Detection of a hypersialylated ß1 integrin endogenously expressed in the human astrocytoma cell line A172

PETR BARTIK[1,2], ANNE MAGLOTT[1], GUSTAV ENTLICHER[2], DIETMAR VESTWEBER[3], KEN TAKEDA[1], SOPHIE MARTIN[1] and MONIQUE DONTENWILL[1]

[1]Département de Pharmacologie et Physicochimie, UMR 7175 CNRS, Université Louis Pasteur - Strasbourg I, Faculté de Pharmacie, BP 60024, 67401 Illkirch, France; [2]Department of Biochemistry, Charles University, Faculty of Science, 12843 Prague, Czech Republic; [3]Max-Planck-Institute of Molecular Biomedicine, D-48161 Muenster, Germany

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Abstract. Gliomas are the most common deadly brain tumors. Human cerebral tumors express high level of α5ß1 integrins. As a potential new target, α5ß1 was investigated here in two human astrocytoma cell lines, A172 and U87MG. We found that a hypersialylated ß1 integrin was endogenously expressed in A172 cells. It forms heterodimers with α5 subunits, localizes at the cell membrane and allows adhesion to fibronectin. This form of ß1 integrin was only recognized by the 9EG7 anti-ß1 antibody and appeared devoid of other specific antibody epitopes (12G10, TS2/16 and mAb13 shown here to be N-glycosylation sensitive). Overexpression of the ß1 integrin subunit in A172 cells not only increased the hypersialylated form but also led to the appearance of a non-hypersialylated ß1 form also addressed to the cell surface. Compared to wild-type A172 cells, ß1-A172 cells showed increased adhesion to fibronectin and decreased sensitivity to SJ749, a non-peptidic α5ß1 antagonist. In addition, ß1-A172 cells exhibited increased matrix dependence for normal cell cycling. Collectively, the data add new evidence for the role of ß1 glycosylation/sialylation in the regulation of integrin functions.

Introduction

Integrins are heterodimeric α/ß transmembrane glycoproteins constituting a family of adhesion receptors. Engagement of integrins with the extracellular matrix (ECM) is crucial for biological processes such as differentiation, proliferation, motility and survival. Thus, integrins participate in various cancer stages such as malignant transformation, tumor growth and progression, invasion and metastasis (1). Repression or overexpression, altered localization and clustering as well as modification in the protein conformation and/or glycosylation have been described for specific integrins in tumor cells versus normal counterparts (2).

Cellular adhesion to fibronectin is mediated primarily by α5ß1 integrins. Changes in the expression of fibronectin-binding integrins have been detected in some transformed cells. For example, high levels of α5ß1 are inversely correlated to hepatocellular carcinoma malignancy (3) and tumorigenicity of CHO (4) and HT29 colon carcinoma cells (5,6). In contrast, gliomas display high levels of α5 subunits which are correlated with pathological malignancy (7). Apart from the level of α5ß1 expression, N-linked glycosylation of α5 and ß1 seems to control integrin function (8,9). Alterations of N-glycans in α5ß1 integrins contribute to changes in the adhesive properties of tumor cells and tumor formation (10). Although compelling evidence shows that modulations of integrin glycosylation play a major role in the variation of the cell phenotype in most cancers, scarce data are available for human cerebral neoplastic tissues.

We characterized α5ß1 integrins in two human astrocytoma cell lines, A172 and U87MG cells given that this integrin species is a potential therapeutic target. We recently showed that U87MG cells expressed twice as much α5ß1 integrins than A172 cells (11). SJ749, a specific non-peptidic α5ß1 antagonist (12,13), differentially affected the growth of the two cell lines (11). To gain further insight into the mechanisms of action of the drug, we aimed to characterize the integrin conformational state by quantifying the proportion of active versus resting integrins in both cell lines. We used LIBS antibodies whose epitopes are related to the activation state of the protein (14). We found a hypersialylated ß1 integrin predominantly expressed in A172 cells which exhibits a lower molecular mass (110 kDa) than the classical integrin (130 kDa) and was only recognized by the 9EG7 antibody. It was able to heterodimerize with α5 addressed to the plasma membrane and to adhere to fibronectin. Overexpression of the ß1 integrin in A172 cells

Correspondence to: Dr Monique Dontenwill, Département de Pharmacologie et Physicochimie, UMR 7175 CNRS, ULP Strasbourg I, BP60024, 67401 Illkirch, France
E-mail: monique.dontenwill@pharma.u-strasbg.fr

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allowed the expression of a non-hypersialylated ß1 integrin at the plasma membrane coexisting with the hypersialylated form. SJ749 was less effective on ß1-A172 than on A172 cells suggesting that the hypersialylated integrin had a lower affinity for fibronectin. ß1-A172 cells also exhibited a dependence towards the matrix for cell cycle progression not observed in A172 cells. Our results are discussed in terms of functional significance of the hypersialylated integrin in cerebral tumor cells.

Materials and methods

Antibodies and reagents. Anti-ß1 integrin antibodies Ab1952 (specific to the cytoplasmic tail, Chemicon, France), mAb13 and 12G10 (Dr M.J. Humphries, Manchester, UK), 9EG7 (15), TS2/16 (Dr. E. Georges-Labouesse, IGBMC, Illkirch, France) and B44 (Dr J.A.Wilkins, Winnipeg, Canada) were used. The anti-α5 integrin antibodies employed were Ab1928 (specific to the cytoplasmic tail, Chemicon) and H104 (Santa Cruz Biotechnology, France).

SJ749 ((S)-2-[(2,4,6-trimethylphenyl) sulfonyl] amino-3-[7-benzyloxycarbonyl-8-(2-pyridinylaminomethyl)-1-oxa-2,7-diazaspiro-(4,4)-non-2-en-3-yl] carbonylamino) propionic acid) was synthesized in our laboratory as described in the patent WO 97/33887. Fibronectin was from Sigma (L'Isle d'Abeau Chesnes, France). Cell Titer 96 Aqueous One Solution cell proliferation assay was from Promega (Charbonniéres les Bains, France).

Cell culture and transfection. Human umbilical vein endothelial cells (HUVEC) were grown in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 0.5 ng/ml EGF, 2 ng/ml FGF, 100 μg/ml streptomycin and 100 U/ml penicillin. A172 and U87MG cell lines were purchased from the ATCC (Walkersville, MD) and maintained in EMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.

When indicated, A172 cells were transfected with the human ß1 subunit subcloned into the pIRES vector (gift of Dr M.J. Humphries, Manchester, UK) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and selected with hygromycin.

Flow cytometry. After detachment with EDTA, cells were preincubated 15 min at 37˚C in washing buffer (TBS/5% BSA/0.1% sodium azide) containing or not 6.25 mM Ca^{2+} or 5 mM Mn^{2+}. Cells (500,000) were then incubated 30 min on ice in the presence of the primary antibodies (10 μg/ml purified mAbs or 1:1 dilution of hybridoma supernatants 9EG7 and TS2/16). After washing, cells were incubated for 30 min on ice in the dark with the secondary antibodies (FITC-conjugated goat anti-rat IgG, 1:50, Jackson ImmunoResearch or RPE- conjugated goat anti-mouse IgG, 1:200, Beckman Coulter). After fixation with 1% paraformaldehyde for 5 min, 20,000 cells were analyzed using a FACScan flow cytometer (Becton-Dickinson, CA).

Immunoprecipitation. After detachment with trypsin, cells were lysed for 1 h at 4˚C in RIPA buffer [1% NP40, 0.5% deoxy-cholic acid, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, supplemented with protease cocktail inhibitors (Roche, Meylan, France)]. After centrifugation at 15,000 g for 20 min at 4˚C, the supernatant protein concentration was determined using the DC Protein Assay kit (Bio-Rad, Marnes la Coquette, France). Total protein extracts (300 μg) were immunoprecipitated with the indicated antibody and Protein G Sepharose beads (GE Healthcare, France) overnight at 4˚C. After 3 washes in RIPA buffer, immunoprecipitated complexes were released from the beads by boiling in Laemmli sample buffer and loaded onto a gel.

Western blot analysis. Cell lysates or immunoprecipitated complexes were separated by SDS-PAGE under non-reducing or reducing conditions respectively and transferred onto PVDF membranes (Amersham Biosciences, Orsay, France). Integrins were probed with indicated antibodies followed by incubation with HRP-conjugated anti-rabbit, anti-mouse or anti-rat antibodies (Promega and Jackson ImmunoResearch, Montluçon, France). The ECL chemiluminescence system (Amersham) followed by exposure to CL-Xposure film (Kodak, Rochester, NY) was used to visualize proteins.

Lectin blotting and lectin precipitation. Immunoprecipitated integrins were separated by SDS-PAGE and transferred onto PVDF membranes. Blots were probed with 2 μg/ml biotinylated lectins (Vector Laboratories, France) for 90 min followed by incubation with streptavidin-HRP (1/5000, Amersham) for another hour at room temperature. Detection was achieved using the ECL chemiluminescence system.

Lectin precipitation was performed by incubation of 300 μg total protein extracts with the biotinylated lectins and streptavidin-agarose CL-4B beads (Fluka) overnight at 4˚C. After washing with RIPA buffer, the material was released from the beads by boiling in Laemmli buffer and loaded onto a gel. Alternatively, 300 μg of total protein extracts were pre-treated with 0.5 U sialidase from Clostridium Perfringens (Sigma) for 4 h at room temperature before performing SNA-lectin precipitation. Reaction was stopped by heating. As a control, mock digestion without enzyme was carried out in parallel.

Deglycosylation and desialylation. Samples suspended in lysis buffer (1% SDS, 25 mM Tris-HCl, 5 mM EDTA, protease inhibitor cocktail, pH 7.0) were boiled for 5 min to allow N-glycosidase F digestion. After cooling, samples were split into two aliquots of 10 μg proteins; one of them was exposed to 1 U of N-glycosidase F (Roche, France) and the other to the solvent. The samples were incubated for 4 h at room temperature (in 15 μl 1% Triton X-100, 0.2% SDS). After digestion, Laemmli buffer was added, samples were boiled for 5 min before SDS-PAGE and Western blotting.

Protein desialylation was performed with 0.05, 0.1 or 0.5 U of sialidase. Total protein extracts (300 μg) were treated for 4 h with the enzyme at room temperature before loading onto a gel and Western blotted with specific antibodies.

Immunoprecipitation of cell surface integrin. Integrins on the cell surface were immunoprecipitated as described recently with slight modifications (16). Briefly, $5x10^5$ cells were incubated with 9EG7 or TS2/16 antibodies at subsaturating concentrations (determined by flow cytometry) for 30 min at

room temperature. After washing, cells were lysed with RIPA buffer containing protease inhibitors. After clarification by centrifugation, integrins were immunoprecipitated by mixing with Protein G Sepharose beads overnight. After washing, the material was released from the beads by boiling in Laemmli buffer before loading onto a gel.

Confocal microscopy. Cells were grown on fibronectin-coated coverslips, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton. Non-specific sites were blocked with 4% BSA/PBS solution for 30 min at room temperature. Primary antibodies [anti-α5 (Ab1928) and anti-ß1 (9EG7)] were diluted in blocking buffer and applied for 3 h at room temperature. Coverslips were washed with 1% BSA/PBS before incubating with FITC- or Rhodamin RedX-secondary antibodies for 1 h at room temperature. Cells were washed with 1% BSA/PBS and confocal images acquired using a MRC1024 laser scanning confocal microscope (Bio-Rad).

Adhesion and detachment assays. To measure cell adhesion on fibronectin, 96-well plates were coated with 5 μg/ml fibronectin for 2 h at room temperature. Non-specific binding sites were blocked with 1% heat denaturated BSA in PBS for 1 h at room temperature. Cells were detached from culture dishes with 0.53 mM EDTA, washed with PBS and resuspended in serum-free HBSS supplemented with 2 mM Ca^{2+} and 2 mM Mg^{2+}. Cells (30,000/well) were allowed to adhere for 30 min. After washing with PBS, adherent cells were fixed with 5% glutaraldehyde for 30 min, washed with PBS and colored with 0.25% crystal violet. The absorbance was read at 595 nm. Alternatively, cells were incubated with solvent or increasing concentrations of SJ749 (10^{-9}-10^{-5} M) before measuring the adhesion as above. IC_{50} were calculated from SJ749 dose-response curves using the GraphPad-Prism computer program.

For detachment experiments, cells were plated in 96-well plates coated with 0.1 μg/ml fibronectin and allowed to adhere for 2 h at 37˚C. Increasing concentrations of SJ749 or solvent (methanol/water, 50/50, vol/vol) were added to the wells. After 4 h, non-adherent cells were removed by washing twice with PBS. Bound cells were fixed with 5% glutaraldehyde, washed and colored with 0.25% crystal violet. Absorbance was recorded at 595 nm.

Proliferation assays and cell cycle analysis. Cell proliferation was measured using the MTS assay (Promega, France) according to manufacturer's description. Cell cycle analysis was performed 48 h after seeding cells on 24-well plates either uncoated (adherent conditions) or coated with poly-HEMA (non-adherent conditions). After collection, cells were resuspended in 300 μl hypotonic fluorochrome solution (50 μg/ml propidium iodide, 3.4 mM sodium citrate and 0.1% Triton X-100 in PBS). DNA content was analyzed on 10,000 cells by flow cytometry and cell cycle repartition was determined using ModFit software.

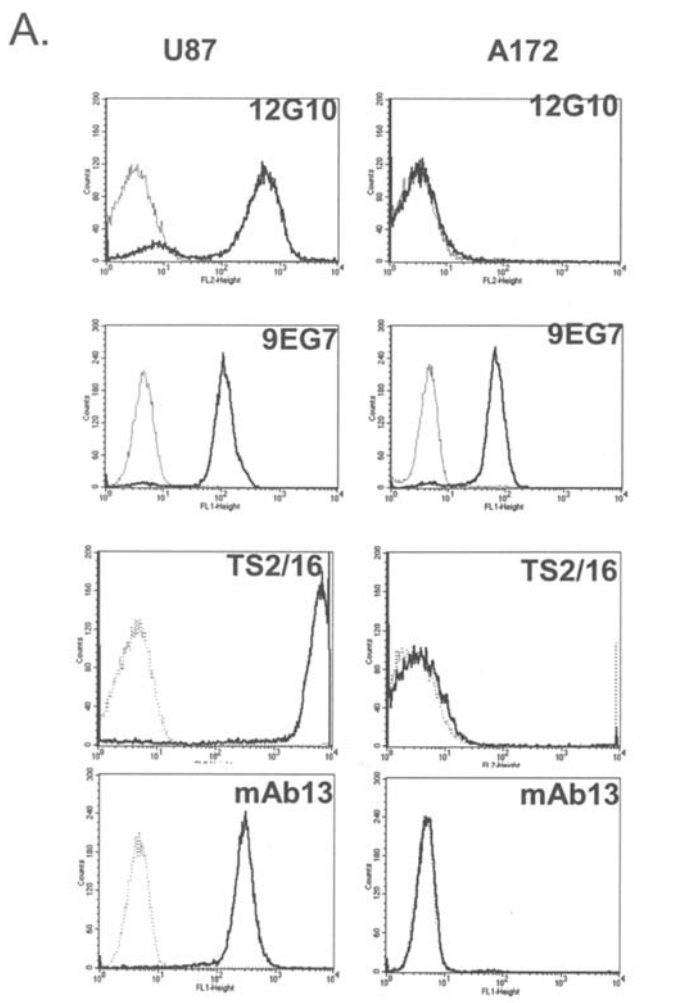
Results

Characterization of α5ß1 integrin in A172 and U87MG cells. We recently reported that both A172 and U87MG cells express functional α5ß1 integrins at levels twice higher in U87MG than in A172 cells (11). The proportion of activated ß1 integrins at the cell surface in both cell lines was determined by flow cytometry using specific function-altering antibodies on non-permeabilized cells. We used an antibody that binds to ß1 in its active and inactive conformation, TS2/16; antibodies that are specific to the active conformation of ß1, 9EG7 and 12G10; and an inhibitory antibody, mAb13. All antibodies were able to reveal ß1 subunits expressed at the cell surface of U87MG cells (Fig. 1A). Surprisingly, the 9EG7 antibody was the only one able to bind to ß1 subunits expressed by A172 cells; its staining was three times higher in U87MG than in A172 cells (MFI values: 47.2±6.3 and 117.5±13.7, n=15, $p<0.0001$, respectively for A172 and U87MG cells; Fig. 1A). These results were confirmed by Western blot analysis (Fig. 1B). Noteworthy, beside the mature form of ß1 integrin (130 kDa), 9EG7 also revealed a form with an increased mobility on SDS-PAGE (110 kDa) (Fig. 1B). Densitometric analysis showed a significantly higher expression of the 110 kDa form of ß1 revealed by 9EG7 in A172 cells compared to U87MG (Fig. 1B, histograms).

As the 9EG7 antibody defines an integrin epitope inducible by Mn^{2+} and inhibited by Ca^{2+} (17), we examined the cation modulation of the 9EG7 epitope in both cells and in HUVEC which express high levels of α5ß1. Neither Mn^{2+} nor Ca^{2+} modified the 9EG7 epitope in A172 cells. In contrast, it was increased by Mn^{2+} and decreased by Ca^{2+} in U87MG cells and HUVEC (Fig. 1C). These data indicate that ß1 integrins expressed in A172 cells markedly differ from those in U87MG cells.

Glycosylation pattern of ß1 integrin in A172 and U87MG cells. To determine whether the 110 kDa form of ß1 detected by 9EG7 might have defects in glycosylation, cell extracts were treated with N-glycosidase F which cleaves both high mannose and complex forms of the N-linked oligosaccharides of the core protein and probed for α5 (using Ab1928) and ß1 (using 9EG7 or Ab1952). Treatment resulted in the detection of single bands at 110 kDa for α5 and 90 kDa for ß1 corresponding to the core protein (Fig. 2A). These data confirmed that the 110 and 130 kDa bands initially detected by 9EG7 correspond in fact to a single protein and suggested that ß1 integrin glycosylation was mainly affected in A172 cells.

Due to the high expression of α5ß1 integrin in HUVEC, we used this cellular model to determine the specificity of the different anti-ß1 antibodies toward the glycosylated form of ß1. Ligation of the different antibodies to ß1 was analyzed in HUVEC total cell extracts untreated and treated with N-glycosidase F. Results were compared afterwards to the profile obtained in A172 cells unexposed to the enzyme. The results showed that mAb13, 12G10 and TS2/16 binding to ß1 was altered by N-glycosidase F in HUVEC (Fig. 2B and data not shown). In contrast, binding of 9EG7 and Ab1952 remain unaffected in HUVEC under these conditions (Fig. 2B). Therefore, mAb13, 12G10 and TS2/16 bind specifically to glycosylated form of ß1. As no binding was observed with these antibodies in A172 cells, this suggests that ß1 subunit has altered glycosylation localized at least in part in its head domain.



Changes in the glycosylation pattern of ß1 were investigated by lectin blotting on immunoprecipitated ß1 subunit. 9EG7 precipitated predominantly the 110 kDa ß1 form in A172 cells and the 130 kDa ß1 form in U87MG cells and as expected TS2/16 precipitated only the 130 kDa ß1 form in U87MG cells (Fig. 3A). Both antibodies were used in the following lectin blotting to immunoprecipitate the ß1 forms.

No striking differences were obtained with L-PHA, E-PHA, LCA or PSA lectins between the two ß1 forms (data not shown). Finally, SNA (a lectin specific for N-acetylneuraminic acid α2,6 sialic acid) stained heavily the 110 kDa ß1 form in A172 compared to the 130 kDa ß1 form in U87MG cells (Fig. 3B, left). When ß1 staining was performed on SNA-precipitated whole cell extract, a large amount of the 110 kDa ß1 form was recovered from A172 cell extract compared to U87MG (Fig. 3B, right). The data suggest that the 110 kDa form of ß1 present in A172 cells carries high levels of α2,6 sialyl acid compared to that in U87MG cells. Sialidase treatment abrogated the lectin precipitation of ß1 integrin confirming the specificity of SNA lectin (data not shown). In addition, treatment of cell extracts with increasing concentrations of sialidase clearly showed that the 110 kDa form of ß1 is largely affected by the enzyme while the 130 kDa protein appears less affected (Fig. 3C). Taken together, data show that the 110 kDa form of ß1 in A172 cells is heavily sialylated.

Membrane expression of the 110 kDa ß1 integrin and interaction with the α5 subunit. We next determined if the 110 kDa

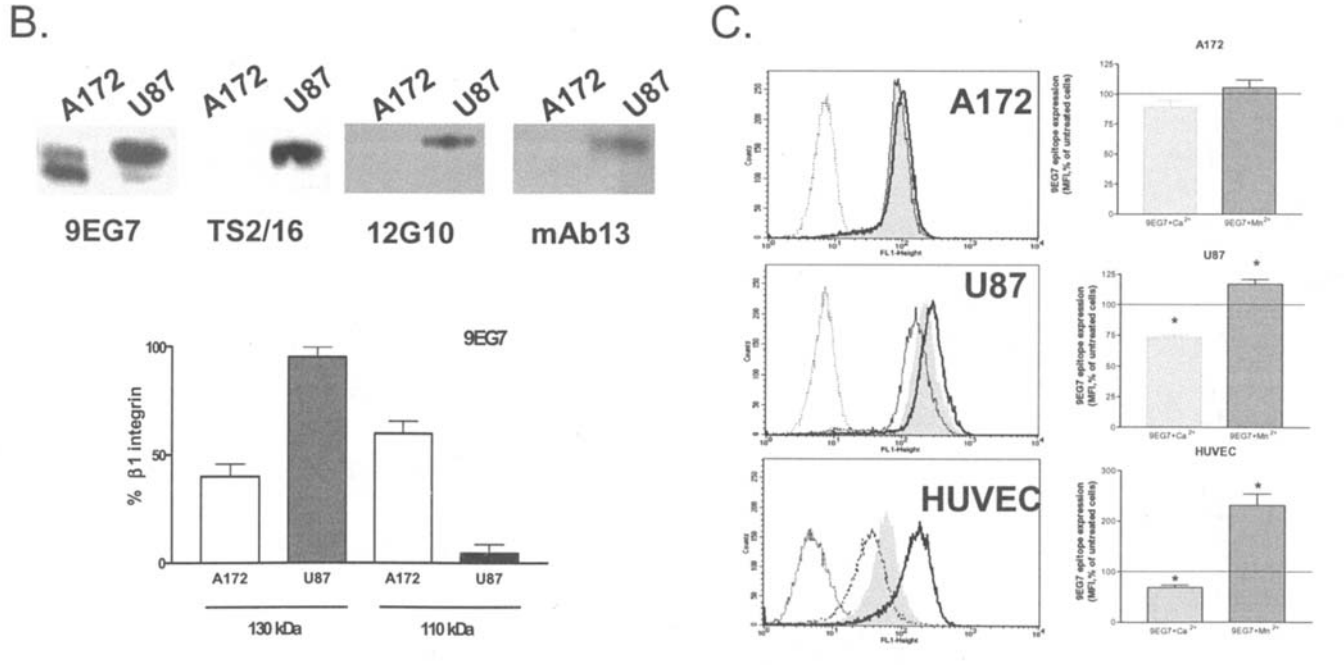
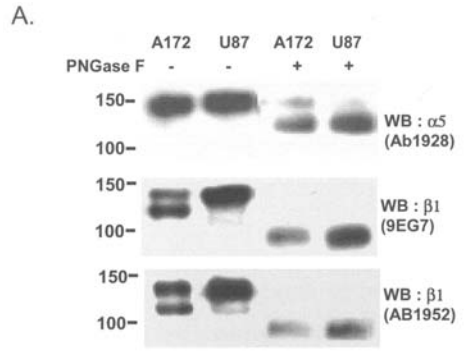
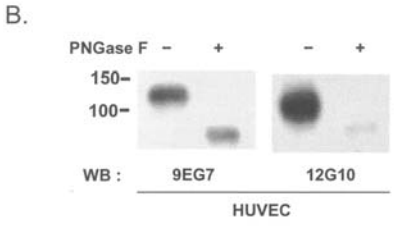


Figure 1. Characterization of ß1 integrin with conformation-sensitive antibodies. (A) Flow cytometry analysis of cell surface integrins: non-permeabilized A172 and U87MG cells were incubated with indicated antibodies (solid line). Non specific binding (dotted line) was determined using secondary antibody alone. (B) Upper panel, Western blot analysis of different epitopes in total protein extracts. Lower panel, densitometric analysis of the proportion of 110 and 130 kDa forms of ß1 in A172 and U87MG cells stained by 9EG7. Results are expressed as mean ± SEM of 6 experiments with 100% corresponding to the densitometry of both forms. Quantification was realized with GeneTool software. (C) A172, U87MG cells and HUVECs were preincubated with 5 mM $MnCl_2$ (solid line), 6.25 mM $CaCl_2$ (broken line) or washing buffer alone (filled area) for 15 min at 37°C prior incubation with 9EG7 and analyzed by flow cytometry. Non-specific binding (dotted line) was determined using secondary antibody alone. Histograms show mean ± SEM of 3 independent experiments.





Antibody	HUVEC / β1 after N-deglycosylation	A172 / β1 without deglycosylation
mAb 13	–/+	–
12G10	–/+	–
TS2/16	–/+	–
9EG7	+	+
AB1952	+	+

Figure 2. Analysis of the ß1 integrin after N-deglycosylation. (A) Total protein extracts were pre-treated with (+) or without (-) PNGase F enzyme for 4 h at room temperature. Western blot analysis were realized using anti-α5 cytoplasmic tail integrin (Ab 1928) or anti-ß1 specific antibodies (9EG7 or Ab1952). (B) Western blot analysis of ß1 in cells untreated or treated with PNGase F to determine the sensitivity of anti-ß1 antibodies toward N deglycosylation. Upper panel, total protein extracts from HUVEC were treated as described in (A) and analyzed with 9EG7 and 12G10 anti-ß1 antibodies. Lower panel, table summarize the ligation of anti-ß1 antibodies in HUVEC exposed to PNGase F compared to untreated A172 cells. (-) meaning no signal, (+) meaning similar signal and (-/+) meaning reduced signal.

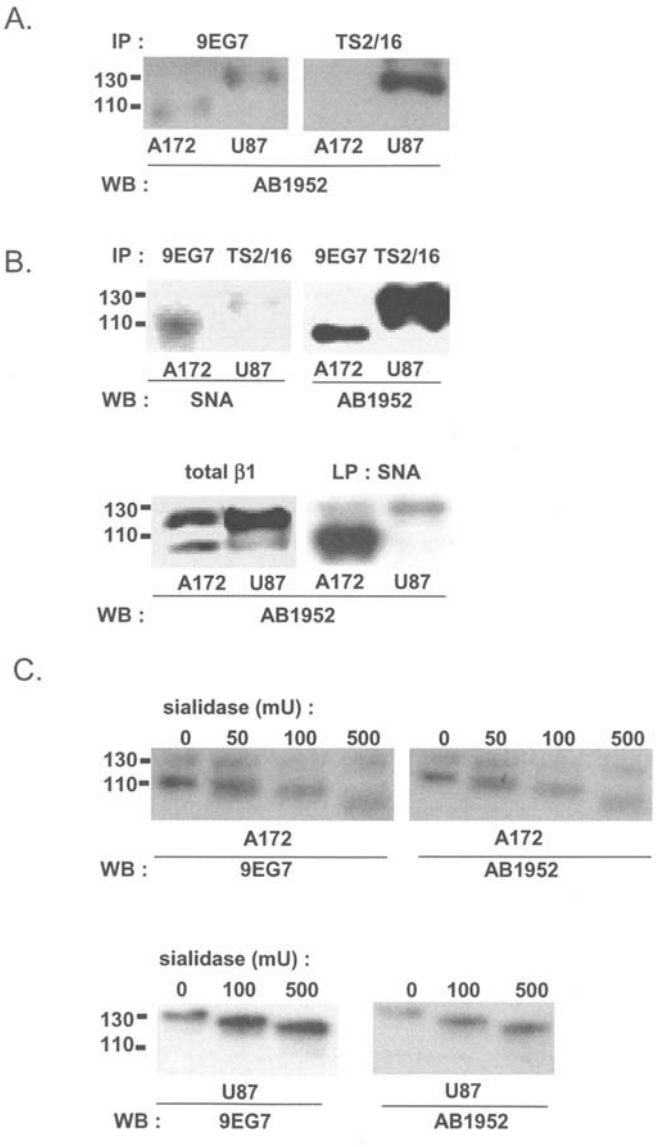


Figure 3. Characterization of ß1 integrin species in A172 and U87MG cells by immuno- and lectin-precipitation. (A) Immunoprecipitated ß1 integrin from A172 and U87MG total protein extracts using 9EG7 and TS2/16 were immunoblotted with Ab1952. (B) Upper panel, immunoprecipitated ß1 integrin from A172 and U87MG total protein extracts using 9EG7 and TS2/16 were lectin blotted with biotinylated-SNA lectin or immunoblotted with Ab1952. Lower panel, precipitated lectin recovered-protein from total extracts using biotinylated SNA and streptavidin-coupled agarose beads from A172 and U87MG cells were immunoblotted with Ab1952. (C) 300 μg of A172 or U87MG total protein extracts were treated with increasing concentrations of sialidase for 4 h at room temperature and analysed with 9EG7 or Ab1952.

integrin expressed in A172 cells was able to form heterodimers with the α5 subunit and to localize at the cell surface. In both cell lines, α5 (detected with H-104) coprecipitated with ß1 (immunoprecipitated by 9EG7) confirming the ability of the 110 kDa ß1 to form α5ß1 dimers (Fig. 4A, upper panel). To determine the presence of the heterodimer at the cell membrane, selective capture of cell surface integrins was performed. Briefly, cells were incubated with subsaturating concentrations of 9EG7 before lysis and precipitation of the solubilized material with protein-G sepharose (16). We found that α5ß1 heterodimers were present at the membrane in both cell lines (Fig. 4A, lower panel). To confirm that 9EG7 concentration was subsaturating, U87MG cell extract (containing the 130 kDa form of ß1 recognized by 9EG7) was added to A172 lysate. No 130-kDa form of ß1 was detected confirming that only cell surface integrins were captured (Fig. 4B). Colocalization of α5 and ß1 at the cell membrane was finally assessed by confocal microscopy using Ab1928 and 9EG7 respectively. Merged images (Fig. 4C) showed that α5 and ß1 colocalized in punctate structures corresponding to focal adhesion points in U87MG cells whereas α5 and ß1 colocalization was restricted to specific membrane portions of A172 cell body. Thus, our results show that 9EG7-sensitive ß1 forms in A172 cells interact with α5 subunits in plasma membrane regions resembling fibrillar adhesions (18).

Characterization of ß1 in A172 cells after overexpression. The ß1 integrin was transfected into A172 cells (ß1-A172). Western blot analysis on whole cell extracts revealed that 9EG7 was able to bind to ß1 in all cell lines, but the ratio 110/130 kDa forms of ß1 was modified in transfected cells

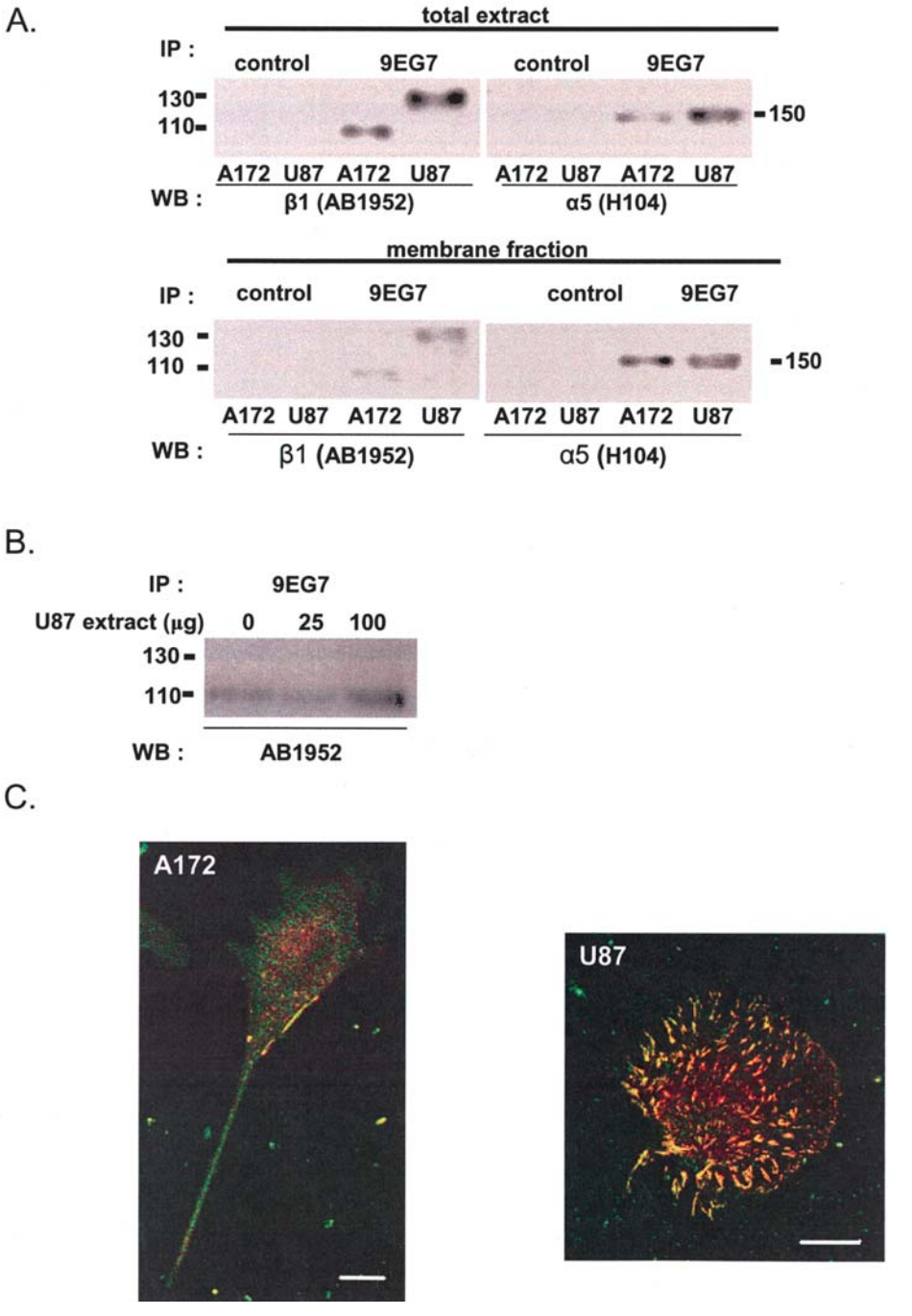


Figure 4. Co-precipitation and co-localization of α5 and ß1 subunits in A172 and U87MG cells. (A) Immunoprecipitated ß1 from A172 and U87MG total cell extracts (upper panel as in Fig. 3A) or membrane preparations (lower panel) using 9EG7 were immunoblotted for ß1 using Ab1952 and α5 using H104. Control antibodies did not precipitate the integrin subunits. For studies performed on membranes, intact A172 and U87MG cells were labelled with subsaturating concentrations of antibodies. After removing free antibodies by extensive washes, cells were lysed and immunocomplexes were precipitated with Protein G-Sepharose beads before immunoblotting. (B) To confirm that antibody concentrations used in (A) was subsaturating, intact A172 cells were incubated with 9EG7, washed and lysed. Before addition of Protein G sepharose beads, 25 or 100 μg of U87MG total protein extract were added to the A172 lysate. Immunocomplexes were blotted with Ab1952. (C) Colocalization of α5 and ß1 subunits by confocal microscopy. Cells were grown on fibronectin, fixed, permeabilized and stained with anti-ß1 antibody 9EG7 (red) and anti-α5 antibody Ab1928 (green). In U87MG cells extensive colocalization of α5 and ß1 subunits was observed in focal adhesion points. In A172, colocalized α5 and ß1 subunits were restricted to particular regions of the membrane. Scale bar, 10 μm.

(Fig. 5A, upper and lower panel). In contrast to A172 and mock-A172 cells, the 130 kDa form of ß1 was detectable by the TS2/16 antibody in ß1-A172 as it was in U87MG cells (Fig. 5A, upper panel). Flow cytometry analysis performed with TS2/16 confirmed that the corresponding epitopes are localized at the cell surface of ß1-A172 (Fig. 5B). In the ß1-A172 cells, the 9EG7 epitope increased upon pre-treatment with Mn^{2+} ions (Fig. 5B) in contrast to the A172 cells (Fig. 1C).

9EG7 precipitated the 110 kDa in whole extracts of both cell lines. Its level was significantly increased in the ß1-A172 extract (205±19%, n=6, p=0.03; Fig. 5C). However, 9EG7

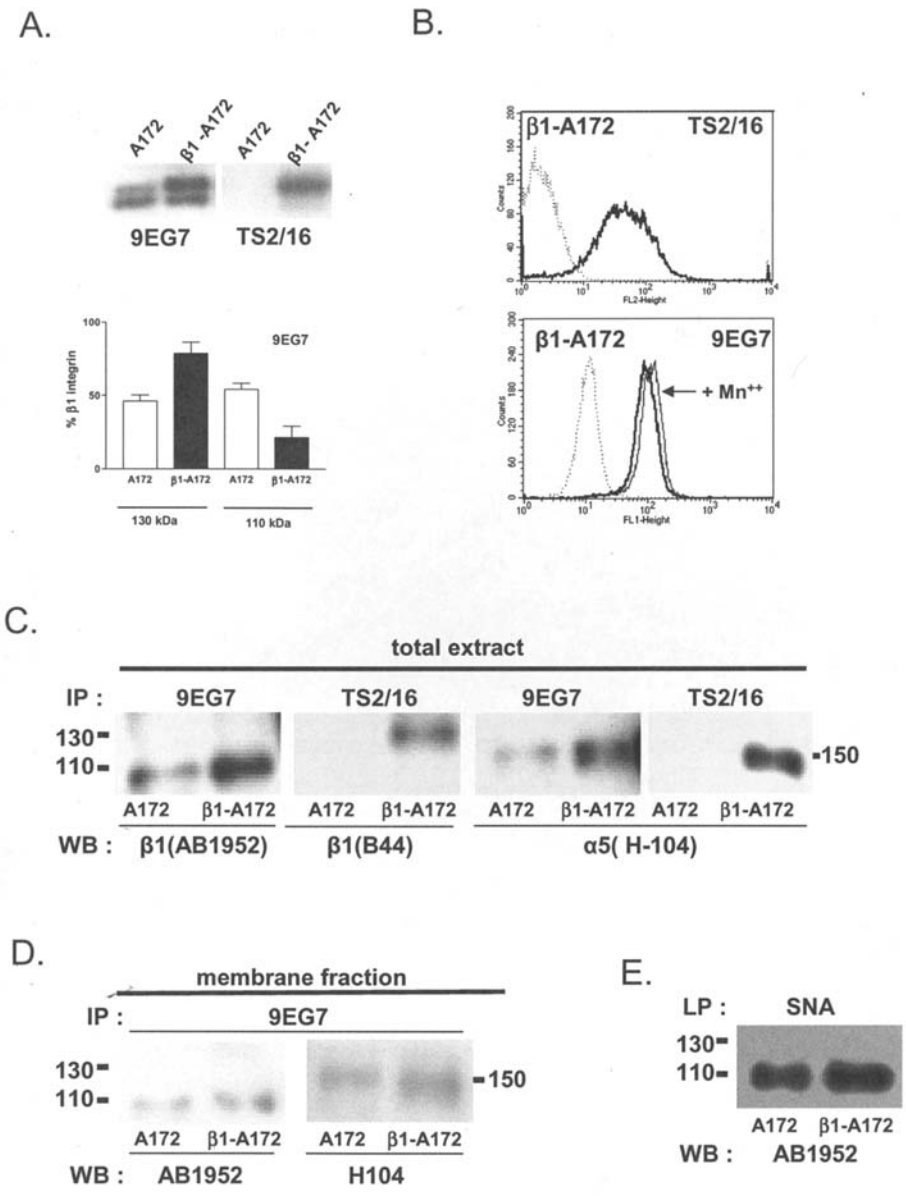


Figure 5. Expression of ß1 integrin epitopes after human ß1 transfection in A172 cells. (A) A172 or ß1-A172 total protein extracts (50 μg) were resolved by SDS-PAGE and immunoblotted with indicated antibodies. Lower panel, densitometric analysis of the proportion of 110 and 130 kDa forms of ß1 in A172 and ß1-A172 cells stained by 9EG7. Results are expressed as mean ± SEM of 3 experiments with 100% corresponding to the densitometry of both forms. (B) Flow cytometry analysis of cell surface integrins: non-permeabilized ß1-A172 cells were incubated with indicated antibodies (solid line). 9EG7 epitope in ß1-A172 cells is increased by Mn^{2+} ions (dotted dark line). Non-specific binding (dotted line) was determined using secondary antibody alone. (C) Immunoprecipitation of ß1 integrin species in A172 and ß1-A172 cells: 300 μg total protein extracts were immunoprecipitated with 9EG7 or TS2/16 and Protein G-Sepharose beads and immunoblotted with anti-ß1 (Ab1952 or B44) and anti-α5 (H104) antibodies. (D) Immunoprecipitation of cell surface ß1 integrin subunits in A172 and ß1-A172 cells using 9EG7. Experiments were done as previously described (Fig. 4A, right). (E) SNA-lectin precipitated A172 and ß1-A172 total protein extracts were immunoblotted with Ab 1952.

was unable to precipitate the 130 kDa protein, even after transfection, suggesting that the antibody has a greater affinity for the sialylated form of ß1. TS2/16 precipitated the 130 kDa form in ß1-A172 in contrast of A172 cells (Fig. 5C). Both ß1 forms coprecipitated α5 (Fig. 5C) although 9EG7 produced twice as much α5 subunit in ß1-A172 as compared to A172 cells (218±53%, n=4, p=0.05). Selective capture of cell surface integrins using 9EG7 showed that the 110 kDa form of ß1 remains at the cell surface and heterodimerizes with α5 (Fig. 5D).

Finally SNA-lectin precipitation showed that only the 110 kDa form of ß1 was recovered in A172 and ß1-A172 cells (Fig. 5E). We failed to detect high sialylation of the 130 kDa form of ß1 in ß1-A172 (Fig. 5E), similarly to U87MG cells (Fig. 3B).

Functional significance of hypersialylated ß1 integrin. Concomitant expression of the hypersialylated 110 and the 130 kDa forms in ß1-A172 cells allowed us to explore the functional significance of both subunits. Adhesion of A172 cells to fibronectin was increased by the overexpression of ß1 (Fig. 6A, left). The specific α5ß1 antagonist, SJ749, hardly prevented attachment to fibronectin of ß1-A172 in contrast to A172 (IC_{50} >10 μM and 67 nM for ß1-A172 and A172 cells

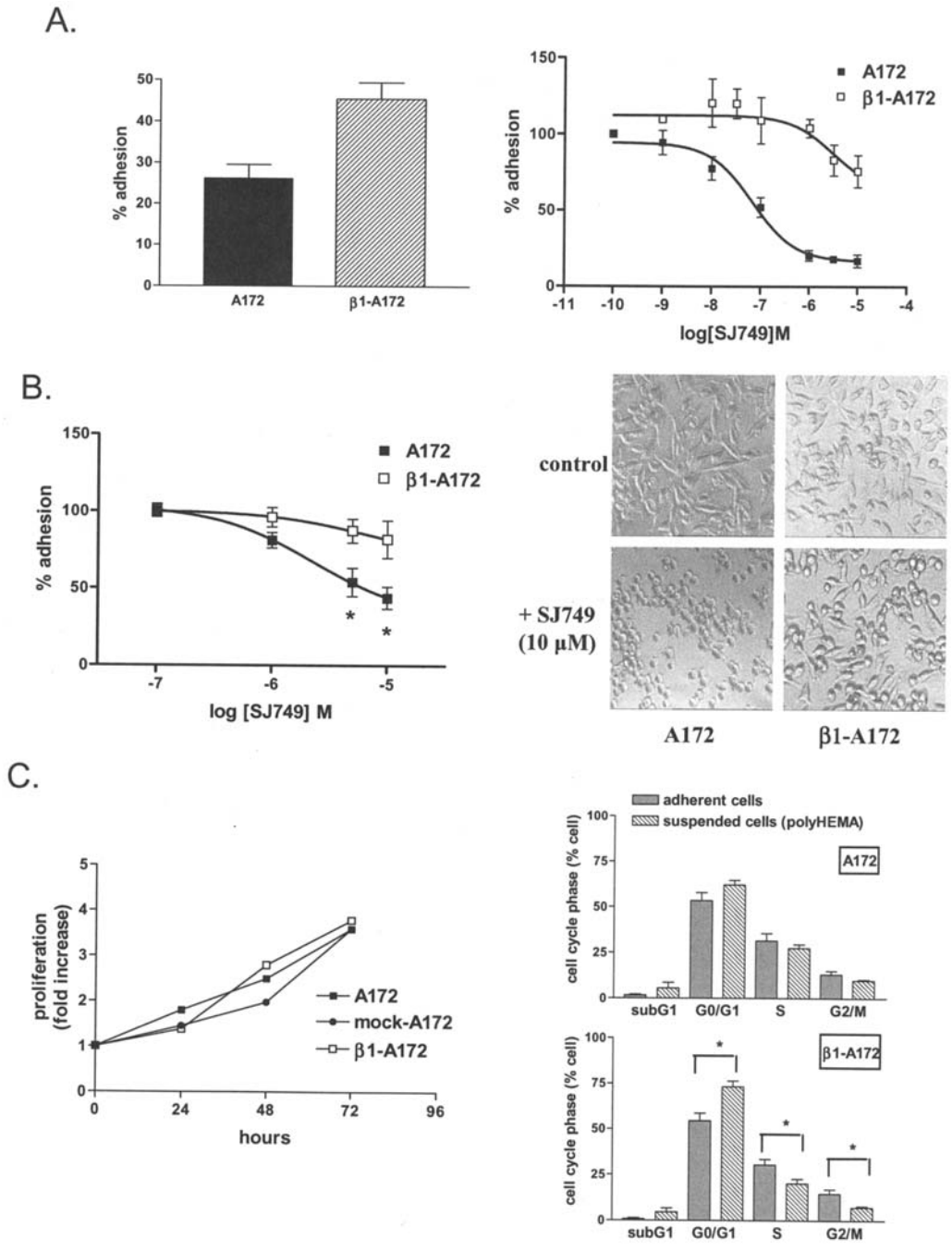


Figure 6. Effects of ß1 integrin overexpression on fibronectin adhesion and growth characteristics. (A) Left, cell adhesion assay: cells were plated in 96-well plates coated with 5 μg/ml fibronectin. After 30-min incubation, unbound cells were washed out and adherent cells fixed and colored with crystal violet. Absorbance was measured at 595 nm. Results are expressed as percentage of adherent cells on fibronectin versus poly-lysine (taken as 100%). Mean ± SEM of 3 experiments in sextuplate. Right, effect of SJ749 on fibronectin adhesion: cells were incubated for 30 min in fibronectin-coated 96-well plates in the absence (100% adhesion) or presence of increasing concentrations of SJ749. ■, A172 cells; □, ß1-A172 cells. Mean ± SEM of 3 experiments in sextuplate. (B) Detachment assays. Cells were allowed to adhere on fibronectin for 2 h and incubated for another 4 h in the absence (100% adhesion) or presence of increasing concentration of SJ749. ■, A172 cells; □, ß1-A172 cells. Mean ± SEM of 3 experiments in quadruplicate. Microphotographs of adherent cells after 2-h incubation with or without 10 μM SJ749. (C) Left, proliferation of parental (■), mock-transfected (•) and ß1-A172 (□) cells in adherent conditions measured by the MTS assay. Right, cell cycle analysis: cells were allowed to grow in adherent or non-adherent conditions (poly-HEMA coated wells) for 48 h. Cell cycle profiles were determined by propidium iodide staining using Modfit software. Mean ± SEM of 3 experiments in duplicate; $^{*}p<0.05$.

respectively; Fig. 6A, right). The capacity of SJ749 to detach cells from fibronectin was next examined. Although SJ749 dose-dependently detached A172 cells from fibronectin, ß1-A172 cells appeared almost insensitive to the drug under the conditions tested. Microphotographs taken after SJ749 treatment revealed morphological differences between the cell lines; A172 were round whereas ß1-A172 cells remained spread out (Fig. 6B). These data suggest that the 130 kDa form of ß1 exhibits a higher affinity for fibronectin and is less sensitive to SJ749.

Proliferation of both cell lines was examined in adherent and non-adherent conditions (poly-HEMA coated wells).

A172 cells were able to proliferate both in adherent and non-adherent conditions without alteration of the cell cycle progression [Fig. 6C; (11)]. In contrast, a significant blockage in G0/G1 phase of the cell cycle was observed when ß1-A172 cells were grown in suspension versus adherent conditions (Fig. 6C). Thus, in non-adherent conditions, expression of the 130 kDa form of ß1 renders cells more dependent on matrix interactions for cell cycling as was the case for U87MG cells (11).

Discussion

The present study aimed to characterize α5ß1 integrins in two human astrocytoma cell lines, A172 and U87MG cells. We reported here for the first time: i) that a functional 110 kDa ß1 integrin form was endogenously expressed at the cell membrane of A172 cells, ii) that this ß1 integrin was highly sialylated and mainly detected by the 9EG7 antibody and iii) finally that this hypersialylated ß1 form might confer a growth advantage to A172 cells especially in non-adherent conditions.

We observed that ß1 forms expressed by A172 and U87MG cells yielded products of the same molecular weight after exposure to N-glycosidase F, suggesting ß1 post-translational N-glycosylation modifications. The biochemical nature of glycans present in this ß1 form contrast with immature ß1 integrin glycans. It is already known that ConA binds both to mature and immature forms of ß1 whereas SNA binds only to mature forms of ß1 (9,19). We found that the 110 kDa form of ß1 had increased α2,6 sialylation compared to the 130 kDa form and that it localized at the plasma membrane.

During oncogenic transformations, changes in the electrophoretic mobility of ß1 integrins have been attributed to differential glycosylation mostly altered abundance of sialic acids or increased ß1,6 branching of N-linked oligosaccharides (reviewed in ref. 9). In A172 cells, sialic acid α2,6-linkage appeared predominant because of the strong reactivity of the 110 kDa form of ß1 with SNA. *In vitro* experiments have shown that during colon cell transformation by Ras oncogene, upregulation of ST6Gal-I led to the hypersialylation of ß1 integrins conferring an increased protein mobility on SDS-PAGE (10). A 6-fold increase in α2,6 sialic acid was observed on ß1 integrins in transformed cells when compared to normal epithelial tissue (20), suggesting a functional role for hypersialylation in colon cancer progression. However, tumors of glial origin and medulloblastoma appeared devoid of either α2,6-sialyltransferase or α2,6-linked sialoconjugates (21) and expressed increased levels of α2,3-sialyltransferase mRNA and α2,3-linked glycoprotein sialylation (22). Thus, A172 cells appear as an interesting model to study the endogenous expression of abnormal α2,6-sialylated ß1 integrins in glioma cells and its functional significance. On the other hand, U87MG cells express ß1 integrins hardly recognized by SNA as already observed for another glioma cell line, U-373 MG cells (23). Work is in progress to compare levels of the different sialyltransferases in the A172 cells.

Changes in sialic acid chains or other glycosylation on ß1 integrins did not affect integrin cell surface expression (10,24,25) but modulate integrin functions. It is currently unknown how altered sialylation could affect integrin conformation and ligand recognition. 9EG7 recognizes the hypersialylated form of ß1 in whole cell extracts as well as at the cell surface. Its epitope has been mapped within the cysteine rich region of ß1 downstream the ligand binding region (amino acids 495-602) (17). Although it was first described as an activation monitoring antibody (15), its binding does not generally correlate with the activation of any ß1 integrin under any conditions (17,26) and seems to be restricted to CLIBS (cation- and ligand-induced binding sites) (27). Increased exposure of such epitopes on ß1 integrins after ligand binding seems to require partial α/ß unfolding (28). Here, we show that the 9EG7 epitope appeared constitutively expressed in A172 cells. Taken together, data show that the 9EG7 epitope is accessible in α5ß1 when ß1 is hypersialylated. In the same way, down regulation of talin was shown to alter α5ß1 glycosylation processing resulting in membrane-localized abnormal heterodimers which had lost many antibody epitopes except the 9EG7 epitope (25,29).

Our results comform to previous work postulating the existence of different glycosylated forms/conformations of ß1 integrins at the cell surface. We show here that overexpression of the ß1 integrin in A172 cells led not only to a 2-fold increase in the 110 kDa protein but also to the expression of the 130 kDa ß1 form coexisting at the cell surface. Our data suggest a modification of the ß1 glycosylation which might be overcome by overexpression of the integrin subunit. On the other hand, we cannot completely exclude that the A172 cells have a mutation in their endogenous ß1 gene. Both hypothesis may coexist in these cells and deserve further studies. It was recently shown (16) that a 110 kDa ß1 integrin was constitutively expressed at the K562 cell surface detected only by 12G10. Thus, two distinct ß1 integrin species, differing by their glycosylation state and by antibody recognition, can coexist at the cell membrane. The mechanisms by which cells generate these different species and how they control their distribution remain to be solved.

Variations in glycosylation have been particularly emphasized in the ß1 subunits compared to ß3 or ß5-subunits (8-10). Changes in the N-glycan structures of ß1 affect its functions and appeared crucial for tumorigenicity. Overexpression by transfection of key enzymes (such as N-acetylglucosaminyltransferases or sialyl transferases), which are implicated in the processing of multiantennary N-glycans, confirmed that adhesion to ECM (20,30,31), integrin clustering (32), cell migration (20,33), cell survival (34) and invasivity (35) were highly modified by glycosylation in a cell type specific context. Taking advantage of the endogenous expression of the hypersialylated form of ß1 in A172 cells, we determined its role in cell adhesion and cell growth. The differences observed in functional assays between A172 and ß1-A172 cells might be attributed to the expression of the low-sialylated integrin form. We showed that adhesion of ß1-A172 cells to fibronectin was increased and the ability of SJ749, a specific α5ß1 antagonist, to block it was impaired, suggesting that hypersialylated integrins exert lower affinities for fibronectin. De-sialylation of ß1 integrin has already been shown to affect adhesive behavior of cells by increasing adhesion to fibronectin (31,36-38). In contrast to α5ß1 and α3ß1 (23,37), desialylation of α1ß1 had diminished collagen-binding capacity (10)

suggesting that sialylation plays a crucial role in ß1 function depending on the nature of the heterodimers concerned.

On the other hand, when the hypersialylated ß1 integrin form is mainly expressed, as is the case in A172 cells, anchorage independent growth seemed to be favored as compared to ß1-A172 or U87MG cells. These results suggested that integrin signaling pathways may be differently activated by the two integrin forms. This could give a selective advantage for growth and invasion to hypersialylated ß1-expressing tumor cells and might explain the enhanced *in vivo* biological aggressiveness of A172 cells as compared to U87MG cells (39).

In conclusion, our results demonstrate that a hypersialylated ß1 integrin is endogenously expressed in a human astrocytoma cell line. They also suggest that the anti-ß1 integrin antibody 9EG7 may be a useful tool to detect abnormally sialylated ß1 integrin. Taken together, our findings add new pieces of evidence supporting that sialylation represent a potential mechanism for the regulation of ß1 integrin signaling in glioma cells.

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