

REGULATION OF  $\beta$ 1 INTEGRIN FUNCTION BY ABERRANT N-LINKED  
GLYCOSYLATION AND VACUOLAR  $H^+$ -ATPASE 16-KDA SUBUNIT EXPRESSION

by

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(UNDER THE DIRECTION OF DR. MICHAEL PIERCE)

Integrins are a family of cell surface glycoproteins that functions as cell adhesion receptors to the extracellular matrix (ECM). The interactions between integrins and ECM play important roles in regulating cell adhesion, migration, and proliferation. In particular,  $\beta$ 1 integrin dimerizes with multiple numbers of integrin  $\alpha$  subunits, leading to the most diverse array of interactions between the cells and ECM. This dissertation research studied how the function of  $\beta$ 1 integrin is affected by vacuolar  $H^+$ -ATPase 16-kDa subunit expression. It was previously shown that overexpression of vacuolar  $H^+$ -ATPase 16 kDa (16K) subunit inhibited  $\beta$ (1,6) branched N-glycans of  $\beta$ 1 integrin, thus blocking  $\beta$ 1 integrin-dependent cell migration toward laminin and fibronectin. Expression of 16K inhibited  $\beta$ 1 integrin-calnexin association in the endoplasmic reticulum, thereby disrupting the folding and subunit assembly of  $\beta$ 1 integrin for functional  $\alpha/\beta$ 1 integrin expression on the cell surface. This inhibition resulted in Triton X-100 insoluble, non-disulfide-linked aggregation of minimally glycosylated  $\beta$ 1 integrin in the endoplasmic reticulum. During the course of this study, we also found that calnexin associates with 16K transiently in the endoplasmic reticulum for 16K biosynthesis. Our results provide a new insight into how 16K- $\beta$ 1 integrin interaction potentially leads to down-regulation of  $\beta$ 1 integrin surface expression and subsequent  $\beta$ 1 integrin-mediated signal transduction, which is highly implicated in resistance of certain tumor cells to cancer chemotherapy. Another focus of this dissertation was to study effect of aberrant glycosylation on neuronal differentiation. N-acetylglucosaminyltransferase VB (GnT-VB) is a Golgi glycosyltransferase that is selectively expressed in brain and neural retina of developing mouse embryo. Expression of GnT-VB in rat pheochromocytoma PC12 cells was used as a model system to investigate if GnT-VB has a significant role in neuromorphogenesis. The results showed that GnT-VB expression promotes an increased rate of neurite outgrowth on collagen and laminin substrates in a  $\beta$ 1 integrin-dependent manner. Using neurite outgrowth by manganese-induced  $\beta$ 1 integrin activation and  $\beta$ 1 integrin function-blocking antibody, it was shown that GnT-VB enhanced neurite outgrowth is most likely mediated by alteration of  $\beta$ 1 integrin-ECM interaction. These results suggested that N-linked glycosylation modification of cell surface glycoproteins by GnT-VB may function during neuronal differentiation.

INDEX WORDS: N-acetylglucosaminyltransferase V,  $\beta$ 1 integrin, Extracellular matrix, Vacuolar  $H^+$ -ATPase, 16-kDa subunit, neurite outgrowth.

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## DEDICATION

I would like to dedicate this dissertation to my parents. Without their continued support and encouragement, this thesis work would have been much more difficult and challenging.

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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

Cell-matrix adhesion is a central behavior of cells that is involved in many, critical events, including cell migration during early development and the invasiveness of oncogenically transformed cells. Most cell-matrix adhesion events are mediated by the class of cell surface receptors known as integrins that bind extracellular ligands and transduce signals intracellularly. Recent studies, including several that are including in this dissertation, demonstrate that changes in the glycosylation of some of the integrins, notably the  $\alpha 5\beta 1$  integrin, can regulate their adhesive and signaling functions, most likely by influencing their clustering behavior in the plasma membrane when they bind ligands, such as fibronectin.

The overall focus of this dissertation is investigating the regulation of cellular adhesive function by changes in the expression of a particular glycosyltransferase, GnT-V, and its brain-specific homolog, GnT-VB. GnT-V expression is known to be up-regulated during many examples of oncogenic transformation. In order to understand the results of experiments contained in these studies, a brief introduction to the currently accepted model of N-linked glycosylation of glycoproteins and the role of N-linked glycans for glycoprotein quality control will be presented. In addition, various functional roles of N-linked glycans will be discussed to give a broader view of how protein glycosylation can affect protein-protein interactions.

Chapter 2 will be a presentation of a recently published manuscript that demonstrates that cell surface expression of functional  $\alpha 5\beta 1$  integrin is regulated by expression levels of the 16K

subunit of the V-ATPase. Chapter 3 is a presentation of a recently submitted paper detailing the regulation by GnT-VB expression of neurite outgrowth on collagen-coated plates in an integrin-dependent manner. Three published papers are included in the Appendix that detail the regulation of integrin-dependent cell-matrix adhesion and cell migration by changes in the expression of GnT-V. Discussion of all of these results will be presented in the Conclusion section.

### **N-linked glycosylation and glycoprotein quality control in the endoplasmic reticulum**

Nascent polypeptides are known to get co-translationally glycosylated by transfer of a highly conserved, dolichol-linked glycan by the oligosaccharyltransferase onto Asn-X-Ser/Thr, the potential consensus sequence in the endoplasmic reticulum (ER) (1). Sequential trimming of two terminal glucose residues by glucosidase I and II then allows  $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$  to be recognized and bound by calnexin/calreticulin, molecular chaperones/lectins, to facilitate folding of the glycosylated polypeptides by another set of ER resident enzymes, glucosidase II and UDP-glucose-glucosyltransferase (UGT) (2, 3). UGT plays a role as a folding sensor of glycosylated polypeptides by binding and transferring a glucose residue to only unfolded or misfolded proteins (Fig.1.1). Calnexin, which facilitates the folding of most membrane bound glycoproteins, has been implicated in not only folding of glycoproteins, but also subunit assembly of multisubunit complex (4, 5) by sequestering and regulating the subunit assembly process. For example,  $\beta 1$  integrin half life in the ER is regulated by its association with calnexin and availability of integrin  $\alpha$  subunits. Calnexin promotes the solubility of  $\beta 1$  integrin and retains the  $\beta 1$  subunit for proper heterodimerization with sufficient number of different  $\alpha$  subunits (6).

Preventing calnexin-integrin association by glucosidase inhibitors drastically reduced half-life of integrin in the ER.

Calnexin and calreticulin also play a central role in glycoprotein degradation in the ER, called “ER-associated degradation” (ERAD) (7, 8). A recent model of ERAD suggests that terminally misfolded glycoproteins are released from calnexin/calreticulin and translocated by Sec61 channel, a retrotranslocon, in an EDEM- an ER-resident, mannose-binding lectin-dependent manner from lumen of ER to cytosol (5). As retrotranslocation of misfolded glycoproteins to the cytosol takes place, the glycosylated polypeptides are ubiquitinated and deglycosylated in the cytosol, followed by degradation of polypeptide chains by proteasomes (5, 9). This model is still a work in progress, because there are several unsolved questions in this process. First, it is not understood how glycosylated transmembrane proteins can be translocated to the cytosol through a transmembrane channel whose diameter is not sufficient to allow passage of large hydrophilic sugar chains bound to partially folded polypeptides (10). Second, there are many examples of glycoproteins that showed no evidence of ubiquitination, but still end up degraded in a proteasome-dependent manner. Furthermore, calnexin was recently shown to have an important role in glycan-independent chaperone activities for protein folding and subunit assembly of transmembrane ion channels (11, 12), suggesting that this ERAD model requires a lot more work to clarify these unresolved issues.

As summarized above, it is clear that N-linked glycosylation of glycoproteins plays essential functions during protein folding and subunit assembly. There also have been numerous studies about diverse roles of N-linked glycosylation during synthesis, maturation, secretion, and even activity of glycoproteins in extracellular space. Therefore, an introduction will be provided to various aspects of functions of N-linked glycosylation that have been documented so far.

### **Cooperative nature of N-linked glycosylation and folding of glycoproteins**

Site occupancy of potential N-X-S/T sites in glycoproteins has been shown to differ, depending on cell types and sources of organism. In one example, Holst *et al* studied how N-glycosylation occupancy on specific sites influences protein folding and its secretion by introducing seven new N-x-S/T (sequons) into carboxypeptidase Y of *S. cerevisiae*, in combination with dithiothreitol (DTT) to disturb disulfide bond formation (13).

Carboxypeptidase Y, in a naturally occurring form, is glycosylated on four different sequons and premature form of CPY from Golgi matures during vacuolar transport by proteolytic cleavage of its propeptide. The mutated protein showed various glycosylation patterns in the newly introduced sequons, depending on either the presence or absence of DTT. When one new sequon (3T) site was glycosylated by addition of DTT in the culture medium, the mutated CPY was not folded to its mature form. Furthermore, deletion of the propeptide segment resulted in a similar glycosylation at the 3T site, indicating that the folding of nascent polypeptides and N-glycosylation compete with each other.

In a similar study by Allen *et al*, tissue-type plasminogen activator (t-PA) expressed in CHO cells was examined for selectivity of its sequons utilization in the presence of various concentration of DTT in growth medium (14). The protein has three glycosylated sequons; two of them (Asn-117 and Asn-448) are completely processed oligosaccharides, while one (Asn184) is partially glycosylated in type I and missing glycosylation in type II t-PA. The two types are different glycoforms of the t-PA, a serine protease, secreted from human and recombinant mammalian cell lines. In this experiment, Asn-184 of secreted t-PA from CHO cells was fully glycosylated in the presence of various concentration of DTT, and this oligosaccharide change

did not cause a failure in protein folding and consequent secretion. Both of these experiments suggested that there is a competition between folding of a nascent polypeptide and N-linked glycosylation of sequons in ER, and that proper N-glycosylation is necessary to achieve a specific protein conformation for unique activity and targeting to specific destinations.

### **N-linked glycosylation of glycoproteins in intracellular glycoprotein trafficking.**

A number of examples of the intracellular trafficking of glycoproteins is also known to be regulated by N-glycosylation. Inside a mammalian cell, hundreds of glycoproteins are constantly synthesized and delivered to appropriate domains of the cell for specific functions. Specific intracellular targeting of proteins was once thought to be mostly regulated in Golgi apparatus by sorting via signal sequences on the proteins, themselves. However, as more evidence is formed by recent researches, it is now evident that the intracellular trafficking of glycoproteins is significantly contributed by the covalently attached oligosaccharides. In a classical example, most lysosomal hydrolases are solely dependent on mannose phosphorylation of their N-linked oligosaccharides. Cuozzo *et al* showed that a lysine-based structural motif exists in a number of lysosomal enzymes and this motif is responsible for the selective mannose phosphorylation (15). The authors compared two lysosomal proteases, Cathepsin D and Cathepsin L, to study how mannose phosphorylation of glycoproteins with no sequence similarity is carried out.

Even though they belong to the same Cysteine protease family, their primary sequences show very small sequence homology. Unique spatial positioning of the N-linked glycans, as well as the 3D conformations of adjacent polypeptides are very similar. In both proteins, two lysine residues are positioned around N-glycans at a specific distance (approx. 34 Å). Site-directed mutagenesis of these two lysines results in a 70% reduction in mannose phosphorylation,

suggesting that evolutionary conservation of lysosomal targeting motifs occurs in conjunction with both specific protein folding and positioning of N-linked glycans in three dimensional spaces.

### **Regulation of glycoprotein half-life by N-linked glycosylation**

Furthermore, the role of N-linked glycans on protection of glycoproteins from early proteolytic degradation, which in many cases determines protein half life, has been reported for many different glycoproteins. Proteins in the cytosol, lysosomes, as well as in circulation, are constantly endangered for fast proteolytic digestion, due to high proteolytic activity in these spaces. In many cases, it was shown that deglycosylation of certain glycoproteins result in a dramatically reduced half-life. For example, Sareneva *et al* studied human interferon-gamma for its protease resistance (16). They showed that N-linked glycans at Asn-25 are essential for maintaining the normal half-life of the glycoproteins in circulation by protecting from proteolysis. In addition, all lysosomal hydrolases are heavily glycosylated to protect themselves from digestion by other lysosomal enzymes (17).

### **N-linked glycosylation plays important roles in cell adhesion activity**

Next, the role of N-linked glycans in cell-cell and cell-matrix interaction will be summarized, since this is a relevant area to this dissertation research.

Mammalian cells are covered with layers of carbohydrates that are conjugated to cell surface membrane-bound proteins and glycolipids. Consequently, researchers in the past speculated that microbial pathogens, including bacteria and virus, must initiate their invasion by interacting with cell surface oligosaccharides in some way. Clearly, it has been shown that many mammalian

pathogens use such oligosaccharides as highly specific cell surface receptors to bind to the surface of mammalian cells. One representative example comes from the influenza viral haemagglutinins that recognize terminal sialic acid residues of N-glycans (18).

Functionality of glycoproteins as a cell adhesion receptor is known to be significantly affected by N-linked glycosylation as well. For example, the neural cell adhesion molecule (N-CAM) contains highly charged oligosaccharide chains with polysialic acids in the embryonic form, reducing homotypic adhesion to neighboring cells. However, in the adult form of N-CAM, sialylation of the chains are reduced substantially and, consequently homotypic adhesion is much more improved (19, 20). Reduction of sialylation was shown to be caused by down-regulation of two polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST)(21, 22). This phenomenon is thought to contribute to the development of the differential plasticity of neural cells, in which the growth of axons and dendrites are variably regulated until optimal neural networks are achieved between the brain and other peripheral system. As this example suggests, N-linked glycosylation of glycoproteins, which function as cell adhesion receptors, undergoes significant changes during embryonic development. One of the best known examples of this phenomenon is catalyzed by a Golgi glycosyltransferase, N-acetylglucosaminyltransferase V, whose expression during embryonic development is hypothesized to promote migration of differentiated cells to the target sites. This enzyme is also highly expressed in many types of malignant cancers, and its expression level is highly correlated with increased motility and invasive potential of the tumor cells

**N-acetylglucosaminyltransferase V activity and cancer progression.**

Aberrant glycosylation occurs when cells become oncogenically transformed and can be characterized as a unique hallmark of cancer progression in many types of cancers, including breast and colon carcinomas (23). One of the most well characterized cancer specific regulation of glycosylation involves  $\beta$  (1,6) branched N-linked glycans whose synthesis is catalyzed by a Golgi-resident glycosyltransferase, N-acetylglucosaminyltransferase V (GnT-V). GnT-V is a 95kDa, type-2 transmembrane glycoprotein which transfers UDP-N-acetylglucosamine (GlcNAc) to the  $\beta$  (1,6) mannose of tri-mannosyl core of N-linked glycans in  $\beta$  (1,6) linkage (Fig.1.2) (24). Expression of GnT-V has been shown to be regulated by the Ras-Raf-MEK-MAPK-Ets pathway and is activated by several oncogenes, including *src*, *her-2/neu*, *H-ras*, and *v-sis* (25, 26). There are 7 members (GnT-I to GnT-VI and GnT-VB) of this family and they are responsible for addition of bi-, tri-, tetra, and pentaantennary N-linked oligosaccharides on glycoproteins (Fig.1.2).

For the last 20 years, GNT-V has been the focus of attention because of its relevancy to tumorigenesis and the metastatic potential of tumor cells (27). A number of studies have shown positive correlation of the expression and activity of GnT-V with the invasive and metastatic potential of various tumor cells (23, 28). An interesting aspect of  $\beta$  (1,6) branched N-linked glycans biosynthesis is the fact that this branch preferentially gives rise to poly N-acetyllactosamine chains, long repeating units of N-acetyllactosamine (29). Poly N-acetyllactosamine chains have been reported to have a role in modulation of cell adhesive activity (30), although it has not been determined whether effects of  $\beta$ (1,6) branching on tumor cell invasion are derived solely from increased poly N-acetyllactosamine. It is not clearly understood how addition of  $\beta$  (1,6) branching can alter tumor cell invasion and metastatic potential, but its strong correlation raised the possibility that cell motility is enhanced due to

increased  $\beta$  (1,6) branched N-linked carbohydrates on cell adhesion receptors, such as integrins and cadherins.

A study by Granovsky and Dennis (31) showed that GnT-V is not essential for life-sustaining cellular functions and GnT-V deficiency during mouse embryonic development was shown to be not lethal in GnT-V knockout (KO) mouse study. However, when GnT-V KO (Mgat5 (-/-)) mice were tested for invasiveness and metastatic potential in the mouse model of breast cancer progression, rate of tumor growth and metastasis were dramatically reduced. The study of Mgat5 (-/-) mouse crossed with mouse mammary tumor virus-polyoma middle T oncogene transgenic mouse, which develops breast tumor at puberty, showed significantly longer delay for the appearance of tumors and reduced tumor mass (31). This result demonstrates strongly that GnT-V expression and activity are not required for oncogenic transformation leading to development of tumors, but plays an important role in the rate of tumor growth and invasion. More recently, it was shown that chemokine receptors with increased  $\beta$  (1,6) branched N-linked glycans are bound by Galectin-3, resulting in delayed internalization and prolonged signal transduction (32), suggesting a novel role of GnT-V in modulating soluble factor-mediated signaling.

Possible roles of GnT-V in colon cancer progression have been particularly well characterized. In a study by Li and Roth, it was reported that cytochemical staining of human colon carcinoma cell lines, HCT-116a and -116b, by digoxigenin (dig) conjugated L-PHA showed much higher staining intensity for the more aggressive subline, HCT-116a (33). L-PHA is a lectin that specifically recognizes and binds to  $\beta$  (1-6) linkages of complex type N-linked oligosaccharides and has been used to detect the change in GnT-V activity of transformed cells (34). In colon cancer patients, increased cytochemical staining of patient tissue samples with L-

PHA corresponded with decreased life expectancy of the patients, supporting the functional role of GnT-V in colon cancer progression (23).

A notable reduction in substratum adhesion and loss of contact inhibition, as well as increased cell migration in the serum-free environment are some of the characteristic changes frequently observed in oncogenically transformed cells. Cell surface receptor proteins, such as integrins and cadherins, regulate adhesion and migration in the extracellular matrix (ECM) and in interaction with other cells. GnT-V transfected Mink lung epithelial cell lines showed significantly increased migration and decreased adhesion on the surface coated with laminin (35). Since effects of GnT-V activity on metastasis and invasiveness of transformed cells are correlated with the role of these important cell surface glycoproteins, researchers tried to answer how adhesion receptors and GnT-V work together in order to bring about the kind of phenotypic changes seen in transformed cells. A fundamental question from this type of study is how selectivity of glycoprotein substrates is determined for GnT-V. In order to answer this question, Do *et al* examined the enzymatic efficiency of GnT-V with a number of different glycoproteins containing bi- and/or triantennary N-linked complex oligosaccharides (36). From the study, Do *et al* concluded that desialylated and degalactosylated forms of bovine fetuin, human transferrin and human fibrinogen, all of which normally contain very small percentage of  $\beta$  (1-6) linkage, became highly efficient substrate for GnT-V. Denaturation of the three glycoproteins by reduction and carboxymethylation yielded 2-3 fold increased transfer of GlcNAc for all three glycoproteins, suggesting that substrate specificity of GnT-V is affected by accessibility of target sequons during protein folding. In addition, when total cell proteins from CHO cells were denatured and used as substrates for GnT-V, other glycoproteins without any sequence

homologies among them showed significant increase in  $\beta$  (1-6) linkage after denaturation. All these results indicated that GnT-V preferentially acts on some glycoproteins because their N-linked oligosaccharides have better accessibility to the enzyme than others, but it is unlikely that there is any consensus recognition sequence or signal patch domain on the substrate glycoproteins (36). This study also implies that there is likely to be a pre-determined set of glycoprotein substrates for GnT-V in a given cell type.

Because integrin is a primary candidate substrate for the effects of GnT-V increase during oncogenesis on tumor cell invasion, a brief review of the integrin family of cell adhesion receptors is provided.

**Integrin: an integrator of extracellular matrix and cell growth and migration.**

Integrins are a family of integral membrane glycoproteins that are involved in cell adhesion, migration and signal transduction of the cellular proliferation pathway (37, 38). They are comprised of heterodimers,  $\alpha$  and  $\beta$  subunits. So far, 16  $\alpha$  subunits and 8  $\beta$  subunits are known to form more than 20 different combinations of  $\alpha\beta$  heterodimers, which have unique binding activities for various extracellular matrix (ECM) proteins, including collagen, fibronectin, laminin and vitronectin. Cell adhesion to the extracellular matrix is necessary to promote cell proliferation and differentiation, and this is now known to be mediated almost exclusively by integrins. Integrin binding to ECM causes cytoplasmic tails of  $\beta$  subunits to promote activation of a number of different associated protein kinases that result in cascade of signal transduction events, as well as formation of intracellular cytoskeletal structures and focal adhesion complexes (Table.1.1) (39). This ability of integrins to integrate the adhesive activity of cells to ECM was the function that this protein family was originally named after (37). Integrin  $\alpha$  subunits are

responsible for ligand binding specificity, while integrin  $\beta$  subunits are involved in facilitation of focal adhesion complex formation. In fact, cytoplasmic tails of integrin  $\beta$  subunits can independently migrate to focal adhesion sites and facilitate aggregation of related proteins, when expressed in chimeric forms (40). The integrin heterodimers with  $\alpha 4$  subunits are preferentially localized in membrane pit, where the caveolins and other glycosphingolipids create relatively more hydrophobic environments. Other integrins are expressed throughout the surface of plasma membrane.

It has been known that integrin expression is regulated in a tissue-specific manner, and different tissues produce different heterodimeric combinations of integrins (37). For example,  $\alpha 6\beta 4$  integrins are expressed in simple and stratified epithelial cells of skin and intestine. This integrin heterodimer preferentially binds laminin 5, which is also more abundantly present in the basement membrane of skin and intestine. In addition,  $\alpha 1\beta 1$  binds preferentially to collagens and fibronectins, while  $\alpha v\beta 3$  integrin binds specifically to vitronectin. In general, there is more than one type of ligand for each integrin heterodimer. This is due to the fact that the ligand specificity of integrins is determined by consensus recognition sequences of ECM proteins that are recognized by I-domains of the integrin  $\alpha$  subunits (41). Though I domains do not exist in all of the integrin  $\alpha$  subunits, most  $\alpha$  subunits have a conserved conformation of specific residues and tertiary structures in the ligand binding sites.

Active turnover events of focal adhesions for cell migration require a complicated communication between ECM and the cells, as well as growth factor-mediated signal transduction (42). Upon binding ligands, integrins are known to switch their conformation into a high affinity conformation, and subsequently cluster together in the plane of the plasma membrane along with specific growth factor receptors and adaptor proteins for signal

transduction pathways. Kim et al recently showed (43) that the integrin conformation change preceded integrin clustering. Integrin clustering was shown to mainly function to strengthen cell adhesion. Since cells need to coordinate their migration across the plane of ECM both via detection of the outside environment and internal regulation of cell cycle, more elaborate schematics of communication is necessary. Outside-in signaling by integrins is essential in that cells must take survey of their surrounding before migration for the presence of appropriate integrin ligands. Binding of specific ECM proteins to cell surface integrins induces conformational changes in the integrins that are propagated to their cytoplasmic tails and thereby results in activation of a number of cytoskeletal component proteins and protein kinases for cellular proliferation signaling to the nucleus (Fig.1.3) (44). Inside-out signaling by integrins is also necessary when cells need to detach from the present position and migration. Although detailed structural analysis of the conformational alteration in integrins is not yet complete, inside-out signaling is proposed to be facilitated by binding of internal signaling molecules to cytoplasmic tail of either  $\alpha$  or  $\beta$  subunits, which causes some conformational changes that are propagated to the extracellular surface or I domain, and results in the release of bound ligand due to reduced binding affinity.

### **Structural features of integrin-ligand interaction**

A present model of integrin-ligand interaction suggests (45-47) that integrin heterodimers are in a steady state between activated, high affinity conformer and inactive, low affinity conformer. An early study by Emsley et al. solved the crystal structure of integrin  $\alpha 2$ -I domain and a synthetic collagen-like tripeptide helix (48). The I domain of integrin  $\alpha 2$  is composed of a center  $\beta$ -sheet and 7 repeat  $\alpha$ -helices and unique C-helix, and a divalent metal ion. At the C-

terminal of  $\beta$ -sheet, there is some structural motif, called “Metal Ion Dependent Adhesion Sites” or MIDAS motif, for the binding of collagen and metal ion coordination (49). The authors proposed that the integrin  $\alpha$ 2-I domain has both an open and a closed conformation. According to the hypothetical model, the I domain is adhered to the  $\alpha$ -R (7 repeat helical repeat region) and a separate  $\alpha$ -I subunit. In closed state, the collagen binding site (MIDAS) is sequestered by  $\alpha$ -R binding, and presumably by  $\alpha$ -I subunit to some degree, as well as C-helix blocking the binding sites on the I domain. The cytoplasmic tail is in inactive state via salt bridge formation between  $\alpha$ -R and  $\alpha$ -I subunits. Upon binding of ligand, the sequential conformational changes result in separation of  $\alpha$ -R and  $\alpha$ -I subunits, while same conformational change propagates to the connecting stalk between the  $\alpha$  subunit and I domain, resulting in rotation of the I-domain to better expose the binding sites to collagen. At the same time, these overall changes on the extracellular domain cause scissor-like action on the cytoplasmic tails of the both subunits (Fig.1.4), resulting in breakage of the salt bridges and allowing access to intracellular signaling molecules (44). Interestingly, Springer’s group demonstrated that this scissor-like conformation change of integrin heterodimers can be stabilized by inserting an N-linked glycan wedge on either  $\beta$ 1 or  $\beta$ 3 integrin subunit, which resulted in trapping of conformation of the mutant integrin heterodimers in high ligand binding affinity conformers (50). This result suggests that it is conceivable that aberrant N-linked glycosylation of integrin  $\beta$  subunit can potentially shift the steady state integrin conformation toward either activated or inactivated state.

**Integrin glycosylation affects integrin function by modulating integrin-ligand binding activity and localization on the plasma membrane**

Important roles of integrin glycosylation in the regulation of integrin function have been studied by a number of researchers. An early study by Akiyama et al reported that  $\alpha 5\beta 1$  integrin glycosylation is essential for proper fibronectin binding and function (51) by showing that inhibition of N-linked glycan maturation with deoxyjirimycin treatment, an inhibitor of Golgi  $\alpha$  mannosidase I and II, results in loss of surface expression of functional  $\alpha 5\beta 1$  integrin which failed to bind fibronectin affinity column. Further study revealed that complete abolition of N-linked glycosylation of  $\alpha 5\beta 1$  integrin by tunicamycin treatment resulted in inhibition of  $\alpha 5$ - $\beta 1$  integrin pairing and surface expression (52), suggesting that integrin N-linked glycosylation is required for subunit assembly and targeting to the cell surface. While these studies established the requirement of integrin glycosylation for their ligand binding activity and proper expression and targeting, authors did not specifically address how changes in distal glycosylation, such as  $\beta(1,6)$  branching, of integrin subunits can alter their activity as cell adhesion receptors. Furthermore, it is still not known how many of 12 potential N-X-S/T sites of  $\beta 1$  integrin are actually utilized, nor which sites are used for variable glycosylation modification. Nakagawa et al. reported that  $\alpha 5\beta 1$  integrin purified from human placenta showed 35 different glycan structures, in which the most predominant glycan structure was diantennary-di- $\alpha(2,3)$ sialyl fucosyl (53). The authors were not able to identify specific sites of each glycan structures nor able to characterize whether a glycan was from  $\alpha 5$  or  $\beta 1$  integrin subunits. Functional regulation of aberrant glycosylation of integrin heterodimers has only recently been documented.

Yamamoto et al showed that  $\alpha 3\beta 1$  integrin with increased  $\beta(1,6)$  branching caused the integrin to localize to leading lamellipodia of migrating glioma (54). Increased  $\alpha(2,6)$  sialylation of  $\beta 1$  integrin has also been reported to stimulate  $\beta 1$  integrin-mediated cell migration on ECM (55). While the mechanism by which this altered adhesive activity is regulated is unknown at

present, selectivity of integrin  $\beta$  subunit sialylation was observed, that is,  $\beta 1$  integrin is sialylated, but not  $\beta 3$  or  $\beta 5$  integrin, suggesting that integrin glycosylation is highly selective (56-58).

Furthermore, there are evidences that glycosylation of integrin  $\alpha$  subunits plays an important role in the interaction of integrin with gangliosides. Kawakami et al reported that ganglioside GM3 promotes tetraspanin CD9- $\alpha 3\beta 1$  integrin interaction in  $\alpha 3$  integrin N-linked glycan-dependent manner, which favors this complex to migrate into lipid rafts on the plasma membrane (59). Another study by Wang et al showed that N-linked glycans of  $\alpha 5$  integrin associates with ganglioside GT1b, altering adhesive activity of  $\alpha 5\beta 1$  integrin on fibronectin (60). These results suggest that integrin glycosylation can modulate receptor-ligand activity by either directly altering ligand binding affinity (i.e. sialylation) or by affecting integrin localization on plasma membrane (i.e.  $\beta$  (1,6) branching) (54, 61). Indeed, it was shown that increased  $\beta(1,6)$  branched N-linked glycans of  $\beta 1$  integrin caused a reduced  $\alpha 5\beta 1$  integrin clustering when cells were allowed to spread on fibronectin coated surface (see appendix). We now have some evidence that level of  $\beta(1,6)$  branched N-glycans of  $\beta 1$  integrin influences  $\beta 1$  integrin internalization upon ligand binding by inhibition of integrin clustering. A new hypothesis involving  $\beta 1$  integrin glycosylation and its effect on integrin recycling and cell migration is discussed in the Chapter.4 conclusion. Finally, I would like to briefly introduce Vacuolar  $H^+$ -ATPase (V-ATPase) and its 16kDa subunit, whose association with  $\beta 1$  integrin in the ER was shown to regulate  $\beta 1$  integrin glycosylation, maturation, and surface expression.

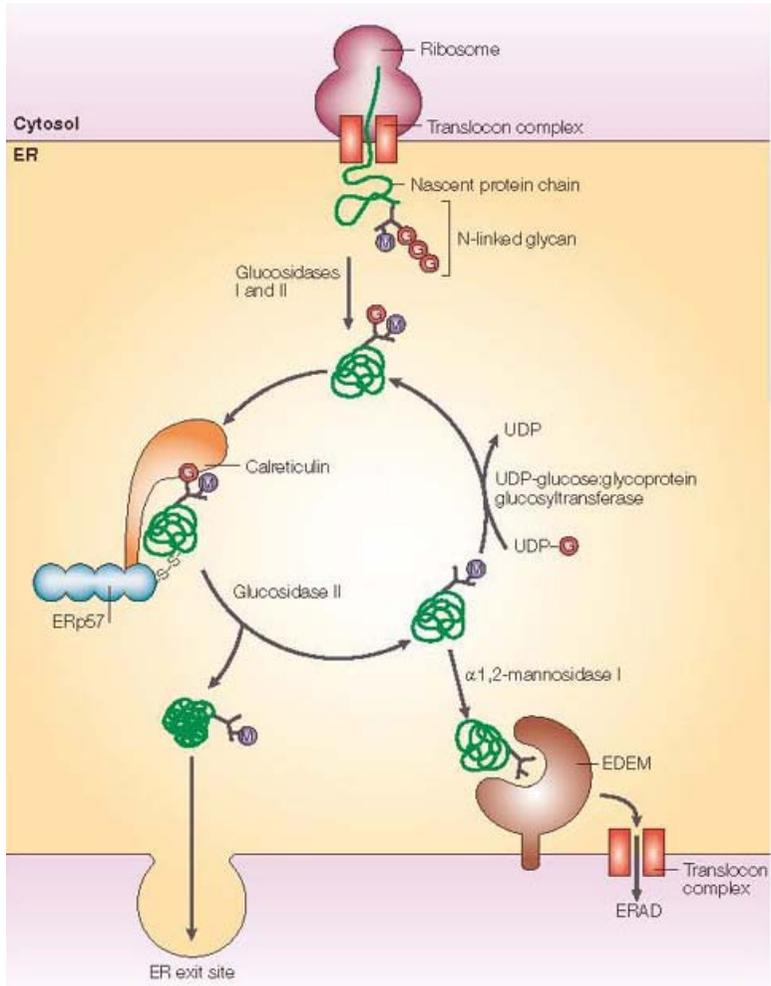
**V-ATPase 16K subunit association with  $\beta 1$  integrin inhibits glycosylation, maturation and surface expression of  $\beta 1$  integrin.**

Integrin is known to interact with a variety of proteins both inside the cells and in extracellular space. Skinner et al reported that  $\beta 1$  integrin and the vacuolar  $H^+$ -ATPase (V-ATPase) 16kDa (16K) subunit associate via transmembrane-transmembrane interaction (62), and later suggested that 16K overexpression causes inhibition of  $\beta$  (1,6) branched N-linked glycans, resulting in suppression of  $\beta 1$  integrin-dependent cell migration toward laminin and fibronectin (63). This conclusion led to the in-depth study to determine the mechanism of this specific inhibition on  $\beta(1,6)$  branching (see Chapter.2). In the same reports, the authors found that transmembrane domain 4 of 16K is directly involved in the binding activity with  $\beta 1$  integrin transmembrane domain. V-ATPase (Fig.1.5) is a multisubunit complex proton pump that is expressed in intracellular membrane bound compartments, such as the Golgi, ER, endosomes, lysosomes, and clathrin-coated vesicles (64). It is mainly responsible for acidification of transport vesicles and Golgi complex, while recent studies revealed that V-ATPase has a few other interesting functions, including the interaction with papilloma virus E5 oncoprotein-PDGF receptor complex in E5-induced PDGF receptor-dependent cell transformation,  $\beta 1$  integrin glycosylation, and a role in endosomal vesicle fusion, as well as multi-drug resistance phenotypes found in cancer chemotherapy (62, 63, 65-69). Surprisingly, all of these known roles of V-ATPase are directly or indirectly mediated by 16K, which forms a hexameric transmembrane proton pore. While there are several examples of interaction between pH regulating ion channels and structural/signaling components of cell adhesion protein complex (70, 71), these reports indicate that intracellular pH regulators are directly involved in cell migration via regulation of both actin cytoskeleton assembly and  $\beta 1$  integrin expression or recycling. Chapter.2 summarizes the results from this study, in which it was shown that the interaction between V-ATPase 16K and  $\beta 1$  integrin results in Triton X-100 insoluble aggregation

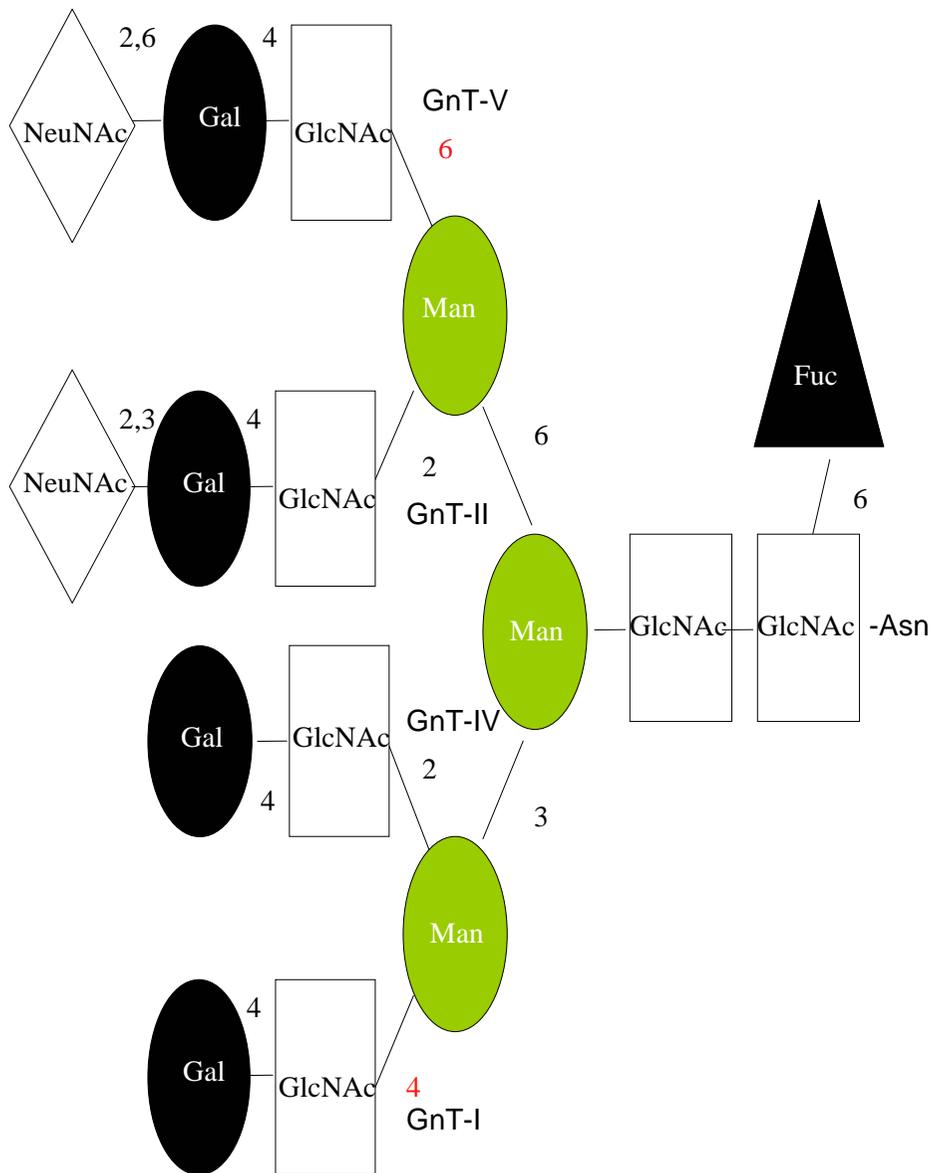
of minimally glycosylated form of  $\beta 1$  integrin in a novel mechanism, involving inhibition of calnexin- $\beta 1$  integrin association by 16K in the ER. A hypothesis on the potential outcome of this effect in the multi-drug resistance of cancer chemotherapy is discussed in Chapter.4 conclusion.

### **Goals**

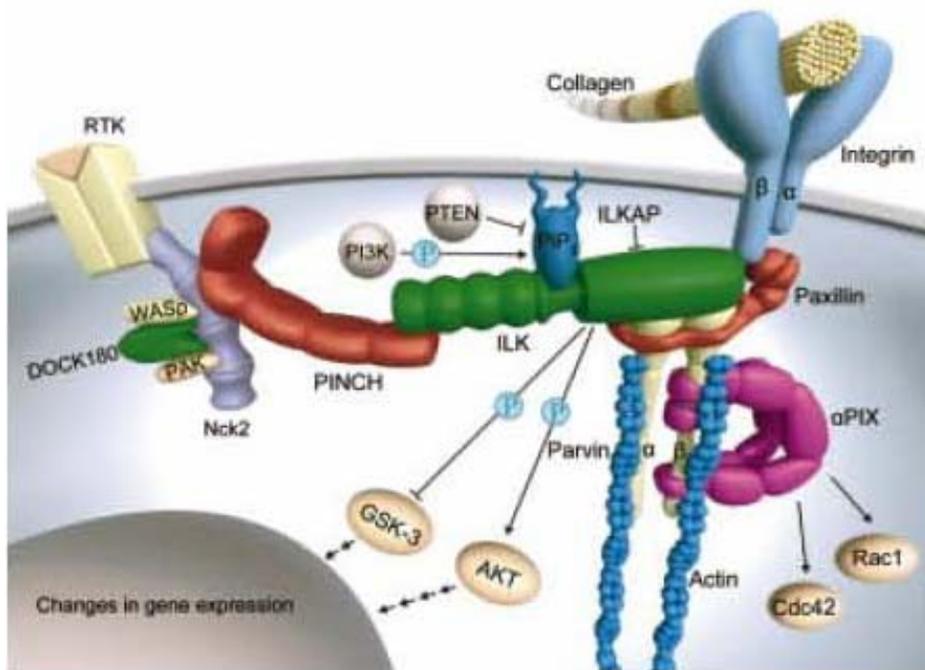
This thesis project focused on (i) the mechanism by which a change in the level of  $\beta$  (1,6) branched N-linked glycans results in regulation of rate of  $\beta 1$  integrin-dependent cell migration; (ii) how  $\beta 1$  integrin with different level of  $\beta$  (1,6) branching can affect integrin recycling; (iii) how V-ATPase 16K subunit interaction with  $\beta 1$  integrin affects biosynthesis, expression and function of  $\beta 1$  integrin; (iv) whether a brain-specific isozyme of GnT-V, GnT-VB, can affect the rate of neurite outgrowth in various neuronal cell lines.



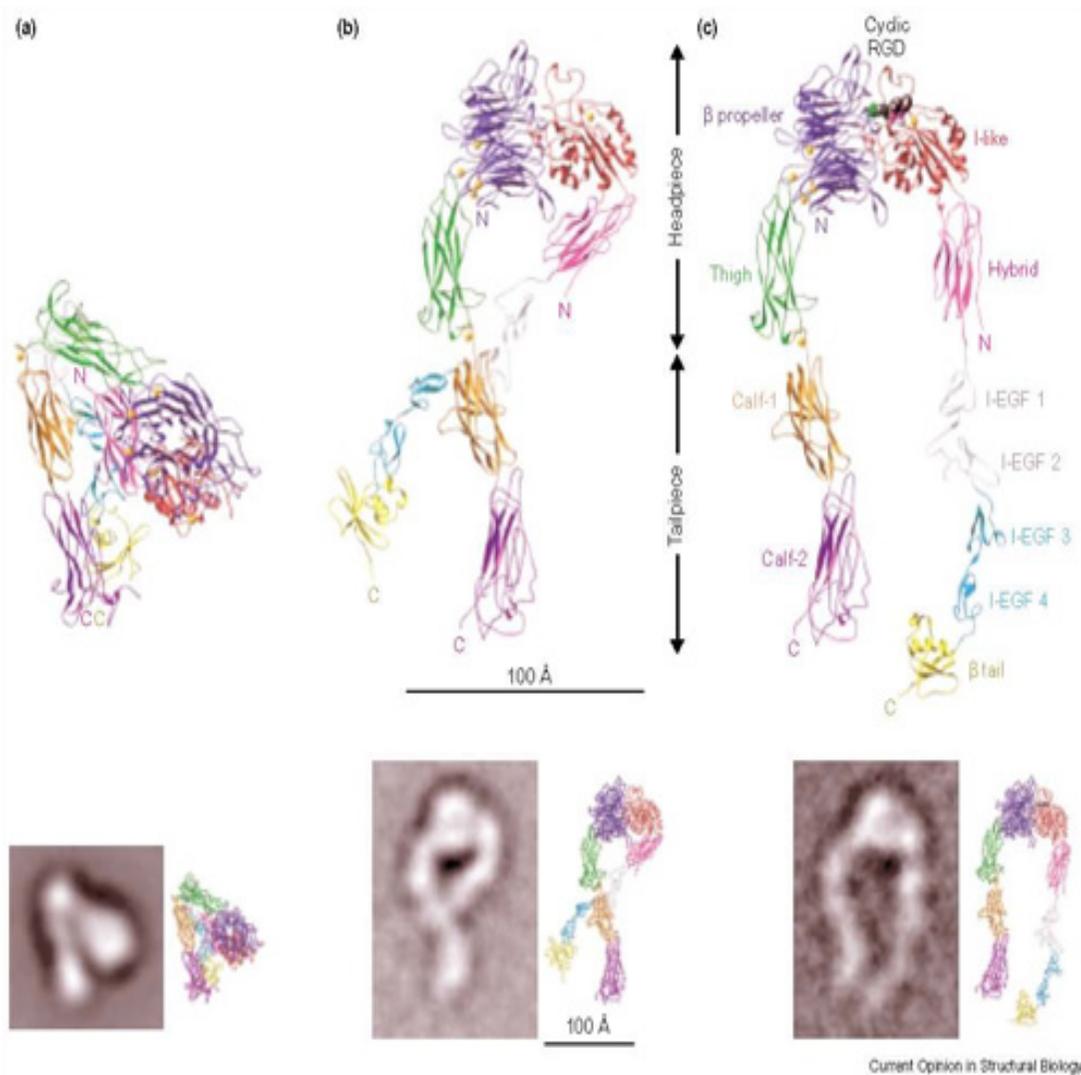
**Fig.1.1** Glycoprotein quality control by calnexin/ calreticulin cycle (Ellgaard and Helenius, Nature review molecular cell biology, 2003)



**Fig.1.2** Specific branching of N-linked glycans by various members of N-acetylglucosaminyltransferases family



**Fig.1.3** Integrins are heterodimeric glycoproteins and function as cell adhesion receptors (Brakebusch and Fassler, EMBO J, 2003)



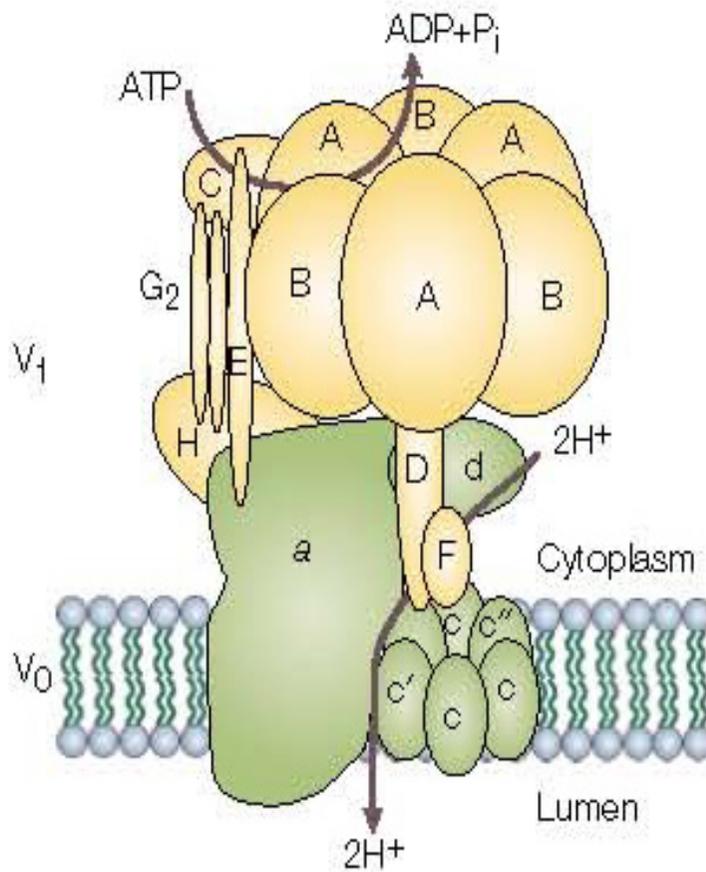
**Low affinity**

**Intermediate affinity**

**high affinity**

**Fig.1.4** Integrin conformation determines integrin-ligand binding affinity (Springer TA, 2002, Current Opinion in Structural Biology)

## V-ATPase



**Fig.1.5** Schematic illustration of V-ATPase subunit assembly. (Nishi and Forgac, Nature review Mol Cell Biol, 2002)

**Table.1.**

**Integrin-mediated Signaling**

ECM-Integrin

Focal Adhesion formation

<i>FAK-associated Kinase activation(<math>\beta</math>1)</i>	<i>Cell adhesion and motility via stress fiber formation</i>	<i>Apoptosis suppression and activation</i>	<i>interaction with growth factor-R</i>
FAK	1) MAPK	Anchorage	RTK Fyn/Shc
Src	MLCK	FAK	Ras
Grb2/mSos	2) EGF/PDGF-R (RTK)	PI3K	Raf
Ras	Cdc42 (Filopodia)	Akt	MEK
Raf	Rac (Lamellipodia)		ERK
MEK	Rho (Actin stress fiber)		Fos
MAPK	RhoK PIP <sub>2</sub>		
Fos	MLC PKC/Akt	Apoptosis	
Jun			
AP-1/ATF2			

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## CHAPTER 2

### EXPRESSION OF THE VACUOLAR H<sup>+</sup>-ATPASE 16 KDA SUBUNIT RESULTS IN THE TRITON X-100- INSOLUBLE AGGREGATION OF $\beta$ 1 INTEGRIN AND REDUCTION OF ITS CELL SURFACE EXPRESSION<sup>1</sup>

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<sup>1</sup>Intack Lee, Mhairi A. Skinner, Hua-bei Guo, Avinash Sujana and Michael Pierce (2004) Journal of Biological Chemistry 279(51), 53007-53014 Reprinted here with permission of publisher, ©The American Society of Biochemistry and Molecular Biology

## **Abstract**

Vacuolar H<sup>+</sup>-ATPase (V-ATPase) functions as a vacuolar proton pump and is responsible for acidification of intracellular compartments such as the endoplasmic reticulum, Golgi, lysosomes and endosomes. Previous reports have demonstrated that 16 kDa (16K) subunit of V-ATPase, via one of its transmembrane domains, TMD4, strongly associates with  $\beta$ 1 integrin, affecting  $\beta$ 1 integrin N-linked glycosylation and inhibiting its function as a matrix adhesion receptor. Because of this dramatic inhibition of  $\beta$ 1 integrin-mediated HEK-293 cell motility by 16K expression, we investigated the mechanism by which 16K was having this effect. Using HT1080 cells, whose  $\alpha$ 5 $\beta$ 1 integrin-mediated adhesion to fibronectin has been extensively studied, expression of 16K also resulted in reduced cell spreading on fibronectin-coated substrates. A pulse-chase study of  $\beta$ 1 integrin biosynthesis indicated that 16K expression down-regulated the level of the 110 kDa biosynthetic form of  $\beta$ 1 integrin (premature form) and, consequently, the level of the 130 kDa form of  $\beta$ 1 integrin (mature form). Further experiments showed that the normal levels of association between the premature  $\beta$ 1 integrin form and calnexin were significantly decreased by expression of either 16K or one of its transmembrane segments, TMD4. Expression of 16K also resulted in a Triton-insoluble aggregation of an unusual 87 kDa form of  $\beta$ 1 integrin. Interestingly, both western blotting and a pulse-chase experiment showed co-immunoprecipitation of calnexin and 16K. These results indicate that 16K expression inhibits  $\beta$ 1 integrin surface expression and spreading on matrix by a novel mechanism that results in reduced levels of functional  $\beta$ 1 integrin.

## Introduction

Vacuolar H<sup>+</sup>-ATPase (V-ATPase) is a multi-subunit complex found in all eukaryotic cells and consists of the V1 domain, which contains the ATPase activity, and the V0 domain, which contains transmembrane proton channel. V-ATPase is ubiquitously expressed in the various intracellular compartments and is responsible for their acidification (1, 2). The 16-kDa subunit (16K) of the V0 domain is a highly hydrophobic protein, consisting of four transmembrane segments, and it forms a homohexamer that functions as the transmembrane proton channel. The 16K can associate with other proteins through interactions with its fourth transmembrane segment, TMD4. For example, the papillomavirus E5 oncogene product associates with the TMD4 of 16K, decoupling 16K from the ATPase V1 and resulting in an elevated pH in the Golgi and facilitation of the oncogenic transformation process. In addition, the complex between bovine papillomavirus E5 and 16K can associate with the PDGF receptor, leading to a ligand-independent activation of PDGF receptor signaling (3).

$\beta$ 1 integrin performs a critical function in the anchorage-dependent growth of many cell types and is transcriptionally regulated by a variety of factors during development and oncogenic transformation (4-9). The TMD4 of 16K has also been shown to mediate an association between 16K and  $\beta$ 1 integrin (10). Subsequently, it was shown that 16K expression in HEK-293 cells caused altered N-linked glycosylation of  $\beta$ 1 integrin, resulting in a significant inhibition of cell migration toward laminin and fibronectin (10, 11). Since altered expression of N-linked glycans on the  $\alpha$ 5 $\beta$ 1 integrin has been shown to significantly affect the binding of cell surface  $\alpha$ 5 $\beta$ 1 to fibronectin, it was reasonable to hypothesize that the expression of 16K had specific effects on

the glycosylation of  $\beta 1$  integrin, thereby inhibiting its adhesive functions that regulate migration on extracellular matrix.

In this study, we investigated the mechanism of how 16K expression levels affect the expression and/or the maturation and glycosylation of  $\beta 1$  integrin during the biosynthesis of the  $\alpha 5\beta 1$  dimer to ultimately inhibit  $\beta 1$  integrin-dependent cell adhesion to fibronectin. The results show that expression of 16K in human fibrosarcoma HT1080 cells down-regulates surface expression of  $\alpha 5\beta 1$  integrin, thereby causing reduced cell spreading on fibronectin-coated surfaces. The loss of cell surface  $\alpha 5\beta 1$  integrin expression was subsequently shown to result from a Triton X100-insoluble aggregation of  $\beta 1$  integrin subunits in the endoplasmic reticulum. Initial results suggested that 16K expression suppressed the maturation of  $\beta 1$  integrin from its premature form that expressed only high-mannose and not complex glycans. Pulse-chase experiments then showed that premature (110 kDa)  $\beta 1$  integrin levels in 16K-transfected cells were greatly decreased, and that this decrease occurred during the first 15 min of  $\beta 1$  integrin biosynthesis. Further experiments using antibodies against calnexin, calreticulin and BiP showed that calnexin, but neither calreticulin nor BiP, associated with the premature  $\beta 1$  integrin. When 16K was expressed, the normal association between the premature  $\beta 1$  integrin and calnexin was almost completely abolished. Expression of the TMD4 alone caused the same level of inhibition on  $\beta 1$  integrin association with calnexin. Surprisingly, both 16K and TMD4 were themselves co-immunoprecipitated with calnexin. Examination of the Triton X-100-insoluble fraction of cells transfected with 16K revealed a new form of  $\beta 1$  with a molecular weight of about 87 kDa. The unusually rapid kinetics of the decrease of the premature (110KDa)  $\beta 1$  integrin and the appearance of a Triton-insoluble 87kDa form of  $\beta 1$  suggests that the decrease of the 110KDa

form was not due primarily to ER-associated degradation (ERAD), but due to an induction of the Triton-insoluble form of  $\beta 1$ . These results suggest a unique mechanism for the regulation of the cell surface expression of  $\beta 1$  integrin by expression of the V-ATPase 16K subunit.

## **Experimental procedures**

***Cell culture, transfections, and materials*** – HT1080 human fibrosarcoma cells were obtained from ATCC and maintained in DMEM containing 10% FBS. Unless otherwise described, all transfections were done transiently using lipofectamine reagent (Gibco) in 100 mm dishes. Cells were harvested typically between 24 hours and 48 hours after transfection and used for further experiments. Rat monoclonal antibodies against human  $\alpha 5$  and  $\beta 1$  integrin, mAb16 and mAb13, respectively, were gifts from Dr. Steven Akiyama, National Institute of Environmental Health Sciences. The anti-T7 and HSV tagged antibodies were purchased from Novagen. Antibodies against calnexin, calreticulin and BiP were purchased from Stressgen. Agarose-conjugated L-PHA, ConA, SNA, and DSA lectins were obtained from Vector Labs, while MG132, an inhibitor of proteasome activity, was from Biomol. PNGase-F was obtained from Sigma, and Streptavidin-HRP from Rockland. NHS-LC-Biotin was purchased from Pierce. Anti-ERK2 antibody, anti-actin antibody, Protein G Plus-Agarose and all secondary antibodies conjugated to HRP were from Santa Cruz Biotechnology. Goat anti-mouse IgG-Alexa 594, and Phalloidin-FITC were all purchased from Molecular Probe. Mammalian expression plasmids, pXJ40-KKO-16K (HSV-tagged 16K), pXJ40-KKO-TMD4 (HSV-tagged TMD4; formerly named  $\alpha 4$ ), and pXJ41- $\beta 1$ -T7 (T7-tagged bovine  $\beta 1$  integrin) were obtained as previously described (11).

***Immunoprecipitation and Western blot analysis*** – Cells were lysed in a lysis buffer consisting of 2% Triton X-100, protease inhibitors and 0.1% SDS. Typically, 100 µg total protein was used for immunoprecipitation and lectin precipitation. Proteins were resolved with SDS-PAGE utilizing 8% gels under non-reducing conditions unless otherwise indicated, transferred to PVDF membranes, blocked in 2% BSA, and probed with antibodies. Following incubation with HRP-conjugated secondary antibodies or streptavidin-HRP, proteins were detected by the ECL reagent (Pierce). Densitometry of the blots was performed using a Bio-Rad Fluor S imager.

***Cell surface biotinylation and immunoprecipitation*** – Cell surfaces were labeled with NHS-LC-Biotin as described previously (12). Briefly, cells were washed three times with ice cold PBS in culture dishes. One mg/ml NHS-LC-Biotin in PBS (1 ml) was added to the cells and incubated at 4 °C for 15 min. Cells were then washed three times in TBS and lysed in lysis buffer. Immunoprecipitation and western blotting were done as described above, except that streptavidin-HRP was directly added with no primary antibody.

***Radiolabeling and autoradiography*** – Cells were pre-incubated with Met/Cys free DMEM (Gibco) for 30 min, followed by labeling with 100 µCi/ml [<sup>35</sup>S]-methionine/cysteine in 3 ml of DMEM lacking methionine and cysteine, but containing 2% dialyzed FBS for 15 min. After removing the radioactive medium, regular growth medium was added and the cells incubated for various times. Labeled cells were then lysed in lysis buffer, and cell lysates was immunoprecipitated with either mAb13 or anti-T7 antibody at 4 °C overnight. For an experiment with MG132, 50 µM final concentration of MG132 in DMSO was added to the cells during both pulse and chase period.

**Realtime PCR analysis** – Cells were transfected with pXJ41- $\beta$ 1-T7 alone (5  $\mu$ g) or pXJ41- $\beta$ 1-T7 plus pXJ40-KKO-16K (5  $\mu$ g of each) and cultured for 24 hours. The RNeasy kit (Qiagen) was used to isolate total RNA. Reverse transcription was done using Superscript III (Stratagene) and random primers using the manufacturer's protocol. Bio-Rad iCycler and iCycler software were used for realtime PCR reactions and data analysis. Primers were designed using Primer3 software. Sequences of primers used in the experiment were as follows: human  $\alpha$ 5 integrin, forward, 5'-CACTGGCCATGATGAGTTTG-3'; reverse, 5'-CGATGGCCACATCATTGTAG-3'; human  $\beta$ 1 integrin, forward, 5'-ATCCCAGAGGCTCCAAAGAT-3'; reverse, 5'-CCCCTGATCTTAATCGCAAA-3'; human 16K, forward, 5'-AGTCCATCATCCCAGTGTG-3'; reverse, 5'-CAGCTGGAGGAAGCTCT TGT-3'; human GAPDH, forward, 5'-CAATGACCCCTTCATTGACC-3'; reverse, 5'-AATCCCATCAC CATCTTCCA-3'. Real-time PCR was performed using the iQ<sup>TM</sup> SYBR Green Supermix (Bio-Rad). The PCR conditions were 95°C, 30 sec, 1 cycle, followed by 40 cycles at 95°C for 10 sec, 65°C for 30 sec; 95°C for 1 min, and 55 °C for 1 min, terminating with 80 cycles at 55°C for 10 sec for melting curve analysis. Standard curves for human  $\alpha$ 5 and  $\beta$ 1 integrin primer characterization were generated by a serial dilution of cDNA obtained from the reverse-transcriptase reaction, while pXJ40-KKO and pT3-GAPDH were used for standard curve generation for 16K and GAPDH, respectively.

**Fluorescent Staining** — Chamberslides (Falcon) were coated with 10  $\mu$ g/ml fibronectin (Sigma) and incubated at 4 °C overnight. Transiently transfected cells were harvested by trypsinization and incubated in suspension in serum free DMEM for 1 hour at 37 °C. Approximately 10<sup>4</sup> cells were added to each well and allowed to spread on fibronectin coated surfaces for 30 min. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS,

permeabilized with 0.5% Triton X-100 in PBS for 15 min, and blocked with 1% BSA for 30 min at 37 °C. Cells were then incubated with anti-HSV tag antibody (1:10000) and mAb13 (10 µg/ml) at 37 °C for 30 min. After washing three times with PBS, the primary antibodies were detected by incubation with either Rhodamine-anti-mouse IgG conjugate or Rhodamine-anti-rat IgG (1:250) at 37 °C for 30 min. For actin staining, phalloidin-FITC (1:250) was used. After washing with PBS, the coverslips were mounted and images were obtained using an inverted fluorescence microscope (Leica).

## **Results**

Expression of the V-ATPase 16K subunit in HEK-293 cells inhibited significantly  $\beta$ 1 integrin-mediated cell adhesion and migration on fibronectin and laminin (11). In addition, expression of 16K appeared to have an inhibitory effect on the complex N-linked glycosylation of  $\beta$ 1 integrin, suggesting that the inhibition of matrix adhesion may have resulted from altered glycosylation of  $\beta$ 1 integrin. In order to test this hypothesis in detail, we used HT1080 cells to examine the effects of 16K expression on  $\beta$ 1 integrin-fibronectin interactions, as well as the biosynthesis of  $\beta$ 1. The predominant  $\beta$ 1 integrin expressed in HT1080 cells is  $\alpha$ 5 $\beta$ 1 integrin (12), and these cells have been used to investigate the effects of altered N-linked glycosylation on  $\alpha$ 5 $\beta$ 1 integrin-mediated cell adhesion and migration on fibronectin (12, 13).

### ***16K expression delays cell spreading and reduces cell surface expression of $\alpha$ 5 $\beta$ 1 integrin. -***

First, we confirmed that 16K expression affected  $\alpha$ 5 $\beta$ 1 integrin-mediated HT1080 cell adhesion and spreading on fibronectin. HT1080 cells were transiently transfected with 16K tagged with

the HSV epitope, harvested, pre-incubated in suspension in serum-free DMEM for 1 hour, added to fibronectin-coated chamber slides, and allowed to spread for 30 min. Phalloidin-FITC was used to visualize the actin cytoskeleton of the cells and anti-HSV used to detect 16K expression. The results (Fig.2.1) show that cells transfected with vector alone (mock) showed extensive spreading on the fibronectin substrate. By contrast, cells transfected with 16K-HSV were attached to the substrate, but were rounded in appearance due to the inhibition of spreading. Experiments were then performed to examine the effects of 16K expression on  $\beta 1$  integrin cell surface expression using cell surface biotinylation. The results showed a gradual decrease of  $\alpha 5\beta 1$  integrin expression on the cell surface as the amount of 16K plasmid used for transfection was increased. Cell surface expression of both the EGF receptor and N-cadherin showed no significant decrease after transfection with 16K plasmid (15  $\mu\text{g}$ ), demonstrating a specific inhibitory effect on  $\alpha 5\beta 1$  integrin (Fig.2.2).

***Characterization of premature and mature forms of  $\beta 1$  integrin***—As a first step to understand the possible effects of 16K expression on  $\beta 1$  integrin cell surface expression, it was necessary to characterize its biosynthesis. Typically,  $\beta 1$  integrin is first expressed as the so-called “premature form” (110 kDa), which dimerizes with  $\alpha$  subunits and is transported to the Golgi complex where it then matures into the 130 kDa form that expresses complex N-linked glycans (7, 14, 15). The premature and mature forms can also be distinguished on Western blots by the binding of specific lectins. Concanavalin A (ConA), which binds high-mannose, hybrid, and diantennary glycans, binds both premature and mature forms of  $\beta 1$ , while L-phytohemagglutinin (L-PHA) and *Sambucus nigra* agglutinin (SNA), which recognize  $\beta$  (1,6) branched and  $\alpha$  (2,6) sialylated glycans, respectively, bind only to the mature  $\beta 1$  form. When T7-tagged bovine  $\beta 1$  integrin ( $\beta 1$ -

T7) was expressed in HT1080 cells, both mature and premature forms of the T7-tagged integrin were detected by Western blotting of cell lysates (Fig.2.3A). The relatively higher levels of the premature form were likely due to limiting levels of integrin  $\alpha$  subunits to allow dimerization with  $\beta$ 1 which is a requirement for Golgi processing and complex N-linked glycosylation. Fig. 2.3A also shows that only the mature (130 kDa) form of  $\beta$ 1 was precipitated with a monoclonal antibody (mAb16) against human  $\alpha$ 5 integrin and the lectins recognizing complex N-linked glycan modifications, L-PHA and SNA, but the premature (110 kDa) form was precipitated only by ConA.

***Premature forms of  $\beta$ 1 integrin were down-regulated by 16K expression*** –When HSV-tagged 16K (16K-HSV) was co-transfected with  $\beta$ 1-T7, expression of the mature  $\beta$ 1 form was preferentially decreased. Immunoprecipitation of the lysate of cells transfected with  $\beta$ 1 integrin or  $\beta$ 1 integrin plus 16K with the antibody specific for the  $\alpha$ 5 subunit (mAb16), revealed that the mature  $\beta$ 1 integrin co-immunoprecipitated with  $\alpha$ 5 integrin was significantly decreased after 16K expression (Fig.2.3B). In order to determine whether increased 16K expression suppressed expression of the premature form as well, the amount of 16K plasmid used for transfections was increased successively, while keeping the amount of  $\beta$ 1-T7 plasmid constant. The results showed that levels of the premature  $\beta$ 1 form were greatly decreased after 16K expression. The levels of  $\alpha$ 5 integrin and  $\beta$ -actin, however, showed no significant decrease as the expression of 16K increased, demonstrating that the inhibitory effect on  $\beta$ 1 expression was specific (Fig.2.3C). Next, when cell lysates from cells co-transfected with  $\beta$ 1 integrin and 16K-HSV were immunoprecipitated with anti-HSV antibody,  $\beta$ 1 co-immunoprecipitated with the anti-HSV tag

antibody, again demonstrating that 16K associated with  $\beta 1$  integrin. After SDS-PAGE and Western blotting, the  $\beta 1$  that co-immunoprecipitated with 16K migrated similarly to  $\beta 1$  integrin treated with Endo H (data not shown).

Normally, the premature  $\beta 1$  integrin form does not persist for more than 2 hours before complete  $\alpha$ - $\beta 1$  dimerization and maturation into the mature 130 kDa form (Fig.2.4A). In order to determine if 16K expression had similar effects on the endogenous  $\beta 1$  integrin expression and maturation as it had on overexpressed  $\beta 1$ , we studied biosynthesis of the endogenous  $\beta 1$  using metabolic labeling and an anti- $\beta 1$  integrin antibody. A pulse-chase experiment with cells transfected with 16K plasmid (10  $\mu$ g) revealed that there was a significant decrease in endogenous premature  $\beta 1$  integrin levels as well, resulting in an overall lower level of  $\alpha 5\beta 1$  integrin after 2 hours of chase (Fig.2.4A). In order to determine the point when the decrease of exogenously expressed  $\beta 1$  integrin began, we performed another pulse-chase study with  $\beta 1$ -T7 and 16K-HSV co-transfected cells. In these cells, premature  $\beta 1$  integrin levels were already down-regulated by approximately 80% at the 0 min chase time (Fig.2.4B). In cells transfected with  $\beta 1$ -T7 alone, there was no significant change of premature  $\beta 1$  integrin level during the 15 min pulse and chase, suggesting that the majority of the premature  $\beta 1$  decrease caused by 16K expression occurred during the 15 min pulse labeling (Fig.2.4B). Since the ER-associated degradation pathway (ERAD) serves as a central pathway for discarding misfolded or incompletely assembled subunits of oligomeric complexes (16-18), we tested whether the decrease of  $\beta 1$  integrin levels is due to degradation by the conventional proteasome-dependent ERAD pathway using a proteasome specific inhibitor, MG132. The result showed that the decrease of  $\beta 1$  integrin levels is not mediated by the ERAD pathway (Fig.2.4B), though the

presence of MG132 (during both pulse and chase) resulted in a slightly increased level of premature  $\beta 1$  integrin. Both this result and the unusually rapid kinetics of the decrease of premature  $\beta 1$  integrin levels (within 15 min) suggested that the decreased  $\beta 1$  integrin level was likely due to degradation by a different mechanism.

***The decreased premature  $\beta 1$  integrin levels resulting from 16K expression were not due to transcriptional down-regulation*** – Since lowered premature  $\beta 1$  integrin levels after 16K expression may be caused by a decrease in the transcription of  $\beta 1$  integrin, we studied whether 16K expression could alter transcription of either  $\alpha 5$  or  $\beta 1$  integrin using real time PCR analysis. There was no significant difference in the transcription levels of either  $\alpha 5$  and  $\beta 1$  integrin (Fig.2.5), indicating that the decrease in premature  $\beta 1$  integrin levels following expression of 16K was not due to transcriptional down-regulation of  $\beta 1$  integrin.

***Expression of either 16K or the transmembrane domain 4 (TMD4) of 16K inhibited association of the premature  $\beta 1$  integrin with calnexin*** – During the biosynthesis of glycoproteins in the ER, protein folding and oligomeric assembly is mediated by various ER-resident lectin and non-lectin chaperones, including calnexin, calreticulin and BiP (18-20). Both integrin  $\alpha$  and  $\beta 1$  premature forms associate with calnexin, which is known to result in a dramatically increased ER resident time of unpaired integrin subunits (21, 22). Therefore, the interaction of  $\beta 1$  integrin with these chaperones was studied to examine whether 16K expression alters chaperone interactions during folding of premature  $\beta 1$  integrin in the ER. Interestingly, co-transfection of  $\beta 1$  integrin and 16K caused a marked decrease in the association of the premature  $\beta 1$  integrin with calnexin, but there was no association with calreticulin or BiP either

before or after expression of 16K (Fig.2.6A). Because of the endogenous function of 16K as a component of V-ATPase, we hypothesized that an intracellular pH change resulting from increased expression of the proton channel might be responsible for the effects on the decreased association between calnexin and the premature  $\beta$ 1. Therefore, as one way to test this hypothesis, since TMD4 serves as the domain of 16K that binds  $\beta$ 1 integrin (10, 11), we expressed the HSV-tagged transmembrane domain 4 (TMD4) of 16K (11) to determine whether TMD4 alone could functionally replace 16K and exert the same level of inhibition on calnexin association with premature  $\beta$ 1 subunits. Expression of TMD4 alone was sufficient to induce all the effects of 16K expression on  $\beta$ 1 integrin reported above, including inhibition of premature  $\beta$ 1 subunit association with calnexin (Fig.2.6B). Furthermore, neither 16K expression nor TMD4 expression showed an effect on  $\alpha$ 5 integrin association with calnexin, demonstrating the specificity of its interaction with the  $\beta$ 1 subunit.

***Calnexin interacts with 16K or TMD4***— A possible explanation for the inhibition of interaction between the premature  $\beta$ 1 subunit and calnexin by 16K was that calnexin could also associate with 16K. The results of experiments to test this possibility showed, surprisingly, that both 16K and TMD4 were co-immunoprecipitated with calnexin (Fig.2.6B), demonstrating an association between 16K and calnexin. This result was obtained both by immunoprecipitation of lysates of cells overexpressing 16K with anti-calnexin antibody, followed Western blotting with anti-HSV tag antibody, and by anti-HSV tag antibody immunoprecipitation, followed by Western blotting with anti-calnexin antibody (Fig.2.7A). To further confirm this interaction, a pulse-chase study with both 16K-HSV and TMD4-HSV expressing cells was performed using an anti-HSV tag antibody (Fig.7B), and in both cases a 90 kDa band was co-immunoprecipitated. This band was

no longer co-immunoprecipitated with 16K after 4 hr. of chase; by contrast, TMD4 was still co-immunoprecipitated with this band after 4 hr.

***Expression of 16K caused a Triton-insoluble non-covalent aggregation of an 87 kDa form of***

***β1 integrin*** – The results presented so far raise a question about the fate of the β1 integrin forms that were not immunoprecipitated with calnexin (Fig.2.6B) after 16K expression. Since the decrease in premature β1 integrin levels was caused neither by transcriptional down-regulation nor ER-associated degradation (Fig. 4 and 5), and since calnexin association with glycoproteins promotes protein solubility, we hypothesized that an association of premature β1 integrin with 16K in the ER could result in an insoluble aggregation of the premature β1 subunits, that would then be excluded from the Triton X-100-soluble fraction of cell lysates used for analysis. To test this hypothesis, cells transfected with β1-T7 alone or β1-T7 plus 16K-HSV were lysed in 1% Triton X-100, and after assay of crude lysates for protein levels, equal amounts of lysate proteins from both transfectants were centrifuged as before. The Triton X-100 soluble (supernatant) fractions were subjected to SDS-PAGE, while the Triton X-100 insoluble pellets were boiled in SDS loading buffer under reducing or non-reducing conditions, subjected to SDS-PAGE, followed by Western blotting with anti-T7 tag antibody. The results showed that when 16K was expressed, a form of β1 integrin of lower apparent molecular weight (about 87 kDa) was detected in the Triton-insoluble pellet, but not in the Triton-soluble fraction (Fig.2.8A). This result suggested that the disappearance of β1 subunits in the soluble fraction of cell lysates after 16K expression was due to an increase in β1 subunit aggregation, which remained in the Triton-insoluble fraction. The aggregated 87 kDa form was detected after boiling the pellet in either reducing or non-reducing SDS sample buffer, demonstrating that the aggregation was not due to

disulfide bond formation. By contrast, neither  $\alpha 5$  integrin nor the cytosolic protein, ERK2, showed signs of this form of aggregation and insolubility in Triton (Fig.2.8A). Furthermore, the 87 kDa form of  $\beta 1$  was not precipitated with Con A, confirming that the shift of the apparent molecular weight of this form was due to incomplete N-linked glycosylation, while both molecular weight forms were precipitated with the anti-T7 antibody (Fig.2.8B).

In conclusion, our results have demonstrated that the V-ATPase 16K subunit can regulate surface expression of  $\beta 1$  integrin, blocking the  $\beta 1$  biosynthetic pathway by inducing a non-disulfide-dependent aggregation. This effect on biosynthesis thereby inhibits  $\beta 1$  integrin-mediated cell spreading on fibronectin.

## **Discussion**

These studies were initiated in order to investigate the mechanism by which the expression of the 16K subunit of the vacuolar  $H^+$  ATPase appeared to have a specific, inhibitory effect on the expression of complex N-glycans on  $\beta 1$  integrin, which, in turn, inhibited cell-matrix interactions. Several investigators have demonstrated that N-glycan expression on  $\beta 1$  integrin subunits is required for proper integrin-matrix interactions (13, 23, 24). Moreover, aberrant N-glycosylation of integrins that often occurs during oncogenic transformation results in modulation of integrin function, cell-matrix interactions, and cell invasiveness (12, 23, 25). Initially, the effects of 16K expression on  $\beta 1$  integrin biosynthesis were investigated, which led to the exploration of the possibility that an association between the 16K subunit and  $\beta 1$  integrin in the ER might be involved in the regulation of  $\beta 1$  subunit maturation and surface presentation. During the course of this study, however, we discovered a novel interaction between calnexin and 16K.

The V-ATPase proton channel consists of a circular arrangement of 6 of the 16K subunits. Each 16K subunit consists of four transmembrane helices, each of which consists of highly hydrophobic amino acids. About 70% of the 16K total amino acids are predicted to be imbedded in the membrane with very small loop regions, typically ranging from 11 to 19 AA in length predicted to lie outside the membrane. Transmembrane domain 4 (TMD4) is unique among the four TM domains of each subunit since it is predicted by homology to the yeast V-ATPase to be on the exterior of the proton channel where it could easily interact with adjacent protein transmembrane domains. The TMD4-HSV used in our experiments consists of 17 amino acids spanning between the third and the fourth TM regions and 22 amino acids of the fourth transmembrane domain, followed by 6 amino acids of the C-terminal region. Based on a recent model (26), the loop preceding the fourth transmembrane domain is found on the cytoplasmic side, while the C-terminal 6 amino acids tail is oriented toward the lumen of ER. Consequently, there are likely to be only relatively small non-membrane-embedded domains that could participate in the interaction of TMD4 with either the lectin domain or stem region of calnexin.

In the present study, it is unclear whether calnexin directly associates with non-glycosylated 16K in the ER, or whether other proteins are involved in this interaction. There are several examples, however, where calnexin seems to show chaperone activity toward highly hydrophobic transmembrane proteins, including CD9 (27, 28), PLP (29), and CD82 (30), suggesting a possible role of calnexin in the quality control of transmembrane domain assembly. These studies, taken with our current results, raise the possibility that calnexin may interact with 16K to function in the ER quality control of 16K biosynthesis.

Since one of the major functions of calnexin is to keep nascent polypeptides soluble for proper folding and oligomeric assembly by preventing aggregation, the inhibitory effect of 16K expression on the interaction between calnexin and  $\beta 1$  integrin likely promotes the subsequent Triton-insoluble aggregation of the  $\beta 1$  subunit. It is possible that the binding of 16K to premature  $\beta 1$  integrin alters the conformation of the  $\beta 1$  subunit so that they can no longer associate with calnexin, leading to aggregation of  $\beta 1$  integrin in the ER. Lack of inhibition of  $\alpha 5$  subunit-calnexin interaction by 16K provide supports for the hypothesis that binding of 16K to the  $\beta 1$  subunit is a prerequisite for the inhibition of integrin association with calnexin in the ER. The Triton-insoluble  $\beta 1$  integrin that resulted from 16K expression exhibited a molecular weight (87 kDa) lower than that of the premature  $\beta 1$  form (110 kDa), and it was not bound by Con A, by contrast to the premature form. The reason for the inability of the 87 kDa form to be bound by Con A is unclear, particularly since the mammalian N-glycanase has been reported only to be present in the cytosol (31).

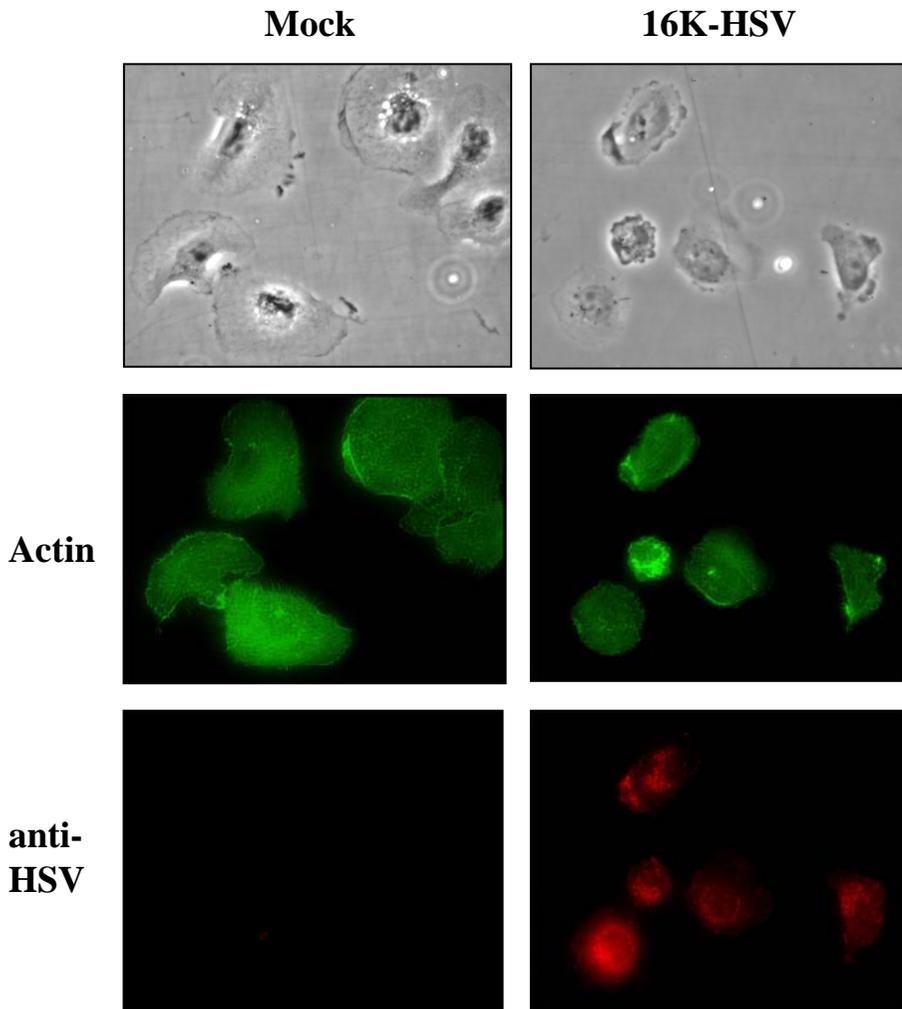
Salicioni *et al* recently reported that the low density lipoprotein-related protein-1 (LRP-1) regulated  $\beta 1$  integrin maturation and surface expression (32). Using LRP-1 deficient MEF cells, these authors demonstrated that LRP-1 deficiency hindered maturation of  $\beta 1$  integrin its surface expression, resulting in accumulation of premature  $\beta 1$  integrin in the ER. Our results demonstrate an additional example of a protein, 16K, whose expression can regulate surface expression of  $\beta 1$  integrin and  $\beta 1$  integrin mediated cell interactions with the ECM.

Several questions arise from our results: does endogenous 16K function in  $\beta 1$  integrin biosynthesis and surface expression? Is there a functional link between a protein involved in vacuolar proton transport and its relatively strong association with a cell adhesion protein such as  $\beta 1$  integrin? Interestingly, a link between another protein that regulates intracellular pH, the  $\text{Na}^+ - \text{H}^+$  exchanger isoform (NHE1), and the modulation of cell-matrix interactions has recently been established. Schwartz *et al* demonstrated that many anchorage dependent cells showed an alteration of their intracellular pH (pHi) upon cell spreading on matrix; however, NIH3T3 cells transformed with *src* or *ras* showed no pHi change when allowed to spread (33). More recently, several groups have reported that cell spreading on ECM caused a consistent intracellular pH (pHi) change that was shown to be mediated by activation of NHE1 via various signal transduction pathways, including RhoA, Cdc42, and  $\text{PIP}_2$ , and that pHi change upon cell spreading was required for stress fiber formation (34-36). Moreover, studies have revealed that the E subunit of the V1 ATPase domain associated with mSos1, resulting in enhanced guanine nucleotide exchange activity of Rac1, while the C subunit of the V1 domain mediated the binding of the V-ATPase to the actin cytoskeleton. Both of these effects are relevant to cell spreading and membrane protrusion (37, 38), suggesting the involvement of V-ATPase in various aspects of actin cytoskeleton rearrangement. In light of these studies, along with the reported ability of the TMD4 deletion mutant of 16K to induce NIH3T3 cell anchorage independent growth (39), our results suggest that there are likely to be additional factors involved in the pHi regulation of cell spreading of anchorage dependent cells and that pHi regulation of both cytosol and vacuolar systems may play an important role for preventing loss of the anchorage-dependent phenotype.

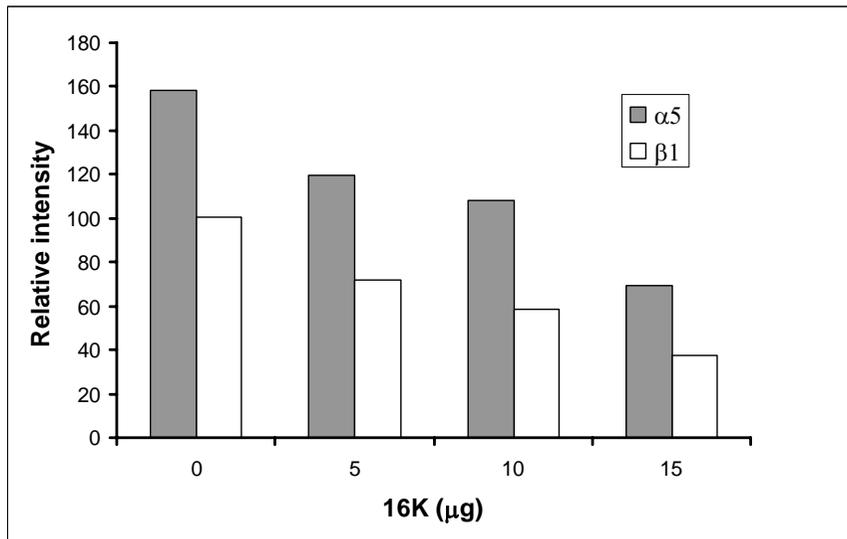
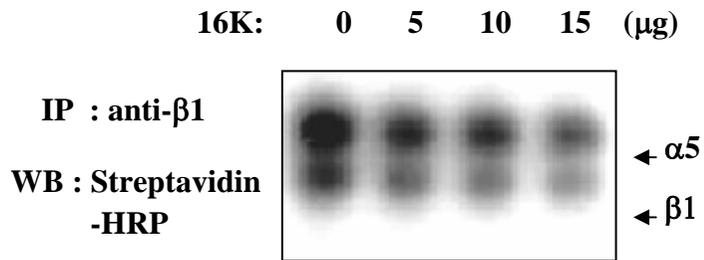
## **Acknowledgements**

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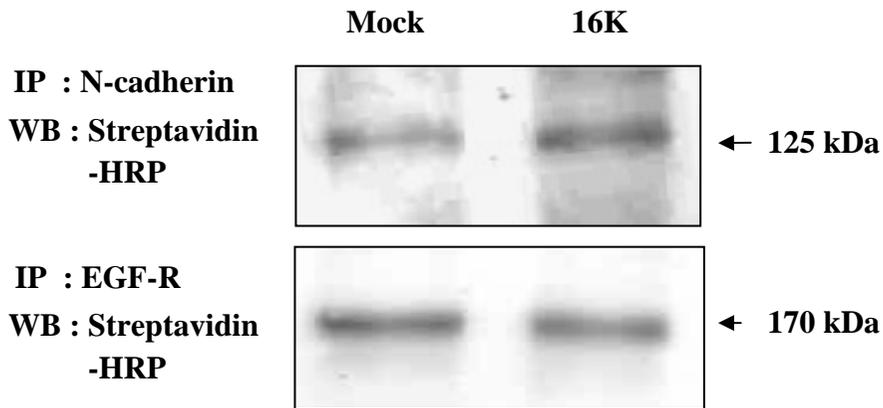
**Abbreviations:** L-PHA, L-phytohemagglutinin; ConA, Concanavalin A; SNA, Sambucus Nigra agglutinin; V-ATPase, vacuolar H<sup>+</sup>-ATPase; 16K, 16-kDa subunit; HSV, herpes simplex virus; PBS, phosphate buffered saline; TBS, Tris-buffered saline; PCR, polymerase chain reaction; ECM, extracellular matrix; ER, Endoplasmic reticulum; TMD4, transmembrane domain 4.



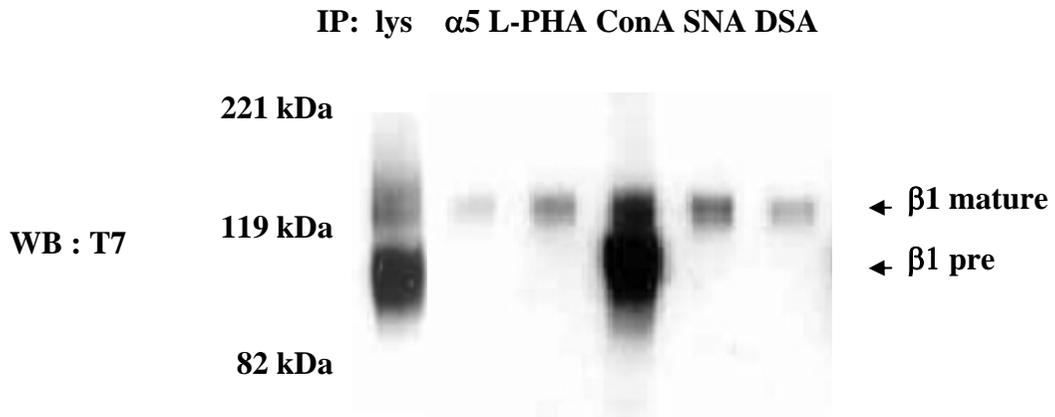
**Fig.2.1** 16K expression significantly inhibits cell spreading on fibronectin coated surface. Transiently transfected HT1080 cells were harvested by trypsinization after 24 hours and incubated in suspension in serum free media for 1 hour to allow the cells to recover. Approximately 10<sup>4</sup> cells were added to fibronectin coated (10  $\mu$ g/ml) chamberslides and allowed to spread for 30 min. Phalloidin-FITC (1:250) was used to visualize the actin cytoskeleton. Mouse monoclonal anti-HSV tag antibody (1:10,000) and Alexa 594 conjugated goat anti-mouse IgG (1:250) were used to detect the expression of 16K-HSV in the transfected cells.



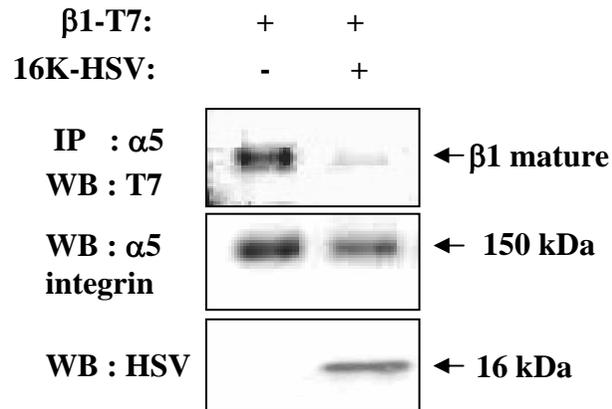
**Fig.2.2A** 16K expression down-regulates surface expression of  $\alpha5\beta1$  integrin in HT1080. Decreased surface expression of  $\alpha5\beta1$  integrin by 16K expression. Cells were transiently transfected with increasing amount of 16K expression plasmid. After 24 hours, cell surface proteins were labeled using NHS-LC-biotin and lysed in a lysis buffer containing 2% Triton X-100. Cell lysates were immunoprecipitated with anti-human  $\beta1$  integrin, mAb13, analyzed by SDS-PAGE and blotted with HRP-conjugated Streptavidin.



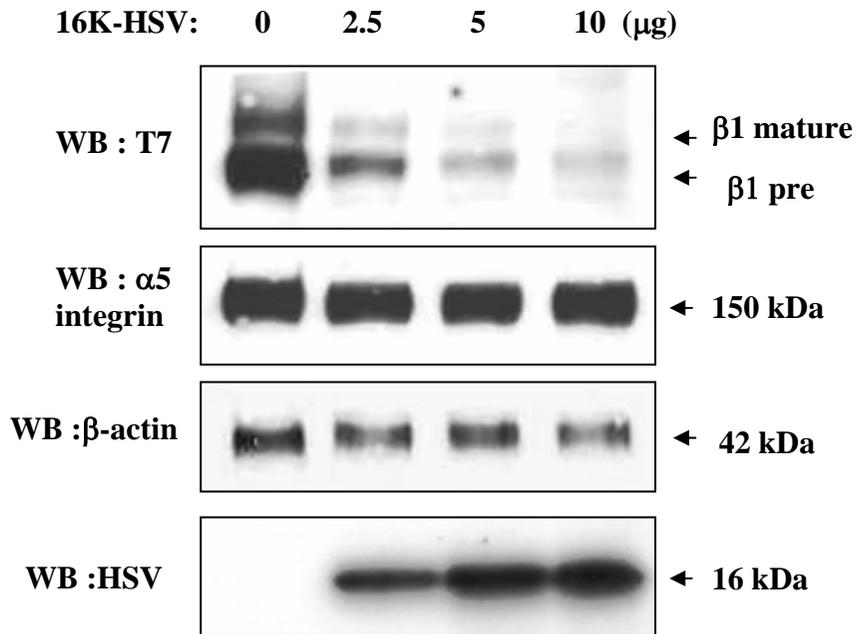
**Fig.2.2B** The panel shows little effect on the levels of surface expression of the EGF receptor and N-cadherin on cells transfected with vector alone or 15  $\mu$ g 16K-HSV. Levels of cell surface N-cadherin were slightly increased when 16K was overexpressed. (IP, immunoprecipitation; WB, western blotting)



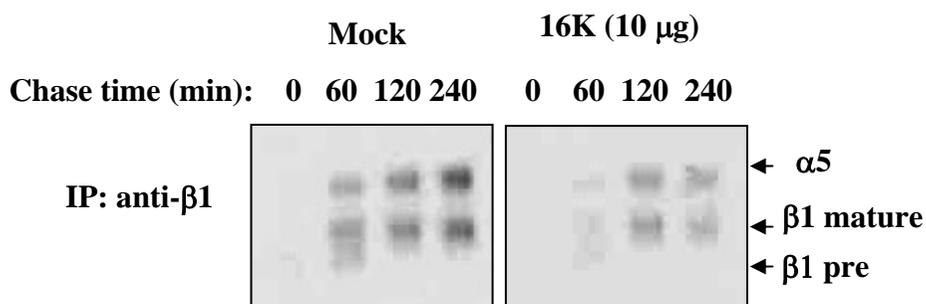
**Fig.2.3A** Effect of 16K expression on  $\alpha$ 5- $\beta$ 1 integrin dimerization and maturation. HT1080 cells were transiently transfected with T7 tagged bovine  $\beta$ 1 integrin ( $\beta$ 1-T7) alone or together with HSV tagged 16K (16K-HSV). (A) Two molecular weight forms of  $\beta$ 1 integrin were detected when  $\beta$ 1 integrin was overexpressed in HT1080 cells. Lysates from cells transfected with  $\beta$ 1-T7 were immunoprecipitated with mAb16, anti- $\alpha$ 5 integrin antibody or various lectins, analyzed by SDS-PAGE and then blotted with anti-T7 antibody.



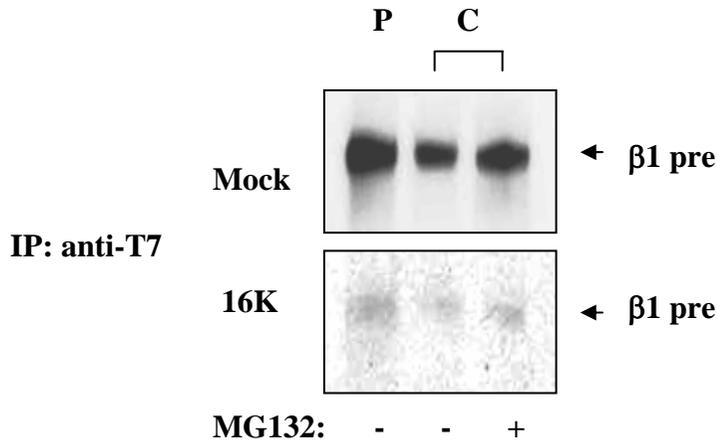
**Fig.2.3B** Only the mature form co-immunoprecipitated with  $\alpha 5$  integrin, and this interaction was inhibited by 16K expression. Cells transfected with  $\beta 1$ -T7 or equal amounts of  $\beta 1$ -T7 plus 16K-HSV were lysed and immunoprecipitated with anti- $\alpha 5$  integrin antibody and blotted with anti-T7 antibody. The lower panel shows western blotting of cell lysates with anti-HSV antibody for 16K expression.



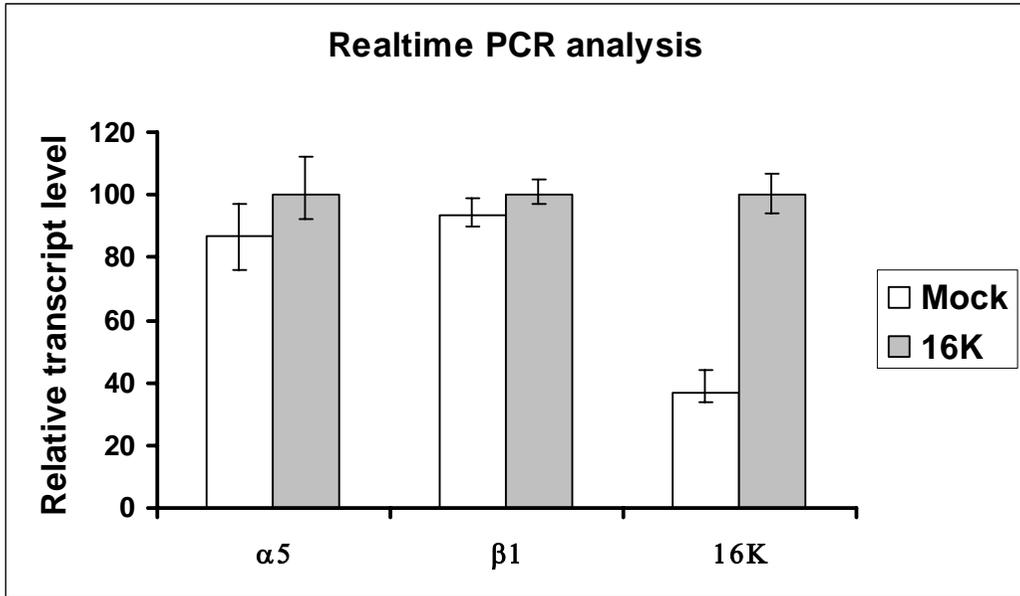
**Fig.2.3C** Both forms of β1 integrin showed decreased levels, as the amount of 16K plasmid was increased. Cells were transfected with 5 μg β1-T7 and increasing amounts of 16K-HSV. Cell lysates were run on SDS-PAGE and blotted with anti-T7 tag, anti-α5 integrin, anti-ERK2 and anti-HSV tag antibodies, respectively.



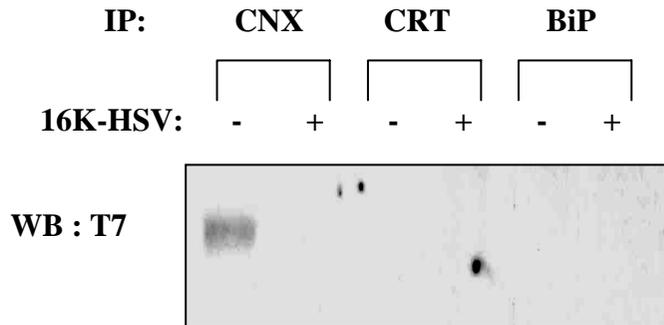
**Fig.2.4A** Endogenous premature  $\beta$ 1 integrin levels are significantly decreased in 16K transfected cells. Pulse-chase study of endogenous  $\beta$ 1 integrin biosynthesis in HT1080. Cells were either transfected with 10  $\mu$ g 16K plasmid or mock transfected and pulse-labeled with [ $^{35}$ S] methionine/Cysteine for 15 min and chased in growth medium for the indicated times. The lysates were immunoprecipitated with anti- $\beta$ 1 integrin antibody and analyzed by SDS-PAGE.



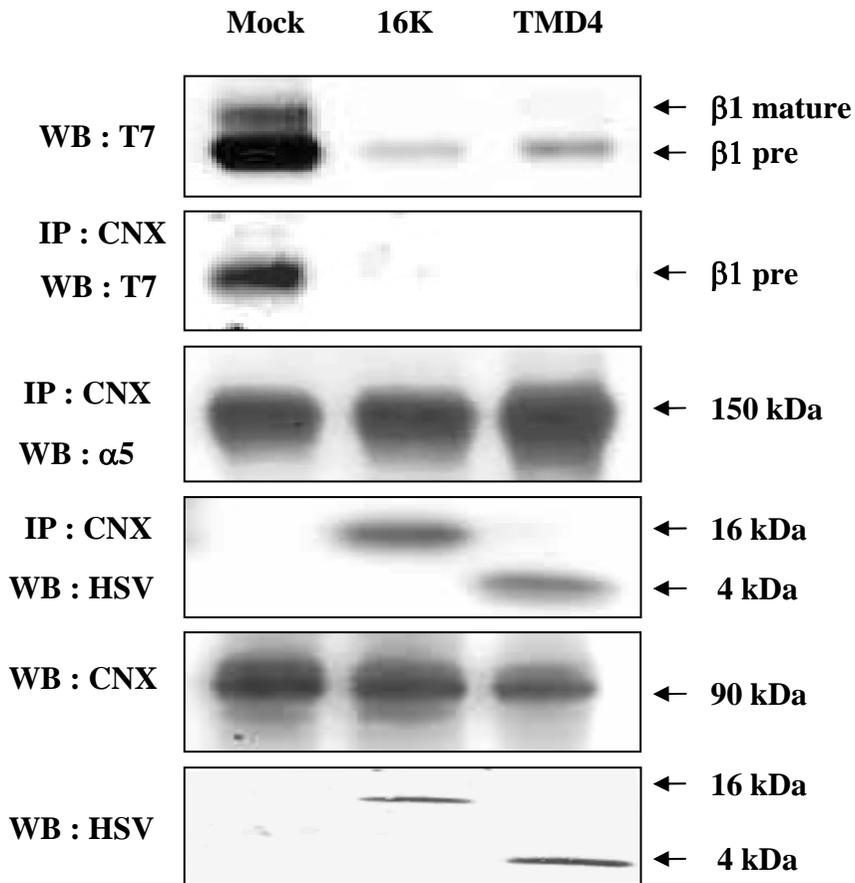
**Fig.2.4B** Pulse-chase study of cells transfected with  $\beta 1$ -T7 alone or  $\beta 1$ -T7 plus 16K-HSV. Cells were transiently transfected and grown overnight. After 24 hours, cells were pulse-labeled with [ $^{35}\text{S}$ ] methionine/cysteine for 15 min and chased for 15 min in growth medium. For lanes with MG132 treatment, a 50  $\mu\text{M}$  final concentration of MG132 in DMSO was added during both pulse and chase periods. Cell lysates were then immunoprecipitated with anti-T7 tag antibody and analyzed by SDS-PAGE. The results indicated that the levels of premature  $\beta 1$  integrin was significantly decreased in 16K-overexpressing cells and this decrease occurs within 15 min of  $\beta 1$  integrin biosynthesis. Addition of MG132 had no effect on the decrease of  $\beta 1$  integrin caused by 16K expression.



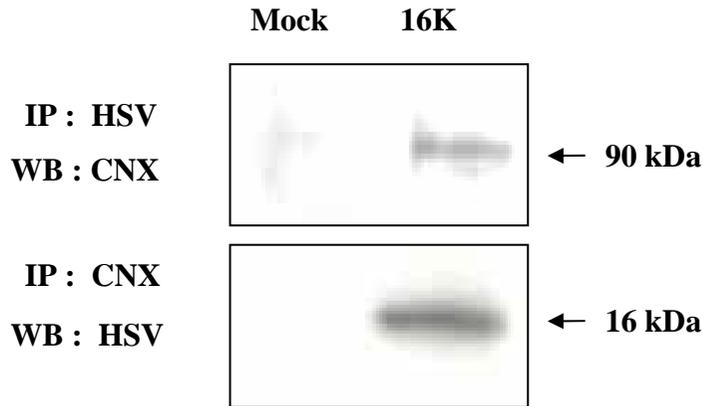
**Fig.2.5** Quantitative analysis of integrin  $\alpha 5$  and  $\beta 1$  and 16K transcription levels before and after transfection with 16K. There was no significant difference in the transcription of  $\alpha 5$  and  $\beta 1$  integrin following 16K expression in HT1080. Real time PCR analysis of mRNA was performed as described in experimental procedures. Results were normalized using GAPDH expression.



**Fig.2.6A** 16K expression inhibits the association between calnexin and premature  $\beta 1$  integrin, but not calnexin and  $\alpha 5$  integrin. (A) Expression of 16K inhibits the association between calnexin and premature  $\beta 1$  integrin. Cells were transfected with either  $\beta 1$ -T7 alone or  $\beta 1$ -T7 plus 16K-HSV. Cell lysates were immunoprecipitated with antibodies against calnexin (CNX), calreticulin (CRT), and BiP, respectively. The samples were then analyzed by SDS-PAGE and western blotting with anti-T7 antibody.

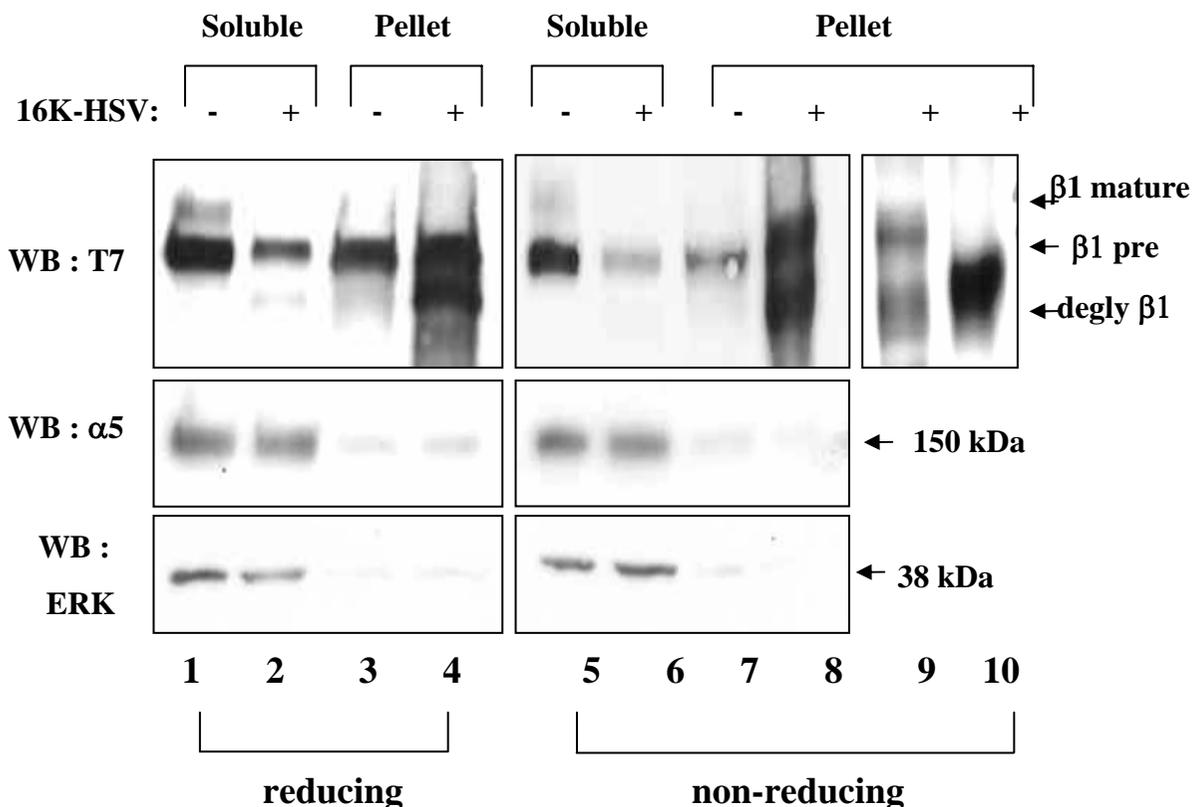


**Fig.2.6B** Expression of TMD4 alone inhibited maturation of  $\beta 1$  integrin as efficiently as 16K. Cells were transfected with  $\beta 1$ -T7 alone or together with 16K or TMD4. The results show that expression of 16K or TMD4 inhibits co-immunoprecipitation of calnexin/ $\beta 1$  integrin. The same lysates were then immunoprecipitated with anti-calnexin antibody. The immunoprecipitates were run on SDS-PAGE and blotted with anti-T7 antibody, anti- $\alpha 5$  integrin antibody or anti-HSV antibody. Note that calnexin and 16K are co-immunoprecipitated. Lower panel shows a western blot with anti-HSV tag antibody demonstrating expression of HSV-tagged 16K and TMD4 in HT1080.

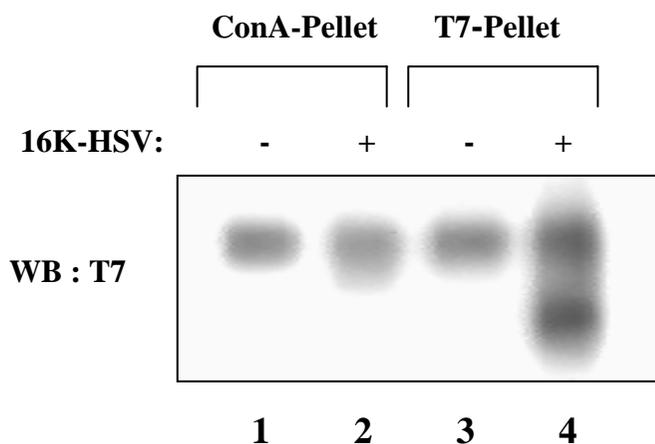


**Fig.2.7A** Calnexin is co-immunoprecipitated with 16K. Cells were transiently transfected with 16K-HSV or vector alone. After 24 hours, cells were lysed in 2% Triton X-100 and immunoprecipitated with either anti-HSV tag antibody or anti-calnexin antibody. The immunoprecipitated proteins were subjected to SDS-PAGE, followed by western blotting with either anti-calnexin or anti-HSV tag antibody, respectively.





**Fig.2.8A** 16K expression causes Triton X-100 insoluble, non-disulfide linked aggregation of the 87 kDa form of  $\beta 1$  integrin. (A) Expression of 16K resulted in a Triton-insoluble aggregation of the 87 kDa form of  $\beta 1$  integrin. Transiently transfected cells were lysed in a lysis buffer containing 1% Triton X-100 and 0.1% sodium deoxycholate. Protein concentration of the crude lysates was determined by BCA protein assay and equal amounts of proteins were pellet by microcentrifugation. The soluble fractions and pellets were boiled in SDS separately and analyzed by SDS-PAGE under either reducing or non-reducing condition, followed by western blotting with anti-T7 antibody and HRP conjugated goat anti-mouse IgG. Lane 1,2,5,6, soluble fraction; lane 3,4,7-10, pellet, while lane 10 only is PNGase-F treated.



**Fig.2.8B**  $\beta$ 1 integrin migrating at 87 kDa on SDS-PAGE after 16K expression is not immunoprecipitated with Con A. Triton insoluble pellets were lysed in 1% SDS, boiled for 5 min, followed by dilution in 2% Triton X-100 for precipitation with Con A. Lanes 1,2 are from the pellets lysed in 1% SDS and immunoprecipitated with Con A; lanes 3,4 are from the pellets lysed in 1% SDS and immunoprecipitated with anti-T7 antibody.

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## CHAPTER 3

# N-ACETYLGLUCOSAMINYLTRANSFERASE VB ENHANCES NERVE GROWTH FACTOR- AND MANGANESE-INDUCED NEURITE OUTGROWTH IN PC12 CELLS IN A $\beta$ 1 INTEGRIN-DEPENDENT MANNER<sup>1</sup>

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<sup>1</sup>Intaek Lee, Huabei Guo, Maria Kamar, Jin-Kyu Lee, Gerardo Alvarez-Manilla, and Michael Pierce. Submitted to Journal of Biological Chemistry. 04.20.2005

## **Abstract**

The role of the Golgi glycosyltransferase, N-acetylglucosaminyltransferase VB (GnT-VB) in neuronal differentiation was investigated, using the rat pheochromocytoma PC12 neurite outgrowth model. PC12 cells stably transfected with GnT-VB consistently showed an enhanced rate of nerve growth factor (NGF) -induced neurite outgrowth on collagen and laminin substrates. Phosphorylation of TrkA receptor and downstream ERK activation by NGF were not influenced by GnT-VB overexpression. It is known that PC12 variant, PC12D cells, demonstrates enhanced rate of neurite extension and can be induced to develop neurites by addition of cAMP activator, forskolin (Sano et al., Brain research, 1990). When PC12/GnT-VB cells were stimulated with forskolin, they did not elicit neurite outgrowth nor induced expression of microtubule-associated protein 1 (MAP1), suggesting that the mechanism of enhanced neurite outgrowth is distinct from that exhibited by PC12D. When cultured on non-coated tissue culture plates, no significant difference was observed in the rate of neurite outgrowth, indicating that integrin-ECM interaction is critical for this stimulatory effect. Neurite outgrowth induced by manganese-dependent activation of  $\beta$ 1 integrin on collagen substrates showed a significant increase in neurite length for PC12/GnT-VB cells, compared to control cells, suggesting that the enhancement is most likely mediated by alteration of  $\beta$ 1 integrin-ECM interaction by GnT-VB. In support of these results, neurite outgrowth induced by either NGF or manganese treatment on collagen substrates was completely abolished by addition of anti- $\beta$ 1 integrin function-blocking antibody in both mock- and GnT-VB transfected PC12 cells. Our results suggest that GnT-VB expression can modulate the rate of neurite outgrowth by altering  $\beta$ 1 integrin-ECM interaction.

**Key words: N-acetylglucosaminyltransferase VB,  $\beta$ 1 integrin, PC12 cells, Extracellular matrix, N-linked glycosylation.**

## Introduction

The cloning of a new brain-specific isoenzyme of UDP-N-acetylglucosamine: $\alpha(1,6)$ -D-mannoside  $\beta(1,6)$ -N-acetylglucosaminyltransferase (GnT-V, Mgat5), named “N-acetylglucosaminyltransferase VB (GnT-VB, also referred to as GnT-IX)” has recently been reported (1, 2). GnT-VB was shown to be highly expressed in brain and testis selectively, while GnT-V was expressed broadly in many tissue types. When transfected in a CHO variant cell line lacking functional GnT-V, Lec4 cells, GnT-VB expression caused increased surface binding of L-phytohemagglutinin (L-PHA), suggesting that GnT-VB was capable of synthesizing N-linked Gal  $\beta(1,4)$ -GlcNAc $\beta(1,6)$ -Man  $\alpha(1,6)$  as was GnT-V (3). An *in situ* staining study of GnT-VB mRNA of developing mouse embryos revealed that GnT-VB is selectively expressed in the brain and neural retina, suggesting that GnT-VB may function in neuronal differentiation during brain development (Matthews et al, manuscript in preparation).

GnT-V transfers UDP-N-acetylglucosamine (GlcNAc) to the  $\alpha(1,6)$  mannose of trimannosyl core of N-linked glycans in  $\beta(1,6)$  linkage and this branch is known to be a preferred acceptor for the formation of poly N-acetyllactosamine chains (4). Increased GnT-V expression has been shown repeatedly to enhance cell motility when overexpressed in several cell types and contribute to tumor cell invasion and metastasis in various malignant cancers (5-7). Since GnT-VB is selectively expressed in brain, we hypothesized that GnT-VB likely play a similar function to enhance neuronal migration and axonal extension of developing neurons during neuromorphogenesis. To test this hypothesis, we utilized the rat pheochromocytoma subline, PC12, as a model system to determine the effect of increased GnT-VB expression on nerve growth factor (NGF)-induced neurite outgrowth. The results showed that overexpression of GnT-VB in PC12 cells results in a significantly enhanced rate of neurite outgrowth when cells were

treated with NGF, compared to mock-transfected cells. Expression of GnT-VB in PC12 cells did not cause a change in NGF-TrkA signaling nor in down-stream ERK signaling. Addition of a cAMP activator, forskolin, which is known to induce neurite outgrowth in a PC12 variant line, PC12D cells, failed to initiate neurite outgrowth in either mock or GnT-VB transfected PC12 cells, suggesting that GnT-VB-transfected PC12 cells differ from PC12D cells in terms of mechanism of “priming” (8, 9). Surprisingly, when cells were treated with NGF on non-coated culture plates, no significant improvement of neurite outgrowth was observed, while cells plated on collagen or laminin displayed enhanced rate of neurite outgrowth, suggesting that integrin-ECM interaction may be involved in the stimulation of neurite outgrowth. Integrin activation-induced neurite outgrowth by manganese treatment on collagen substrates (10) suggested that enhanced neurite outgrowth seen in PC12 cells overexpressing GnT-VB was most likely mediated by alteration of  $\beta 1$  integrin function. Addition of  $\beta 1$  integrin function-blocking antibody caused complete abolition of NGF-induced and manganese-induced neurite outgrowth in both control and GnT-VB overexpressing PC12 cells. Our results demonstrate that GnT-VB expression stimulates neurite extension in a  $\beta 1$  integrin-dependent manner and that alteration of  $\beta 1$  integrin-ECM interaction is critical for this enhancement. ,

## **Experimental Procedures**

***Cell culture, transfection, and materials*** – Rat pheochromocytoma PC12 cells were purchased from ATCC and grown in RPMI1640 medium supplemented with 10% horse serum, 5% fetal bovine serum on collagen I coated 100 mm dishes (BD Biosciences). Nerve growth factor-induced differentiation was typically performed on collagen I or Laminin-coated 6 well plates or chamberslides under low (2%) serum condition for the indicated times. Human GnT-

VB/pcDNA3.1 expression plasmid was prepared as described previously (1). Lipofectamine 2000 (Life Technology) was used for stable transfection of human GnT-VB into PC12 cells following standard transfection procedures provided by the manufacturer. Cells were co-transfected with pEGFP vector (Invitrogen) with 5 GnT-VB:1 pEGFP ratio and selected for 3 weeks under 800  $\mu\text{g/ml}$  G418. Cells with the highest GFP expression were isolated using fluorescent activated cell sorting (Dakocytometer *MOFLO*). The 2.5S Nerve Growth Factor (NGF) was obtained from Chemicon. Biotinylated L-phytohemagglutinin (L-PHA) was from Vector Labs (CA). Rabbit anti phospho-TrkA (Tyr490) antibody was purchased from Cell Signaling Technology. Rabbit anti-TrkA antibody, anti-ERK1/2 antibody, anti-phosphoERK antibody, and all secondary antibodies conjugated with horse-radish peroxidase were from Santa Cruz Biotechnology. Rabbit anti- $\beta$ 1 integrin antibody, mouse monoclonal antibody against MAP1, and anti-rat  $\beta$ 1 integrin function-blocking antibody, Ha2/5, were obtained from Chemicon. Rhodamine-streptavidin was from Molecular Probes, while forskolin was purchased from Calbiochem.

**Fluorescent Staining** — PC12 cells were grown in chamberslides coated with rat tail collagen I (BD Biosciences) overnight. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min, and blocked with 1% BSA for 30 min at 37 °C. Biotinylated L-PHA (1  $\mu\text{g/ml}$ ) in blocking buffer was added to the chamber at 37 °C for 30 min. After washing three times with PBS, rhodamine-streptavidin (1:1000) was used to detect L-PHA bound to cell surface glycoproteins by incubating at 37 °C for 30 min. After washing with PBS, the coverslips were mounted and images were obtained using an inverted fluorescence microscope (Leica). For swansonine

treatment, culture medium containing 2% serum was supplemented with 1  $\mu$ M swansonine and added to the cells for 72 hours.

***Immunoprecipitation and Western blot analysis*** – Cells were lysed in a buffer consisting of 1% Triton X-100, protease inhibitors and 0.1% SDS. Typically, 100  $\mu$ g total protein was used for immunoprecipitation and lectin precipitation. Proteins were resolved by SDS-PAGE utilizing 7.5% or 4-20% gels under non-reducing conditions unless otherwise indicated, transferred to PVDF membranes, blocked in 2% BSA, and probed with antibodies. Following incubation with HRP-conjugated secondary antibodies or streptavidin-HRP, proteins were detected by the ECL reagent (Pierce). For detection of TrkA and ERK Phosphorylation, the blot was probed with anti-TrkA Tyr-490 antibody (1:1000) or anti-TrkA (1:500) and developed by chemiluminescence method. Densitometry of the blots was performed using a Bio-Rad Fluor S imager.

***Treatment of PC12 cells with NGF or Manganese*** – Typically, cells were plated on collagen-coated 6 well plates or chamberslides (BD Biosciences) overnight. After 24 hours, cells were washed once with PBS, culture medium was replaced with medium containing 2% FBS supplemented with NGF (50ng/ml) or manganese (200  $\mu$ M), either in the presence or absence of anti- $\beta$ 1 integrin function-blocking antibody, Ha2/5 (10  $\mu$ g/ml). Normal rabbit IgG was used as a control. For longer periods of incubation, the NGF treated media was replaced every 3 days.

***GnT-V activity assay*** - Cells were trypsinized, pelleted, and lysed with 50 mM MES (pH 6.5), 150 mM NaCl, and 1% Triton X-100. Insoluble debris was pelleted by microcentrifugation (10 min. at 4°C), and the supernatant was used for the GnT-V activity assay using UDP-[ $^3$ H]-GlcNAc as acceptor substrate in the presence of  $Mn^{2+}$ . Sep-Pak columns (Waters) were used to

separate the substrates and products as described (11). The column methanol eluant was added to scintillation fluid and radioactivity measured by scintillation counting. Assays were performed in duplicate, the data averaged, and results were expressed as specific activity (nmol/h/mg). Protein concentration in the cell lysates was determined using the BCA assay (Pierce).

**Measurement of Neurite outgrowth-** Typically, cells were plated on collagen-coated plates and cultured overnight. NGF (50 ng/ml) was added to the cells in culture medium containing 2% serum. After stimulation for 7 days with NGF, cells were washed and fixed with 3.5% formaldehyde. Ten random fields were photographed with a phase contrast microscope at 100X magnification. A bar that is as long as average diameter of cell body in a field was placed in each pictures and used as a standard to determine the length of neurites from each cell. Approximately total 300 cells were measured for neurite lengths, and cells with different lengths of neurites were grouped together such as one-cell body long, two-cell body long, and three-cell body long. Experiments were repeated twice for confirmation.

## Results

We reported the cloning of a brain-specific isoenzyme of N acetylglucosaminyl-transferase V (GnT-V), named GnT-VB (1, 2) with 53% similarity and 41% identity at amino acid level to GnT-V. When either enzymes were expressed in Lec4 cells that lacked endogenous GnT-V and GnT-VB activity, L-phytohemagglutinin (L-PHA), a plant lectin that binds  $\beta(1,6)$  branched N-linked oligosaccharides, bound the transfected cells. A GnT-VB expression profile study using realtime PCR and an *in situ* study of GnT-VB mRNA in mouse embryos indicated that this glycosyltransferase is highly expressed in brain and neural retina, while GnT-V is

broadly expressed in many tissues (12). Because of this narrow expression pattern of GnT-VB, we decided to explore the possibility that GnT-VB expression is important for neuronal differentiation and migration during embryonic development. A rat pheochromocytoma cell subline, PC12, has been extensively used as a model system for studies of nerve growth factor (NGF) induced-neuronal cell differentiation into sympathetic neuron-like morphology (13, 14). Typically, PC12 cells stimulated with NGF displays initial cell spreading, initiation of short membrane outgrowth, followed by extension of neurites between day 3 and day 14 before maximal neurite outgrowth. This process of neuron-like differentiation is known to involve transcriptional regulations to synthesize a set of proteins necessary to promote and maintain neurite outgrowth (14, 15). Therefore, we used PC12-NGF neuronal differentiation model to test the hypothesis that GnT-VB expression plays a role in neuromorphogenesis.

### ***Expression of GnT-VB causes an increased surface binding with L-PHA***

First, we made stable PC12 cells overexpressing human GnT-VB and determined whether increased GnT-VB expression resulted in increased  $\beta(1,6)$  branched N-linked glycans on the cell surface, as expected. Using a GnT-V enzymatic assay with radiolabeled UDP- $[^3\text{H}]\text{GlcNAc}$  and the synthetic trisaccharide acceptor,  $\text{GlcNAc}\beta(1,2)\text{Man}\alpha(1,2)\text{Glc-O-octyl}$ , PC12 overexpressing GnT-VB (PC12/GnT-VB) showed a significant increase in activity (Fig.1A), compared to mock transfected cells. The parental PC12 cells showed very low endogenous GnT-V activity. Fluorescent staining with L-PHA indicated increased  $\beta(1,6)$  branched N-linked glycans on the surface of PC12/GnT-VB, compared to control cells (Fig.1B). As a control, inhibition of the formation of complex type N-linked glycan expression by swansonine, an inhibitor of  $\alpha$ -mannosidase II, reduced L-PHA binding to the cell surface for

both mock- and GnT-VB-transfected PC12 cells, as expected. This result showed that increased L-PHA binding to PC12/GnT-VB cells can be inhibited by blocking formation of complex type N-linked glycans, suggesting that GnT-VB is capable of producing N-linked glycans which binds the L-PHA in PC12 cells.

***PC12 cells overexpressing GnT-VB show stimulation of nerve growth factor induced-neurite outgrowth, compared to control PC12 cells.***

Next, in order to test whether increased GnT-VB expression in PC12 cells would affect NGF-induced neurite outgrowth, both mock-transfected and GnT-VB-transfected PC12 cells were plated on collagen-coated 6 well plates and treated with 50 ng/ml NGF for up to 14 days. The rate of neurite outgrowth was examined and analyzed at day 3, 7, and 14 using phase contrast microscopy, as described in experimental procedures. Interestingly, PC12/GnT-VB cells showed a significantly enhanced rate of neurite outgrowth, compared to control cells (Fig.2A). In GnT-VB overexpressing PC12 cells, initial neurite outgrowth was detected between 36 hours and 48 hours, compared to approximately 72 hours for control PC12 cells, and the rate of neurite extension was significantly increased. When cells were counted as separate groups, such that cells with different neurite lengths (i.e. two-cell body, three-cell body, four-cell body) were individually grouped for analysis, GnT-VB overexpressing PC12 cells showed much higher numbers of cells with neurites of two, three, or four-body length, compared to mock-transfected cells. Overall, quantitative analysis based on the ratio of lengths of neurites relative to cell body length indicated that PC12/GnT-VB cells showed more than triple the percentage of cells with at least two-body length neurites at day 7 (Fig.2B). At day 14, no significant differences in neurite lengths were observed, suggesting that GnT-VB modulates the rate, but not the extent of neurite

outgrowth. In addition, number of neurites from each cell stayed the same; on average, both mock- and GnT-VB-transfected PC12 cells contained approximately 4-5 neurites when stimulated with NGF. These results indicated that increased GnT-VB expression led to stimulation of neurite extension in NGF-induced PC12 cells. We next characterized possible mechanisms for this stimulatory effect.

***Overexpression of GnT-VB does not influence NGF-TrkA and downstream ERK signaling.***

A neurotrophin receptor, TrkA, is highly expressed in PC12 cells and functions as a NGF receptor that, upon ligand binding, activates a downstream signaling pathway leading to ERK activation, an essential signaling intermediate for NGF-induced PC12 neurite outgrowth (16-18). We hypothesized that the enhanced neurite outgrowth observed in PC12/GnT-VB cells may be mediated by alteration of NGF-TrkA signaling. To test this hypothesis, cells were plated on collagen-coated dishes and treated with NGF for the indicated times. The results showed that addition of NGF yielded a similar level of TrkA phosphorylation of Tyr490 (Fig.3A) at both 20 min and 60 min of incubation in both mock- and GnT-VB-transfected cells. The same cell lysates were used to assess ERK activation levels upon NGF stimulation. The level of ERK phosphorylation also did not show significant differences (Fig.3B), suggesting that the NGF-induced signal transduction pathway is not likely to contribute significantly to enhanced neurite outgrowth seen in PC12/GnT-VB cells. These results showed that GnT-VB expression did not affect NGF-TrkA and downstream ERK signaling in PC12 cells.

***Nerve growth factor, but not forskolin, induces neurite outgrowth in both mock- and GnT-VB transfected PC12 cells.***

Enhanced neurite outgrowth has been described for a PC12 variant, PC12D cells (8, 19), which has been characterized as “being primed” for neurite extension. A few unique features of this “priming” effect on neurite outgrowth in PC12D cells have been characterized, including (i) increased expression of microtubule associated protein 1 (MAP1), compared to conventional PC12 cells, that promotes faster microtubule assembly (15); (ii) induction of neurite outgrowth by a cAMP activator, forskolin, in PC12D cells, but not in conventional PC12 cells (8, 9). One other interesting observation was that PC12D cells appeared much better spread on culture dishes than PC12 cells. Since NGF treatment promotes PC12 cell spreading prior to differentiation, it was suggested that more extensive cell spreading in the absence of NGF was a sign of PC12D cells being “primed” for neurite outgrowth (8). To test if GnT-VB overexpressing PC12 cells show “primed” behavior similar to PC12D cells, we first investigated whether addition of forskolin can induce neurite extension in GnT-VB transfected PC12 cells. The results showed that treatment of forskolin (10  $\mu$ M) for 7 days failed to induce any significant neurite outgrowth in either mock or GnT-VB transfected cells (Fig.4). Unlike PC12D cells, forskolin treated GnT-VB/PC12 cells did not show increased protein expression of MAP1 (data not shown), indicating that the mechanism of neurite outgrowth stimulation was different from that of PC12D cells (8). These results suggested that GnT-VB-dependent enhancement of NGF-mediated neurite outgrowth is likely distinct from that which is operative in the PC12D cells.

#### ***GnT-VB-mediated enhancement of neurite outgrowth requires integrin-ECM interaction***

There have been reports that neurite outgrowth of neuronal cells is directly mediated by integrin-ECM interactions in developing nervous system, as well as in the neuritogenesis model system, PC12 cells (20-22). To test if PC12/GnT-VB cells show enhanced neurite outgrowth in

the absence of ECM ligands, cells were plated on non-treated tissue culture plates or plates coated with either collagen or laminin and treated with NGF for 7 days. Surprisingly, under these conditions, PC12/GnT-VB cells showed no significant difference in the rate of neurite outgrowth, compared to control cells (Fig.5A), while PC12/GnT-VB cells plated on collagen or laminin showed significant enhancement of NGF-induced neurite outgrowth, suggesting integrin-ECM interaction is critical for this stimulation. Several differences were observed between the cells plated on ECM and on non-treated culture plates, including (i) absence of ECM ligands resulted in an increased rate of neurite outgrowth for control PC12 cells, but slightly a decreased rate for PC12/GnT-VB cells, leading to canceling out of the difference in the rate of neurite outgrowths; (ii) cells plated on either collagen or laminin displayed morphologically straight neurites, whereas neurites from cells on non-treated culture plates appeared to be more curved, when stimulated with NGF. These changes may have resulted from different combinations of the adhesion receptors used in the neurite outgrowth between ECM-coated and non-treated plates. Furthermore, addition of  $\beta 1$  integrin function-blocking antibody, Ha2/5, (10  $\mu\text{g}/\text{ml}$ ) completely abolished NGF-induced neurite outgrowth of both control and GnT-VB overexpressing cells grown on either collagen- or laminin-coated wells (Fig.5B). No significant inhibition was observed with addition of control normal rabbit IgG (data not shown). These results suggested that enhanced neurite outgrowth of PC12 cells by GnT-VB expression is most likely mediated by a  $\beta 1$  integrin-collagen dependent mechanism.

***Enhancement of neurite outgrowth mediated by manganese-induced  $\beta 1$  integrin activation of PC12/GnT-VB cells***

The regulation of neurite outgrowth by integrin activation has been well documented (10, 21-23). In particular, the manganese-dependent activation of  $\beta 1$  integrin was shown to promote neurite outgrowth in PC12 cells (10). Therefore, we studied if activation of  $\beta 1$  integrin by manganese treatment was sufficient for PC12/GnT-VB cells to show enhanced neurite outgrowth on collagen substrates, compared to control cells. Cells were plated on collagen-coated plates and treated with manganese for 72 hours in the presence or absence of  $\beta 1$  integrin function-blocking antibody. The results from this experiment showed that manganese-induced neurite outgrowth on a collagen substrate was significantly enhanced in the PC12/GnT-VB cells, and was inhibited by addition of anti- $\beta 1$  integrin function-blocking antibody (Fig.6A). In control PC12 cells, only small processes and occasional short neurites were observed, compared to well-developed neurites of PC12/GnT-VB cells in response to the manganese treatment. When number of cells with neurites were quantitatively analyzed, PC12/GnT-VB cells showed more than twice the percentage of cells with one body length neurites, compared to control cells (Fig.6B), providing an additional support for the hypothesis that GnT-VB expression alters  $\beta 1$  integrin-collagen interaction, leading to enhanced neurite outgrowth. Next, we examined whether  $\beta 1$  integrin was a substrate for GnT-VB in PC12 cells and two other neuronal cell lines, human neuroblastoma U373 and rat gliosarcoma 9L cells, using lectin precipitation and western analysis using  $\beta 1$  integrin antibody. Cell lysates from control and GnT-VB overexpressing cells were lectin-precipitated with L-PHA to bind  $\beta(1,6)$  branched N-linked glycans. Bound glycoproteins were run on SDS-PAGE and blotted onto PVDF membrane. When probed with anti- $\beta 1$  integrin antibody, the results indicated that L-PHA bound significantly more  $\beta 1$  integrin in PC12/GnT-VB cells as well as U373 and 9L cells transfected with GnT-VB, suggesting that  $\beta 1$  integrin is a substrate for GnT-VB glycosylation in neuronal cells (Fig.6C). These results

demonstrated that GnT-VB may promote enhanced neurite outgrowth by altering  $\beta 1$  integrin-ECM interaction, rather than by affecting NGF-TrkA and downstream signal transduction pathway.

## **Discussion**

Our results showed that expression of GnT-VB in PC12 cells promotes enhanced NGF-induced neurite outgrowth on collagen and laminin substrates, and that this enhancement may be mediated by alteration of  $\beta 1$  integrin function in its interaction with ECM ligands. Since GnT-VB is strongly expressed in the developing mouse embryo brain (Matthews et al, unpublished data), it is of particular interest that GnT-VB is capable of stimulating neurite extension since integrin-ECM interaction plays an important role in axonal extension during neuronal differentiation (20, 24). It is possible that GnT-VB expression plays an important role for proper timing of neurite extension to target innervations during nervous system development by modulation of integrin function. Manganese-induced neurite outgrowth of PC12 cells has been known to be strictly mediated by integrin activation and requires the presence of ECM for the initiation of neurite outgrowth (10). Therefore, it is reasonable to assume that enhanced neurite outgrowth by manganese treatment in PC12/GnT-VB cells demonstrates a direct involvement of modulation of  $\beta 1$  integrin function by GnT-VB expression.

Important functions of  $\beta 1$  integrin N-linked glycosylation have been examined by several previous studies. Akiyama et al. reported that inhibition of N-linked glycosylation maturation of  $\alpha 5\beta 1$  integrin by 1-deoxymannojirimycin treatment (MNJ), an inhibitor of Golgi  $\alpha$ -mannosidase IA/IB, renders the purified integrin heterodimers unable to bind fibronectin affinity column (25). Furthermore, inhibition of N-linked glycosylation by tunicamycin treatment blocked not only  $\alpha 5$ -

$\beta$ 1 integrin pairing, but also the surface expression of functional  $\alpha$ 5 $\beta$ 1 integrin (26), suggesting that N-linked glycosylation of  $\beta$ 1 integrin plays critical functions from  $\alpha$ - $\beta$ 1 subunit dimerization to surface expression of functional integrin receptors. PC12 cells express a number of integrin heterodimers, including a collagen receptor and a laminin receptor,  $\alpha$ 1 $\beta$ 1 and  $\alpha$ 3 $\beta$ 1 integrin, respectively (10, 27), suggesting that collagen receptor function may have been modulated by GnT-VB expression.

In similar studies using PC12 cells, expression of GnT-III, a Golgi glycosyltransferase that synthesizes a bisecting complex type N-linked glycans, was shown to inhibit PC12 neurite outgrowth induced by either NGF or a combination of EGF/integrin signaling (28, 29). Different from our results, the authors demonstrated that introduction of a bisecting N-linked glycans on TrkA or the EGF receptor causes down-regulation of growth-factor mediated signaling, resulting in inhibition of neurite outgrowth in PC12 cells transfected with GnT-III. In our study, no apparent changes in NGF-TrkA and downstream ERK activation signaling were observed, indicating that the mechanism of modulation of neurite outgrowth in PC12/GnT-VB is unique compared to that of PC12/GnT-III.

Effects of aberrant N-linked glycosylation of cell adhesion receptors on cell motility has been documented (5, 6, 30, 31). In particular,  $\beta$ 1 integrin was shown to be a target for increased  $\beta$ (1,6) branched N-linked glycans, as well as  $\alpha$ (2,6) sialylation that lead to reduced integrin-dependent cell adhesion and increased cell migration on ECM ligands (5, 32). The role of integrins in neurite outgrowth and axon guidance during nervous system development has only recently been characterized (24, 33, 34). The interaction of axon guidance proteins (e.g. Netrin)

with integrins (35) suggested that the basic mechanism of directional migration of various cell types is, in fact, similar to the mechanism of neurite outgrowth in neuronal cell types. These studies provide a plausible hypothesis that aberrant glycosylation of integrin receptors can lead to a direct modulation of neuronal differentiation in integrin-dependent manner. While integrin glycosylation would not affect a specific integrin-ECM preference during target innervations, it would presumably regulate the timing or the rate of neural network formations.

Regarding the role of integrin-ECM interaction in neuronal differentiation, Guan et al. demonstrated that sensory neurons differentiate and extend neurites for different target innervations, based on the types of integrin receptors expressed by the neurons and subsequent integrin-ECM interactions, rather than the types of neurotrophin receptor expressed (20). Furthermore, the authors also showed that the expression levels of various integrin subunits were transcriptionally regulated as neuronal differentiation progressed. Interestingly, while target preference of sensory neurons was dependent of integrin expression profiles, neurotrophin receptors expression did not show a significant influence both in the presence and absence of neurotrophins during target innervations. These results strongly supported the concept that innervations of sensory neurons during nervous system development are substantially regulated by specific integrin-ECM interactions.

In summary, our results demonstrated that alteration of  $\beta 1$  integrin function by GnT-VB causes  $\beta 1$  integrin-dependent enhancement of neurite extension in both NGF- and manganese-induced neurite outgrowths on collagen and laminin substrates. However, any other possibility, such as alteration of signal transduction other than NGF-TrkA (i.e. cAMP signaling in PC12D

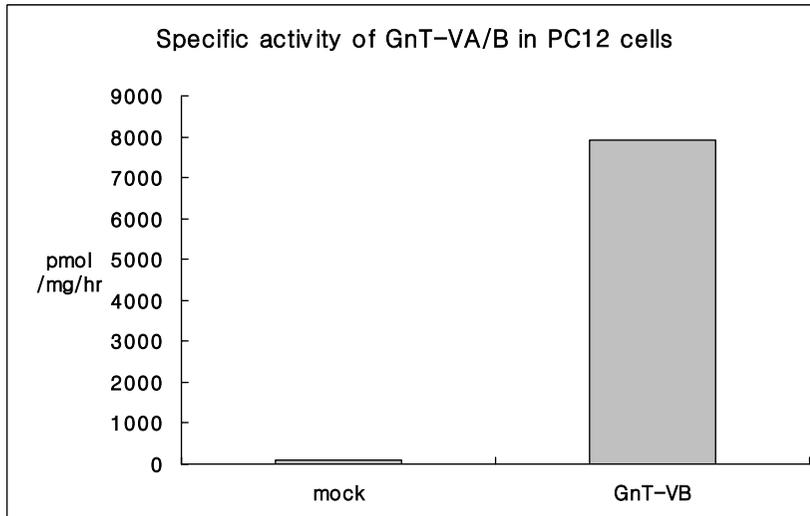
cells (36)), may not be excluded at present. In addition, GnT-VB is reported to be a branching enzyme for O-linked mannosyl glycans, an important brain-specific glycan structure (37). The presence of O-linked mannosyl glycans was shown to be necessary for  $\alpha$ -dystroglycans/laminin mediated neuronal cell adhesion to ECM. While it is not known how the branching of O-mannosyl glycans affects its function for  $\alpha$ -dystroglycans/laminin interaction, the HNK-1 epitope was found on O-linked mannosyl glycans with 2-mono- and 2,6-di-substituted mannose, suggesting the potential role of branching of O-linked mannosyl glycans for this epitope (38).

Thus, future aims would be to evaluate (i) the effect of GnT-VB deficiency during mouse embryonic development to determine if GnT-VB expression contributes to normal nervous system development; (ii) whether increased branching of O-mannosyl glycans by GnT-VB modulates  $\alpha$ -dystroglycans/laminin interaction.

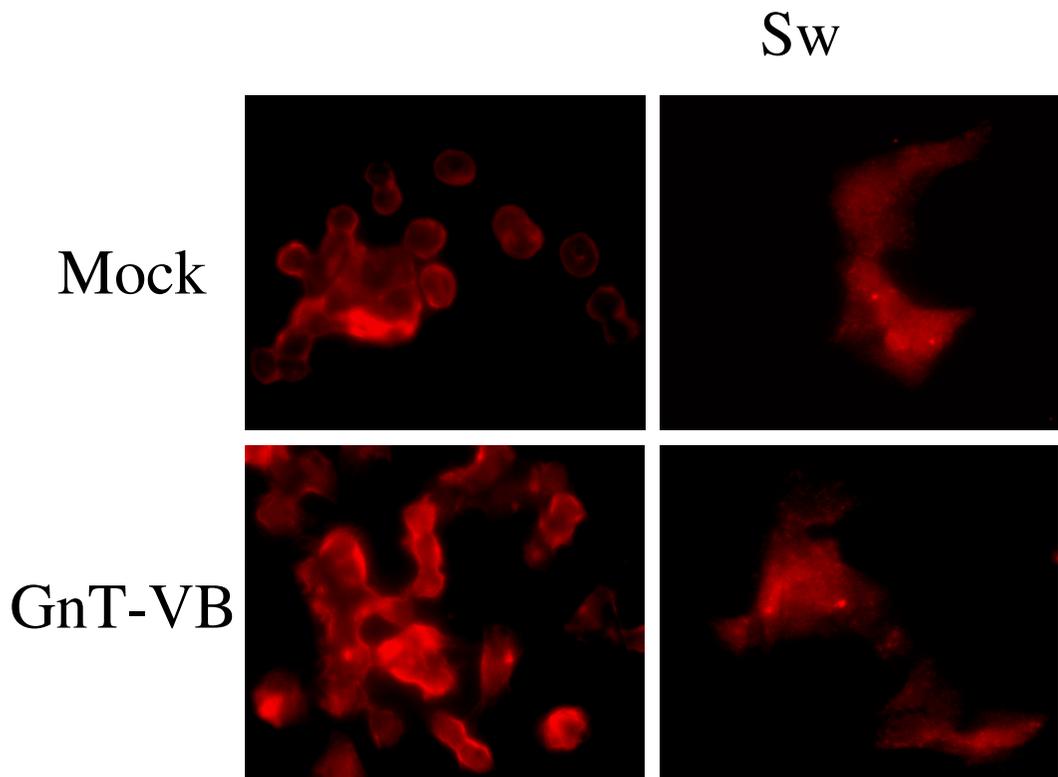
### **Acknowledgement**

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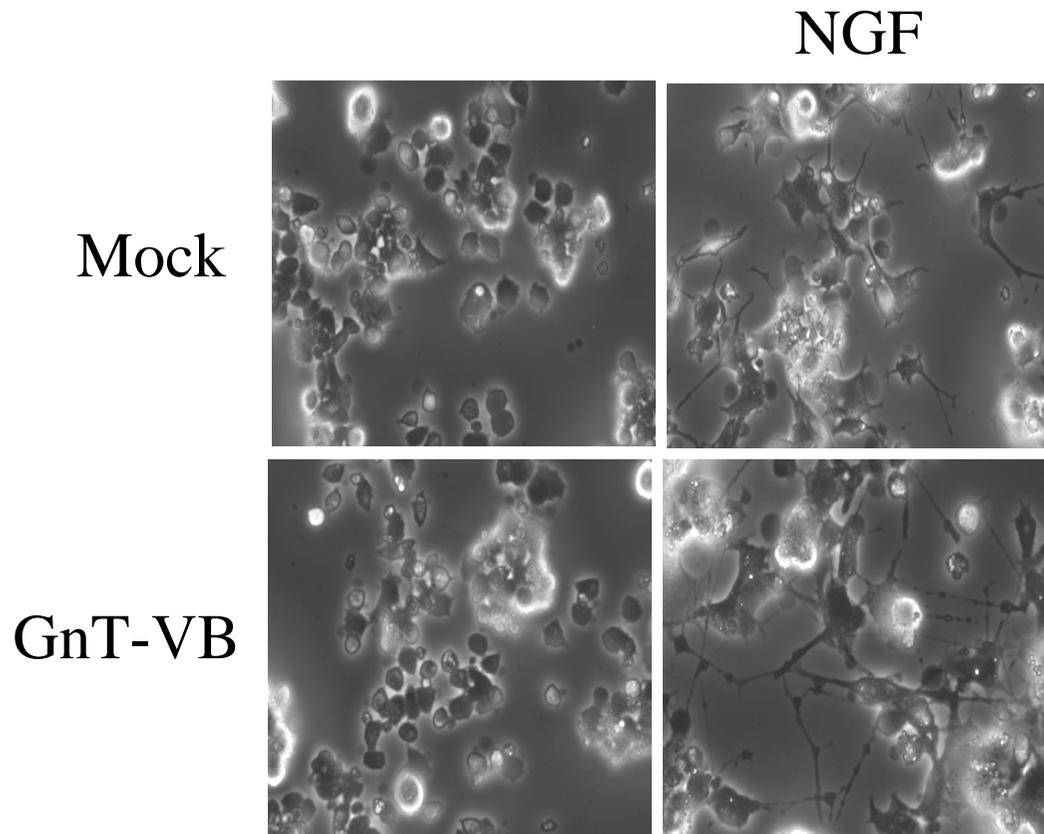
**Abbreviations:** L-PHA, L-phytohemagglutinin; PBS, phosphate buffered saline; NGF, Nerve growth factor; GnT-V, N-acetylglucosaminyltransferase V; SDS, sodium dodecyl sulfate; FBS, fetal bovine serum; BSA, bovine serum albumin; GlcNAc, N-acetylglucosamine; Man, mannose; Sw, swansonine.



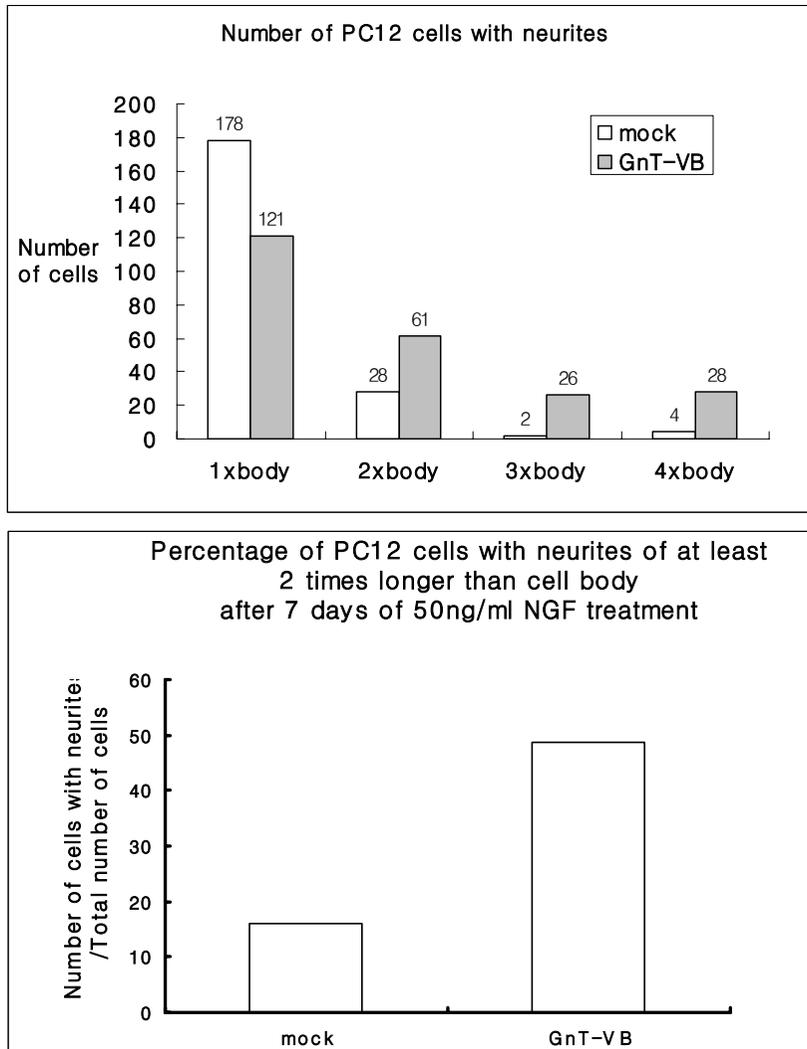
**Fig.3.1A** PC12 cells were stably transfected with human GnT-VB. GnT-V enzymatic assay using radiolabeled UDP-[3H]GlcNAc and the synthetic trisaccharide acceptor in the presence of Mn<sup>2+</sup>



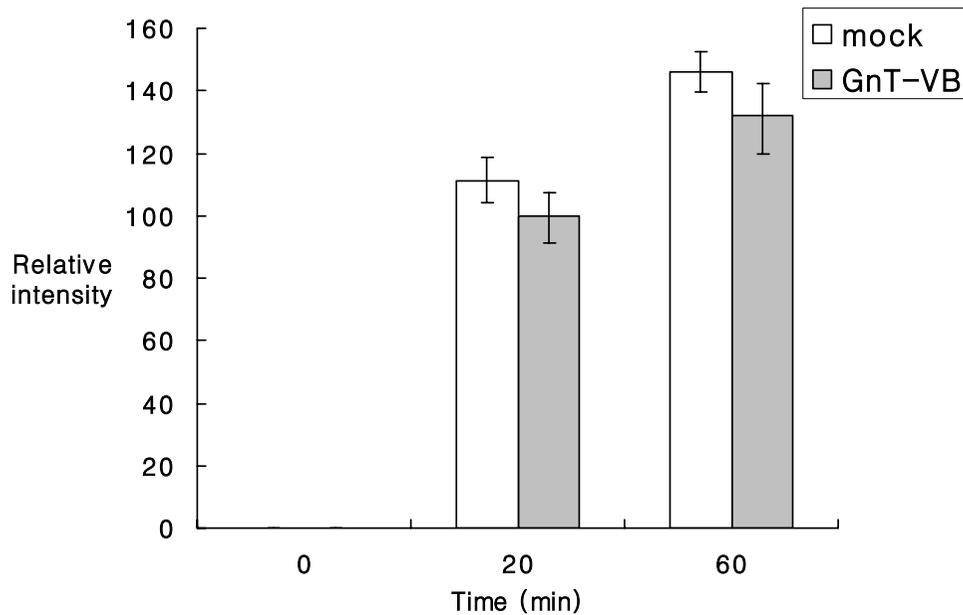
**Fig.3.1B** Fluorescent staining of control PC12 cells and PC12/GnT-VB cells with L-PHA-rhodamine. Cells were plated on chamberslides coated with rat tail collagen I (10  $\mu\text{g/ml}$ ). For swansonine treatment, cells were treated with 1  $\mu\text{M}$  swansonine for 72 hours, before staining with L-PHA. The images were obtained with Leica inverted fluorescent microscope at 400x magnification.



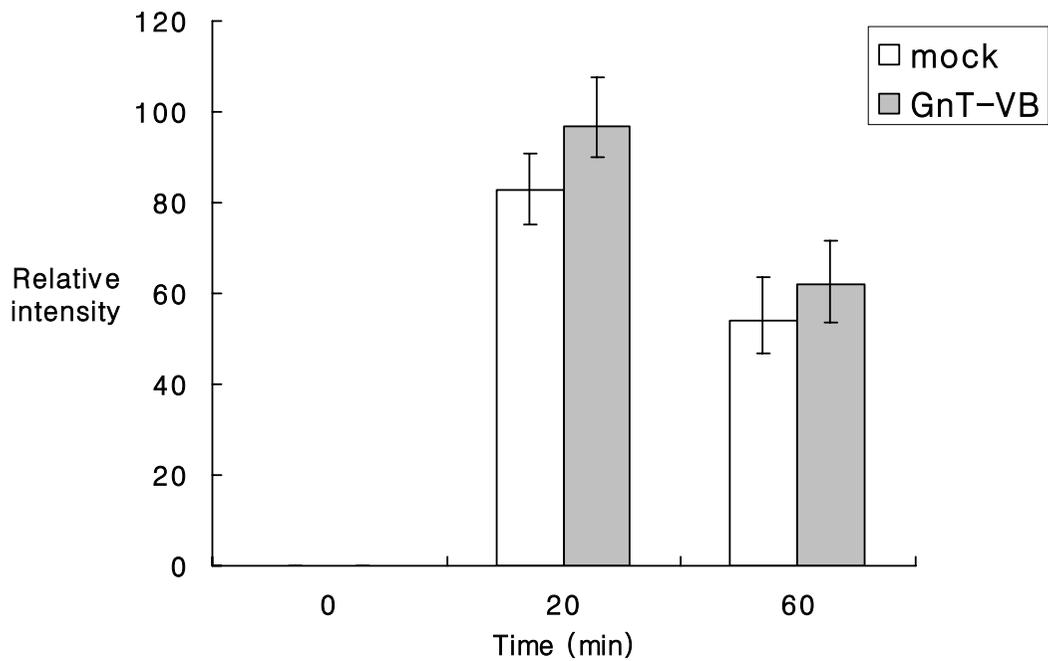
**Fig.3.2A** Cells were plated on 6 well plates coated with collagen I and cultured overnight, followed by treatment with NGF (50 ng/ml) for up to 14 days in culture medium containing 2% serum. The difference in neurite outgrowth was most prominent between day 5 and day 7, but both mock- and GnT-VB transfected PC12 cells showed maximal neurite outgrowth after day 14. The image was obtained at day 5.



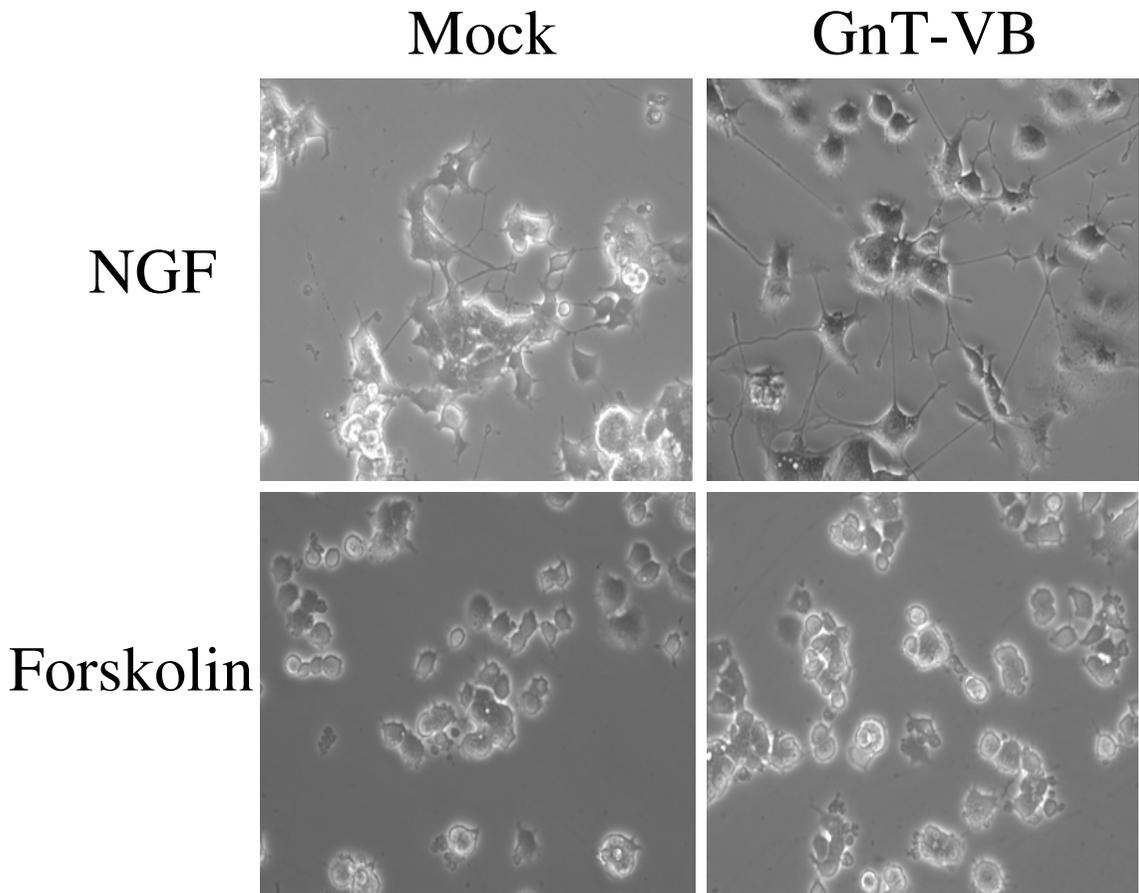
**Fig.3.2B** Cells with different lengths of neurites were quantitatively analyzed. Most cells developed neurites of at least one body length long at day 7. Phase contrast images of ten random fields were captured at 10x magnification and the number of cells with different lengths of neurites was counted and regrouped for analysis at day 7. Approximately, total 300 cells were counted. Two independent experiments were carried out to confirm the result. The numbers shown in the figures were averaged from two experiments.



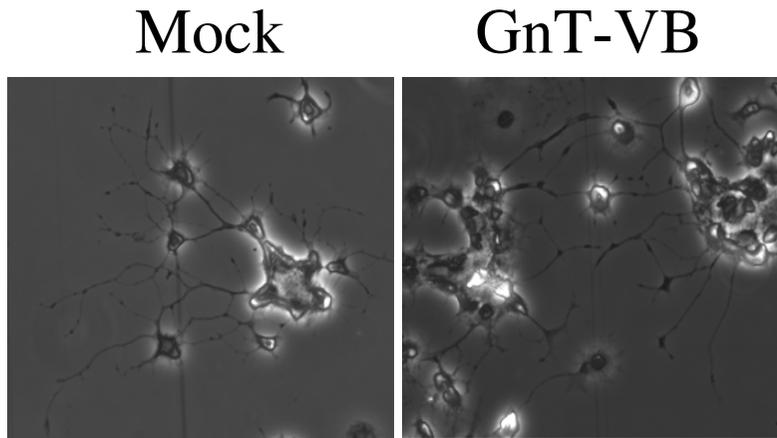
**Fig.3.3A** Densitometry of western blot for TrkA phosphorylation at Tyr490. Cells were plated on collagen-coated 100 mm dishes and cultured overnight. Culture medium was replaced with medium containing 2% serum and incubated for 18 hours before NGF (100 ng/ml) in serum free medium was added to the cells for the indicated times. After stimulation with NGF, cells were washed twice with ice cold PBS, followed by lysis on culture dish with 1% Triton X-100 lysis buffer in PBS, containing protease inhibitor cocktail and 1 mM sodium orthovanadate. The densitometry of X-ray film was performed with Versadoc (Bio-rad) using Quantity One software.



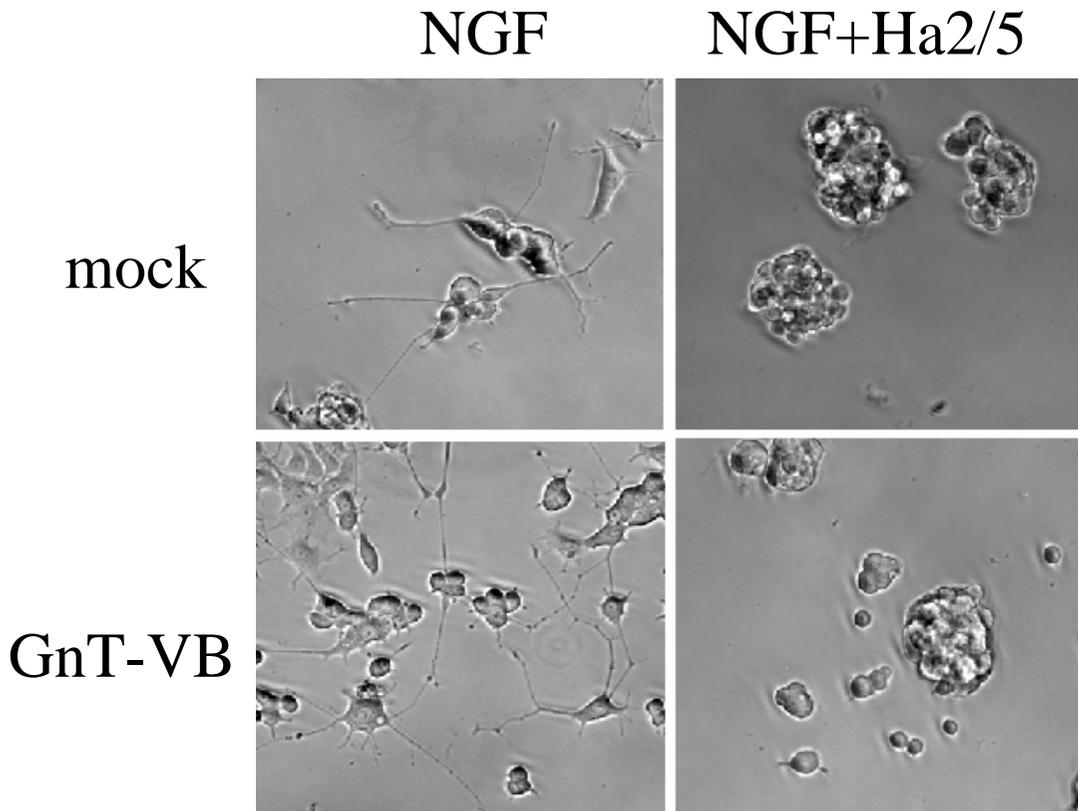
**Fig.3.3B** The same cell lysates were used to load onto 4-20% SDS-PAGE and probed with anti-phospho-ERK or anti-ERK antibodies after western transfer. The densitometry of the blots was performed as described above. Similar result was obtained when cells were stimulated with 50 ng/ml NGF.



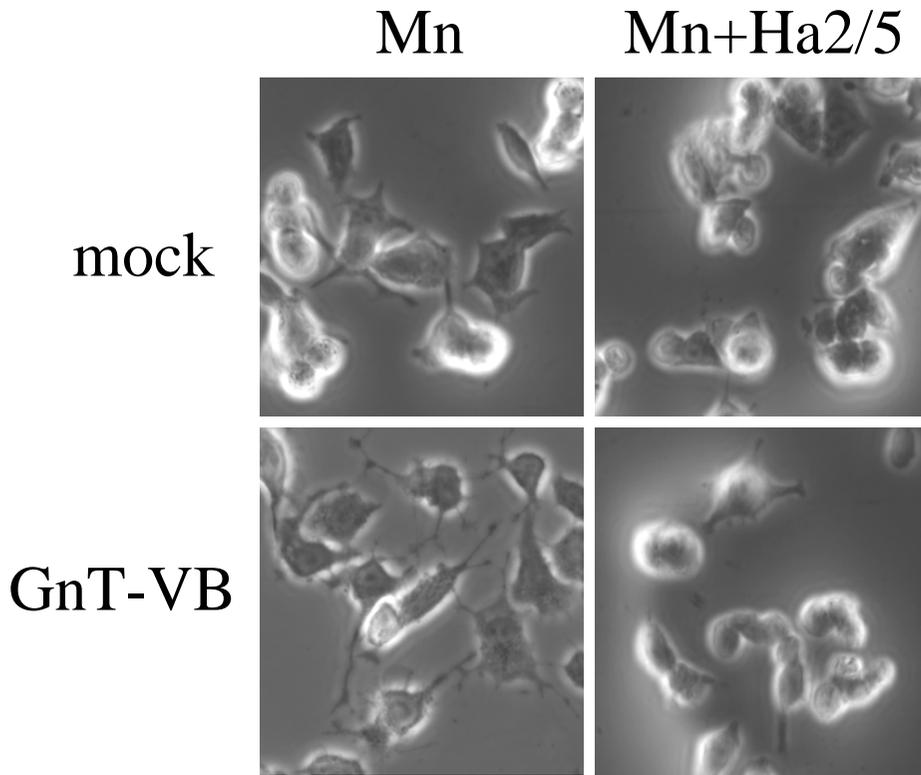
**Fig.3.4** Forskolin, a potent activator of cAMP signaling, does not induce neurite outgrowth in PC12/GnT-VB cells on collagen substrate. Cells were plated on collagen-coated 6 well plates and cultured overnight. After 24 hours, the culture medium was replaced with medium containing 1% serum, supplemented with either 100 ng/ml NGF or 10  $\mu$ M Forskolin and cultured for 7 days, before examined by using phase contrast microscopy.



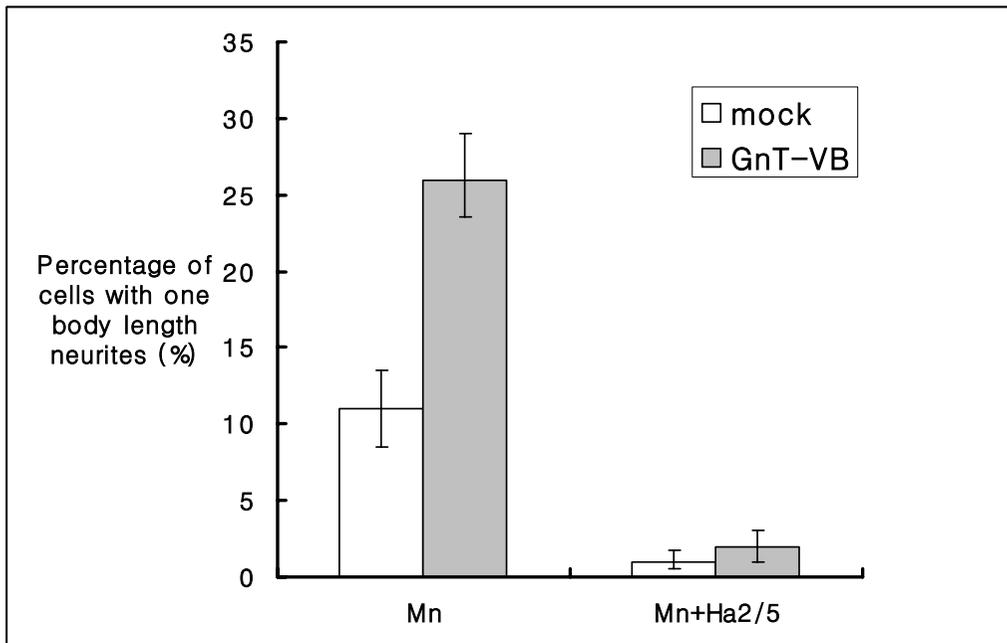
**Fig.3.5A** PC12/GnT-VB cells show no significant difference in NGF-induced neurite outgrowth when cultured on non-treated tissue culture plates. Cells were plated either on non-treated culture 6 well plates or culture plates coated with collagen or laminin and cultured overnight. After 24 hours, cells were stimulated by addition of NGF (50 ng/ml) in culture medium containing 2% serum for 7 days, followed by examination under phase contrast microscope.



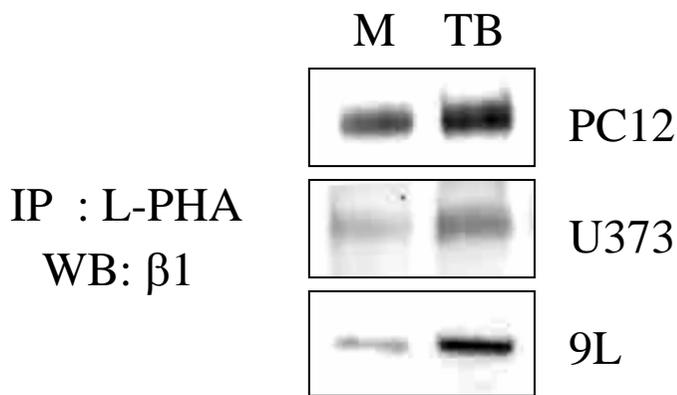
**Fig.3.5B** NGF-induced neurite outgrowth on collagen substrate is completely inhibited by addition of anti- $\beta$ 1 integrin function-blocking antibody. Cells were plated on collagen-coated 6 well plates overnight. Next day, culture medium was replaced with medium containing 2% serum supplemented with NGF (100 ng/ml) either in the presence or absence of anti-rat  $\beta$ 1 integrin function-blocking antibody and cultured for 7 days, before neurite outgrowth of cells was examined.



**Fig.3.6A** PC12/GnT-VB shows enhanced neurite outgrowth in manganese-induced neurite outgrowth, compared to mock transfected PC12 cells. Cells were plated on collagen-coated 24 well plates and cultured overnight. Next day, cells were treated with 200 uM manganese in culture medium containing 2% serum with or without addition of anti-rat  $\beta$ 1 integrin function blocking antibody, Ha2/5, (10  $\mu$ g/ml) for 72 hours, followed by examination under phase contrast microscope at 400x magnification.



**Fig.3.6B** Six random fields from duplicate wells were photographed, and numbers of cells with one-body length neurites were quantitated. Experiments were repeated twice.



**Fig.3.6C** Human neuroblastoma U373 and rat gliosarcoma 9L cells were transiently transfected with human GnT-VB. Cells were harvested by scraping, washed twice with PBS, followed by lysis in 1% Triton X-100 lysis buffer containing protease inhibitors. Cell lysates were then precipitated overnight with L-PHA and loaded onto 7.5% SDS-PAGE. After western transfer to PVDF membrane, the blots was probed with anti- $\beta$ 1 integrin antibody and developed by chemilluminescence method. The densitometry of the blot was done as described earlier.

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## CHAPTER 4 CONCLUSION

### *N-Acetylglucosaminyltransferase V Regulates $\beta 1$ integrin clustering by affecting level of $\beta(1,6)$ branched N-linked glycans on $\beta 1$ integrins*

A strong correlation between level of  $\beta(1,6)$  branched glycoprotein expression and tumor invasiveness/metastasis has been known for more than 20 years (1). Unfortunately, due to the complex and diverse nature of carbohydrate-carbohydrate and carbohydrate-protein interaction on the cell surface, it has been difficult to determine the exact molecular mechanism of increased tumor cell invasion/metastasis by  $\beta(1,6)$  branching. Evidence has accumulated, however, suggesting that one of the main mechanism is most likely increased level of  $\beta(1,6)$  branching on cell surface adhesion receptors such as integrin and cadherin. In order to study whether these adhesion receptors are in fact the substrates for modification with  $\beta(1,6)$  branching and if so, how this modification leads to changes in cell motility, we studied human fibrosarcoma HT1080 cells infected with a retroviral expression vector containing N-acetylglucosaminyltransferase V. HT1080 cells express high levels of  $\beta 1$  integrin mainly as the  $\alpha 5\beta 1$  integrin heterodimer, which was a putative target acceptor for GnT-V and a fibronectin receptor (2, 3). Important findings in the project were two-fold; (i) the  $\beta 1$  integrin subunit showed  $\beta(1,6)$  branching, but not the  $\alpha 5$  integrin subunit, suggesting that GnT-V likely has a specific set of substrate proteins in a given cell type, rather than broadly glycosylates glycoproteins with complex type N-glycans, (ii) immunostaining results with anti- $\beta 1$  integrin and  $\alpha 5$  integrin subunits antibodies revealed that clustering of  $\alpha 5\beta 1$  integrin was significantly impaired when GnT-V overexpressing HT1080 cells were allowed to spread onto fibronectin coated surface, compared to control cells (4). This result led to a hypothesis that  $\beta 1$  integrin with increased  $\beta(1,6)$  branching has reduced ability to

cluster to focal adhesion complex, where the integrin and other structural and signaling proteins form a large aggregation on the plasma membrane, physically linking extracellular matrix to the intracellular actin cytoskeleton. In current models of cell adhesion, the more stable the physical linkage becomes during cell spreading, the stronger the cell adhesive strength is to the ECM (5, 6). Therefore, it is likely that increased  $\beta(1,6)$  branching of  $\beta 1$  integrin leads to unstable or a reduced number of  $\beta 1$  integrin-dependent linkages between actin cytoskeleton and ECM, causing the cells to become more motile, and, in the case of oncogenically transformed cells, easily detach from original sites and invade surrounding tissues. This hypothesis is supported by a recent study by Springer's group where the role of integrin clustering in integrin function was investigated, compared to the role of integrin affinity regulation upon integrin-ligand engagement (7). The authors showed that integrin clustering had a negligible effect on initial ligand-binding affinity, but later clustering led to a significant strengthening of already established cell adhesion. This study provides a strong support for our hypothesis that GnT-V expression likely decreases cell adhesive strength by reducing integrin clustering.

***Regulation of integrin clustering by  $\beta(1,6)$  branching influences the rate of integrin internalization***

Even though the mechanism of how increased  $\beta(1,6)$  branching causes a decrease in integrin clustering is not clear, studies of integrin recycling between plasma membrane and transport vesicles of endocytosis/exocytosis system seem to provide unique insights on this issue. Normally, integrin-ligand complexes are internalized shortly after their engagement and recycled back and forth between, presumably, multi-vesicular body complexes and the plasma membrane (8). The current understanding of this model suggests the following: (i) different integrin

subunits in the same cell types or same integrin subunits in different cell types utilize distinct internalization pathways, including lipid raft-mediated endocytosis (9), clathrin-dependent endocytosis (10), and caveolar-mediated endocytosis (11). (ii) The ability of integrin to cluster and migrate into distinct lipid microdomains, such as lipid rafts and caveolae, determines the rate of integrin internalization and eventually integrin recycling back to plasma membrane (8, 11). According to this model, reduced integrin clustering most likely leads to enhanced internalization of integrins in focal adhesion complexes upon ligand binding. Our data also showed that ligand-bound  $\beta 1$  integrins in GnT-V deficient HT1080 and Hela cells prepared by GnT-V siRNA tend to have longer retention time in the plasma membrane, compared to control cells, suggesting that  $\beta 1$  integrin recycling is significantly slowed in GnT-V deficient cells (Lee et al. unpublished observation).

How would increased surface retention time of  $\beta 1$  integrin result in decreased  $\beta 1$  integrin-dependent cell motility? Gundersen's group at Columbia University made an interesting observation regarding how mobility of integrin-containing focal adhesion complexes on the plasma membrane are affected when stationary cells are stimulated to migrate (12). In stationary fibroblastic cells, a  $\beta 1$  integrin-GFP chimeric protein showed a high mobility on the cell surface, suggesting reduced engagement with the actin cytoskeleton in stationary mode. By contrast, when cells were induced to migrate by monolayer wound or after cell division, the integrins tended to be static in terms of position on the plasma membrane. In this study, moving focal adhesions in stationary cells were shown to remain associated with contracting actin fibers, suggesting that contraction of actin fibers is likely to be responsible for movement of integrin-focal adhesion complex. The authors were able to correlate mobility of integrin-focal adhesion complexes with generation of traction force for migration. That is, during directional migration,

inhibition of integrin-focal complex mobility via engagement of stable actin cytoskeleton linkages functions as a “molecular clutch” that allows the integrin-focal adhesion to form the optimal number of stable adhesion sites, thereby simultaneously coordinating traction force and directional polarization. This counter-intuitive phenomenon creates an innate ambiguity in appropriate interpretation of our data, because optimal cell migration is achieved when the rate of integrin recycling and actin cytoskeleton-integrin-ECM interaction are best balanced. The rate of integrin recycling is an important factor in regulating membrane movement of polarized, migrating cells, whereas stability of actin-integrin-ECM determines the traction force generated by a particular group of focal adhesion complex. When a focal complex is not stable enough, then there is not enough traction force for causing membrane movement. If integrin recycling is compromised, the polarized leading edge of migrating cells would be supplied with an insufficient number of integrins for new engagement with ECM proteins.

***A hypothesis on how the rate of cell migration is affected by integrin glycosylation, rate of integrin internalization and subsequent recycling.***

In regard to this hypothesis, we found that GnT-V overexpressing fibroblastic cells showed a very different cell shape during migration, compared to control cells (Lee et al, unpublished observation). Normally, migrating control cells were characterized by a highly elongated morphological phenotype. By contrast, GnT-V overexpressing cells were much shorter and round up even during migration. This phenotype has been documented by recent studies (13, 14), showing that invasive tumor cells tend to show an amoeboid-like movement during migration through three dimensional ECM, much resembling lymphocyte migration in ECM, rather than a typical, longer cell shape displayed by non-invasive cells. There are obviously

advantages for tumor cells to display this morphology, since it would greatly decrease the time of integrin recycling during rapid migration without actually having to enhance the rate of recycling.

This model, in fact, provides us with a very interesting theory, when reversed. That is, the faster the rate of integrin recycling (i.e. integrin with increased  $\beta(1,6)$  branching), the more compact the cell morphology would become during cell migration. Therefore, one of the future aims of this project would be to examine the integrin recycling in migrating GnT-V-deficient cells in a three dimensional matrix (15).

***Vacuolar H<sup>+</sup>-ATPase (V-ATPase) 16 kDa subunit associates with  $\beta 1$  integrin in the endoplasmic reticulum and causes  $\beta 1$  integrin aggregation by inhibition of  $\beta 1$  integrin-calnexin association.***

V-ATPase expression has recently been associated with tumor cell invasion and multi-drug resistance during cancer chemotherapy (16-19). Due to alkaline charges of most anticancer agents (17), a high intracellular pH is favored by tumor cells to block the binding of cancer drugs to their intracellular targets, thus abrogating the effect of chemotherapy. V-ATPase in some cancers, such as breast cancer, is targeted to cell surface and function as a proton pump (19). Interestingly, several studies reported that most subunits of V-ATPase is transcriptionally up-regulated in response to anticancer agents, to five-fold (20, 21). In particular, Torigoe et al. demonstrated that 16K is highly up-regulated by both cisplatin and TAS-103. Then, how would increased 16K expression at the protein level influence anti-cancer treatment? As described in Chapter 2, we showed that overexpression of 16K results in down-regulation of functional  $\beta 1$  integrin on the cell surface. This, in turn, would lead to reduced  $\beta 1$  integrin-dependent signal transduction for cell proliferation and survival. Sethi et al's study on the role of  $\beta 1$  integrin-

dependent signaling in drug resistance of small cell lung carcinoma (22) suggests a novel function that 16K- $\beta$ 1 integrin interactions may play in relation to drug resistance. There seems to be two different aspects that need to be considered; (i) 16K- $\beta$ 1 integrin interaction clearly leads to both decreased  $\beta$ 1 integrin surface expression and  $\beta$ 1 integrin-dependent signaling. Ironically, it is consistently reported that 16K expression is up-regulated only in drug-resistant cancer cells. (ii) A recent study suggests that small cell lung cancer exploits  $\beta$ 1 integrin-dependent signaling for survival against anticancer agents. These considerations would lead to the logical conclusion that inhibition of  $\beta$ 1 integrin-dependent signaling or  $\beta$ 1 integrin expression by 16K should counteract an important tumor cell-survival mechanism.

This conclusion raises an obvious question. That is, what would be the benefits for tumor cells to up-regulate 16K expression in response to anticancer agents? Further study is obviously required to address this complicated issue. One way to study this question is by overexpressing 16K in cancer cells, followed by cisplatin treatment to see if increased 16K expression enhances or compromises the anti-cancer drug treatment for inducing apoptosis in cancer cells.

***GnT-VB expression in rat pheochromocytoma PC12 cells stimulates nerve growth factor-induced neurite outgrowth.***

Neurite extension by growth cone elongation is carried out by a mechanism essentially identical to those found in membrane protrusion of migrating fibroblastic cells (23, 24). Since GnT-VB is selectively expressed in brain (25), we hypothesized that GnT-VB would perform a similar function as GnT-VA in stimulation of cell motility (i.e. enhancing neurite outgrowth in neuronal cells). Therefore PC12 cells were stably transfected with human GnT-VB and NGF-induced neurite outgrowth was studied. As described in Chapter 3, we observed a significant

enhancement in the rate of neurite outgrowth for GnT-VB expressing PC12 cells, compared to control cells. This enhancement was neither caused by increased expression of microtubule associated proteins nor by the alteration of NGF-dependent signal transduction pathway. Interestingly, the differences in the rate of neurite outgrowth were only observed on collagen coated surfaces, but not on non-treated tissue culture plates. This result suggested that GnT-VB may be affecting integrin-mediated neurite outgrowth by differential glycosylation. This was further supported by a study of  $\beta 1$  integrin activation-dependent neurite outgrowth induced by manganese treatment along with an  $\beta 1$  integrin function blocking antibody, which completely inhibited neurite outgrowth in both control and GnT-VB overexpressing PC12 cells. Unlike the previously reported PC12 variant cell line, PC12D (26), PC12/GnT-VB showed no neurite outgrowth in response to a cAMP activator, forskolin, confirming that GnT-VB enhancement of PC12 neurite outgrowth is not likely dependent on NGF-TrkA and downstream signal transduction pathway. We found no evidence of altered glycosylation of TrkA receptor in PC12/GnT-VB cells, however there was an indication that  $\beta 1$  integrin, a collagen receptor, was glycosylated by GnT-VB. These results demonstrated that  $\beta 1$  integrin glycosylation by GnT-VB likely exerts a significant influence on enhanced neurite extension during neuronal differentiation.

In summary, our results clearly showed that N-linked glycosylation of integrin receptors plays important roles in regulating cell motility, which has a critical impact during tumor progression and differentiation.

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## APPENDIX

### ABERRANT N-GLYCOSYLATION OF $\beta 1$ INTEGRIN CAUSES REDUCED $\alpha 5\beta 1$ INTEGRIN CLUSTERING AND STIMULATES CELL MIGRATION<sup>1</sup>

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Hua-Bei Guo, <sup>1</sup>Intaek Lee, Maria Kamar, Steven K. Akiyama, and Michael Pierce (2002) Cancer Research 62, 6837-6845 Reprinted here with permission of publisher, ©American Association of Cancer Research

**The abbreviations used are:** GnT-V, N-acetylglucosaminyltransferase V (Mgat5); GlcNAc, N-acetylglucosamine; N-glycan, asparagine-linked glycans; ECM, extracellular matrix; L-PHA, leucoagglutinating phytohemagglutinin; Con A, concanavalin A; SW, swainsonine; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ECL, enhanced chemiluminescence.

## ABSTRACT

Altered expression of cell surface N-linked oligosaccharides is associated with the oncogenic transformation of many types of animal cells. One of the most common forms of glycosylation in transformed cells and human tumors is the highly elevated  $\beta$ 1,6 branching of N-linked oligosaccharides caused by increased transcription of N-acetylglucosaminyltransferase V (GnT-V). To characterize the effects of increased  $\beta$ 1,6 branching on cell-matrix adhesion mediated phenotypes, human fibrosarcoma HT1080 cells were transfected with retroviral systems encoding GnT-V that utilized both non-inducible and tetracycline-inducible (tet-off) promoters. Increased GnT-V expression resulted in a >25% inhibition of cell attachment to and a >50% inhibition of cell spreading on fibronectin. Both cell adhesion and spreading were suppressed by function-blocking antibodies specific for the  $\alpha$ 5 and  $\beta$ 1 integrin subunits of the fibronectin receptor. Cell migration towards fibronectin and invasion through Matrigel were both substantially stimulated in cells with induced expression of GnT-V. Induction of GnT-V had no effect on the level of cell surface expression of  $\alpha$ 5 and  $\beta$ 1 integrin subunits, but did, however, result in a more diffuse staining of the  $\alpha$ 5 and  $\beta$ 1 integrin subunits on the cell surface, suggesting that inhibition of integrin clustering may be causing these cells to be less adhesive and more motile. Surprisingly, there was no detectable expression of N-linked  $\beta$ 1,6 branching on the  $\alpha$ 5 subunit purified from HT1080 cells before and after induction of GnT-V; by contrast, however, the  $\beta$ 1 subunit showed a basal level of  $\beta$ 1,6 branching that was greatly increased after induction of GnT-V. These results suggest that changes in N-linked  $\beta$ 1,6 branching that occur during oncogenesis alter cell-matrix adhesion and migration by modulating integrin clustering

and subsequent signal transduction pathways. These effects most likely result from altered N-linked carbohydrate expression on the  $\beta$ 1 integrin subunit.

## INTRODUCTION

Alterations of cell surface glycan structures are often associated with malignant transformation of cells. One of the most common is a significant increase in the levels of N-glycans containing the  $\beta$ 1,6 branch,  $\beta$ 1,6-GlcNAc linked to the  $\alpha$ 1,6- mannose of the trimannosyl core (1). This GlcNAc $\beta$ 1,6Man structure is synthesized by N-acetylglucosaminyltransferase V (GnT-V or Mgat5, E.C.2,1,4,155 ), a key enzyme in the processing of multi-antennary N-glycans during the synthesis of glycoproteins (2, 3). Results from previous studies showed the expression of  $\beta$ 1,6 GlcNAc branched N-linked oligosaccharides in human mammary, colon, hepatic, and glial tumors (4-8), and suggest that a relationship exists between N-linked  $\beta$ 1,6 branching glycans on one hand, and tumorigenesis and tumor invasion on the other hand.

Recent experiments have revealed the mechanisms by which oncogenesis causes changes of GnT-V activity. GnT-V transcription was stimulated by several oncogenes including *src*, *her-2/neu*, *H-ras* and *v-sis* (9-11), and this over-expression was regulated through the ras-raf-ets pathways. Stimulation of GnT-V activity has also been observed via the PKC and PI3K-PKB signaling pathways (9-13). GnT-V deficient mice (GnT-V  $-/-$  or Mgat5  $-/-$ ) have been used to study the effects of eliminating GnT-V activity on tumor progression (14). When crossed with mice that express the polyoma middle T-antigen under control of the mouse mammary tumor virus promoter, the progression of mammary tumors in the GnT-V ( $-/-$ ) mice was significantly

reduced compared to that in the GnT-V (+/-) mice. This study provides evidence that changes in N-linked  $\beta$ 1,6 branching can affect carcinoma progression *in vivo*.

Since GnT-V cDNA was cloned by our and Taniguchi's group (15, 16), several studies have transfected GnT-V sense or anti-sense cDNA into cultured cells, selected for clones with altered GnT-V expression, and investigated cell adhesive properties (8, 17-19). Although these studies show that alterations in N-linked  $\beta$ 1,6 branching may affect cell adhesive behaviors, including cell adhesion and migration, the exact relationship were often inconsistent among the clones under study and mechanisms involved in these events remain unclear. In one study, for example, two clones over-expressing GnT-V were selected and shown to have altered cell migration on plastic, high levels of apoptosis, and decreased adhesion to fibronectin and laminin, but the clone with intermediate GnT-V expression and  $\beta$ 1,6 branching levels showed a greater alteration of adhesive properties than the clone with higher levels of GnT-V and  $\beta$ 1,6 branches (17).

Altered cell-cell and cell-extracellular matrix interactions are clearly critical during the acquisition of the invasive phenotype. Cell surface integrins, especially those binding to fibronectin and laminin, play essential roles in tumor cell invasion and metastasis. The fibronectin receptor  $\alpha$ 5 $\beta$ 1 contains a total of 26 putative N-linked glycosylation sites (14 on  $\alpha$ 5 and 12 on  $\beta$ 1), although it is not known which of these sites are utilized. Inhibitors of N-linked oligosaccharide processing have been shown to inhibit the ability of  $\alpha$ 5 $\beta$ 1 to bind fibronectin but not to affect its cell surface levels or ability to dimerize (20, 21). The mechanisms by which changes in distal glycosylation, such as  $\beta$ 1,6 branching, could affect cell adhesion and migration are, however, only now being understood.

To investigate in detail the relationship between increased N-linked  $\beta$ 1,6 branching and  $\alpha$ 5 $\beta$ 1 -mediated adhesion to fibronectin and explore its possible mechanisms, we have used an inducible retroviral expression system. Our results show that increased expression of GnT-V activity and resulting increased levels of  $\beta$ 1,6 branching inhibits attachment and spreading of cells on fibronectin, but stimulates cell migration towards fibronectin and invasion through a reconstituted Matrigel basement membrane. Increased GnT-V expression cause increased levels of N-linked  $\beta$ 1,6 branching on the  $\beta$ 1 subunit of the fibronectin receptor, but there is no evidence of these oligosaccharide structures on the  $\alpha$ 5 subunit, even after increased GnT-V activity. Increased  $\beta$ 1,6 oligosaccharide branching inhibits clustering of the  $\alpha$ 5 $\beta$ 1 integrins and organization of F-actin into extended microfilaments when cells are plated on fibronectin-coated plates.

## **MATERIALS AND METHODS**

**Cell lines and materials.** HT1080 human fibrosarcoma cells (CCL-121) and Phoenix-producer cells (SD3443) were obtained from ATCC; Bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM), human plasma fibronectin, swainsonine (SW), BES, SB3-10 and hexadimethrine bromide (polybrene) were products of Sigma; Retroviral plasmid (pTJ66) and the Tet-inducible (tet-off) retroviral plasmid (pTJ68-IZG) were kindly provided by Dr. T.J. Murphy, Emory University. NHS-LS-Biotin, Ultralink immobilization kit and ECL assay kit were products of Pierce. Streptavidin-HRP was obtained from Rockland. Biotinylated L-PHA and Con A were products of Vector Labs. Rhodamine-phalloidine was from Molecular Probes. Protein A-agarose, protein L-agarose and Rhodamine-conjugated anti-rat IgG were from Santa

Cruz Biotechnology. 1% Gelatin gel was obtained from Bio-Rad company. The 12-well chemotaxis Boyden chambers (Transwell) and 24-well matrigel coated chemotaxis Boyden chamber (8.0  $\mu$ m) were products of BD Falcon™.

**Construction of retrovirus plasmids containing GnT-V cDNA.** A 2.5 kb fragment containing the full-length mouse GnT-V cDNA was cut from the pMMVGnT-V plasmid with *Sma* I. This fragment was then ligated with *Sfi* I adapters (formed using 3' GATCCGGA and 5'GTAGGCCTACA) and cloned into the non-cohesive universal cloning *Sfi* I site of retroviral plasmid pTJ66 to produce pTJ66GnT-V. The orientation of the insert was confirmed by *BstX* I restriction digestion mapping. This vector uses a 5'-LTR promoter, includes an internal ribosome entry signal (IRES), and produces a chimera mRNA that simultaneously expresses the protein of interest and the Zeo:eGFP reporter/selection marker. pTJ68-IZG denotes the retroviral plasmid with inducible CMV/tet-operon (tetO-CMV) promoter, which expresses the tetracycline-transactivator (tTA) protein (tet-off) and uses Zeo:eGFP as the reporter marker. For the inducible expression of GnT-V, pTJ68-IZG was linearized by digestion with *BstX* I, followed by dephosphorylation, and then ligated, after its 3'-overhang was removed using T4 polymerase, with the 2.5 kb fragment of GnT-V to produce pTJ68-IZGGnT-V. *BstX* I and *Sap* I digestions were used to identify the orientation of the insert.

**Cell culture, retroviral production and infection.** HT1080 and Phoenix-producer cells were maintained and passaged routinely at 37°C in 5% CO<sub>2</sub> in DMEM growth media containing 10% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals) supplemented with 0.1 mM non-

essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µg streptomycin (Sigma).

Retroviral production and infection were performed as described previously (22). Briefly, infectious retroviral supernatants were produced by a transient, helper virus-free protocol with pTJ66GnT-V and pTJ68-IZGGnT-V plasmids. Phoenix-producer cells were grown to 50-80% confluence in DMEM growth media in 100 mm culture dishes and transfected with retroviral plasmids (pTJ66GnT-V and pTJ68-IZGGnT-V, respectively) using 2 M  $\text{CaPO}_4$  and 25 mM chloroquine in 50 mM BES buffer, pH 7.0, for 6-8 h, and then the dishes were re-fed with 25 ml growth media. Twenty four hours after transfection, the growth media was aspirated, replaced with 9 ml growth media, and incubated in 5%  $\text{CO}_2$  at 32°C to increase the retroviral titer. Supernatants containing infectious retrovirus were collected every 12 h up to 106 h after transfection and then filtered using a 0.45 µm filter, aliquoted, snap frozen in liquid nitrogen, and stored at -80°C. At the same time, pTJ66 and pTJ68-IZG plasmids without GnT-V cDNA insert were used to generate control retroviral supernatants.

For the infection, HT1080 cells were grown in 6-well plates and infected by adding retroviral supernatant and polybrene (8 µg/ml). The plates were then incubated at 32°C for 15 min, followed by centrifuging at 2500 rpm for 30 min at 32°C. The infectious supernatant was aspirated, the cells were re-fed with growth media, and then placed back to the incubator with 37 °C. The infection was repeated 8-12 h later. Cells were then cultured for 48 h after the second infection before selection in media containing 800 µg/ml Zeocin for 3-5 days. The surviving cells (expressing GFP) were transferred into a culture flask and used for experiments. After two rounds of retroviral infections, over 95% of the cells showed GFP fluorescence in each infection experiment when viewed with an inverted fluorescence microscope.

Transfected cells were maintained in DMEM growth media plus 10% FBS. The cells with tet-inducible promoter were maintained in growth media with 0.1 µg/ml tetracycline to suppress exogenous GnT-V expression (non-induced). Prior to each experiment, the cells containing the inducible promoter were induced to express GnT-V by the withdrawal of tetracycline from the culture media (induced), and levels of expression were determined by altering the duration of time cells were maintained in the media without tetracycline. Unless specified, induction time was at least 36 h. For non-induced cells, parallel cultures were maintained with growth media containing 0.1 µg/ml tetracycline.

**GnT-V activity assay.** Cells were trypsinized, pelleted, and lysed with 50 mM MES (pH 6.5), 150 mM NaCl, and 1% Triton X-100. Insoluble debris was pelleted by microcentrifugation (10 min. at 4°C), and the supernatant was used for the GnT-V activity assay using UDP-[<sup>3</sup>H]-GlcNAc as acceptor substrate. Sep-Pak columns (Waters) were used to separate the substrates and products as described (23). The column methanol eluant was added to scintillation fluid and radioactivity measured by scintillation counting. Assays were performed in duplicate, the data averaged, and results were expressed as specific activity (nmol/h/mg). Protein concentration in the cell lysates was determined using the BCA assay (Pierce).

**Lectin blotting.** Cells were harvested, rinsed with PBS, and lysed with 1% Triton X-100 in PBS. Cell lysates containing 30 µg of protein were boiled in SDS-sample buffer with or without β-mercaptoethanol, loaded on 4-20% SDS-PAGE gels, and then transferred onto a PVDF membrane. After blocked with 1% BSA, the membrane was incubated with 1 µg/ml biotinylated L-PHA in blocking solution for 1 h followed by the incubation with a 1:5000

dilution of streptavidin-HRP for another 30 min at room temperature. The blots were washed and developed with the ECL detection system using X-ray film. Quantification was obtained using a Fluor-S scanner (Bio-Rad).

**Assays of cell-fibronectin adhesion and spreading.** Cell attachment was assayed as described (24). Briefly, 96-well microtiter plates were coated with 0.1 ml of human plasma fibronectin in PBS, incubated at 37°C for 1 h, and blocked by 1% BSA at 37°C for 30 min after washing. Cells ( $3 \times 10^4$ ) suspended in 100  $\mu$ l serum-free DMEM culture media were added to each coated well and incubated at 37 °C for 30 min. Wells were gently washed 3 times with 100  $\mu$ l ice cold PBS to remove unbound cells, followed by fixation of adherent cells using 3.5% formaldehyde for 15 min. Cells were then stained with a 0.5% crystal violet solution. After washing the wells twice with PBS, the absorbance of each well at 595 nm was measured using an automated microtiter plate spectrophotometer. For the antibody inhibition experiments (25), function-blocking mAb 13 (50  $\mu$ g/ml) against  $\beta$ 1 integrin or mAb16 (2 mg/ml) against  $\alpha$ 5 integrin was added to the cell suspension in 100  $\mu$ l PBS prior to addition of the cell suspension to the fibronectin-coated wells. The data were expressed as the mean of triplicate wells.

For assessment of cell spreading on fibronectin, the same procedure as above was used. After the adherent cells were fixed and stained with crystal violet, the number of spread cells was quantitated by counting the percentage of spread cells versus the total number of cells. Cells were considered as spread if they became flattened and lost nuclear refractivity. Each experimental point was the result of counting cells in 3-5 fields/ well in at least 2 wells viewed at 100X magnification. The values from 6-10 fields were used to calculate the mean and standard error. Typically 50-150 cells were present per field.

**Wound healing assay.** Cells ( $3 \times 10^5$ ) were seeded into fibronectin (10  $\mu\text{g/ml}$ )-coated 6-well plates in 2 ml of serum-free DMEM culture media overnight. A clear area was then scraped in the monolayer with a 200  $\mu\text{l}$  yellow plastic tip. After washing with serum-free DMEM, the plate was incubated for 6 h at 37°C in serum-free DMEM media. In some experiments, 1  $\mu\text{g/ml}$  swainsonine was added to the cells for 24 h as GnT-V was induced to express. For inhibitory experiments, mAb13 (50  $\mu\text{g/ml}$ ), or mAb16 (2 mg/ml) was added into the well after wounding. Migration of cells into wounded areas was evaluated with an inverted microscope and photographed.

**Transwell cell migration and invasion assays.** Migration assays were performed using 12-well Transwell units with 8- $\mu\text{m}$  pore size polycarbonate inserts. Briefly, the undersides of insert membranes were coated with fibronectin (10  $\mu\text{g/ml}$ ) overnight at 4°C and blocked with 1% BSA for 1 h at 37 °C. Cells ( $3 \times 10^4$ ) suspended in 400  $\mu\text{l}$  DMEM plus 0.1% BSA were added to the upper compartment of the transwell unit, and 1.5 ml DMEM plus 0.1% BSA was added into the lower chamber. Cells were allowed to migrate for 6 h at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells on the upper side of the membrane were removed using a cotton swab, while the cells that migrated to the underside were fixed and stained with crystal violet. The number of cells on the underside of the membrane was counted in 5 different fields with a light microscope at 100X magnification, and the mean and standard deviation calculated. Typically, each field contained ~300 cells. For some experiments, mAb13 (50  $\mu\text{g/ml}$ ) or mAb16 (2 mg/ml) was added into the cells before they were added to the Transwell apparatus. For swainsonine-treated cells, 1  $\mu\text{g/ml}$  swainsonine was added into the culture media for 24 h before

cells were removed for addition to the upper chamber of the transwell apparatus. Using Matrigel-coated 24-well Boyden chambers, invasion assays were performed using the same procedure as the migration assays, except that the incubation time of the experiment was prolonged to 21 h. Cells that migrated through the membrane were stained and counted as described above.

**Gelatin gel zymography.** Cells were cultured to subconfluence in 6-well plates coated with fibronectin (10 µg/ml). Then, cells were washed and incubated with 2 ml serum-free media for 24 h. The media were collected, centrifuged and frozen until experiment. To detect gelatinase, 10 µl of conditioned media were loaded to 10% SDS-PAGE gel containing 1% gelatin at 4°C under nonreducing condition. After electrophoresis, the gels were rinsed in renaturing buffer (2.5% Triton X-100) for 1 h and incubated at 37°C overnight in buffer containing 50 mM Tris-HCl (pH7.7), 5 mM CaCl<sub>2</sub>, and 0.02% NaN<sub>3</sub>. MMP-9 and MMP-2 were indicated by clear bands at 92 kDa and 72 kDa that appeared after staining with Coomassie Brilliant Blue. Gels were then resined and dried.

**Cell surface labeling and immunoprecipitation.** Cell labeling with biotin and immunoprecipitation were performed as described by Rigot *et al* (26) with minor modifications. Sub-confluent cells were washed and detached using 2 mM EDTA. Cells were then washed twice with ice-cold PBS and incubated with 1 mg/ml NHS-LC-biotin in PBS for 20 min at 4°C on a rocking platform. After washing with PBS, cells were lysed by incubation with buffer I (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, Complete™ mini protein inhibitor cocktail, 0.7 µg/ml pepstatin, and 1% Triton X-100). Lysates were cleared by centrifugation and incubated with protein-L agarose (50 µl of beads/ml lysate) at 4°C under

agitation for 3-5 h to remove nonspecific adsorption to the agarose beads. After the determination of total protein using a BCA assay, cell lysates were incubated with 5 µg/ml mAb13 or mAb16 overnight at 4°C under agitation, followed by incubation with 50 µl/ml protein L-agarose at 4 °C for 3-5 h under agitation. The pellets were washed twice in buffer I, followed by two washes in buffer II (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P40, 0.05% sodium deoxycholate), and once in buffer III (50 mM Tris-HCl, pH 7.5, 0.1 % Nonidet P40, 0.05% sodium deoxycholate), and subjected to SDS-PAGE under reducing and non-reducing conditions. The gels were then transferred onto PVDF membrane and probed with streptavidin-HRP (1:5000).

**Fluorescence staining.** Cells were plated onto fibronectin coated chamberslides (10 µg/ml) at 37 °C in serum-free media for either 30 min or 120 min. Cells were then fixed with 3.5% formaldehyde in PBS for 15 min followed by three times washes with PBS, permeabilized with 0.5% Triton X-100 (for actin staining only) for 15 min and blocked with 1% BSA. For the localization of  $\alpha 5\beta 1$  integrins, chamberslides were incubated with 10 µg/ml mAb13 for  $\beta 1$  or mAb11 for  $\alpha 5$  integrin at 37°C for 30 min, followed by the incubation with Rhodamine-conjugated anti-rat IgG (1:250) at 37 °C for another 30 min. For actin filament staining, chamberslides were incubated with Rhodamine-phalloidin (1:40). After washing with PBS, the chamberslides were mounted and imaged using an immunofluorescence confocal microscope (Bio-Rad).

**$\alpha 5\beta 1$  integrin purification.** Purification of  $\alpha 5\beta 1$  integrin was performed according to the procedure of Pytela *et al* (27) with minor modifications. Briefly,  $2 \times 10^8$  cells were harvested and

lysed in PBS buffer containing mini protease inhibitor cocktail (1 tablet/10ml), 0.7  $\mu\text{g/ml}$  pepstatin, and 100 mM N-Octylglucoside plus 0.5% SB3-10 (NDSB, Sigma). Lysed cells were loaded onto an immunoaffinity column consisting of mAb16 (against  $\alpha 5$  integrin) coupled to agarose beads using the Ultralink immobilization kit. Briefly, 2 mg of mAb 16 in 200  $\mu\text{l}$  PBS were mixed with 0.25 g dry beads in 4 ml MOPS coupling buffer and allowed to swell and couple for 1 h. The reaction was terminated by incubating beads in 5 ml of 3 M ethanolamine for 3 h. Non-coupled protein was removed by washing the beads with 8 M urea in PBS, followed by rinsing with at least 10 ml of equilibration buffer (PBS containing 0.5% SB3-10). Beads were incubated on ice for 1 h prior to use. After cell lysate was applied to the 1 ml column of beads at 4  $^{\circ}\text{C}$ , the column was washed with 10 column volumes of equilibration buffer and eluted with low pH elution buffer (0.1 M glycine adjusted to pH 2.9 with acetic acid) at 4  $^{\circ}\text{C}$ . 1 ml fractions were collected and subjected to SDS-PAGE under non-reducing conditions. The identity of the two distinct bands corresponding to 140 kDa and 120 kDa under non-reducing condition was confirmed to be the  $\alpha 5$  and  $\beta 1$  subunits, respectively, by western blotting with a combination of 20  $\mu\text{g/ml}$  mAb11 and 20  $\mu\text{g/ml}$  mAb13.

## RESULTS

In order to develop an experimental system in which GnT-V activity could be over-expressed without the need for selection of single cell clones, we developed two retroviral expression plasmids to be used to infect HT1080 human fibrosarcoma cells. A cDNA encoding mouse GnT-V was inserted into two retroviral expression vectors with green fluorescent protein (GFP) expression to monitor the infection efficiency, one of which utilized tet-off inducible

expression. In all experiments to be described, >95% of each population of cells expressed GFP fluorescence 24 h after infection with retroviral supernatants.

After infection of HT1080 cells with a plasmid with no GnT-V sequence and a non-inducible promoter (mock transfected cells) showed a GnT-V specific activity of  $0.18 \pm 0.02$  nmol/h/mg while cells infected with a similar plasmid that encoded GnT-V (GnT-V transfected cells) showed a specific activity of  $10.7 \pm 1.3$  nmol/h/mg. As shown in Fig 1A, cells infected with the plasmid containing GnT-V insert and the tet-off inducible promoter (GnT-V transfected cells) showed an increase in GnT-V activity as early as 3 h after the removal of tetracycline and reached maximum induction (about 10-12 fold) 24 h after withdrawal of tetracycline from culture media. These cells also showed a dose-dependent increase in GnT-V activity as the concentration of tetracycline in the culture media was reduced (Fig. 1B). When maintained in tetracycline concentrations of  $> 0.1 \mu\text{g/ml}$ , these cells showed about 1.5-fold higher GnT-V activity compared to mock transfected cells, showing that the promoter activity of the GnT-V plasmid could not be completely suppressed. In these cells, mRNA that encoded both GnT-V and GFP showed a tetracycline dose-dependent induction as the concentration of tetracycline in the culture media decreased (data not shown).

The level of expression of cell surface N-linked oligosaccharides that contained  $\beta$ 1,6 oligosaccharide branches increased after transfection of many cells types by several oncogenes, including, *src*, *her-2/neu*, *ras* and *sis* (9-11), caused by the up-regulation of GnT-V transcription. To determine whether the over-expression of GnT-V showed the expected increase in  $\beta$ 1,6 oligosaccharide branching on the cell surface of infected HT1080 cells, the levels of  $\beta$ 1,6 branched oligosaccharides present on the glycoproteins of GnT-V transfected cells were compared before and after induction by using the L-PHA, which specifically binds to N-linked

oligosaccharides containing [Gal( $\beta$ 1,4)GlcNAc $\beta$ (1,6)Man $\alpha$ (1,6)]. Cell lysates were subjected to SDS-PAGE, followed by lectin blotting and ECL detection of L-PHA binding. The results (Fig. 1C) confirmed that there were substantial increases in the levels of  $\beta$ 1,6 branching of glycoproteins after induction of GnT-V activity.

We first studied the growth properties of HT1080 cells over-expressing GnT-V. The growth rate of GnT-V transfected cells cultured on plastic in serum-containing medium was similar to that of mock transfected cells, and no significant difference in number of colonies was observed when both types of transfected cells were grown in soft agar (data not shown), showing that high levels of GnT-V activity did not influence cell proliferation. Then, we measured the effects of GnT-V increased expression on cell spreading and adhesion to fibronectin. A population of HT1080 cells over-expressing GnT-V activity showed significantly reduced (> 50%) spreading on fibronectin-coated plates after 30 min (Fig. 2A). Spreading on fibronectin was inhibited by the addition of either of two function-blocking monoclonal antibodies, mAb 13, specific for the human  $\beta$ 1 integrin subunit, and mAb 16, specific for the  $\alpha$ 5 subunit. These results demonstrate that the spreading of the cells on fibronectin resulted from adhesion through the  $\alpha$ 5 $\beta$ 1 integrin receptors and that increased expression of GnT-V caused significantly reduced spreading. When HT1080 cells transfected with GnT-V under control of the tet-off promoter were induced to over-express GnT-V (Fig. 2B), inhibition was also observed on spreading on fibronectin. In fact, some inhibition of spreading could be observed when non-induced cells with GnT-V insert were compared to mock transfected cells, suggesting that even relatively small increases in GnT-V activity, caused by the leaky Tet-off promoter, could affect spreading on fibronectin.

The effects of GnT-V over-expression were next tested on simple cell attachment to fibronectin. The results of these experiments show that inducing the expression of GnT-V in HT1080 cells caused decreased cell attachment to fibronectin (Fig. 3). The attachment of non-induced cells was slightly but consistently inhibited compared to that of control vector-infected cells. Adhesion of the HT1080 cells was inhibited by mAb 13 and mAb 16, showing that the adhesion was  $\alpha 5\beta 1$ -dependent (data not shown). These results show that the adhesion of HT1080 cells to fibronectin was significantly inhibited by increased GnT-V expression.

Experiments were then designed to determine if GnT-V over-expression could affect cell migration, assayed by both wound healing on fibronectin substrates and haptotaxis toward fibronectin. The results in Fig. 4 show that induction of GnT-V activity caused a striking increase of cell migration, and that this effect was strongly inhibited by antibodies against the integrin  $\beta 1$  subunit and to a lesser extent by antibodies against the integrin  $\alpha 5$  subunit.

Swainsonine inhibits the Golgi  $\alpha$ -mannosidase II, ultimately causing the inhibition of N-linked  $\beta 1,6$  oligosaccharide expression upstream of the action of GnT-V. Cells pre-incubated in swainsonine before wounding but after induction of GnT-V showed little or no increase in migration.

In the Boyden chamber (Transwell) migration assays, HT1080 cells induced to over-express GnT-V showed about a two-fold increase in rate of migration compared to mock transfected cells (Fig. 5A). These increased rates were inhibited by antibodies to  $\alpha 5$  and  $\beta 1$  and by pre-incubation with swainsonine. Taken together, these results are consistent with the conclusion

that increased  $\beta$ 1,6 oligosaccharide branching caused altered adhesion to fibronectin mediated by the  $\alpha$ 5 $\beta$ 1 integrin that results in increased rates of migration.

We next examined if increased GnT-V increased expression could alter *in vitro* invasive behavior through a reconstituted Matrigel basement membrane. Fig. 5B shows that HT1080 cells with induced GnT-V activity showed almost a 3-fold increase in *in vitro* invasiveness after 21 h incubation, and that this effect was inhibited by antibodies to  $\alpha$ 5 and  $\beta$ 1, or by pre-treatment of cells with swainsonine. Non-induced cells, which showed a 1.5-fold increase in GnT-V activity, migrated only slightly faster than control cells, suggesting that the ability of cells to penetrate and to migrate through Matrigel was very sensitive to relatively small increases in GnT-V activity.

We also detected the secretion of the metalloproteinases MMP-9 and MMP-2 in the culture media of HT1080 cells grown on the fibronectin coated plates, but no significant difference in the MMP-9 or MMP-2 levels were found after induction of GnT-V over-expression (Fig. 5C). Taken together, these results show that the increased ability of HT1080 cells over-expressing GnT-V to migrate through Matrigel resulted from increased expression of  $\beta$  1,6 branching.

To investigate the mechanisms underlying the effects of GnT-V expression on cell adhesive behavior, F-actin filaments were first visualized in GnT-V over-expressing cells. At early times (30 min) after plating cells on fibronectin-coated wells, phalloidin staining of permeabilized cells revealed increased F-actin staining in membrane protrusions in cells over-expressing GnT-V, suggesting increased motility of these cells (Fig. 6). After longer times on fibronectin (120 min), the network of F-actin stress fibers appeared to be less organized in the

GnT-V induced cells, as compared to mock transfected cells, suggesting that increased  $\beta$ 1,6 branching had a destabilizing effect on F-actin filaments.

One possible explanation of the effects of GnT-V levels on fibronectin-mediated cell adhesion and migration could be that changes in  $\beta$ 1,6 oligosaccharide branching affect levels of  $\alpha$ 5 $\beta$ 1 integrin expression on the cell surface. To test this possibility, cell surface  $\alpha$ 5 $\beta$ 1 integrin receptors were quantitated by biotinylation, immunoprecipitation, and blotting using streptavidin-peroxidase. As shown in Fig. 7A, levels of  $\alpha$ 5 or  $\beta$ 1 on HT1080 cells were similar after induction of GnT-V expression. In addition, the total mRNA levels of  $\beta$ 1 integrin determined by RT-PCR were also found unchanged in GnT-V expressing cells compared to control cells (data not shown). These results suggest that the effects observed on adhesion/migration after induction were not the result of altered levels of  $\alpha$ 5 $\beta$ 1 on the cell surface.

Fluorescent staining patterns of cell surface  $\alpha$ 5 and  $\beta$ 1 using monoclonal antibodies, however, showed clear differences after induction of GnT-V activity (Fig. 7B). Cells with induced GnT-V grown on fibronectin plates for 2 h demonstrated a more diffuse surface staining of both  $\alpha$ 5 and  $\beta$ 1 subunits compared to mock transfected cells, suggesting decreased  $\alpha$ 5 $\beta$ 1 integrin clustering. This observation is consistent with a less adhesive and more motile cellular phenotype (28).

To determine if levels of  $\beta$ 1,6 branching on the  $\alpha$ 5 $\beta$ 1 subunits were specifically altered after induction of GnT-V, the  $\alpha$ 5 $\beta$ 1 receptor was purified from HT1080 cells before and after induction of GnT-V using an antibody affinity column, followed by chromatography on

immobilized wheat germ agglutinin (WGA). Equal amounts of the purified receptor were subjected to SDS-PAGE under non-reducing conditions, blotted, and probed with various antibodies and lectins. The results of these experiments (Fig. 7C) showed that the subunits could be distinguished and that each bound the lectin Concanavalin A (Con A). Surprisingly, however, only the  $\beta$ 1 subunit and not the  $\alpha$ 5 subunit was bound by L-PHA, demonstrating that  $\beta$ 1,6 branching could be detected only on the  $\beta$ 1 subunit. When GnT-V activity was induced, significant increases in L-PHA staining (>3-fold) were observed on the  $\beta$ 1 subunit. Even after induction, however, no L-PHA binding to the  $\alpha$ 5 subunit was observed, suggesting a selective glycosylation of the  $\beta$ 1 subunit by GnT-V. These results demonstrate that induction of GnT-V activity caused increased  $\beta$ 1,6 branching on the  $\beta$ 1 subunit and that the effects of increased GnT-V expression on fibronectin-mediated adhesive phenotypes likely result from this altered glycosylation.

## **DISCUSSION**

To test directly the hypothesis that changes in GnT-V activity and  $\beta$ 1,6 branching can cause altered cell-matrix adhesion and migration and allow study of the underlying mechanisms, we transfected mouse GnT-V cDNA into human HT1080 cells using retroviral vectors, one of which contained a tetracycline-inducible (Tet-off) promoter. Compared to standard plasmid transfection used in previous studies (8, 17-19), retroviral infection is a highly efficient gene transfer method applicable to many cell types (29), that yields high expression efficiency and the ability to modulate expression (29, 30). In the present study, both retroviral and tet-inducible retroviral systems were used to express GnT-V, at efficiencies >95%, as judged by GFP

fluorescence of infected cells. Because of the high efficiency, we were able to examine total populations of transfected cells, ensuring that any observed alterations in cell behaviors could be attributed to GnT-V expression and not to phenotypic variation of selected clones. Stable populations of HT1080 cells were produced whose expression of GnT-V could be regulated by changing levels of tetracycline in the growth media. In both infected cell types, increased expression of  $\beta$ 1,6 branching followed increased activity of GnT-V.

We first investigated the effect of GnT-V expression on cell proliferation and found that GnT-V levels had minimal effects on the rate of cell growth on plastic or anchorage-independent growth in soft agar. In addition, no effect of GnT-V over-expression was observed on the apoptotic behavior of cell populations (data not shown), by contrast to the results of an earlier report (17). Altering GnT-V expression did, however, cause significant changes in cell-matrix interaction. We found that both cell adhesion to and cell spreading on fibronectin was markedly decreased after the induction of GnT-V expression in HT1080 cells, and these events were most likely mediated by  $\alpha$ 5 $\beta$ 1 integrin confirmed by antibodies that blocked  $\alpha$ 5 $\beta$ 1 adhesion. After exogenous expression of GnT-V, reduced cell-matrix adhesion was observed in mink lung epithelial (17) and hepatocarcinoma cells (31), but not in mouse mammary cancer cells (19). This difference in cell adhesion might result from the variation between individual clones used in the experiment. Our results together with previous studies suggest strongly, therefore, that changes in N-linked glycosylation caused by increased  $\beta$  1,6 branching had direct effects on  $\alpha$ 5 $\beta$ 1-mediated cell spreading and adhesion.

We used different types of assays to test if GnT-V over-expression could affect rates of migration. We observed with both the wound healing assay and the haptotaxis assay that cell motility was significantly increased after induced expression of GnT-V. The increased

migration of HT1080 cells induced by GnT-V expression could be suppressed by adding function-blocking antibodies against  $\alpha 5$  or  $\beta 1$ , indicating that this receptor was involved in regulating this migratory behavior. The increased migration of HT1080 cells over-expressing GnT-V was inhibited by swainsonine treatment, consistent with the conclusion that  $\beta 1,6$  oligosaccharide branching may play a direct role in  $\alpha 5\beta 1$  integrin-mediated cell motility. Cell migration is a complex behavior regulated by multiple mechanisms (32). A critical factor that regulates the rate of cell migration is the degree of adhesion of cells to their ECM substrata. Migration is maximal under conditions of intermediate levels of cell adhesion (33). Thus, the increased rate of migration on fibronectin is consistent with decreased cell adhesion and spreading observed for GnT-V over-expressing cells.

Increased  $\beta 1,6$  branching also promoted the ability of cells to invade through Matrigel. Antibodies against both the  $\beta 1$  and  $\alpha 5$  integrin subunits and swainsonine treatment blocked increased HT1080 invasion caused by the induced expression of GnT-V, indicating the involvement of  $\alpha 5\beta 1$  integrin and  $\beta 1,6$  branching in the invasion process. HT1080 cells produce matrix metalloproteinases (MMPs) including both MMP-2 and MMP-9, which can degrade the components of basement membranes and extracellular matrix and increase cell invasion (34). To determine if GnT-V over-expression enhanced the secretion of MMP-2 and MMP-9 in HT1080 cells, levels of MMP-2 and MMP-9 in culture media were analyzed by zymography using gelatin as substrate. No significant differences were observed in levels of either MMP-2 or MMP-9 after induction expression of GnT-V in HT1080 cells. This result supports the conclusion that increased cell invasion due to increased  $\beta 1,6$  branching is the result of increased cell migration rather than an increased secretion of MMPs.

Focal adhesions are formed when cells contact the extracellular matrix (ECM), linking the

cytoplasmic tails of integrins to the actin cytoskeleton, and play a pivotal role in cell spreading and migration (35, 36). In our study, we found increased F-actin staining in membrane protrusion at early times and decreased stress fiber formation in the cells with over-expressed GnT-V. These observations are consistent with a less adhesive, more motile phenotype, as suggested by Duband *et al* (28). It was shown that following cell spreading on fibronectin, activation of focal adhesion kinase (FAK) and transient inhibition of a small GTPase, RhoA, proved to be essential for promoting membrane protrusion and polarity (37, 38); Moreover, FAK-dependent transient inhibition of RhoA is required to promote focal adhesion turnover and actin filament reorganization (39, 40). It is reasonable, therefore, to postulate that the signaling pathway mediated by FAK and/or RhoA activity might be affected in GnT-V over-expressing cells.

Integrin receptors regulate cell adhesion, spreading, migration, proliferation, as well as gene transcription (25, 32, 41, 42). The  $\alpha5\beta1$  integrin, a primary receptor of fibronectin, contains N-linked glycans. Previous studies demonstrated that  $\alpha5\beta1$  function can be modulated by N-linked oligosaccharide expression (20, 43). To investigate further glycosylation differences after GnT-V induction, the  $\alpha5\beta1$  integrin was purified from induced and non-induced cells and subjected to lectin blotting with L-PHA. Increased  $\beta1,6$  branching was observed on the  $\beta1$  subunit after induction of GnT-V, but no  $\beta1,6$  branching could be detected on the  $\alpha$  subunit either before or after induction. ConA bound to both  $\alpha5$  and  $\beta1$  subunits, by contrast, and little change in binding could be observed before and after the induction of GnT-V expression. More information on the N-linked oligosaccharide expression on the integrin subunits will await further detailed structural characterization. The effect of increased  $\beta1,6$  oligosaccharide branching on  $\alpha5\beta1$  function, however, appears to result solely from changes in  $\beta1$  glycosylation. Kobata's laboratory suggested that changes in  $\beta1,6$  branching of the  $\beta1$  subunit of transformed

3T3 cell lines correlated well with increased tumorigenicity and metastatic potential (44). More recently, Skinner *et al* (45) reported that over-expression of the 16-kDa, the membrane subunit of vacuolar ATPase could inhibit  $\beta 1,6$  branching of  $\beta 1$  integrin subunit, resulting in decreased tumor cell invasiveness. These studies are consistent with our direct demonstration of increased  $\beta 1,6$  branching on the  $\beta 1$  subunit after induction of GnT-V expression.

In our study, the levels of both  $\alpha 5$  and  $\beta 1$  subunits on HT1080 cell surfaces remained relatively unchanged when GnT-V was induced (Fig. 7). We did observe changes in cell surface  $\alpha 5\beta 1$  integrin clustering on fibronectin, however, after induction of GnT-V over-expression, using antibodies to visualize separately the  $\alpha 5$  and  $\beta 1$  subunits. Formation of focal adhesions is a critical event as cells attach to matrix components, initiated by the binding of integrins to specific extracellular matrix ligands and subsequent clustering of these receptors (46). We found more diffuse staining of  $\alpha 5\beta 1$  integrins on cells spreading on fibronectin after induced GnT-V expression, indicating that the clustering of  $\alpha 5\beta 1$  integrins was reduced in GnT-V over-expressing cells. This conclusion was further supported by our recent experiments with 12G10, an activating anti- $\beta 1$  integrin mAb which was reported to induce cell-cell adhesion and  $\beta 1$  integrin clustering in HT1080 cells (47). We found that over-expression of GnT-V in HT1080 cells could significantly inhibit  $\beta 1$  integrin clustering induced by 12G10 (unpublished data), suggesting that N-linked glycosylation of the  $\beta 1$  integrin may be a critical factor for regulating integrin clustering. In another study (8), human glioma cells transfected with GnT-V resulted in different localization pattern of  $\alpha 3\beta 1$  integrin, where the immunostaining of  $\alpha 3\beta 1$  integrin showed a strong localization of the integrin heterodimer to leading lamellipodia of GnT-V transfected cells, but not of parental or mock transfected cells. It is therefore clear that

glycosylation of integrins by GnT-V causes functional alteration in  $\beta$ 1-containing integrin heterodimers.

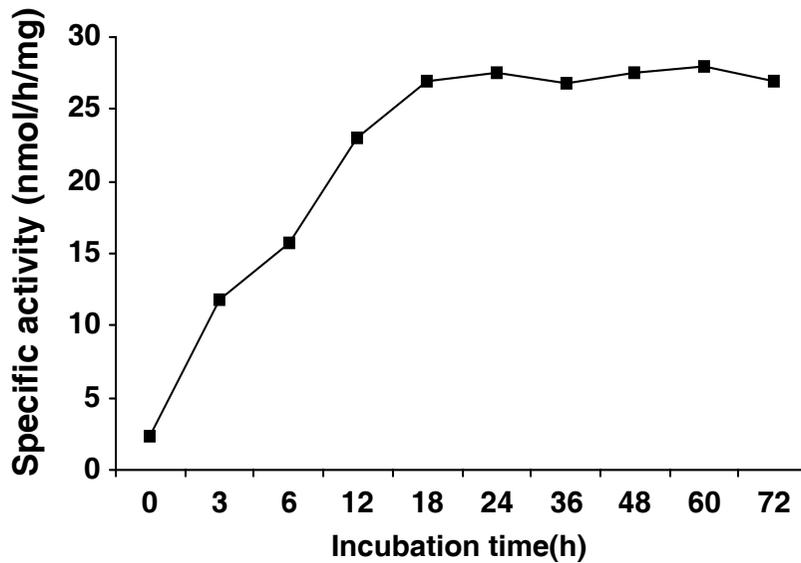
The function of integrin clustering in the regulation of cell adhesion strength has been studied by several groups. Yauch *et al* (48) reported that the deletion of the  $\alpha$ 4 integrin tail region significantly impaired static cell adhesion by severely restricting  $\beta$ 1 integrin clustering. Without affecting ligand-binding affinity, integrin-mediated adhesion appeared to be regulated by the rate of receptor diffusion/clustering. Another study showed that LFA-1 or integrin  $\alpha$ <sub>L</sub> $\beta$ <sub>2</sub> ligand binding strength to ICAM-1 was regulated by lateral diffusion of the integrins and their interaction with the cytoskeleton (49). Kassner *et al* (50) showed that an  $\alpha$ 4 integrin chimera expressing  $\alpha$ 2 or  $\alpha$ 5 tails greatly enhanced localization of the chimeric VLA-4 into focal adhesions and concluded that relatively more mobile  $\alpha$ 4 integrin, less engaged with cytoskeletal interaction, is likely to augment cell migration and diminish cell spreading and adhesion strengthening. Consistent with these reports, our data suggest that altered integrin clustering is one of the major consequences of increased  $\beta$  1,6 branching of  $\beta$ 1 integrin and results in a less adhesive and more motile phenotype through regulating cell spreading, adhesion, as well as actin-filament reorganization.

There are now clear examples of the inhibition of signal transduction pathways by mutations in specific N-acetylglucosaminyltransferases. Altered glycosylation of the *Notch* cell surface receptor during *Drosophila* development can clearly affect the signaling function of this receptor (51) and is caused by mutations in the *Fringe* N-acetylglucosaminyltransferase in the O-linked pathway. In addition, a recent study has documented that inactivating mutations in a human N-acetylglucosaminyltransferase which also functions in the biosynthesis of specific O-linked glycans, causes an inhibition of a particular cell surface signaling pathway, which, in turn,

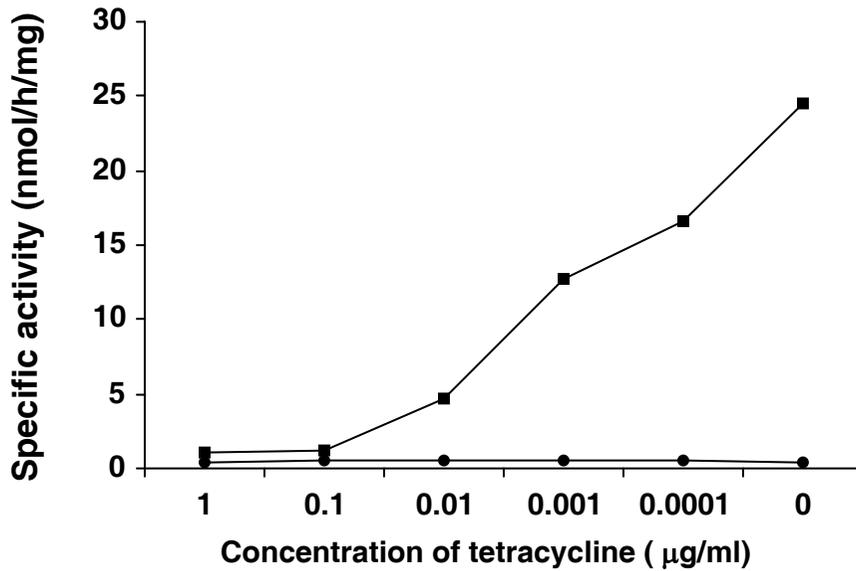
results in a form of muscular dystrophy known as muscle-eye-brain disease(52). Glycosylation of the CD8 $\alpha\beta$  co-receptor stalks on native thymocytes has recently been shown to regulate interactions between the CD8 “head” domain and HMCI tetramers, thereby modulating thymic selections (53). In light of these results, it is reasonable to suggest that increased  $\beta$ 1,6 branching of the  $\beta$ 1 integrin subunit by GnT-V that is up-regulated after oncogenesis results in increased cell migration and invasion by modulating integrin clustering and subsequent signal transduction pathways.

## **ACKNOWLEDGMENTS**

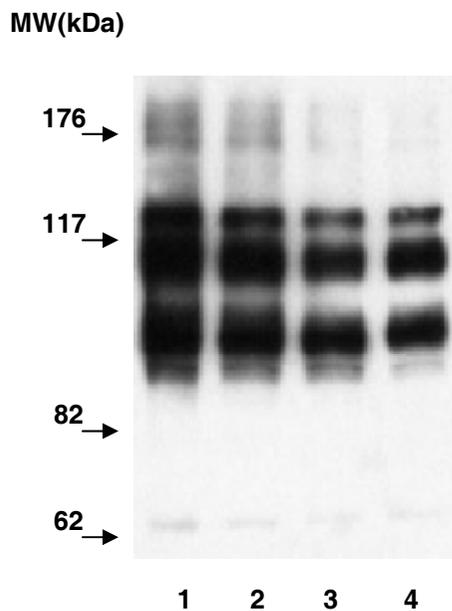
We thank Dr. T.J. Murphy of Emory University for his kind gifts of the retroviral expression vectors and Karen Abbott for her excellent technical assistance.



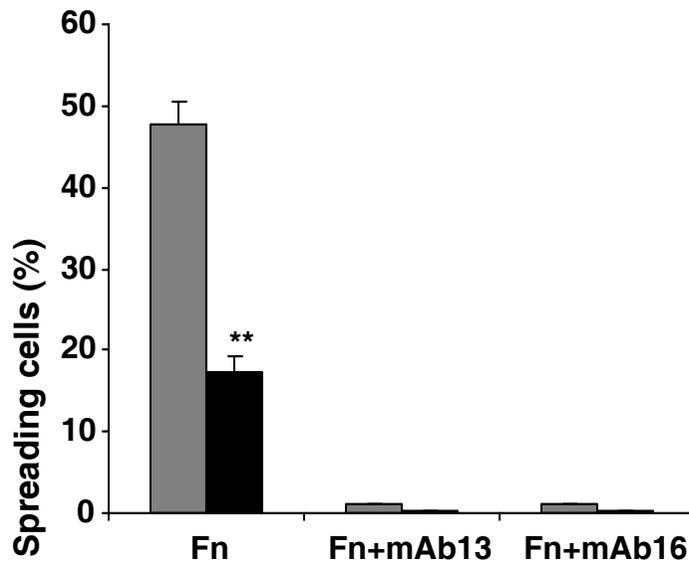
**Fig.1A** Time course of induction of GnT-V activity. GnT-V transfected HT1080 cells were grown in the presence of tetracycline (0.1  $\mu\text{g/ml}$ ) and induced by tetracycline withdrawal (0 h); cells were harvested at indicated times for GnT-V activity assay.



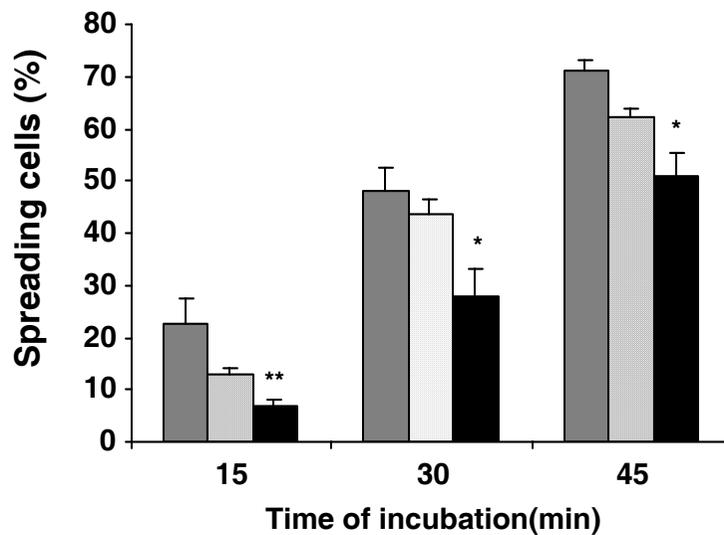
**Fig.1B** Dependence of GnT-V activity on tetracycline concentration. Transfected cells were cultured in the various tetracycline concentrations for > 36 h and harvested for assay of GnT-V activity. Circles, mock transfected HT1080 cells; Squares, GnT-V transfected HT1080 cells.



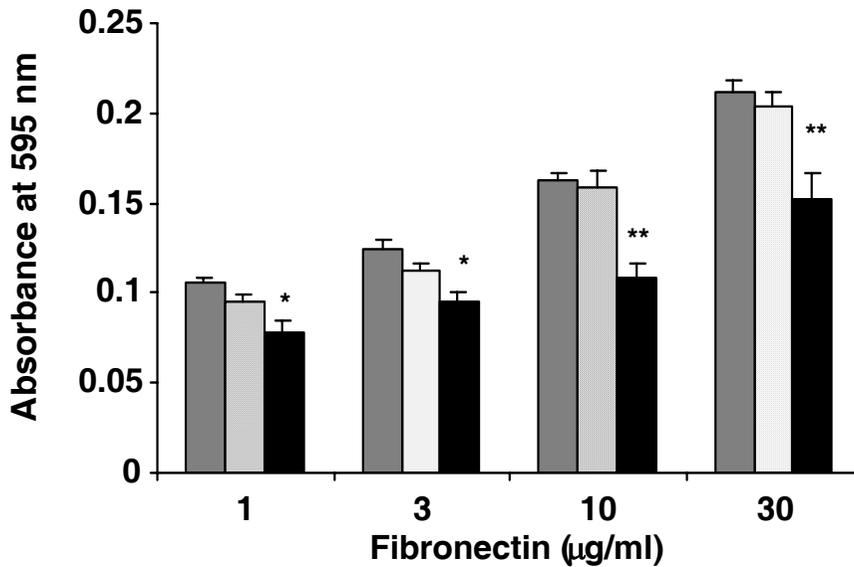
**Fig.1C** Lectin blotting was performed with biotinylated L-PHA using cell lysates from GnT-V transfected cells cultured in tetracycline-free (lane 1) and tetracycline-containing (0.001  $\mu\text{g/ml}$ , lane 2; 1  $\mu\text{g/ml}$ , lane 3) media and mock transfected HT1080 cells (lane 4).



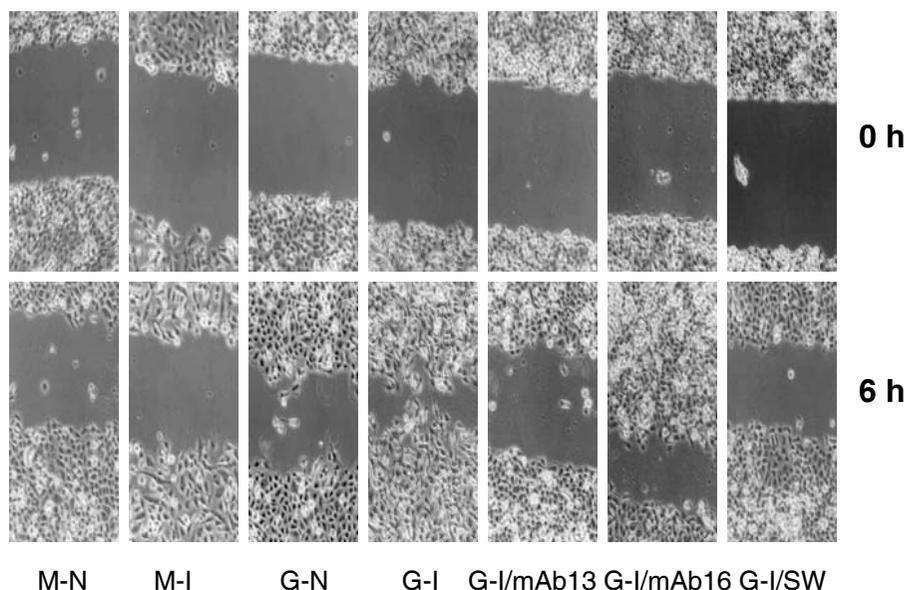
**Fig.2A** Cells ( $3 \times 10^4$ ) were added to 96-well plates coated with  $10 \mu\text{g/ml}$  of fibronectin either alone or in the presence of mAb 13 ( $50 \mu\text{g/ml}$ ) or mAb 16 ( $2 \text{ mg/ml}$ ), and cultured at  $37^\circ\text{C}$  for 30 min. The cells were rinsed, fixed, stained with crystal violet.



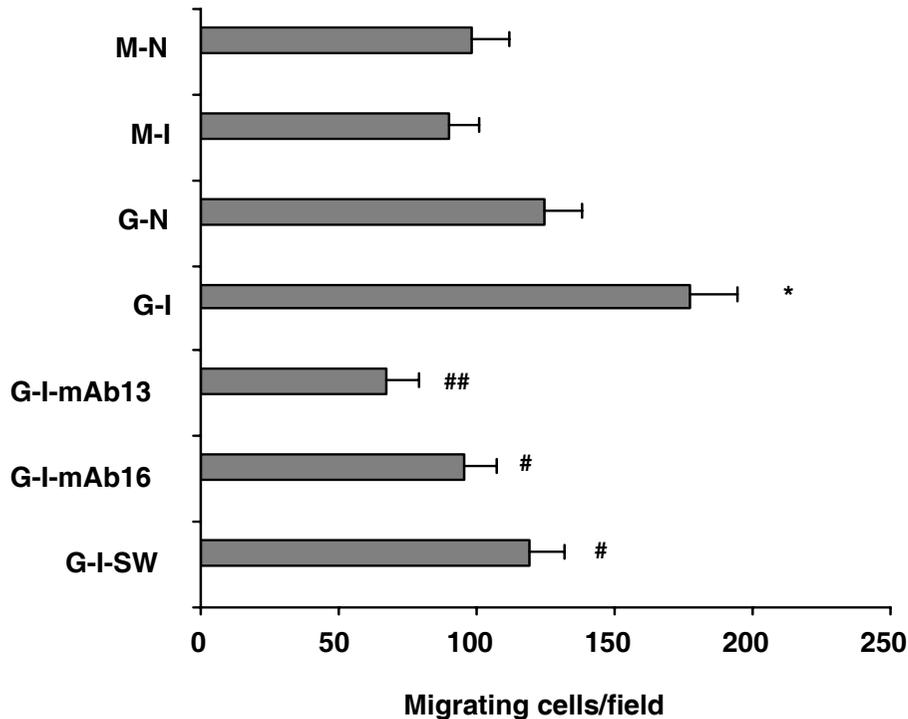
**Fig.2B** Time course of HT1080 cell spreading on fibronectin (10  $\mu\text{g/ml}$ ). Gray bars, mock transfected cells; hatched bars, non-induced GnT-V transfected cells; and black bars, induced GnT V transfected cells. Each bar represents the mean ( $\pm$ S.D.) of the percentage of spreading cells in 6-10 randomly selected fields. Similar results were obtained in three separate experiments. \*:  $P \leq 0.05$  and \*\*:  $P \leq 0.01$  vs. mock transfected cells (Student's t-test).



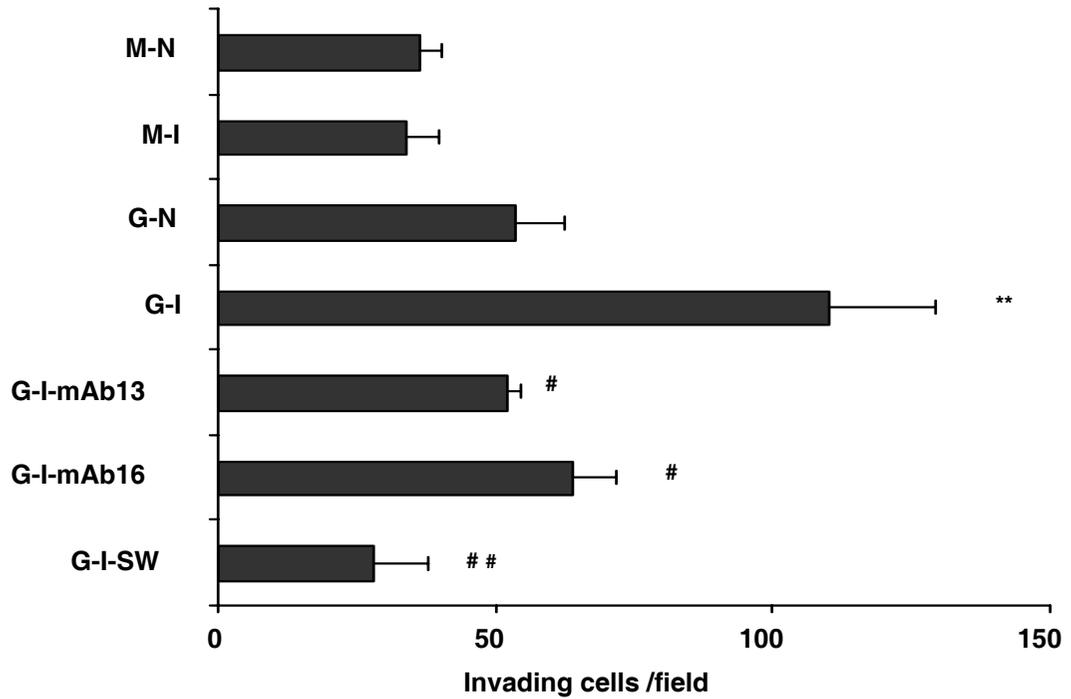
**Fig.3** Adhesion of GnT-V over-expressing cells to fibronectin. Cells ( $3 \times 10^4$ ) were applied to fibronectin-coated 96-well plates and incubated at 37 °C for 30 min. Adherent cells were stained with crystal violet and absorbance of each well determined at 595 nm. Gray bars, mock transfected cells; hatched bars, non-induced GnT-V transfected cells; black bars, induced GnT-V transfected cells. Each bar represents the mean ( $\pm$ S.D.) of triplicate determinations. Similar results were obtained from three separate experiments. \*:  $P \leq 0.05$  and \*\*:  $P \leq 0.01$  vs. mock transfected cells.



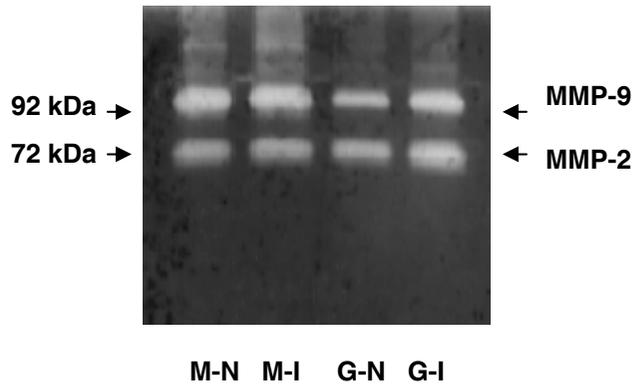
**Fig.4** Scratch-wound assay using confluent HT1080 cells on fibronectin plates. Cells ( $3 \times 10^5$ ) were seeded into a fibronectin-coated ( $10 \mu\text{g/ml}$ ) 6-well plate in serum-free medium for 24 h and the monolayer was then scratched with a yellow plastic pipette tip ( $0.2 \text{ ml}$ ). The plate was then incubated for 6 h at  $37^\circ\text{C}$  in serum-free media and areas of migration photographed. M-N, non-induced mock transfected cells; M-I, induced mock transfected cells; G-N, non-induced GnT-V transfected cells; G-I, induced GnT-V transfected cells; G-I-mAb13, induced GnT-V transfected cells with mAb13 ( $50 \mu\text{g/ml}$ ); G-I-mAb16, induced GnT-V transfected cells with mAb16 ( $2 \text{ mg/ml}$ ); G-I-SW, induced GnT-V transfected cells with swainsonine ( $1 \mu\text{g/ml}$ ) for 24 h prior to scratching and during migration period.



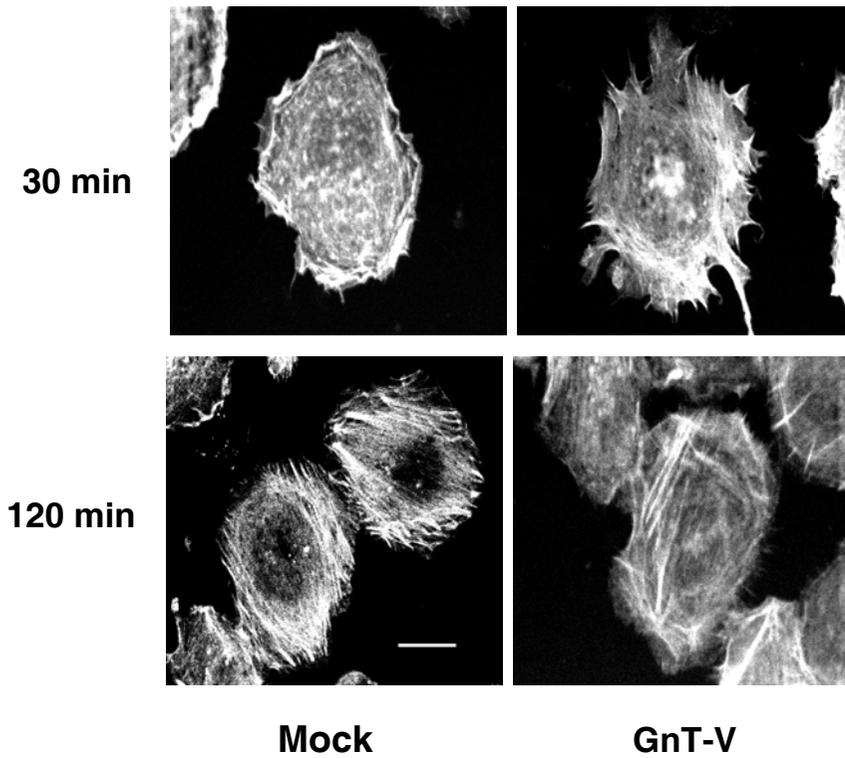
**Fig.5A** Cell migration determined using a Transwell apparatus with the underside of membrane insert coated with fibronectin (10  $\mu$ g/ml). Cells were incubated with indicated inhibitors in the upper chamber and allowed to migrate for 6 h. Migrating cells on the underside of the membrane were then fixed, stained, and counted using a light microscope. M-N, non-induced mock transfected cells; M-I, induced mock transfected cells; G-N, non-induced GnT-V transfected cells; G-I, induced GnT-V transfected cells; G-I-mAb13, induced GnT-V transfected cells with mAb13 (50  $\mu$ g/ml); G-I-mAb16, induced GnT-V transfected cells with mAb16 (2 mg/ml); G-I-SW, induced GnT-V transfected cells with swainsonine (1  $\mu$ g/ml) prior to and during migration. \*,  $P \leq 0.05$  and \*\*,  $P \leq 0.01$  vs. mock transfected cells; #,  $P \leq 0.05$  and ##,  $P \leq 0.01$  vs induced GnT-V transfected cells.



**Fig.5B** Cell invasion was determined using a Transwell membrane coated on the upper sides with Matrigel, and cells were allowed to migrate through Matrigel for 21 h. Each bar represents the mean ( $\pm$ S.D.) number of migrating cells from 5 randomly selected fields. Similar results were obtained in three separate experiments.



**Fig.5C** MMP activity was determined using gelatin gel zymography. Cells were cultured in 6-well plates pre-coated with fibronectin (10  $\mu\text{g/ml}$ ) in 2 ml serum-free media for 24 h. The media were collected, centrifuged and 10  $\mu\text{l}$  of each was analyzed for MMP activity by zymography.

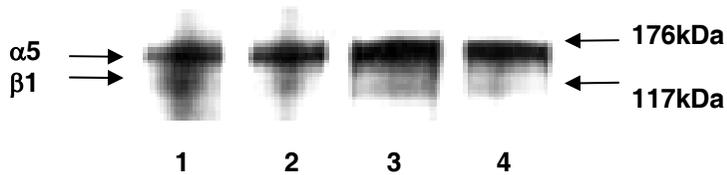


**Fig.6** Filamentous actin staining of HT1080 cells after GnT-V over-expression. Cells were cultured on fibronectin-coated (10  $\mu\text{g/ml}$ ) chamber slides for 30 min (top panels) or 120 min (lower panels) in serum-free media. After cells were fixed and blocked with BSA, F-actin was visualized using Rhodamine-phalloidin (1:40). Mock, mock transfected cells; GnT-V, GnT-V transfected cells. Bar, 10  $\mu\text{m}$

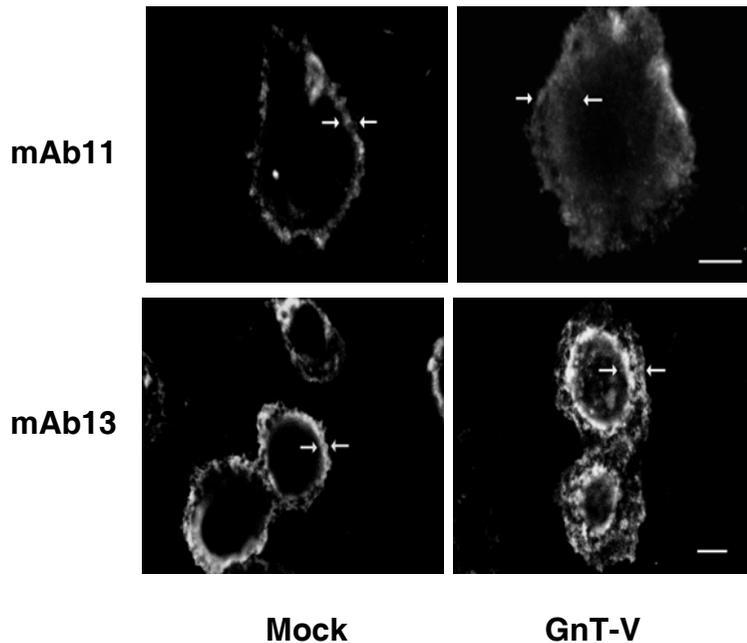
**IP: mAb13**



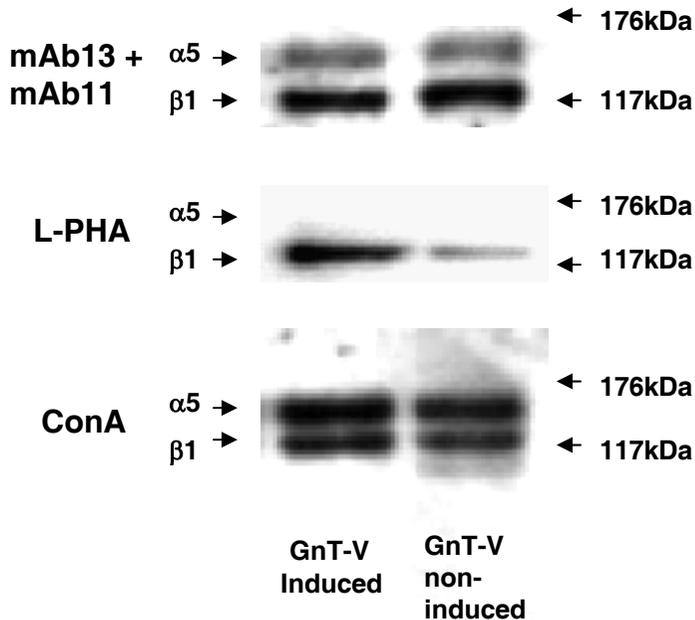
**IP: mAb16**



**Fig.7A** Cell surface expression of  $\alpha 5\beta 1$  integrin subunits before and after induction of GnT-V expression. Cells were harvested and cell surface proteins were biotinylated with NHS-LC-Biotin. Immunoprecipitation (IP) using mAb13 (top) and mAb16 (bottom) and detection of  $\alpha 5\beta 1$  integrins after SDS-PAGE were performed. SDS-PAGE was performed under non-reducing (lane 1-2) and reducing (lane 3-4) conditions using cell lysates from non-induced (lane 1, 3) and induced (lane 2, 4) GnT-V transfected cells.



**Fig.7B** Localization of cell surface  $\alpha 5$  and  $\beta 1$  integrins. Cells were cultured on fibronectin-coated coverslips for 2h in serum-free media, fixed, blocked with BSA and then stained with mAb11 and mAb13 (10  $\mu\text{g}/\text{ml}$ ) for  $\alpha 5$  and  $\beta 1$  respectively. Arrows indicate more diffused integrin clustering in GnT-V expressing cells compared to mock transfected cells. Bar, 10  $\mu\text{m}$ .



**Fig.7C** Glycosylation of  $\alpha 5\beta 1$  integrin purified from GnT-V transfected cells.  $\alpha 5\beta 1$  integrins were immunopurified from HT1080 cells before and after induction of GnT-V. The same amounts (2.0 mg) of purified  $\alpha 5\beta 1$  integrin were subjected to SDS-PAGE (4-20%) and transferred to PVDF membranes. Blot was probed using a combination of mAb13 and mAb11 (top panel), HRP-L-PHA (1:5000) (middle panel), and HRP-Con A (1:5000) (bottom panel). Mock, mock transfected cells; GnT-V, GnT-V transfected cells.

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