Journal of Cellular Biochemistry 100:230–241 (2007)

Increase in β1-6 GlcNAc Branching Caused by *N*-Acetylglucosaminyltransferase V Directs Integrin β1 Stability in Human Hepatocellular Carcinoma Cell Line SMMC-7721

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Abstract In this study, an enzymatic inactive mutant of GnT-V (ΔcGnT-V) was constructed and transfected in SMMC 7721 cell line. Integrin β1 in ΔcGnT-V transfectants (Δc-7721) showed attenuation of the number of β1-6 GlcNAc branching, whereas those in wtGnT-V transfectants (wt-7721) presented a β1-6 GlcNAc-rich pattern. High integrin β1 expression was observed in wt-7721 compared with mock cells (7721 cell transfected with the vector pcDNA3), while transfection of ΔcGnT-V decreased the integrin β1 expression, despite of no significant changes on integrin β1 mRNA level in these cell lines. Pulse-chase experiment showed that Integrin β1 in Δc-7721 was prone to quick degradation and its half-life was less than 3 h, on the contrary, the alleviating degradation of β1 subunit was observed in wt-7721 where the β1 subunit half-life was about 16 h, meanwhile, the degradation rate of β1 subunit in mock cells was in between, about 10 h. More effective in promoting cell migration toward fibronectin and invasion through Matrigel was observed in wt-7721 while this was almost suppressed in Δc-7721. Our results suggest that the addition of β1-6 GlcNAc branching caused more fully glycosylated mature form on integrin β1 and inhibited β1 protein degradation. Glycosylation caused by GnT-V directs integrin β1 stability and more delivery to plasma membrane, subsequently promotes Fn-based cell migration and invasion. J. Cell. Biochem. 100: 230–241, 2007.

Key words: β1-6-*N*-acetylglucosaminyltransferase V; cell migration; protein glycosylation; protein stability; pulse-chase assay

Malignant behaviors in transformed cells and human tumors such as migration and invasion are highly associated with the significant increase of asparagine-linked glycans (*N*-glycans) containing β1-6 GlcNAc branching [Dennis et al., 1987; Yagel et al., 1989; Miyoshi et al., 1993]. The β1-6 branching of carbohydrates is the result of β1-6 *N*-acetylglucosaminyltransferase V (GnT-V, E.C. 2.1.4.155), a trans Golgi enzyme encoded by the mannoside acetylglucosaminyltransferase 5 (*Mgat5*) gene, which adds *N*-acetylglucosamine (GlcNAc) to the mannose of the trimannosyl core in a β1-6 linkage [Granovsky et al., 2000]. Results from recent studies showed the expression of β1,6-GlcNAc branched *N*-linked oligosaccharides in human mammary, colon, hepatic, and glial tumors [Seelentag et al., 1998; Ito et al., 2001] and confirm the notion that the β1-6 GlcNAc branched structure acquires the properties of cancer invasion and metastasis. Therefore, GnT-V was taken as one of the most important

Abbreviations used: GnT-V, β1-6 *N*-acetylglucosaminyltransferase V; PHA-L, phytohemagglutinin-leukoagglutinin; GlcNAc, *N*-acetylglucosamine; GnT-III, β1,4-*N*-acetylglucosaminyltransferase III; ECM, cell-extracellular matrix; Fn, fibronectin; GNA, Galanthus nivalis agglutinin; DSA, Datura stramonium agglutinin.

Grant sponsor: National Natural Science Foundation of China; Grant number: 30370342.

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Received 20 April 2006; Accepted 15 June 2006
DOI 10.1002/jcb.21071

glycosyltransferases in tumor growth and metastasis [Dennis et al., 1987; Demetriou et al., 1995; Granovsky et al., 2000]. Consequently, mice that lack GnT-V expression due to a targeted deletion (GnT-V or Mgat5 −/−) have been used to study the effects of eliminating GnT-V activity on tumor progression [Granovsky et al., 2000].

Integrins are composed of α and β subunits, which are all major carriers of *N*-glycans [Kim et al., 1992; Moss et al., 1994; Leppa et al., 1995; Veiga et al., 1995; Ringeard et al., 1996; Yan et al., 1997; Semel et al., 2002; Seales et al., 2003]. Changes in *N*-glycans of these integrins have the key effects on cell–cell and cell-extracellular matrix (ECM) interactions [Dennis et al., 1999]. Over expression of GnT-V in HT1080 cells, the β1 but not the α5 integrin chains of these cells altered glycosylation patterns, with an increase in β1,6 branching. β1 subunit has been identified as the target of GnT-V [Guo et al., 2002]. A number of studies reported that cells expressed some β1 integrin isoforms with altered electrophoretic motility during events dictate a marked change of metastatic and invasive phenotypes in melanoma cells, hepatocarcinoma cells, colorectal carcinoma cells [Veiga et al., 1995; Ringeard et al., 1996; Guo et al., 2001]. PGNase-F-mediated deglycosylation of integrins β1 resulted in species with molecular masses of approximately 90 kDa [Albiges-Rizo et al., 1995; Meng et al., 2005]. These results indicated that both the 105 and 125 kDa forms are differentially *N*-glycosylated. An altered β1 integrin carbohydrate profile most commonly results from a change in β1-6 branching of oligosaccharides, or in the degree of sialylation [Bellis, 2004]. Altered sialylation of β1 integrins regulates immune cell behavior, an increase in β1,6 GlcNAc branching on integrin β1 caused by GnT-V enhanced invasion and metastasis [Kim et al., 1992; Bellis, 2004; Gu and Taniguchi, 2004]. However, a detailed explanation of how GnT-V promotes integrin β1 glycosylation with integrin β1 stability is not currently available. Reportedly, the minimal catalytic domain of GnT-V was existed in its carboxyl-terminus, and deletion of as few as 4–8 amino acids from its carboxyl-terminus destroys its catalytic activity [Korczak et al., 2000]. To investigate in detail the relationship between the catalytic activity of GnT-V and integrin β1, we constructed a GnT-V inactive mutant ΔcGnT-V with deletion of six amino acids at C-terminal extreme end of the original GnT-V (pcDNA3-ΔcGnT-V). The cellular transfectant was designated as Δc-7721. We investigated the integrin β1 expression pattern in the mock, wt-7721 and Δc-7721 cells. Our results suggest that integrin β1 was increased in wt-7721 cells compared with mock cells, while decreased in Δc-7721 cells and up-regulation of β1 integrin induced by GnT-V was due to resistance of β1 subunit to degradation.

MATERIALS AND METHODS

Cell Culture and Reagents

A human hepatocellular carcinoma cell line (SMMC-7721) was obtained from the Liver Cancer Institute in Zhongshan Hospital, Fudan University (Shanghai, China). Cells were cultured in RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 10% of calf bovine serum. pcDNA3-wtGnT-V plasmid (i.e., pcDNA3Flu-HuTV in the reference [Guo et al., 2001]) containing full-length cDNA of human GnT-V gene was kindly provided by Prof. Huili Chen (Key Laboratory of Glycoconjugate Research, Fudan University). Antibodies against human GnT-V (S-15) and α-tubulin (B-7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal antibody against integrin β1 subunit was from BD Transduction Laboratories (San Jose, CA). Horseradish peroxidase-labeled anti-mouse or rabbit IgG secondary antibodies were purchased from Calbiochem. Pfu DNA polymerase was purchased from TaKaRa (Dalian, China). Fibronectin (Fn), neuraminidase (*clostridium perfringens*), Galanthus nivalis agglutinin (GNA), phytohemagglutinin-leukoagglutinin (PHA-L), Datura stramonium agglutinin (DSA) were purchased from Sigma (Saint Louis, MI); GNA, PHA-L and DSA were labeled with horseradish peroxisidase as described previously [Osaw and Tsuji, 1987]. β peptide (DLYYLMDLSYSMK), a blocking peptide, derived from a conserved sequence of the β subunit of integrins [Liu et al., 1997], was used to inhibit the functional β1 integrins; meanwhile, nonsense peptide (MKGGDLYYLMDLS) was used as a control [Liu et al., 1997].

Plasmid Construction of pcDNA3-ΔcGnT-V

A specific set of primers was synthesized in order to generate C-terminal deletion mutant of

GnT-V DNA. The primer pair was 5′-GGG GTA CCG GAC AGG TGA AGT TGC CAG AGA GCA-3′ (F) and 5′-GCT CTA GAC TAA GCC ACC TGG CCC TTG AT-3′ (R). The forward primer was designed according to the upstream sequence just before the ATG starting coden of GnT-V with a Kpn I restriction enzyme site included at the 5′. The reverse primer was designed according to the sequence of the C-terminal of GnT-V with the deletion of last six amino acid residues, in which the stop conden TAG and a Xba I site were included. The plasmid pcDNA3FluHuTV which encoding the full-length of human GnT-V gene was used as a template for PCR amplification. The PCR amplified fragment (2,205 bp) was thus contained the whole GnT-V gene with the deletion of the C-terminal six amino acid residues. The PCR product was cut with Kpn I/Xba I and cloned into the eukaryotic expression plasmid pcDNA3. The constructed plasmid was designated as pcDNA3-ΔcGnT-V which was confirmed by DNA sequencing.

Stable Transfections

pcDNA3, pcDNA-wtGnT-V and pcDNA3-ΔcGnT-V plasmids were stably transfected to SMMC-7721 using Lipofectamine 2000 reagent (LF2000, Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Selection was performed via the addition of 1 mg/ml G418. Positive (pcDNA-wtGnT-V or pcDNA3-ΔcGnT-V) and negative (empty vector) clones were selected, and transfectants with higher expression levels of wt- or Δc- GnT-V were used for the experiments described herein. The transfectants from pcDNA3, pcDNA-wtGnT-V, and pcDNA3-ΔcGnT-V were designated as mock, wt-7721 and Δc-7721 cells, respectively.

Semi-Quantitative and Real-Time Reverse Transcriptase-Polymerase Chain Reaction

For RT-PCR, total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's manual. RNA was treated with Dnase I (Invitrogen) for 15 min at room temperature followed by the addition of EDTA and heated at 65°C for 10 min to remove degraded genomic DNA. Semi-quantitative RT-PCR was basically performed as described elsewhere [Cai et al., 2000]. The first appropriate primer pair specific for total *Mgat5* (including both wild-type and the C-terminal deletion mutant ΔcGnT-V) was 5′- AAT GTG TAT TGC CTC CTA TG-3′ (1F) and 5′- AAC TGC TGA GGG TTC AGA TT -3′ (1R), which amplified the fragment from 464 to 1,265 of *Mgat5* (802 bp). The second primer pair for wild-type *Mgat5* only was 5′-TGC CCA ACT GTA GGA GAC-3′ (2F) and 5′-TAT AGG CAG TCT TTG CAG AG-3′ (2R), which amplified the fragment from 1,045 to 2,251 of *Mgat5* (product size: 1,207 bp, Genebank accession number AF113921). β-actin was used as an internal control for RT-PCR, the primers were designed according to the reference [Takano et al., 2000]. Integrin β1 mRNA levels were detected using real-time PCR as described previously [Hernandez-Pigeon et al., 2004], and the primers used were 5′-TTG CCC TCC AGA TGA CAT AGA-3′ (F) and 5′-AAG ACA GGT CCA TAA GGT AGT AG-3′ (R). The primers for GAPDH were 5′-AAG GTC GGA GTC AAC GGA TT-3′ (F) and 5′-CTG GAA GAT GGT GAT GGG ATT-3′(R). The product sizes of integrin β1 and GAPDH amplified were 236 and 222 bp, respectively. Real-time PCR was performed using an iCycler thermal cycler (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Reactions were performed with 0.2 μM primers. Nucleotides, Taq DNA polymerase, and buffer were included in SYBR Green JumpStart Taq ReadyMix for quantitative PCR. cDNA amplification consisted of one cycle at 95°C for 1.5 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The threshold cycle (C_T) values were determined by iCycler software (Bio-Rad) and the quantification data were analyzed following the $\Delta\Delta C_T$ method using GAPDH as reference.

Immunocytochemical Staining With HRP-Lectin Complexes

Immunocyto-chemical staining with lectins was described elsewhere [Guo et al., 1999]. Briefly, cells cultured on the plates (Nunc, Wiesbaden, Germany) were sequentially treated with PBS, 0.3% H_2O_2 and 1% BSA, each for 30 min, and then incubated with 4 μg/ml with HRP-GNA [Shibuya et al., 1988], HRP-PHA-L [Cummings and Kornfeld, 1982] and HRP-DSA (cells were initially treated with neuraminidase 0.5 U/ml, pH5.0, 37°C for 3 h to remove the sialic acids at the terminal of *N*-glycans) for 2 h at

37°C. Finally, cells were stained with diaminobenzene (DAB)-H_2O_2 methods.

Immunoprecipitation, Lectin Blotting Assay

Immunoprecipitation analysis was carried out as described elsewhere [Guo et al., 2002]. For lectin blotting assays [Ihara et al., 2002], the cultured mock and transfectant cells were washed three times with ice-cold PBS and solubilized with 1 ml of lysis buffer (50 mM HEPES/KOH, pH 7.9, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1.5 mM EGTA, 1 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Detergent-insoluble materials were removed by centrifugation at 12,000 rpm for 15 min at 4°C. The whole cell lysates (0.5 mg) were immunoprecipitated with anti-integrin β1 monoclonal antibody at 4°C for 3 h. Pre-equilibrated protein A/G PLUS-agarose beads (20 μl) were then added and incubated overnight. They were collected by centrifugation and then gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in two times SDS sample buffer and resolved on a 10% SDS–PAGE gel. The proteins were transferred onto a PVDF membrane and probed with the appropriate horseradish peroxidase labeled-lectin complex, then detected using the ECL kit.

Flow Cytometry Analysis for Cell Membrane Surface β1 Integrin

Cultured cells were grown for 24 h, detached with 2 mM EDTA, and then collected by centrifugation at 300*g* for 5 min. The cells were blocked with 1 mg/ml BSA in PBS, and successively incubated with primary antibody β1-mAb against human β1 expressed onto the cell plasma membrane and a secondary antibody conjugated with fluorescein for 2 h at 4°C, respectively. After washes with PBS, the resulting suspensions were analyzed on the FACS (Becton Dickinson). A suspension of 1×10^4 cells was analyzed for each sample, and each experiment was repeated at least twice.

Immunofluorescent Staining

Cultured cells were plated at densities of 2×10^4 cells/cm^2 onto glass coverslips and grown for overnight. Cells were washed twice in 1 × PBS, and fixed in 4% (v/v) paraformaldehyde for 10 min at room temperature. The subsequent immunofluorescent staining was carried out as described previously [Nonclercq et al., 2004]. Briefly, the fixed cells were incubated with primary antibody against human integrin β1 subunit (diluted 1:500), washed three times with 1× PBS followed by treatment with FITC-conjugated secondary antibody (diluted 1:200). Negative control was produced as mentioned above except for omitting the primary antibody. The immunofluorescently stained cells were examined on the OLYMPUS^{TM} microscope.

Pulse-Chase Experiments

Mock, wt-7721 and Δc-7721 cells in 60-mm tissue culture plates were preincubated for 1 h at 37°C with methionine- and cysteine-free medium containing 10% calf bovine serum. Pulse-chase studies were carried out as described previously [Ihara et al., 2002]. Briefly [35S] methionine/cystenine (Amersham, Piscataway, NJ) was added at a concentration of 100 μCi/ml to the culture medium, followed by incubation for 30 min for protein labeling. Cells were then incubated for 0, 2, 4, 8, 16, and 24 h in RPMI 1640 supplemented with 2 mM methionine and 2 mM cysteine. At the various incubation times, cell lysates were collected and protein concentration was assayed by the Lowry method. The subsequent immunoprepitation was performed described above. After SDS–PAGE, gels were dried, and the radioactivity was measured with a Fujifilm FLA-5100 Bio-Imaging Analyzer system (Fuji Photo Film).

Transwell Cell Migration and Invasion Assays

Migration assays using fibronectin-coated insert membrane and invasion assays using Matrigel-coated chambers were carried out described previously with minor modifications [Guo et al., 2002]. Migration assays were performed using 24-well Transwell units with 8 μm pore size polycarbonate inserts (BD Biosciences). Transwells were coated overnight with 10 μg/ml of Fn in PBS at 4°C, followed by incubation with 1% BSA for 1 h at 37°C. The cells were detached with trypsin/EDTA, washed once with DMEM containing 10% FBS, and resuspended in DMEM containing 1% FBS at 2×10^5 cells/ml. Aliquots (100 μl) of mock and Δc-7721 cell suspensions were directly added to the upper side of each chamber, whereas

wt-7721 suspensions were pre-incubated with or without the β peptide or nonsense peptide (100 μM) [Liu et al., 1997] and then added into the chamber. Following incubation for 12 h, the cells on the upper side of the membrane were removed, whereas the cells that migrated to the underside were fixed with 3% formaldehyde and stained with 0.3% crystal violet for 10 min. The number of cells on the underside of the membrane was counted in five different fields with a light microscope at ×100, and the mean and SD was calculated from three independent experiments.

Invasion assays were performed by using the similar procedure as the migration assays, except that Matrigel (BD) was coated on the 24-well chambers. Cells that penetrated through the membrane were stained and counted as described above.

RESULTS

Characterization of GnT-V and ΔcGnT-V Transfectants

To investigate in detail the effects of GnT-V on integrin β1 levels, we constructed its inactive mutant. Reportedly, the minimal catalytic domain of GnT-V was existed in its carboxyl-terminus, and deletion of as few as 4–8 amino acids from its carboxyl-terminus destroys its catalytic activity [Korczak et al., 2000]. Based on the above characteristic, we produced ΔcGnT-V with deletion of six amino acids at its C-terminal extreme end (pcDNA3-ΔcGnT-V), and its cellular transfectant was designated as Δc-7721. As shown in Figure 1A, one set of primer amplifies both the endogenous and exogenous transcripts (802 bp, lanes 2,4,6) and the other only the endogenous, native transcripts (1,207 bp, lanes 3,5,7). β-actin was used as an internal control and yielded a product of 412 bp.

Immunoblotting experiments revealed that GnT-V and ΔcGnT-V were overexpressed in the wt-7721 and Δc-7721 cells, respectively. Although GnT-V proteins in Δc-7721 included the wild-type and C-terminal deleted mutants (Fig. 1C), this type of GnT-V antibody (S-15) recognizes the epitope mapping within an internal region of GnT-V of human origin, so it can bind both wild-type and the extreme C-terminal deletion mutant of GnT-V in the present study. These results showed GnT-V and ΔcGnT-V were overexpressed in wt-7721 and Δc-7721 cells, respectively.

Glycan Profile of Glycoproteins in the Transfected Cells

Lectin-staining assay was performed using GNA-, PHA-L-, and DSA horseradish peroxidase complex. GNA specifically binds to high-mannose type glycans (called here GNA reactivity) [Shibuya et al., 1988], whereas PHA-L and DSA strongly recognize tri- and tetra-antennary complex type *N*-glycans, especially PHA-L specific for β1-6 GlcNAc branching, the product of GnT-V [Guo et al., 2002; Ihara et al., 2002]. Compared with the mock cells, plenty of glycoproteins in wt-7721 were stained positively with DSA (for the tri- and tetra-antennary complex type *N*-glycans) and especially showed PHA-L reactivity (specific for GlcNAc β1-6 branched), while those proteins in Δc-7721 cells illustrated that the preferential glycosylation was GNA-positive, high-mannose oligosaccharide of *N*-glycans (Fig. 1D). As mentioned above, Δc-7721 cells mainly expressed the inactive mutant of GnT-V. Therefore, this result suggested that β1-6 GlcNAc-rich oligosaccharides were prevalent under the control of GnT-V, and indicated GnT-V activity in Δc-7721 cells is very lower compared with the wt-7721 and mock cells.

Integrin β1 Protein Levels Vary in the Different Transfectants

To evaluate exactly the effects of GnT-V on β1 integrin, we investigated integrin β1 expression pattern in the mock, wt-7721 and Δc-7721 cells. Typically, β1 integrin is initially expressed as the so-called premature form (105 kDa), which dimerizes with α subunits and is transported to the Golgi complex where it then matures into the 125-kDa forms with complex type *N*-linked glycans [Albiges-Rizo et al., 1995]. In this study, the mature integrin β1 (hyperglycosylated form) was increased in wt-7721 cells, but decreased in Δc-7721 cells (Fig. 2A, middle and right lanes). Compared with the mock and wt-7721 cells, the premature β1 subunit was also suppressed in Δc-7721 cells (Fig. 2A, right lane). Next, to exclude the transcriptional control of integrin β1 by GnT-V, we examined the mRNA levels of integrin β1 in these transfectants. Our findings showed that integrin β1 mRNA was leveled off compared to GAPDH in three kinds of cells using real-time RT-PCR (Fig. 2B). Since

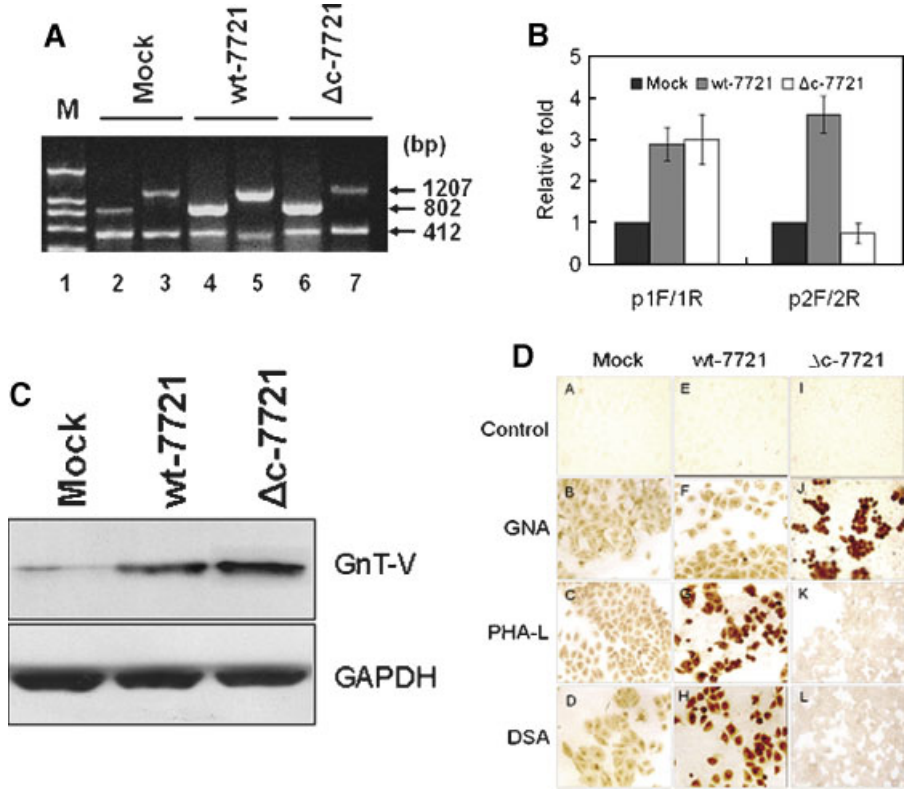

Fig. 1. Expression level of C-terminal deleted mutant ΔcGnT-V and its wild-type GnT-V in stable transfectants. **A**: Three independent cell clones of each transfectant were employed to detect the mRNA levels of GnT-V and its C-terminal mutant ΔcGnT-V using RT-PCR, and the results from each clone are similar. Shown is the representative one. The first primer pair (1F/1R) could amplify both the wild-type and C-terminal deleted mutant mRNA (**lanes 2** and **4**, only containing the wild-type mRNA in mock and wt-7721 cells, and **lane 6** containing both wild-type and mutant mRNA in Δc-7721; 802 bp), and the second pair (2F/2R) only for the wild-type mRNA (**lanes 3**, **5**, and **7**, 1,207 bp). β-actin (412 bp) were performed as an internal control. DNA molecular marker (M) was DL2000 from Takara. **B**: Graph of the results from (A). The band area of GnT-V was measured and normalized by that of β-actin, and then the specific GnT-V mRNA level was estimated. Total GnT-V mRNA levels (**lanes 2** and **4**) were almost the same as the wild-type (**lanes 3** and **5**) in mock and wt-7721 cells (compared **lane 2** with **3**, and **lane 4** with **5**), and wild-type mRNA of GnT-V were elevated in wt-7721 (**lane 4** or **5**) and mutant ones were increased in Δc-7721 cells (the intensity from **lane 6** minus that from **lane 7**). The results were expressed from three independent cell clones of each transfectant. The data represent the mean ± SD of three independent analyses. **C**: GnT-V and Δc GnT-V proteins were detected using Western blot assay, and its levels from wt-7721 and Δc-7721 cells were higher than control, although GnT-V proteins in Δc-7721 was the sum of the wild-type and C-terminal deleted mutants. **D**: Lectin staining of cellular glycoproteins in the transfected cells. Cells were cultured on the plates, then washed with PBS, followed by detection with HRP-GNA, HRP-PHA-L, and HRP-DSA complexes. The stained cells were observed microscopically. Mock, wt-7721 and Δc-7721 represented the transfectants with pcDNA3-empty, pcDNA-wtGnT-V and pcDNA3-ΔcGnT-V, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the mature β1 was mainly translocated from the Golgi complex to cytoplasm membrane, we investigated the subcellular localization of β1 subunit using immunocytochemical-staining and flow cytometry assays. Actually, it was true for the mature form of integrin β1 in wt-7721 cells (Fig. 2C, middle panel; Fig. 2D, curve 3). Conversely, the membrane staining of β1 subunit was hardly detected in Δc-7721 cells (Fig. 2C, bottom panel; Fig. 2D, curve 1), and even lower than mock cells (Fig. 2C, upper panel; Fig. 2D, curve 2). Because GnT-V was suppressed in Δc-7721 cells, the relative higher level of the mature form in wt-7721 was probably due to the higher GnT-V activity. So the changes of β1 integrin protein levels were mainly attributed to the post-translational modification, that is, *N*-linked glycosylation in this research.

Pulse-Chase Experiment to Monitor Protein Degradation of Integrin β1

To further investigate the mechanisms by which integrin β1 protein level was decreased in

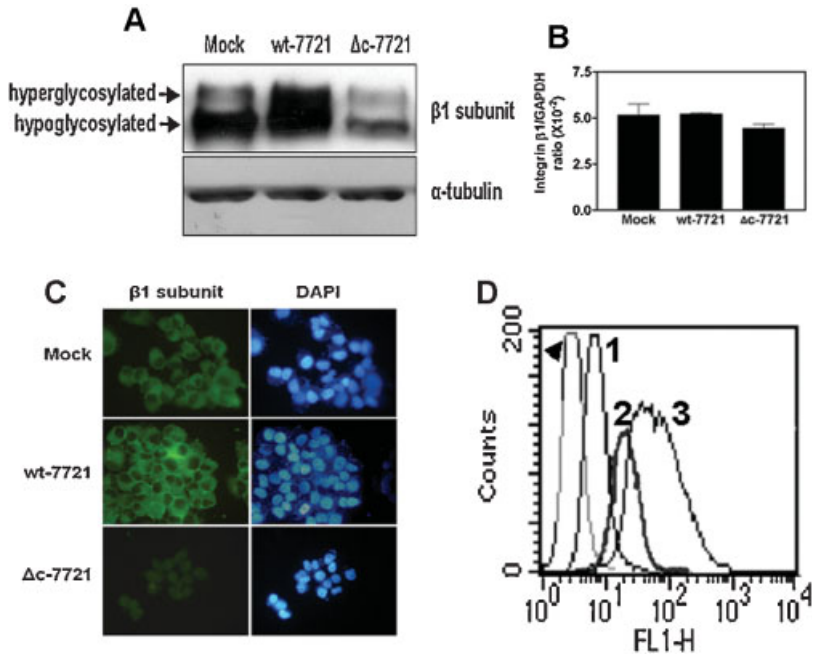

Fig. 2. Integrin β1 expression levels in the transfected cells. **A**: Cells were cultured to 80% confluence, harvested and lysed in 1× SDS lysis buffer. The samples were boiled and clarified by centrifugation, and lysates were then resolved by 10% SDS–PAGE, followed by transferring onto polyvinylidene difluoride membranes. The protein signals were detected with the appropriate antibody and visualized using enhanced chemiluminescent kit (ECL). The different forms of β1 subunit were shown and due to the different levels of β1 glycosylation. The hypoglycosylated lower band was tentatively identified as biosynthetic precursor (premature) of β1 subunit; the hyperglycosylated upper band was mature subunits, exposed in part on the cell surface (the 125-kDa product). **B**: Integrin β1 mRNA level was also determined by real-time PCR. The levels of expression of target genes were standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene detected in the identical cDNA samples. The results were expressed as averages of duplicate assays of each transfectant, *error bars* indicated variations from the mean. **C**: Immuno-cytochemical staining of cell surface β1 integrin. The nuclei were counterstained with DAPI. **D**: The cell surface integrin β1 levels in the transfectant cells were detected with flow cytometry assay, which was described in detail under ''Materials and Methods''. The negative control (*arrowhead*) showed background fluorescence which was detected as same as the other three samples except for absence of the primary antibody against human β1 integrin. Curve 1, the fluorescent level of integrin β1 subunit in Δc-7721 cells; curve 2, the fluorescent level in mock-7721 cells; and curve 3, the fluorescent level in wt-7721 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Δc-7721 cells, we exploited pulse-chase experiment to monitor protein degradation of integrin β1 subunit. In Δc-7721 cells, integrin β1 levels were markedly decreased with time, and its half-life was less than 3 h. On the contrary, the alleviating degradation of β1 subunit was observed in wt-7721 where the β1 subunit half-life was about 16 h. Meanwhile, the degradation rate of β1 subunit in mock cells was expectedly in between (Fig. 3A). Taken together, these findings suggested that ΔcGnT-V promoted integrin β1 degradation at the posttranslational level.

Altered Integrin β1 Levels Were Correlated With its Different Glycoform

Integrin β1 subunit contains several potential sites for *N*-linked oligosaccharides [Gu and Taniguchi, 2004]. To understand changes in carbohydrate structure of integrin β1 in these transfectants, immunoprecipitation with anti-integrin β1 antibody and lectin-blotting assay with GNA-, PHA-L, and DSA-peroxidase complex were performed. The level of immunoprecipitated β1 subunit was changed in three transfectants in the same manner as Figure 2A. The β1 integrin from Δc-7721 showed the GNA reactivity that was disappeared in the mock and wt-7721 cell, since the GnT-V activity was dramatically inhibited in Δc-7721, and subsequently the *N*-linked β1-6 branching was almost diminished. Nevertheless, the β1 integrin from wt-7721 showed DSA and PHA-L reactivity (Fig. 4A). Specifically, the PHA-L activity in wt-7721 was greatly higher than the β1 integrin level in this kind of cells, and this

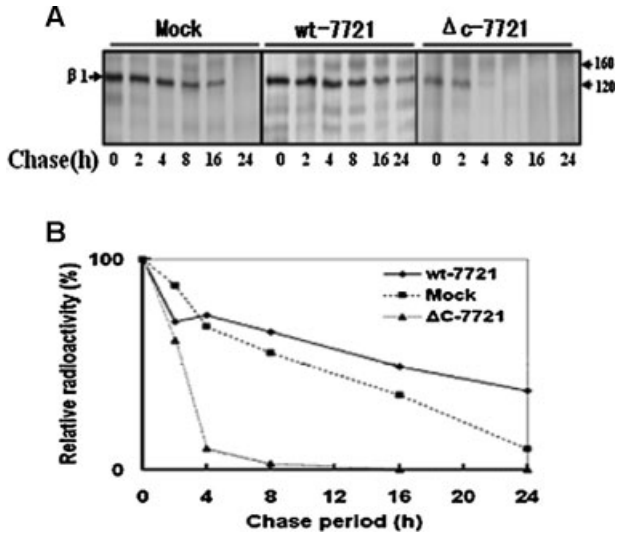

Fig. 3. Degradation assay and pulse-chase study of integrin β1. **A**: Integrin β1 protein degradation was carried out using pulse-chase experiment which described in ''Materials and methods''. Mock, wtGnT-V and ΔcGnT-V transfectants were radiolabeled with [35S] methionine for 30 min. At 0, 2, 4, 8, 16, and 24 h after pulse labeling, integrin β1 was immunoprecipitated from cell lysates using the anti-integrin β1 antibody. After the separation of immunoprecipitated integrin β1 by SDS–PAGE, the gel was dried and the radioactivity was measured with a Fujifilm FLA-5100 Bio-Imaging Analyzer system (Fuji Photo Film) (*arrow*). **B**: Diagram of the half-life of integrin β1 in mock, wt-7721 and Δc-7721 cells. Quantitative analysis based on densitometry is shown as a graph.

discrepancy could be regarded as the result of overexpressed GnT-V. In contrast, the PHA-L activity was dramatically lower than β1 level in Δc-7721 cells, and this may be attributed to the lack of GnT-V function (Fig. 4B). These results revealed the strong binding of PHA-L lectin to integrin β1 precipitation pellets in GnT-V transfectants and indicated *N*-glycans of integrin β1 were glycosylated by the overexpressing GnT-V. Taken together with the integrin β1 protein levels in three transfectants, altered integrin β1 levels may be attributed to the different glycosylation pattern and the integrin β1 bands that were resistant to degradation coincided with those containing β1-6 GlcNAc branching.

Functional β1 Integrins are Associated With FN-based Cell Migration and Invasion

Cell migration and invasion induced by integrin signaling are always correlated with interaction of integrins and their matrix ligands, and integrin α5β1 is the canonical receptor of fibronectin in many cell types including hepatocytes. Moreover, β1 subunit has been identified as the target of GnT-V, but there is no solid evidence of *N*-linked β1-6 branching oligosaccharide structures on the α5 subunit [Guo et al., 2002]. So, we supposed that Fn-based cell behaviors induced by GnT-V may be mainly attributed to the β1 integrin. In this experiment, we observed Δc-7721 cells migrating to the lower surface of the membrane were considerably decreased using Transwell methods and its invasive ability was almost inhibited under the experiments of Transwell-matrigel assays (Fig. 5A, compared b and g with a and f, respectively). Conversely, GnT-V transfectants (wt-7721) showed the increased level of migrating and invasive cells compared with the mock transfectants (Fig. 5A, compared c and h with a and f, respectively). Remarkably, however, β peptide blocking the functional β1 integrins could also obstruct migration and invasion of wt-7721 cells (Fig. 5A, compared e and j with d and i, respectively); the changes were similar as Δc-7721 cells. These results showed that ΔcGnT-V had the opposite effects on cell migration and invasion and these discrepancies would be attributed to the inactive form of ΔcGnT-V, and that cell behaviors induced by ΔcGnT-V might be mainly attributed to the decrease of functional β1 integrin.

DISCUSSION

In the present study, we found that the addition of β1-6 GlcNAc branching on integrin β1 caused by GnT-V inhibited its degradation, resulting in the up-regulation of β1 integrin

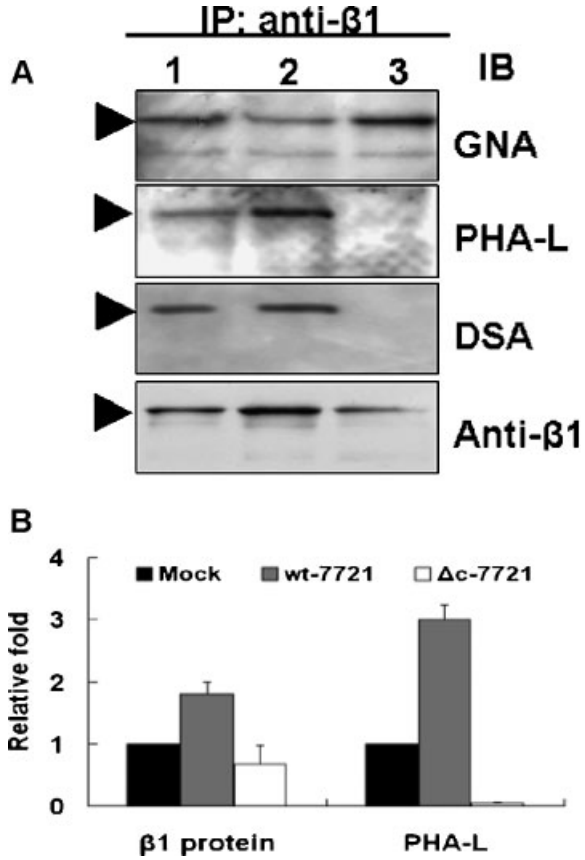

Fig. 4. The different glycoform of integrin β1 in the different transfectants. **A**: The glycoform of β1 integrin from each of three transfectants was detected using immunoprecipitation and lectin blotting assays. Cultured cells were washed and lysed in the appropriate lysis buffer. The whole cell lysates (1 mg) were immunoprecipitated with anti-integrin β1 monoclonal antibody. Pre-equilibrated protein A/G PLUS-agarose beads (20 μl) were then added and incubated overnight. The bound proteins were eluted by boiling in 2× SDS sample buffer and resolved on a 10% SDS–PAGE gel. The proteins were transferred onto a PVDF membrane and probed with the appropriate horseradish peroxidase labeled-lectin complex. **Lanes 1–3**: mock, wt-7721 and Δc-7721 cells. GNA specifically binds to high-mannose type glycans, whereas PHA-L and DSA strongly recognize tri- and tetra-antennary complex type *N*-glycans, especially PHA-L specific for β1-6 GlcNAc branching. IP, immunoprecipitation; IB, immunoblot. **B**: The β1 protein level and its PHA-L activity were separately measured in each of three transfectants. The assays were performed in triplicate measurements of the same sample. The data represent the mean ± SD of three independent analyses.

expression on the cell surface despite the fact that no significant changes in integrin β1 mRNA expression were observed using real-time RT-PCR. As previously reported, differentially electrophoretic motility bands of β1 integrin are typically observed [Albiges-Rizo et al., 1995; Semel et al., 2002]. These bands represent integrin β1 isoforms that are in different stages of maturation. During protein synthesis on rough endoplasmic reticulum (ER), β1 integrins are partially glycosylated by ER glycosyltransferases, generating a high mannose, premature form, and show GNA reactivity [Shibuya et al., 1988]. This pre-integrin β1 is complexed with a premature form of α subunit within the ER, and then transported to the Golgi apparatus, where α and β subunits undergo further processing by Golgi glycosyltransferases to generate the fully glycosylated mature form. This mature form is subsequently translocated to cell surface, where it becomes functional. Recent studies indicate that protein stability affected by N-Linked glycans are diverse. Species that are permanently misfolded or premature glycoprotein, orphan subunits of oligomers, and some heterologously expressed or mutant glycoprotein in the endoplasmic reticulum are prevented from exit to the Golgi apparatus. They are selectively transported to the cytosol, where they are degraded in the proteasomes [Parodi, 2000; Helenius and Aebi, 2001; Wujek et al., 2004]. An interesting role of mature glycans in stabilizing proteins has been found in the protein aquaporin 2 (AQP2) [Buck et al., 2004; Nivedita et al., 2006]. These studies suggest that mature N-glycosylation is advantageous for proteins folding correctly instead of targeting them to the endoplasmic reticulum degradation pathway [Helenius and Aebi, 2001]. Taniguchi's group recently observed that the addition of β1-6 GlcNAc branching by GnT-V on matriptase, an epithelium-derived, integral membrane serine protease, inhibited its degradation, resulting in the high matriptase expression [Ihara et al., 2002; Ihara et al., 2004; Ito et al., 2006]. In the present study, we found that the addition of β1-6 GlcNAc branching caused more fully glycosylated mature form on integrin β1 and inhibited β1 protein degradation, resulting in the up-regulation of β1 integrin expression on the cell surface.

It is known that premature *N*-glycosylation of some proteins serves as a targeting signal to eliminate intracellular glycoproteins by Fbx2-dependent ubiquitination and proteasomal degradation [Yoshida et al., 2002]. Pre-integrin β1 subunit is indeed a target of Fbx2, which binds specifically to proteins attached to *N*-linked high-mannose oligosaccharides and subsequently contributes to ubiquitination of

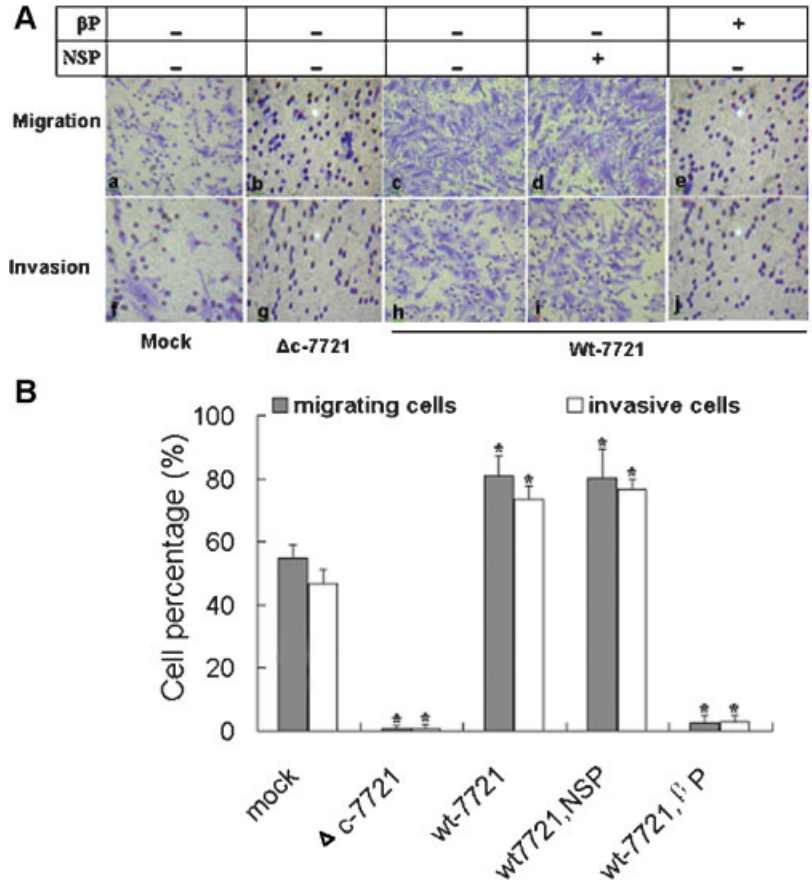

Fig. 5. Migration and invasion of the mock, wt-7721 and Δc-7721 cells with or without the blocking peptide of β1 integrin. **A**: Cell migration was determined using a Transwell membrane coated on the upper sides with fibronectin (10 μg/ml). Cell invasion was determined using a Transwell membrane coated on the upper sides with Matrigel, and cells were allowed to migrate through Matrigel for 12 h. Aliquots (100 μl) of 2×10^4 cells suspension were pre-incubated with the β peptide or nonsense peptide (100 μM) and then added to the upper side of each chamber. After 12 h of incubation at 37°C, The cells that had migrated to the lower surface of the membrane were fixed with 3% formaldehyde and stained with 0.3% Crystal Violet for 10 min. **B**: Diagrams of migrating and invading cells from the different transfectants are shown, which from more than three independent experiments. *Indicates $P < 0.01$ compare to mock cells (one-way ANOVA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

N-glycosylated proteins [Yoshida et al., 2005]. The three cells were treated with 10 μM MG132 (a proteasome inhibitor) respectively in indicated times, MG132 increased significant the level integrin β1 after 1.5 h treatment in Δc-7721 cells (Fig. 6). As described in this study, overexpression of an enzymatic inactive mutant of GnT-V (ΔcGnT-V) blocks conversion of the pre-integrin β1 into the mature form. The pre-integrin β1 was mainly attached with the high mannose oligosaccharides and degraded quickly compared with counterparts of integrin in wt-7721 and mock cells (Fig. 3). The forced expression of ΔcGnT-V could decrease both premature and mature levels of β1 integrin, and mature integrin β1 in wt-7721 was more resistant to degradation coincided with those containing β1-6 GlcNAc branching.

Integrins link the cell to extracelluar-matrix ligands. A growing number of evidence indicates that the presence of the appropriate oligosaccharide can modulate α5β1 integrin activation and integrin-ligand binding affinities [Zheng et al., 1994; Guo et al., 2001]. Previous reports suggested that overexpression of GnT-V led to excessive β1-6 *N*-glycosylation, which changed α5β1 interaction with specific adhesion molecules of extracelluar-matrix and then caused these cells more motile [Guo et al., 2002; Bellis, 2004]. Taniguchi's group observed that bisecting GlcNAc could be introduced into α5β1 by GnT–III (GnT–III considered to be an

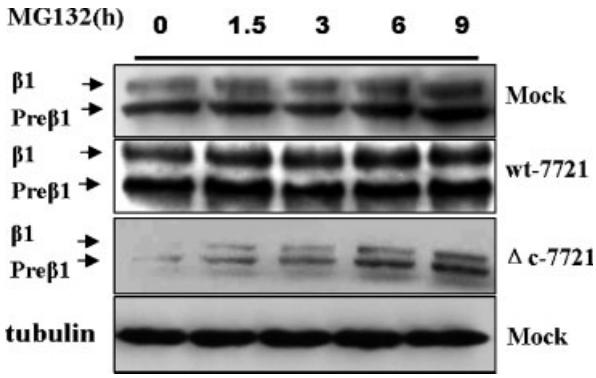

Fig. 6. Degradation of pre-integrin β1 protein was proteasome-dependent in Δc-7721 cells. The three cells were treated with 10 μM MG132 (a proteasome inhibitor) respectively in indicated times.

antagonist to GnT-V) and substantially blocked α5β1 integrin-mediated cell migration [Isaji et al., 2004]. In present study, as for FN-based cell behaviors, cell migration and invasion were significantly blocked in ΔcGnT-V transfectants, while it was dramatically increased in GnT-V-overexpressing cells. Moreover, β peptide, the blocking peptide of functional β1 integrin, could also inhibit cell migration and invasion of wtGnT-V transfectants, which mimicked the behaviors of ΔcGnT-V transfectants. These results also suggest that the deficits in cell motility observed in ΔcGnT-V transfectants result mainly from diminished cell surface expression of α5β1 integrin heterodimers. The cell migration speed depends on integrin-ligand optimum adhesiveness and integrin level [Palecek et al., 1997], which are both associated with β1-6 GlcNAc branching of integrin β1.Taken together, our findings support that GnT-V up-regulates β1 integrin preferably through enhancing its stability, subsequently promotes Fn-based cell migration and increases cell invasion ability to penetrate through Matrigel.

ACKNOWLEDGMENTS

We thank Prof. Hui-Li Chen (Key Laboratory of Glycoconjugate Research, Fudan University) for his kindly providing of the pcDNA3Flu-HuTV (pcDNA3/GnT-V) plasmid, Prof. Jianxin Gu and Dr. Yanzhong Yang (Gene Research Center, Shanghai Medical Colloge, Fudan University) for their generous guidance in pulse-chase assay, Prof. Yinkun Liu and Dr. Songmei Wang for their providing the blocking peptides of β1 integrin.

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