Integrin α9β1 is a receptor for nerve growth factor and other neurotrophins

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Summary

The integrin α9β1 is a multifunctional receptor that interacts with a variety of ligands including vascular cell adhesion molecule 1, tenascin C and osteopontin. We found that this integrin is a receptor for nerve growth factor (NGF) and two other neurotrophins, brain-derived neurotrophic factor and NT3, using a cell adhesion assay with the α9SW480 cell line. Interaction of α9β1 with NGF was confirmed in an ELISA assay by direct binding to purified integrin. α9β1 integrin binds to neurotrophins in a manner similar to another common neurotrophin receptor, $p75^{NTR}$ (NGFR), although α9β1 activity is correlated with induction of pro-survival and pro-proliferative signaling cascades. This property of α9β1 resembles the interaction of NGF with a high affinity receptor, TrkA, however, this integrin shows a low affinity for NGF. NGF induces chemotaxis of cells expressing α9β1 and their proliferation. Moreover, α9β1 integrin is a signaling receptor for NGF, which activates the MAPK (Erk1/2) pathway. The α9β1-dependent chemotactic ability of NGF appears to result from the activation of paxillin.

Key words: Integrins, Neurotrophins, Nerve growth factor, Cell adhesion, Proliferation, Migration

Introduction

Nerve growth factor (NGF) belongs to the neurotrophin (NT) family that is involved in the functional regulation of many organs including nervous, immune and vascular systems. The NT family contains four members: NGF (Levi-Montalcini et al., 1996), BDNF (brain-derived neurotropic growth factor) (Barde et al., 1987), NT3 (Hohn et al., 1990) and NT4/5 (also known as NTF5) (Berkemeier et al., 1991), which promote neuronal cell survival, differentiation and cell death (Huang and Reichardt, 2001). NTs are highly basic proteins that in the active, circulating stage form homodimers composed of non-covalently associated subunits with molecular masses of about 13 kDa (Bradshaw et al., 1993). NGF is produced by the murine or rat submaxillary gland as a precursor complex (pro-NGF; also known as 7S) of about 130 kDa. This complex is composed of three subunits α, β and γ, which disassociate at a very acidic pH, enabling the isolation of the active β-subunit (Shooter, 2001). The β-subunit, known as β-NGF (2.5S), is the active subunit of NGF and exhibits all of the biological activities classically described for NGF. The properties of α and γ subunits are probably irrelevant to NGF functions because they have been only found in the mouse and rat submaxillary gland (Shooter, 2001).

The neurotrophins have receptors on the cell surface that have been classified according to binding specificity. High affinity receptors belong to the class of tyrosine receptor kinases (Trks; also known as NTRKs, neurotrophic tyrosine kinase receptors), and NGF has been recognized as a specific ligand for TrkA (also known as NTRK1), BDNF and NT4 for TrkB (also known as NTRK2), and NT3 for TrkC (also known as NTRK3) (Chao, 2003). The association of neurotrophins with these receptors usually leads to an increase of cell proliferation ratio and the induction of pro-survival mechanisms. Although prevention of neuronal death is one of the major functions of neurotrophins, in certain situations they may induce cell apoptosis (Frade and Brade, 1998). This situation occurs when neurotrophins, especially NGF binds to the low affinity receptor $p75^{NTR}$. This receptor belongs to the tumor necrosis receptor family, and following association with the ligand induces an apoptotic signal. By contrast, $p75^{NTR}$ (also known as NGFR) is also involved in the creation of a high affinity complex receptor with Trks and can enhance its specificity for neurotrophins (Bibel et al., 1999). However, in the absence of Trks, $p75^{NTR}$ acts as a death receptor indicating that neurotrophins function as cell survival-death regulatory factors. In this paper we report, for the first time, that α9β1 integrin is a common low affinity receptor for neurotrophins, but in contrast to $p75^{NTR}$, it is involved in triggering pro-survival signals.

Integrins are cell surface receptors that in active form are composed of two subunits: α and β. Currently, 18 α and 8 β subunits have been identified and they form, in a restricted manner, at least 24 heterodimers (Sheppard, 2000; Hynes, 2002). Integrins are the major structural receptors that maintain proper tissue organization through cell-cell and cell-extracellular matrix (ECM) interaction. Despite having structural functions, integrins also participate in the transferring of cellular signals leading to modulation of cell death or survival, proliferation and migration (Frisch and Ruoslahti, 1997; Stupack and Cheresh, 2002). Integrin-dependent adhesion to the ECM proteins increased the pro-survival ability of cells, whereas any detaching or adhesion to degraded ECM leads to induction of apoptosis. Cell signaling induced by integrins is also associated with cytoskeleton reorganization following cell spreading and migration (Hood and Cheresh, 2002; Liddington and Ginsberg, 2002), as well as with modulation of cell

proliferation. These cell properties are important in the pathological changes and progression of many diseases including cancer angiogenesis and metastasis, autoimmune disorders, and cardiovascular diseases (Marcinkiewicz, 2005).

The integrin α9 subunit has been identified as a heterodimer, only in association with the β1 subunit (Palmer et al., 1993). The α subunit of this integrin consists of a single polypeptide, whereas most α subunits are composed of two chains. This integrin is widely distributed in the human body and is expressed on many types of cells including epithelial cells, muscle cells, neutrophils and endothelial cells (Palmer et al., 1993; Tarui et al., 2001; Vlahakis et al., 2005; Staniszewska et al., 2007). The wide distribution of α9β1 integrin could be related to its high cross-reactivity with a variety of endogenous ligands such as vascular cell adhesion molecule 1 (VCAM1) (Taooka et al., 1999), tenascin C (Yokosaki et al., 1998), and osteopontin (Smith et al., 1996). It was also characterized as a receptor for ADAM (a disintegrin and metalloproteinase) family protein members such as ADAM12 and ADAM15 by interaction with their disintegrin-like domain (Eto et al., 2002; Lafuste et al., 2005). We also found that this integrin is a receptor for thrombospondin 1 (TSP-1; also known as THBS1) and has a binding site on the N-terminal (NoC1) domain of this ECM protein (Staniszewska et al., 2007). Recently, α9β1 integrin was also reported to interact with vascular endothelial growth factor isoforms (Vlahakis et al., 2005; Vlahakis et al., 2007). The work presented in this paper showed that this integrin can directly interact with another growth factor, NGF, and induce proliferation and migration of cells.

Results

The wide distribution of α9β1 integrin among mammalian tissues has been known for some time (Palmer et al., 1993). These results obtained by immunostaining of frozen sections of a variety of mouse and human tissues were confirmed by us through western blot analysis (Fig. 1). We focused on rat tissues that have been isolated from an adult female and lysed to obtain cell extracts for SDS-PAGE separation. We detected the α9 integrin subunit in all the tested organs except brain (Fig. 1Ae). Interestingly, all lysates contained protein with a molecular mass of about 100 kDa that strongly cross-react with anti-α9 antibody. This band is not related to a degradation product of the α9 subunit, because it is also present in mock-SW480 cells (Fig. 1Aa). Therefore, we assume that this band is not associated with an interaction between polyclonal antibody and α9 integrin, but with a protein that contains fragments with an identical sequence to the cytoplasmic domain of this integrin subunit. The control IgG isolated from pre-bleed serum showed no cross reactivity with any proteins detected by anti-α9 antibody, although some negligible bands were present in the lower molecular mass area of the gel (Fig. 1B).

Interaction of α9β1 integrin with NGF and other neurotrophins

SW480 colon cancer cell line transfected with α9β1 integrin potently adhered to immobilized NGF, whereas control mock-transfected cells showed no adhesion (Fig. 2). The adhesion of α9SW480 cells was observed in a dose-dependent manner for two types of NGFs isolated from mouse submaxillary glands (Fig. 2A). Both low molecular mass mNGF (2.5S two β subunit complex) and high molecular mass pro-NGF (7S NGF, three subunits α, β, γ complex), showed a similar pattern of adhesion although mNGF promoted adhesion at a significantly lower concentration than pro-NGF. Using preparations isolated from natural sources there is the possibility of nonspecific interactions because of contaminants that are difficult to remove during the purification process. To avoid this kind of misinterpretation of our results we compared the activity of mNGF with a human recombinant form of this growth factor. The adhesion of α9SW480 cells to mNGF and hrNGF was almost identical, showing no statistically important differences in the adhesion to a specific anti-α9β1 monoclonal antibody (clone Y9A2), or a well characterized ligand for this integrin, VCAM1. Adhesion of mock-transfected cells to both factors was negligible. Further, we explored the possibility of an interaction of α9β1 integrin with other neurotrophins. We selected BDNF and NT3, which specifically interact with two other receptors TrkB and TrkC, respectively. Both neurotrophins potently interact with α9SW480 cells in adhesion assay to a similar extent as NGF, as well as another endogenous ligand for α9β1 integrin, VCAM1 (Fig. 2B). A little adhesion of mock cells was observed for BDNF, suggesting that the wild-type of colon adenocarcinoma SW480 cell line may contain a high affinity receptor for this neurotrophin, TrkB. Although there are no reports showing the presence of TrkB on colon cancer, the overexpression of this receptor was found in many other types of adenocarcinomas including lung, prostate and pancreas (Ricci et al., 2001; Desmet and Peeper, 2006). The adhesion of cells expressing α9β1 integrin to immobilized neurotrophins was dependent on divalent cations. Elimination of calcium and magnesium from the reaction buffer abolished adhesion of α9SW480 cells to NGF, BDNF and NT3 (data not shown). The specificity of the interaction between NGF and α9β1 integrin in adhesion assays was also confirmed in competition studies (Fig. 2C). Adhesion of α9SW480 cells was inhibited by

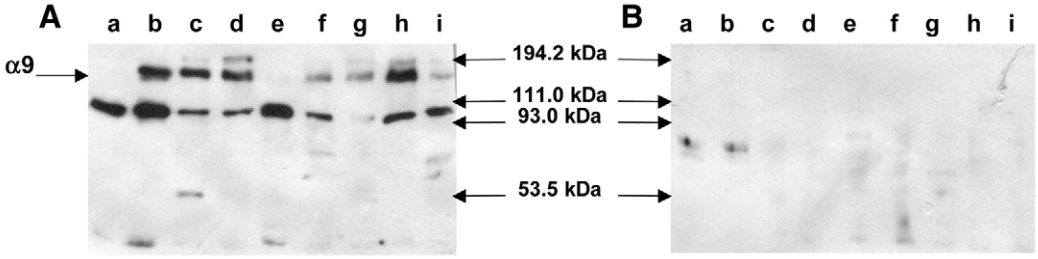

Fig. 1. Detection of α9 integrin subunit in a variety of tissues isolated from adult rat. The isolated tissues were cut into small pieces and lysed. Lysates were separated under reduced conditions by SDS-PAGE (7.5% gel) and electrotransferred into a PVDF membrane. Membrane was incubated with anti-α9 polyclonal antibody against the cytoplasmic domain of integrin (A) or with IgG isolated from pre-bleed serum of the same rabbit (B). The bands were visualized using a chemiluminescent western detection kit. The molecular mass markers are indicated by arrows. Lane a, lysate of mock-SW480 cells – negative control; lane b, lysate of α9SW480 cells – positive control; lane c, vena cava; lane d, aorta; lane e, brain; lane f, kidney; lane g, heart; lane h, lung; lane i, liver.

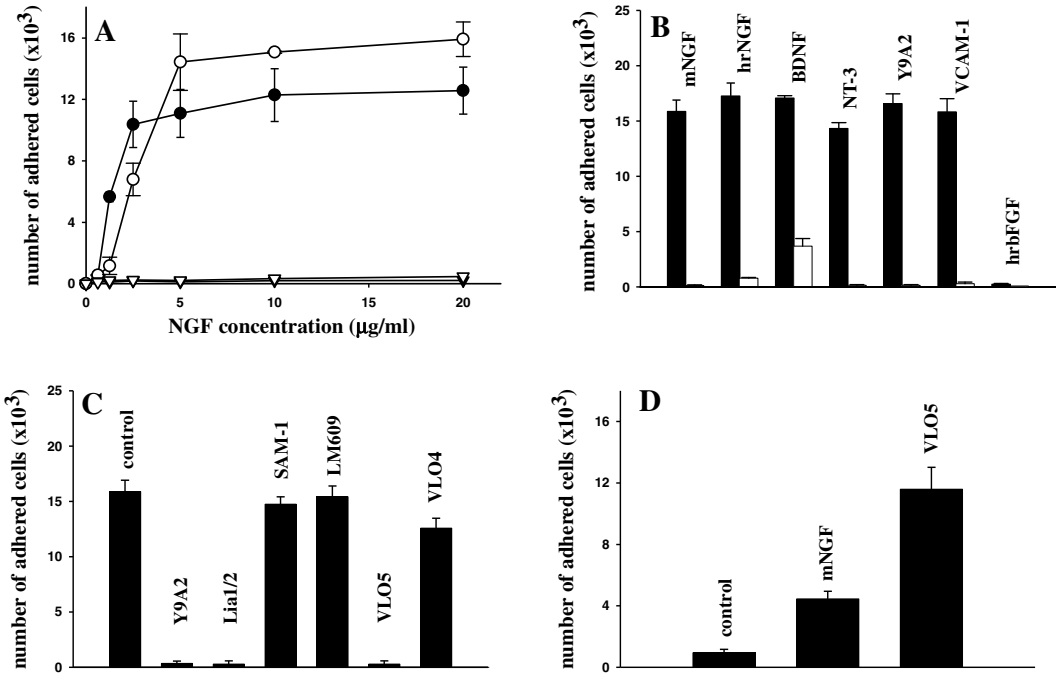

Fig. 2. Interaction of α9β1 integrin with neurotrophins in an adhesion assay. (A) Adhesion of α9SW480- (circles) and mock-SW480- (triangles) transfected cells to immobilized mNGF (filled symbols) and pro-NGF (open symbols). mNGF or pro-NGF was immobilized in a 96-well microplate in PBS by overnight incubation at 4°C. Cells were labeled with CMFDA and added to the wells, previously blocked by BSA. Incubation was performed at 37°C for 30 minutes in HBSS buffer containing calcium and magnesium. After washing off unbound cells, Triton X-100 was added to the wells and the plate was read using a fluorescence microplate reader. The number of adhered cells was calculated from the standard curve prepared in parallel in the same plate from a known number of cells. (B) Adhesion of α9SW480 cells (filled bars) and mock-SW480 cells (open bars) to immobilized neurotrophins, growth factors, monoclonal antibody and adhesion molecule. All proteins were immobilized on 96-well plates at a concentration of 10 μg/ml in PBS by overnight incubation at 4°C. Adhesion experiments were performed as described above. (C) Effect of various monoclonal antibodies and snake venom disintegrins on adhesion of α9SW480 cells to immobilized (10 μg/ml) mNGF. Monoclonal antibodies Y9A2 (anti-α9β1), Lia1/2 (anti-β1), SAM-1 (anti-α5) LM609 (anti-αvβ3), or snake venom disintegrins at concentrations of 10 μg/ml, were preincubated with CMFDA-labeled cells for 15 minutes before adding to the wells for adhesion. Adhesion experiments were performed as described above. (D) Effect of mNGF and VLO5 on expression of LIBS epitope on the β1 subunit of integrin in α9SW480 cells. Anti-LIBS, anti-human monoclonal antibody (B44), was immobilized in 96-well plates at a concentration of 10 μg/ml in PBS, overnight at 4°C. VLO5 or mNGF at a concentration of 1 μM was preincubated with CMFDA-labeled α9SW480 cells for 15 minutes before addition to the wells for adhesion. Adhesion experiments were performed as described above. Error bars indicate the standard deviation from three independent experiments.

blocking with anti-α9β1 and anti-β1 monoclonal antibodies, whereas blocking antibodies against other integrins expressed on the SW480 cell line such as anti-α5β1 and anti-αvβ3 had no inhibitory effect. Snake venom MLD-disintegrin, VLO5 that antagonizes α4β1 and α9β1 integrins, also inhibited adhesion of α9SW480 cells to NGF. However, snake venom disintegrin VLO4 that potently binds to α5β1 integrin through its RGD motif had no effect on α9β1-NGF interaction. α4β1 and α9β1 integrins show a high level of structural homology and share some ligands including VCAM1. We tested the possibility of an interaction between α4β1 integrin and NGF in an adhesion assay using two cell lines, Jurkat and Ramos, which both highly express this integrin. However, our results did not show any cross-reactivity of these cell lines with NGF (data not shown). Interaction of NGF with α9β1 integrin was confirmed using an activating anti-LIBS (ligand-induce binding site) antibody. In the adhesion assay, mNGF and VLO5 significantly increased the binding of α9SW480 cells to anti-β1 (B44) monoclonal antibody, reflecting induction of conformational changes in the integrin (Fig. 2D).

The direct binding of NGF to α9β1 integrin was monitored by ELISA (Fig. 3). In this assay, we reversed the immobilization strategy used in cell adhesion, coating the plate with purified integrin. The soluble NGF also bound to α9β1 integrin in a dose-dependent manner showing saturation at a concentration below 1 μg/ml. The calculated K_d in this assay was 4.5±0.8 nM (±s.d.). In this experiment we used hrNGF instead of mNGF to excluding any non-specificity related to trans-specie interaction. This result indicated that NGF is a very specific ligand for α9β1 integrin interacting in both soluble and immobilized forms. We used α9β1 integrin purified from another α9-transfected cell line, K562, in the ELISA assay, to show that this interaction is not just related to one cell line. Regarding this point, we should mention that in an adhesion assay α9K562 cells also adhere to immobilized NGF, as well as other cell lines, including G361 melanoma and LN229 glioblastoma that naturally express α9 integrin (data not shown).

Identification of TrkA and p75^{NTR} on SW480 cells

Integrin α9β1 in the context of its direct interaction with NGF appears to be a signaling receptor that may regulate cell functions such as migration and proliferation. However, investigation of these functions that depend on α9β1-NGF interaction required the elimination of other NGF receptors involved in these processes. Using RT-PCR analysis we found the presence of two other NGF receptors, TrkA and p75^{NTR} at the mRNA level in SW480 cells. Interestingly, the amount of TrkA mRNA in mock-SW480 cells was similar to that in human neuroblastoma cell line (SH-SY5Y),

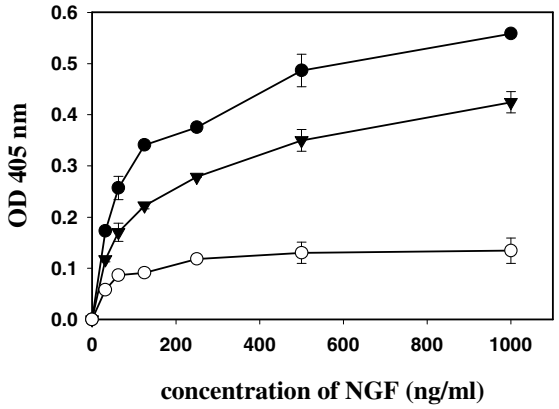

Fig. 3. Binding of mNGF to purified α9β1 integrin in ELISA assay. α9β1 integrin isolated from α9K562 cells was immobilized in a 96-well plate at a concentration of 0.5 μg/ml by overnight incubation at 4°C in PBS. After blocking with 5% non-fat milk, a range of concentrations of hrNGF were added to the wells, which were incubated for 30 minutes at 37°C. The wells were then washed, and bound ligand was detected by adding the primary anti-NGF polyclonal antibody and incubation for 60 minutes at 37°C. After washing, the goat anti-rabbit alkaline-phosphatase (AP)-conjugated IgG was added and incubation was continued for another 60 minutes at 37°C. The color was developed with AP substrate (4-nitrophenylphosphate) and the plate was read using an ELISA plate reader at a 405 nm single wavelength. The specific binding (▲) was estimated by subtraction of binding of NGF to the blocker (○) from binding of NGF to immobilized integrin (●). The error bars represent the standard deviation from three duplicated independent experiments.

whereas it was significantly downregulated in cells transfected with α9 integrin subunit (Fig. 4A,B). The RT-PCR results were confirmed by western blot analysis of these cell lysates with polyclonal antibody against TrkA (Fig. 4D). The radioreceptor binding studies of ^{125}I-NGF with cells revealed a strong interaction of this growth factor with rat neuronal cells (PC12 cell line) and a negligible interaction with mock- and α9SW480 cell lines (Fig. 4C). High expression of TrkA protein was observed in the PC12 cell (Fig. 4Da). Since the affinity of ^{125}I-NGF to cell binding sites is very high (around 10^{-11} M), the activity of this reagent only reflects the interaction with the high affinity complex receptor TrkA-p75^{NTR}. Therefore, the binding of the low affinity receptors p75^{NTR} and α9β1 integrin to ^{125}I-NGF was not reflected in the value measured. The very low density of ^{125}I-NGF binding sites on the SW480 cell lines compared with PC12 neuronal cells, is probably not sufficient to support NGF-induced, TrkA-mediated signals as previously observed (Jiang et al., 1997). Lack of competition of NGF with VLO5 in binding assay with neuronal cells further supports the conclusion that this disintegrin is not an antagonist of TrkA and its inhibitory effects are strictly mediated by α9β1 antagonism.

Binding of NGF activates an α9β1 integrin-dependent pathway of cell signaling

Previously published studies revealed that α9β1 integrin is involved in the activation of a signaling pathway that includes phosphorylation of Erk1/2 (MAPK) and paxillin (Young et al., 2001; Vlahakis et al., 2005). NGF as an immobilized ligand for α9β1 integrin activates both signaling molecules following the adhesion to α9SW480 cells (Fig. 5). However, in the presence of α9β1 integrin inhibitors such as Y9A2 and VLO5, phosphorylation of Erk1/2 (Fig. 5A) and paxillin (Fig. 5B) was significantly decreased. Interestingly, activation of Erk1/2 was strongly inhibited by VLO5, whereas a much more potent inhibitory effect of Y9A2 was observed for paxillin phosphorylation. The increased activation of these proteins was not observed for mock-SW480 cells (data not shown). The NGF-induced, α9β1 integrin-dependent signal transduction was confirmed with the GD10 cell line. In this experiment we used soluble mNGF for the stimulation of the cells in the culture. Co-transfection of this cell line with α9 and β1 integrin subunits revealed significant increase of Erk1/2 phosphorylation as a response to stimulation with NGF in comparison with wild-type cells (Fig. 5C). However, integrin with a hybrid β subunit (β1/β3), which is composed of a β1 extracellular and transmembrane domain and a β3 cytoplasmic domain, showed no significant NGF-induced Erk1/2 phosphorylation.

Chemotaxis of α9β1 integrin-expressing cells induced by NGF

NGF appeared to be a potent inducer of the migration of α9SW480 cells (Fig. 6A), whereas mock-SW480 cells migrated toward this growth factor at the random level (data not shown). The migratory properties of cells strongly depend on the concentration of NGF and the time of migration. The lower concentrations (2.5 and 5 μg/ml) of NGF produced maximum migration at 4 hours, whereas at higher concentrations (10 and 20 μg/ml) maximum migration occurred at 2 hours. Y9A2 was significantly effective in blocking migration of α9SW480 cells to mNGF (5 μg/ml), whereas the effect of VLO5 was not significant (Fig. 6B). As negative controls for this assay, neutral mouse IgG and the disintegrin eristostatin were used, because they do not cross-react with any receptor on SW480 cells. Eristostatin is a specific disintegrin for platelet fibrinogen receptor, αIIbβ3 integrin (Marcinkiewicz et al., 1996).

α9β1 integrin-dependent cell proliferation induced by NGF

The proliferation of cells expressing α9β1 integrin was potently induced by hrNGF in a dose-dependent manner, showing a saturation point at 250 ng/ml (Fig. 7A), whereas proliferation of mock-SW480 cells was not changed by this growth factor. This clear difference between α9-transfected cells and mock-transfected cells suggests that binding of NGF to α9β1 integrin is involved in the induction of cell proliferation. To reach the saturation point of mNGF in proliferation experiment required using more than a fourfold higher concentration of this growth factor (up to 1 μg/ml). This species selectivity was observed in ELISA (Fig. 3), because using hrNGF and purified human receptor increased affinity about 100-fold when compared with mNGF. NGF-induced proliferation of α9SW480 cells is dependent on its interaction with α9β1 integrin, because inhibitors of this integrin, Y9A2 and VLO5 blocked this process to the level of non-stimulated cells (Fig. 7B). In the proliferation experiment, we used mNGF (1 μg/ml) to show that the blocking of α9β1 integrin is independent of the source of NGF, although the same inhibitory results were obtained for hrNGF. Vincristine was used as a positive control in this assay, and neutral IgG and eristostatin were negative controls. The polyclonal blocking serum against TrkA showed no effect on proliferation of α9SW480 cells, suggesting that the major NGF receptor on these cells is α9β1 integrin. The α9β1 integrin-dependent proliferation induced by mNGF was also confirmed by transient transfection of the SW480 cell line by α9 integrin subunit (data not shown). This procedure avoided any possible uncertainty related to the use of a stably transfected cell line maintained in culture for many generations. Moreover, transient transfection of the GD10 cell line

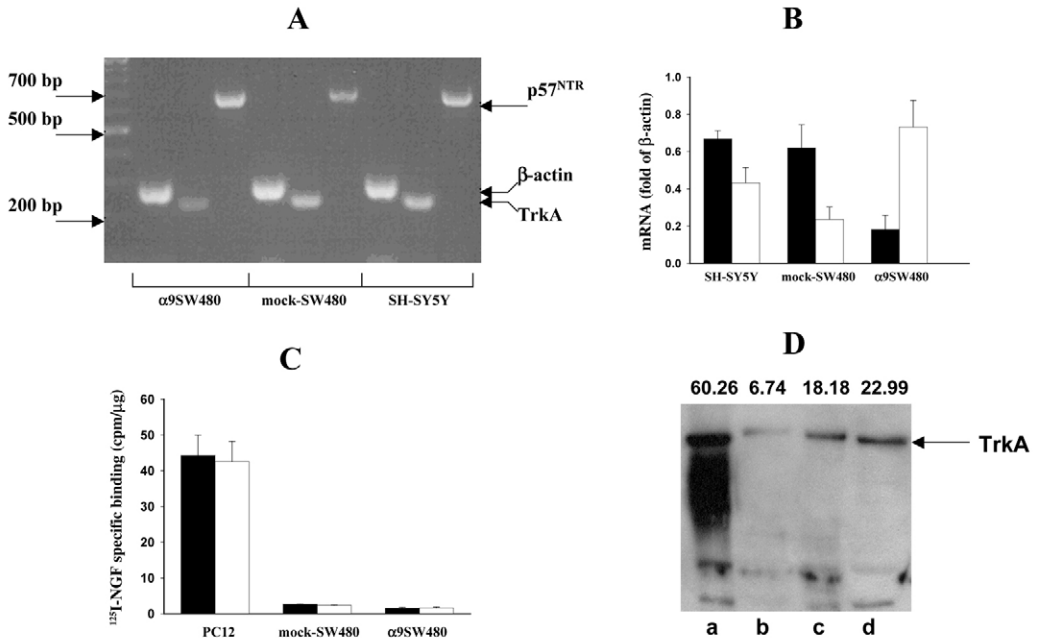

Fig. 4. Identification of TrkA and $p75^{NTR}$ receptors on SW480 cells. (A) RT-PCR analysis of β-actin, TrkA and $p75^{NTR}$ mRNA; the first lane shows markers of a DNA ladder. (B) Comparison of the intensity of bands from the RT-PCR gel in relation to the intensity of β-actin. Bands that are visualized in A were scanned into Uni-Scan-It software and digitalized to obtain the pixel numbers. The relative presence of mRNA of analyzed receptors was calculated relative to β-actin mRNA. Filled bars, TrkA; open bars, $p75^{NTR}$. Error bars indicate s.d. from five measurements. (C) Binding of ^{125}I-NGF to three cell lines in the presence or absence of VLO5. VLO5 (1 μM) was added to the cell suspension and experiments were performed as described in Materials and Methods. Filled bars, specific binding of NGF in the absence of VLO5; open bars, in the presence of VLO5. Error bars indicate s.d. from three experiments, each performed in triplicate. (D) Western blot analysis of cell lysates obtained from PC12 (lane a), α9SW480 (lane b), mock-SW480 (lane c), and SY-SY5Y (lane d) using anti-TrkA polyclonal serum. The bands were visualized using a chemiluminescent western detection kit. The numbers above the bands represent the value of the average pixels, reflecting intensity of the bands, digitalized using Un-Scan-It gel software.

expressing β1 with the α9 subunit also confirmed that signals transferred through α9β1 integrin in response to NGF are not associated with any degeneration of α9SW480 cells with long-term culture.

Chemotaxis of human neutrophils to α9β1 integrin ligands

Previous reports described the expression of α9β1 integrin on neutrophils and the participation of this integrin in migration of these granulocytes (Taooka et al., 1999; Marcinkiewicz et al., 2000). In the presented work, we tested different α9β1 integrin ligands, including NGF as possible chemoattractants for neutrophils (Fig. 8A). NGF appears to be a potent inducer of cell migration with levels similar to those of the disintegrin VLO5 or of Y9A2, a monoclonal antibody against α9β1 integrin. As a positive control 2% FBS was used. Transmigration of neutrophils to mNGF was blocked by Y9A2 monoclonal antibody, whereas the inhibitory effect of VLO5 in this assay was not significant (Fig. 8B).

Discussion

In this study we found that neurotrophins such as NGF, BDNF and NT3 contain a third common receptor, α9β1 integrin. Currently characterized receptors for these molecules belong to the highly specific tyrosine kinase family (Trks), and common to all the neurotrophins, the low affinity receptor $p75^{NTR}$. Very specific interaction of this integrin with these three molecules was observed in adhesion assays of cells transfected with the α9 integrin subunit, verifying the expectation that α9β1 integrin is another common, low affinity receptor for neurotrophins similar as $p75^{NTR}$. However, these two cell surface receptors, following binding to NGF appeared to show completely opposite functions. Integrin induces pro-proliferative and pro-migratory activity of cells, whereas $p75^{NTR}$ is involved in transferring pro-apoptotic signals. Interaction of NGF with α9β1 does not require any integrin stimulators or binding enhancers such as monoclonal antibodies (e.g. TS2/16) or high concentrations of Mn^{2+} (Bazzoni et al., 1995; Byzova and Plow, 1998). This observation increases the relevancy of α9β1-neurotrophin interaction in the physiology and pathology of various organs.

The deficiency of this integrin on brain cells (Fig. 1) (Staniszewska et al., 2007) is a little intriguing in the context of our major discovery that it is a receptor for neurotrophins. This suggests that the interaction of NGF with α9β1 integrin does not have a regulatory effect on the central nervous system, however, it may influence the physiology of other organs affecting the peripheral nervous system. The binding of neurotrophins to α9β1 integrin may be important in the development of pathology in the brain. A majority of malignant gliomas express this integrin and NGF potently induces α9β1-dependent proliferation and invasion of these cancer cells (our unpublished data).

The binding of NGF to α9β1 integrin is promoted similarly to other natural ligands such as VCAM1 or ECM proteins. The necessity of divalent cations for this interaction indicates that subunits of α9β1 need to form an active conformation of their ligand binding pocket. This requirement is strictly essential for specific integrin-ligand interactions in cell physiology. Moreover,

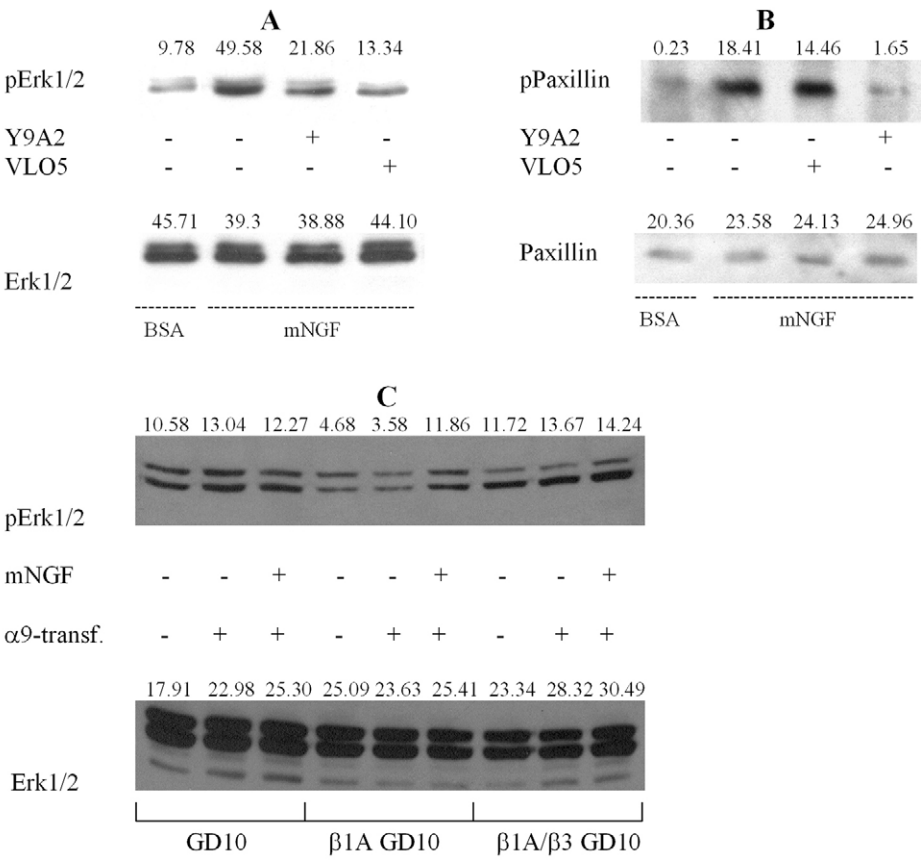

Fig. 5. α9β1 integrin-dependent signaling induced by mNGF. (A) α9SW480 cells were allowed to adhere to immobilized mNGF (20 μg/ml) or BSA for 30 minutes in the absence or presence of α9β1 integrin inhibitors, Y9A2 (10 μg/ml) or VLO5 (10 μg/ml). Cell lysates were obtained and equal amounts of protein were separated under reducing conditions by 10% SDS-PAGE. The proteins from the gel were electro-transferred onto a PVDF membrane and incubated with primary anti-phospho-Erk1/2 (Thr202/Tyr204) and anti-Erk1/2 polyclonal antibodies. The bands were visualized using chemiluminescent western detection kit. The numbers above the bands represent the average number of pixels, reflecting intensity of the bands in the presented scans, digitalized using Un-Scan-It gel software. (B) Paxillin phosphorylation in α9SW480 cells induced following binding to immobilized mNGF. Anti-phospho-paxillin (Tyr31) and anti-paxillin polyclonal antibodies were used. (C) Effect of mNGF on phosphorylation of Erk1/2 in GD10 cells transfected with β1 subunit or hybrid β1/β3 subunit and co-transfected with α9 integrin subunit. Cells were cultured on the plate and stimulated with or without mNGF (50 ng/ml) for 1 hour. Cells were lysed and detection of phosphor-Erk1/2 and total Erk1/2 was performed as described above. All signaling experiments were repeated at least three times.

the ability of NGF to induce the LIBS epitope on the β subunit of α9β1 exhibits a similar mechanism of binding to integrins as endogenous (ECM) and exogenous (disintegrins) ligands, which in association with their receptor express a neo-epitope that is recognized by an anti-LIBS antibody (Marcinkiewicz et al., 1997). Finally, the ELISA assay confirmed the direct binding of hrNGF to α9β1 integrin in a manner opposite to that of immobilized mNGF and integrin in solution. Interestingly, we found species selectivity for soluble neurotrophin in ELISA. Experiment with hrNGF showed two orders of magnitude of increasing binding affinity in comparison with mNGF (K_d 4.5 vs 440 nM). By contrast, NGF in the immobilized form appears to have species-independent pro-adhesive properties for cells expressing α9β1 integrin, suggesting that ligand-receptor interaction in the solid phase may have different conformational requirements.

We found in cell migration and proliferation assays, the importance of α9β1-NGF interaction in cell physiology. Young et al. (Young et al., 2001) reported that the cytoplasmic domain of the α9 integrin subunit may be important in enhancing the rate of cell migration, which agrees with the α9β1 integrin-dependent pro-

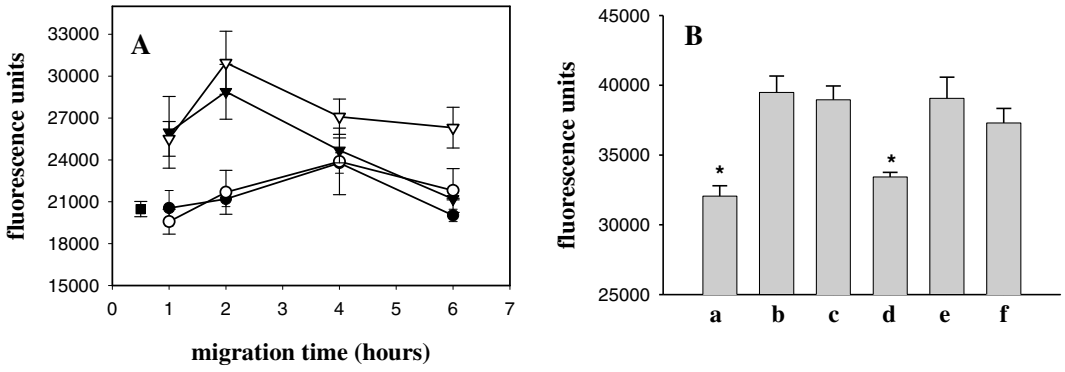

Fig. 6. Chemotaxis of α9SW480 cells to mNGF. (A) Chemotaxis was assessed in a Boyden chamber with fluoroblock membranes (8.0 μm). Calcein-labeled cells were added to the upper chamber of the membrane with immobilized collagen IV, and mNGF was added to the lower chamber as a chemoattractant. Random migration is indicated by ■; ●, 2.5 μg/ml; ○, 5 μg/ml; ▲, 10 μg/ml; △, 20 μg/ml. (B) Inhibition of chemotaxis of α9SW480 cells to mNGF (5 μg/ml). The measurement of migration was assessed after 4 hours. Random migration is represented by bar a; control migration to mNGF without inhibitors, bar b; inhibition by control mouse IgG (10 μg/ml), c; by Y9A2 (10 μg/ml), d; by disintegrins eristostatin (1 μM), e: and by VLO5 (1 μM), f. Error bars indicate s.d. from three separate experiments. *Significant difference (P<0.001) in relation to the group treated with 5 μg/ml of mNGF alone (b).

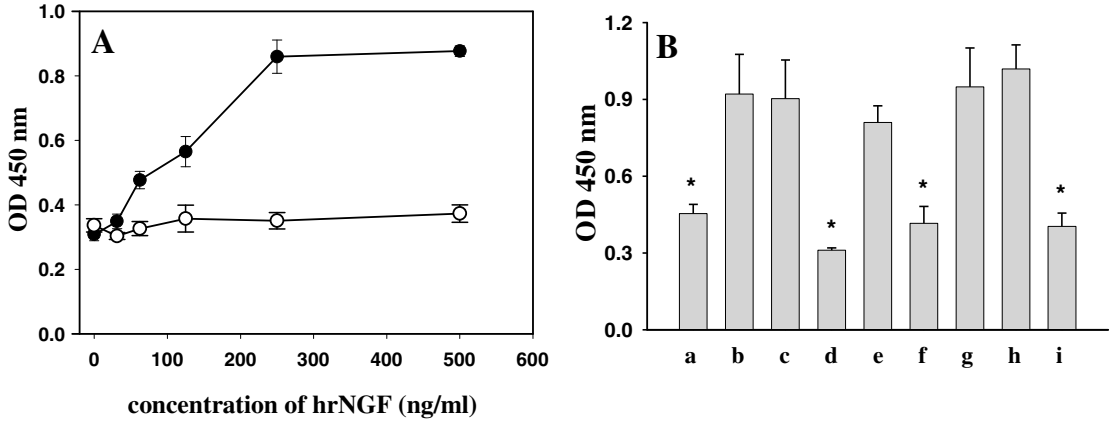

Fig. 7. Effect of NGF and α9β1 integrin inhibitors on cell proliferation in BrdU assay. (A) α9SW480 cells (●) or mock-SW480 cells (○) were grown in 96-well plates to 70% confluence and starved for 48 hours in FBS-free medium. Cells were not treated or treated with different concentrations of hrNGF in serum-free medium for 48 hours. BrdU color development assay was performed according to the manufacturer's instruction (Roche). Error bars indicate s.d. from triplicate experiments. (B) Effect of α9β1 integrin and TrkA inhibitors on mNGF-induced proliferation of α9SW480 cells. Cells were non-treated (a) or treated with 1 μg/ml of mNGF (b) in the presence of 10 μg/ml control mouse IgG (c) 10 μg/ml Y9A2 (d), 1 μM disintegrins eristostatin (e) and VLO5 (f), 10 μg/ml control rabbit serum (g), 10 μg/ml anti-TrkA polyclonal serum (h), or 50 μg/ml vincristine (i). Error bars indicate s.d. from three separate experiments. *Significant difference (P<0.001) in relation to the group treated with 1 μg/ml of mNGF alone (b).

migratory activity of NGF. However, the mechanism that involves α9β1 integrin in the promotion of cell migration is still unclear. Our results confirmed that α9β1 integrin, following binding to its ligand NGF, induces activation of a focal adhesion adaptor protein, paxillin (Fig. 4B). Binding of paxillin to the α9 subunit is sufficient to modulate cell spreading, but it has no effect on the regulation of cell migration. However, using two different inhibitors of this integrin, we observed a correlation between phosphorylation of paxillin and cell migration. The NGF-dependent chemoattraction of α9SW480 cells was potently inhibited by blocking with an anti-α9β1 monoclonal antibody, whereas the effect of the MLD-disintegrin, VLO5 was not significant. The same activity of α9β1 integrin inhibitors was obtained in the transmigration assay of human neutrophils. Accordingly, inhibition of NGF-induced paxillin phosphorylation was more potently inhibited by Y9A2 than by VLO5. These data may suggest that growth factor-induced α9β1 integrin-dependent cell migration is associated with paxillin activation, whereas extracellular matrix (Tenascin-C)-induced cell migration is paxillin independent (Young et al., 2001). Further work with paxillin-deficient cells will verify this hypothesis.

The major difference between α9β1 and the other common receptor for neurotrophins, $p75^{NTR}$, is an effect on cell proliferation. $p75^{NTR}$ without association with TrkA is an inhibitor of this process and an inducer of pro-apoptotic signals (Lee et al., 2001). Following binding to α9β1, NGF induces the activation of MAPK Erk1/2, which is generally characterized as a signaling molecule mediating pro-proliferative and pro-survival signals. Interestingly, this growth factor caused increased phosphorylation of Erk1/2 only in transfected cells expressing α9β1 integrin, whereas no effect was observed in control cells. The α9β1-dependent mediation of cell signaling upon binding to NGF was also confirmed by specific integrin inhibitors, as well as by interaction with GD10 cells

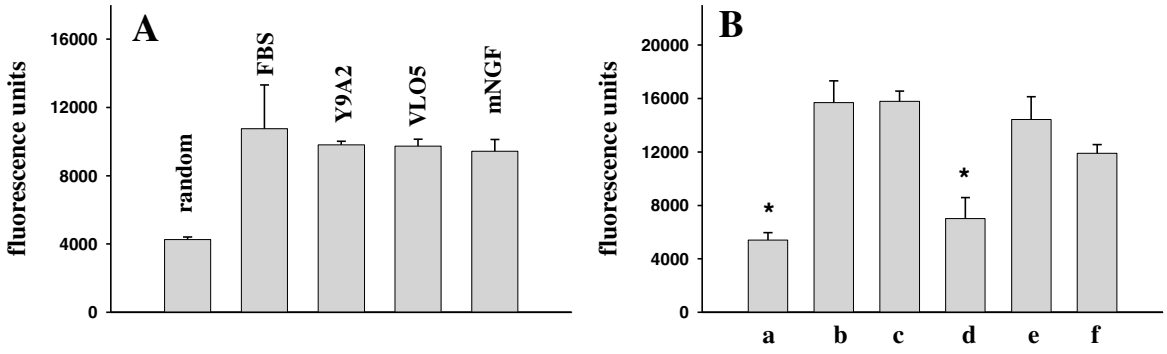

Fig. 8. Chemotaxis of neutrophils to α9β1 integrin ligands. (A) Chemotaxis was assessed in a Boyden chamber with fluoroblock membranes (3.0 μm). Neutrophils isolated from human blood were labeled with calcein by incubation at 37°C for 30 minutes. Neutrophils were added to the upper chamber, and integrin ligands such as Y9A2 (5 μg/ml), VLO5 (1 μM) and mNGF (5 μg/ml), or 2% FBS were applied to the lower chamber. The plate was incubated at 37°C for 2 hours. Migration level was estimated as described in Fig. 5. Error bars indicate s.d. from three separate experiments. All chemoattracted groups were significantly different in comparison with random migration (P<0.001). (B) Inhibition of chemotaxis of neutrophils to mNGF. Migration was assessed after 4 hours. Random migration is represented by bar a and control migration to mNGF without inhibitors by bar b. Inhibition of chemotaxis of neutrophils to mNGF (5 μg/ml) by control mouse IgG (10 μg/ml), c; Y9A2 (10 μg/ml), d; disintegrins eristostatin (1 μM), e and VLO5 (1 μM), f. Error bars indicate s.d. from three separate experiments. *Significant difference (P<0.001) in relation to the group treated with 5 μg/ml of mNGF alone (b).

transfected with α9 subunit and hybrid β1/β3 subunits. VLO5 and Y9A2 significantly decreased Erk1/2 phosphorylation in α9SW480 cell adhesion to immobilized NGF, whereas soluble NGF increased signal transduction in GD10 cells transfected with wild-type α9β1 integrin. This effect was not observed in the cells that were transfected with a hybrid β subunit containing extracellular and transmembrane domains from β1 and the cytoplasmic domain from β3 subunit. It suggests that NGF, similarly to ECM proteins (e.g. fibronectin) (Danen et al., 2002), induces cell signaling pathways through the β1 subunit of the integrin.

The signal transduction activity links α9β1 integrin with the high affinity NGF receptor, TrkA, which is also involved in transferring signals inside the cells utilizing the Erk1/2 pathway (Edsjo et al., 2001; Slack et al., 2005). In this context, expression of $p75^{NTR}$ appears to be less important, because this is a pro-apoptotic receptor that is not involved in the stimulation of cell proliferation and Erk1/2 signaling. Although RT-PCR and western blot results showed a very low expression of TrkA on α9SW480 cells, and a radioisotope study confirmed an almost entire lack of high affinity-dependent interaction of these cells with NGF, we are not able to exclude the possibility that both receptors may cooperate in signal transduction. In this context, the observation that cells transfected with α9 integrin subunit significantly decrease expression of TrkA is very interesting. It may suggest that both NGF receptors are in a functional relationship and the deficiency of one is compensated by the other. Although blocking experiments with anti-TrkA serum and TrkA siRNA (data not shown) proved that α9β1 integrin is independent in transferring pro-survival signals, the possibility of participation of TrkA in the cross-talk with this integrin may occur under certain physiological conditions.

Previously published reports showed a high and selective expression of α9β1 integrin on neutrophils and the involvement of this integrin in chemotaxis of these granulocytes across an activated endothelial monolayer (Taooka et al., 1999; Marcinkiewicz et al., 2000). Our findings may contribute to the explanation of mechanisms that are involved in NGF-dependent interaction of neutrophils in the progression of certain autoimmune diseases. Increased NGF levels have been observed in various inflammatory states including asthma (Braun et al., 1999) and arthritis (Aloe et al., 1992) and this upregulation was associated with a significant influx of neutrophils. Gee et al. (Gee et al., 1983) also observed activity of NGF as an inducer of polymorphonuclear leukocyte chemotaxis at nanomolar concentrations. However, the mechanism of this pro-migratory effect was not evaluated. The interaction of NGF with integrin as proposed by us may contribute to the explanation of this phenomenon. Another very important pathology induced by NGF is thermal hyperalgesia. Injection of NGF into experimental animals induced neutrophil accumulation and hyperalgesia in skin (Bennett et al., 1998). A recent report showed that the involvement of certain integrins, especially the β1 subunit, is important in developing mechanical hyperalgesia induced by NGF (Malik-Hall et al., 2005). However, NGF protected murine neutrophils from apoptosis and enhanced their survival (Kannan et al., 1991). Based on the data presented in this work the mechanism of neutrophil accumulation and induction of pro-survival signals may be dependent on the interaction of NGF with α9β1 integrin.

NGF has been detected in body fluids as well as in a majority of tissues, but in different concentrations (Katoh-Semba et al., 1989). Our finding that α9β1 integrin binds this growth factor in immobilized and soluble form suggests the availability of this receptor-ligand interaction in circulatory systems and solid tissues of the body. Published studies show that the effective concentration of NGF required to stimulate neurite outgrowth in vitro is in the range of 10-100 ng/ml (Levi-Montalcini, 1982), whereas the NGF level in blood plasma was estimated to be approximately 100 pg/ml. Therefore, this in vitro activity that is dependent on a high affinity NGF receptor, TrkA, requires a concentration of NGF at least a two orders of magnitude higher than those observed in vivo. The affinity interaction of isolated human recombinant α9β1 integrin with hrNGF in ELISA (K_d=4.5 nM) is in the same range as for $p75^{NTR}$, which was K_d=1.4 nM in a Scatchard analysis of ^{125}I-NGF binding to cells expressing this receptor (Hempstead et al., 1991). The same radioactive analysis showed K_d=25 pM for a high affinity complex of TrkA. Therefore, the concentrations of NGF that we used in our in vitro experiments corresponds to this ratio, because α9β1 integrin is a low affinity receptor and in all cell signaling and proliferation experiments its effective doses did not exceed 1 μg/ml. Higher amounts of mNGF used in the cell migration and adhesion assays resulted from specificity of assays. Immobilization of protein on the plastic is not 100%, especially for proteins of low molecular mass, such as NGF.

Materials and Methods

Antibodies, neurotrophins, snake venom disintegrins

Anti-α9β1 integrin (clone Y9A2) monoclonal antibody was prepared as described previously (Wang et al., 1996) and kindly provided by D. Sheppard (University of California, San Francisco, CA). Anti-α5 (SAM-1) and anti-β1 (Lia1/2) were purchased from Beckman Coulter Inc. (Fullerton, CA); anti-β1 (B44) and anti-αvβ3 (LM609) was purchased from Chemicon (Temecula, CA). Antibodies against signaling molecules were purchased as follow: anti-pErk (Thr202/Tyr204) and anti-Erk polyclonal antibodies (Cell Signaling Inc., Beverly, MA), anti-p-paxillin (Tyr31) (Biosource, Camarillo, CA), anti-paxillin (Chemicon). Anti-TrkA polyclonal serum was kindly provided by L. Reichardt (University of California, San Francisco, CA). Polyclonal serum against the α9 subunit of the integrin cytoplasmic domain was developed in a rabbit. The synthetic peptide representing the entire amino acid sequence of the cytoplasmic domain was used as an antigen. The development of this polyclonal antibody was performed commercially by Chemicon. Control, neutral mouse and rabbit IgGs, and human recombinant FGF were purchased from Sigma Inc. (St Louis, MO). All neurotrophins, native and recombinant, were purchased from PeproTech Inc. (Rocky Hill, NJ). Two snake venom dimeric disintegrins, VLO4 and VLO5 were purified from the venom of *Vipera lebetina obtusa* using two steps of reverse phase HPLC, as described previously (Bazan-Socha et al., 2004) and monomeric disintegrin eristostatin was purified using the same method from *Eristocophis macmahoni* venom (Latoxan, Valence, France).

Cell lines and transfection

α9- and mock-transfected SW480 cells were kindly provided by D. Sheppard. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1 mg/ml G418 (Mediatech Inc., Herndon, VA). α9K562 cells were provided by P. Weinreb (Biogen Inc.) and cultured in RPMI-1640 medium containing 10% FBS, 1% penicillin-streptomycin supplemented with 1 mg/ml G418. Rat pheochromacytoma PC12 and human neuroblastoma SH-SY5Y neuronal cells were kindly provided by G. Guroff (NIH, Bethesda, MA) and grown in DMEM supplemented with 7.5% horse serum, 7.5% FBS and DMEM supplemented with 10% FBS, respectively.

GD10 cells deficient in the integrin β1 subunit (Bae et al., 2004) as well as cells transfected with the β1A splice variant gene β1A-GD10 (Wennerberg et al., 1996) and β1A/β3-GD10 (Danen et al., 2002) were provided by D. Mosher (University of Wisconsin, Madison, WI). Stable transfectants were grown in media containing 10 μg/ml puromycin. The pBlueScript (BS)-SKα9 cDNA construct (Yokosaki et al., 1994) for transient transfection of cells with α9 integrin subunit, was provided by D. Sheppard. Lipofectamine (Invitrogen, Carlsbad, CA) was used for transfection of GD10, β1A-GD10 and β1A/β3-GD10 cells with pBlueScript (BS)-SKα9 cDNA plasmid according to the manufacturer's instruction. After 72 hours α9-GD10 cells were selected with 1 mg/ml G418, whereas α9β1A-GD10 and α9β1A/β3-GD10 cells were selected with 1 mg/ml G418 and 10 μg/ml puromycin.

Analysis of α9 integrin subunit expression in rat tissues by western blot

The various tissues were removed from an adult female rats and washed with ice-cold PBS containing protease inhibitors (Sigma). Tissues were cut into small pieces and incubated at room temperature for 30 minutes in lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 25 mM NaF, 10%

glycerol, 1 mM PMSF, 1 mM Na_3VO_4. Insoluble debris were removed by centrifugation and the supernatant was separated (20 μg proteins per sample) on a 7.5% SDS-PAGE under reducing conditions. The protein bands were electrotransferred into a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with 5% non-fat milk in TBST and probed with anti-α9 integrin subunit polyclonal antibody. After washing, the membrane was incubated with HP-conjugated secondary antibodies and developed using chemiluminescence (Cell Signaling Inc.).

Cell adhesion and ELISA studies

Adhesion studies of cultured cells labeled with 5-chloromethyl fluorescein diacetate (CMFDA; Invitrogen) were performed using 96-well microtiter plates (BD Falcon, Frankin Lakes, NJ) as described previously (Marcinkiewicz et al., 1997). ELISA assay with purified α9β1 integrin was performed according to the procedure described earlier (Bazan-Socha et al., 2004).

Identification of TrkA and $p75^{NTR}$ on SW480 cells

RT-PCR assay was performed as described earlier (Jiang et al., 1997). Briefly, total RNA was isolated using the SV total RNA isolation system (Promega, Madison, WI). 1 μg of total RNA was reverse transcribed using the Reverse Transcription System (Promega), according to the manufacturer's instruction. Then PCR was applied in the presence of 5 μg cDNA, 50 pmoles upstream sense and downstream antisense primers, using GoTaq Green Master Mix (Promega). The cDNA for β-actin was amplified for 35 cycles; for TrkA and $p75^{NTR}$ for a 40 cycles. To generate the various cDNA fragments, a Mastercycler gradient (Eppendorf, Germany) was used. To identify, the following primers were used: β-actin (285 bp) sense: 5′-TCAT-GAAGTGTGACGTTGACATCCGT-3′ and antisense: 5′-CTTAGAAGCATTTGC-GGTGCACGATG-3′; TrkA (232 bp) sense: 5′-TGCCTGCCTCTTCCTTTCTA-3′ and antisense: 5′-GTGGTGAACACAGGCATCAC-3′; $p75^{NTR}$ (663 bp) sense: 5′-AGCCAACCAGACCGTGTGTG-3′ and antisense: 5′-TTGCAGCTCTTCCAC-CTCTT-3′. PCR products were analyzed by electrophoresis on an agarose gel (2%) containing ethidium bromide for UV visualization.

Binding of ^{125}I-NGF to cell suspension was performed as described earlier (Lazarovici et al., 1997). Briefly, the cells were grown on collagen (200 μg/ml) and polylysine (10 μg/ml) coated 6-well plates. The monolayer was washed twice with DMEM containing 0.1% BSA (binding medium). The medium was replaced with 1 ml of binding medium containing 50,000 cpm ^{125}I-NGF with or without 4 nM cold NGF to measure total and nonspecific binding, respectively. The specific binding represents the difference between the total and nonspecific binding. The incubation was carried out at 37°C for 45 minutes, and then the binding buffer was removed and the cells were washed with ice-cold PBS. The monolayer of cells was solubilized with 1 ml of 1 N NaOH overnight at room temperature. The cell-associated radioactivity was counted in a γ-counter and a portion of the solublized mixture was used for protein determination. All binding assays were performed in triplicates and repeated at least three times using cells from different passages.

Cell chemotaxis assay

Collagen IV (10 μg/ml) was immobilized on a 8.0 or 3.0 μm pore size membrane (HTS FluoroBlok inserts, Falcon) by incubation in PBS for 2 hours at room temperature. Neutrophils were isolated from the human blood according to the procedure described previously (Marcinkiewicz et al., 2000). Cells were labeled with calcein (Invitrogen) by incubation in culture for 1 hour. After trypsinization cells were suspended in DMEM (5×10^4 cells per 300 μl), pre-incubated in the presence or absence of α9β1 inhibitors for 30 minutes at room temperature and applied on the top surface of inserts (upper chamber). Inserts were placed into 24-well plates containing 700 μl of DMEM with mNGF, VLO5, Y9A2 or 2% FBS used as a chemoattractants (bottom chamber). The plate was placed into a CO_2 incubator and measurement of fluorescence units was performed after 1, 2, 4 and 6 hours, using a fluorescence microplate reader (Bio-Tek, Winooski, VT) with the bottom reading option, at an excitation wavelength of 485 nm using a 530 nm emission filter.

Cell proliferation assay

α9SW480 or mock-SW480 cells were seeded on 96-well plates and grown to 70% confluence in complete medium. Complete medium was changed to serum-free DMEM and cells were starved for 48 hours. Then serum-free medium was changed again to medium containing no mNGF or containing different concentrations of mNGF. Stimulation was conducted for 48 hours, and the proliferation ratio was determined using the BrdU proliferation assay kit according to the manufacturer's instruction (Roche, Mannheim, Germany).

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