CD155/PVR Enhances Glioma Cell Dispersal by Regulating Adhesion Signaling and Focal Adhesion Dynamics

Kevin E. Sloan,[1] Jean K. Stewart,[1] Allison F. Treloar,[2] Russell T. Matthews,[2] and Daniel G. Jay[1]

[1]Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts and [2]Department of Neurobiology, Yale University School of Medicine, New Haven, Connecticut

Abstract

We recently identified the immunoglobulin-CAM CD155/PVR (the poliovirus receptor) as a regulator of cancer invasiveness and glioma migration, but the mechanism through which CD155/PVR controls these processes is unknown. Here, we show that expression of CD155/PVR in rat glioma cells that normally lack this protein enhances their dispersal both *in vitro* and on primary brain tissue. CD155/PVR expression also reduced substrate adhesion, cell spreading, focal adhesion density, and the number of actin stress fibers in a substrate-dependent manner. Furthermore, we found that expression of CD155/PVR increased Src/focal adhesion kinase signaling in a substrate-dependent manner, enhancing the adhesion-induced activation of paxillin and p130Cas in cells adhering to vitronectin. Conversely, depletion of endogenous CD155/PVR from human glioma cells inhibited their migration, increased cell spreading, and down-regulated the same signaling pathway. These findings implicate CD155/PVR as a regulator of adhesion signaling and suggest a pathway through which glioma and other cancer cells may acquire a dispersive phenotype. (Cancer Res 2005; 65(23): 10930-7)

Introduction

In glioblastoma, the most aggressive form of brain cancer, tumor cells disperse so extensively that current treatment approaches such as surgical resection or radiation therapy have little effect in checking progression (1, 2). This is a significant problem and it is recognized as a major priority to better understand glioma dispersal and to develop therapies that limit this process (2). A first step in this development is the identification of proteins that function aberrantly in dispersal-relevant processes such as cell migration, which is frequently deregulated in cancer cells (3–6).

Cell migration requires a coordinated orchestration of complex events including polarization, protrusion, adhesion, de-adhesion, and retraction (4). Upon encountering a migration-promoting agent, a cell becomes polarized and will extend protrusions in the direction of migration. These protrusions can take on two forms: large, broad lamellipodia and spike-like filopodia. Both forms are driven by actin polymerization and are stabilized by adhering to the extracellular matrix (ECM) or adjacent cells via transmembrane receptors linked to the actin cytoskeleton through focal adhesion complexes. These adhesion points provide a foundation for traction forces that enable the cell to move forward. Disassembly of adhesion points and retraction at the rear of the cell completes the process, enabling net forward migration (7).

Many cell-surface proteins have been implicated in the regulation of cell migration. Growth factor receptors receive environmental cues and can initiate signaling cascades resulting in polarization and directional migration (6). Adhesion molecules such as integrins, cadherins, and immunoglobulin family proteins can regulate adhesion and de-adhesion between a cell and its neighbors or the ECM and can also contribute to polarization and directional motility in response to the ECM (8–10). Adhesion molecules connect to signaling cascades that regulate actin and microtubule dynamics and ultimately control cell behavior (8, 10). The expression of adhesion molecules is frequently deregulated in cancer, resulting in changes to downstream signaling pathways and enhanced motility (5).

We previously reported the identification of CD155/PVR (the poliovirus receptor), as a regulator of cancer cell invasiveness through a functional proteomic screen (11). CD155/PVR is a member of the immunoglobulin family of cell adhesion molecules, first identified based on its ability to mediate the binding of poliovirus to host cells (12). Little is known, however, regarding its endogenous function in cells. We previously found that CD155/PVR was highly expressed in both U87 human glioma cells and primary glioblastoma tumor tissue, and that inactivation of CD155/PVR reduced cell migration *in vitro* (11). These findings suggested a novel role for CD155/PVR in regulating motility and prompted us to explore its contribution to glioma dispersal in more detail.

Here, we confirm the role of CD155/PVR in regulating glioma dispersal, both *in vitro* and on primary brain tissue. We report that expression of CD155/PVR in rodent glioma cells that normally lack the protein results in increased motility and substrate-dependent reductions in adhesion and cell spreading. Conversely, we show that depletion of CD155/PVR from human glioma cells that express high levels of the protein results in decreased migration and increased cell spreading. Furthermore, we show that CD155/PVR can modulate an adhesion-induced Src/focal adhesion kinase (FAK)/paxillin/p130Cas signaling pathway to destabilize focal adhesions at the cell-substrate interface.

Materials and Methods

Cells, reagents, and antibodies. All reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise indicated. 9L and CNS-1 rat glioma cells, described elsewhere (13), were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (9L) or RPMI (CNS-1) supplemented with 10% FCS (Hyclone, Logan, UT) and 100 units/mL penicillin/streptomycin. A172 human glioma cells, described elsewhere (14), were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FCS and 100 units/mL penicillin/streptomycin. Purified vitronectin was obtained from Promega (Madison, WI); purified fibronectin and collagen-1 were obtained from BD Biosciences (San Jose, CA); bovine serum albumin (BSA) was from Sigma (St. Louis, MO);

Requests for reprints: Daniel G. Jay, Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. Phone: 617-636-6714; Fax: 617-636-0445; E-mail: daniel.jay@tufts.edu.

doi:10.1158/0008-5472.CAN-05-1890

anti-CD155/PVR (clone D171), used for immunocytochemistry, was obtained from Lab Vision (Fremont, CA); anti-CD155, used for immunoblotting, was a kind gift from Dr. Eckard Wimmer; antivinculin (clone 7F9) was from Chemicon (Temecula, CA); AlexaFluor 594 phalloidin and AlexaFluor 488 goat anti-mouse IgG secondary antibody were from Molecular Probes (Eugene, OR); anti-phospho-Src (Tyr^{416}), anti-phospho-paxillin (Tyr^{118}), anti-phospho-p130Cas (Tyr^{165}), anti-phospho-CrkII (Tyr^{221}), anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), and anti-phospho-Akt (Ser^{473}) were obtained from Cell Signaling Technology (Beverly, MA); anti-phospho-FAK (Tyr^{397}, clone 14) was from BD Biosciences; anti-β-actin (clone AC-15) was from Sigma. Peroxidase-conjugated antimouse and antirabbit IgG secondary antibodies were obtained from Cell Signaling Technology.

Expression constructs, small interfering RNA, and transfection. A full-length wild-type human CD155/PVR cDNA (accession no. BC015542) in the pCMV-SPORT6 expression vector was purchased from Invitrogen and confirmed by sequencing. The enhanced green fluorescent protein expression vector pEGFP-N1 was obtained from BD Biosciences. Expression constructs were transfected into 9L cells using LipofectAMINE 2000 following the manufacturer's instructions using 0.5 μg DNA per 35 mm dish. Cells were incubated with DNA in OptiMEM for 6 hours after which time normal growth medium was added. Cells were then incubated for 24 to 48 hours to achieve robust expression. Control cells were subjected to identical handling but did not receive DNA. We previously reported the development of a small interfering RNA duplex that efficiently knocked down CD155/PVR protein expression (11). Briefly, a double-stranded siRNA oligonucleotide targeting CD155/PVR (5′-CAA-CUU-UAA-UCU-GCA-ACG-UdTdT-3′) was chemically synthesized (Dharmacon, Lafayette, CO) and transfected into A172 cells using Oligofect-AMINE following the manufacturer's instructions using 200 nmol/L siRNA per 35 mm dish. Cells were incubated with siRNA in OptiMEM for 6 hours after which time normal growth medium was added. Cells were then incubated for 72 hours to achieve >80% knockdown of CD155/PVR. Control cells were transfected with a scrambled siRNA oligonucleotide at matching concentrations.

Adhesion signaling assay and immunoblotting. Mock- or CD155-transfected cells were grown for 48 hours in 100 mm tissue culture dishes. Cells were detached with Versene and 1×10^6 cells plated onto 100 mm culture dishes previously coated with vitronectin (2 μg/mL), fibronectin (2 μg/mL), or PBS and blocked overnight with 1% BSA in PBS. Cells were allowed to adhere at 37°C for 90 minutes and then washed with room temperature PBS and incubated in NP40 lysis buffer [0.2% NP40, 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% glycerol] supplemented with protease inhibitors (Roche, Nutley, NJ) and phosphatase inhibitors (Sigma) for 30 minutes at 4°C. Lysates were collected by scraping, cleared by centrifugation for 15 minutes at 10,000 × *g* and quantified using the DC Protein Assay (Bio-Rad, Hercules, CA). Lysate (30 μg) was separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% nonfat dry milk in PBS and probed with primary antibody diluted in 5% BSA in PBS overnight. Antibody binding was detected with peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence substrate (Perkin-Elmer, Boston, MA).

Cell morphology and immunocytochemistry. After transfection, cells were detached with Versene and 1×10^4 cells were deposited on glass coverslips that had been previously coated with 2 μg/mL vitronectin, fibronectin, or collagen-1 and blocked with 1% BSA in PBS. Cells were incubated for 2 hours at 37°C, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 and blocked with 1% BSA in PBS. Cells were incubated with primary antibody for 1 hour at room temperature and washed extensively in PBS. Appropriate species-specific secondary antibodies conjugated to AlexaFluor 488 or 594 were used to visualize antibody staining.

Substrate adhesion assay. Cells were detached with Versene and 2×10^4 cells were deposited into replicate wells of a microtiter plate that had been previously coated with 2 μg/mL vitronectin or collagen-1 and blocked with 1% BSA in PBS. Cells were incubated for 90 minutes at 37°C, briefly shaken at 200 rpm, washed twice with medium, and fixed with 4% paraformaldehyde in PBS. Attached cells were stained for 1 hour with crystal violet (5 mg/mL in 2% ethanol), washed extensively with water, and allowed to dry overnight. Stained cells were solubilized in 50 μL of 2% SDS and absorbance was measured on a SpectraFluor Plus automated plate reader (Tecan, Research Triangle Park, NC).

Transwell migration assay. Migration assays with 9L and A172 cells were done as described previously (11). Briefly, cells were transfected with cDNA for 24 hours, or siRNA for 72 hours, prior to the assay and maintained at subconfluence under normal growth conditions. On the day of the assay, cells were labeled with cell tracker orange (Molecular Probes), and 5×10^4 cells were loaded onto replicate 8 micron, 96-well Fluoroblok membranes (BD Biosciences) with 5% serum chemoattractant in the bottom chamber. The membrane apparatus was incubated at 37°C and cell migration to the underside of each light-impermeable membrane was quantified at various time points using an automated plate reader to measure fluorescence, and was confirmed by visualization using an inverted fluorescence microscope. In a subset of experiments, the membrane surface was coated with 2 μg/mL of vitronectin for 2 hours at room temperature, washed, and blocked with 1% BSA in PBS before loading cells.

Cell proliferation assay. Cell proliferation was measured using the CellTiter 96 AQueous Assay (Promega) following the manufacturer's instructions. Briefly, 2,000 cells from various treatment groups were plated in replicate wells of a 96-well plate and grown overnight. CellTiter 96 AQ reagents were added to a subset of wells at 0, 24, 48, and 72 hours, incubated for 2 hours at 37°C, and absorbance was quantified on an automated plate reader.

Brain slice dispersal assay. The use of organotypic brain slice culture to model glioma dispersal has previously been described in detail (15). All studies were approved by the Yale University Institutional Animal Care and Use Committee. Briefly, brains from postnatal day 2 CD-1 mice (Charles River Laboratories, Wilmington, MA) were obtained by rapid decapitation of anesthetized pups and removal of the brain into ice-cold HBSS containing penicillin, streptomycin, and amphotericin B. The meninges were removed and 350 μm coronal slices prepared using a McIlwain Tissue Chopper vibratome (Brinkmann Instruments. Westbury, NY). Brain slices were dissociated in HBSS and placed on the porous membrane of a 0.4 mm Millicell-CM insert (Millipore, Billerica, MA) in a 35 mm culture dish containing 800 μL of medium (50% Neurobasal-A, 25% HBSS, 20 mmol/L HEPES, 10% FCS, supplemented with B-27, G5, penicillin, streptomycin, and amphotericin B) below the insert. All media above the insert were removed. Brain slice cultures were incubated at 37°C, 5% CO_2 in a humid incubator. A single-cell suspension of 9L or CNS-1 cells was prepared and transiently transfected with 0.5 μg full-length CD155/PVR cDNA and 0.5 μg green fluorescent protein (GFP) cDNA, or GFP alone, using LipofectAMINE 2000. The cell suspension was seeded into six-well culture plates coated with a thin layer of sterile 1% GPG agarose (American Bioanalytical, Natick, MA) dissolved in growth medium and incubated at 37°C for 24 hours to encourage the formation of tumor cell spheroids. Using a dissecting microscope, one spheroid was removed from the culture using a pulled glass capillary pipette and gently deposited onto individual brain slices. The initial size of the tumor spheroid was recorded by fluorescence microscopy of the GFP-transfected cells and measured using NIH Image (NIH, Bethesda, MD). The total area of cell migration was subsequently measured at 24, 48, and 72 hours.

Results

CD155/PVR expression enhances glioma cell migration. We addressed the role of CD155/PVR in glioma migration and dispersal by expressing it in 9L rat glioma cells that normally lack the protein, as CD155/PVR expression is restricted to primates (16, 17). 9L cells are nondispersive in primary brain tissue (13) and provide a convenient cell system to study the effects of exogenous CD155/PVR expression. Expression of CD155/PVR by 9L cells after transfection was confirmed by immunocytochemistry and Western blot (Fig. 1*A, inset*). 9L cells expressing CD155/PVR exhibited increased transwell migration towards a serum chemoattractant

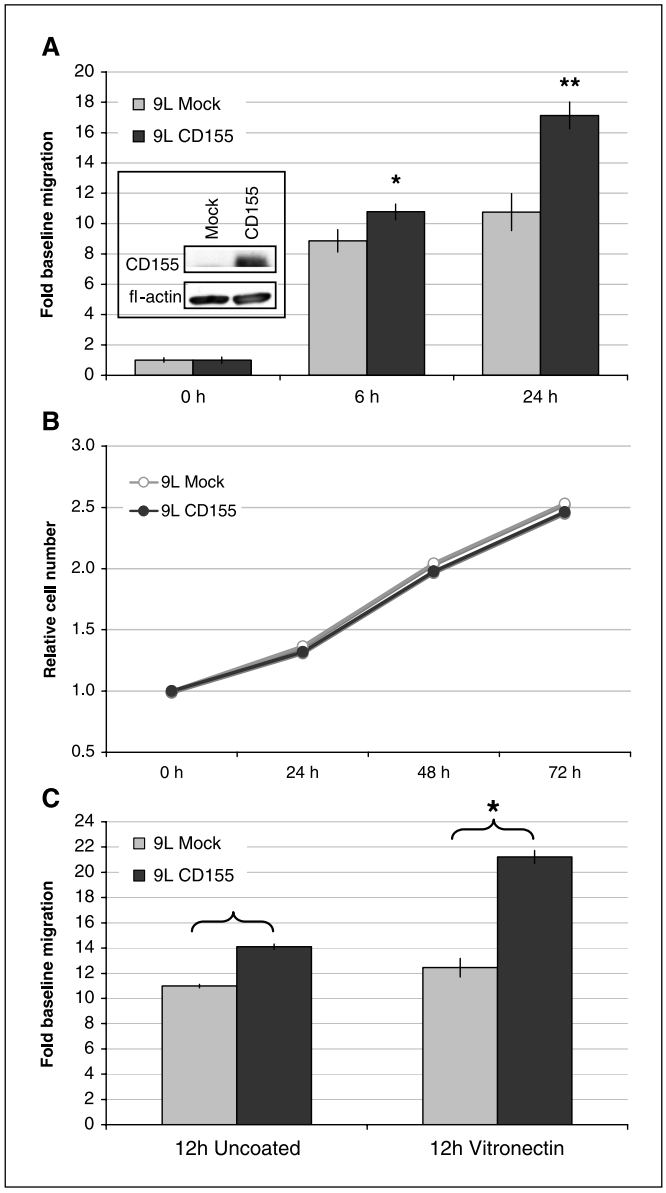

Figure 1. Expression of CD155/PVR enhances the transwell migration of 9L glioma cells. *A,* full-length CD155/PVR was transfected into 9L rat glioma cells and resulted in efficient CD155 protein expression (*inset*). Mock- and CD155-transfected 9L cells were labeled with a fluorescent tracking dye, seeded into the top chambers of a transwell migration chamber, and challenged to migrate towards a 5% FCS chemoattractant. CD155-transfected 9L cells (*dark columns*) had migrated significantly more than mock-transfected controls (*light columns*) at 6 hours (*, $P < 0.02$; $n = 6$) and 24 hours (**, $P < 0.01$; $n = 6$). *Columns,* mean; *bars,* ± SE. *B,* expression of CD155/PVR did not alter the doubling time of 9L cells. Growth curves of mock-transfected (○) and CD155-transfected (●) 9L cells, as measured by MTS assay, were indistinguishable. *C,* enhancement of migration by CD155/PVR was potentiated by vitronectin. The increase in 9L transwell migration over 12 hours due to CD155/PVR expression was significantly greater in cells migrating through vitronectin-coated membranes than through uncoated membranes (*, $P < 0.01$; $n = 9$). *Columns,* mean; *bars,* ± SE.

compared with mock-transfected controls at both 6 and 24 hours after plating (Fig. 1*A*). This difference was not due to changes in cell proliferation as the growth kinetics of 9L cells were unaffected by CD155/PVR expression over 72 hours (Fig. 1*B*). These results indicate that CD155/PVR expression is sufficient to up-regulate 9L cell migration *in vitro*. CD155/PVR has been reported elsewhere to interact with the ECM protein vitronectin (18), but the functional significance of this interaction has been unclear. To explore this link, we tested the effects of adding a thin layer of vitronectin to the top of each transwell membrane and found that this enhanced the ability of CD155/PVR to potentiate transwell migration over 12 hours (Fig. 1*C*). This result suggested a substrate dependence for CD155/PVR function.

CD155/PVR expression reduces substrate adhesion. Given the effects of CD155/PVR on cell migration, we were curious as to whether it might also play a role in substrate adhesion. We allowed mock- or CD155-transfected 9L cells to adhere to microtiter plate wells coated with vitronectin or collagen, and then challenged them to remain attached through several washes. Mock-transfected 9L cells adhered readily to vitronectin-coated wells but adhered poorly to collagen-coated wells. Expression of CD155/PVR in these cells markedly reduced their adhesion to vitronectin (Fig. 2*A*). In contrast, CD155/PVR did not alter the ability of 9L cells to adhere to collagen. These results suggest a substrate-dependent role for CD155/PVR in regulating adhesion.

CD155/PVR expression reduces cell spreading and destabilizes focal adhesions. We further explored the substrate dependence of CD155/PVR function by assessing the behavior of mock- or CD155-transfected 9L cells on glass coverslips coated with purified ECM proteins. In CD155-transfected 9L cells adhering to vitronectin, CD155/PVR localized to the cell membrane and was recruited to peripheral sites of active membrane ruffling (Fig. 2*B*), a finding consistent with our previous observations of endogenous CD155/PVR localization in a number of human cancer cell lines (11). Two hours after seeding, 9L cells expressing CD155/PVR were much smaller and less spread on a vitronectin substrate than controls (Fig. 2*C*). Mock-transfected cells developed many large focal adhesions (Fig. 2*C*) and a dense meshwork of actin stress fibers (Fig. 2*D*). In contrast, these structures were seldom observed in 9L cells expressing CD155/PVR (Fig. 2*C* and *D*). These observations were consistently made when cells were attached to either vitronectin or fibronectin (data not shown) but not in cells adhering to collagen (Fig. 2*C*). On collagen, 9L cells exhibited similar morphology and similar focal adhesion density in the presence or absence of CD155/PVR. Taken together, these results suggest a substrate-dependent role for CD155/PVR in regulating adhesion dynamics and, in particular, the formation of mature focal adhesions linked to the actin cytoskeleton.

CD155/PVR expression enhances adhesion signaling. Given the observed effect of CD155/PVR on focal adhesions, we hypothesized that CD155/PVR might alter the dynamics of focal adhesion complexes such that cells could not develop mature, stable adhesions. CD155/PVR might inhibit the formation of focal adhesions or enhance their turnover. Upon integrin binding to substrate proteins in the ECM, FAK and Src are recruited into developing focal adhesions. FAK is autophosphorylated at Tyr^{397}, exposing an SH2 binding site that recruits and activates Src via a conformational change (19). After Src phosphorylates FAK, the active Src/FAK complex recruits and activates numerous downstream targets including paxillin and p130Cas (20). Activation of paxillin has been shown to be required for the disassembly of focal adhesions (21) and efficient motility (22). Upon activation, both paxillin and p130Cas have been shown to recruit and activate Crk, which can subsequently recruit Dock and activate Rac, eventually leading to increased membrane ruffling and lamellipodial extension, processes required for efficient migration (23, 24).

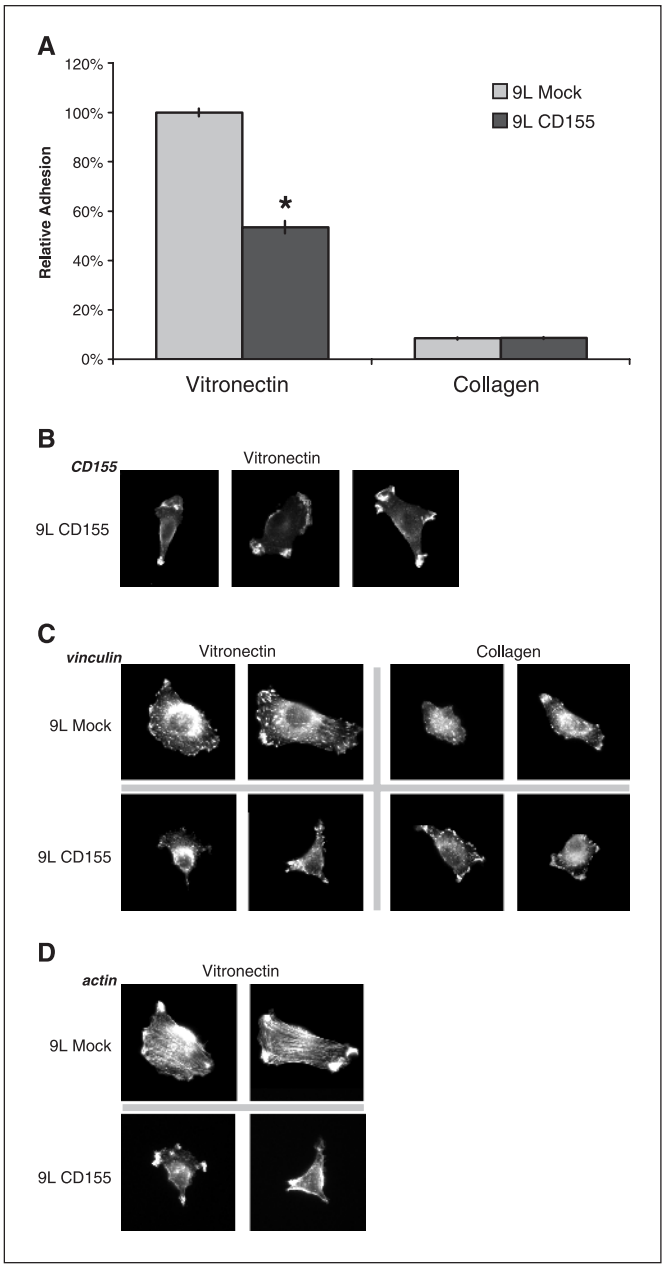

Figure 2. Expression of CD155/PVR reduces adhesion, spreading, focal adhesion density, and actin stress fiber formation in 9L cells adhering to vitronectin but not collagen. *A,* mock- or CD155-transfected 9L cells were challenged to adhere to microtiter plates previously coated with vitronectin or collagen and then washed as described in Materials and Methods. Mock-transfected 9L cells adhered strongly to vitronectin-coated wells and weakly to collagen-coated wells. Expression of CD155/PVR significantly reduced adhesion to vitronectin-coated wells (*, $P < 0.01$; $n = 5$) but did not affect adhesion to collagen. Mock- or CD155-transfected 9L cells were also challenged to adhere to glass coverslips previously coated with vitronectin or collagen and immunostained to visualize CD155/PVR, vinculin, and actin. *B,* CD155/PVR immunocytochemistry in 9L cells adhering to vitronectin. CD155/PVR localized to membrane ruffles at the periphery of CD155-transfected 9L cells. *C,* focal adhesion complexes visualized by vinculin immunocytochemistry. Mock-transfected 9L cells were well spread and exhibited many large focal adhesions on vitronectin-coated coverslips. CD155-expressing 9L cells were much less spread on vitronectin and exhibited almost no large focal adhesions. In contrast, mock- and CD155-transfected 9L cells exhibited similar size and similar number of focal adhesions after adhering to collagen. *D,* actin stress fibers as visualized by rhodamine-phalloidin. Mock-transfected cells adhering to vitronectin contained a dense meshwork of actin stress fibers. In contrast, CD155-expressing cells exhibited few, if any, stress fibers.

We asked whether expression of CD155/PVR in 9L cells might alter the activation of focal adhesion complex proteins after substrate adhesion. Mock- or CD155-transfected 9L cells were challenged to adhere to tissue culture dishes coated with ECM substrate proteins for 90 minutes before lysates were prepared. Immunoblot analysis revealed that expression of CD155/PVR resulted in increased phosphorylation of Src, FAK, paxillin, p130Cas, and Crk after adhesion to vitronectin (Fig. 3). The phosphorylation of other signaling proteins including ERK1/2 and Akt were not affected by CD155/PVR expression. For all signaling proteins analyzed, the total levels of each protein were unchanged (data not shown). Similar results were observed at 30 minutes after adhesion and in cells adhering to fibronectin (data not shown). In 9L cells adhering to uncoated tissue culture plastic, we observed much smaller changes in protein phosphorylation due to CD155/PVR expression, with the exception of paxillin, which continued to exhibit a large increase in phosphorylation after adhesion in cells expressing CD155/PVR (Fig. 3). These findings implicate CD155/PVR in modulating a signaling pathway known to control focal adhesion turnover and migration (20). Although these findings suggest a substrate-specific requirement for enhanced adhesion-induced activation of Src, FAK, p130Cas, and Crk, they also suggest a substrate-independent link between CD155/PVR and paxillin.

CD155/PVR expression enhances glioma dispersal on primary brain tissue. To establish the physiologic importance of CD155/PVR in regulating glioma dispersal, we employed an *ex vivo*

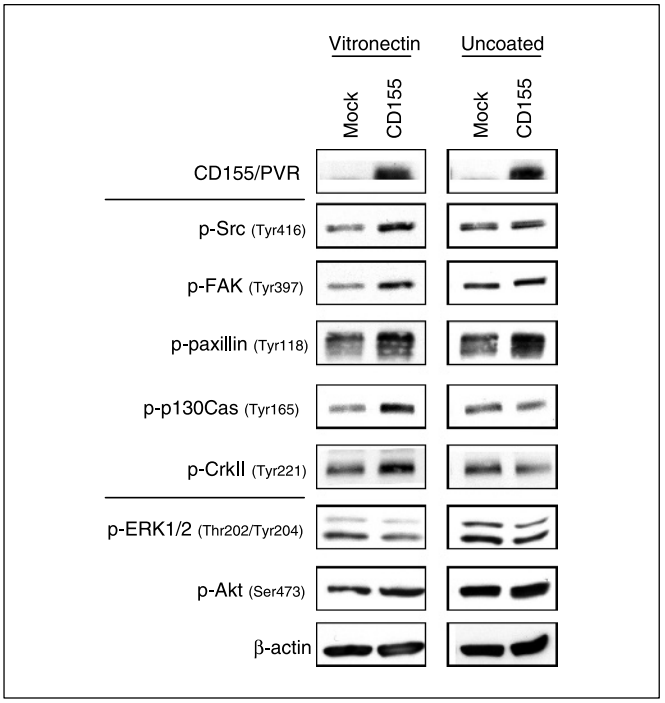

Figure 3. Expression of CD155/PVR enhances adhesion signaling in 9L cells. Mock- or CD155-transfected 9L cells were challenged to adhere to vitronectin-coated or uncoated tissue culture dishes for 90 minutes. Cell lysates were prepared and analyzed by immunoblot, using phospho-specific antibodies to candidate signaling proteins. Representative immunoblots show increased phosphorylation of Src, FAK, paxillin, p130Cas, and Crk in CD155-expressing 9L cells adhering to vitronectin compared with control cells (*left*). Phosphorylation of ERK1/2 and Akt was unaffected by CD155/PVR expression. β-Actin is shown as a loading control. In contrast, much smaller changes in the phosphorylation state of these signaling proteins due to CD155/PVR expression were observed in 9L cells adhering to uncoated tissue culture plastic, with the exception of paxillin (*right*).

brain slice assay. This assay more closely mimics *in vivo* dispersal because glioma cells must migrate on primary brain tissue (15). Mock- and CD155-transfected 9L cells were cotransfected with GFP and grown in agar to generate spheroids. These spheroids were deposited onto coronal brain sections prepared from postnatal day 2 CD-1 mice as described in Materials and Methods. The radius of dispersal from each spheroid was measured at 0, 24, 48, and 72 hours. Mock-transfected 9L control cells, which are noninvasive *in vivo* (13), did not disperse far beyond their origin in this assay. In contrast, CD155-transfected 9L cells migrated extensively from their origin (Fig. 4*A* and *B*). The extent of CD155-expressing 9L cell dispersal was nearly equivalent to that observed with wild-type rat CNS-1 glioma cells (Fig. 4*A*), which disperse readily *in vivo* (13). These results indicate that CD155/PVR expression is sufficient to convert a cell that is nondispersive on primary brain tissue into one that readily migrates.

CD155/PVR depletion inhibits the migration of human glioma cells. To test whether our findings were predictive of the role of endogenous CD155/PVR that is frequently expressed by human cancer cells, we used the A172 human glioma cell line that expresses high levels of CD155/PVR. We previously reported the generation of siRNA duplexes specifically targeting CD155/PVR (11) and found that these could efficiently deplete CD155/PVR from A172 cells (Fig. 5*A, inset*). Knockdown of CD155/PVR by RNA interference in A172 cells resulted in reduced transwell migration compared with cells transfected with a scrambled siRNA control at 6 and 24 hours after seeding (Fig. 5*A*). This result is consistent with our previous observation that knockdown of CD155/PVR by fluorophore-assisted light inactivation reduced the transwell migration of U87MG glioma cells (11). The observed inhibition of A172 migration was not due to changes in proliferation as depletion of CD155/PVR did not alter the growth kinetics of these cells over 72 hours (Fig. 5*B*). These results confirm a role for CD155/PVR in enhancing the migration of human glioma cells.

CD155/PVR depletion increases cell spreading and reduces adhesion signaling. Depletion of CD155/PVR also affected the morphology of A172 human glioma cells. On vitronectin, normal A172 cells typically take on a stellate morphology after 2 hours, with several points of active membrane ruffling stretching the cell in multiple directions (Fig. 5*C*). CD155/PVR localizes to these sites of active ruffling (Fig. 5*C, insets*). In contrast, CD155-depleted cells were more spread and exhibited a much rounder and more uniform morphology that lacked focal areas of membrane ruffling (Fig. 5*C*), suggesting that CD155/PVR might normally function to disrupt substrate adhesion in these cells.

CD155-depleted A172 cells were also found to show reduced activation of Src, paxillin, p130Cas, and Crk compared with control cells after adhesion to vitronectin (Fig. 5*D*). The activation states of Erk and Akt were unaffected by depletion of CD155/PVR. The total levels of each signaling protein examined were unchanged (data not shown). In contrast to our observation with expression of exogenous CD155/PVR in 9L cells, FAK phosphorylation at Tyr^{397} did not seem to be significantly affected by depletion of CD155/PVR in these human glioma cells. Similar results were observed using another human glioma cell line, U87MG (data not shown). This may suggest that CD155/PVR functions downstream of FAK. Residual CD155/PVR may be sufficient for FAK phosphorylation at this site or phosphorylation may be maintained by other proteins in these cells. Importantly, CD155/PVR depletion consistently inhibited the adhesion-induced activation of two established targets of the Src/FAK signaling complex, paxillin and p130Cas.

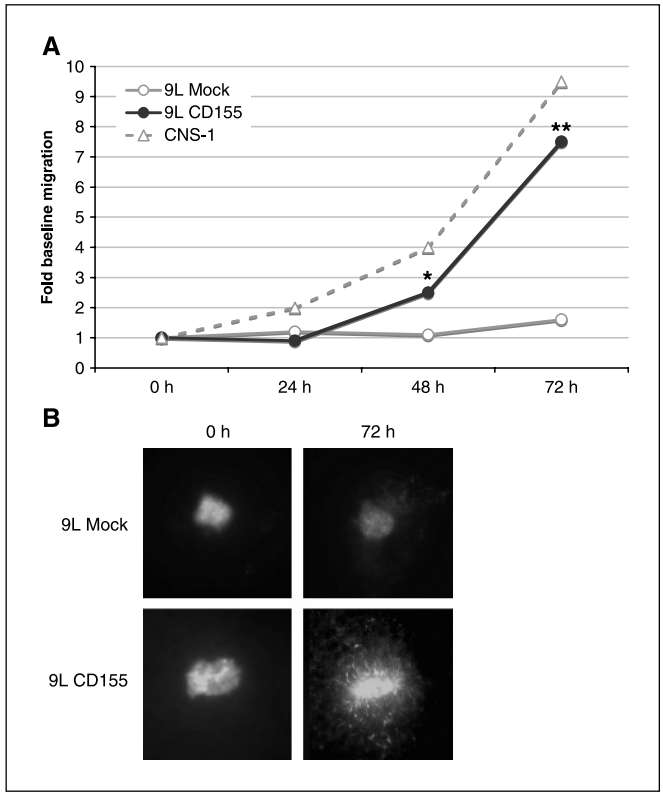

Figure 4. Expression of CD155/PVR enhances the dispersal of 9L cells on brain tissue. Spheroids of mock- or CD155-transfected 9L cells expressing GFP were seeded onto coronal sections freshly prepared from postnatal mouse brain as described in Materials and Methods. Cells were imaged at 0, 24, 48, and 72 hours and dispersal away from the origin was quantified. *A,* CD155-transfected 9L cells (●) had migrated significantly farther than mock-transfected 9L control cells (○) at 48 hours (*, $P < 0.05$; $n = 6$) and 72 hours (**, $P < 0.01$; $n = 6$). The relative migration of wild-type CNS-1 cells (*dashed line*) is shown for comparison (see text). *B,* representative images showing relative dispersal of mock- and CD155-transfected 9L cells at 0 and 72 hours after seeding on a brain slice.

Together, these results implicate CD155/PVR in regulating the migration of human glioma cells and suggest that this is accomplished by the modulation of an adhesion-induced Src/FAK/paxillin/p130Cas signaling pathway known to regulate focal adhesion turnover and membrane ruffling (20). Taken together with our earlier observations using exogenous expression of CD155/PVR in 9L cells, these results strongly implicate CD155/PVR in glioma dispersal and suggest a mechanism through which this may be accomplished.

Discussion

Our findings suggest that up-regulation of CD155/PVR expression may be one mechanism through which cancer cells can acquire an invasive phenotype. These results show that CD155/PVR can alter adhesion dynamics, resulting in fewer mature focal adhesions in cells adhering to vitronectin or fibronectin substrates, and suggest that this is achieved through modulation of a signaling pathway implicated in the turnover of focal adhesions (20). Cell migration is a highly regulated process that is required for many aspects of normal and disease biology (3–6). In normal cells, the continuous formation and disassembly of focal adhesions is a tightly controlled, dynamic process essential for migration (21), and the deregulation of this process has been linked to tumor

invasion (25). Migration plays a key role in the invasion and dispersal of tumor cells, and proteins that regulate migration are often up-regulated in these cells (5). We have now established that CD155/PVR can promote the dispersal of glioma cells both *in vitro* and in primary brain tissue. Given that we initially identified CD155/PVR in a screen for fibrosarcoma invasiveness (11), and that CD155/PVR is frequently expressed at high levels in a variety of cancers (11, 26), it seems that this protein may also play a critical role in the migration and invasion of other cancers. Our findings link cellular behavior to molecular signaling events and provide mechanistic insight into the function of CD155/PVR.

We found that CD155/PVR could enhance the adhesion-induced activation of Src, FAK, paxillin, and p130Cas, all central effectors of adhesion signaling. Upon integrin-mediated substrate adhesion, an activated Src/FAK complex activates paxillin and p130Cas (20). Paxillin is required for the disassembly of focal adhesions (17) and for efficient motility (18). Both paxillin and p130Cas have been shown to recruit and activate Crk (24, 27), which can subsequently recruit the guanine exchange factor Dock180 and activate Rac, resulting in increased membrane ruffling and lamellipodial extension, processes required for efficient migration (20, 28, 29). We determined that enhanced activation of this pathway by CD155/PVR correlated with fewer mature focal adhesions, reduced cell spreading, reduced substrate adhesion, and increased cell migration both *in vitro* and on primary brain tissue.

How might CD155/PVR regulate the Src/FAK/paxillin/p130Cas pathway? Given the substrate-dependence of the observed CD155/PVR effects, we speculate that CD155/PVR could exist in a tripartite complex with an ECM protein such as vitronectin and an integrin α/β pair (Fig. 6). CD155/PVR has been shown to interact with vitronectin (18) and we have observed a colocalization of CD155/PVR with αv-integrin in cancer cells (11). As such, vitronectin could act as an extracellular scaffold to bring CD155/PVR, integrins, and their associated proteins into close proximity. Once recruited to a nascent focal adhesion, CD155/PVR could interact directly with members of the Src/FAK/paxillin/p130Cas

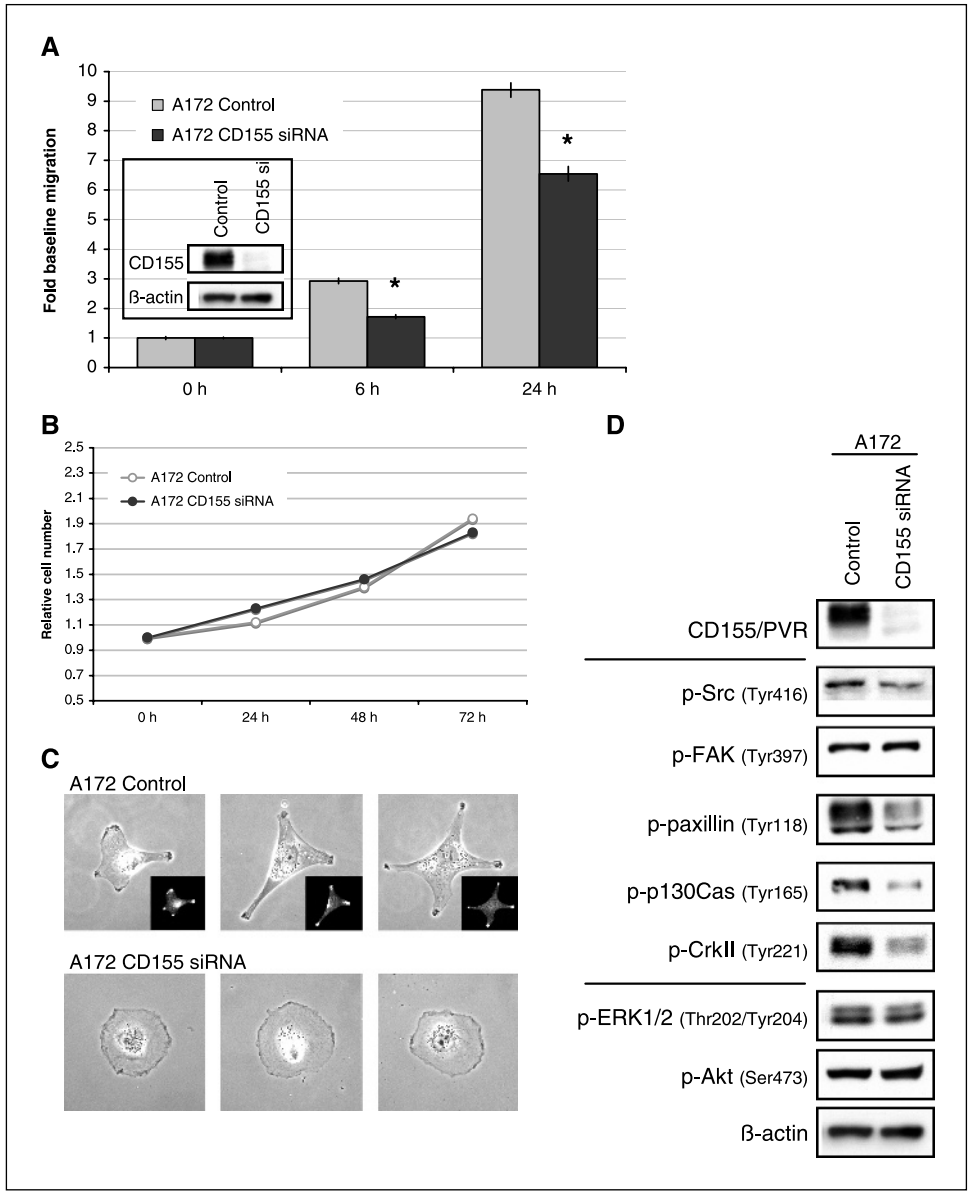

Figure 5. Depletion of CD155/PVR from A172 human glioma cells inhibits migration, alters cell morphology, and reduces adhesion signaling. *A,* knockdown of CD155/PVR by siRNA-reduced transwell migration of A172 cells. Treatment of A172 cells with CD155-specific siRNA efficiently reduced CD155/PVR protein expression (*inset*). CD155-depleted cells (*dark columns*) migrated significantly less than control cells (*light columns*) at both 6 and 24 hours (*, $P < 0.01$; $n = 12$). *Columns,* mean; *bars,* $\pm$ SE. *B,* depletion of CD155/PVR did not alter the proliferation of A172 cells. Growth curves of cells receiving control siRNA (○) and CD155-specific siRNA (●) were indistinguishable. *C,* knockdown of CD155/PVR alters the morphology of A172 cells adhering to vitronectin. Two hours after adhering to vitronectin, control cells exhibited a typical stellate morphology with CD155/PVR localizing to distal points of active membrane ruffling (*insets*). Under identical conditions, CD155-depleted cells were much more uniform in shape, exhibiting a rounder, more spread morphology, and lacking points of focused membrane ruffling. *D,* knockdown of CD155/PVR inhibits adhesion signaling in A172 cells. Control and CD155-depleted cells were challenged to adhere to vitronectin-coated tissue culture dishes for 90 minutes. Lysates were prepared and analyzed by immunoblot, using phospho-specific antibodies to candidate signaling proteins. Representative immunoblots show decreased phosphorylation of Src, paxillin, p130Cas, and Crk in A172 cells in which CD155/PVR had been depleted. The phosphorylation levels of ERK, Akt, and FAK were unaffected (see text).

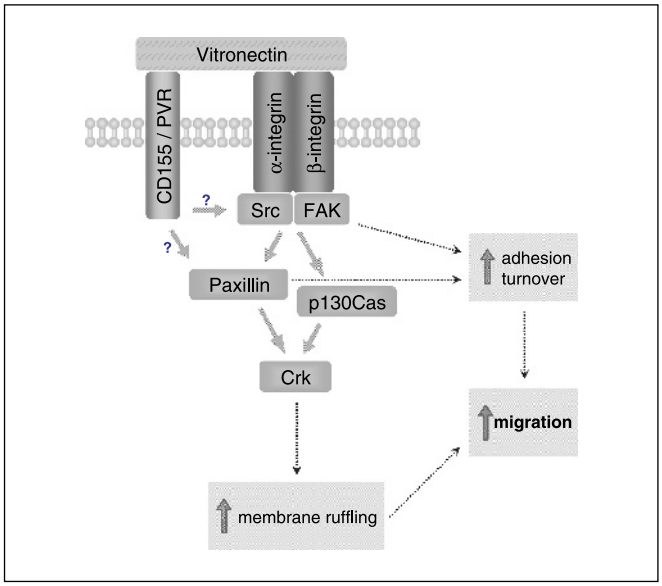

Figure 6. Src/FAK/paxillin/p130Cas pathway model. Our data suggests a role for CD155/PVR in enhancing the adhesion-induced activation of paxillin and p130Cas. CD155/PVR could be recruited to focal complexes through its interaction with ECM substrates such as vitronectin, resulting in a tripartite complex including an α/β integrin pair. CD155/PVR could then act directly on proteins in the focal complex such as paxillin or p130Cas, or it could recruit other proteins into the complex. Reports in the literature predict that enhanced activation of paxillin would result in increased turnover of focal adhesions, whereas enhanced activation of p130Cas would result in increased membrane ruffling and lamellipodial extension. Both effects would contribute to increased cell migration. Because CD155/PVR expression is commonly up-regulated in cancer, this may be a common pathway through which cancer cells can acquire an invasive phenotype (see text).

signaling cascade or could recruit other proteins into focal complexes.

Our finding that depletion of CD155/PVR from human glioma cells reduced the adhesion-induced activation of Src, paxillin, and p130Cas, but not FAK, favors a link between CD155/PVR and Src. Recently, the protein phosphatase Shp2 has been reported to interact with CD155/PVR (30), raising the possibility that Shp2 recruited to a focal complex could alter the phosphorylation state of complex members. For example, dephosphorylation of Src at Tyr^{527} is required to relieve autoinhibition and permit its maximal activation (31). Recruitment of Shp2 by CD155/PVR into a developing focal adhesion could increase Src activity by relieving repression and subsequently lead to enhanced activation of Src/FAK targets including paxillin and p130Cas, ultimately resulting in increased motility.

Alternatively, CD155/PVR could interact with downstream focal complex proteins such as paxillin, an idea supported by our observation that paxillin phosphorylation was highly up-regulated by CD155/PVR expression in a manner independent of substrate availability (Fig. 3). Although we have been unable to coimmunoprecipitate paxillin or Shp2 with CD155/PVR, the possibility of an indirect association remains. Enhanced activation of paxillin or increased recruitment of paxillin into nascent focal adhesions would likely result in increased focal adhesion turnover and enhanced motility (21, 22). Future work will seek to define the interactions between CD155/PVR and focal complex members to better understand how CD155/PVR regulates adhesion signaling.

In summary, we have now extended our earlier studies by demonstrating a key role for CD155/PVR in regulating glioma dispersal. In addition, we have identified a novel mechanism through which CD155/PVR can regulate cancer cell migration. We propose that CD155/PVR may control migration in a substrate-dependent manner by decreasing the stability of focal adhesions at the cell-substrate interface, resulting in cells that are less adherent and more motile. CD155/PVR is up-regulated in many cancers (11, 26, 32). Thus, up-regulation of CD155/PVR expression may be a common pathway through which cancer cells can acquire a more invasive or dispersive phenotype, a requirement for cancer progression (33). CD155/PVR is not normally expressed in the adult brain but has been detected at high levels in glioma patient tissue by immunohistochemistry (11) and microarray analyses (34). Given its accessibility as a surface protein and its differential tissue expression, CD155/PVR may have potential as a therapeutic target for drugs to limit the dispersal of brain tumor cells or the invasion of other cancer cell types.

Acknowledgments

Received 5/31/2005; revised 9/12/2005; accepted 9/23/2005.

Grant support: National Cancer Institute grant (CA-81668; D.G. Jay), Goldhirsh Foundation grant (D.G. Jay), and a training grant from the NIH (DK-07542; K.E. Sloan).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank R. Hauptschein, L. Liscum, and S. Schonhoff for helpful discussions, and E. Wimmer for kindly providing the polyclonal antibody to CD155/PVR.

References

1. Demuth T, Berens ME. Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 2004;70:217–28.
2. Louis DN, Posner JB, Jacobs T, Kaplan R. Report of the Brain Tumor Progress Review Group; 2000.
3. Guo W, Giancotti FG. Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 2004;5:816–26.
4. Ridley AJ, Schwartz MA, Burridge K, et al. Cell migration: integrating signals from front to back. Science 2003;302:1704–9.
5. Bogenrieder T, Herlyn M. Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 2003;22:6524–36.
6. Hsu MY, Meier F, Herlyn M. Melanoma development and progression: a conspiracy between tumor and host. Differentiation 2002;70:522–36.
7. Horwitz R, Webb D. Cell migration. Curr Biol 2003;13:R756–9.
8. Cavallaro U, Christofori G. Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer 2004;4:118–32.
9. Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002;2:91–100.
10. Juliano RL. Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Annu Rev Pharmacol Toxicol 2002;42:283–323.
11. Sloan KE, Eustace BK, Stewart JK, et al. CD155/PVR plays a key role in cell motility during tumor cell invasion and migration. BMC Cancer 2004;4:73.
12. Mendelsohn CL, Wimmer E, Racaniello VR. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell 1989;56:855–65.
13. Jaworski DM, Kelly GM, Piepmeier JM, Hockfield S. BEHAB (brain enriched hyaluronan binding) is expressed in surgical samples of glioma and in intracranial grafts of invasive glioma cell lines. Cancer Res 1996;56:2293–8.
14. Giard DJ, Aaronson SA, Todaro GJ, et al. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973;51:1417–23.
15. Ohnishi T, Matsumura H, Izumoto S, Hiraga S, Hayakawa T. A novel model of glioma cell invasion using organotypic brain slice culture. Cancer Res 1998;58:2935–40.
16. Gromeier M, Solecki D, Patel DD, Wimmer E. Expression of the human poliovirus receptor/CD155 gene during development of the central nervous system: implications for the pathogenesis of poliomyelitis. Virology 2000;273:248–57.
17. Ida-Hosonuma M, Sasaki Y, Toyoda H, et al. Host

range of poliovirus is restricted to simians because of a rapid sequence change of the poliovirus receptor gene during evolution. Arch Virol 2003;148:29–44.
18. Lange R, Peng X, Wimmer E, Lipp M, Bernhardt G. The poliovirus receptor CD155 mediates cell-to-matrix contacts by specifically binding to vitronectin. Virology 2001;285:218–27.
19. Parsons JT. Focal adhesion kinase: the first ten years. J Cell Sci 2003;116:1409–16.
20. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol 2005;6:56–68.
21. Webb DJ, Donais K, Whitmore LA, et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004;6:154–61.
22. Subauste MC, Pertz O, Adamson ED, Turner CE, Junger S, Hahn KM. Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. J Cell Biol 2004;165:371–81.
23. Hsia DA, Mitra SK, Hauck CR, et al. Differential regulation of cell motility and invasion by FAK. J Cell Biol 2003;160:753–67.
24. Cho SY, Klemke RL. Purification of pseudopodia from polarized cells reveals redistribution and activation of Rac through assembly of a CAS/Crk scaffold. J Cell Biol 2002;156:725–36.
25. Carragher NO, Frame MC. Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. Trends Cell Biol 2004;14:241–9.
26. Masson D, Jarry A, Baury B, et al. Overexpression of the CD155 gene in human colorectal carcinoma. Gut 2001;49:236–40.
27. Schaller MD. Paxillin: a focal adhesion-associated adaptor protein. Oncogene 2001;20:6459–72.
28. Feller SM. Crk family adaptors-signalling complex formation and biological roles. Oncogene 2001;20:6348–71.
29. Valles AM, Beuvin M, Boyer B. Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells. J Biol Chem 2004;279:44490–6.
30. Oda T, Ohka S, Nomoto A. Ligand stimulation of CD155α inhibits cell adhesion and enhances cell migration in fibroblasts. Biochem Biophys Res Commun 2004;319:1253–64.
31. Larsen M, Tremblay ML, Yamada KM. Phosphatases in cell-matrix adhesion and migration. Nat Rev Mol Cell Biol 2003;4:700–11.
32. Merrill MK, Bernhardt G, Sampson JH, Wikstrand CJ, Bigner DD, Gromeier M. Poliovirus receptor CD155-targeted oncolysis of glioma. Neuro-oncol 2004;6:208–17.
33. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
34. Gromeier M, Lachmann S, Rosenfeld MR, Gutin PH, Wimmer E. Intergeneric poliovirus recombinants for the treatment of malignant glioma. Proc Natl Acad Sci U S A 2000;97:6803–8.