important functional proteins.

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of the cells. The integrin family consists of α and β heterodimeric transmembrane receptors for ECM and connects many biological functions, such as development, the control of cell proliferation, protection against apoptosis, and malignant transformation (8). For example, α3β1 integrin, the major receptor for laminin 5 (LN5), is widely distributed in almost all tissues, and α3 knock-out mice have been reported to show the defects in kidney, lung, and skin (9). It has been reported that G-like repeats of LN5 constitute the favored ligand for α3β1 integrin, triggering haptotaxis (10). Especially, the G3 domain is essential for the unique activity of LN5, such as promotion of cell migration (11). Furthermore, α3β1 integrin has been proposed to be involved in tumor invasion (12, 13): the interaction

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[3] The abbreviations used are: EGF, epidermal growth factor; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen; mAb, monoclonal antibody; PBS, phosphate-buffered saline; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; MEF, mouse embryonic fibroblast; ERK, extracellular signal-regulated kinase; AAL, *Aleuria aurantia* lectin; LC, liquid chromatography; MS, mass spectrometry; FT, Fourier transform; GM3, NeuAcα2,3Galβ1,4Glc-ceramide.

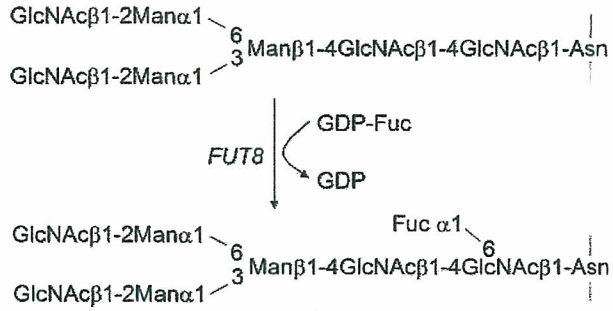

FIGURE 1. **Reaction pathway for the biosynthesis of core fucose by *Fut8*.** *Man*, mannose; *Fuc*, fucose; *GDP-Fuc*, guanosinediphospho-fucopyranoside; *Asn*, asparagine.

of α3β1 integrin with LN5 in exposed basement membrane provides both a molecular and a structural basis for cell arrest during pulmonary metastasis (14). In some malignant tumors, α3β1 integrin is found to be the most predominant integrin expressed (15), and cell invasion on ECM could be inhibited by antibodies against α3 integrin (13) and β1 integrin (14). Thus, α3β1 integrin, which mediates to laminins of basement membrane, preferentially promotes cell migration and metastasis (16–18). Given its various biological functions, α3β1 integrin, as one of most important extracellular adhesive molecules, deserves the more detailed investigation.

It has long been known that various factors can modulate integrin functions, including the status of glycosylation of integrin (19), the partnerships with tetraspanins, growth factor receptors (20–22), and the association with ganglioside GM3 (22), and others. Cell surface integrins are all major carriers of *N*-glycans, therefore *N*-glycosylation of integrins plays an important role in their biological functions (23). For example: the α3 and β1 subunits expressed by the metastasis human melanoma cell lines carry β1,6-branched structures, and these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand properties of α3β1 integrin (23). The linkage and expression levels of the terminal sialic acids of α3β1 integrin play an important role in cell-ECM interactions (24, 25). An increase in β1,6-GlcNAc sugar chains of β1 integrin resulted in the stimulation of cell migration and the organization of F-actin into extended microfilaments in cells plated on FN-coated plates (26). Moreover, a recent study has shown that introduction of bisecting GlcNAc into α5β1 integrin down-regulates cell adhesion and cell migration (27). These previous papers listed above have shown that the functions of integrins were positively or negatively regulated by *N*-glycans catalyzed by GnT-III, GnT-V, sialyltransferases, and others.

However, until now the effect of core fucosylation on integrin functions remains unclear. Here, we described studies comparing embryonic fibroblasts from wild-type and $Fut8^{-/-}$ mice to elucidate the role of core fucosylation in α3β1 integrin-stimulated events, and our finding for the first time showed that core fucosylation is required for the functions of α3β1 integrin.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—A polyclonal antibody against mouse α3 integrin and functional blocking monoclonal antibody (mAb) against α2β1 integrin were obtained from Chemicon International, Inc. (Temecula, CA). mAbs against α3 integrin, FAK, FAK (pY397), and functional blocking mAbs against integrin α6 and β1 subunits were from BD Transduction Laboratories (Lexington, KY). A polyclonal antibody against rabbit ERK1/2 and peroxidase-conjugated goat antibody against rabbit IgG were obtained from Cell Signaling (Beverly, MA). A mouse control IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A peroxidase-conjugated goat antibody against mouse IgG was obtained from Promega (Madison, WI), and biotinylated *Aleuria aurantia* lectin (AAL) was from Seikagaku Corp., Japan.

Cell Culture—$Fut8^{+/+}$ and $Fut8^{-/-}$ mouse embryonic fibroblasts (MEFs) and restored cells were previously established in our laboratory (6). $Fut8^{+/+}$ and $Fut8^{-/-}$ embryonic fibroblasts and restored cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of 400 μg/ml Zeocin, and restored cells were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μg/ml Zeocin and 400 μg/ml hygromycin.

Western Blot and Lectin Blot Analysis—Cell cultures were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 × *g* for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit (Pierce). Proteins were then immunoprecipitated from the lysates using a combination of 2 μg of anti-α3 integrin antibody and Protein G-Sepharose beads. Immunoprecipitates were suspended in nonreducing buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti-α3 integrin antibody and biotinylated AAL, respectively. Immunoreactive bands were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and an ECL kit (Amersham Biosciences).

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously (28). Briefly, cells were rinsed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfosuccinimidobiotin (Pierce) for 2 h at 4 °C. After incubation, 50 mM Tris-HCl (pH 8.0) was used for the initial wash to quench any unreacted biotinylation reagent, and the cells were washed three times with ice-cold PBS and then solubilized in lysis buffer. The resulting cell lysate was then immunoprecipitated with the anti-α3 integrin antibody as described above. The biotinylated proteins were visualized using a Vectastain ABC kit and an ECL kit.

Migration Assay and Functional Blocking Assay—Transwells (BD Bioscience) were coated with 5 nM of recombinant LN5, as described previously (29), or 15 nM of human plasma FN, 50 μg/ml collagen I (COL, Sigma) in PBS by an overnight treatment at 4 °C followed by an incubation with 1% bovine serum albumin for 1 h at 37 °C. Serum-starved cells (2×10^5) per well in 500 μl of fetal calf serum-free medium were seeded in the upper compartment of the plates. After incubation for 3 h, the cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and

stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well. To identify which specific integrin mediates cell migration on LN5, monoclonal antibodies against different types of integrins at concentrations of 10 μg/ml were preincubated individually with fibroblasts for 10 min at 37 °C. Then cells were transferred into Transwells coated with LN5 and then incubated for 2 h 37 °C. The migrated cells were then quantified as described above.

Construction of Small Interference RNA Vector and Retroviral Infection—Small interfering oligonucleotides specific for integrin α3 subunit were designed on the Takara Bio website, and the oligonucleotide sequences used in the construction of the small interference RNA vector were as follows: 5′-GATCCGCTATGGAGAATCACACTGATTCAAGAGATCAGTGTGATTCTCCATAGCTTTTTTG-3′ and 5′-AATTCAAAAAAGCTATGGAGAATCACACTGATCTCTTGAATCAGTGTGATTCTCCATAGCG-3′. The oligonucleotides were annealed and then ligated into BamHI/EcoRI sites of the RNAi-Ready pSIREN-Retro Q vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using a Retrovirus Packaging Kit Eco (Takara Bio) according to the manufacturer's protocol. Embryonic fibroblasts cells were infected with the viral supernatant, and the cells were then selected with 15 μg/ml puromycin for 2–3 weeks. Stable α3 integrin knockdown clones were therefore selected.

Tyrosine Phosphorylation Assay of FAK—Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK (pY397) antibody. Then the equal loading was confirmed by blotting with an antibody against total FAK.

Purification of α3β1 Integrin—The purification of α3β1 integrin was performed as described previously (30). Briefly, confluent cells were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl-β-D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of GD6 peptide of laminin α1 chain (30) (KQNCLSSRASFRGCVRNLRLSR residues numbered 3011–3032, Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound α3β1 integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl-β-D-glucopyranoside. The elutes containing α3β1 integrin were further purified on 1 ml of a wheat germ agglutinin-agarose column (Seikagaku Corp.) and eluted with 0.2 M *N*-acetyl-D-glucosamine containing 100 mM octyl-β-D-glucopyranoside. The purity of the integrin was verified by SDS-PAGE by means of a silver staining kit (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Analysis of N-Glycan Structure by Liquid Chromatography (LC/Tandem Mass Spectrometry (MS/MS))—Purified α3β1 integrin was applied to SDS-PAGE and excised from the gel then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced and carboxymethylated with dithiothreitol and monoiodoacetic acid according to the reports described by Kikuchi *et al.* (31) with some modifications. *N*-Glycans were released and extracted from the gel pieces as reported by Kustar *et al.* (32). The extracted oligosaccharides were reduced with $NaBH_4$. LC/MS was performed using a quadrupole liner ion trap-Fourier transform (FT) ion cyclotron resonance mass spectrometer (Finnigan LTQ FT™, Thermo Electron Corp., San Jose, CA) connected to a nanoLC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6/2% CH_3CN (pump A), and 5 mM ammonium acetate, pH 9.6/80% CH_3CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb (0.1 × 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% of B in 45 min and 20–50% of B in 45 min. FT-full MS scan (*m/z* 450–2000) followed by data-dependent MS/MS for the most abundant ions was performed in both negative and positive ion modes as described in the previous report (33).

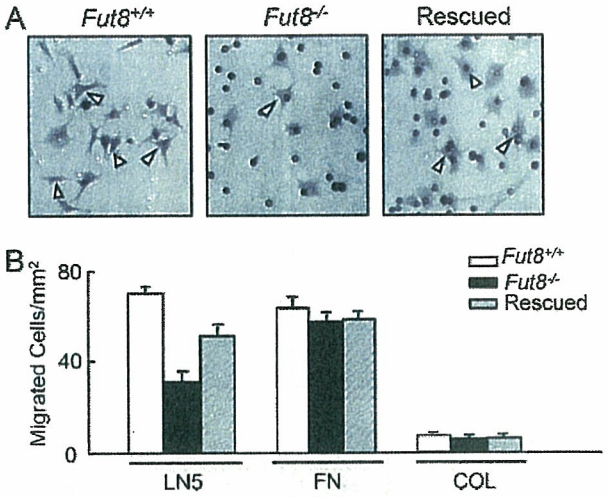

FIGURE 2. **Effects of deficient core fucosylation on cell migration on LN5 but not on FN.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were replated on the upper chamber coated with LN5 (5 nM), FN (15 nM), or 50 μg/ml COL. Cell migration was determined using the Transwell assay described under "Experimental Procedures." *A*, representative fields on LN5 were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, the numbers of migrated cells on LN5, FN, or COL were quantified and expressed as the means ± S.D. from three independent experiments.

RESULTS

Impaired α3β1 Integrin-mediated Cell Migration Was Found in Fut8$^{-/-}$ *Cells*—One of the major functions of α3β1 integrin is promotion of cell migration. In some malignant tumors, α3β1 integrin was found to be the most predominant integrin expressed (15), and it has made an important contribution to metastasis (14); therefore, cell motility on different ECMs was firstly examined by utilizing a Transwell assay. Cells were applied into the chambers, the bottoms of which had been coated with LN5, FN, or COL. As shown in Fig. 2 (*A* and *B*), *Fut8*$^{-/-}$ cells showed impaired migration on LN5 by a decrease to 44% relative to *Fut8*$^{+/+}$ cells. Consistently, reintroduction of *Fut8* partly restored cell migration by an increase in the percentage of migrating cells from 44% to 74%, indicating that core fucosylation is required for LN5-stimulated cell migration. But in the case of cell migration on FN, a specific ligand for α5β1

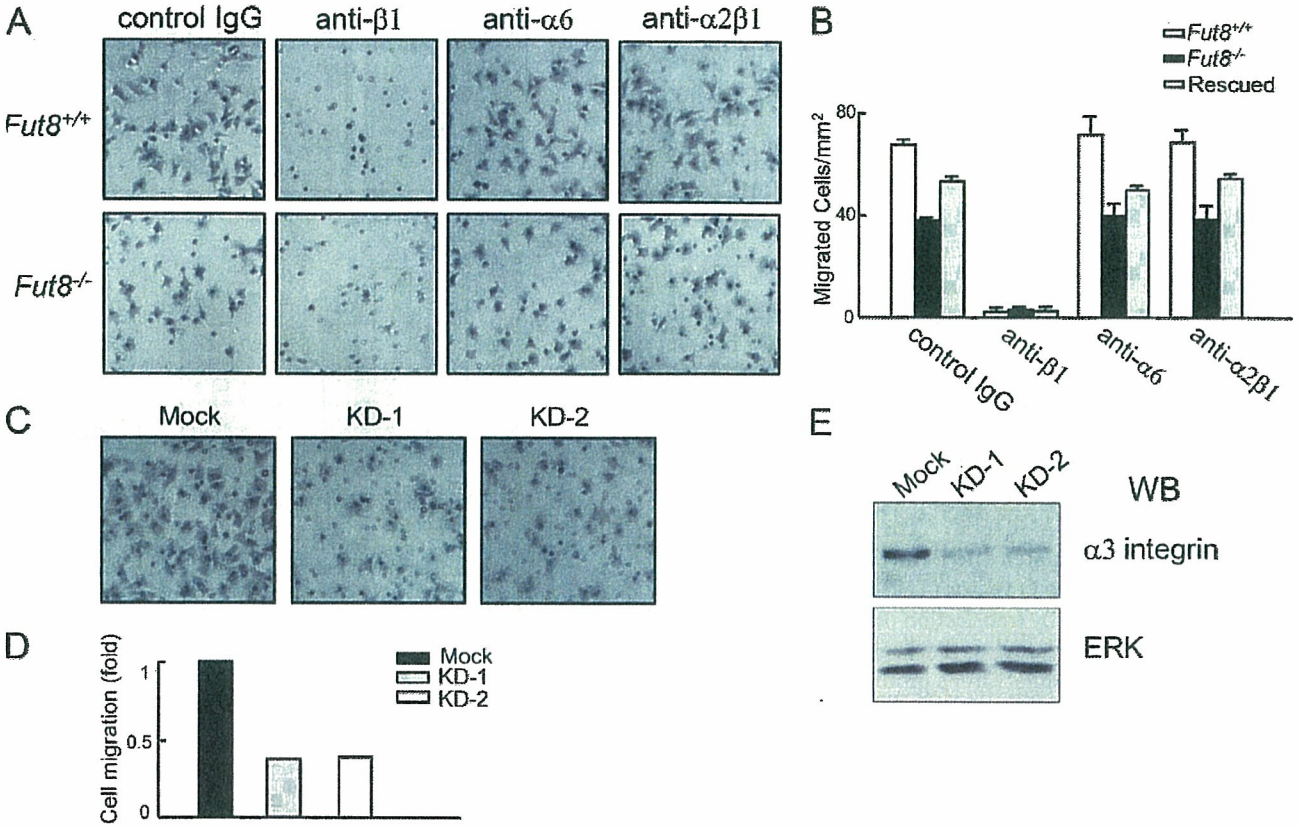

FIGURE 3. **Cell migration on LN5 was mediated by α3β1 integrin.** *A*, *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were detached, preincubated with mouse control IgG or function-blocking mAbs against β1, α6, or α2β1 integrin for 10 min, replated on the upper chamber coated with LN5 (5 nM), and checked by Transwell assay. Representative fields were photographed using a phase-contract microscope. *B*, the numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *C*, cell migration of α3-knockdown cells on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. *Arrowheads* indicate migrated cells. *D*, quantification of migration of mock and α3-knockdown cells. The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *E*, α3-knockdown was confirmed by blotting total cell lysates with anti-α3 antibody (*upper panel*), and equal loading was confirmed by probing with an antibody against total protein ERK1/2 (*lower panel*). *KD1* and *KD2*, α3-knockdown cells.

integrin, the obvious difference among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells was not found. In addition, the motility of these three types of cells on COL, a ligand for α1β1 and α2β1 integrins, was barely detectable (Fig. 2*B*). This suggested that α5β1, α1β1 and α2β1 integrins, unlike receptor of laminin 5, may be not strongly affected by *Fut8*. So MEF cells may favor LN5 as an ECM for cell migration. Furthermore, the cell migration on LN5 was completely blocked by the presence of function-blocking antibodies against β1 but not by α6 or α2β1 integrin antibodies (Fig. 3, *A* and *B*), further excluding the involvement of α6 and α2β1 integrin on LN5-stimulated cell migration. However, so far the function-blocking antibody against mouse α3 is unavailable. To definitely confirm the important function of integrin α3 subunit for the cell migration on LN5, we utilized an RNA interference strategy to silence α3 in MEF cells. After retroviral infection, the cells were selected based on their resistance to puromycin as described under "Experimental Procedures." Expression of α3 but not α5 (data not shown), or other proteins such as ERK, was effectively down-regulated, compared with those in mock cells (Fig. 3*E*). We then tested cell migration on LN5 and found that α3-knockdown resulted in a significant decreased cell migration compared with mock cells (Fig. 3, *C* and *D*). Together with the data in Fig. 3 (*A* and *B*), these results provided the evidence that in this study the cell migration on LN5 was mediated by α3β1 integrin. This result was consistent with the view of previous study that α3β1 integrin is distinct from other integrins and preferentially promotes cell migration (16). The result above was also supported by the previous observation that LN5 as well as LN10/11 promoted cell migration is mainly mediated by α3β1 integrin, but not α6β1 or α6β4 integrins (34). However, we cannot definitely exclude the involvement of syndecan-1 and -4, because it has been reported to have an interaction with LN5 (35, 36); therefore, they might regulate integrin functions in an indirect way. Collectively, these results suggested that α3β1 integrin is a key molecule for cell migration on LN5 in the embryonic fibroblasts and that core fucosylation regulates α3β1 integrin-mediated cell migration.

Integrin-stimulated Phosphorylation of FAK Was Reduced in Fut8$^{-/-}$ *Cells*—ECM-integrin signaling events are prominently involved in regulating cell migration (16). In particular, the protein-tyrosine kinase FAK plays a prominent role in integrin signaling (37–39). To address the effects of *Fut8* on α3β1 integrin-mediated signaling, we examined FAK phosphorylation in adherent cells on LN5. As shown in Fig. 4, the level of tyrosine phosphorylation was reduced in the *Fut8*$^{-/-}$ cells compared with *Fut8*$^{+/+}$ cells, moreover the down-regulation of phosphorylation in *Fut8*$^{-/-}$ cells was restored in the rescued cells, suggesting that deficient core fucosylation was able to neg-

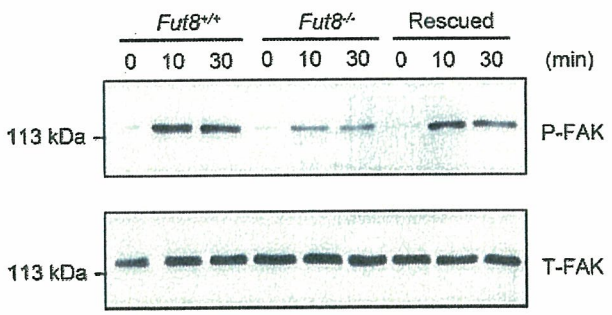

FIGURE 4. **Comparison of tyrosine phosphorylation levels of FAK among *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ and rescued cells on LN5.** Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK antibody to detect the amount of phosphorylation. Then the equal loading was confirmed with an antibody against total protein FAK.

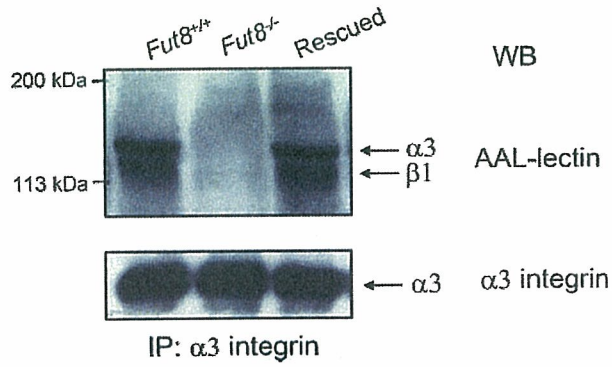

FIGURE 5. **Glycosylation analysis of α3β1 integrin from *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells.** Whole cell lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under nonreducing condition. After electroblotting, the blots were probed, respectively, by AAL (*upper panel*) and an anti-α3 integrin antibody (*lower panel*).

atively regulate α3β1 integrin-mediated signaling pathway. Considerable evidence implicates FAK in the regulation of cell migration. Most notably, FAK-deficient cells exhibit poor migration ability in response to chemotactic and haptotactic migration (40, 41). Therefore, based on such evidence we suggested that the deficient signal transduction may account for the deficient cell migration on LN5 in *Fut8*$^{-/-}$ cells.

Expression of α3β1 Integrin on the Cell Surface Was Not Influenced by Fut8—Some important glycosyltransferases have been reported to modify and further regulate the functions of integrins by modulating the status of glycosylation on them such as GnT-III and GnT-V; however, there is no such data so far to show the relation of *Fut8* and integrins. Therefore, in Fig. 5, the fucosylation on α3β1 integrin among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells has been examined by using blotting of α3 integrin-immunoprecipitated lysates with AAL lectin (*upper panel*). Equal loadings were verified by blotting with α3 integrin antibodies (*lower panel*). As shown in Fig. 5, the levels of core fucosylation in both α3 and β1 subunits were abolished in *Fut8*$^{-/-}$ cells consistent with no *Fut8* activity in these cells (7), whereas they were rescued by reintroduction of *Fut8*, suggesting α3β1 integrin is the target of *Fut8*. Furthermore, the effect of deficiency of core fucosylation on the expression of α3β1 integrin on the cell surface was also determined, because *N*-glycosylation plays an important role in the quality control of the expression of glycoproteins. The biotinylation of cell surface proteins followed by immunoprecipitation of α3 integrin was examined by blotted. As shown in Fig. 6, the expression levels of α3β1 integrin on the cell surface remained unchanged among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells, indicating that the expression of α3β1 integrin on cell surface was not influenced by *Fut8*. Collectively, we suggested that the deficiency of core fucosylation resulted in the malfunctions of α3β1 integrin but not its expression level.

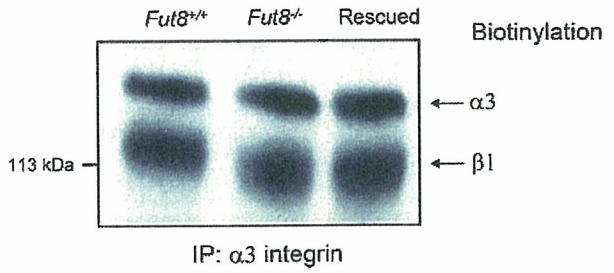

FIGURE 6. **Effects of core fucosylation on expression levels of α3β1 integrin on cell surface.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were biotinylated, whole lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were then detected as described under "Experimental Procedures."

Purified α3β1 Integrin, Rich in Core Fucosylation, Was Shown by LC/MS/MS—The analysis of glycan structural alteration in glycoproteins is becoming increasingly important in the discovery of therapies and diagnostic markers (42). To better understand the detailed modification of *Fut8* for α3β1 integrin, we purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells by using a GD6 peptide affinity column combined with a wheat germ agglutinin affinity column. The purity was evaluated by SDS-PAGE followed by silver staining. Two major bands, migrating at 150 and 110 kDa on SDS-PAGE under nonreducing conditions (Fig. 7*A*, *inset, right panel*), corresponding to the immunoreactivity with the anti-α3 and anti-β1 antibodies, were detected, respectively (data not shown). Then we analyzed *N*-glycan profiles of purified α3β1 integrin by LC/MS and LC/MS/MS. The profiles of the *N*-linked oligosaccharides extracted from purified α3β1 integrin of *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$, respectively, are shown in Fig. 7*A*. They were obtained by full MS scan (*m/z* 450–2000) in the negative ion mode. The FT MS spectra of the *peaks 1–7* (from *Fut8*$^{+/+}$) and *peaks 1′–7′* (from *Fut8*$^{-/-}$) are shown in Fig. 7*B*, respectively. The structures of carbohydrates in these peaks could be deduced from the *m/z* values of protonated ions obtained by FT MS and data-dependent MS/MS spectra. The oligosaccharides released from α3β1 integrin of *Fut8*$^{+/+}$ (*peaks 1–7*) were assigned to fucosylated complex and hybrid type oligosaccharides, whereas those released from α3β1 integrin of *Fut8*$^{-/-}$ (*peaks 1′–7′*) were nonfucosylated forms. The data correspond to that of AAL lectin blot, revealing that α3β1 integrin derived from *Fut8*$^{+/+}$ is highly modified by *Fut8* and suggesting loss of core fucosylation will result in the deficiency of α3β1 integrin function.

DISCUSSION

The physiological importance of fucose modification on proteins has been highlighted by the description of human congen-

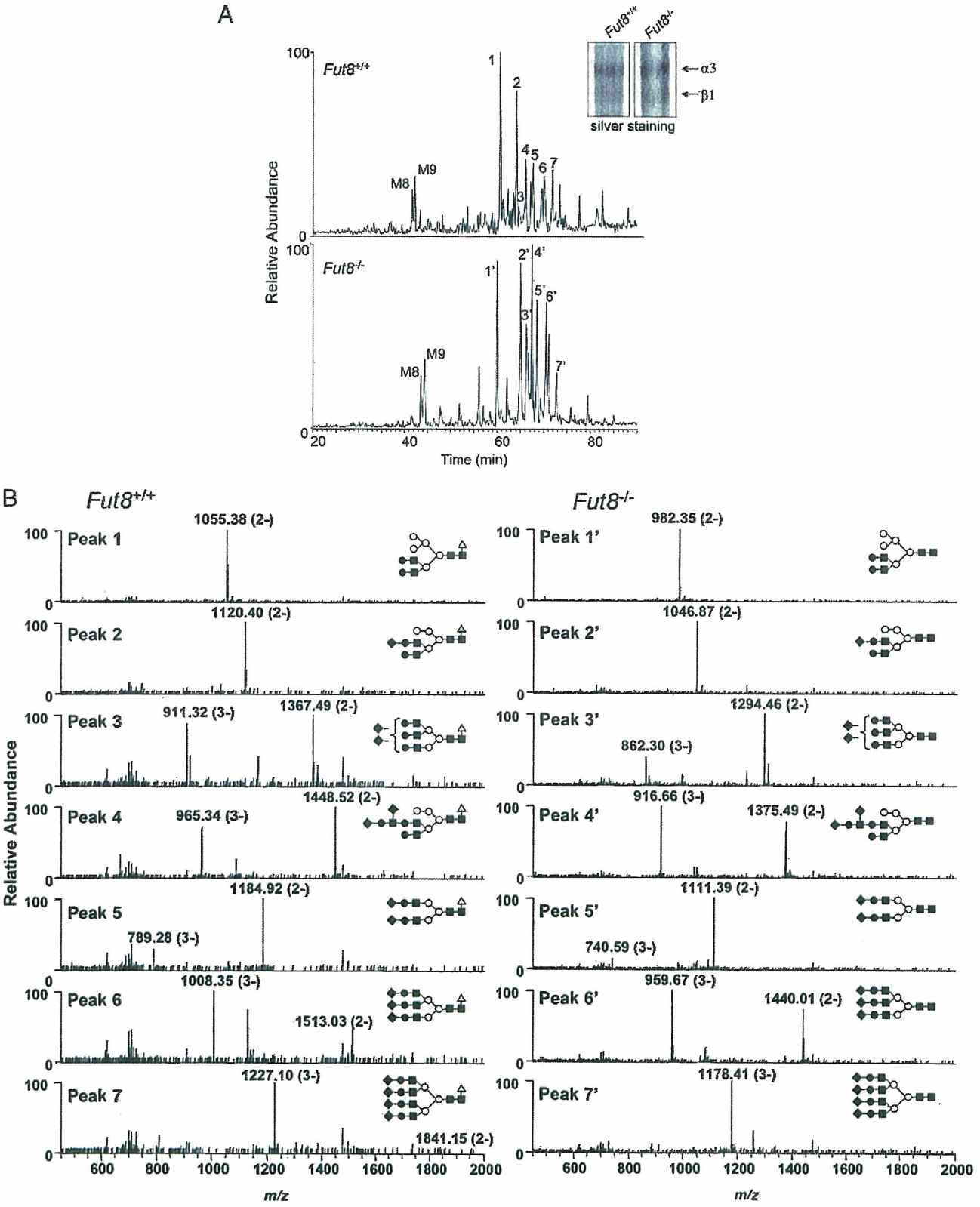

FIGURE 7. **Chromatograms of *N*-linked oligosaccharides extracted from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells.** In *A*: MS, full MS scan (*m/z* 450–2000) in the negative ion mode. LC, pump A, 5 mM ammonium acetate, pH 9.6/2% CH_3CN; Pump B, 5 mM ammonium acetate, pH 9.6/80% CH_3CN; column, hypercarb (0.1 × 150 mm); gradient, 5–20% of B (0–45 min) and 20–50% of B (45–90 min). The purity of α3β1 integrin was verified by silver staining under nonreducing condition as shown in the *right panel* of the *inset*. *B*, FT MS spectra of *N*-glycans from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells. *Peaks 1–7* in *Fut8*$^{+/+}$ cells, *peaks 1′–7′* in *Fut8*$^{-/-}$ cells, and carbohydrate compositions assigned by *m/z* values of protonated ions and MS/MS spectra. △, fucose; ●, galactose; ○, mannose; ■, *N*-acetylglucosamine; ♦, *N*-acetylneuraminic acid.

ital disorders of glycosylation (6). The congenital disorders of glycosylation-IIc disease is due to lack of the GDP-fucose transporter activity (43, 44), which mainly caused reduced terminal fucosylation of *N*-glycans (45, 46), and the core fucosylation is speculated to be responsible for the phenotype of congenital disorders of glycosylation-IIc (6). Recently, the loss of core fucosylation has been reported to down-regulate transforming growth factor-β1 receptor and EGF receptor functions, which is thought to be related to the phenotype of emphysema and growth retardation of *Fut8*$^{-/-}$ mice. In the present study, we found that the deficient core fucosylation results in the blockage of α3β1 integrin-mediated cell migration and cell signaling. These results showed for the first time that in addition to the important physiological functions mentioned above, core fucosylation is also essential for the functions of α3β1 integrin.

Several lines of evidence suggest that *N*-glycans are required for integrin activation. An increase in β1,6-branched sugar chains on α5β1 integrin by GnT-V promotes cell migration on FN (26). Although the overexpression of GnT-III has been reported to inhibit α5β1 integrin-mediated functions in HeLa S3 cells (27). It has also been reported that GnT-III and GnT-V can positively and negatively regulate α3β1 integrin-mediated cell migration on LN5 (47). The modification of β1 integrin by sialyltransferase makes this integrin capped with the negatively charged sugar, sialic acid, and contributes to cell motility and invasion (25). We found that cell migration on COL was barely detectable, suggesting that MEFs did not favor COL as an ECM for cell migration. In fact, we found that different cells may favor specific ECM for cell migration (27). We also found that core fucosylation had no significant difference in the cell migration on FN among wild-type, Fut8-KO, and rescued cells. This suggests that α1,6-fucose modification has little or only mild effects on α5β1 integrin, which is a receptor for FN. Actually, we previously reported that the introduction of the bisecting GlcNAc to the α5 subunit resulted in a reduced affinity in the binding of α5β1 integrin to FN, therefore resulting in decreased cell migration (27). Thus, we assumed that the core fucosylation affected α3 subunit in a similar manner, which caused the decreased cell migration on LN5. However, the modification of α1,6-fucose to α5 subunit may not affect their binding to FN. As described before, only *N*-glycans on some important domains of integrins, can contribute to the regulation of their functions (48). For example, the addition of a glycan at the β1 or β3 subunit I-like domains caused an increase in the distance between the head and stalk domains, therefore inducing the integrin dimer to adopt a more activated integrin conformation. Furthermore, it has recently been reported that the *N*-glycans only located on some specific sites of integrin α5 subunit play key roles in functional expression (49).

It has been reported that purified α5β1 integrin from human placenta and purified α3β1 integrin from the human ureter epithelium cell line HCV29 exhibited a highly heterogenous glycosylation pattern, and >50% of these were fucosylated (50, 51). In this study, the α3β1 integrin we purified from mouse embryonic fibroblast carried the bi-, tri-, and tetra-antennary complex types, and the majority of these were core-fucosylated. So it is easily postulated that core fucosylation may be important to integrin functions due to the abundance of it. However, to our knowledge, no reports showing that core fucosylation regulates integrin functions have appeared to date. The fact that integrin-mediated migration and cell signaling were decreased in *Fut8*$^{-/-}$ cells, and such inhibition was partly rescued by re-introduction of the *Fut8* gene to *Fut8*$^{-/-}$ cells, strongly suggested that core fucosylation is important to α3β1 integrin, and *Fut8*, like other important glycosyltransferases, plays an essential role in the regulation of integrin functions.

Although the precise reason for why the core fucosylation modifies these molecular functions remains to be elucidated, we proposed some possible mechanisms: *Fut8* may affect the cross-talk between growth factor receptors and integrin. It is well known that integrin mediated functions cooperatively with growth factor receptors in the control of cell proliferation, cell differentiation, cell survival, and cell migration in epithelial cells and fibroblasts (52), because integrins and growth factor receptors share many common elements in their signaling pathway (19). PC12 cells in a serum-free medium were plated on the plates without ECM coating and, when treated with EGF alone, failed to induce neurite formation (53), suggesting that the integration of the signaling pathway triggered by receptor and integrins is required for the regulation of PC12 cell differentiation. In our study, the association of integrin with EGF receptor was indicated by co-precipitation, and we found that the complex of α3β1 integrin and EGF receptor in *Fut8*$^{-/-}$ cells was decreased compared with *Fut8*$^{+/+}$ cells.[4] This may affect the signal integration of both partners and, thus, further affect the α3β1 integrin-stimulated signal and cell migration, or deficient core fucosylation may cause the conformation of integrin to change. Luo *et al.* (48) have suggested that the changes in the glycan structures of integrin can affect its conformation and activity. They reported that in Chinese hamster ovary-K1 cells, the addition of a glycan at β1 I-like domain caused an increase in the distance between the β1 head and stalk domains, therefore inducing the integrin dimmer to be a more extended (activated) integrin conformation (48). Consistently, the affinity of the binding of α5β1 integrin to fibronectin was significantly reduced by the introduction of the bisecting GlcNAc (27). So we supposed that core fucosylation contributes to stable conformation and normal activity of α3β1 integrin to its ligand. However, we cannot exclude additional reasons that still remain to be determined.

The α3 integrin gene is expressed during the development of many epithelial organs, including the kidney (54), lung (55), and others. As a major basement membrane receptor in both kidney and lung during embryogenesis, α3β1 integrin is likely to be involved in mediating signals between the mesenchyme and epithelial cells in the kidney and lung. The glomeruli of α3-KO mice showed the abnormality in kidney, including disorganized glomerular basement membrane and a dramatic absence of foot process formation by podocytes (9). Therefore, it could be worthy to extensively examine the effects of core fucosylation on α3β1 integrin *in vivo* in the future.

In conclusion, we demonstrate here some aspects of the biological significance of the core fucosylation of α3β1 integrin-medi-

[4] Y. Zhao, S. Itoh, X. Wang, T. Isaji, E. Miyoshi, Y. Kariya, K. Miyazaki, N. Kawasaki, N. Taniguchi, and J. Gu, unpublished data.

ated cell migration and signaling. This study provides new insights into the biological functions of core fucosylation and the significance of the modification of *N*-glycans for α3β1 integrins.

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REFERENCES

1. Wilson, J. R., Williams, D., and Schachter, H. (1976) *Biochem. Biophys. Res. Commun.* **72,** 909–916
2. Miyoshi, E., Noda, K., Yamaguchi, Y., Inoue, S., Ikeda, Y., Wang, W., Ko, J. H., Uozumi, N., Li, W., and Taniguchi, N. (1999) *Biochim. Biophys. Acta* **1473,** 9–20
3. Noda, K., Miyoshi, E., Gu, J., Gao, C. X., Nakahara, S., Kitada, T., Honke, K., Suzuki, K., Yoshihara, H., Yoshikawa, K., Kawano, K., Tonetti, M., Kasahara, A., Hori, M., Hayashi, N., and Taniguchi, N. (2003) *Cancer Res.* **63,** 6282–6289
4. Ito, Y., Miyauchi, A., Yoshida, H., Uruno, T., Nakano, K., Takamura, Y., Miya, A., Kobayashi, K., Yokozawa, T., Matsuzuka, F., Taniguchi, N., Matsuura, N., Kuma, K., and Miyoshi, E. (2003) *Cancer Lett.* **200,** 167–172
5. Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., and Shitara, K. (2003) *J. Biol. Chem.* **278,** 3466–3473
6. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M., Uozumi, N., Ihara, S., Lee, S. H., Ikeda, Y., Yamaguchi, Y., Aze, Y., Tomiyama, Y., Fujii, J., Suzuki, K., Kondo, A., Shapiro, S. D., Lopez-Otin, C., Kuwaki, T., Okabe, M., Honke, K., and Taniguchi, N. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15791–15796
7. Wang, X., Gu, J., Ihara, H., Miyoshi, E., Honke, K., and Taniguchi, N. (2006) *J. Biol. Chem.* **281,** 2572–2577
8. Hynes, R. O. (2002) *Cell* **110,** 673–687
9. Kreidberg, J. A., Donovan, M. J., Goldstein, S. L., Rennke, H., Shepherd, K., Jones, R. C., and Jaenisch, R. (1996) *Development* **122,** 3537–3547
10. Hintermann, E., and Quaranta, V. (2004) *Matrix Biol.* **23,** 75–85
11. Kariya, Y., Tsubota, Y., Hirosaki, T., Mizushima, H., Puzon-McLaughlin, W., Takada, Y., and Miyazaki, K. (2003) *J. Cell. Biochem.* **88,** 506–520
12. Melchiori, A., Mortarini, R., Carlone, S., Marchisio, P. C., Anichini, A., Noonan, D. M., and Albini, A. (1995) *Exp. Cell Res.* **219,** 233–242
13. Plopper, G. E., Domanico, S. Z., Cirulli, V., Kiosses, W. B., and Quaranta, V. (1998) *Breast Cancer Res. Treat* **51,** 57–69
14. Wang, H., Fu, W., Im, J. H., Zhou, Z., Santoro, S. A., Iyer, V., DiPersio, C. M., Yu, Q. C., Quaranta, V., Al-Mehdi, A., and Muschel, R. J. (2004) *J. Cell Biol.* **164,** 935–941
15. Tysnes, B. B., Larsen, L. F., Ness, G. O., Mahesparan, R., Edvardsen, K., Garcia-Cabrera, I., and Bjerkvig, R. (1996) *Int. J. Cancer* **67,** 777–784
16. Gu, J., Sumida, Y., Sanzen, N., and Sekiguchi, K. (2001) *J. Biol. Chem.* **276,** 27090–27097
17. Gu, J., Fujibayashi, A., Yamada, K. M., and Sekiguchi, K. (2002) *J. Biol. Chem.* **277,** 19922–19928
18. Kreidberg, J. A. (2000) *Curr. Opin. Cell Biol.* **12,** 548–553
19. Gu, J., and Taniguchi, N. (2004) *Glycoconj. J.* **21,** 9–15
20. Giancotti, F. G., and Ruoslahti, E. (1999) *Science* **285,** 1028–1032
21. Nishiuchi, R., Sanzen, N., Nada, S., Sumida, Y., Wada, Y., Okada, M., Takagi, J., Hasegawa, H., and Sekiguchi, K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1939–1944
22. Toledo, M. S., Suzuki, E., Handa, K., and Hakomori, S. (2005) *J. Biol. Chem.* **280,** 16227–16234
23. Pochec, E., Litynska, A., Amoresano, A., and Casbarra, A. (2003) *Biochim. Biophys. Acta* **1643,** 113–123
24. Yamamoto, H., Oviedo, A., Sweeley, C., Saito, T., and Moskal, J. R. (2001) *Cancer Res.* **61,** 6822–6829
25. Bellis, S. L. (2004) *Biochim. Biophys. Acta* **1663,** 52–60
26. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K., and Pierce, M. (2002) *Cancer Res.* **62,** 6837–6845
27. Isaji, T., Gu, J., Nishiuchi, R., Zhao, Y., Takahashi, M., Miyoshi, E., Honke, K., Sekiguchi, K., and Taniguchi, N. (2004) *J. Biol. Chem.* **279,** 19747–19754
28. Sato, Y., Takahashi, M., Shibukawa, Y., Jain, S. K., Hamaoka, R., Miyagawa, J., Yaginuma, Y., Honke, K., Ishikawa, M., and Taniguchi, N. (2001) *J. Biol. Chem.* **276,** 11956–11962
29. Kariya, Y., Ishida, K., Tsubota, Y., Nakashima, Y., Hirosaki, T., Ogawa, T., and Miyazaki, K. (2002) *J. Biochem. (Tokyo)* **132,** 607–612
30. Gehlsen, K. R., Sriramarao, P., Furcht, L. T., and Skubitz, A. P. (1992) *J. Cell Biol.* **117,** 449–459
31. Kikuchi, M., Hatano, N., Yokota, S., Shimozawa, N., Imanaka, T., and Taniguchi, H. (2004) *J. Biol. Chem.* **279,** 421–428
32. Kuster, B., Wheeler, S. F., Hunter, A. P., Dwek, R. A., and Harvey, D. J. (1997) *Anal. Biochem.* **250,** 82–101
33. Itoh, S., Kawasaki, N., Hashii, N., Harazono, A., Matsuishi, Y., Hayakawa, T., and Kawanishi, T. (2005) *J. Chromatogr. A* **1103,** 296–306
34. Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (1998) *J. Biol. Chem.* **273,** 15854–15859
35. Okamoto, O., Bachy, S., Odenthal, U., Bernaud, J., Rigal, D., Lortat-Jacob, H., Smyth, N., and Rousselle, P. (2003) *J. Biol. Chem.* **278,** 44168–44177
36. Utani, A., Nomizu, M., Matsuura, H., Kato, K., Kobayashi, T., Takeda, U., Aota, S., Nielsen, P. K., and Shinkai, H. (2001) *J. Biol. Chem.* **276,** 28779–28788
37. Parsons, J. T. (2003) *J. Cell Sci.* **116,** 1409–1416
38. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 549–599
39. Yamada, K. M., and Miyamoto, S. (1995) *Curr. Opin. Cell Biol.* **7,** 681–689
40. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377,** 539–544
41. Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D. (1999) *J. Cell Sci.* **112,** 2677–2691
42. Hashii, N., Kawasaki, N., Itoh, S., Hyuga, M., Kawanishi, T., and Hayakawa, T. (2005) *Proteomics* **5,** 4665–4672
43. Lubke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K., and Korner, C. (2001) *Nat. Genet.* **28,** 73–76
44. Luhn, K., Wild, M. K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001) *Nat. Genet.* **28,** 69–72
45. Sturla, L., Rampal, R., Haltiwanger, R. S., Fruscione, F., Etzioni, A., and Tonetti, M. (2003) *J. Biol. Chem.* **278,** 26727–26733
46. Sturla, L., Fruscione, F., Noda, K., Miyoshi, E., Taniguchi, N., Contini, P., and Tonetti, M. (2005) *Glycobiology* **15,** 924–934
47. Zhao, Y., Nakagawa, T., Itoh, S., Inamori, K. I., Isaji, T., Kariya, Y., Kondo, A., Miyoshi, E., Miyazaki, K., Kawasaki, N., Taniguchi, N., and Gu, J. (2006) *J. Biol. Chem.* **281,** 32122–32130
48. Luo, B. H., Springer, T. A., and Takagi, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2403–2408
49. Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, N., and Gu, J. (2006) *J. Biol. Chem.* **281,** 33258–33267
50. Litynska, A., Przybylo, M., Pochec, E., and Laidler, P. (2002) *Acta Biochim. Pol.* **49,** 643–650
51. Nakagawa, H., Zheng, M., Hakomori, S., Tsukamoto, Y., Kawamura, Y., and Takahashi, N. (1996) *Eur. J. Biochem.* **237,** 76–85
52. Schwartz, M. A., and Ginsberg, M. H. (2002) *Nat. Cell Biol.* **4,** E65–E68
53. Gu, J., Zhao, Y., Isaji, T., Shibukawa, Y., Ihara, H., Takahashi, M., Ikeda, Y., Miyoshi, E., Honke, K., and Taniguchi, N. (2004) *Glycobiology* **14,** 177–186
54. Korhonen, M., Ylanne, J., Laitinen, L., Cooper, H. M., Quaranta, V., and Virtanen, I. (1991) *Lab. Invest.* **65,** 347–356
55. Mette, S. A., Pilewski, J., Buck, C. A., and Albelda, S. M. (1993) *Am. J. Respir. Cell Mol. Biol.* **8,** 562–572

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N-Acetylglucosaminyltransferase III Antagonizes the Effect of *N*-Acetylglucosaminyltransferase V on α3β1 Integrin-mediated Cell Migration*

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N-Acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of β1,6-GlcNAc branching of *N*-glycans, which contributes to metastasis. *N*-Acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting GlcNAc structure in *N*-glycans, resulting in the suppression of metastasis. It has long been hypothesized that the suppression of GnT-V product formation by the action of GnT-III would also exist *in vivo*, which will consequently lead to the inhibition of biological functions of GnT-V. To test this, we draw a comparison among MKN45 cells, which were transfected with GnT-III, GnT-V, or both, respectively. We found that α3β1 integrin-mediated cell migration on laminin 5 was greatly enhanced in the case of GnT-V transfectant. This enhanced cell migration was significantly blocked after the introduction of GnT-III. Consistently, an increase in bisected GlcNAc but a decrease in β1,6-GlcNAc-branched *N*-glycans on integrin α3 subunit was observed in the double transfectants of GnT-III and GnT-V. Conversely, GnT-III knockdown resulted in increased migration on laminin 5, concomitant with an increase in β1,6-GlcNAc-branched *N*-glycans on the α3 subunit in CHP134 cells, a human neuroblastoma cell line. Therefore, in this study, the priority of GnT-III for the modification of the α3 subunit may be an explanation for why GnT-III inhibits GnT-V-induced cell migration. Taken together, our results demonstrate for the first time that GnT-III and GnT-V can competitively modify the same target glycoprotein and furthermore positively or negatively regulate its biological functions.

Malignant transformation is accompanied by increased β1,6-GlcNAc branching of *N*-glycans attached to Asn-*X*-Ser/Thr sequences in mature glycoproteins (1–3). *N*-Acetylglucosaminyltransferase V (GnT-V)[3] catalyzes the addition of β1,6-linked GlcNAc (see Fig. 8) and defines this subset of *N*-glycans (4, 5). A relation between GnT-V and cancer metastasis has been reported by Dennis *et al.* (6) and Yamashita *et al.* (1). Studies on transplantable tumors in mice indicate that the product of GnT-V directly contributes to the growth of cancer and subsequent metastasis (7, 8). On the other hand, somatic tumor cell mutants that are deficient in GnT-V activity produce fewer spontaneous metastases and grow more slowly than wild-type cells (6, 9). The suppression of tumor growth and metastasis has been reported in GnT-V-deficient mice (3). Moreover, Partridge *et al.* (10) reported that GnT-V-modified *N*-glycans with poly-*N*-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors oppose their constitutive endocytosis and result in promoting intracellular signaling and consequently cell migration and tumor metastasis. These results indicate that inhibition of GnT-V might be useful in the treatment of malignancies by targeting their roles in metastasis.

N-Acetylglucosaminyltransferase III (GnT-III) participates in the branching of *N*-glycans (see Fig. 8), catalyzing the formation of a unique sugar chain structure-bisecting GlcNAc (11). GnT-III is generally regarded to be a key glycosyltransferase in the *N*-glycan biosynthetic pathway, since *in vitro* the introduction of the bisecting GlcNAc results in the suppression of further processing and the elongation of *N*-glycans as the result of catalysis by other glycosyltransferases, which are unable to use the bisected oligosaccharide as a substrate (12, 13). It is interesting to note that the metastatic capabilities of B16 mouse melanoma cells are down-regulated by introduction of the GnT-III gene (14). E-cadherin, a homophilic type of adhesion molecule (15), is highly associated with the prevention of metastasis (16), and E-cadherin on GnT-III-transfected cell

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[3] The abbreviations used are: GnT-V, *N*-acetylglucosaminyltransferase V; GnT-III, *N*-acetylglucosaminyltransferase III; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen I; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; LC, liquid chromatography; MS, mass spectrometry; FT-ICR, Fourier transform ion cyclotron resonance.

surfaces was found to be resistant to proteolysis, resulting in an extended half-life of turnover (17). Thus, GnT-III, contrary to GnT-V, has long been thought to inhibit cancer metastasis.

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of cells. Cell surface transmembrane glycoprotein-integrin is a major receptor for ECM and connects many biological functions, such as development, control of cell proliferation, protection against apoptosis, and malignant transformation (18). Integrin $\alpha3\beta1$, the major laminin 5 (LN5) receptor, is widely distributed in almost all tissues, and it has been proposed to be involved in tumor invasion (19–21). In some malignant tumors, $\alpha3\beta1$ integrin was found to be the most predominant integrin expressed (22) and made an important contribution to pulmonary metastasis (23). On the other hand, the glycosylation of integrins contributes to the tumor metastasis. Guo *et al.* reported that an increase in $\beta1$,6-GlcNAc sugar chains of the integrin $\beta1$ subunit resulted in the stimulation of cell migration (24). Interestingly, it has also been reported that the $\alpha3\beta1$ integrin expressed by the metastasis human melanoma cell lines, contained a higher level of $\beta1$,6-branched structures than that expressed in a nonmetastasis parent cell line (25).

Although it had been assumed that the reaction of GnT-V can be inhibited by the action of GnT-III, as evidenced by substrate specificity studies *in vitro*, the hypothesis of competition between GnT-III and GnT-V in cell migration and tumor metastasis has not been directly verified so far. In the present study, we examined the functions of $\alpha3\beta1$ integrin, which is believed to be highly associated with tumor metastasis, and found that $\alpha3\beta1$ integrin can be modified by either GnT-III or GnT-V. Our finding clearly shows that GnT-III inhibits the effects of GnT-V on $\alpha3\beta1$ integrin-mediated cell migration by competing with GnT-V for the modification of $\alpha3$ subunit.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies against integrin $\alpha3$ subunit (P1B5, I-19), monoclonal antibody against β-actin, mouse control IgG, and peroxidase-conjugated rabbit antibody against goat IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Functional blocking antibody against the integrin $\beta1$ subunit was purchased from Chemicon International, Inc. (Temecula, CA). A peroxidase-conjugated goat antibody against mouse IgG was from Promega (Madison, WI). Biotinylated leukoagglutinating phytohemagglutinin (L_4-PHA), biotinylated erythroagglutinating phytohemagglutinin (E_4-PHA), and monoclonal antibodies against GnT-III and GnT-V were from Seikagaku Corp.

Cell Culture—Transfected MKN45 Cells were established as previously reported (26). Human gastric cancer cell line MKN45 cells were cultured in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) under a humidified atmosphere containing 5% CO_2. Human GnT-V cDNA (27) or GnT-III cDNA was inserted into a mammalian expression vector pCXNII (28). Vectors were then transfected into MKN45 cells by means of Lipofectamine (Invitrogen). Selection was performed by the addition of 500 μg/ml G418 (Sigma). CHP134 cells, a human neuroblastoma cell line expressing endogenous GnT-III and GnT-V, were cultured in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml) under a humidified atmosphere containing 5% CO_2.

Plasmids and Transient Virus Transfection—cDNAs encoding full-length human GnT-III or GnT-III inactive mutant (D317A) were ligated into adenoviral vector, constructed using an adenoviral expression vector kit (Takara Bio). The 3×10^5 MKN45 GnT-V transfectants were then infected with 150 μl of virus solution (2×10^9 plaque-forming units/ml). After a 24-h incubation, the cultured medium was replaced with a fresh medium. 48 h later after infection, cells were subjected to various experiments.

Construction of Small Interfering RNA Vector and Retroviral Infection—Small interfering oligonucleotides specific for GnT-III were designed on the Takara Bio site on the World Wide Web, and the oligonucleotide sequences used in the construction of the small interfering RNA vector were as follows: 5′-GATCCGTCAACCACGAGTTCGACCTTCAAGAGAGGTCGAACTCGTGGTTGACTTTTTTAT-3′ and 5′-CGATAAAAAAGTCAACCACGAGTTCGACCTCTCTTGAAGGTCGAACTCGTGGTTGACG-3′. The oligonucleotides were annealed and then ligated into BamHI/ClaI sites of the pSINsi-hU6 vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using the retrovirus packaging kit Ampho (Takara Bio) according to the manufacturer's protocol. CHP134 cells were infected with the viral supernatant, and the cells were then selected with 500 μg/ml G418 for 2–3 weeks. Stable GnT-III knockdown clones were selected and confirmed by GnT-III activity and gene expression. Quantitative real time PCR analyses of GnT-III mRNA expression in these clones were performed with a Smart Cycler II System and the SYBR premix Taq (Takara Bio). Reverse transcription was carried out at 42 °C for 10 min, followed by 95 °C for 2 min using random primers, followed by PCR for 45 cycles at 95 °C for 5 s and 60 °C for 20 s with the following primers: 5′-GCGTCATCAACGCCATCAA-3′ 5′-TGGACTCGCACACCACAAAG-3′. Normalization of the data were performed using the glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

GnT-III and GnT-V Activity Assay—The activities of GnT-III and GnT-V were assayed as described previously (29, 30). Briefly, cell lysates were homogenized in phosphate-buffered saline (PBS) containing protease inhibitors. The supernatant, after removal of the nucleus fraction by centrifugation for 15 min at 900 × *g*, was used in the assays, which involved high performance liquid chromatography methods using a pyridylaminated biantennary sugar chain as an acceptor substrate. Protein concentrations were determined using a bicinchoninic acid kit (BCA kit) (Pierce) with bovine serum albumin as a standard.

Western Blot and Lectin Blot Analysis—Cell cultures were harvested in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 × *g* for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit. Proteins were then immunoprecipitated from

the lysates using a combination of 2 μg of anti-integrin α3 subunit antibody and 15 μl of protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 1 h at 4 °C. Immunoprecipitates were suspended in reducing sample buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti-α3 antibody or biotinylated E_4- or L_4-PHA. Immunoreactive bands were visualized using the Vectastain ABC kit (Vector Laboratories, CA) and an ECL kit (Amersham Biosciences). For GnT-III, GnT-V, cell lysate, and actin blotting, an equal amount of cell lysates was subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with the corresponding primary antibodies and secondary antibodies for 1 h each, and detection was performed by an ECL kit.

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously with minor modifications (31). Briefly, various semiconfluent transfected MKN45 cells were washed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfosuccinimidobiotin (Pierce), for 3 h at 4 °C. After incubation, the cells were washed three times with ice-cold PBS, scraped, and lysed with radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The resulting cell lysates were immunoprecipitated with anti-α3 antibody, as described above. The immunocomplex was subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking the membranes with 3% (w/v) skim milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST, pH 7.5), the biotinylated proteins were visualized using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and an ECL kit.

Migration Assay—Transwells (BD Biosciences) were coated with 5 nM recombinant LN5 as described previously (32), 10 μg/ml human plasma FN, and collagen I (COL) (Sigma) in PBS by an incubation overnight at 4 °C. Serum-starved cells (2×10^5 cells/well in 500 μl of 5% fetal calf serum medium) were seeded in the upper chamber of the plates. After incubation overnight at 37 °C, cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well.

Functional Blocking Assay—To identify which integrin is involved in cell migration on LN5, functional blocking antibodies against different types of integrins were individually preincubated with cells for 10 min at 37 °C. The preincubated cells were transferred into transwells coated with LN5 and then incubated overnight at 37 °C. The migrated cells were then quantified as described above.

Statistical Analysis—Statistical evaluations were performed using Student's *t* test; differences among experimental groups were considered significant for $p < 0.05$. Data were expressed as mean values ± S.D.

Purification of α3β1 Integrin—The purification of α3β1 integrin was performed as described previously (33). Briefly, cells in confluent were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl-β-D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of the GD6 peptide of laminin α1 chain (33) (KQNCLSSRASFRGCVRNLRLSR residues numbered 3011–3032) (Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound α3β1 integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl-β-D-glucopyranoside. The elutes containing α3β1 integrin were further purified on a 1-ml wheat germ agglutinin-agarose column (Seikagaku Corp., Tokyo, Japan) and eluted with 0.2 M *N*-acetyl-D-glucosamine containing 100 mM octyl-β-D-glucopyranoside.

Analysis of N-Glycan Structures by Mass Spectrometry (LC/MS^n)—Purified α3β1 integrin was applied to SDS-PAGE, and the α3 subunit was excised from the gel and then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced and carboxymethylated by the incubation with dithiothreitol and sodium monoiodoacetate (34). *N*-Glycans were extracted from the gel pieces as reported by Kustar *et al.* (35) and reduced with $NaBH_4$. Half of the extracted oligosaccharides were incubated with α-neuraminidase from *Arthrobacter ureafaciens* in 50 mM phosphate buffer, pH 5.0, at 37 °C for 18 h and desalted with Envi-carb (Supelco, Bellefonte, PA). LC/MS and LC/multistage MS (MS^n) was carried out on a quadrupole liner ion trap-Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS; Finnigan LTQ FTTM, Thermo Electron Corp., San Jose, CA) connected to a nano-LC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6, 2% CH_3CN (pump A) and 5 mM ammonium acetate, pH 9.6, 80% CH_3CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb (0.1 × 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% B in 45 min and 20–50% B in 45 min. A full MS^1 scan (*m/z* 450–2000) by FT-ICR MS followed by data-dependent $MS^{2,3}$ for the most abundant ions was performed in both negative and positive ion modes as previously reported (36).

RESULTS

Overexpression of GnT-V Stimulated α3β1 Integrin-mediated Cell Motility—It has been reported that overexpression of GnT-V in epithelial cells results in a loss of contact inhibition, increased cell motility in athymic nude mice (7), and an enhanced metastasis (8). In this study, experiments were first designed to determine whether GnT-V overexpression could affect cell migration on different ECMs. The extent of haptotaxis toward LN5, FN, and COL, specific ligands for α3β1, α5β1, and α1β1 and α2β1 integrin, respectively, was observed in MKN45 cells transfected with mock, GnT-III, or GnT-V. In the case of the GnT-V transfectants on LN5, the number of transwell cells migrating to the lower surface of the membrane was considerably increased ($p = 0.001$), the overexpression of

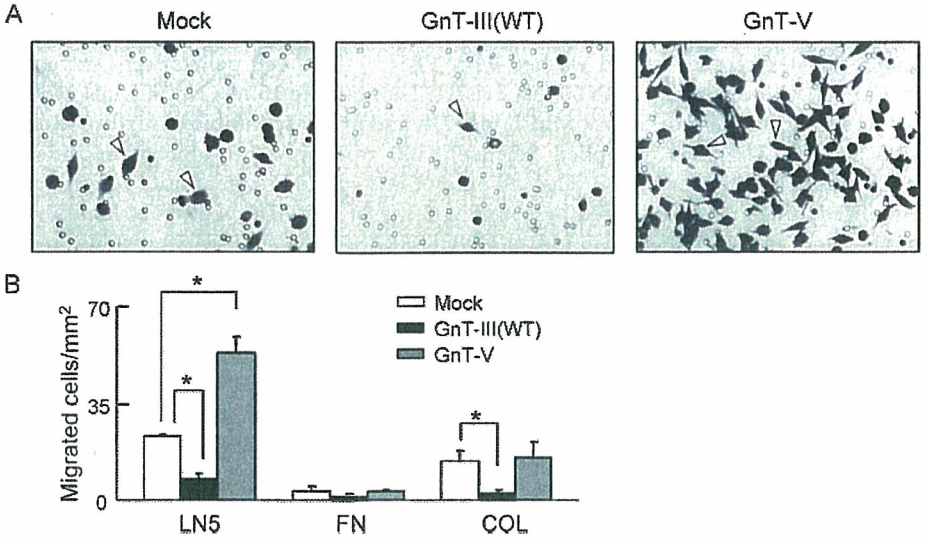

FIGURE 1. **Increased cell migration induced by GnT-V on LN5.** MKN45 cells were replated on the upper chamber in the presence of 5% fetal bovine serum. Cell migration was determined using the Transwell assay as described under "Experimental Procedures." After incubation overnight, the cells that had migrated to the lower surface of the membrane were fixed and stained with 0.3% Crystal Violet. *A*, cell migration on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, quantification of migration on LN5 (5 nM), COL (15 nM), and FN (15 nM). The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *WT*, wild type.

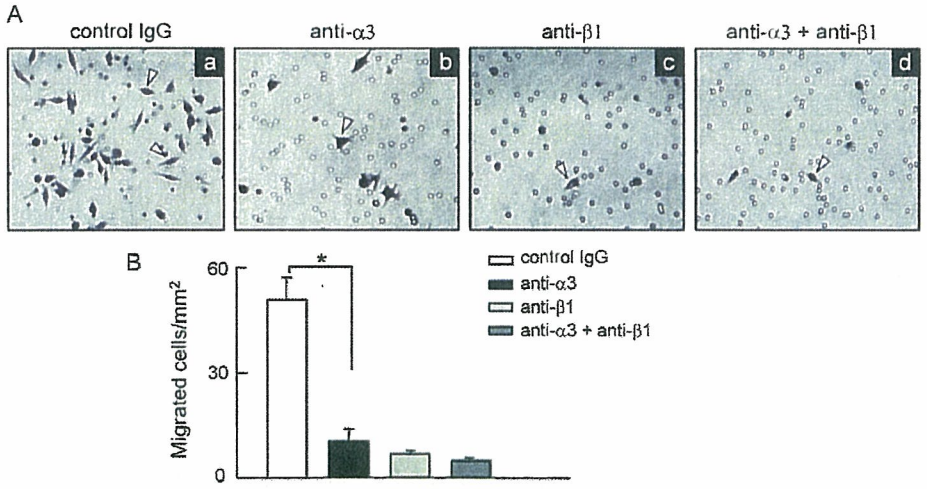

FIGURE 2. **GnT-V induced cell migration was mediated by $\alpha3\beta1$ integrin.** *A*, GnT-V-transfected MKN45 cells were detached, preincubated with mouse control IgG (*a*) or function-blocking monoclonal antibodies against $\alpha3$ (*b*) or $\beta1$ (*c*) or both (*d*) for 10 min, and then replated on the upper chamber coated with LN5 (5 nM) and checked by Transwell assay. Representative fields were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, quantification of migration on LN5 (5 nM). The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments.

GnT-III resulted in a decrease in cell migration on LN5 compared with mock ($p = 0.0013$) (Fig. 1*A*). However, the migration of these three types of cells on FN was barely detectable. Although GnT-III transfection resulted in a decreased cell migration on COL compared with mock ($p = 0.007$), GnT-V transfection failed to induce a significant increase in cell migration on COL (Fig. 1*B*), suggesting that MKN45 cells may favor LN5 as an ECM for cell migration induced by GnT-V. These results further supported the view that $\alpha3\beta1$ integrin, one of the most abundant integrins in epithelial cells, is distinct from other integrins, such as $\alpha5\beta1$ integrin, and preferentially promotes cell migration (37). Moreover, the cell migration of GnT-V transfectant on LN5 was strongly inhibited by the presence of function-blocking antibodies against integrin $\alpha3$ or/and $\beta1$ subunit, suggesting that the GnT-V-induced cell migration on LN5 was mainly mediated by $\alpha3\beta1$ integrin (Fig. 2). These results indicated that overexpression of GnT-V resulted in an increase in $\alpha3\beta1$ integrin-mediated cell motility.

Overexpression of GnT-III Inhibited $\alpha3\beta1$ Integrin-mediated Cell Migration Induced by GnT-V—The Overexpression of GnT-III has been reported to inhibit cell migration by enhancement of E-cadherin-mediated homotypic adhesion (17) and by inhibiting $\alpha5\beta1$ integrin-mediated cell migration (38). In addition, *in vitro* GnT-V cannot use the product of GnT-III, a bisected oligosaccharide, as a substrate (12), so experiments were then designed to determine whether the introduction of GnT-III prevents $\alpha3\beta1$ integrin-mediated cell migration enhanced by GnT-V. The efficiency of transfection was confirmed by immunostaining with anti-GnT-III antibody and determined to be more than 80% (data not shown). As shown in Fig. 3, the transfection of GnT-III into the GnT-V transfectant resulted in a significant decrease in cell migration compared with the GnT-V transfectant ($p = 0.002$). However, the inhibition was not observed after transfection of the GnT-III-inactive mutant, suggesting that the activity of GnT-III was essential for the negative regulation of GnT-V-induced cell migration. Therefore, we proposed that GnT-III directly counteracted the effect of GnT-V on $\alpha3\beta1$ integrin-mediated cell migration.

Transfection of GnT-III Had No Effect on the Expression of GnT-V and Integrin $\alpha3$ Subunit—To explore the possible mechanisms involved in the inhibition of GnT-III- to GnT-V-induced cell migration, we first attempted to determine whether the overexpression of GnT-III affected the expression of GnT-V and $\alpha3$ subunit expressed on the cell surface by means of blotting a total cell lysate with the GnT-III antibody and the biotinylation of cell surface proteins followed by immunoprecipitation of $\alpha3$ using the corresponding antibody, since *N*-glycosylation plays an important role in the quality control of

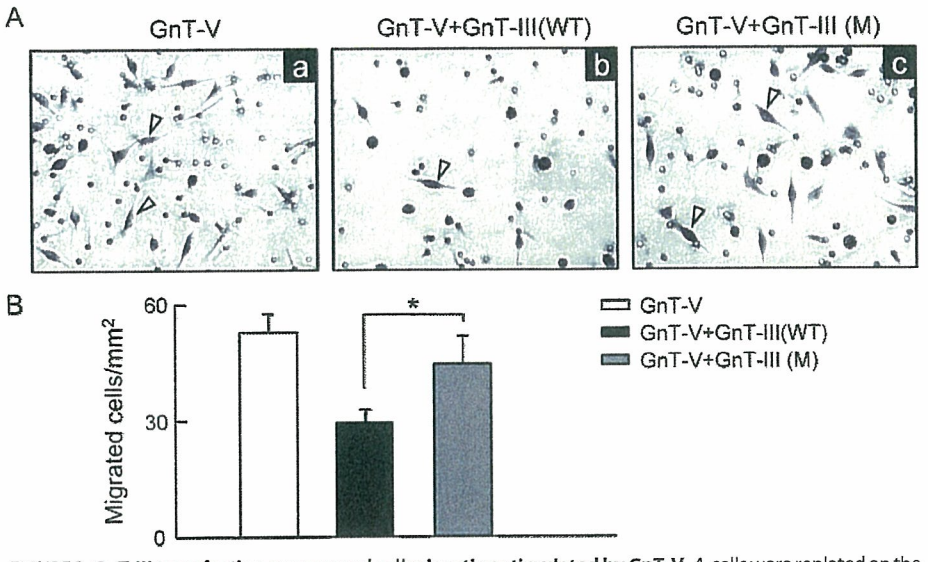

FIGURE 3. **GnT-III transfection suppressed cell migration stimulated by GnT-V.** *A*, cells were replated on the upper chamber coated with LN5 (5 nM). Cell migration was investigated by the GnT-V transfectant (*a*), GnT-III transfection to GnT-V transfectant (*b*), and GnT-III mutant transfection to GnT-V transfectant (*c*). Representative fields were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, the numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *WT*, wild type.

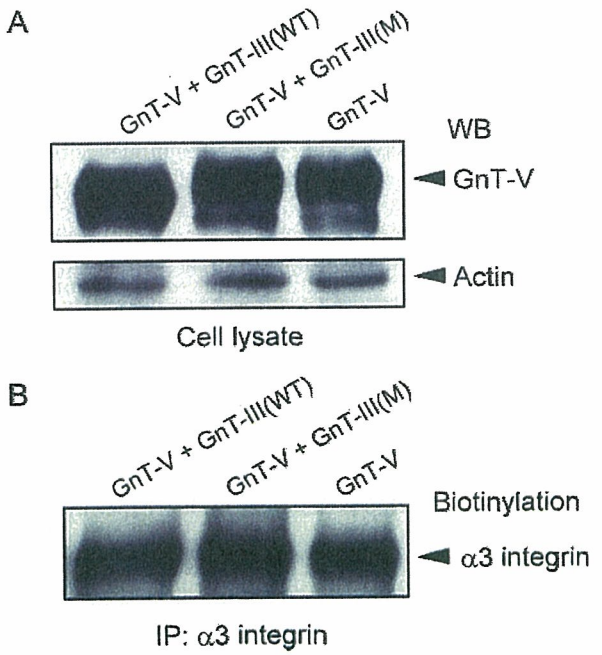

FIGURE 4. **No effects of GnT-III transfection on expression levels of both GnT-V and integrin α3 subunit expressed on cell surface.** *A*, double-transfected cells were lysed, and whole lysates were subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane and blotted with GnT-V antibody (*top*) or actin antibody (*bottom*). *B*, transfected cells were biotinylated, whole lysates were immunoprecipitated (*IP*) with anti-α3 antibody, and the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The biotinylated proteins were then detected as described under "Experimental Procedures." *WT*, wild type; *WB*, Western blot.

the expression of glycoproteins. As shown in Fig. 4*A*, the levels of expression of GnT-V were not influenced by the introduction of GnT-III, and equivalent amounts of loaded proteins were verified by blotting an actin antibody. On the other hand, the expression of integrin α3 subunit on the cell surface also remained unchanged among the transfectants of GnT-III plus GnT-V, GnT-III mutant plus GnT-V, and GnT-V (Fig. 4*B*). These results suggested that the inhibition of GnT-III- to GnT-V-induced cell migration could not be ascribed to a change in the expression levels of GnT-V and/or α3 subunit on the cell surface.

Transfection of GnT-III Had No Effect on the Activity of GnT-V—Since the introduction of GnT-III had no effect on the expressions of GnT-V and α3 subunit, we further determined if the overexpression of GnT-III suppressed the activity of GnT-V. Since this was a transient transfection, the activity of GnT-III was checked at six time points from 24 to 144 h after the transfection. We found that GnT-III activity reached the highest level 48 h after transfection (Fig. 5*A*), and there was no corresponding activity in GnT-III mutant (data not shown). The expression level of GnT-III mutant was similar to that of wild-type GnT-III confirmed by blotting with GnT-III antibody, and equivalent amounts of loaded proteins were verified by blotting with anti-actin antibody (Fig. 5*B*). As shown in Fig. 5*C*, GnT-V activity was found to be stable, even in the period (48 h after transfection) where the activity of GnT-III reached the highest level in these double-transfected cells. This result indicated that GnT-III inhibited GnT-V-induced cell migration not due to the suppression of GnT-V activity.

Increased GnT-III Product but Decreased GnT-V Product on Integrin α3 Subunit—The modification of *N*-glycosylation contributes to the functions of integrins (39). Here, we checked whether changes of α3β1 integrin modification had occurred in these transfectants. The integrins were immunoprecipitated from these transfectants and then probed with E_4-PHA lectin, which preferentially binds to bisecting GlcNAc residues in *N*-glycans, or L_4-PHA lectin, which binds to β1,6-branched GlcNAc. Fig. 6*A* (*top*) shows that the transfection of GnT-III to the GnT-V transfectant resulted in an increase in the GnT-III product on the integrin α3 subunit. More interestingly, the level of GnT-V product on α3 was decreased in the double transfectants (Fig. 6*A*, *middle*). Consistent with this observation, transfection of the GnT-III mutant failed to induce such changes. Equivalent amounts of the α3 subunit were verified by blotting α3-immunoprecipitated lysates (Fig. 6*A*, *bottom*). Moreover, cell lysates were subjected to SDS-PAGE, followed by a lectin blot. A comparison of bands especially around 117–200 and 60–89 kDa among these transfectants consistently indicated that increased GnT-III products but decreased GnT-V products presented on the glycoproteins after the introduction of GnT-III to the GnT-V transfectant (Fig. 6*B*). Furthermore, to further confirm such competition on the α3 sub-

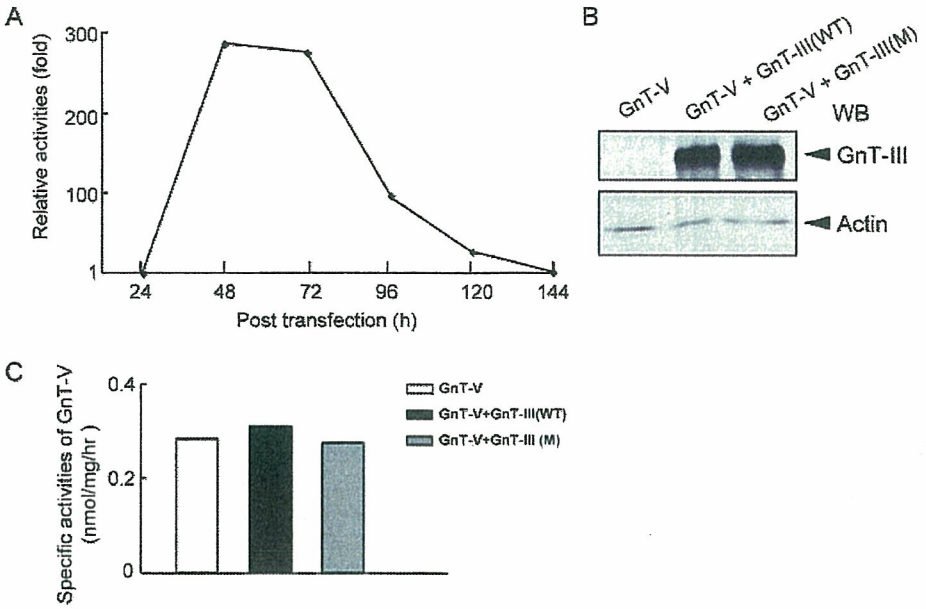

FIGURE 5. **No effects of GnT-III transfection on activity of GnT-V.** *A*, GnT-III activity of transient transfection of adenoviral expression vector to the GnT-V transfectant was examined by high performance liquid chromatography using a fluorescence-labeled agalactobiantennary sugar chain as a substrate. GnT-III activity was determined at various time points (6 days). Data are expressed as a ratio to the first time point (24 h after transfection). *B*, double-transfected cells were lysed, and whole lysates were subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane and blotted with GnT-III antibody (*top*) or actin antibody (*bottom*). *C*, GnT-V activity of the transient transfection of the adenoviral expression vector to GnT-V transfectant was investigated using the method mentioned in *A* at the time point of 48 h after transfection. Data are expressed as specific activity (nmol of product/mg of lysate/h). *WT*, wild type; *WB*, Western blot.

unit, we purified this integrin from GnT-III, GnT-V, and GnT-III plus GnT-V transfectants using a GD6 peptide affinity column combined with a wheat germ agglutinin affinity column. The purity was evaluated by SDS-PAGE followed by silver staining (data not shown). The purified α3 subunit was cut from gels and then subjected to LC/MS^n as described under "Experimental Procedures." As shown in Fig. 6, *C* and *D*, mass spectra of desialylated *N*-glycans were obtained from the α3 expressed in GnT-III, GnT-V, and GnT-III plus GnT-V transfectants, respectively, by a full MS1 scan (*m/z* 450–2000). Carbohydrate structures of the major peaks were deduced from the *m/z* values of protonated ions in the full MS^1 spectra obtained by FT-ICR MS and product ions in MS^{2,3} spectra (Fig. 6*D*). Based on the presence of $[HexNAc\text{-}Hex\text{-}HexNAc\text{-}HexNAc\text{-}OH + H]^+$ (*m/z* 792) and $[HexNAc\text{-}Hex\text{-}HexNAc\text{-}(dHex)HexNAc\text{-}OH + H]^+$ (*m/z* 938) in MS2,3 spectra, peaks 4, 5, 7, 8, 10, and 11 were determined as bisected glycans. Peak 4 was deduced to be a biantennary oligosaccharide, the major peak in the GnT-III transfectant. After the transfection of GnT-III into the GnT-V transfectant, peak 4 was increased compared with that of the GnT-V transfectant, whereas peak 6, which is the major peak in the GnT-V transfectant, was decreased. For the present technique, the branched form is determined by analyzing the sialylated oligosaccharides by LC/MS^n in the negative ion mode. Referring to the result of the L_4-PHA lectin blot and the fact that peak 6 is the major one in the GnT-V transfectant, peak 6 could be deduced the β1,6-branched GlcNAc form, although only bisialylated forms were detected by MS. The MS data also revealed that peaks 7 or 8 and 9, 10, or 11 were triantennary and tetraantennary oligosaccharides, respectively, from the presence of their corresponding trisialylated and tetrasialylated forms. Peaks 1 and 2 were high mannose oligosaccharides. To further quantify the competition, we used the MS results to show that for GnT-III products (represented by the sum of the peaks 4, 5, 7, 8, 10, and 11), the proportion was, respectively, 79.5, 29.5, and 48.5% among the transfectants of GnT-III, GnT-V, and GnT-III plus GnT-V; for GnT-V products (represented by the sum of peaks 6 and 9), the proportion was 1.2, 34.9, and 18.1%, respectively, among the transfectants of GnT-III, GnT-V, and GnT-III plus GnT-V. Consistent with the results shown in Fig. 6*A*, these data strongly suggested that GnT-III transfection resulted in increased bisecting GlcNAc but decreased β1,6-branched GlcNAc on the α3 subunit. However, the *N*-glycan proportions partially, but not totally, are correlated with the extent of the modification in cell migration observed (Fig. 3), since only *N*-glycans located on some motifs of integrins have been proposed to influence their conformations and therefore to regulate their functions (40).[4] Taken together, these results suggested the following; α3 was a common target of GnT-III and GnT-V, and the priority taken by GnT-III in the competition resulted in the inhibition of GnT-V modification.

Increased β1,6-Branched GlcNAc as Well as Cell Migration in GnT-III Knockdown Cells—To further identify the competition of GnT-III and GnT-V definitely, we developed an RNA interference strategy to efficiently silence GnT-III expression in CHP134 cells, which express endogenous GnT-III and GnT-V. After retroviral infection, CHP134 cells were selected based on their resistance to G418 as described under "Experimental Procedures." GnT-III activity was effectively down-regulated by 70%, compared with those in parent and mock cells (Fig. 7*A*), whereas GnT-V activity, as a control, showed no significant changes (data not shown). A quantitative real time PCR analysis also indicated the down-regulation of RNA interference-directed GnT-III mRNA expression in these cells (Fig. 7*B*). We then tested cell migration on LN5 and found that GnT-III knockdown resulted in an increased cell migration compared with mock cells (Fig. 7, *C* and *D*). We further investigated the *N*-glycans on the α3 subunit. As shown in Fig. 7*E*, increased β1,6-branched GlcNAc but decreased bisecting GlcNAc on α3 was found in the GnT-III knockdown cells, compared with those in the mock cells. Together with the data in Fig. 6, these data provided the evidence to show that GnT-III inhibited

[4] Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, N., and Gu, J. (2006) *J. Biol. Chem.*, in press.