Increased Glycosylation of \(\beta_1\) Integrins Affects the Interaction of Transformed S115 Mammary Epithelial Cells with Laminin-1

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Abstract

The effect of transformation on the expression and the functions of \(\beta_1\) integrins was studied using an in vitro cell transformation model. S115 mammary epithelial tumor cells undergo transformation into tumorigenic fibroblastoïd cells in the presence of steroids.

Transformation was found to reduce the attachment and the spreading of S115 cells on laminin-1 but not fibronectin. Adhesion of S115 cells to laminin-1 was inhibited in the presence of an antibody against the \(\beta_1\) integrin subunit. Both nontreated and transformed S115 cells expressed at least two putative laminin-1-binding \(\beta_1\) integrins at the same level. In transformed cells, however, the mature integrin subunits appeared to be structurally altered, showing a slower electrophoretic mobility. Treatment with \(N\)-glycosidase-F and tunicamycin abolished this mobility difference, suggesting that the presence of complex-type \(N\)-linked oligosaccharides was responsible. Detailed enzymatic analysis of the oligosaccharides present on the \(\beta_1\) subunits revealed that the difference in glycosylation is, at least partially, due to poly-N-lactosaminoglycan chains on \(\beta_1\) integrin from transformed cells. Removal of this difference in glycosylation by either cleavage of the polylactosaminoglycan chains with endo-\(\beta\)-galactosidase or inhibition of complex-type glycan formation with swainosine repeatedly enhanced the spreading of transformed cells on laminin-1. Thus, the increased size of complex-type oligosaccharides on \(\beta_1\) integrin may affect cell-laminin-1 interactions. Similar changes may contribute to the altered adhesion of cancer cells during the invasion and metastasis.

Introduction

Malignant transformation leading to invasion and metastasis is a complex process that requires multiple changes in the interactions between cells and extracellular matrices. These interactions are mediated by cell surface molecules, such as integrins (1), and cell surface proteoglycans (2). Integrins are heterodimeric transmembrane glycoproteins composed of \(\alpha\) and \(\beta\) subunits. At least 21 noncovalently bound \(\alpha\beta\) heterodimers are known, of which 14 are shown to be matrix receptors (1, 3). A single matrix molecule can be recognized by several different integrins. All together, eight integrin heterodimers can bind fibronectin. The integrin family also contains at least seven putative laminin-1 receptors and multiple receptors for collagens and vitronectin. It is not totally understood why a single ligand requires so many receptors. One reason could be that different integrins recognize different domains within the ligand molecule. Furthermore, binding of the same ligand molecule to different integrins leads to different cellular responses (4), possibly due to differences in the intracellular sequences of the \(\alpha\) subunits.

It has been suggested that integrin-type adhesion receptors have a critical role in determining the behavior of tumor cells (5, 6). Integrins mediate tumor cell adhesion to interstitial components and transmit signals into the cells. In this way, they regulate cell migration, differentiation, and protein synthesis. Thus, transformation-related changes in the properties of integrins may have important consequences. Malignant transformation may alter the cellular pattern of integrin expression (7, 8). In general, transformation does not appear to be associated with the suppression or up-regulation of any specific integrin. Instead, different malignancies seem to have unique changes in their integrin patterns. For example, the expression of the \(\alpha_5\beta_1\) integrin shows inverse correlation with the invasive potential of murine melanomas (9), whereas both chemical and viral transformation induces the expression of the \(\alpha_5\beta_1\) in normally negative human osteosarcoma cells (10).

Malignant transformation, however, does not always result in changes in the level of integrin expression. The glycosylation of integrins may also change during transformation. Adhesion of mouse B-16 melanoma cells to laminin-1 and the cell binding domain of fibronectin has been demonstrated to correlate with the glycosylation of \(\beta_1\) integrins. A weakly metastatic mutant was found to be less adherent than the parental B-16 melanoma cells, and cell spreading in both cases was inhibited by an antibody against the \(\beta_1\) chain (11). Further characterization of \(\alpha_5\beta_1\) integrin identified \(\alpha\)-galactosyl residues in the \(\alpha_5\) subunit as laminin-1 binding determinants, whereas complex-type oligosaccharides in the \(\beta_1\) chain were associated with changes in cell spreading rather than adhesion (12). The mechanism by which these changes in sugar chains affect the function of \(\beta_1\) integrins is not yet known. It may be that such alterations in glycosylation cause a local conformational change in the recognition domain of the integrin that affects its biological activity.

In the present study, we have analyzed the effect of transformation on \(\beta_1\) integrins in S115 epithelial tumor cells. Studies with this steroid-responsive S115 mammary tumor cell line have demonstrated that steroids can affect both cell proliferation and morphology (13). In the presence of testosterone, the cells express a transformed phenotype exhibiting an increased growth rate, fibroelastic morphology, and an ability to grow in suspension. Removal of
Integrin Glycosylation and Transformation

Fig. 1. Morphology of S115 cells on laminin-1 and fibronectin. S115 cells were cultured for 5 days in DMEM in 2% dextran-coated charcoal-FCS without (A and C) or with (B and D) 10 nm testosterone, trypsinized, and allowed to spread on laminin-1 (A and B) or fibronectin (C and D)-coated wells at 37°C for 2.5 h.

hormone from these cells results in a reduced growth rate, a dramatic change from fibroblastic to epithelioid morphology, and a loss of anchorage independency. We have previously shown that a cell surface proteoglycan, syndecan-1, is suppressed during this epithelioid-fibroblastic conversion (14, 15). Here we report that steroid-induced transformation of S115 cells results in reduced attachment and spreading on laminin-1 and increased N-linked glycosylation of β1 integrins. Specifically, this increased glycosylation is due to the presence of polylactosaminoglycan chains on β1 integrins in transformed cells. Agents that abolish the change in β1 glycosylation also restore cell spreading on laminin-1. Our data suggest that β1 integrins are involved in the altered behavior of steroid-transformed S115 mammary epithelial tumor cells.

Results

Steroid-induced Transformation Selectively Decreases the Adhesion of S115 Cells to Laminin-1. S115 cells have been used previously to study the expression of a transmembrane proteoglycan, syndecan-1, during hormone-induced malignant transformation (14, 15). We have now extended this work to analyze the effect of S115 cell transformation on cell-matrix interactions. Under serum-free conditions, transformation resulted in a difference in S115 cell spreading on laminin-1 during a 2.5-h incubation. S115 cells cultured with testosterone spread less well than cells without testosterone (Fig. 1, A and B). The phenomenon appeared to be substratum specific, since both epithelial and fibroblastic cells spread equally well on fibronectin (Fig. 1, C and D). These findings were quantified by measuring the proportion of spread cells, and the data are shown in Fig. 2A. Fig. 2B shows that the initial attachment after 1 h incubation of S115 cells to laminin-1 was also reduced in transformed cells, whereas the attachment of cells to fibronectin was not altered by transformation. The assays were repeated five times with similar results.

Although transformation of S115 cells has been shown to reduce the expression of the matrix-binding proteoglycan, syndecan-1, laminin-1 is not thought to be a ligand for syndecan-1 in epithelial cells. Therefore, we studied the effect of anti-β1 integrin antiserum on spreading of S115 cells on laminin-1. When the cells were allowed to spread on laminin-1-coated wells in the presence of anti-β1 antibody, the spreading of both transformed and untransformed cells was totally inhibited (Fig. 3A). Furthermore, when anti-β1 antibody was added into established cultures of S115 cells, it was possible to see cell rounding and partial detachment (data not shown), suggesting that S115 cell spreading and morphology were also regulated by β1 integrins. To investigate the involvement of β1 integrins in primary attachment, the cells were allowed to attach for 1 h in the presence of rabbit IgG or the purified IgG fraction of anti-β1 integrin antiserum. Rabbit IgG did not alter initial attachment to laminin-1, but the IgG fraction of anti-β1
integran antiserum inhibited attachment in a concentration-dependent manner (Fig. 3, B and C), confirming that S115 cell attachment to laminin-1 also requires active β1 integrins.

**Altered Structure of β1 Integrins in Hormone-treated S115 Cells.** Reduced cell adhesion to laminin-1 could reflect differences in cell surface expression of β1 integrins between testosterone-treated and nontreated cells. Therefore, we quantitated the levels of β1 and α5 subunits at the cell surface. Results from FACS analysis by staining with antibodies against β1 and α5 integrins showed that both subunits were equally expressed by both testosterone-treated and nontreated S115 cells (Fig. 4). In contrast, control stainings using antibody against syndecan-1 showed that nontreated S115 cells expressed high levels of syndecan-1, which was clearly reduced on testosterone-treated cells (Fig. 4). These results indicate that the difference in cell adhesion to laminin-1 does not correlate with the amount of β1 integrins at the cell surface.

To determine whether structural modifications of β1 integrins might be associated with altered adhesion of S115 cells on laminin-1, we immunoprecipitated [125I]methionine-labeled cells with a polyclonal antiserum against the β1 subunit. This antiserum can coprecipitate β1-related α subunits in the presence of divalent cations (16). Electrophoretic analysis in nonreducing conditions (Fig. 5A) revealed three different bands with apparent molecular masses of 140, 125, and 115 kDa. These bands corresponded to α5, β1, and pre-β1 subunits of integrin molecules, respectively, as previously shown (16). Further immunoprecipitations with specific anti-α chain antibodies (Fig. 5B) revealed that S115 cells expressed at least two putative laminin-1 receptors, i.e., α5β1 and α6β1.

Although the integrin expression levels remained unaltered by transformation, structural alterations were induced. Both β1 subunits and α5 subunits from testosterone-treated cells migrated more slowly in SDS-PAGE gels than those from nontreated S115 cells or from normal mouse mammary epithelial (NMuMG) cells (Fig. 5). The mobility shift was detected only in mature integrins but not in the pre-β1 subunit, suggesting that the size difference is due to post-translational modifications. Steroid treatment of NMuMG

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1. The abbreviation used is: kDa, kilodalton(s).
Integrin Glycosylation and Transformation

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**A**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>S115</th>
<th>S115</th>
<th>NMuMG</th>
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<tr>
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<td>NRS</td>
<td>β1</td>
</tr>
<tr>
<td>Testosterone</td>
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<td>-/+</td>
<td>-/+</td>
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</table>

Mr kDa

- 200
- 92.5
- 69

**B**

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</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>-/+</td>
<td>-/+</td>
</tr>
</tbody>
</table>

Mr kDa

- 200
- 92.5
- 69

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**Fig. 5.** The expression and the altered structure of integrin subunits in S115 cells. Cells were cultured for 4 days with (+) or without (-) testosterone and metabolically labeled with [14C]methionine. Labeled cells were harvested and immunoprecipitated with anti-β1 integrin antiserum or normal rabbit serum (NRS) (A) or with antibodies against α1 and α2 integrin subunits (B). Immunoprecipitated proteins were analyzed by SDS polyacrylamide electrophoresis in a 6% gel under nonreducing conditions, followed by fluorography. Normal mouse mammary epithelial (NMuMG) cells were used as a control.

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cells left the integrins unaltered (Fig. 5A). As another control, we used syndecan-1-transfected S115 cells that restore the transformed phenotype to a more epithelioid one, also in the presence of testosterone (15). Interestingly, overexpression of syndecan-1 in testosterone-treated cells also removed the altered glycosylation of mature integrin subunits, suggesting that testosterone itself does not regulate integrin structure but that the alterations are due to transformation (data not shown).

**The Differences in β1 Subunit Size Are a Result of Altered Glycosylation.** To study whether the structural alterations in integrins following testosterone treatment involved differences in glycosylation, we analyzed anti-β1 integrin immunoprecipitates using endoglycosidase H (Fig. 6A). Endoglycosidase H, which can remove N-linked high mannose and some hybrid oligosaccharides from glycoproteins, was partially able to digest oligosaccharides from the mature integrins, but it could not remove the difference in their electrophoretic mobility. Digestions with endoglycosidase F enzyme gave similar results (data not shown). Treatment of immunoprecipitates with N-glycosidase F, however, abolished the size difference (Fig. 6B). Furthermore, immunoprecipitation of β1 integrins from tunicamycin-treated cells resulted in bands of 75 kDa for both transformed and nontransformed cells (Fig. 6C). These results indicate that the altered mobility of integrins from transformed S115 cells was due to the presence of complex-type N-linked oligosaccharides. Altered glycosylation was detected both in α and β subunits, which is consistent with previous data, suggesting that β1 integrins form heterodimers in the endoplasmic reticulum before they reach the Golgi apparatus (16). Thus, the same enzymes are thought to modulate glycoconjugates of both subunits.

**Inhibition of Complex-Type Glycan Formation Increases S115 Cell Spreading on Laminin-1.** To investigate whether the changes in integrin glycosylation can be related to the observed changes in cell adhesion, we assayed the behavior of swainsonine-treated cells on laminin-1. Swainsonine is an inhibitor of Golgi α-mannosidase II, which blocks the formation of complex oligosaccharides, yet allows the formation of high-mannose and hybrid chains. Swainsonine did not alter the spreading of nontransformed S115 cells grown without testosterone (Table 1). In contrast, when transformed cells were treated with swainsonine, the proportion of cells that spread on laminin-1 was significantly increased (Table 1). Analysis of β1 integrin-immunoprecipitates showed that swainsonine treatment of S115 cells decreased the size of mature α and β subunits in both testosterone-treated and untreated cells (Fig. 7A). This confirms that β1 integrins in S115 cells contain multibranch, complex-type N-linked oligosaccharides. A small mobility difference between β1 integrins from transformed and untransformed cells was, however, still apparent. To study whether increased terminal sialylation of β1 integrins affected the spreading of transformed cells, neuraminidase-treated cells were also assayed. Desialylation did not significantly affect the spreading of S115 cells on laminin-1 (Table 1). Immunoprecipitation analysis showed that neuraminidase treatment of S115 cells also slightly increased the electrophoretic mobilities of mature α and β subunits, but it did not abolish the size difference (Fig. 7B). Cotreatment with swainsonine and neuraminidase was also unable to abolish this size difference (data not shown).

The data for swainsonine treatment suggested that β1 integrin from transformed S115 cells contains N-linked, complex-type structures that inhibit the interaction of cells with laminin-1. To analyze whether β1 integrins carry poly-N-lactosaminoglycan chains, immunoprecipitates were treated with endo-β-galactosidase. As shown in Fig. 7C, treatment of untransformed cells did not affect the mobility of β1 integrins. However, endo-β-galactosidase-treated β1 integrins from transformed cells migrated identically to β1 integrins from untransformed cells. When transformed cells were treated with endo-β-galactosidase, cell spreading on laminin-1 was also improved (Table 1). In all of these experiments, neither swainsonine nor the glycosidases affected the spreading of S115 cells on fibronectin (data not shown).

In attachment assays, a small increase of initial attachment to laminin-1 was observed in testosterone-treated cells after the removal of polyglactosaminoglycan chains by endo-β-galactosidase. However, swainsonine and neuraminidase did not have any effect (Fig. 8, A and B).
Fig. 6. Altered glycosylation of β1 integrins in S115 cells. [35S]Methionine-labeled cells were cultured with (+) or without (−) testosterone (Te) and harvested; then aliquots of cell extracts were immunoprecipitated with anti-β1 antiseraum. A, samples treated with (+) or without (−) endo-β-galactosidase H (Endo H). B, samples treated with N-glycosidase-F (N-gly F). C, immunoprecipitation with anti-β1 antiseraum from cells grown in the absence (−) or presence (+) of tunicamycin (TM). The immunoprecipititates were analyzed by gel electrophoresis and fluorography.

Table 1  Effect of glycosylation on the spreading of S115 cells on laminin-1

<table>
<thead>
<tr>
<th>S115 cells</th>
<th>Swaixonine-</th>
<th>Neuraminidase</th>
<th>Endo-β-</th>
<th>Exp.* no.</th>
<th>% of total cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>treated S115 cells</td>
<td>treated S115 cells</td>
<td>galactosidase</td>
<td>treated S115 cells</td>
<td>+te</td>
</tr>
<tr>
<td>I</td>
<td>93 ± 5</td>
<td>63 ± 3</td>
<td>94 ± 2</td>
<td>80 ± 3</td>
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<tr>
<td>II</td>
<td>87 ± 1</td>
<td>41 ± 2</td>
<td>86 ± 6</td>
<td>61 ± 3</td>
<td>84 ± 1</td>
</tr>
<tr>
<td>III</td>
<td>97 ± 3</td>
<td>56 ± 4</td>
<td>95 ± 5</td>
<td>57 ± 7</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>IV</td>
<td>67 ± 4</td>
<td>24 ± 4</td>
<td>67 ± 8</td>
<td>29 ± 3</td>
<td>69 ± 6</td>
</tr>
</tbody>
</table>

*Exp., experiment; te, testosterone.

Furthermore, attachment to fibronectin was not markedly altered by deglycosylation (Fig. 8, C and D). These results suggest that the differences in a relative migration of β1 integrins can be due to poly-N-lactosaminoglycans. They also suggest that cell spreading on laminin-1 is modulated by the glycosylation state of β1 integrins.

Discussion

Cell-Cell and Cell-Matrix Interactions Are Important Mediators of Cell Behavior. The adhesive properties of a cell depend on a repertoire of cell adhesion receptors expressed at its surface and the composition of the matrix that surrounds it. During malignant transformation, many of these tightly regulated interactions are disturbed. Invasive tumor cells dissociate from the initial tumor and break through the basement membrane and blood vessel walls. The cells then circulate in the blood, adhere to endothelia, escape from capillaries, and proliferate at distant organs to form metastases. These processes involve both the loss of controlled cell-matrix interactions and the acquisition of new ones. Indeed, the amount of secreted extracellular matrix proteins or the pattern of matrix receptors expressed are often seen to change during transformation.

Integrins are the largest family of matrix receptors. Several studies have shown an altered expression pattern of integrin α subunits in virally or chemically transformed cells (7, 8, 10). Although these studies provide circumstantial evidence for either loss or induction of certain β1 integrins leading to increased malignancy, the relationship between the expression of β1 integrins and tumorigenicity is not clear. Direct evidence for the involvement of specific β1 integrins in malignancies comes from experiments in which their expression levels have deliberately been altered. For example, overexpression of α5β1 integrin suppressed the tumorigenicity of CHO cells (17), transfection of α5 integrin subunit cDNA into rhabdomyosarcoma cells conferred a metastatic potential (18), and the overexpression of αvβ3 integrin reduced the invasive potential of melanoma cells (9). However, transformation does not necessarily result in altered levels of integrin expression but does increase the rate of integrin processing, decrease the size of the intracellular pool of immature integrins, or alter the distribution of integrins (19).

We have reported previously that another group of matrix-binding proteins, cell surface proteoglycans, are affected by malignant transformation. The loss of syndecan-1 expression is associated with the transformed phenotype of S115 mouse mammary tumor cells, whereas syndecan-1 reexpression can restore the epithelial phenotype, leading to decreased growth (14, 15). We have also suggested that syndecan-1 might not be the only matrix-binding receptor altered in the transformation of S115 cells. This was evident in experiments where altered cell adhesion could not be explained by syndecan-1 expression, since laminin-1 is not a known ligand for syndecan-1 in epithelial cells. Instead, S115 cell-laminin-1 interactions are primarily mediated by β1 integrins, since an antiserum against β1 subunit inhibited cell adhesion. Both steroid-treated and untreated S115 cells expressed the same levels of β1, αv, and αv integrin subunits. However, β1 integrins were found to be structurally altered in transformed cells due to changes in their complex-type, N-linked oligosaccharides. Our results are in agreement with the finding that cell spreading, but not necessarily adhesion, on laminin-1 is associated with the glycosylation state of the β1 integrin chain (12). β1 integrins from different cell types have been shown to differ in the extent of N-linked carbohydrate processing and ligand affinity (20-22). Thus, the level and the type of N-linked glycosylation of β1 integrin may regulate its biological interaction with the extracellular matrix.
Malignant transformation has been correlated with a shift towards larger complex-type, N-linked oligosaccharides (23–26). This is due to increased activity of several glycosyltransferases, which promote branching and polylactosaminoglycan formation (27–29). In the case of S115 cells, the differences in β integrin structure between testosterone-treated and nontreated cells were not due to either increased branching or increased terminal sialylation. Instead, β integrins from transformed S115 cells contain polylactosaminoglycan chains, which contribute the size difference described here. Several observations suggest that polylactosamine is preferentially added to the β1,α1 branched, complex-type, N-linked oligosaccharides in transformed cells (25, 30). Therefore, the regulation of the enzymes that promote branching appears to be the mechanism of controlling polylactosamine addition.

Our data also suggest that the structure of the complex-type N-linked oligosaccharide present on an integrin can modify its binding capacity. Polylactosamine may reduce cell-substratum adhesion, which could then enhance cell motility and facilitate invasion. Similarly, polylactosamine sequences in the embryonic form of fibronectin have been shown to reduce fibronectin binding to collagen (31).

How increased glycosylation of β integrin would result in a change in cell adhesion is not yet understood. It is possible that the polylactosaminoglycan chain on β integrin may sterically mask a specific binding site for laminin-1. Similarly, polysialylation of N-CAM inhibits cell-cell interactions (32), whereas the size of unspecified N-linked oligosaccharides on ICAM-1 affects its binding to the integrin MAC-1 (33). Another possible mechanism to account for the decrease in β integrin-mediated spreading of the transformed S115 cells on laminin-1 is a conformational change in the αβ integrin heterodimer as a result of the increased glycosylation of the β integrin chain. This putative conformational change might specifically affect the α chains mediating laminin-1 binding since spreading on fibronectin was not affected. In fact, conformational changes due to altered glycosylation of the β integrin chain have been suggested to occur in the laminin-1 binding αβ integrin (12). Analogously, in other model systems, the function of integrins seems to be highly dependent on their conformational state. For example, activating monoclonal antibodies may change the conformation of β integrin and induce cell spreading (34). Likewise, the change in conformation caused by altered glycosylation may contribute to the activation or inactivation of β integrin. Thus, modulation of the oligosaccharides on β integrin may result in changes in cell behavior that are highly significant during the processes of invasion and metastasis by tumor cells.

Materials and Methods

Cell Culture and Labeling. S115 mouse mammary epithelial cells were routinely cultured in DMEM (GIBCO) supplemented with 5% heat-inactivated FCS (GIBCO), 1 mM pyruvate (GIBCO), 1 mM glutamine, 100 IU/ml penicillin, and 10 nM testosterone (Sigma Chemical Co.). Normal mouse mammary epithelial (NuMuMG) cells were cultured in DMEM with 5% FCS. In experiments involving hormone treatment, FCS was replaced with 2% dextran charcoal-treated-FCS, with or without 10 nM testosterone.

For metabolic labeling, cells were grown for 16 h in methionine-free DMEM supplemented with 1% dialysed FCS with 50 μCi/ml [35S]methionine (Trans35S-label; ICN). Tunicamycin (5 μg/ml; Sigma) was added to the labeling medium prior to the addition of [35S]methionine. After metabolic labeling, the cells were subjected to immunoprecipitations.

Synthesis of complex-type glycan formation was inhibited by the addition of swainsonine (Sigma) to the culture medium for 20 h at a final concentration of 1 μg/ml. For deglycosylation of intact cells, monolayers were harvested with trypsin, which was then inactivated with 500 μg/ml trypsin inhibitor (Sigma). The trypsinized cells were washed with PBS (pH 7.4), resuspended in PBS with 25 milliunits/ml endo-β-galactosidase from Escherichia freundii (Seikagaku Kogyo) or 50 milliunits/ml neuraminidase from Vibrio cholerae (Behring), and 1 mM phenylmethylsulfonyl fluoride (Sigma), and incubated at 37°C for 60 min. Control cells were treated identically but without the glycosidases. The treated cells were then washed and used in adhesion assays.

Antibodies. For immunostaining and immunoprecipitation assays, an antisera against human β integrin subunit (16), antisera against a synthetic peptide corresponding to the intracellular domain of chicken α2 subunit (kindly provided by Dr. R. Hynes, MIT, Cambridge, MA; Ref. 35), a monoclonal antibody for C9H7, against human α2 (kindly provided by Dr. A. Sonnenberg, The Netherlands Cancer Institute, Amsterdam, the Netherlands; Ref. 36), and a
monoclonal antibody 281–2 against mouse syndecan-1 (37) were used. In cell attachment assays, the IgG fraction of the anti-β1 antisera was purified using a protein A-Sepharose (Pharmacia, Sweden) column, followed by elution with 0.2 M sodium citrate. Normal rabbit serum and purified rabbit IgG (Sigma) were used as controls.

**Cell Attachment and Spreading Assays.** Plastic plates were coated with 30 μg/ml laminin-1 (mouse EHS-laminin; UBI) or fibronectin (human plasma fibronectin; GIBCO) in PBS for 2 h at 37°C or overnight at 4°C. Wells were saturated with 1% BSA (Sigma) in PBS for 1 h before the assay and subsequently washed with PBS. BSA-coated wells (10 mg/ml) were used as controls. For spreading assays, cells were detached with trypsin, which was inactivated with 500 μg/ml trypsin inhibitor. Cells were then washed twice in serum-free DMEM, resuspended in DMEM with or without testosterone, and allowed to spread for 2.5 h. Nonadherent cells were removed by aspiration, followed by three washes with PBS. Adherent cells were fixed with 3% paraformaldehyde. Photographs were taken under phase contrast optics. The percentage of spread cells was determined by counting the number of spread cells in samples of 100–200 cells. Flattened cells with regular margins were defined as “spread.” The statistical significance of differences seen in cell spreading assays was assessed by a general mixed model ANOVA (BMDP/Dynamic, ver. 7.0; BMDP Statistical Software, Inc., Cork, Ireland) for multiple independent experiments. (The random factor was the experiment, and the fixed factor was the treatment.)

Cell attachment assay was carried out basically as described above. Cells were detached by mild trypsin treatment; then trypsin was inactivated, and 5 × 10^4 cells/well were allowed to attach for 60 min at 37°C on protein-coated 96-well plates. The IgG fraction of the immunosera against β1 integrin subunit or normal rabbit IgG were added to some wells prior to the addition of cells.

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**Fig. 8.** The primary attachment of S115 cells on laminin-1 after treatment with agents that reduce N-linked glycosylation. Cells were cultured with (S115+) or without (S115−) testosterone the last 20 h in the absence or presence of swainsonine (SW), detached with trypsin, and subsequently treated with or without neuraminidase (Nase) or endo-β-galactosidase (Endo-β-gal). After washes, cells were resuspended in DMEM and allowed to attach on laminin-1 (A and B) and fibronectin (C and D) for 60 min. Adherent cells were fixed, stained, and enumerated from dye uptake. The data shown are the mean values from a representative experiment done in triplicate; bars, SD.
Nonadherent cells were washed away. Adherent cells were fixed, stained with 0.5% crystal violet in 10% acetic acid, and washed with distilled water. The plates were allowed to dry, after which the cells were suspended into 10% acetic acid. Absorbance was read at 595 nm.

**Flow Cytometry.** Monolayer cells were washed with ice-cold PBS, detached with 0.5 mM EDTA in PBS, resuspended in PBS, and centrifuged. Cells were then incubated with an antiserum against β1 integrin subunit, a mAb G4.5 against α5 integrin subunit or mAb 281–2 against syndecan-1 in 5% FCS-1% BSA-PBS at 4°C for 30 min, washed with PBS, and resuspended in 5%FCS-1% BSA-PBS containing FITC-conjugated anti-rat or anti-rabbit IgG. After another 30-min incubation at 4°C, the cells were washed twice with PBS and analyzed using a quantitative FACScan cytometer (Becton Dickinson, Mountain View, CA).

**Immunoprecipitations.** For immunoprecipitations, [35S]-methionine-labeled monolayers were washed twice with buffer containing 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 25 mM Tris-HCl (pH 7.4), scraped from the culture dishes, washed twice, and lyzed with the same buffer containing 100 mM n-octylglucoside (Boehringer-Mannheim). Detergent-soluble aliquots were preclarified by incubation with protein A-Sepharose beads for 2 h. The resulting supernatants were incubated for 12 h at 4°C with antibodies against β1, α5, or α5 integrin subunits. Normal rabbit serum was used as a control. Immunocomplexes were coupled to protein A-Sepharose beads for 2 h at 4°C and washed four times with 25 mM Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100 and 0.1% BSA, followed by two washes with Tris-buffered saline alone. Immunoprecipitated proteins were analyzed by electrophoresis using a 6% homogenous polyacrylamide gel (SDS-PAGE) and fluorography.

**Analysis of Integrin Glycosylation.** Metabolically labeled cells were harvested and immunoprecipitated with anti-β1 integrin antibody as described above. Immunoprecipitates bound to protein A-Sepharose were incubated overnight at 37°C in a 0.2 mM sodium citrate buffer (pH 5.5), with or without 5 milliunits of Endoglycosidase H (Boehringer-Mannheim); in PBS containing 0.5 mM EDTA, 0.2% SDS, and 1% octylglucoside, with or without 10 units of N-glycosidase F (Boehringer-Mannheim); in 50 mM sodium acetate buffer (pH 5.8), with or without endo-β-galactosidase (25 milliunits/ml); or in 50 mM sodium acetate buffer (pH 5.5) containing 1 mM CaCl2, with or without neuraminidase (50 milliunits/ml). The following protease inhibitors were also included: 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 2 mM aminobenzamidine, and 20 mM N-ethylmaleimide (Sigma). After two washes with PBS, proteins were separated on a 6% SDS-PAGE gel and detected by fluorography.

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