Suppression of Tumor-related Glycosylation of Cell Surface Receptors by the 16-kDa Membrane Subunit of Vacuolar H⁺-ATPase*

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The glycosylation of integrins and other cell surface receptors is altered in many transformed cells. Notably, an increase in the number of β1,6-branched N-linked oligosaccharides correlates strongly with invasive growth of cells. An ectopic expression of the Golgi enzyme N-acetylglucosaminyltransferase V (GlcNAc-TV), which forms β,6 linkages, promotes metastasis of a number of cell types. It is shown here that the 16-kDa transmembrane subunit (16K) of vacuolar H⁺-ATPase suppresses β1,6 branching of β1 integrin and the epidermal growth factor receptor. Overexpression of 16K inhibits cell adhesion and invasion. 16K contains four hydrophobic membrane-spanning α-helices, and its ability to influence glycosylation is localized primarily within the second and fourth membrane-spanning α-helices. 16K also interacts directly with the transmembrane domain of β1 integrin, but its effects on glycosylation were independent of its binding to β1 integrin. These data link cell surface tumor-related glycosylation to a component of the enzyme responsible for acidification of the exocytic pathway.

Integrins and other cell surface receptors are extensively N-glycosylated by enzymes that reside in the endoplasmic reticulum (ER)1 and Golgi complex. Upon the insertion of newly synthesized polypeptides into the ER, oligosaccharyltransferase adds Glc₃Man₉GlcNAc₂-P-P-dolichol to asparagine residues. The three glucose and all but the last five mannose residues on the oligosaccharide core are progressively trimmed off as the protein moves from the ER to the cis-Golgi by a series of glucosidases and mannosidases. Upon entering the medial Golgi, the N-acetylglucosaminyltransferases GlcNAc-TI and GlcNAc-TII begin the process of rebuilding the oligosaccharides with GlcNAc residues. In various cells and tissues, GlcNAc-TI, -TII, -TIV, and/or -TV make subsequent GlcNAc additions, and this rebuilt backbone serves as a structure to which other complex sugars are added.

Many cancer cells exhibit altered glycosylation patterns on surface receptors, but the complexity and differences in oligosaccharides in normal cells have made it difficult to establish causal relationships between sugar modifications and abnormal cell growth properties. Nevertheless, there is a growing body of convincing evidence that hyperactivity of GlcNAc-TV promotes a metastatic phenotype (1–9). β1 integrin and the epidermal growth factor receptor (EGF-R) are both targeted by GlcNAc-TV (10–13), which adds GlcNAc to the oligosaccharide backbone via a β,1,6 linkage. In addition, both receptors can have significant involvement in cancer cells. β1 integrin is implicated in the invasive processes of many tumor cells (14–19), and an elevated expression of the EGF receptor is an indicator of poor prognosis for many cancers including breast, ovarian, and uterine (20–23).

We showed recently that the transmembrane domain of β1 integrin interacts with the 16K subunit of vacuolar H⁺-ATPase (V-ATPase) (24), the enzyme that acidifies the Golgi and exocytic and endocytic compartments (25, 26). 16K is a membrane-spanning protein that folds as a four-helix bundle and assembles into a hexamer, forming the membrane proton channel of the enzyme. In addition to its role in the V-ATPase, 16K has been reported to form gap junctions and neurotransmitter release channels (27–29). It is believed to play a role in the function of the beta form of platelet-derived growth factor receptor (PDGF-βR) with which it interacts and participates in trimeric complexes with the E5 protein of papillomaviruses (30). Deletion of the fourth transmembrane helix of 16K leads to reduced interactions with E5, the PDGF-βR, and β1 integrin (31, 24), and this truncated protein induces anchorage-independent growth in 3T3 cells and enhances their growth as tumors in nude mice (31). Even less is known about how 16K might regulate β1 integrin functions. A role for 16K was suggested by experiments showing that fibronectin induces a redistribution of 16K-containing vesicles into proximity of the points of contact between the cell and the extracellular matrix (24).

Considering that integrin functions are dependent upon glycosylation and because all of the molecules of interest including E5, 16K, the PDGF-βR, the EGF-R, and β1 integrin pass through the ER and Golgi, we conducted a study to assess whether 16K regulates receptor processing. Using lectins specific for different processing intermediates, we found that 16K inhibits the addition of β1,6-branched oligosaccharides to both β1 integrin and the EGF-R. This result correlated with a loss of invasive growth. Each individual transmembrane helix of 16K was expressed, and both the second and fourth helices were able to inhibit β1,6 branching. This inhibition occurred independently of direct binding to the receptors. These and other
data highlight a critical role for the β1 integrin transmembrane domain in the glycosylation of the protein and reveal that 16K can regulate processing events that are implicated in cancer.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Transfections, Antibodies, and Lectins—Human embryonic kidney cells (HEK293) constitutively expressing T7 polymerase (a gift from Dr. M. A. Billeter, Institut für Molekularbiologie, Abteilung, Switzerland) were grown in α-minimum Eagle’s medium in 10% fetal bovine serum at 37 °C in 5% CO2. All transfections were done using a standard calcium phosphate procedure. Unless otherwise described, HEK293 cells were incubated with precipitate for 3 h, and lysates were made after 24 h of expression. The HSV and T7 antibodies were obtained from Novagen, and the anti-PDGFRβ was from Santa Cruz Biotechnology Inc. Alexa 488-conjugated goat anti-mouse was obtained from Molecular Probes, Inc. Agarose-conjugated Phalloidin (Bovine, Sigma) and recanalized T7 (Con A) and alkaline phosphatase-conjugated rabbit anti-mouse IgG were purchased from Sigma. Alkaline phosphatase-conjugated goat anti-rabbit was was from Chemicon.

Assembly of T7-tagged Wild Type and W7286G Mutant β1 Integrin—Oligos encoding the 22 amino acid rat β1 integrin signal sequence (32) followed by the amino acid T7 epitope (33) were generated, annealed, and ligated into the BamHI and EcoRI sites of pXJ41 to make XJ41-T7. The desired calcium phosphate precipitate was then added to the standard calcium phosphate procedure. Unless otherwise described, HEK293 cells were incubated with precipitate for 3 h, and lysates were made after 24 h of expression. The HSV and T7 antibodies were obtained from Novagen, and the anti-PDGFRβ was from Santa Cruz Biotechnology Inc. Alexa 488-conjugated goat anti-mouse was obtained from Molecular Probes, Inc. Agarose-conjugated Phalloidin (Bovine, Sigma) and recanalized T7 (Con A) and alkaline phosphatase-conjugated rabbit anti-mouse IgG were purchased from Sigma. Alkaline phosphatase-conjugated goat anti-rabbit was was from Chemicon.

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forms was dependent on the levels of 16K in the cell (Fig. 1, B–E). Increasing amounts of the vector encoding HSV-tagged 16K were co-transfected with a constant amount of T7-tagged \( \beta_1 \) integrin. As more 16K was made (Fig. 1D), the largest form of the integrin disappeared (Fig. 1B). This corresponded with a loss of L-PHA-reactive \( \beta_1 \) integrin molecules (Fig. 1C). There was not a simultaneous build-up of ER-resident forms of the integrin, suggesting that the decrease was because of an alteration in \( \beta_1 \) integrin processing rather than retention of the integrin in the ER. Western blot analysis of the same lysates for endogenous PDGF-\( \beta \)R (Fig. 1E) showed that 16K does not affect overall expression levels of other glycosylated cell surface receptors.

Inhibition of 1,6 Branching by 16K Correlates with a Loss of Migratory Abilities of HEK293 Cells—In transwell invasion assays (Fig. 2), the expression of 16K abrogated migration through fibronectin and laminin (LN) matrices, demonstrating a correlation between the loss of 1,6 branching and the invasive abilities of cells. HEK293 cells transiently transfected with 16K were split, and aliquots were plated on plastic (Fig. 2, A and D), fibronectin (B and E), or LN (C and F). Cells expressing 16K, identified by immunofluorescence using anti-HSV antibody, were abundant on plastic but nearly absent from the population that attached to fibronectin or LN. This finding suggests that the inhibition of migration results from an inhibition of attachment.

The Effect of 16K on \( \beta_1 \) Integrin Processing Is Independent of V-ATPase Activity—One possible explanation for the effect of 16K on glycosylation is that GlcNAc-TV activity is highly sensitive to the pH of the Golgi, and that by overexpressing 16K, the activity of the V-ATPase is altered. If glycosylation was in fact sensitive to pH, it would be predicted that a major disruption of V-ATPase function would lead to altered processing. Dicyclohexylcarbodiimide (DCCD) is a potent inhibitor of V-ATPase (39), and it was used to treat cells that had been transfected with T7-tagged \( \beta_1 \) integrin. After 2 h in DCCD, no changes in the forms of integrin or in the amount of L-PHA-reactive molecules were seen (Fig. 3A). Acridine orange was used to confirm that the DCCD had dramatically inhibited acidification of cellular compartments (Fig. 3B). Pulse-chase labeling of transfected cells demonstrated that within 1 h newly synthesized \( \beta_1 \) integrin polypeptides are processed into all three prominent molecular mass forms (Fig. 3C). Therefore, we concluded that the lack of any effect on glycosylation of a 2-h treatment with DCCD was strong evidence that 16K is not altering glycosylation by altering V-ATPase activity. The pulse-chase experiments also showed that over an 18-h period the 1,6-branched form of \( \beta_1 \) integrin is never present when 16K is expressed.

The Inhibition of Glycosylation by 16K Is Not Dependent upon a Direct Association with \( \beta_1 \) Integrin—Although 16K is primarily a V-ATPase component, its interaction with \( \beta_1 \) integrin raised the possibility that it also functions as a shuttling molecule that can direct the integrin to bypass GlcNAc-TV. There are many ER/Golgi components implicated in the trafficking of molecules, such as ERGIC-53, Rab6, the ADP-ribosylation factor family of proteins, COP1, COP2, and SNARES (39–47). ERGIC-53, for example, transports glycoproteins through compartments, presumably via direct association with glycan intermediates (48). Because glycosylation occurs sequentially through different compartments, we examined whether the inhibitory effect of 16K on glycosylation of \( \beta_1 \) integrin was dependent on their association. A series of HSV epitope-tagged truncated 16K molecules (Fig. 4, upper panel) was made, and their ability to bind \( \beta_1 \) integrin in co-immunoprecipitation experiments was compared with their ability to promote the loss of 1,6 branching.

The interaction of 16K with \( \beta_1 \) integrin required the region of the protein spanning helices 2–4 with helix 4 contributing the most to the interaction (Fig. 4C, lanes 8–10). This finding confirmed our earlier results using the yeast two-hybrid assay and direct protein interaction studies, showing the importance of helix 4 for the interaction (24). Although no individual helices formed stable interactions with \( \beta_1 \) integrin, helices 2 and 4 suppressed 1,6 branching as effectively as full-length 16K.
Amino Acids within the Transmembrane Domain of \( \beta_1 \) Integrin Are Required for Control of \( \beta_1,6 \) Branching—These results, which show that membrane-spanning hydrophobic fragments of 16K could influence \( \beta_1 \) integrin processing, led us to predict that the transmembrane domain of \( \beta_1 \) integrin played a role in processing. We created mutations spanning the integrin transmembrane domain, expressed them as T7-tagged proteins,\(^2\) and assessed their effect on glycosylation. Whereas most mutations had little effect, the conversion of Trp\(^{728}\), which is buried in the membrane near the cytoplasmic surface (Fig. 5A), to glycine caused processing to become strikingly similar to that seen when 16K was co-expressed with wild type \( \beta_1 \) integrin. That is, there was a significant reduction of \( \beta_1,6 \) branching of this mutant (lanes 1–6). At the same time, the core protein form of this mutant retained its ability to interact with 16K (lanes 7–10). The mutation of Trp\(^{728}\) to glycine does not affect the ability of the integrin to get to the cell surface (Fig. 5B) nor its ability to bind to the integrin matrices fibronectin, laminin, or vitronectin (data not shown) and, therefore, is probably not altering the conformation of the integrin. These data further demonstrate that glycosylation can be orchestrated from within the membrane and in addition suggest that 16K might target an intermediary factor whose interaction with \( \beta_1 \) integrin is abrogated by this tryptophan mutation.

\(^2\) M. A. Skinner and A. G. Wildeman, manuscript in preparation.
Glycosylation of the EGF-R Is also Inhibited by 16K—The receptor for the EGF-R also has β1,6-branched oligosaccharides, and it too was T7 epitope-tagged and expressed in HEK293 cells along with T7-tagged β1 integrin and the 16K derivative (lanes 1–4). Integrin expression was assessed by Western blot analysis of RIPA lysates probed with anti-T7 antibody (panel A), and glycosylation by GlcNAc-TV was assessed by incubating extracts with agarose-conjugated L-PHA and analyzing recovered proteins with anti-T7 antibody (panel B). Proteins interacting with 16K or 16K mutants were isolated by immunoprecipitation using anti-HSV antibody followed by agarose-conjugated protein A, and recovery of β1-T7 integrin was monitored by Western blot analysis using anti-T7 antibody (panel C).

**Fig. 4.** Inhibition of glycosylation does not depend on a direct association between β1 integrin and 16K. **Upper panel,** 16K derivatives comprised of specific hydrophobic helices were generated with carboxyl-terminal HSV tags. **Lower panel,** HEK293 cells were transfected with β1-T7 only (lane 1) or co-transfected with β1-T7 and the 16K derivative (lanes 2–10). Integrin expression was assessed by Western blot analysis of RIPA lysates probed with anti-T7 antibody (panel A), and glycosylation by GlcNAc-TV was assessed by incubating extracts with agarose-conjugated L-PHA and analyzing recovered proteins with anti-T7 antibody (panel B). Proteins interacting with 16K or 16K mutants were isolated by immunoprecipitation using anti-HSV antibody followed by agarose-conjugated protein A, and recovery of β1-T7 integrin was monitored by Western blot analysis using anti-T7 antibody (panel C).

**Discussion**

Integrins and other surface receptors undergo extensive post-translational modification throughout the exocytic pathway with glycosylation being a major determinant of receptor functions. Considering that the acidic environment of this pathway is regulated by V-ATPase, it is intriguing that one of the 16K and the EGF-R, again confirming that modulation of glycosylation by 16K occurs without direct interaction with the glycosylation substrate.
the subunits of this enzyme, 16K, can play a role in processing. As we have shown, this involvement appears to be independent of V-ATPase activity, indicating that 16K in fact has multiple roles. There is little known about the cellular regulators of glycosylation, and our data show that for GlcNAc-TV, a specific subdomain of 16K, can have a major impact on β1 integrin processing. Furthermore, to add to the complexity of these data, 16K can form a stable complex with β1 integrin, but the effect of 16K on glycosylation is not dependent upon this interaction.

There are several events that might explain these data. On one hand, 16K may act as a trafficking molecule to assist in the movement of β1 integrin through the Golgi, and 16K overexpression could cause the integrin to be diverted prematurely to proteasomes or the cell surface without full processing occurring. On the other hand, 16K may somehow regulate GlcNAc-TV or the mechanisms, which determine how the transferase finds its targets.

For various reasons, the first of these explanations is not satisfactory. The fact that an α-helical subdomain of 16K, which has no ability to bind β1 integrin, can regulate processing and that the EGF-R is also affected even though it does not bind to 16K makes a cargo receptor role for 16K seem unlikely. ER to Golgi cargo receptors, such as ERGIC-53, are also structurally unrelated to 16K, functioning as mannose-specific lectins (40). In addition, the overall levels of β1 integrin or the EGF-R are not significantly reduced by the addition of 16K, indicating that proteolysis is not being enhanced.

Nevertheless, it should be noted that the V₀ subunit of the yeast V-ATPase, which is comprised of a hexamer of 16K, Vma6, Vph1, Vma11, and Vma16, has recently been found to catalyze membrane fusion of vacuoles in a calcmodulin/calcium-dependent and V-ATPase-independent manner (53). The authors propose that V₀ subunits from opposing membranes form a pore spanning both membranes, providing a mechanism for membrane fusion. Thus, the possible involvement of 16K in the trafficking of molecules within compartments should not be excluded.

The possibilities that 16K regulates GlcNAc-TV or that it plays a role in the identification of glycosylation targets of the transferase must be considered in light of the fact that 16K is a highly hydrophobic membrane protein. GlcNAc-TV has a single membrane-spanning hydrophobic domain compared with the four within a 16K monomer or the twenty-four within the 16K hexamer that constitutes the proton channel of V-ATPase. Although there is as yet no supporting evidence, 16K could act as a docking site in the membrane to cluster molecules with complementing functions. The transmembrane 4 superfamily family of tetraspan proteins is believed to carry out such a role in plasma membranes (54, 55). It is relevant in this regard that the transmembrane domain of β1 integrin was found in our study to play a key role in the ability of that molecule to become β1,6-branched. Notably, the mutation of Trp728 resulted in levels of β1,6 branching that were identical to those seen when 16K was overexpressed. The most direct interpretation is that Trp728 is required for a direct interaction between GlcNAc-TV and β1 integrin or for the interaction with another molecule(s) that is needed for GlcNAc-TV to have access to β1 integrin. Do et al. (56) showed that β1,6 branching is in fact dependent upon GlcNAc-TV having access to suitable oligosaccharide acceptors. Increasing amounts of 16K could compete for GlcNAc-TV or for this other molecule, thereby altering glycosylation of β1 integrin. It is predicted that other β1,6-branched receptors such as the EGF-R also participate in similar complexes with which 16K can compete. Clearly, it will now be necessary to search for other putative transmembrane partners of β1 integrin, the EGF-R, GlcNAc-TV, and 16K. We recently identified in a yeast two-hybrid screen two additional uncharacterized binding partners for β1 integrin (24).

The reduction in β1,6 branching in response to increasing amounts of 16K may also be the result of the inhibition of GlcNAc-TV expression. The GlcNAc-TV gene is itself sensitive to signaling pathways with ets-dependent activation by src kinase and by her-2/neu stimulation of the Ras-Raf signaling

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**Figure 5.** The transmembrane domain of β1 integrin contributes to β1,6 branching. A, HEK293 cells were transfected with wild type T7-tagged β1 integrin (lanes 2, 3, 5, 6, 8, and 10) or the W728G transmembrane mutant (lanes 1, 4, 7, and 9) β1 integrin. Four of the wild type T7–β1 samples (lanes 2, 5, 8, and 10) and two of the W728G mutant samples (lanes 7 and 9) had HSV-16K included in the transfection. Western blots of RIPA lysates probed with anti-T7 as shown in lanes 1–3 and lanes 9 and 10 confirm that the mutant and wild type are expressed at similar levels. β1,6-Branched proteins, recovered using L-PHA-conjugated agarose, are reduced similarly by co-transfection with 16K or by the mutant (lanes 4–6). Immunoprecipitations with anti-HSV antibody from co-transfected cells (lanes 7 and 8) show that the W728G mutation does not interfere with binding of the core integrin protein to 16K. B, flow cytometry experiments revealed that the integrin mutant W728G is expressed on the surface of the cell at levels comparable with those of the wild type-tagged β1 integrin.

**Figure 6.** 16K inhibits β1,6 branching of the EGF-R. HEK293 cells were co-transfected with T7-tagged β1 integrin and the EGF-R. Lanes 1–4 contain equal amounts of T7-tagged EGF-R and β1 integrin with increasing amounts of HSV-tagged 16K. Lanes 5 and 6 show lysates transfected with only β1 integrin or EGF-R, respectively. A, Western blot analysis of RIPA lysates treated with anti-T7 antibody. B, increasing amounts of 16K suppress β1,6 branching of both β1 integrin (lower band) and the EGF-R (upper band), as detected using L-PHA-conjugated agarose and Western blot analysis with anti-T7 antibody.
pathway playing a role (57, 58). In addition, the processing of \( \beta_1 \) integrin can be accelerated by TGF-\( \beta_1 \) through the stimulation of Ras (59), suggesting that any effects of 16K on signaling pathways using Ras could affect glycosylation. It is also a reasonable expectation that the involvement of 16K in cell surface receptor glycosylation will in turn perturb gene expression pathways by altering receptor function. In support of this finding, it has been suggested that a role of 16K-linked glycosylation is to prevent surface receptors from dimerizing in the absence of an appropriate ligand (60). Additionally, the inhibition of \( \beta_1,6 \) branching in GlcNAc-TV knockout mice enhances the clustering of T cells and lowers T cell activation (61).

There are very few reports in the literature of cellular regulators of GlcNAc transferases. Guo et al. (52) reported a modest (<50%) decrease in GlcNAc-TV activity in response to the metastasis-suppressor gene nm23-H1 and the tumor suppressor gene p16, but they did not examine specific receptors such as \( \beta_1 \) integrin or the EGF-R. In our experiments, we observed a >90% loss of \( \beta_1,6 \) branching of these targets and a concurrent dramatic reduction in migration through extracellular matrix components. 16K may provide unique opportunities for the intervention in \( \beta_1,6 \) branching and reduction of invasive cell growth.

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