

Sialic Acid Associated with $\alpha_v\beta_3$ Integrin Mediates HIV-1 Tat Protein Interaction and Endothelial Cell Proangiogenic Activation*

Received for publication, December 23, 2011, and in revised form, April 3, 2012. Published, JBC Papers in Press, April 23, 2012, DOI 10.1074/jbc.M111.337139

Paola Chiodelli[‡], Chiara Urbinati[‡], Stefania Mitola[‡], Elena Tanghetti[§], and Marco Rusnati^{‡1}

From the Units of [‡]General Pathology and Immunology and [§]Histology, Department of Biomedical Sciences and Biotechnology, School of Medicine, University of Brescia, 25123 Brescia, Italy

Background: HIV-1 Tat engages $\alpha_v\beta_3$, leading to endothelial cell (EC) proangiogenic activation.

Results: Sialic acid (NeuAc)-binding lectins and neuraminidase partially inhibit Tat/ $\alpha_v\beta_3$ interaction and consequent EC proangiogenic activation.

Conclusion: Endothelial $\alpha_v\beta_3$ -associated NeuAc is involved in Tat interaction and consequent EC proangiogenic activation.

Significance: Integrin-associated NeuAc can be considered a target for the development of new treatments for angiogenesis/AIDS-associated pathologies.

Sialic acid (NeuAc) is a major anion on endothelial cells (ECs) that regulates different biological processes including angiogenesis. NeuAc is present in the oligosaccharidic portion of integrins, receptors that interact with extracellular matrix components and growth factors regulating cell adhesion, migration, and proliferation. Tat is a cationic polypeptide that, once released by HIV-1⁺ cells, accumulates in the extracellular matrix, promoting EC adhesion and proangiogenic activation by engaging $\alpha_v\beta_3$. By using two complementary approaches (NeuAc removal by neuraminidase or its masking by NeuAc-binding lectin from *Maackia amurensis*, MAA), we investigated the presence of NeuAc on endothelial $\alpha_v\beta_3$ and its role in Tat interaction, EC adhesion, and proangiogenic activation. $\alpha_v\beta_3$ immunoprecipitation with biotinylated MAA or Western blot analysis of neuraminidase-treated ECs demonstrated that NeuAc is associated with both the α_v and the β_3 subunits. Surface plasmon resonance analysis demonstrated that the masking of $\alpha_v\beta_3$ -associated NeuAc by MAA prevents Tat/ $\alpha_v\beta_3$ interaction. MAA and neuraminidase prevent $\alpha_v\beta_3$ -dependent EC adhesion to Tat, the consequent FAK and ERK1/2 phosphorylation, and EC proliferation, migration, and regeneration in a wound-healing assay. Finally, MAA inhibits Tat-induced neovascularization in the *ex vivo* human artery ring sprouting assay. The inhibitions are specific because the NeuAc-unrelated lectin from *Ulex europaeus* is ineffective on Tat. Also, MAA and neuraminidase affect only weakly integrin-dependent EC adhesion and proangiogenic activation by fibronectin. In conclusion, NeuAc is associated with endothelial $\alpha_v\beta_3$ and mediates Tat-dependent EC adhesion and proangiogenic activation. These data point to the possibility to target integrin glycosylation for the treatment of angiogenesis/AIDS-associated pathologies.

Polyanionic macromolecules are extremely abundant in the extracellular environment, readily accessible to many proteins for interactions implicated in various biological functions. Among polyanions, sialic acid (NeuAc)-bearing gangliosides and glycoproteins are widely distributed in biological fluids, extracellular matrix, and cell membrane, where they act as receptors for various physiological ligands and for many human viruses, bacteria, and protozoa (1–3).

The term NeuAc encompasses a large family of sugars characterized by a nine-carbon sugar acid common in higher animals and some microorganisms (4). NeuAc is found mainly as a terminal component of glycoprotein and gangliosides, where it regulates various molecular and cellular interactions (5).

NeuAc is the major surface anion on the endothelial cell (EC)² surface. Accordingly, the lectin from *Maackia amurensis* (MAA), which specifically binds NeuAc residues attached to galactose through an $\alpha(2\rightarrow3)$ linkage, binds to ECs of retina, brain, and myocardium (6). NeuAc expression on ECs is regulated during ontogenesis, inflammation (7–9), and possibly neovascularization, as suggested by the observation that the binding of the NeuAc-binding lectin from *Limax flavus* to ECs increases during angiogenesis in the chick embryo chorioallantoic membrane (8).

NeuAc is involved in different physiological and pathological functions of the endothelium; in its ganglioside- or glycoprotein-associated form, it mediates EC infection by different microorganisms (10) and the transport of HIV-1 or of its proteins across the blood-brain barrier (11, 12). In its ganglioside-associated form, NeuAc takes part in the regulation of neovascularization (13–15). When associated with integrin subunits (including α_E (16), α_2 (17), α_3 (18), α_4 (19), α_5 and α_v (20), β_1

* This work was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca, Istituto Superiore di Sanità (AIDS Project), and Cassa di Risparmio delle Provincie Lombarde (to M. R.).

¹ To whom correspondence should be addressed: General Pathology and Immunology, Dept. of Biomedical Sciences and Biotechnology, viale Europa 11, 25123 Brescia, Italy. Tel: 39-30-3717315; Fax: 39-30-3717747; E-mail: rusnati@med.unibs.it.

² The abbreviations used are: EC, endothelial cell; FAK, focal adhesion kinase; FN, fibronectin; MAA, lectin from *M. amurensis*; UEA, lectin from *U. europaeus*; PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PPPP, D-1-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl; SPR, surface plasmon resonance; VEGFR2, vascular endothelial growth factor receptor-2; WB, Western blot; TRITC, tetramethylrhodamine isothiocyanate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

(17, 18, 20), β_2 (21), and β_4 (16, 20)), NeuAc contributes to leukocyte and tumor cell extravasation during inflammation and metastasization, respectively.

Integrins are widely distributed receptors that interact with extracellular matrix components, growth factors, and microbial proteins regulating adhesion, migration, and proliferation of various normal and transformed cell types (22). Among the various integrins, $\alpha_v\beta_3$ expressed on the surface of ECs plays a central role in neovascularization (23). Interestingly, NeuAc has been found associated with $\alpha_v\beta_3$ integrin from melanoma metastatic cell surface (18), but no data are available for $\alpha_v\beta_3$ from ECs.

HIV-1 Tat is a cationic protein that, once released by HIV-1-infected cells (24), targets ECs, causing a variety of pathological effects that, in turn, lead to different angiogenesis-related AIDS-associated diseases such as Kaposi sarcoma and ocular microangiopathies. Extracellular Tat accumulates in the extracellular matrix where, by binding to endothelial $\alpha_v\beta_3$, it promotes EC adhesion and proangiogenic activation (25–27). Tat/ $\alpha_v\beta_3$ interaction occurs both via the RGD motif and the basic domain (RKKRRQRRR) of Tat (25). On the basis of what is described above, in this study, we decided to evaluate the presence of NeuAc on integrin $\alpha_v\beta_3$ expressed at the EC surface and to investigate its role in Tat engagement and consequent biological activities.

EXPERIMENTAL PROCEDURES

Chemicals—Synthetic 86-amino acid Tat was from Xeptagen (Venezia, Italy). The recombinant wild type 86-amino acid form of HIV-1 Tat and its mutants Tat 1e (characterized by the deletion of the amino acid sequence that contains the RGD sequence) and Tat R→A (in which the arginine residues 49, 52, 53, 55, 56, and 57 within the basic domain were mutated to alanine residues) were purified from *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins (28). GST moiety does not interfere with Tat molecular interactions and biological activities (25). Anti-vascular endothelial growth factor receptor-2 (VEGFR2) antibody was gifted by Prof. H. A. Weich, National Research Centre for Biotechnology, Braunschweig, Germany. The heptapeptides GRGDSPK and GRADSPK were from Neosystems Laboratoires, Strasbourg, France, K5NOSH was from Glycores 2000, Milan, Italy, specific $\alpha_v\beta_3$ antagonist SCH221153 and its inactive analog SCH21668 (27) were from Schering-Plough (Kenilworth, NJ), anti-phospho-FAK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-ERK1/2 antibody and anti-phospho-VEGFR2 antibody were from Cell Signaling Technology (Danvers, MA), biotinylated MAA was from Vector Laboratories (Burlingame, CA), streptavidin-Sepharose, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and *N*-hydroxy-succinimide were from GE Healthcare, anti-paxillin antibody was from Upstate Biotech Millipore (Lake Placid, NY), purified human $\alpha_v\beta_3$ integrin, anti- $\alpha_v\beta_3$ LM 609, anti-fascin, anti- α_v and anti- β_3 antibodies were from Chemicon, Millipore (Billerica, MA), glucosyl ceramide synthase inhibitor *D*-threo-1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol (PDMP) and *D*-1-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (PPPP) were from Matreya, LLC (Pleasant Gap,

PA), anti- α -tubulin antibody, TRITC-phalloidin, FITC-conjugated anti-mouse IgG, neuraminidase from *Clostridium perfringens*, MAA, lectin from *Ulex europaeus* (UEA), poly-L-lysine, fibrinogen, fibronectin (FN), phorbol myristate acetate, 4-6-diamidino-2-phenylindole (DAPI), phenylmethylsulfonyl fluoride (PMSF), amino-*n*-caproic acid, leupeptin, Na_3VO_4 , and NaF were from Sigma.

Surface Plasmon Resonance (SPR) Analysis—A BIAcore X instrument (GE Healthcare) was used. Two different immobilizations were used to study the Tat/ $\alpha_v\beta_3$ interaction. (i) As described previously (25), synthetic Tat (40 $\mu\text{g}/\text{ml}$) was allowed to react with a CM5 sensor chip activated with 50 μl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.5 M *N*-hydroxy-succinimide, leading to the immobilization of 6,470 resonance units (0.35 pmol/ mm^2) of protein. Similar results were obtained for the immobilization of bovine serum albumin (BSA), used as a negative control and for blank subtraction. Increasing concentrations of integrin $\alpha_v\beta_3$ in 10 mM Tris, pH 7.8, containing 10 nM Mn^{2+} were injected over the Tat or BSA surfaces in the absence or in the presence of MAA (250 nM) for 4 min and then washed until dissociation. In parallel experiments, increasing concentrations of integrin $\alpha_v\beta_3$ in 10 mM Tris, pH 7.8, containing 10 nM Mn^{2+} were incubated for 1 h with neuraminidase (500 milliunits/ml) before injection. Samples containing $\alpha_v\beta_3$ to which neuraminidase was added only before injection were used as controls, demonstrating that the presence of the enzyme does not interfere significantly with Tat/ $\alpha_v\beta_3$ interaction. After every run, the sensor chip was regenerated by injection of 2.0 M NaCl in 10 mM HEPES, 3 mM EDTA, 150 mM NaCl, 0.005% surfactant P20. The dissociation constant (K_d) of the Tat/ $\alpha_v\beta_3$ interaction was calculated by the Scatchard plot analysis of the steady-state SPR data. (ii) Anti-GST antibody was immobilized on a CM5 surface using standard amine-coupling chemistry allowing the immobilization of 25,000 resonance units, equal to 0.98 pmol. Then, wild type GST-Tat, GST-Tat 1e, and GST-Tat R→A (120 $\mu\text{g}/\text{ml}$ in Tris 10 mM pH 7.8 containing 10 nM Mn^{2+}) were injected over the anti-GST surface at a flow rate of 10 $\mu\text{l}/\text{min}$, allowing the immobilization of about 800 resonance units (equal to about 0.023 pmol) for wild type GST-Tat and GST-Tat R→A, and 1,300 resonance units (equal to 0.037 pmol) for GST-Tat 1e and for the GST moiety alone, used for blank subtraction.

Cell Culture—Transformed fetal bovine aortic endothelial GM7373 cells (obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) (29), were grown in Dulbecco's modified minimum essential medium (DMEM), 10% fetal calf serum (FCS), and antibiotics (Invitrogen, Paisley, UK). Removal of NeuAc from the cell surface was obtained by a 1-h incubation at 37 °C of cells with phosphate-buffered saline (PBS) containing neuraminidase from *C. perfringens* (from 125 to 500 milliunits/ml) and used for the various assays described below.

Detection of NeuAc on Integrin $\alpha_v\beta_3$ —GM7373 ECs (1×10^6 cells/sample) were treated with neuraminidase (from 125 to 500 milliunits/ml), washed, scraped in 50 μl of 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 4 mM amino-*n*-caproic acid,

Endothelial $\alpha_v\beta_3$ Sialic Acid and HIV-1 Tat

10 $\mu\text{g/ml}$ leupeptin, 1 mM Na_3VO_4 , 50 mM NaF (radioimmuno-precipitation modified lysis buffer) and centrifuged (10 min at 12,000 rpm). Cell extracts (30- μg aliquots) were analyzed on nonreducing SDS-6% PAGE followed by Western blot (WB) with anti- α_v or anti- β_3 antibodies. Human purified $\alpha_v\beta_3$ (100 ng) was incubated for 1 h at 37 °C with neuraminidase (125 milliunits/ml in PBS) and used as control.

For immunoprecipitation analysis, GM7373 EC cultures were lysed in radioimmunoprecipitation modified lysis buffer and centrifuged (10 min at 12,000 rpm). Cell extracts (400 μg) were incubated for 1 h at 25 °C with biotinylated MAA (1 $\mu\text{g/sample}$) and for an additional 16 h at 4 °C with streptavidin-Sepharose (30 $\mu\text{l/sample}$), centrifuged (1 min at 3,000 rpm), and analyzed on nonreducing SDS-6% PAGE followed by WB with anti- α_v or anti- β_3 antibodies. Human purified $\alpha_v\beta_3$ integrin (250 ng) incubated with biotinylated MAA was used as control.

Cell Adhesion Assay—Adhesion assay was performed with GM7373 ECs on polystyrene nontissue culture microtiter plates coated with Tat, with the $\alpha_5\beta_1$ -ligand FN, or with the $\alpha_v\beta_3$ ligand fibrinogen as described (30).

Immunocytochemistry—GM7373 ECs (40,000/ cm^2) were allowed to adhere to glass coverslips coated with Tat or FN for 4 h in DMEM containing 1% FCS. Cells were then treated with neuraminidase (125 milliunits/ml), washed, fixed, permeabilized, saturated (27), stained for actin by a 30-min incubation at room temperature with 0.9 $\mu\text{g/ml}$ TRITC-phalloidin in PBS containing 3% BSA (PBS/BSA), co-stained for paxillin by a 1-h incubation at room temperature with anti-paxillin antibody (1:800 in PBS/BSA), further incubated for 45 min at room temperature with FITC-conjugated anti-mouse IgG (1:200 in PBS/BSA), and photographed under an Axioplan 2 microscope equipped for epifluorescence (Carl Zeiss, Gottingen, Germany).

FAK, ERK1/2, and VEGFR2 Phosphorylation Analysis—Confluent GM7373 EC cultures were maintained in serum-free DMEM for 16 h, detached, and resuspended in DMEM 1% FCS. Aliquots of 1,000,000 cells were treated with neuraminidase (125 milliunits/ml), maintained in suspension, or allowed to adhere for 30 min to Tat- or FN-coated 35-mm nontissue culture plates. Alternatively, cells preincubated for 60 min with the lectins were allowed to adhere to Tat- or FN-coated 35-mm nontissue culture plates for a further 30 min. At the end of the incubations, cells were lysed in radioimmunoprecipitation modified lysis buffer and centrifuged (10 min at 13,000 rpm). Twenty μg of protein/sample were analyzed on reducing 8% SDS-PAGE followed by WB with anti-phospho-FAK, anti-phospho-ERK1/2, or anti-phospho-VEGFR2 antibodies. Equal loading of the different lanes was confirmed by WB with anti- α -tubulin antibody. The extent of FAK, ERK1/2, or VEGFR2 phosphorylation was quantified by using the Image Pro-Plus analysis system (Media Cybernetics, Silver Spring, MD). Briefly, the autoradiographies for phosphorylated second messengers or tubulin were digitized on a high resolution monitor and stored within the memory of the Pro-Plus analysis system. The integrated densities of the bands were then calculated, and the values of those corresponding to phosphorylated second messengers were normalized to tubulin levels.

EC Proliferation Assay—GM7373 ECs (25,000/well) were seeded onto Tat- or FN-coated polystyrene nontissue culture microtiter plates and incubated for 4 h in DMEM 1% FCS. Then, cells were treated with neuraminidase (125 milliunits/ml) and further incubated for 24 or 48 h in DMEM, 0.4% FCS containing 5-bromo-2-deoxyuridine in the presence of MAA or UEA (62 nM). Incorporation of 5-bromo-2-deoxyuridine was detected with the colorimetric cell proliferation ELISA kit (Roche Applied Science, Mannheim, Germany).

EC Membrane Ruffling and Wound-healing Assays—Confluent cultures of GM7373 ECs were allowed to adhere to Tat- or FN-coated 35-mm polystyrene nontissue culture plates, treated with neuraminidase (125 milliunits/ml), wounded with a rubber policeman, and incubated with DMEM, 0.4% FCS for 3 or 48 h for membrane ruffling and wound-healing assay, respectively. Alternatively, neuraminidase-untreated ECs were incubated as described above in the presence of MAA or UEA (62 nM). For membrane ruffling assay, at the end of the 3-h incubation, ECs were stained for nuclei (DAPI), immunostained for fascin (by a 1-h incubation with anti-fascin antibody followed by a further 45-min incubation with TRITC-conjugated anti-mouse IgG antibody), and photographed under an Axioplan 2 microscope equipped for epifluorescence (Carl Zeiss). Then, the number of ruffling-positive cells was counted. For the wound-healing assay, at the end of the 48-h incubation, wounded monolayers were photographed under the inverted microscope. The extent of wound repair (due to both EC migration and proliferation (31)) was evaluated by measuring the area of the wound by computerized image analysis using the Image Pro-Plus analysis system (Media Cybernetics).

MTS Assay—Tat-adherent GM7373 ECs were treated with neuraminidase or lectins as described above and then incubated for 24 or 48 h in DMEM, 0.4% FCS. Then, cell viability was assessed by using the CellTiter 96 AQ_{ueous} kit from Promega (Madison, WI).

Human Artery Ring Sprouting Assay—This assay was performed as already described (32). Briefly, 1-mm-thick human umbilical artery rings were embedded in fibrin gel and cultured in human EC serum-free medium (Invitrogen) in the absence or in the presence of Tat (5 nM) and the lectins MAA or UEA (62 nM). After 6 days, rings were photographed at 100 \times magnification using an AxioVert 200M microscope equipped with a 20 \times objective (LD A PLAN 20 \times /0.30PH1, Zeiss), and EC sprouts were counted.

RESULTS

NeuAc Is Associated with Endothelial $\alpha_v\beta_3$ Integrin—To evaluate the presence of NeuAc on glycan(s) linked to $\alpha_v\beta_3$ of the endothelial surface, ECs were lysed, immunoprecipitated with the NeuAc-binding lectin MAA, and blotted with anti- α_v or anti- β_3 antibodies. As shown in Fig. 1A, MAA binds to both α_v and β_3 subunits, similarly to the α_v subunit from human placenta integrin. In a second set of experiments, ECs were treated with neuraminidase (500 milliunits/ml), lysed, and analyzed by WB with anti- α_v or anti- β_3 antibodies. Neuraminidase treatment causes a decrease of the molecular mass of both α_v (from 134 to 126 kDa) and β_3 (from 70 to 67 kDa). Similar results were obtained with $\alpha_v\beta_3$ purified from human placenta (Fig. 1B). The

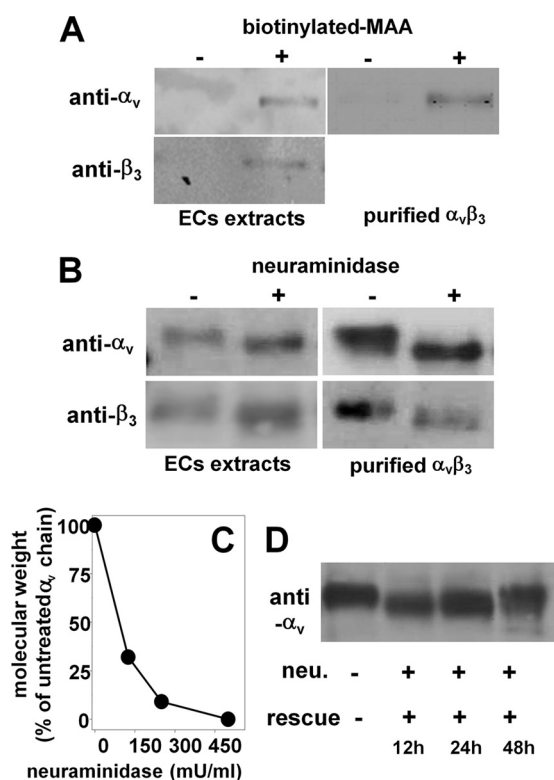


FIGURE 1. Detection of NeuAc on integrin $\alpha_v\beta_3$. *A*, GM7373 ECs or purified human integrin $\alpha_v\beta_3$ were immunoprecipitated with biotinylated MAA and analyzed in WB with anti- α_v or anti- β_3 antibodies. *B* and *C*, alternatively, they were incubated with 500 milliunits/ml (*mU/ml*) (*B*) or with the indicated concentrations (*C*) of neuraminidase, lysed, and analyzed in WB with anti- α_v or anti- β_3 antibodies. *D*, GM7373 ECs were treated with neuraminidase (*neu.*, 500 milliunits/ml), washed, further incubated for the indicated periods of time in the absence of the enzyme (*rescue*), lysed, and analyzed in WB with anti- α_v antibodies. In *panels A, B, and D*, the data shown are representative of 2–3 other experiments that gave similar results. In *C*, data are expressed as the percentage of the molecular mass of the α_v subunit from neuraminidase-treated ECs with respect to the intact protein from untreated ECs.

effect of neuraminidase is dose-dependent, with a partial removal of NeuAc residues obtained for concentrations of the enzyme lower than 500 milliunits/ml (Fig. 1*C*). After its enzymatic removal, NeuAc is fully re-exposed on endothelial $\alpha_v\beta_3$ only after 48 h (Fig. 1*D*).

NeuAc Is Involved in $\alpha_v\beta_3$ /Tat Interaction—Tat binds to $\alpha_v\beta_3$ (25). The contribution of the RGD motif and of the basic domain to $\alpha_v\beta_3$ binding and cell-adhesive capacity was here characterized by two experimental approaches. First, the mutants GST-Tat 1e (in which the RGD sequence has been deleted) and GST-Tat R→A (in which the arginine residues of the basic domain have been substituted with alanine residues) were evaluated for their $\alpha_v\beta_3$ binding capacity in SPR. As shown in Fig. 2*A* and Table 1, the two mutants retain the capacity to bind to $\alpha_v\beta_3$, although decreased in respect to wild type GST-Tat. Second, a cell adhesion assay was performed in the presence of the peptide GRGDSPK (which competes with the RGD motif of Tat for the binding to $\alpha_v\beta_3$) or in the presence of the K5 derivative K5NOSH (which inhibits EC adhesion to Tat (33) by binding to the basic domain of the transactivating factor). As shown in Fig. 2*B*, when assayed at the doses of 12 μM (GRGDSPK) and 75 nM (K5NOSH), the two compounds slightly inhibit EC adhesion to Tat, but when assayed together,

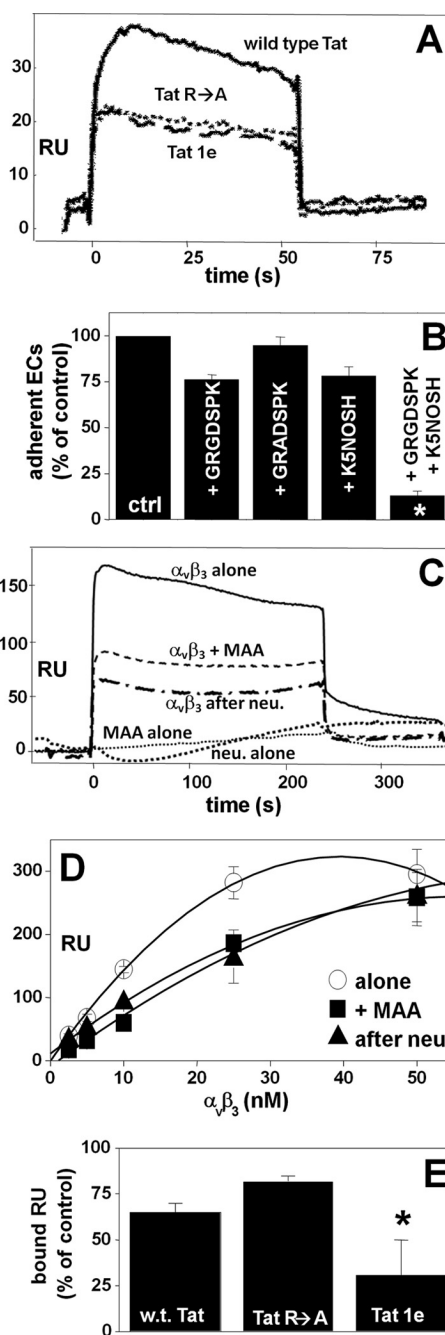


FIGURE 2. Role of $\alpha_v\beta_3$ -associated NeuAc in Tat interaction. *A*, overlay of blank-subtracted sensorgrams showing the binding of $\alpha_v\beta_3$ (10 nM) to sensor chip-immobilized wild type GST-Tat or its mutants GST-Tat 1e or GST-Tat R→A. *B*, GM7373 ECs were allowed to adhere to Tat in the absence (*ctrl*) or in the presence of the indicated inhibitors. *C*, overlay of blank-subtracted sensorgrams showing the binding of native or neuraminidase-treated (*neu.*) $\alpha_v\beta_3$ (12.5 nM) to sensor chip-immobilized synthetic Tat in the absence or in the presence of MAA (250 nM). The sensorgrams generated by injecting MAA (250 nM) or neuraminidase (500 milliunits/ml) on the Tat surface are also shown. *D*, saturation curves obtained using the values of resonance units (RU) bound at equilibrium from injection of increasing concentrations of native or neuraminidase-treated $\alpha_v\beta_3$ onto immobilized synthetic Tat in the absence or in the presence of MAA (250 nM). *E*, $\alpha_v\beta_3$ (50 nM) was injected onto the sensor chip containing the indicated GST-Tat proteins in the absence or in the presence of MAA (250 nM). In *A* and *C*, the sensorgrams shown are representative of three others that gave similar results. In *D*, each point is the mean \pm S.E. of 3 independent injections. In *B* and *E*, each point is the mean \pm S.E. of 3 independent experiments in duplicate and is expressed as the percentage of ECs adherent to Tat in the absence of any inhibitor or of $\alpha_v\beta_3$ bound to the sensor chip at the equilibrium in the absence of MAA, respectively (* = $p < 0.05$, Student's *t* test).

TABLE 1
Affinity of the interaction of $\alpha_v\beta_3$ to the various Tat mutants immobilized to a BIAcore sensor chip

The values of dissociation constant (K_d) reported have been calculated by the Scatchard plot analysis of the steady-state SPR data in the different experimental conditions adopted. For a comparison, the K_d of the Tat/heparin interaction previously calculated by Scatchard plot analysis of the steady-state SPR (33) is also reported.

Ligand	Analyte	K_d
Synthetic Tat	Native $\alpha_v\beta_3$	19.9
	$\alpha_v\beta_3$ after neuraminidase treatment	157.1
	$\alpha_v\beta_3$ + MAA	49.5
GST-Tat wild type	Native $\alpha_v\beta_3$	40.3
	GST-Tat R→A	102.6
	GST-Tat 1e	136.2
	GST-Tat wild type	16.0
	Heparin	16.0

they completely inhibit the same process. Taken together, these data indicate that both the RGD and the basic domain of Tat contribute to its cell-adhesive capacity, the presence of one of the two domains being enough to ensure a partial $\alpha_v\beta_3$ binding and cell-adhesive capacity to Tat.

Besides $\alpha_v\beta_3$, the basic domain also mediates the binding of Tat to the polyanionic heparin/heparan sulfate (28), the two interactions occurring with similar affinities (Table 1). These observations suggest that the basic domain of Tat, besides interacting with the negatively charged sulfated groups of heparin, may as well make contact with the negatively charged NeuAc residues of $\alpha_v\beta_3$. To evaluate this possibility, two different experimental approaches were exploited. (i) We first evaluated the effect of neuraminidase and MAA on $\alpha_v\beta_3$ /Tat interaction. Preliminary SPR analyses demonstrated that neuraminidase treatment of the integrin, as well as the presence of MAA, significantly inhibits its interaction with Tat. It is important to note that MAA and neuraminidase do not bind directly to Tat (Fig. 2C). In a second set of experiments, increasing concentrations of native or neuraminidase-treated $\alpha_v\beta_3$ were injected onto the Tat surface in the absence or in the presence of MAA. Then, the values of steady-state SPR data were used to generate the saturation curves shown in Fig. 2D. Scatchard plot analysis demonstrated that neuraminidase treatment, as well as MAA, decreases the affinity of the $\alpha_v\beta_3$ /Tat interaction (Table 1). Interestingly, the reduction of the affinity observed for $\alpha_v\beta_3$ /Tat interaction in the absence or in the presence of MAA (2.48 times) is in the same order of magnitude as the difference of the affinity measured for the interaction of $\alpha_v\beta_3$ with wild type GST-Tat or with GST-Tat R→A (2.53 times) (Table 1), suggesting that MAA hampers the interaction of NeuAc residues of $\alpha_v\beta_3$ to the basic domain of Tat. (ii) The two Tat mutants GST-Tat 1e and GST-Tat R→A were evaluated for their capacity to induce EC adhesion in the presence of MAA. Similarly to what was observed with synthetic Tat, at 250 nM, MAA partially inhibits the binding of $\alpha_v\beta_3$ to wild type GST-Tat (Fig. 2E). At the same concentration, MAA exerts a weak inhibition on $\alpha_v\beta_3$ /GST-Tat R→A interaction (possibly because this occurs only via the RGD sequence), whereas it efficiently inhibits $\alpha_v\beta_3$ /GST-Tat 1e interaction (possibly because this can occur only via the basic domain) (Fig. 2E). Taken together, these data indicate that at low concentrations, MAA binds to NeuAc residues of $\alpha_v\beta_3$, inhibiting the

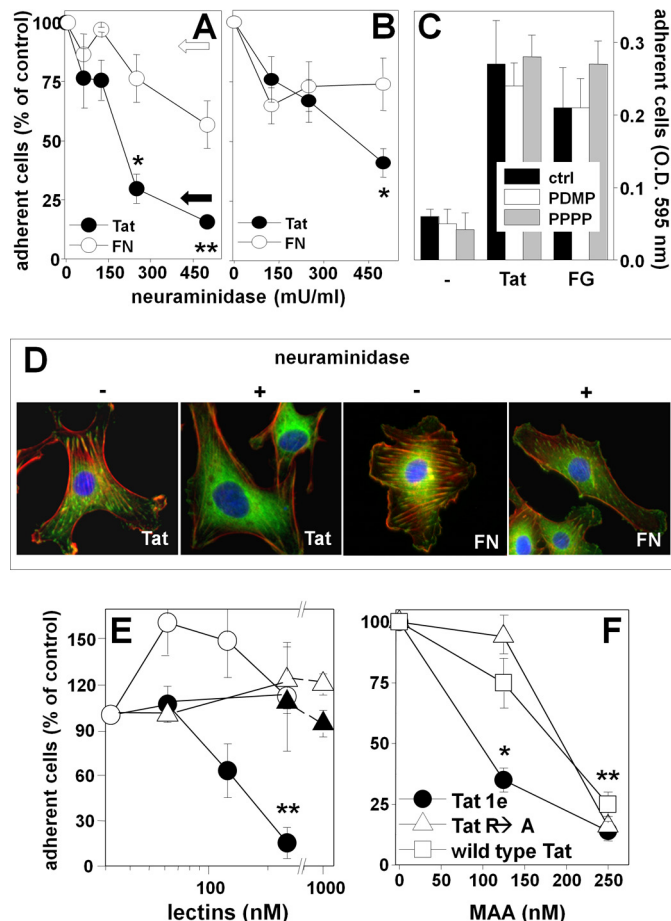


FIGURE 3. Role of NeuAc in $\alpha_v\beta_3$ -dependent EC adhesion to Tat and cytoskeleton organization. A, GM7373 ECs were treated with the indicated concentrations of neuraminidase and subjected to cell adhesion assay on Tat or FN. Alternatively, cells were subjected to adhesion assay in the presence of anti-VEGFR2 antibody (400 $\mu\text{g/ml}$, white arrow) or with anti- $\alpha_v\beta_3$ antibody (100 $\mu\text{g/ml}$, black arrow). B, GM7373 ECs were allowed to adhere to Tat or FN, incubated for 1 h with increasing concentrations of neuraminidase, and then further incubated in the absence of the enzyme for 24 h. C, GM7373 ECs were treated with PDMP (10 μM for 72 h), PPPP (1 μM for 48 h), or vehicle (ctrl) and subjected to cell adhesion assay on wells without coating (–) or coated with Tat or fibrinogen (FG). At the end of the incubations, adherent cells were counted. D, GM7373 ECs were treated with neuraminidase, allowed to adhere to Tat or FN, co-stained for nuclei (blue), paxillin (green), and actin (red), and photographed (630 \times). E, GM7373 ECs were incubated for 2 h at 37 $^{\circ}\text{C}$ with increasing concentrations of MAA (circles) or UEA (triangles) and then allowed to adhere to Tat (black symbols) or FN (white symbols). F, GM7373 ECs were incubated for 2 h at 37 $^{\circ}\text{C}$ with increasing concentrations of MAA and allowed to adhere onto the indicated GST-Tat proteins. In panels A–C, E, and F, each point is the mean \pm S.E. of 3–4 independent experiments in duplicate (* = $p < 0.05$, ** = $p < 0.01$, with respect to untreated controls, Student's *t* test).

interaction of the integrin with the basic domain of Tat but leaving unaffected that with the RGD motif.

NeuAc Mediates $\alpha_v\beta_3$ -dependent EC Adhesion to Substrate-immobilized Tat—Substrate-immobilized Tat induces EC adhesion in an $\alpha_v\beta_3$ -dependent manner (25). Accordingly, by using specific anti- $\alpha_v\beta_3$ or anti-VEGFR2 antibodies, here we confirmed that EC adhesion to Tat specifically depends on the integrin but not on VEGFR2 (Fig. 3A). Also, our unpublished experiments with silencing RNAs directed against enzymes of the biosynthetic pathway of heparan sulfate demonstrated that these receptors are not involved in EC adhesion to Tat (data not shown).

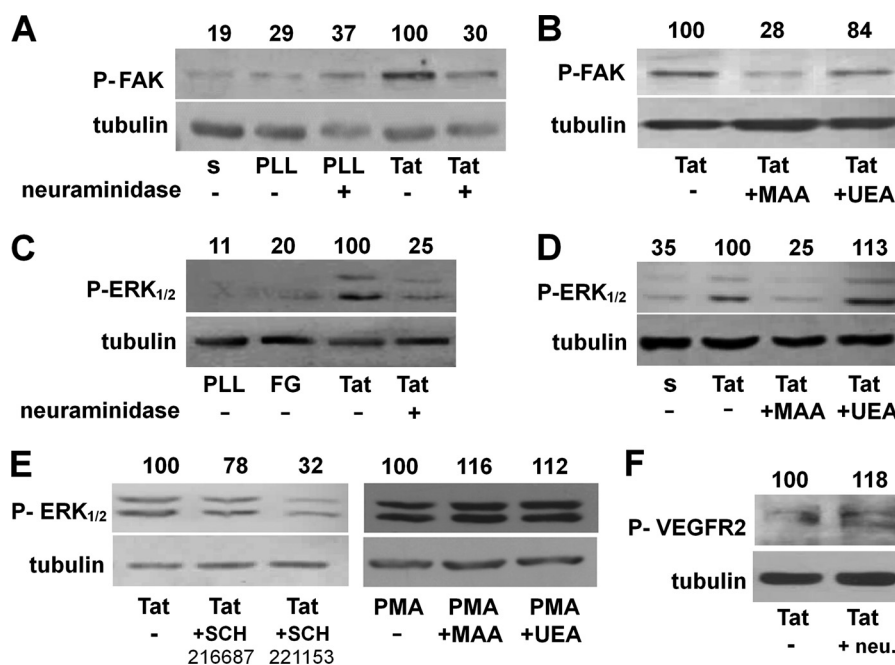


FIGURE 4. Role of NeuAc in Tat/ $\alpha_v\beta_3$ -triggered signal transduction in ECs. A–E, serum-starved GM7373 ECs were allowed to adhere to the indicated proteins in the presence of MAA or UEA (62 nM) or treated with neuraminidase (*neu.*, 125 milliunits/ml) and then maintained in suspension (s) for 30 min or allowed to adhere for 1 h to the indicated proteins. Alternatively, cells were allowed to adhere to Tat and incubated in the presence of the $\alpha_v\beta_3$ antagonist SCH221153 or of its inactive analog SCH216687 (0.3 μ M) or allowed to adhere to tissue culture plates and treated with free phorbol myristate acetate (PMA, 10 ng/ml) in the presence of MAA or UEA (62 nM) (E). At the end of the incubations, cells were lysed and analyzed for FAK (A and B), ERK1/2 (C–E), or VEGFR2 (F) phosphorylation (indicated by P). Tubulin was used as a loading control. The integrated densities of the bands were evaluated, normalized to tubulin, and expressed as the percentage with respect to the bands from EC adherent to Tat in the absence of any inhibitor. The data shown are representative of 2–3 additional experiments that gave similar results. PLL, poly-L-lysine.

The removal of NeuAc from the EC surface by neuraminidase prevents EC adhesion to Tat, only slightly affecting that to the $\alpha_5\beta_1$ -ligand FN, here used as a control (Fig. 3A). Neuraminidase also causes the detachment of ECs already adhered to Tat over a 24-h period of incubation (Fig. 3B), without causing significant cell death (as assessed by MTS assay, data not shown).

Besides integrins, NeuAc is also associated with gangliosides. To evaluate the possible involvement of these structures in EC adhesion to Tat, the cells were treated with PDMP or PPPP (which prevent ganglioside biosynthesis without affecting intracellular levels of ceramide (34)) and then evaluated for their capacity to adhere to Tat. When used at doses that effectively hamper the expression of NeuAc-bearing gangliosides (34), PDMP and PPPP do not affect ECs adhesion to Tat or to fibrinogen (another $\alpha_v\beta_3$ ligand here used as a control, Fig. 3C). These results rule out the possibility that ganglioside-associated NeuAc is responsible for the observed EC adhesion to Tat.

EC adhesion to Tat induces cytoskeleton organization with the assembly of actin stress fibers and focal adhesion plaques containing integrins and paxillin (35). We then evaluated the involvement of NeuAc in cytoskeleton organization of Tat-adherent ECs. When tested at concentrations that do not hamper EC adhesion (125 milliunits/ml), neuraminidase prevents the proper organization of actin stress fibers and paxillin-containing focal adhesion plaques. The effect is specific because neuraminidase did not alter focal adhesion plaque formation in FN-adherent ECs (Fig. 3D).

In a second set of experiments, we also evaluated whether MAA affects $\alpha_v\beta_3$ -dependent EC adhesion to Tat. As shown in Fig. 3E, MAA inhibits EC adhesion to Tat in a dose-dependent

way. The inhibition is specific because MAA does not affect EC adhesion to FN and UEA (a lectin that specifically binds to α -linked fucose) does not affect EC adhesion to Tat. In agreement with the results of the SPR analyses, MAA inhibits EC adhesion to GST-Tat 1e with a potency ($ID_{50} < 125$ nM) that is higher than those with which it inhibits EC adhesion to GST-Tat R \rightarrow A and to wild type GST-Tat ($ID_{50} = 198$ and 182, respectively) (Fig. 3F).

NeuAc Is Required for Signal Transduction Triggered by Tat/ $\alpha_v\beta_3$ Interaction in ECs—We evaluated the role of NeuAc in Tat/ $\alpha_v\beta_3$ -dependent phosphorylation of FAK, a second messenger involved in Tat/ $\alpha_v\beta_3$ -dependent EC cytoskeleton organization and proangiogenic activation (25, 36). Preliminary experiment confirmed that FAK undergoes phosphorylation in ECs adherent to Tat but not in ECs maintained in suspension or adherent to poly-L-lysine (an integrin-independent adhesive molecule). When used at 125 milliunits/ml (a dose that prevents cytoskeleton organization without hampering EC adhesion to Tat; Fig. 3), neuraminidase inhibits FAK phosphorylation in Tat-adherent ECs (Fig. 4A). Accordingly, at 62 nM, MAA, but not UEA, prevents FAK phosphorylation (Fig. 4B). It is relevant to note that at 62 nM, MAA does not hamper EC adhesion to Tat (Fig. 3E). Besides FAK, EC adhesion to Tat induces the activation of ERK1/2, another second messenger involved in Tat-dependent EC proangiogenic activation (37). Neuraminidase pretreatment (Fig. 4C) and MAA, but not UEA (Fig. 4D), prevent ERK1/2 phosphorylation in Tat-adherent EC.

In the same experimental conditions, ERK1/2 phosphorylation is inhibited by the specific $\alpha_v\beta_3$ antagonist SCH221153 but not by its inactive analog SCH21668, indicating that ERK1/2

Endothelial $\alpha_v\beta_3$ Sialic Acid and HIV-1 Tat

activation is dependent, at least in part, on $\alpha_v\beta_3$. Also, MAA does not affect ERK1/2 phosphorylation induced by phorbol myristate acetate in EC adherent to tissue culture plastic (Fig. 4E), suggesting that the specificity of the inhibitory effect exerted by MAA is likely due to its interaction with $\alpha_v\beta_3$, which prevents the binding of the integrin to Tat (Fig. 2).

Besides integrin $\alpha_v\beta_3$, the proangiogenic activity of Tat is also mediated by VEGFR2 activation (38). Interestingly, this receptor bears NeuAc residues (39). On these bases, we evaluated the effect of the removal of NeuAc residues of VEGFR2 on its phosphorylation driven by Tat. As shown in Fig. 4F, neuraminidase effectively removes NeuAc from VEGFR2, as assessed by the decrease of the molecular mass of the band corresponding to the receptor. However, neuraminidase treatment does not hamper VEGFR2 phosphorylation in response to Tat, which is instead even increased (Fig. 4F).

EC NeuAc Is Involved in $\alpha_v\beta_3$ -dependent Proangiogenic Activity of Tat—EC adhesion, proliferation, and migration represent essential steps of neovascularization (40). Accordingly, ECs adherent to substrate-immobilized Tat are induced to migrate and proliferate in an $\alpha_v\beta_3$ -dependent way (27). Preliminary experiments confirmed that Tat-adherent ECs proliferate more efficiently than those adherent to FN both at 24 h and at 48 h (Fig. 5A). Pretreatment with neuraminidase (125 milliunits/ml) inhibits the proliferation of Tat-adherent cells in a specific way because it leaves unaffected the basal proliferation of FN-adherent ECs or of ECs adherent on tissue culture plates (data not shown). Besides neuraminidase, MAA (62 nM), but not UEA (assayed at both 62 nM and 100 nM), inhibits the proliferation of Tat-adherent ECs (Fig. 5A).

Cell membrane ruffling, which precedes the migration of EC body, is considered a morphological phenotype of motile cells (41) and has been already exploited to characterize the migration of Tat-adherent ECs (27). As shown in Fig. 5D, when adherent to Tat or to FN, ECs rapidly form membrane ruffles at the edge of a wounded monolayer. Pretreatment with neuraminidase (125 milliunits/ml) inhibits membrane ruffling in Tat-adherent ECs and, to a lesser extent, in FN-adherent ECs (Fig. 5B). Accordingly, MAA (62 nM), but not UEA, inhibits membrane ruffling only in Tat- but not FN-adherent ECs (Fig. 5C).

The ability of a substrate-immobilized protein to stimulate proliferation and motility in adherent ECs leads to an increased capacity of a mechanically wounded EC monolayer to cover the denuded area, a biological activity referred to as “motogenic activity” that has been used as a surrogate marker of angiogenesis (31). Substrate-immobilized Tat induces motogenesis of adherent ECs in an $\alpha_v\beta_3$ -dependent way (25).

When allowed to adhere to Tat, treated with neuraminidase (250 milliunits/ml), and wounded, ECs show a decreased motogenic activity. The effect is specific because, in the same experimental conditions, neuraminidase does not affect the motogenic activity of FN-adherent ECs (Fig. 6A). In this regard, it is important to recall that when added after EC adhesion has occurred, neuraminidase at 250 nM does not cause the detachment of Tat-adherent ECs (Fig. 3B) nor affect their viability over a 48-h period of time (data not shown). MAA (62 nM)

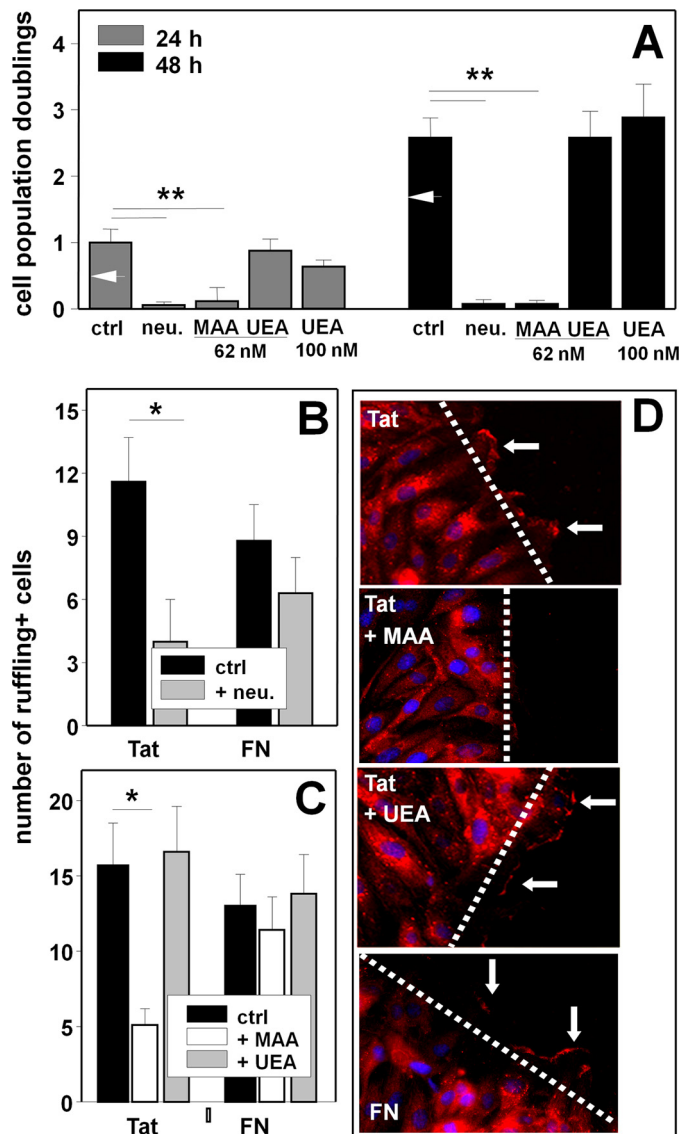


FIGURE 5. Role of NeuAc in Tat/ $\alpha_v\beta_3$ -dependent proliferation and migration of ECs. GM7373 ECs adherent to the indicated proteins were treated as follows. *A*, cells were treated with neuraminidase (*neu.*, 125 milliunits/ml) and incubated in the absence of the enzyme for an additional 24 or 48 h. Alternatively, cells were directly incubated for 24 or 48 h with the indicated concentrations of MAA or UEA. *B*, cells were incubated in the absence (*ctrl*) or in the presence of neuraminidase (125 milliunits/ml), wounded, and further incubated for 30 min. *C*, cells were wounded and incubated for 30 min in the absence (*ctrl*) or in the presence of MAA or UEA (62 nM). At the end of the incubations, EC proliferation (*A*) or the number of ruffling-positive ECs (*B* and *C*) were evaluated. Each point is the mean \pm S.E. of 3–4 independent experiments in duplicate ($* = p < 0.01$, $** = p < 0.001$, Student's *t* test). In *panel A*, white arrowheads point to the proliferation measured in cells adherent to FN. *D*, representative epifluorescence microphotographs (630 \times) of ECs at the edge of a wounded monolayer incubated in the absence (*ctrl*) or in the presence of MAA or UEA (62 nM) and stained with DAPI (blue) and with anti-fascin antibody (red). Arrows point to the most prominent ruffles.

inhibits the motogenic activity of Tat. The effect is specific because MAA does not affect viability of Tat-adherent ECs (data not shown) nor the motogenic activity of FN-adherent ECs (Fig. 6, *B* and *C*). Also, UEA is ineffective on Tat-adherent ECs (Fig. 6, *B* and *C*).

Tat induces neovascularization via $\alpha_v\beta_3$ (27). We then decided to evaluate the involvement of NeuAc in Tat proangiogenic

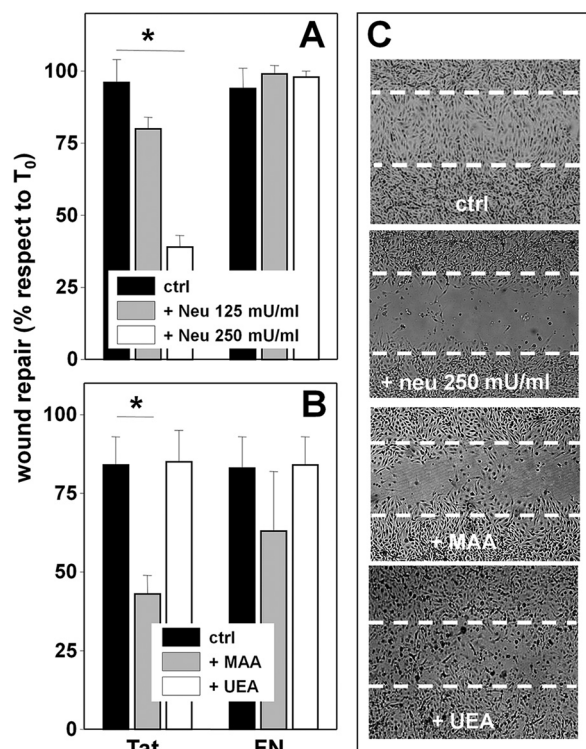


FIGURE 6. Role of NeuAc in Tat/ $\alpha_v\beta_3$ -dependent motogenesis of ECs. A, GM7373 ECs adherent to the indicated proteins were left untreated (*ctrl*) or treated with neuraminidase (*neu*) at 125 or 250 milliunits/ml, wounded, and further incubated for 48 h. B, GM7373 ECs adherent to the indicated proteins were wounded and incubated for 48 h in the absence (*ctrl*) or in the presence of MAA or UEA (62 nM). At the end of the incubations, the extension of the area of the wound repaired was evaluated. Each point is the mean \pm S.E. of 3–5 fields measured in one experiment out of 2–3 that gave similar results (* = $p < 0.05$, Student's *t* test). C, microphotographs (50 \times) of wounded monolayers of Tat-adherent GM7373 ECs taken at the end of the 48-h period of incubation with the indicated treatments. Dashed lines mark the edge of the wound at the beginning of the experiment.

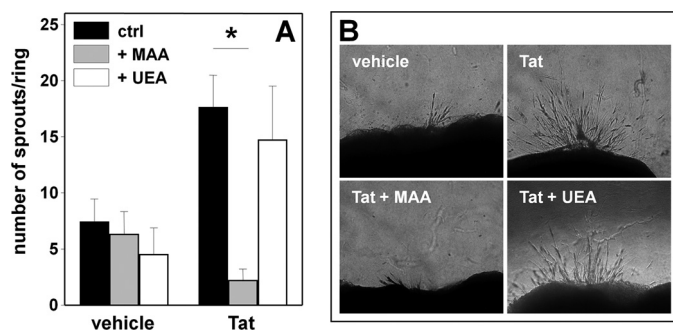


FIGURE 7. Effect of MAA on Tat-induced neovascularization. A, human umbilical artery rings were embedded in fibrin gel and incubated without (*vehicle*) or with Tat in the absence (*ctrl*) or in the presence of MAA or UEA. After 6 days, EC sprouts were counted. Each point is the mean \pm S.E. of 10–29 artery rings randomly chosen (* = $p < 0.001$, Student's *t* test). B, representative aorta rings in the different experimental conditions photographed under an inverted microscope at 200 \times magnification.

genic activity by exploiting the human artery ring sprouting assay, an *ex vivo* model used to characterize the pro- or antiangiogenic properties of various angiogenic growth factors and inhibitors (32). As shown in Fig. 7, Tat effectively induces an increase of the number of EC sprouts that generate from a human artery ring, and this activity can be inhibited by MAA, but not by UEA.

DISCUSSION

$\alpha_v\beta_3$ mediates EC adhesion and proangiogenic activation by binding cationic angiogenic growth factors including FGF2 and HIV-1 Tat (27, 30). On the other hand, NeuAc has so far been identified on $\alpha_v\beta_3$ of melanoma metastatic cell surface, where it regulates cell adhesion (18, 20). We thus decided to investigate whether NeuAc is also associated with $\alpha_v\beta_3$ integrin of ECs and whether it is involved in $\alpha_v\beta_3$ /Tat interaction and in the consequent proangiogenic activation of ECs. These possibilities were investigated by two complementary approaches, consisting of the use of neuraminidase from *C. perfringens* (an enzyme that removes NeuAc from the cell surface) and of MAA (a lectin that specifically binds NeuAc residues).

In ECs, the removal of $\alpha_v\beta_3$ -associated NeuAc by neuraminidase is rapid and efficient, occurring in less than 2 h. The biochemical features of bacterial neuraminidase surely contribute to this (43), but so does the rapid recycling of integrins that are classically internalized to the early endosomes to be immediately returned to the plasma membrane (44, 45). In this way, endogenous $\alpha_v\beta_3$ remains continuously exposed at the cell surface, accessible to exogenous neuraminidase.

Fully sialylated $\alpha_v\beta_3$ integrins are re-exposed on ECs only 48 h after neuraminidase treatment. The long-lasting nature of $\alpha_v\beta_3$ desialylation is likely due to the fact that after *de novo* synthesis, integrins must undergo sialylation by sialyltransferases in the Golgi apparatus (46). Whatever its cause, the long-lasting desialylation of $\alpha_v\beta_3$ by neuraminidase is in agreement with the capacity of the enzyme to inhibit processes, such as EC proliferation and motogenesis, that occur over 24–48-h periods.

MAA immunoprecipitates both α_v and β_3 subunits from ECs, indicating the presence of $\alpha(2\rightarrow3)$ -linked NeuAc on the two chains. We also obtained similar results with the lectin from *Sambucus nigra* that binds instead to $\alpha(2\rightarrow6)$ -linked NeuAc (data not shown). Accordingly, neuraminidase from *C. perfringens*, which hydrolyzes both $\alpha(2\rightarrow3)$ -linked and $\alpha(2\rightarrow6)$ -linked NeuAc, causes a decrease of the molecular mass of both α_v subunit (–7 kDa, corresponding to about 25 NeuAc residues) and β_3 subunit (–3 kDa, corresponding to about 10 NeuAc residues). These data are in agreement with the observations that α_v and β_3 subunits possess 13 and 6 putative *N*-glycosylation sites, respectively, with biantennary structures (UniProt accession number P05106) (18).

Neuraminidase cleaves $\alpha(2\rightarrow3)$ -linked NeuAc residues faster than $\alpha(2\rightarrow6)$ linkages (47). Accordingly, neuraminidase operates a complete removal of $\alpha_v\beta_3$ -associated NeuAc only when used at high concentration (>250 milliunits/ml), whereas when used at suboptimal concentrations (125 milliunits/ml), desialylation of $\alpha_v\beta_3$ remains incomplete. Interestingly, when used at higher concentrations, neuraminidase directly prevents (and even disrupts) $\alpha_v\beta_3$ -dependent EC adhesion to Tat (Fig. 3, A and B), whereas at lower concentrations, it leaves EC adhesion unaffected, although inhibiting Tat/ $\alpha_v\beta_3$ -dependent proangiogenic activation of Tat-adherent ECs. Taken together, these data suggest that the complete removal of $\alpha_v\beta_3$ -associated NeuAc prevents the binding of the integrin to Tat and the consequent EC adhesion. Instead, a partial removal of NeuAc

residues allows an “unproductive” Tat/ $\alpha_v\beta_3$ interaction enough for cell adhesion, but that does not mediate the signal transduction cascade required for EC proangiogenic activation.

By removing NeuAc residues from $\alpha_v\beta_3$, neuraminidase causes a decrease of the affinity of $\alpha_v\beta_3$ /Tat interaction and its inhibition (Fig. 2). Accordingly, by binding NeuAc residues of $\alpha_v\beta_3$, MAA causes the same effects, also if at a lesser extent. The inhibition exerted by neuraminidase and MAA on $\alpha_v\beta_3$ /Tat interaction is mirrored by their capacity to inhibit several $\alpha_v\beta_3$ -dependent biological activities of Tat, connecting the two processes. In effect, MAA shares with neuraminidase the capacity to differently affect Tat/ $\alpha_v\beta_3$ interaction and consequent biological activities in a concentration-dependent way. At higher concentrations (125–250 nM), it directly inhibits $\alpha_v\beta_3$ -mediated EC adhesion to Tat, whereas at a lower concentration (less than 62 nM), it leaves cell adhesion unaffected, although inhibiting the $\alpha_v\beta_3$ -dependent signal transduction and proangiogenic activation of Tat-adherent ECs. Interestingly, MAA scarcely affects $\alpha_v\beta_3$ interaction with (and EC adhesion to) GST-Tat R→A (in which the intact RGD motif mediates integrin interaction and EC adhesion), whereas it exerts a stronger inhibition on GST-Tat 1e that, lacking the RGD motif, binds to integrin and mediates EC adhesion via its basic domain. These findings, together with the notion that $\alpha_v\beta_3$ interaction occurs via both the RGD motif and the basic domain of Tat (25) (Fig. 2B), suggest that at appropriate concentrations, MAA succeeds in inhibiting the interaction of the basic domain of Tat with NeuAc residues of $\alpha_v\beta_3$, leaving unaffected the RGD-dependent $\alpha_v\beta_3$ /Tat interaction. This generates an unproductive Tat/ $\alpha_v\beta_3$ interaction enough to promote cell adhesion but not adequate to trigger signal transduction and hence EC proangiogenic activation.

Besides integrin $\alpha_v\beta_3$, a wide variety of cell surface sialoglycoproteins can be affected by neuraminidase and MAA, possibly impacting the phenomena reported here. Although we cannot completely rule out this possibility, it is, however, relevant to point out that neuraminidase does not significantly affect (or even increase) Tat-driven phosphorylation of VEGFR2, a tyrosine kinase receptor that bears NeuAc residues (39) and whose activation is required for Tat proangiogenic activity (38). Curiously, the proangiogenic fibroblast growth factor (FGF) receptor-1 also possesses NeuAc-bearing glycans whose removal leads to an increased capacity to bind its ligand FGF2 (48). Also, hypo- or desialylation of $\alpha_5\beta_1$ integrin increases its affinity for the natural ligand FN (49). Relevant to this point, FN acts as a proangiogenic factor (50), and $\alpha_5\beta_1$ can act as a Tat receptor on ECs (26). Taken together, these results indicate that although neuraminidase and MAA can act on sialoglycoproteins different from $\alpha_v\beta_3$, the overall inhibitory effect exerted on the Tat proangiogenic potential depends on their action on NeuAc linked to $\alpha_v\beta_3$.

The results presented in this work open new fields of research, some of which have been totally unexplored so far. First, mammals have four types of endogenous sialidases, among which the plasma membrane-associated sialidase NEU3 and the secreted form of NEU2 (51) could be involved in the fine-tuning of the acidic sugar on the cell surface. On the other hand, sialidases are expressed by ECs (52), and neutrophil-de-

rived endogenous sialidase(s) have already been demonstrated to induce desialylation of the EC surface with implications in the process of inflammation (53). Taken together, these observations point to a possible role of neuraminidases as regulators of the process of neovascularization. Second, besides Tat, VEGF (54) and FGF2 (14, 15) also bind to integrins and to NeuAc-bearing gangliosides (55), suggesting a broader involvement of integrin-associated NeuAc in angiogenesis. Third, sialyltransferases are a family of enzymes located in the Golgi apparatus that mediate sialylation of various glycoconjugates (56) including integrins (46). Also, they are expressed by ECs (57). Thus, besides neuraminidases, sialyltransferases may also be involved in the regulation of angiogenesis. Fourth, integrin $\alpha_v\beta_3$ is a target for antitumor therapies based on antibodies or RGD-based compounds that prevent the interaction of the integrin with its proangiogenic ligands (58). The $\alpha_v\beta_3$ antagonist activity of MAA points to NeuAc-binding lectins as templates for the design of novel integrin antagonists endowed with antiangiogenic potential. Fifth, in the field of virology, integrins act as entry receptors for several viruses (59), suggesting the possibility of blocking viral infection by means of NeuAc-binding lectin-like compounds or by NeuAc analogues. Relevant to this point, the NeuAc derivative NMSO3 has been demonstrated to exert a potent inhibition against HIV-1 (42).

In conclusion, the results presented in this study open up the possibility that modulation of integrin glycosylation could be a promising strategy for regulating angiogenesis and viral infection.

Acknowledgments—We thank Mauro Giacca (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) for the *E. coli* strain expressing the various GST-Tat mutants, Pasqua Oreste for the K5NOSH, Alessandra Armato for technical assistance, and Eugenio Monti and Marco Presta (University of Brescia) for helpful discussion.

REFERENCES

1. Urbinati, C., Chiodelli, P., and Rusnati, M. (2008) Polyanionic drugs and viral oncogenesis: a novel approach to control infection, tumor-associated inflammation, and angiogenesis. *Molecules* **13**, 2758–2785
2. dos Santos, W. L., Rahman, J., Klein, N., and Male, D. K. (1995) Distribution and analysis of surface charge on brain endothelium *in vitro* and *in situ*. *Acta Neuropathol.* **90**, 305–311
3. Vorbodt, A. W. (1989) Ultracytochemical characterization of anionic sites in the wall of brain capillaries. *J. Neurocytol.* **18**, 359–368
4. Traving, C., and Schauer, R. (1998) Structure, function, and metabolism of sialic acids. *Cell. Mol. Life Sci.* **54**, 1330–1349
5. Schauer, R. (2009) Sialic acids as regulators of molecular and cellular interactions. *Curr. Opin. Struct. Biol.* **19**, 507–514
6. Mureşan, V., and Simionescu, N. (1987) High and low molecular weight tracers for the electron microscopical detection of sialoglycoconjugates. *Histochem. J.* **19**, 170–178
7. Welim, H. B., Thies, M., and Herken, R. (1989) Appearance of lectin-binding sites during vascularization of the primordium of the central nervous system in 10–12-day-old mouse embryos. *Cell Tissue Res.* **255**, 627–630
8. Henry, C. B., and DeFouw, D. O. (1996) Distribution of anionic sites on microvascular endothelium of the chick chorioallantoic membrane. *Tissue Cell* **28**, 449–454
9. Doiron, A. L., Kirkpatrick, A. P., and Rinker, K. D. (2004) TGF- β and TNF- α affect cell surface proteoglycan and sialic acid expression on vas-

- cular endothelial cells. *Biomed. Sci. Instrum.* **40**, 331–336
10. Karlsson, K. A. (1991) Glycobiology: a growing field for drug design. *Trends Pharmacol. Sci.* **12**, 265–272
 11. Banks, W. A., Robinson, S. M., Wolf, K. M., Bess, J. W., Jr., and Arthur, L. O. (2004) Binding, internalization, and membrane incorporation of human immunodeficiency virus-1 at the blood-brain barrier is differentially regulated. *Neuroscience* **128**, 143–153
 12. Banks, W. A., and Kastin, A. J. (1998) Characterization of lectin-mediated brain uptake of HIV-1 GP120. *J. Neurosci. Res.* **54**, 522–529
 13. Chung, T. W., Kim, S. J., Choi, H. J., Kim, K. J., Kim, M. J., Kim, S. H., Lee, H. J., Ko, J. H., Lee, Y. C., Suzuki, A., and Kim, C. H. (2009) Ganglioside GM₃ inhibits VEGF/VEGFR2-mediated angiogenesis: direct interaction of GM₃ with VEGFR2. *Glycobiology* **19**, 229–239
 14. Rusnati, M., Tanghetti, E., Urbinati, C., Tulipano, G., Marchesini, S., Ziche, M., and Presta, M. (1999) Interaction of fibroblast growth factor-2 (FGF-2) with free gangliosides: biochemical characterization and biological consequences in endothelial cell cultures. *Mol. Biol. Cell* **10**, 313–327
 15. Rusnati, M., Urbinati, C., Tanghetti, E., Dell'Era, P., Lortat-Jacob, H., and Presta, M. (2002) Cell membrane GM₁ ganglioside is a functional coreceptor for fibroblast growth factor 2. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 4367–4372
 16. Kajiji, S., Tamura, R. N., and Quaranta, V. (1989) A novel integrin ($\alpha_5\beta_4$) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO J.* **8**, 673–680
 17. Graham, K. L., Halasz, P., Tan, Y., Hewish, M. J., Takada, Y., Mackow, E. R., Robinson, M. K., and Coulson, B. S. (2003) Integrin-using rotaviruses bind $\alpha_2\beta_1$ integrin α_2 I domain via VP4 DGE sequence and recognize $\alpha_x\beta_2$ and $\alpha_v\beta_3$ by using VP7 during cell entry. *J. Virol.* **77**, 9969–9978
 18. Kremser, M. E., Przybylo, M., Hoja-Lukowicz, D., Pocheč, E., Amoresano, A., Carpentieri, A., Bubka, M., and Lityńska, A. (2008) Characterization of $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrin N-oligosaccharides in metastatic melanoma WM9 and WM239 cell lines. *Biochim. Biophys. Acta* **1780**, 1421–1431
 19. Woodard-Grice, A. V., McBrayer, A. C., Wakefield, J. K., Zhuo, Y., and Bellis, S. L. (2008) Proteolytic shedding of ST6Gal-I by BACE1 regulates the glycosylation and function of $\alpha_4\beta_1$ integrins. *J. Biol. Chem.* **283**, 26364–26373
 20. Chiang, C. H., Wang, C. H., Chang, H. C., More, S. V., Li, W. S., and Hung, W. C. (2010) A novel sialyltransferase inhibitor AL10 suppresses invasion and metastasis of lung cancer cells by inhibiting integrin-mediated signaling. *J. Cell Physiol.* **223**, 492–499
 21. Morova, J., Osicka, R., Masin, J., and Sebo, P. (2008) RTX cytotoxins recognize β_2 integrin receptors through N-linked oligosaccharides. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5355–5360
 22. Mousa, S. A. (2008) Cell adhesion molecules: potential therapeutic and diagnostic implications. *Mol. Biotechnol.* **38**, 33–40
 23. Eliceiri, B. P., and Cheresh, D. A. (1998) The role of α_v integrins during angiogenesis. *Mol. Med.* **4**, 741–750
 24. Noonan, D., and Albini, A. (2000) From the outside in: extracellular activities of HIV Tat. *Adv. Pharmacol.* **48**, 229–250
 25. Urbinati, C., Bugatti, A., Giacca, M., Schlaepfer, D., Presta, M., and Rusnati, M. (2005) $\alpha_v\beta_3$ integrin-dependent activation of focal adhesion kinase mediates NF- κ B activation and motogenic activity by HIV-1 Tat in endothelial cells. *J. Cell Sci.* **118**, 3949–3958
 26. Barillari, G., Sgadari, C., Fiorelli, V., Samaniego, F., Colombini, S., Manzari, V., Modesti, A., Nair, B. C., Cafaro, A., Stürzl, M., and Ensolì, B. (1999) The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins and by mobilizing sequestered basic fibroblast growth factor. *Blood* **94**, 663–672
 27. Urbinati, C., Mitola, S., Tanghetti, E., Kumar, C., Waltenberger, J., Ribatti, D., Presta, M., and Rusnati, M. (2005) Integrin $\alpha_v\beta_3$ as a target for blocking HIV-1 Tat-induced endothelial cell activation *in vitro* and angiogenesis *in vivo*. *Arterioscler. Thromb. Vasc. Biol.* **25**, 2315–2320
 28. Rusnati, M., Tulipano, G., Urbinati, C., Tanghetti, E., Giuliani, R., Giacca, M., Ciomei, M., Corallini, A., and Presta, M. (1998) The basic domain in HIV-1 Tat protein as a target for polysulfonated heparin-mimicking extracellular Tat antagonists. *J. Biol. Chem.* **273**, 16027–16037
 29. Grinspan, J. B., Mueller, S. N., and Levine, E. M. (1983) Bovine endothelial cells transformed *in vitro* by benzo(a)pyrene. *J. Cell Physiol.* **114**, 328–338
 30. Rusnati, M., Tanghetti, E., Dell'Era, P., Gualandris, A., and Presta, M. (1997) $\alpha_v\beta_3$ integrin mediates the cell-adhesive capacity and biological activity of basic fibroblast growth factor (FGF-2) in cultured endothelial cells. *Mol. Biol. Cell* **8**, 2449–2461
 31. Lauder, H., Frost, E. E., Hiley, C. R., and Fan, T. P. (1998) Quantification of the repair process involved in the repair of a cell monolayer using an *in vitro* model of mechanical injury. *Angiogenesis* **2**, 67–80
 32. Mitola, S., Moroni, E., Ravelli, C., Andres, G., Belleri, M., and Presta, M. (2008) Angiopoietin-1 mediates the proangiogenic activity of the bone morphogenic protein antagonist Drm. *Blood* **112**, 1154–1157
 33. Urbinati, C., Bugatti, A., Oreste, P., Zoppetti, G., Waltenberger, J., Mitola, S., Ribatti, D., Presta, M., and Rusnati, M. (2004) Chemically sulfated *Escherichia coli* K5 polysaccharide derivatives as extracellular HIV-1 Tat protein antagonists. *FEBS Lett.* **568**, 171–177
 34. Kopitz, J., Bergmann, M., and Gabius, H. J. (2010) How adhesion/growth-regulatory galectins-1 and -3 attain cell specificity: case study defining their target on neuroblastoma cells (SK-N-MC) and marked affinity regulation by affecting microdomain organization of the membrane. *IUBMB Life* **62**, 624–628
 35. Urbinati, C., Ravelli, C., Tanghetti, E., Belleri, M., Giacomuzzi, E., Monti, E., Presta, M., and Rusnati, M. (2012) Substrate-immobilized HIV-1 Tat drives VEGFR2/ $\alpha_v\beta_3$ integrin complex formation and polarization in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **32**, e25–e34
 36. Angelucci, A., and Bologna, M. (2007) Targeting vascular cell migration as a strategy for blocking angiogenesis: the central role of focal adhesion protein tyrosine kinase family. *Curr. Pharm. Des.* **13**, 2129–2145
 37. Rusnati, M., Urbinati, C., Musulin, B., Ribatti, D., Albini, A., Noonan, D., Marchisone, C., Waltenberger, J., and Presta, M. (2001) Activation of endothelial cell mitogen-activated protein kinase ERK1/2 by extracellular HIV-1 Tat protein. *Endothelium* **8**, 65–74
 38. Albini, A., Soldi, R., Giunciuglio, D., Giraudo, E., Benelli, R., Primo, L., Noonan, D., Salio, M., Camussi, G., Rockl, W., and Bussolino, F. (1996) The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nat. Med.* **2**, 1371–1375
 39. Nacev, B. A., Grassi, P., Dell, A., Haslam, S. M., and Liu, J. O. (2011) The antifungal drug itraconazole inhibits vascular endothelial growth factor receptor 2 (VEGFR2) glycosylation, trafficking, and signaling in endothelial cells. *J. Biol. Chem.* **286**, 44045–44056
 40. Folkman, J., and Klagsbrun, M. (1987) Angiogenic factors. *Science* **235**, 442–447
 41. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401–410
 42. Terada, M., Fujita, S., Suda, I., and Mastico, R. (2005) Polysulfated sialic acid derivatives as anti-human immunodeficiency virus. *Biomed. Pharmacother.* **59**, 423–429
 43. Bouwstra, J. B., Deyl, C. M., and Vliegthart, J. F. (1987) Purification and kinetic properties of sialidase from *Clostridium perfringens*. *Biol. Chem. Hoppe Seyler* **368**, 269–275
 44. Caswell, P. T., Vadrevu, S., and Norman, J. C. (2009) Integrins: masters and slaves of endocytic transport. *Nat. Rev. Mol. Cell Biol.* **10**, 843–853
 45. Roberts, M., Barry, S., Woods, A., van der Sluijs, P., and Norman, J. (2001) PDGF-regulated rab4-dependent recycling of $\alpha_v\beta_3$ integrin from early endosomes is necessary for cell adhesion and spreading. *Curr. Biol.* **11**, 1392–1402
 46. Christie, D. R., Shaikh, F. M., Lucas, J. A., 4th, Lucas, J. A., 3rd, and Bellis, S. L. (2008) ST6Gal-I expression in ovarian cancer cells promotes an invasive phenotype by altering integrin glycosylation and function. *J. Ovarian Res.* **1**, 3
 47. Corfield, A. P., Higa, H., Paulson, J. C., and Schauer, R. (1983) The specificity of viral and bacterial sialidases for $\alpha(2\rightarrow3)$ - and $\alpha(2\rightarrow6)$ -linked sialic acids in glycoproteins. *Biochim. Biophys. Acta* **744**, 121–126
 48. Duchesne, L., Tissot, B., Rudd, T. R., Dell, A., and Fernig, D. G. (2006) N-Glycosylation of fibroblast growth factor receptor 1 regulates ligand and heparan sulfate co-receptor binding. *J. Biol. Chem.* **281**, 27178–27189
 49. Semel, A. C., Seales, E. C., Singhal, A., Eklund, E. A., Colley, K. J., and Bellis,

Endothelial $\alpha_v\beta_3$ Sialic Acid and HIV-1 Tat

- S. L. (2002) Hyposialylation of integrins stimulates the activity of myeloid fibronectin receptors. *J. Biol. Chem.* **277**, 32830–32836
50. Scatena, M., Almeida, M., Chaisson, M. L., Fausto, N., Nicosia, R. F., and Giachelli, C. M. (1998) NF- κ B mediates $\alpha_v\beta_3$ integrin-induced endothelial cell survival. *J. Cell Biol.* **141**, 1083–1093
51. Monti, E., Bonten, E., D'Azzo, A., Bresciani, R., Venerando, B., Borsani, G., Schauer, R., and Tettamanti, G. (2010) Sialidases in vertebrates: a family of enzymes tailored for several cell functions. *Adv. Carbohydr. Chem. Biochem.* **64**, 403–479
52. Renkonen, R., Mattila, P., Majuri, M. L., Rabinä, J., Toppila, S., Renkonen, J., Hirvas, L., Niittymäki, J., Turunen, J. P., Renkonen, O., and Paavonen, T. (1997) *In vitro* experimental studies of sialyl Lewis x and sialyl Lewis a on endothelial and carcinoma cells: crucial glycans on selectin ligands. *Glycoconj. J.* **14**, 593–600
53. Sakarya, S., Rifat, S., Zhou, J., Bannerman, D. D., Stamatou, N. M., Cross, A. S., and Goldblum, S. E. (2004) Mobilization of neutrophil sialidase activity desialylates the pulmonary vascular endothelial surface and increases resting neutrophil adhesion to and migration across the endothelium. *Glycobiology* **14**, 481–494
54. Lang, Z., Guerrero, M., Li, R., and Ladisch, S. (2001) Ganglioside GD1a enhances VEGF-induced endothelial cell proliferation and migration. *Biochem. Biophys. Res. Commun.* **282**, 1031–1037
55. Rusnati, M., and Presta, M. (2006) Extracellular angiogenic growth factor interactions: an angiogenesis interactome survey. *Endothelium* **13**, 93–111
56. Harduin-Lepers, A., Mollicone, R., Delannoy, P., and Oriol, R. (2005) The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. *Glycobiology* **15**, 805–817
57. Brockhausen, I., Lehotay, M., Yang, J. M., Qin, W., Young, D., Lucien, J., Coles, J., and Paulsen, H. (2002) Glycoprotein biosynthesis in porcine aortic endothelial cells and changes in the apoptotic cell population. *Glycobiology* **12**, 33–45
58. Auzzas, L., Zanardi, F., Battistini, L., Burreddu, P., Carta, P., Rassa, G., Curti, C., and Casiraghi, G. (2010) Targeting $\alpha_v\beta_3$ integrin: design and applications of mono- and multifunctional RGD-based peptides and semi-peptides. *Curr. Med. Chem.* **17**, 1255–1299
59. Stewart, P. L., and Nemerow, G. R. (2007) Cell integrins: commonly used receptors for diverse viral pathogens. *Trends Microbiol.* **15**, 500–507