Changes in the Fibronectin-specific Integrin Expression Pattern Modify the Migratory Behavior of Sarcoma S180 Cells In Vitro and in the Embryonic Environment

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Abstract. The molecules that mediate cell–matrix recognition, such as fibronectins (FN) and integrins, modulate cell behavior. We have previously demonstrated that FN and the $\beta1$-integrins are used during neural crest cell (NCC) migration in vitro as well as in vivo, and that the FN cell-binding domains I and II exhibit functional specificity in controlling either NCC attachment, spreading, or motility in vitro. In the present study, we have analyzed the effect of changes in the integrin expression patterns on migratory cell behavior in vivo. We have generated, after stable transfection, S180 cells expressing different levels of $\alpha4\beta1$ or $\alpha5\beta1$ integrins, two integrins that recognize distinct FN cell-binding domains. Murine S180 cells were chosen because they behave similarly to NCC after they are grafted into the NCC embryonic pathways in the chicken embryo. Thus, they provide a model system with which to investigate the mechanisms controlling in vitro and in vivo migratory cell behavior. We have observed that either the overexpression of $\alpha5\beta1$ integrin or the induction of $\alpha4\beta1$ expression in transfected S180 cells enhances their motility on FN in vitro. These genetically modified S180 cells also exhibit different migratory properties when grafted into the early trunk NCC migratory pathways. We observe that $\alpha5$ and low $\alpha4$ expressors migrate in both the ventral and dorsolateral paths simultaneously, in contrast to the parental S180 cells or the host NCC, which are delayed by 24 h in their invasion of the dorsolateral path. Moreover, the $\alpha4$ expressors exhibit different migratory properties according to their level of $\alpha4$ expression at the cell surface. Cells of the low $\alpha4$ expressor line invade both the ventral and dorsolateral pathways. In contrast, the high expressors remain as an aggregate at the graft site, possibly the result of $\alpha4\beta1$-dependent homotypic aggregation. Thus, changes in the repertoire of FN-specific integrins enable the S180 cells to exploit different pathways in the embryo and regulate the speed with which they disperse in vivo and in culture. Our studies correlate well with known changes in integrin expression during neural crest morphogenesis and strongly suggest that neural crest cells that migrate into the dorsolateral path, i.e., melanoblasts, do so only after they have upregulated the expression of FN receptors. Some of our in vivo results are at variance with what was expected, based on the in vitro behavior of the S180-transfected clones, and they demonstrate the importance of examining cell behavior in the tissue environment.

INTERACTIONS between cells and their environment play a crucial role in the control of cell behavior during embryogenesis and tissue remodeling, as well as in numerous pathological states (Hynes and Lander, 1992; Edelman et al., 1990; Horwitz and Thiery, 1994; Adams and Watt, 1993). Cell–cell and cell–matrix adhesion molecules

are important mediators of these interactions and are developmentally regulated. The ontogeny of the neural crest is one particularly striking example of exquisite regulation of cell adhesion during morphogenesis. Neural crest cells (NCC)[1] are initially integrated in the neuroepithelium before undergoing an epithelial-mesenchymal conversion, followed by extensive migration throughout the embryo to give rise to a plethora of cell types and structures (reviewed by

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1. *Abbreviations used in this paper:* FN, fibronectin; HPSG, heparan-sulfate proteoglycan; NCC, neural crest cell.

Le Douarin, 1982; Le Douarin et al., 1993). Their detachment from the neuroepithelium, subsequent migration, and cessation of migration are regulated to a considerable degree by changes in cell–cell and cell–matrix attachments (reviewed by Erickson and Perris, 1993). Numerous experiments have emphasized the role of fibronectin (FN), in particular, in the adhesion and migration of NCC in vitro and in vivo (e.g., Boucaut et al., 1984; Bronner-Fraser, 1985; Duband et al., 1986; Dufour et al., 1988).

FN is a dimeric glycoprotein comprised of two major cell-binding domains that are recognized by different receptors belonging to the integrin family (reviewed by Hemler, 1991; Hynes, 1990, 1992; Yamada, 1989). The cell-binding domain I, or the central cell-binding domain, contains the RGDS recognition motif and an additional site, which act in synergy to regulate cell adhesion, migration, and matrix assembly (Akiyama et al., 1990; Obara et al., 1988; Aota et al., 1991; Nagai et al., 1991). This central cell-binding domain is recognized by many of the integrins including $\alpha5\beta1$, $\alpha3\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, and $\alpha IIb\beta3$.

The second cell-binding domain in FN, called cell-binding domain II, is comprised of sequences located in the IIICS region, and in the adjacent heparin-binding domain II (see Fig. 4). The amino acid sequences that mediate adhesion are LDV and REDV, located in the CS1 and CS5 regions of the IIICS domain, respectively, and one IDAPS sequence located in the heparin-binding domain II. These sequences are recognized by the $\alpha4\beta1$ integrin (Garcia-Pardo et al., 1990; Guan and Hynes, 1990; Mould and Humphries, 1991; Mould et al., 1991; Komoriya et al., 1991). Sequences that interact with cell surface heparan sulfate proteoglycans, and that promote focal adhesion formation, are also found in the heparin II domain (Woods et al., 1993; Woods and Couchman, 1994).

The cell-binding domains of FN have distinct functional activities, and are used by different cell types in various combinations. We have demonstrated that NCC in vitro interact with the cell-binding domains I and II of FN through $\beta1$-containing integrins (Duband et al., 1986; Dufour et al., 1988). The CS1 sequence in cell-binding domain II is mainly used for attachment and motility, whereas the central binding site (cell-binding domain I) is used for spreading, as well as for attachment and motility. Thus, based on what is currently known about integrin specificity, NCC must use at least one of the RGDS-sensitive integrins ($\alpha5\beta1$, $\alpha v\beta1$, and $\alpha3\beta1$), as well as $\alpha4\beta1$, when they attach and migrate in tissue culture. Moreover, both of the cell-binding domains are required to obtain the maximum adhesive and migratory capabilities on FN. In contrast, fibroblasts interact preferentially with the cellular binding domain I, whereas melanoma cells, which are malignant derivatives of the NCC, use primarily the cellular binding domain II (Humphries et al., 1986, 1987).

Although we know a great deal about integrin function from in vitro studies, little is known about how the integrins are involved in migratory events in the embryo. Indeed, our understanding of in vivo events is complicated by the fact that tissue-derived cells frequently alter the complement of proteins produced, including integrins, even after only a short exposure to culture conditions. For example, normal human melanocytes express a different repertoire of integrins in situ than when isolated and cultured (Zambruno et al., 1993).

Thus, the relevance of the many tissue culture studies to the behavior of the same cells during embryogenesis is unclear.

One way to address this issue would be to alter the expression of specific integrins one at a time and to test the effect of these alterations on cell behavior in the embryo. It has not yet been possible to alter stably the repertoire of integrins on early migratory neural crest cells. However, the mouse sarcoma S180 cell line has been shown to behave similarly to neural crest cells when grafted into the NCC migratory pathways at the thoracic level (Erickson et al., 1980). Therefore, we have chosen S180 cells as a model system in which to modify the integrin expression pattern and subsequently test cell behavior in the embryonic environment.

In this report, we have focused on the $\alpha4\beta1$ and $\alpha5\beta1$ integrins, since these interact specifically with the two distinct adhesion domains of FN known to be crucial for NCC motility in vitro. We have generated stable transfectants of the parent S180 cell line that express the human $\alpha4$ or $\alpha5$ chain. Expression of either the $\alpha4$ or the $\alpha5$ chain at the surface of S180 cells promotes an increase in cell attachment, spreading, and motility on FN fragments containing the cell-binding domain II or on intact FN, respectively. These genetically modified S180 cells exhibit different migratory properties when grafted into the early crest migratory pathways. We demonstrate that the parent S180 cells migrate into the anterior half of the somite (the ventral pathway), but they do not invade the space between the ectoderm and dermamyotome (the dorsolateral path) during the first 24 h in the embryo, thus mimicking the behavior of the endogenous neural crest cells. The $\alpha5$ expressors migrate in both the ventral and dorsolateral paths simultaneously, in contrast to the host neural crest cells, which are delayed in their invasion of the dorsolateral path until another 24–26 h (Serbedzija et al., 1990; Erickson et al., 1992; Oakley et al., 1994). Moreover, the transfected cells exhibit long processes, in contrast to the rounded morphology and short processes of the parent cell line. Finally, the $\alpha4$ expressors exhibit different migratory properties according to their level of $\alpha4$ expression at the cell surface. Cells of the low expressor line invade both the ventral and dorsolateral pathways, but they are generally spread on the basement membranes and are infrequently found in the interstitial matrix. In contrast, the high expressor cells remain as an aggregate at the site of grafting, even though they exhibit obvious cell processes. Thus, the complement of integrins expressed enables the S180 cells to exploit different pathways in the embryo, as well as to regulate the speed with which they disperse. Some of these results are contrary to what was expected, based on the behavior of the same cells in culture, and they demonstrate the importance of examining cell behavior in the tissue environment.

Materials and Methods

Antibodies and Reagents

Dr. G. Tarone (Università di Torino, Italy; DeFilippi et al., 1991a,b; Arcangeli et al., 1993). Control rat IgG and mouse IgG were purchased from Sigma Immunochemicals (St. Louis, MO). Peptides H120, H89, H95, and H0 (see Fig. 4) corresponding to different spliced forms of the IIICS region of human fibronectin (herein referred to as IIICS-spliced fragments) were made as described in Mould et al. (1994). The pFNRα5 eukaryotic expression vector coding for the human α5 chain of integrin was a generous gift of Drs. F. G. Giancotti and E. Ruoslahti (Cancer Research Institute, La Jolla, CA: Giancotti and Ruoslahti, 1990).

Construction of the Human α4 Expression Vector

The pKSα4 cloning vector coding for the full-length α4 cDNA was a generous gift from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA; Takada et al., 1989). A KpnI-AflIII insert (KA insert) was amplified from pKSα4 by PCR using the (5'-TCAGCACATGTTCATTAATCATCATTG-CTTTT-3') and the (5'-TAATGGTACCAAATTCTTTT-3') oligonucleotides (antisense and sense, respectively) and digested with KpnI and AflIII restriction enzymes. This was done to create an AflIII restriction site after the stop codon of the α4 cDNA sequence. The SalI-KpnI insert (SK insert) of the pKSα4 was purified from an agarose gel. The pFNα5 expression vector was digested with SalI and NcoI restriction enzymes to eliminate the α5 cDNA translated sequence. Both the SK insert and PCR-primed KA insert from pKSα4 were inserted into the SalI-NcoI sites of the pFNRα5 vector to obtain the expression vector pFNα4 coding for the human α4 integrin subunit. In this construct, the coding sequence for the human α4 chain was linked to the 3' noncoding sequence of the α5 subunit to abolish possible regulatory effects of different 3' noncoding sequences on the mRNA stability or subunit expression. The integrity of the pFNRα4 construct was confirmed by sequence analysis.

Cell Culture and Transfection

The sarcoma S180 cells used in this study are a subclone derived by Dr. K. Yamada (National Institutes of Health, Bethesda, MD) from the original parental cell line, which was obtained from American Type Culture Collection (Rockville, MD). These cells were selected for their inability to assemble an FN matrix at their cell surface. The cells were cultured in DME supplemented with 10% (vol/vol) FCS (Polylabo, Strasbourg, France). Sarcoma cells were stably transfected with either the pFNRα5 or pFNRα4 expression vector by the phosphate calcium method according to Gorman (1985). The cells were cotransfected with pAG60, which codes for the neomycin selection marker. Neomycin-resistant cells were analyzed for the expression of human α5 or human α4 by FACScan® analysis (Becton Dickinson) using the mAb16 and L25 monoclonal antibodies, respectively. The L27 expressor clone for human α5 chain and the P22 control neomycin-resistant clone were chosen for further in vitro and in vivo analysis. Stable expressor clones for the human α4 chain that were selected initially showed heterogenous levels of expression of this chain at their cell surface. They were subsequently subcloned to obtain a homogenous population. The D711 (high) and D828 (low) expressor clones and the B8 control neomycin-resistant clone were subsequently chosen for further in vitro and in vivo analysis. The stability of the expression level of human integrin α chain in the transfectants was verified by flow cytometry every several passages.

Cell Surface Labeling and Immunoprecipitation

Cells were surface-labeled with ^{125}I (Amersham Corp., Arlington Heights, IL) as previously described (Rossino et al., 1990). Briefly, cells were released from culture dishes by 5 mM EDTA treatment in PBS and washed three times after centrifugation with culture medium. Cells were resuspended in PBS containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and labeled with 1 mCi of ^{125}I in the presence of lactoperoxidase (200 μg/ml; Sigma) and H_2O_2 (0.002%). To determine which integrins are expressed in S180 and transfected clones, labeled cells were treated as previously described (Defilippi et al., 1991a). Briefly, labeled cells were washed with cold PBS and extracted for 20 min at 4°C with 0.5% Triton X-100 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml of aprotinin (all from Sigma). After centrifugation at 10,000 g for 10 min, extracts were incubated with specific antibodies conjugated to protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h at 4°C with gentle agitation. After washing, bound material was eluted by boiling the beads in 1% SDS, separated using 6% SDS-PAGE, and autoradiographed. The autoradiogram was digitized using a video camera (612F Data Copy) linked to an acquisition module and a graphic control unit, which were designed by the staff of the Computer Science Laboratory of the Ecole Normale Supérieure. A band profile was obtained for each α subunit, and these were subsequently analyzed using the HERMeS software (Vincent et al., 1986) to quantify the intensity of each band (arbitrary units).

Assays for Cellular Adhesion and Spreading

Cell adhesion of the L27, P22, B8, D711, and D828 clones to FN-coated substrata and to IIICS-containing fragments (H120, H89, H95, and H0; see Fig. 4) were tested as described in Dufour et al. (1988). Cellular attachment and spreading assays were performed on bacteriological petri dishes. 20-μl droplets of hpFN (10 μg/ml) or IIICS-spliced fragments (2–20 μg/ml in PBS) were deposited on bacteriological dishes and incubated at 37°C for 1 h, followed by a 30-min incubation with 3 mg/ml solution of BSA in PBS (previously heat inactivated for 3 min at 80°C). After extensive washing, the substrata were maintained in PBS until used. Cells were harvested with a solution of 0.25% trypsin and 1 mM EDTA in PBS (GIBCO BRL, Gaithersburg, MD) for 5 min at 37°C. After centrifugation at 1,200 rpm, cells were incubated for 1 h in DME supplemented with 10% (vol/vol) FN-depleted fetal calf serum to allow recovery from trypsin treatment. 2,000 cells were deposited on the precoated substrata. The dishes were incubated at 37°C for various times, rinsed with PBS to remove the nonadherent cells, and fixed in 3.7% formaldehyde in PBS. Attached and spread cells (any cell that had extended cell processes and had begun to flatten) were counted with an inverted phase contrast microscope (E. Leitz, Inc., Rockleigh, NJ). At least three independent experiments were done for each substratum and for each of the clones tested.

Specificity of adhesion was tested by challenging the cells with antifunctional antibodies against the α5 or α4 subunit. Transfected cells were plated on either FN or the IIICS-spliced fragments for 30 min. The medium was subsequently rinsed, and adherent cells were incubated in the presence of monoclonal antibodies mAb16 or L25 at various concentrations for 30 min. The cultures were then rinsed with PBS to remove nonadherent cells, fixed with 3.7% formaldehyde in PBS, and counted as described above. For all compared values, the statistical significance was determined using a χ_1^2 test, with a level of confidence >95%.

Assay for Cell Migration

Speed of movement was assessed using two different assays. In the first, the rate of individual cell movement was analyzed using time-lapse videomicroscopy. Glass coverslips were coated with hpFN (30 μg/ml) or with IIICS fragments (6 μg/ml), followed by an incubation with 3 mg/ml of BSA. S180-transfected cells were deposited on these substrata in DME containing 10% FN-depleted fetal calf serum. For time-lapse videomicroscopy, cultures were maintained at 37°C in a plexiglass chamber on the stage of a Leitz Laborlux phase-contrast microscope and their behavior was recorded using a time-lapse videorecorder (Panasonic; Matsushita Electric Industrial Co., Ltd., Japan). Paths of migration of 20 cells were traced from the video monitor onto transparent paper. This was done for two independent experiments. The total distance of migration was measured and the speed of locomotion was calculated. The results of cells migrating on intact FN are expressed as an increase in speed of movement of the transfected cells when compared to that of the control. This increase is calculated as the ratio between the mean speed of locomotion calculated for the α-expressor clones (tv) and the mean speed calculated for the control clones (cv).

In the second test, cell migration was quantitated by determining the extent of migration from agarose droplets using a modification of the method described by Varani et al. (1978). Briefly, cells were harvested as described previously and resuspended at 33 × 10⁶ cells/ml in bicarbonate-free DME containing 25 mM Hepes, pH 7.4 (GIBCO BRL), and 0.2% agarose (Sea Plaque; FMC Corp. BioProducts, Rockland, ME) maintained at 39°C. The cells were plated as 0.8-μl droplets on flat-bottom wells (microtest III flexible 96-well assay plates, nontissue culture treated; Falcon Plastics, Cockeysville, MD) that were precoated with 10 μg/ml hpFN. After the agarose was gelled, 100 μl of culture medium was added with care to avoid detachment of droplets. To specifically inhibit attachment mediated by α5β1, either the monoclonal antibody mAb16 or control rat IgG was added to the culture medium at a final concentration of 0.1 mg/ml. Cells were cultured at 37°C for various times and then fixed with 3.7% formaldehyde. After rinsing, the cells were stained with 1% crystal violet in ethanol, destained in water, and air dried. The cultures were photographed, and total area of cell outgrowth was measured. The results are expressed in arbitrary units. For all the compared values, the statistical significance was determined using a χ_1^2 test, with a level of confidence >95%. In all the figures, the error bars represent SD.

Labeling of Cells for In Vivo Analysis

The cells to be grafted were cultured in 30-ml tissue culture flasks (Corning Inc., Corning, NY) in DME until 50% confluent. Just before grafting, the medium was removed and replaced with 4 ml of culture medium containing 24 μl of Fluoro-gold stock solution (2% in Locke's saline; Fluorochrome Inc., Englewood, CO). The cells were incubated with the Fluoro-gold for 4 h, at which time the labeling medium was removed and the cells washed three times in Hanks' balanced salt solution (GIBCO BRL) to remove all residual label. The cells were rinsed with 2.5% Pancreatin in PBS (GIBCO BRL) and the flasks gently rapped against the palm of the hand to detach the cells from the substratum. Culture medium was added to the flasks to stop the tryptic action and to suspend the cells. The cell suspension was gently centrifuged, the cell pellet resuspended in fresh culture medium, and incubated for 2 h in a 37°C water bath for the cells to reconstitute membrane proteins. The cells were then repelleted by centrifugation, the pellet transferred to a petri dish containing sterile Locke's saline, and lightly stained with a few drops of 0.2% neutral red.

Grafting Experiments

White Leghorn chicken embryos were incubated at 38°C until they had developed 20–23 somite pairs, corresponding to stages 13–14 (according to the criteria of Hamburger and Hamilton, 1951). The eggs were cracked into a finger bowl of sterile Locke's saline and the blastoderms were cut out and transferred with a wide-mouth pipette to an agar-coated dish. The embryo was oriented dorsal side–up and spread so that it lay flat and the somites could be readily counted. A small portion of the cell pellet (50–100 cells) was pipetted onto the embryo with a Spemann pipette and gently maneuvered with a tungsten needle through a slit in the ectoderm made between the neural tube and somite at the axial level five somites from the last formed somite. This is the axial level where neural crest cells have just migrated off the dorsal surface of the neural tube (cf. Loring and Erickson, 1987). To aid in the relocation of the graft site after sectioning, a small amount of blood charcoal was inserted into the center of the somite two segments posterior to the graft site. The embryo was then carefully picked up with a ring of filter paper and suspended on a nucleopore filter (polycarbonate, 8 μm pores) over the well of an organ culture dish (Falcon) that was filled with L15 medium (GIBCO BRL) supplemented with 10% FCS. The embryos were returned to the 38°C incubator for an additional 18 h and then fixed.

Histology

Embryos were fixed in 4% paraformaldehyde for 2 h, washed with PBS, and a region of the embryo six somites in length encompassing the graft cut out with tungsten needles. Embryo pieces were washed for 3 h in 3% BSA in PBS and then incubated overnight at 4°C in the HNK-1 antibody (hybridoma ascites; 1:250 in PBS/BSA) to label the neural crest cells. The unbound antibody was washed out during a 3-h incubation in 0.5% BSA in PBS, and the specimens were immersed overnight in the secondary antibody (rhodamine-conjugated goat anti–mouse whole antibody, 1:50 in PBS; Cappell Laboratories, Cochranville, PA). After several rinses in PBS the specimens were postfixed in 0.1% paraformaldehyde for 1 h, dehydrated in acetone, and embedded in Spurr's plastic. The specimens were sectioned at 3 μm on a Histodiamond knife from Diatome U.S. (Fort Washington, PA) using an ultramicrotome (MT-2; Sorvall Instruments, Wilmington, DE), and the serial sections were collected on glass slides in individual drops of water. After the sections dried to the slide, they were mounted in 70% glycerol containing 0.1 M NaHCO$_3$, pH 7.8, and 2% n-propyl gallate as an antioxidant, and were viewed with a Leitz Dialux 20 microscope equipped with epifluorescence and a Vario Orthomat camera.

Results

### Cell Transfections and Integrin Pattern Expression at the Cell Surface

At least in vitro, NCC use different members of the integrin family, apparently including α4β1 and α5β1, to adhere to and migrate on FN (Dufour et al., 1988). To study the potential role of these two integrins in in vivo migration, we have modified their expression in S180 cells, since these cells are
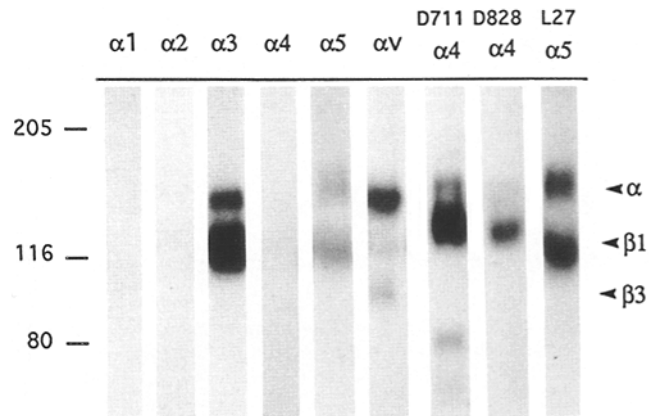
Figure 1. Repertoire of integrins expressed by the parental S180 cells and transfected clones. [125]I surface-labeled extracts of parental S180 cells were immunoprecipitated with polyclonal antibodies directed against the cytoplasmic sequence of α1, α2, α3, α4, α5, and αv integrin chains. [125]I surface-labeled extracts of high (*D711*) and low (*D828*) α4-expressor cells were immunoprecipitated with a polyclonal antibody directed against the α4 integrin chain. Similarly, ^{125}I surface-labeled cells of the α5 expressor clone L27 were immunoprecipitated with a polyclonal antibody directed against the α5 integrin chain. Note that the S180 cells do not express detectable levels of α1, α2, and α4 chains. These cells express high levels of α3 and αv and low levels of the α5 integrin chain. The β1 chain coprecipitates with the α3 and α5 chains; αv coprecipitates with both the β1 and β3 chains. The β5 subunit was not detected in the αv immunoprecipitate. There is a fourfold difference in expression of the α4 integrin chain between the D711 and D828 cells, as measured by scanning autoradiography. The lower band detected at 80 kD corresponds to a proteolytic cleavage product of the α4 chain, as previously observed (Teixidó et al., 1992). The L27 cells express high levels of the α5β1 integrin at their surface. A fivefold increase in the level of the α5 chain in L27 cells was measured by scanning the autoradiograph. Electrophoretic mobility of high molecular mass markers (kD) is indicated on the vertical axes.

known to mimic some of the migratory behavior of early migrating trunk NCC (Erickson et al., 1980). Before transfection, it was essential to determine the prevalence of the various integrins expressed at the surface of S180 cells. We performed surface labeling and immunoprecipitation of cell extracts with different polyclonal antibodies specific for α1, α2, α3, α4, α5, and αv subunits (Arcangeli et al., 1993; De Filippi et al., 1991a,b). As shown in Fig. 1, control S180 cells do not express detectable levels of α1, α2, or α4 chains. They express high levels of the α3 chain and low levels of α5. The β1 chain coprecipitates with both the α3 and α5 subunits. S180 cells express high levels of the αv chain, which coprecipitates with the β1 or β3 subunits. We could not detect the β5 chain (which can also associate with αv) in the immunoprecipitates.

Since the S180 cells produce low levels of α5β1 and virtually no α4β1, we cotransfected S180 cells with pFNα4 or pFNα5 expression vectors, which code for the human α4 and α5 integrin chains, respectively, and with the pAG60 vector coding for neomycin resistance. After selection, resistant clones were immunolabeled with the L25 or mAb16 monoclonal antibodies, which are directed against the human α4 and α5 chain, respectively, and were subsequently

A

B8

D828

D711

relative cell number

B

P22

L27

log fluorescent intensity
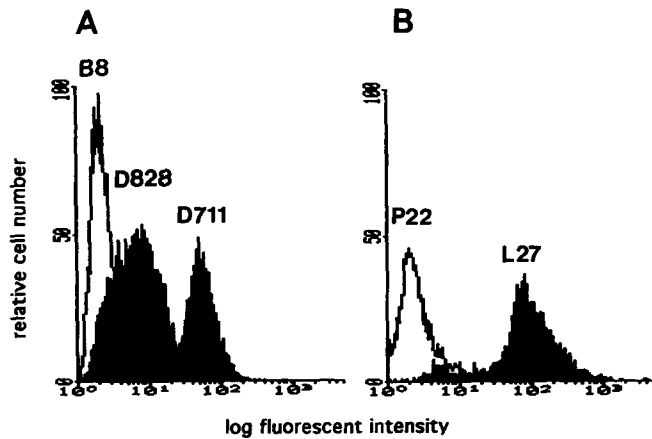
Figure 2. Flow cytometry was used to quantitate the expression of human α4 and α5 integrin chains at the surface of mouse S180 transfected cells. The abscissa represents the logarithm of the fluorescence intensity, and the ordinate represents the number of cells at each level of intensity. (*A*) Histogram obtained after immunolabeling of two pFNRα4-transfected clones with L25 monoclonal antibody specific for the human α4 chain. The control neomycin-resistant clone B8 (*unfilled peak*) does not express the human α4 integrin chain. The two expressor clones D828 and D711 (*filled peaks*) express moderate and high levels of human α4 chain, respectively, at their cell surface. (*B*) Histogram obtained after immunolabeling of a pFNRα5-transfected clone with mAb16 monoclonal antibody directed against the human α5 chain. The P22 control neomycin-resistant clone (*unfilled peak*) does not express the human α5 integrin chain. The L27 cells (*filled peak*) express high levels of the human α5 integrin chain.

analyzed by flow cytometry (Fig. 2). Initially, the clones selected for α4 expression exhibited heterogeneous levels of this subunit at the cell surface. Subcloning allowed us to select for low and high human α4 expressors, named D828 and D711 (Fig. 2 *A*). The D828 cells contain low amounts of α4β1, whereas the D711 cells express four times as much α4β1 as the D828 cells (Fig. 1). These clones were used for further analysis, as well as the B8 control neomycin-resistant clone. The highest expressor clone obtained after stable transfection with pFNα5, called L27, expressed five times the level of α5β1 integrin as the parent S180 line (Figs. 1 and 2 *B*). L27 and a control neomycin-resistant clone, P22, were selected for further analysis. All the α4 and α5 expressor cells, as well as the control cells, expressed the same level of α3 and αv integrin chain at their cell surface, as revealed by a flow cytometry analysis (data not shown).

In Vitro Attachment and Spreading of α5 and α4 Expressor S180 Clones

Functional activity resulting from an increased expression of α4 or α5 integrin chain at the surface of transfected S180 cells was first tested in attachment and spreading assays on specific substrata. The L27 (α5 expressor) cells and the control P22 cells were tested on an FN-coated substratum. As shown in Fig. 3 *A*, 56.3% of the P22 cells are able to adhere and 29.5% to spread on an FN-coated substratum in <20 min. Cells of the L27 clone, which express five times the amount of the α5β1 integrin at their cell surface, are able to interact more efficiently with the FN-coated substratum; 85.7% of

L27 cells are attached and 67.5% are spread after 20 min. The percentage of cells that are attached increases with time, and by 120 min, there is no significant difference in the percentage of attached cells between the two cell lines (92% of P22 attached vs 98% of L27 cells attached).

In contrast, the percentage of cells that are spread is consistently different between the two cell lines at all time points; by 120 min, 82.6% of the P22 cells are spread, in contrast with 96% of the L27 cells. Not only do the L27 cells spread faster, but they also display a flatter morphology and greater degree of spreading (Fig. 3 *Bb*) compared to the P22 cells (Fig. 3 *Ba*). This increase in spreading can be abolished in the presence of the monoclonal antibody mAb16, which is specifically directed against the human integrin α5 chain (0.1 mg/ml; Fig. 3 *Bc*), whereas control IgG at the same concentration (Fig. 3 *Bd*) has no effect on L27 cell morphology. Thus, the change in morphology is caused by the elevated expression of α5 chain.

The S180 cells cannot assemble FN matrix at their cell surface, except at high density. We have observed that the L27 cells can initiate FN fibril formation at their cell surface, even at subconfluency, whereas control S180 cells or α4 expressor cells do not assemble FN fibrils under these conditions. L27 cells also incorporate FN into their pericellular matrix at confluency to a greater extent than control cells (data not shown).

The functional activity resulting from the cell surface expression of α4β1 integrin on cells from the D828 and D711 clones was tested in in vitro attachment and spreading assays on different spliced variants of the adhesive domain II (Fig. 4), as well as on intact FN. All of these variants contained the amino acid sequences corresponding to the 14th, 15th, 16th, and 17th type III homology domains of human FN. The 16th and 17th type III homology sequences were interrupted either by the entire IIICS domain (H120), the CS5-spliced IIICS domain (H89), the CS1-spliced IIICS domain (H95), or directly joined to each other (H0; without the IIICS domain). The H120 spliced variant corresponds to the entire cell-binding domain II of FN, whereas the REDV- or the LDV-cell-binding site is removed, in H89 and H95, respectively. The LDV and REDV sites are both missing in H0. All of these spliced variants still contain the IDAPS sequence recognized by α4β1 integrin and also heparan-sulfate-proteoglycan–binding sites located in the heparin-binding II domain.

The attachment and spreading assays were performed for 30 min on substrata made using 2- or 10-μg/ml coating solutions of each IIICS-spliced variant. The B8 control cells adhere poorly to H120-, H89-, H95-, and H0-coated substrata (<5%) and cannot spread on them (Fig. 5 *A*). This is consistent with the fact that we could not detect the α4β1 integrin at the surface of S180 cells (Fig. 1). Expression of α4β1 on D828 and D711 cells (low and high expressors, respectively) promotes cell attachment on H120-, H89-, and H95- coated substrata, which contain the LDV and/or the REDV sequence. The percentage of adherent cells depends on the concentration of the solution used to coat the substratum (Fig. 5 *A*). It is noteworthy that D711 cells attach much more efficiently (58%) than the D828 cells (17%) on the H120-coated substratum (2 μg/ml). Similar values were observed on the H89-coated substratum. In contrast, these cells cannot adhere to the H95- and H0-coated substrata (2 μg/ml).
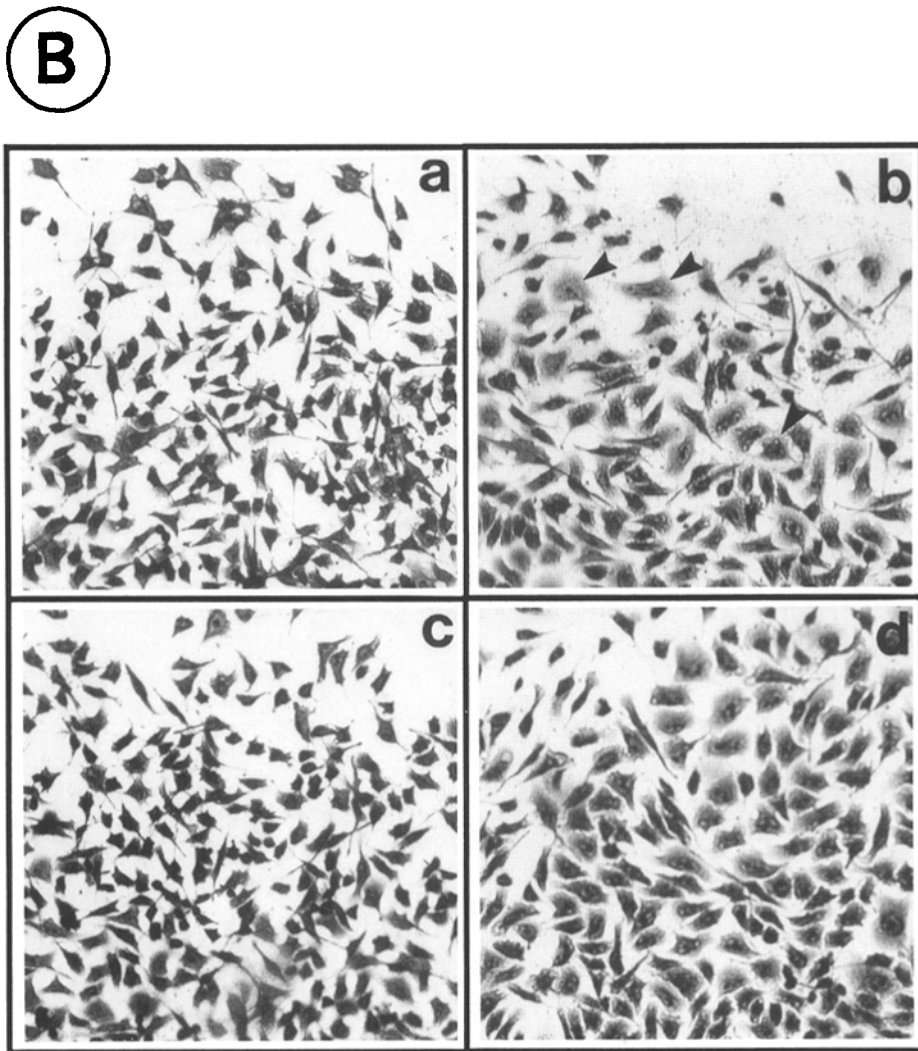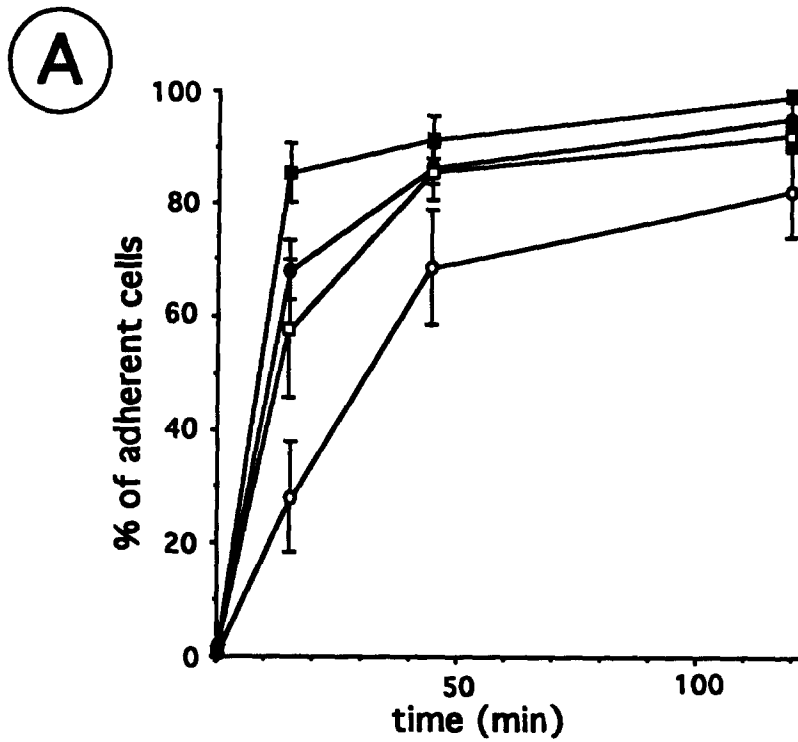
Figure 3. Adhesion and morphology of L27 and P22 cells on fibronectin-coated substrata. (*A*) Adhesion assay performed with L27 (*black*) and P22 (*white*) cells on fibronectin-coated substrata. At 20, 45, or 120 min of the assay, nonadherent cells were removed, and adherent cells fixed and counted to determine the percentage of attached (*square*) or spread (*circle*) cells. Note that the L27 cells are able to attach and spread more efficiently than the control cells on a fibronectin-coated substratum. The difference is more clearly seen during short periods of incubation (20 and 45 min); at 120 min, there is not a significant difference seen between the control and L27 cell lines. For all compared values, the statistical significance was determined using a χ_1^2 test, with a level of confidence superior to 95%. (*B*) Morphology of L27 cells (*b–d*) and P22 cells (*a*) at 24 h. On a fibronectin-coated substratum, L27 cells have a more flattened and highly spread morphology (*b, arrowheads*) when compared to the control cells (*a*). The increase in spread morphology of the L27 cells can be reversed when 0.1 mg/ml of mAb16 antibody (specifically directed against the human $\alpha 5$ chain) is added to the culture medium (*c*), whereas control Igs have no effect (*d*).
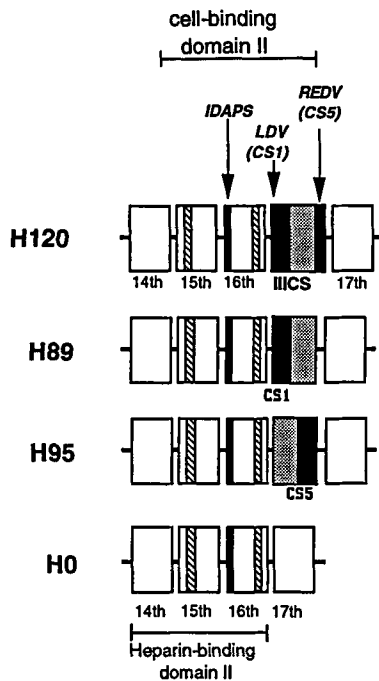
Figure 4. Variants of the human FN cell-binding domain II containing different spliced forms of the IIICS region. All the fragments contain the 14th, 15th, and 16th FN type III homology regions (*white rectangles*), which together correspond to the heparin-binding domain II and the 17th FN type III homology region. The heparin-binding domain II possesses one adhesion site recognized by the α4β1 integrin (*black rectangle*). It also contains adhesion sites that interact with heparan-sulfate proteoglycans (*hatched rectangles*). The H120 fragment corresponds to the entire cell-binding domain II of FN, with the IIICS region (120 amino acids) inserted between the 16th and 17th FN type III regions. The IIICS region contains two adhesion sites (*black*) specific for the α4β1 integrin: the LDV sequence in the CS1 domain and the REDV sequence in the CS5 domain. The H89 and the H95 fragments contain, respectively, the CS5-spliced form and the CS1-spliced form of the IIICS region in the cell-binding domain II. The H0 fragment does not contain the IIICS region.

Increasing the concentration of the H120 and H89 fragments used to coat the substratum (10 µg/ml) resulted in an increase in the percentage of adherent cells to 85 and 78% for D828 cells and to 82 and 60% for D711 cells, respectively. On the H95-coated substratum (10 µg/ml), 60% of the D828 cells and 67% of the D711 cells adhere. In contrast, only 1% of either of these two expressor cells was able to interact with the H0-coated substratum. The percentage of cell attachment can be increased when the assay is performed for a longer period of time (4–6 h) without any significant increase in cell spreading. These results suggest that attachment of these expressor clones by way of the α4β1 integrin is largely mediated by the LDV sequence and, to a lesser extent, by the REDV sequence.

At a higher coating concentration of the H0 fragment (20 µg/ml), an increase in cell attachment that was independent of the transfected cells used was observed (B8, D828, and D711; data not shown). This is probably caused by interaction of cell surface proteoglycans with the heparin-binding II domain of the peptides.

Cell spreading was also markedly different, depending on the level of α4β1 expression at the cell surface. 2% of the D828 cells vs 42% of the D711 cells were spread on substrata coated with a 2 µg/ml solution of H120. Similarly, 38% of the D828 cells vs 78% of the D711 cells were spread on substrata coated with 10 µg/ml of H120. Comparable results were observed on the H89-coated substrata. No cell spreading was observed on substrata coated with 2 µg/ml solution of H95. In contrast, 3% of D828 cells vs 29% of D711 cells were spread on substrata coated with the 10 µg/ml H95 solution. No spreading was observed on H0-coated substrata.

The attachment and spreading of D711 cells on H120- and H89-coated substrata could be specifically inhibited by the monoclonal antibody L25 directed against the human α4 chain, or by the CS1 peptide (Fig. 5 *B*). 70–80% inhibition of cell attachment and spreading was observed using 10 µg/ml of L25 on H120- and H89-coated substrata, respectively, whereas control IgG had no effect. In contrast, the inhibitory effect obtained with the CS1 peptide was more pronounced on cell spreading than on cell attachment; D711 cell attachment and spreading were inhibited by 23 and 40%, respectively, on H120-coated substratum when treated with 1 mg/ml of CS1 peptide. These results indicate that cell adhesion and spreading observed on H120- and H89-coated substrata are mediated by the α4β1 integrin, which is expressed in an active conformational state at the surface of D828 and D711 cells.

In Vitro Motility of α5 and α4 Expressor S180 Clones

L27, D828, D711, and control P22 and B8 cells were sparsely plated on FN-coated substrata or substrata coated with IIICS fragments, and the mean of the speed of locomotion was calculated for the expressor clones (*tv*) and the control clones (*cv*; Fig. 6 *A*). Overexpressor cell lines for the α5β1 or α4β1 integrins increase their speed of movement on FN-coated substrata. A marked difference is observed for D711 cells (high α4 expressors), which exhibit a 3.2-fold increase in cell motility over controls, compared to a 1.3- and 1.4-fold increase obtained for L27 and D828 cells, respectively. The increase in D711 cell motility can be substantially inhibited when either the L25 antibody (specific to α4 subunit) or CS1 peptide is added to the culture medium (Fig. 6 *A*).

The B8, D828, and D711 cells were also tested for their ability to use the cell-binding domain II as a substratum for locomotion (Fig. 6 *B*). B8 cells adhere poorly on all the IIICS fragment–coated substrata used, and they do not use these substrata for locomotion. In contrast, we observed that H120- and H89-coated substrata can promote cell motility of α4 expressor clones, depending on the level of expression of this integrin at the cell surface. The D711 cells (high α4 expressors) migrate at a speed of 46 µm/h, whereas the D828 cells (low α4 expressors) migrate at 19 µm/h on H120-coated substrata. A similar result was observed on H89-coated substrata; we recorded speeds of 39 and 12 µm/h for D711 and D828 cells, respectively. These cells can use the H95- or the H0-coated substrata for locomotion only to a limited extent, probably owing to the IDAPS found on the heparin-binding domain of these fragments.

The Varani assay was also employed in our studies since it uses cells embedded at high density in agarose droplets,
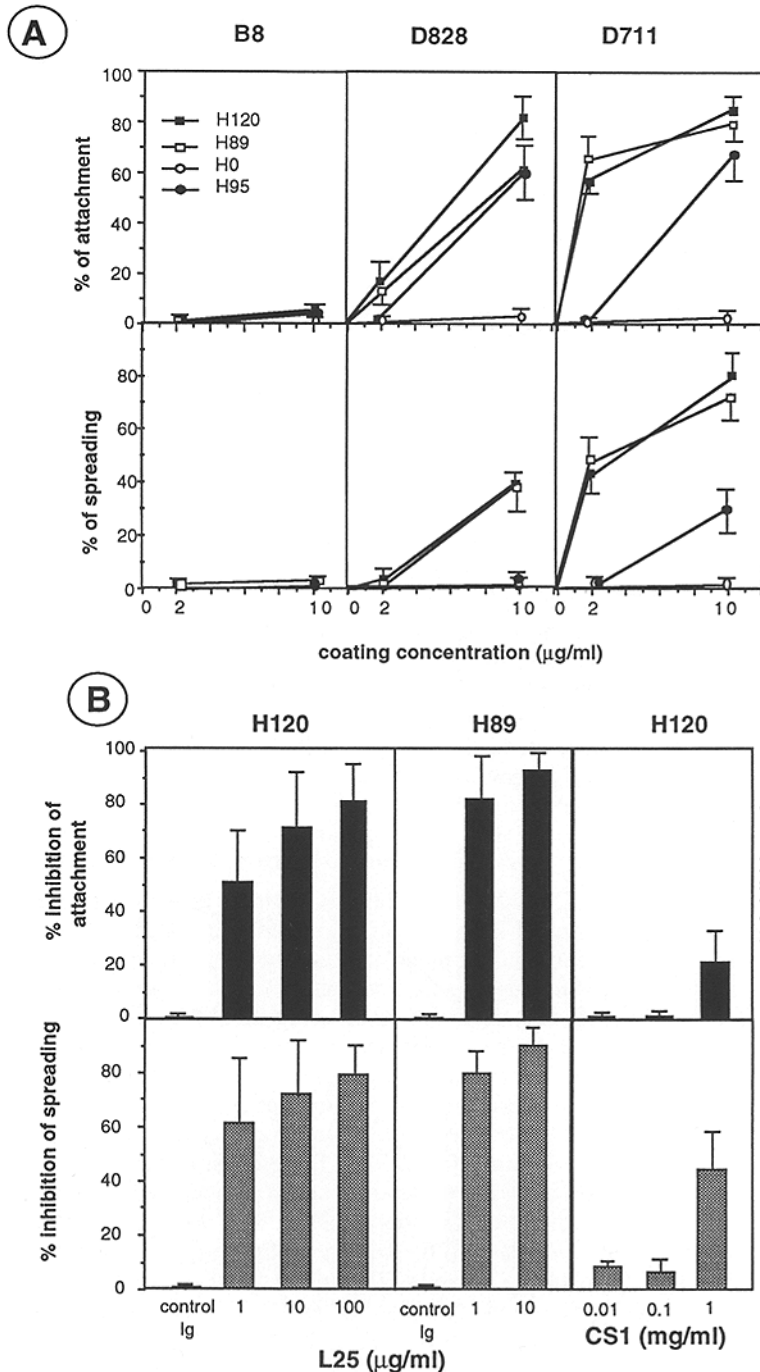
Figure 5. Adhesion of α4 expressors D828 and D711 cells and control B8 cells on IIICS fragments (A) and inhibition of D711 cell adhesion on these coated substrata by CS1 peptide or L25 monoclonal antibody (B). (A) Adhesion assay for control B8 cells and α4 low and high expressor cells (D828 and D711) on substrata coated with solutions of IIICS fragments at 2 or 10 μg/ml. The cells were incubated for 30 min at 37°C, then fixed and the adherent cells were counted. Upper and lower panels indicate the means and standard deviations of the percentages of total cell attachment and spreading, respectively, for each clone. H120 (black square), H89 (white square), H95 (black circle), and H0 (white circle). Note that the B8 control cells cannot adhere significantly to any of these substrata. On CS1-containing fragments (H120 and H89), the α4 expressor clones adhere proportionally to the level of α4β1 at their cell surface; a marked difference in attachment is observed between the D828 and D711 cells on substrata coated with 2 μg/ml solution. Similarly, there is a difference between the two cell lines in the ability to spread on substrata coated with either 2- and 10-μg/ml solutions; the high expressor α4β1 cells have a greater ability to attach and spread. On CS1-spliced fragments (H95 and H0), no cells can adhere on substrata coated with 2 μg/ml solution. At higher concentrations of these substrata, the α4 expressor clones can adhere only on the CS5-containing fragment, but to a lesser extent than on the CS1-containing fragments. (B) The CS1 peptide and the L25 monoclonal antibody can inhibit the attachment (black rectangle) and spreading (hatched rectangle) of D711 cells on H120- and H89-coated substrata. The cells are plated on the substratum for 30 min, then the medium is removed and adherent cells are incubated in the presence of antibodies for 30 min. The L25 antibody (even at the very low concentration of 1 μg/ml) inhibits more efficiently than the CS1 peptide. For all compared values, the statistical significance was determined using a χ^2 test, with a level of confidence >95%.

which may mimic the escape of cells from an aggregate that has been implanted in the embryo (see results below). The area of outgrowth was measured at two time points. As shown in Fig. 7, L27 cells exhibit a 30% increase in their area of outgrowth when compared to that of control P22 cells. This increase is statistically significant, and it can be specifically inhibited when mAb16 is added to the culture medium, whereas control IgG has no effect. An enhanced difference between L27 and P22 areas of outgrowth (100% increase) was observed when measured after a longer period of time (40 h). In contrast, no difference in the area of cell outgrowth is observed between the control B8 cells and the

D828 and D711 α4 expressor cells in this assay either at 24 or 40 h (data not shown).

In Vivo Behavior of Transfected Clones

Both the parent S180 cell line and the transfected clones were grafted into the early neural crest migratory pathway to assess their behavior in an embryonic environment. All cells were inserted into the embryo at the axial level where neural crest cells have just reached the dorsal margin of the somites (i.e., the fifth somite anterior to the last formed somite). Behavior of the cells was assessed by fixing the host embryos 18–24 h after receiving the graft.
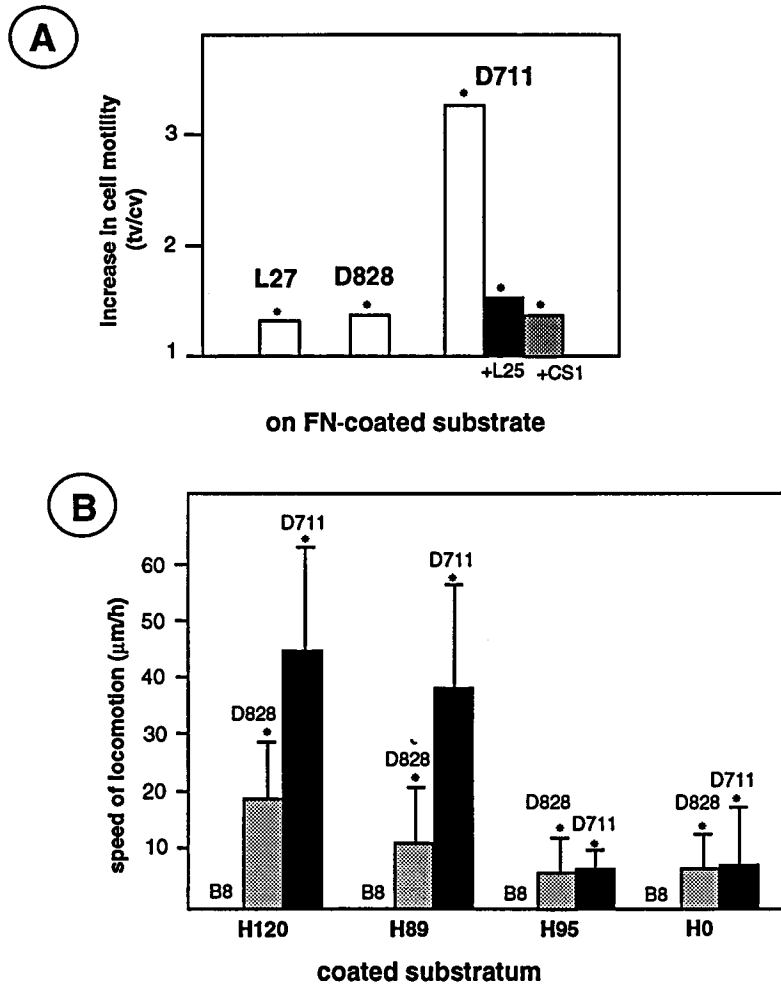
Figure 6. Motility assay of transfectants on FN-coated substrata (A) or IIICS fragment–coated substrata (B). (A) The speed of locomotion of 20 isolated cells in culture was measured using time-lapse videomicroscopy. The mean speed of locomotion (cv) obtained for control cells P22 and B8 was used as the reference to calculate the increase of speed of locomotion obtained with the expressor cells (tv). The ratio tv/cv corresponds to the value of increase of cell motility. Note that the D711 cells exhibit a marked increase in their speed of locomotion on a FN-coated substratum when compared to that obtained for D828 or L27 cells. The high cell motility of the D711 cells can be greatly reduced when specific competitors are added to the medium; L25 monoclonal antibody at 100μg/ml (black rectangle) or CS1 peptide at 1 mg/ml (hatched rectangle). * The increase of the speed of locomotion measured for the α expressors vs the control cells and the inhibition obtained with the competitors is statistically significant. (B) The speed of locomotion of 20 α4 expressor cells and control cells was measured on IIICS fragment–coated substrata. The few B8 cells adhering to these substrata cannot migrate on them in contrast to D828 and D711 cells, which can locomote actively on CS1-containing fragments. The ability of cells to migrate on H120- or H89-coated substrata depends on the level of α4 expression at the cell surface. On the H0- or H95-coated substrata, the D828 or D711 cells exhibit the same residual migratory activity, which is probably caused by the interaction of α4β1 integrin with the IDA site present in the heparin-binding II domain. * The speed of locomotion obtained for the D828 and D711 α4 expressors is significantly increased when compared to that of B8 control cells. In contrast, the value obtained for the same cell line on H120- and H89-coated substrata are not statistically significant. This was determined using a χ_1^2 test, with a level of confidence >95%.

The parental S180 cells migrate in a pattern similar to the host neural crest cells, confirming some observations made previously (Erickson et al., 1980). The S180 cells only invade the anterior half of a somite (Fig. 8 A), and they never invade the posterior half (Fig. 8 B). Moreover, they invade the somite along the interface between the myotome and sclerotome. Neural crest cells during their first 24 h of migration do not enter the space between the ectoderm and dermatome (the dorsolateral space),and, likewise, the S180 cells do not invade this path either (Table I; Fig. 8, A and B). S180 cell behavior differs from the host neural crest cells in two respects, however. S180 cells can migrate ventrally in the extracellular space between the somite and neural tube, as

Figure 7. The Varani migration assay performed using L27 and P22 cells. The cells were incubated at high density in culture medium containing 0.2% low-melting agarose (final concentration) at 39°C for a few minutes, and small droplets of the cell suspension were deposited on FN-coated substrata and allowed to form an artificial cell aggregate as the droplets gelled. These droplets containing L27 cells (grey rectangle) or P22 cells (white rectangle) were then incubated in culture medium for 24 or 40 h, fixed, and the area of
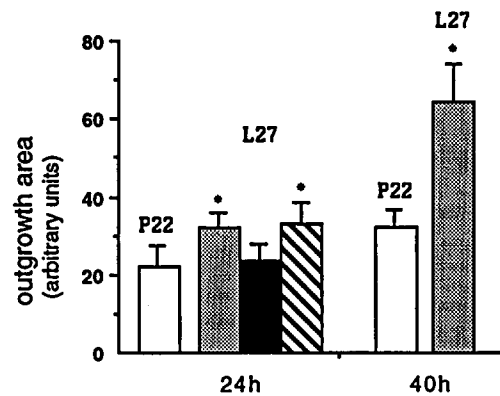
cell outgrowth was measured. In the case of L27 cells, the assay was also performed in the presence of mAb16 antibody (black rectangle) or control Ig (hatched rectangle) at 0.1 mg/ml. Note that the L27 cells exhibit a greater ability than the P22 cells to escape from the aggregate, and their migration can be inhibited when the specific antibody mAb 16 is added to the culture medium. *All the values compared (speed of locomotion of L27 cells with or without Igs vs that of the P22 cells) were significantly different as determined by a χ_1^2 test, with a level of confidence >95%.
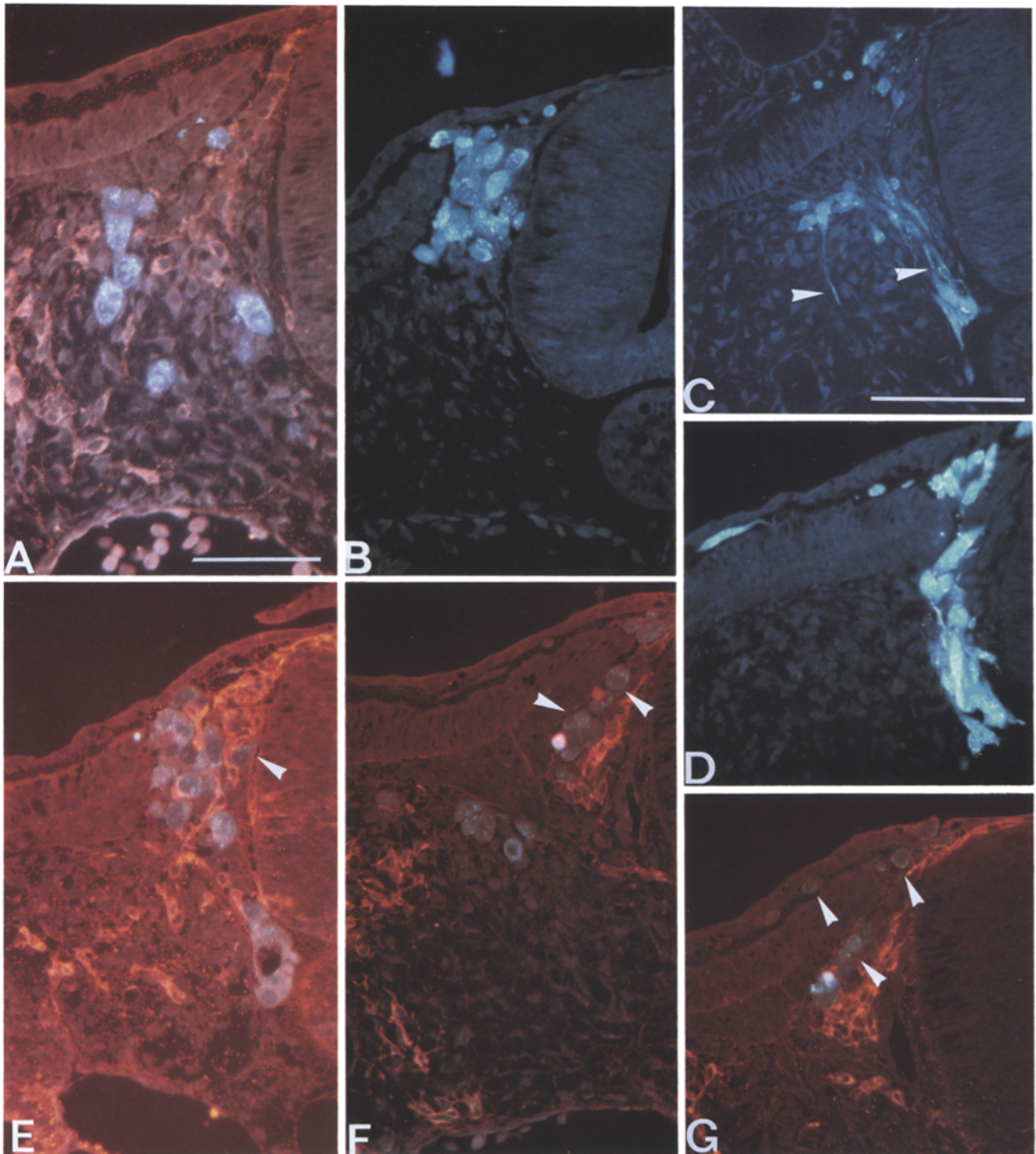
Figure 8. Fluoro-gold–labeled S180 cells or transfected cells lines grafted into the trunk neural crest migratory pathway. Embryos were fixed 18 h after receiving the graft and were subsequently sectioned. The S180 cells and transfected cells are labeled with Fluoro-gold and appear blue (*A–G*), whereas the neural crest cells are labeled with HNK-1 and a rhodamine-conjugated secondary antibody and appear red (*A*, *E*, *F*, and *G*). (*A*) Section taken through the anterior half of a somite reveals that the S180 cells and the neural crest cells follow similar migratory paths, although neural crest cells are generally dispersed farther. Note that neither neural crest cells nor S180 cells are in the dorsolateral path. Bar, 50 μm (also for *B*, *D*, *E*, *F*, and *G*). (*B*) Section through the posterior half of a somite showing that the S180 cells do not invade that portion of the somite and remain aggregated at the graft site. (*C*) Section through the anterior somite half reveals α5 expressors at the interface between the myotome and sclerotome, in the dorsolateral path, and in the space between the sclerotome and neural tube. Note the very long cell processes that these cells extend (*arrowheads*). Bar, 100 μm. (*D*) Higher magnification of α5 expressor cells migrating extensively in the dorsolateral path. This section passes through the posterior half of the somite, and no grafted cells are found in the sclerotome. (*E*) Section through an embryo into which α4 high expressor cells were grafted. Although these cells display short cell processes (*arrowheads*), they do not disperse as single cells from the graft site. The small cluster of S180 cells near

Cell type	S180	L27	D828 (low)	D711 (high)
No. of embryos	10	7	7	7
No. of cells/embryo in dorsolateral path (mean ± SE)	1.80 ± 0.69	9.85 ± 1.26	7.85 ± 1.14	1.14 ± 0.55

There is a highly significant increase ($P = 0.0001$) in the number of L27 and D828 cells found in this particular pathway when compared to control or D711 cells using a Student's t test assuming unknown and unequal variances.

far ventral as the notochord. Neural crest cells are generally not found in this space any farther ventral than the myotome at this stage of migration. A second difference is that the S180 cells do not disperse as far (and therefore as fast) as the host neural crest cells.

The L27 cells migrate through the anterior sclerotome in a pattern similar to the S180 cells (Fig. 8, *C* and *D*). In addition, they also migrate ventrally, often along the basal surface of the neural tube. Their patterns and mode of migration diverge from the parental line in two important respects. First, the L27 cells were observed in relatively large numbers in the dorsolateral path, where host crest cells will not appear for at least another 24 h (Table I; see Erickson et al., 1992). Thus, the α5-transfected cells appear to be able to exploit a pathway that is not accessible to early migratory neural crest cells. Second, the L27 cells exhibit extremely long cell processes, whereas the S180 cells are relatively rounded and have very short processes.

The α4-transfected cell lines behave differently than either the S180 parental line or the α5-transfected clones. Cells of the high expressor line scarcely move from their graft site, even though the cells clearly display very short processes (Fig. 8 *E*). Occasionally, a mass of the cells can be seen spreading along the basal surface of the myotome, but it invades only one or two cell lengths into the somite. Likewise, the graft as a whole occasionally spreads ventrally along the basement membrane of the neural tube.

Cells of the low expressor α4 line disperse farther than their high expressor counterparts, although they do not migrate nearly as far as either the parental S180 cells or the α5-transfected cells. Two interesting features were noted about the pathways they exploit. Like the α5-transfected cells, the low expressor α4 cells are abundant in the dorsolateral path (Table I; Fig. 8 *G*). Second, these cells are nearly always adherent to basement membranes, either on the ectoderm or closely apposed to the myotome (Fig. 8, *F* and *G*). They are rarely dispersed in the sclerotome, in contrast to the neural crest cells or the α5-transfected cells.

Discussion

We have observed that modification of the expression pattern of FN-binding integrins at the surface of S180 cells produces changes in their in vitro and in vivo migratory behavior. These changes are dependent upon the types of integrins expressed as well as their prevalence. Interestingly, some aspects of the behavior observed in the embryo were not predicted based on the behavior of the same cells in tissue culture, reinforcing the importance of examining cell behavior in the tissue environment.

Characterization of In Vitro Cell Behavior of α5-expressing Cells

The parental S180 cells can theoretically interact with a FN-coated substratum using the α3β1, αvβ1, αvβ3, and α5β1 integrins, all of which they express. And, in fact, the untransfected control cells readily adhere to and spread on fibronectin. However, when the level of α5β1 is increased in L27 cells, they adhere and spread more rapidly than the parent cell line. In addition, they exhibit a more flattened morphology. This is consistent with previous studies that show that increased affinity to fibronectin, whether by increasing the concentration of integrins, the concentration of the matrix, or the strength with which integrins and matrix interact, results in an increase in attachment, spreading, and a modification in speed of movement and dispersion (Erickson and Turley, 1983; Rovasio et al., 1983; Tucker and Erickson, 1984; Duband et al., 1991; Ffrench-Constant et al., 1991). Since there is only a fivefold increase in the concentration of $α_5β_1$ integrin on L27 cells, there is only a moderate increase in speed of movement.

We can draw some interesting comparisons between our work and several other studies. Bauer and colleagues (1992) selected variants of CHO cells that expressed reduced levels of α5β1 at their surface, a condition reversed from ours. The CHO variants exhibited slower migration than the parental line. Taken together with our data, there appears to be a direct correlation between the concentration of α5β1 integrin and speed of movement, at least in this range of expression levels. Giancotti and Ruoslahti (1990) have also generated CHO clones by transfection that express either 18 or 33 times more α5β1 than is expressed by control CHO cells. However, they observed a decrease in cell motility using a wound healing repair assay. There are several possible explanations for this result, which at first glance seems contrary to ours. One possibility is based on the fact that our transfected cells only express five times the level of α5 subunit, compared to the parental line, whereas the CHO cells expressed 18–33 times more α5 than the control cells. In the latter case, such a high level of α5 integrin may have immobilized the cells, as Duband et al. (1991) demonstrated for neural crest cells when affinity is increased to very high levels. Thus, at some

the notochord is revealed in adjacent sections to be contiguous with the rest of the graft. (*F* and *G*) Sections through the anterior half of a somite into which the low expressor α4 cells (*D828*) have migrated. These cells are almost always found contacting basement membranes (*arrowheads*), either of the ectoderm or the myotome. These cells are relatively rounded with short cell processes, and they have not dispersed as extensively as the host neural crest cells.

level of $\alpha 5$ expression, an increase may produce such a high affinity to the substratum that motility is reduced rather than increased.

Alternatively, as a result of increased expression of the $\alpha 5$ subunit, both the CHO cells (Giancotti and Ruoslahti, 1990) and S180 cells (this report) demonstrate enhanced FN assembly at their surface. CHO cells may assemble so much matrix at their surface that they may become immobilized, perhaps because there are no longer any free receptors with which to generate motility. Thus, enhanced matrix assembly may be antagonistic to motility in some physiological conditions.

In the Varani assay, the difference in rate of dispersion between P22 and L27 cells is more pronounced than their speed of movement measured in sparse and planar cultures. In the former assay, cells disperse from a droplet, where they are at high density, into a region of few cells. The assay does not measure simply the intrinsic migratory properties of cells, but a more complex behavior of cells in a mass. Thus, the greater difference observed between the migratory capability of L27 vs P22 cells in this assay may reflect an enhanced effect owing to population pressure and contact inhibition of locomotion, giving rise to a more directional outward migration when compared to that of isolated cells. Indeed, contact inhibition of migration has been proposed as the most plausible explanation for the directional movement of cells from a mass (Abercrombie, 1970; Heaysman, 1978; Erickson, 1988; Newgreen and Erickson, 1986). Recently, another phenomenon coined "contact stimulation of migration" has been described (Thomas and Yamada, 1992), where cells in high density pellets migrated outward with a greater velocity than when plated in sparse culture. Thomas and Yamada suggest that "contact inhibition of locomotion" and "contact-stimulated migration" can act synergistically in controlling cell velocity and directional movement.

Characterization of In Vitro Cell Behavior of $\alpha 4$ Expressor Cells

We examined the role of $\alpha 4 \beta 1$ expression in the attachment and spreading, as well as migration, of S180 cells on FN. $\alpha 4 \beta 1$ clearly mediates attachment and spreading to FN-cell-binding domain II, depending on its expression level at the cell surface. The LDV sequence within this domain appears to be the major recognition site, since the $\alpha 4$-expressing clones displayed identical levels of adhesion to the LDV-spliced variant (H95 and H0: 2 μg/ml) as the nonexpressing clones. The REDV site is probably involved, but to a lesser extent, since there is little difference in adhesion and spreading using the H120 variant (REDV+) and H89 (REDV−), and because the functional activity of this site is only revealed when H95 is used at high coating concentrations (10 μg/ml). The IDAPS site in the heparin-binding domain does not facilitate binding since D828 and D711 cells adhere to the heparin-binding domain fragment (H0) minimally and to the same extent as cells of the B8 line, which do not express $\alpha 4 \beta 1$. The very slight adhesion observed on H0 fragments is likely caused by binding to the heparan-sulfate proteoglycan (HSPG)-binding site, and it suggests that S180 cells express cell surface HSPG.

The CS1 peptides do not dramatically reduce attachment and spreading on the spliced variants, whereas the L25 anti-

body reduces attachment and spreading by >89%. This is probably caused by relatively inefficient competition by CS1. Alternatively, it suggests the intriguing possibility that there is some synergism between the LDV site and sites in the heparin-binding domain, since the antibody, which is directed against the integrin, probably interferes with several integrin-binding sites to FN. Binding assays to a spliced variant that does not contain the heparin-binding domain or the IDAPS site might address this possibility.

The $\alpha 4 \beta 1$ integrin also facilitates motility in addition to adhesion. The speed of movement is a function of the level of expression of $\alpha 4 \beta 1$ at the surface, since the high expressors move more rapidly than the low expressors. Moreover, $\alpha 4 \beta 1$ may be more efficient than $\alpha 5 \beta 1$ in regulating motility since a fivefold increase in $\alpha 5$ produces only a 1.3-fold increase in L27 motility on fibronectin, whereas a fourfold increase in $\alpha 4 \beta_1$ between the D711 cells (high expressors) and D828 cells (low expressors) results in a 2.5-fold increase in motility (which can be abolished in the presence of L25 antibody).

In the Varani assay, the area of outgrowth is similar for the $\alpha 4$ expressors and control cells, which is surprising in light of the fact that in sparse culture, $\alpha 4$ expressors are dramatically more motile than the controls. We have noted that the $\alpha 4$ expressor cells adhere to each other tenaciously, possibly owing to homotypic adhesive interactions (see discussion below). Speed of dispersion as measured in the Varani assay is dependent not only on cell–matrix affinity, but also on cell–cell affinity. Thus, the balance between the two types of adhesion will determine rate of dispersion from a mass. In the two migration assays we used in this study, the results obtained in the Varani assay are consistent with the in vivo ability of cells to migrate. However, it cannot replace the direct implantation of cells in the embryo, where the cells are exposed to a more complex environment than the planar substratum with which the cells are confronted in the Varani assay.

D828 cells and D711 cells migrate on FN cell-binding domain II, and the speed of locomotion on these substrata is also dependent on the level of $\alpha 4 \beta 1$ expression. LDV appears to be the major site for generation of motility since there is a drastic reduction of speed of movement when this site is removed (compared motility on H89 and H0). The REDV site apparently does not act synergistically with the LDV sequence to promote cell migration. The slight decrease in speed of movement of cells measured on H120 vs H89 substrata is not statistically significant, and the presence of the REDV site on the substratum (H95 vs H0) does not induce an increase in cell motility.

We observed that there is a low level of motility of D828 and D711 cells on H0 fragments, which could be caused by either the IDAPS site recognized by $\alpha 4 \beta 1$ or the heparan sulfate proteoglycan-binding sites. It seems likely that motility on the H0 fragment results from $\alpha 4 \beta 1$ interaction with the IDAPS site since B8 cells, which lack the $\alpha 4$ subunit, can adhere poorly to, but do not migrate on, H0-coated substrata. We have no evidence to support or exclude the idea that the HPSG-binding sites and the IDAPS site can act synergistically to promote cell motility, nor can we rule out the possibility that other unidentified binding sites that regulate motility may yet exist in this domain.

Changes in the Integrin Expression Pattern Modify the In Vivo Cell Behavior

Three aspects of S180 cell behavior in vivo were altered by their acquisition of additional integrins: their morphology, their speed of movement, and the pathways they take in the embryo. Morphological changes appear to be similar in vitro and in vivo, whereas speed of motility was not predictable from our observations in culture.

In culture, α5 expressor cells on FN have a more flattened and elongated morphology when compared to that of the parent cells or α4 expressors. It was interesting to observe that these cells had similar morphological characteristics in vivo. α5 expressor cells exhibited long, thin processes that could extend many times the length of their cell bodies, whereas α4 expressors remained rounded, although clearly extending short, stubby processes. These in vivo studies confirm the in vitro observation that α5β1 is more functionally active in promoting cell spreading than α4β1.

The rate at which these grafted transfected cells migrate in vivo was not predictable, based on their behavior in culture. In culture on FN, α4β1 integrin is a more potent inducer of cell motility, and speed of movement of the α4 expressors increases dramatically with only a fourfold increase in the concentration of the integrin. However, in vivo, the α5 expressors disperse more readily. There are several explanations for these differences.

The rate of dispersion, within a certain range, is proportional to the avidity of attachment between cells and their substratum. And in fact, it has been demonstrated in vitro that the rate of migration is regulated by both the affinity of the integrin receptors for their ligands, by the number of receptors occupied, or the ligand density (Duband et al., 1991; Lauffenburger, 1989). Thus, the in vivo behavior of the cells is as likely to be controlled by the number of integrins as by the concentration and type of matrix with which they are confronted. Thus, one might imagine the possibility that the cell-binding domains recognized by α5 or α4 integrins are differentially exposed or available in the FN matrices in vivo than on FN-coated substrata in vitro. For example, the affinity to FN via α5 may be relatively greater in the embryo than in our culture conditions due to the greater availability of the RGD-binding site. There is no evidence currently concerning the relative distribution of various spliced variants of FN in the chicken embryo, although we are at least assured that the FN-spliced variant containing the LDV sequence is found in abundance in young (2.5–11-d old) chick embryos (Norton and Hynes, 1987). A second possibility is that with a very large increase in affinity, cells are too strongly adherent to detach, and they therefore become immobilized (Duband et al., 1991; Erickson and Turley, 1983; Tucker and Erickson, 1994). Thus, the high α4-transfected S180 cells may adhere so well in the embryonic environment that they are fixed in place, whereas in contrast, the low α4 or the α5 expressors are still able to migrate rapidly.

Alternatively, the α4 expressor cells may disperse less well from the aggregates owing to homotypic adhesive interactions generated by the expression of the α4 integrin. In fact, we consistently noted that pellets of D711 cells that were used in the grafting experiments always held together more tenaciously than pellets of the other cell lines, which

fell apart readily. Moreover, in sections through embryos into which D711 cells were grafted, the cells appear to adhere to each other in aggregates in the position where they were grafted. This lack of migration appears not to be caused by poor health or inability to migrate since small cell processes were nevertheless produced. Such homotypic interactions could also explain the slow dispersal of the α4 expressors in the Varani assay. A similar homotypic aggregation has also been reported for other cells expressing α4β1 integrins (Bednarczyk et al., 1993; Campanero et al., 1992; Pulido et al., 1991; Qian et al., 1994; Vonderheide and Springer, 1992), and at least in the case of melanoma cells, this homotypic aggregation is correlated with decreased motility and invasiveness in vivo (Qian et al., 1994). While VCAM-1 and α4β1 have been documented to bind heterotypically, S180 cells and the transfectants do not express VCAM-1 (data not shown).

Perhaps the most provocative results of this study are that pathways of migration taken by different S180 cell lines are dependent on the integrins they express. Cells of the S180 parent line, like the neural crest cells, do not invade the dorsolateral path, whereas the α5 expressor line and the low α4 expressor line invade this pathway immediately upon grafting and 24 h before the host neural crest cells. This is particularly interesting in light of the observation that only melanoblasts are able to invade the dorsolateral path and newly migrating neural crest cells acquire this invasive ability only after 24 h either in vivo or in culture (Erickson and Goins, 1995). It is not known what cellular changes must occur to confer this migratory ability on melanoblasts, but several recent reports suggest that melanoblasts in the dorsolateral path or NCC express either the α4 (in mice, Sheppard et al., 1994; in chicks, Stepp et al., 1994) or α5 integrin (in chicks, see Fig. 3 in Muschler and Horwitz, 1991). Our work demonstrates that either α5β1 or α4β1 permits invasion, and taken together with the studies just cited, suggests that only after neural crest cells begin to express high levels of FN receptors will they exploit the dorsolateral path. Perhaps the receptors allow crest cells to adhere more tightly to the FN in this path (Newgreen and Thiery, 1980; Thiery et al., 1982) and to overcome what is initially a less satisfactory, or perhaps even inhibitory, migratory substratum (e.g., Erickson et al., 1992; Oakley et al., 1994).

Definitive studies using transgenic mice may shed more light on the role of integrins in neural crest migration and in melanoblast migration in particular. In fact, mice with heterozygous null mutations in the genes encoding FN or the α5 integrin chain have been obtained (George et al., 1993; Yang et al., 1993). The heterozygous mice lacking one allele of these genes appear to be normal, whereas those lacking two allelic genes coding for FN or α5 die by days 10–11 and present strong morphogenetic defects, indicating the essential function of these molecules during early development (George et al., 1993; Yang et al., 1993). Although the malformations obtained in the α5-null embryos suggest an important role for this integrin during mesoderm formation and movement, it has not been reported if migratory properties of specific cell populations such as NCC were perturbed in these mouse embryos.

These results do not indicate precisely which integrins can produce changes in neural crest behavior, or which account for their enhanced motility compared with other embryonic

cells. Nevertheless, they are informative since at the very least, these results indicate how various integrins interact with the complex extracellular matrix environment of the embryo, and they can be used to verify or refute results obtained from culture experiments. Moreover, our in vivo behavioral assay, as described in this report, can be used to test directly the migratory role of particular integrins that are expressed by NCC and that are proposed from immunocytochemical studies to be correlated with neural crest behavior.

References

Abercrombie, M. 1970. Contact inhibition in tissue culture. *In Vitro.* 6:128-142.

Adams, J. C., and F. M. Watt. 1993. Regulation of development and differentiation by the extracellular matrix. *Development (Camb.).* 117:1183-1198.

Akiyama, S. K., S. S. Yamada, W.-T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* 109:863-875.

Akiyama, S. K., K. Nagata, and K. M. Yamada. 1990. Cell surface receptors for extracellular matrix components. *Biochim. Biophys. Acta.* 1031:91-110.

Aota, S., T. Nagai, and K. M. Yamada. 1991. Characterization of regions of fibronectins besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using the site-directed mutagenesis. *J. Biol. Chem.* 266:15938-15943.

Arcangeli, A., A. Becchetti, A. Mannini, G. Mugnai, P. DeFilippi, G. Tarone, R. Del Bene, E. Barletta, E. Wanke, and M. Olivotto. 1993. Integrin-mediated neurite outgrowth in neuroblastoma cells depends on the activation of potassium channels. *J. Cell Biol.* 122:1131-1143.

Bauer, J. S., C. L. Schreiner, F. G. Giancotti, E. Ruoslahti, and R. L. Juliano. 1992. Motility of fibronectin receptor-deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. *J. Cell Biol.* 116:477-487.

Bednarczyk, J. L., J. N. Wygant, M. C. Szabo, L. Molinari-Storey, M. Renz, S. Fong, and B. W. McIntyre. 1993. Homotypic leukocyte aggregation triggered by a monoclonal antibody specific for a novel epitope expressed by the beta1 subunit: conversion of nonresponsive cells by transfecting human integrin alpha 4 subunit cDNA. *J. Cell Biochem.* 51:465-478.

Boucaut, J. C., T. Darribère, T. J. Poole, H. Aoyama, K. M. Yamada, and J. P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J. Cell Biol.* 99:1822-1830.

Bronner-Fraser, M. 1985. Alteration in neural crest cell migration by a monoclonal antibody that affects cell adhesion. *J. Cell Biol.* 101:610-617.

Campanero, M. R., A. G. Arroyo, R. Pulido, A. Ursa, M. S. De Matias, P. Sanchez-Mateos, P. D. Kassner, B. M. Chan, M. E. Hemler, A. L. Corbi, et al. 1992. Functional role of alpha2/beta1 and alpha4/beta1 integrins in leukocyte intercellular adhesion induced through the common beta1 subunit. *Eur. J. Immunol.* 22:3111-3119.

Defilippi, P., V. van Hinsbergh, A. Bertolotto, P. Rossino, L. Silengo, and G. Tarone. 1991a. Differential distribution and modulation expression of alpha1/beta1 integrin on human endothelial cells. *J. Cell Biol.* 114:855-863.

Defilippi, P., G. Truffa, G. Stefanuto, F. Altruda, L. Silengo, and G. Tarone. 1991b. Tumor necrosis factor alpha and interferon gamma modulate the expression of the vitronectin receptor (integrin beta3) in human endothelial cells. *J. Biol. Chem.* 266:7638-7645.

Duband, J. L., S. Rocher, W.-T. Chen, and K. M. Yamada. 1986. Cell adhesion and migration in the early vertebrate embryo: location and possible role

of the putative fibronectin receptor complex. *J. Cell Biol.* 102:160-178.

Duband, J. L., S. Dufour, S. Yamada, K. M. Yamada, and J. P. Thiery. 1991. Neural crest cell locomotion induced by antibodies to b1 integrins. A tool for studying the roles of substratum molecular avidity and density in migration. *J. Cell Sci.* 98:517-532.

Dufour, S., J. L. Duband, M. J. Humphries, M. Obara, K. M. Yamada, and J. P. Thiery. 1988. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2661-2671.

Edelman, G. M., B. A. Cunningham, and J. P. Thiery. 1990. Morphoregulatory Molecules. John Wiley & Sons, Inc., New York. 648 pp.

Erickson, C. A., K. W. Tosney, and J. A. Weston. 1980. Analysis of migratory behavior of neural crest and fibroblastic cells in embryonic tissues. *Dev. Biol.* 77:142-156.

Erickson, C. A., and E. A. Turley. 1983. Substrata formed by a combination of extracellular matrix components alter neural crest cell motility in vitro. *J. Cell Sci.* 61:299-323.

Erickson, C. A. 1988. Control of path finding by avian trunk neural crest. *Development (Camb.).* 103:63-80.

Erickson, C. A., T. D. Duong, and K. W. Tosney. 1992. Descriptive and experimental analysis of the dispersion of neural crest cells along the dorsolateral path and their entry into ectoderm in the chick embryo. *Dev. Biol.* 151:251-272.

Erickson, C. A., and R. Perris. 1993. The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest. *Dev. Biol.* 159:60-74.

Erickson, C. A., and T. Goins. 1995. Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes. *Development (Camb.).* In press.

Ffrench-Constant, C., A. Hollingsworth, J. Heasman, and C. C. Wylie. 1991. Response to fibronectin of mouse primordial germ cells before, during and after migration. *Development (Camb.).* 113:1365-1373.

Garcia-Pardo, A., E. A. Wayner, W. G. Carter, and O. C. Ferriera. 1990. Human B lymphocytes define an alternative mechanism of adhesion to fibronectin: the interaction of the $\alpha 4\beta 1$ integrin with the LHGPEILDVPST sequence of the type III-connecting segment is sufficient to promote cell attachment. *J. Immunol.* 144:3361-3366.

George, E. L., E. N. Georges-Labouesse, R. S. Patel-King, H. Rayburn, and R. O. Hynes. 1993. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development (Camb).* 119:1079-1091.

Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha 5\beta 1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849-859.

Gorman, C. 1985. High efficiency gene transfer into mammalian cells. *In* DNA Cloning: A Practical Approach. Vol. II. IRL Press, Oxford. pp. 143-190.

Guan, J.-L., and R. O. Hynes. 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha 4\beta 1$. *Cell.* 60:53-61.

Hamburger, V. and H. L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49-92.

Heaysman, J. E. M. 1978. Contact inhibition of locomotion: a reappraisal. *Int. Rev. Cytol.* 55:49-66.

Hemler, M. E. 1991. Structures and functions of VLA proteins and related integrins. *In* Receptors for Extracellular Matrix Proteins. R. P. Mecham, J. A. M., editors. Academic Press, Inc., San Diego, CA.

Horwitz, A., and J. P. Thiery. 1994. Cell-to-cell contact and extracellular matrix. *Curr. Opin. Cell Biol.* In press.

Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1986. Identification of an alternative spliced site in human plasma fibronectin that mediates cell-type specific interaction. *J. Cell Biol.* 103:2637-2647.

Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. Identification of two distinct regions of the type III-connecting segment of plasma fibronectin that promote cell-type specific adhesion. *J. Cell Biochem.* 262:6886-6892.

Hynes, R. O. 1990. Fibronectins. Springer Verlag Publishing Co., New York. 546 pp.

Hynes, R. O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* 69:11-25.

Hynes, R. O. and A. D. Lander. 1992. Contact and adhesive specificities in the associations, migration, and targetting of cells and axons. *Cell.* 68:303-322.

Komoriya, A., L. J. Green, M. Mervic, S. S. Yamada, K. M. Yamada, and M. J. Humphries. 1991. The minimal essential sequence for a major cell-type specific adhesion site (CS1) within the alternatively spliced type III-connecting-segment domain of fibronectin is leucine-aspartic acid-valine. *J. Biol. Chem.* 266:15075-15079.

Lauffenburger, D. 1989. A simple model for the effects of receptor-mediated cell-substratum adhesion on cell migration. *Chem. Eng. Sci.* 44:1903-1914.

Le Douarin, N. M. 1982. The Neural Crest. Cambridge University Press, Cambridge. 246 pp.

Le Douarin, N. M., C. Ziller, and G. F. Couly. 1993. Patterning of neural crest derivatives in the avian embryo: in vivo and in vitro studies. *Dev. Biol.* 159:24-49.

Loring, J. F., and C. A. Erickson. 1987. Neural crest cell migratory pathways

in the trunk of the chick embryo. *Dev. Biol.* 121:220-236.

Matsumoto, A., and M. E. Hemler. 1993. Multiple activation states of VLA-4. *J. Biol. Chem.* 268:228-234.

Mould, A. P., and M. J. Humphries. 1991. Identification of a novel recognition sequence for the integrin α4β1 in the COOH-terminal heparin-binding domain of fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:4089-4095.

Mould, A. P., A. Komoriya, K. M. Yamada, and M. J. Humphries. 1991. The CS5 peptide is a second site in the IIICS region of fibronectin recognized by the integrin alpha4-beta1: inhibition of alpha4-beta1 function by RGD peptide homologue. *J. Biol. Chem.* 266:3579-3585.

Mould, A. P., J. A. Askari, S. E. Craig, A. N. Garratt, J. Clements, and M. J. Humphries. 1994. Cell adhesion and migration mediated by the integrin α4β1: effect of fibronectin alternative splicing and the role of ligand affinity. *J. Biol. Chem.* 269:27224-27230.

Muschler, J. L., and A. F. Horwitz. 1991. Down-regulation of the chicken α5β1 integrin fibronectin receptor during development. *Development (Camb.).* 113:327-337.

Nagai, T., N. Yamakawa, S.-I. Aota, S. S. Yamada, S. K. Akiyama, K. Olden, and K. M. Yamada. 1991. Monoclonal antibody characterization of two distant sites required for function of the central cell-binding domain of fibronectin in cell adhesion, cell migration, and matrix assembly. *J. Cell Biol.* 114:1295-1305.

Newgreen, D., and J. P. Thiery. 1980. Fibronectin in the early avian embryo: synthesis and distribution along migratory pathways of neural crest cells. *Cell Tissue Res.* 211:269-291.

Newgreen, D. F., and C. A. Erickson. 1986. Migration of neural crest cells. *Int. Rev. Cytol.* 103:89-144.

Norton, P. A., and R. O. Hynes. 1987. Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. *Mol. Cell. Biol.* 7:4297-4307.

Oakley, R. A., C. J. Lasky, C. A. Erickson, and K. W. Tosney. 1994. Glycoconjugates mark a transient barrier to neural crest migration in the chicken embryo. *Development (Camb.).* 120:103-114.

Obara, M., M. S. Kang, and K. M. Yamada. 1988. Site-directed mutagenesis of the cell-binding domain of fibronection: separable, synergistic sites mediate adhesive function. *Cell.* 53:649-657.

Pulido, R., M. J. Elices, M. R. Campanero, L. Osborn, S. Schiffer, A. Garcia-Pardo, R. Lobb, M. E. Hemler, and F. Sanchez-Madrid. 1991. Functional evidence for three distinct and independently inhibitable adhesion activities mediated by the human integrin VLA-4. Correlation with distinct alpha 4 epitopes. *J. Biol. Chem.* 266:10241-10245.

Qian, F., D. L. Vaux, and I. L. Weissman. 1994. Expression of the integrin α4β1 on melanoma cells can inhibit the invasive stage of metastasis formation. *Cell.* 77:335-347.

Rossino, P., I. Gavazzi, R. Timpl, M. Aumailley, M. Abbadini, F. G. Giancotti, L. Silengo, P. C. Marchisio, and G. Tarone. 1990. Nerve growth factor induces increased expression of a laminin-binding integrin in rat pheochromocytoma PC12 cells. *Exp. Cell Res.* 189:100-108.

Rovasio, R. A., A. Delouvée, K. M. Yamada, R. Timpl, and J. P. Thiery. 1983. Neural crest cell migration: requirement for exogenous fibronectin and high cell density. *J. Cell Biol.* 96:462-473.

Ruoslahti, E. 1988. Fibronectin and its receptors. *Annu. Rev. Biochem.* 57:375-413.

Serbedzija, G. N., S. E. Fraser, and M. Bronner-Fraser. 1990. Pathways of trunk neural crest cell migration in the mouse embryo as revealed by vital dye labelling. *Development (Camb.).* 108:605-612.

Sheppard, A. M., M. D. Onken, G. D. Rosen, P. G. Noakes, and D. C. Dean. 1994. Expanding roles for α4 integrin and its ligands in development. *Cell. Adhes. Commun.* 2:27-43.

Stepp, M. A., L. A. Urry, and R. O. Hynes. 1995. Expression of α4 integrin mRNA and protein and fibronectin in the early chick embryo. *Cell Adhes. Commun.* 2:359-375.

Takada, Y., M. J. Elices, C. Crouse, and M. E. Hemler. 1989. The primary structure of the α4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1361-1368.

Teixido, J., M. E. Parker, P. D. Kassner, and M. E. Hemler. 1992. Functional and structural analysis of VLA-4 integrin α4 subunit cleavage. *J. Biol. Chem.* 267:1786-1791.

Thiery, J. P., J. L. Duband, and A. Delouvée. 1982. Pathways and mechanism of avian trunk neural crest cell migration and localization. *Dev. Biol.* 93:324-343.

Thomas, L. A., and K. M. Yamada. 1992. Contact stimulation of cell migration. *J. Cell Sci.* 103:1211-1214.

Tucker, R. P., and C. A. Erickson. 1984. Morphology and behavior of quail neural crest cells in artificial three-dimensional extracellular matrices. *Dev. Biol.* 104:390-405.

Varani, J., W. Orr, and P. R. Ward. 1978. A comparison of the migratory pattern of normal and malignant cells in two assay systems. *Am. J. Pathol.* 90:159-171.

Vincent, P., N. Paris, J.-L. Pujol, C. Gaboriaud, T. Rabilloud, J.-L. Pennetier, P. Matherat, and P. Tarroux. 1986. HERMeS: a second generation approach to the automatic analysis of two-dimensional electrophoresis gels. Part I: Data acquisition. *Electrophoresis.* 7:347-356.

Vonderheide, R. H., and T. A. Springer. 1992. Lymphocyte adhesion through very late antigen 4: evidence for a novel binding site in the alternatively spliced domain of vascular cell adhesion molecule 1 and an additional alpha4 integrin counter-receptor on stimulated endothelium. *J. Exp. Med.* 175:1433-1442.

Wayner, E. A., and N. L. Kovach. 1992. Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V region of fibronectin. *J. Cell Biol.* 116:489-497.

Woods, A., J. B. McCarthy, L. T. Furcht, and R. Couchman. 1993. A synthetic peptide from the COOH-terminal heparin-binding domain of fibronectin promotes focal adhesion formation. *Mol. Biol. Cell.* 4:605-613.

Woods, A., and J. R. Couchman. 1994. Syndecan 4 heparan sulfate proteoglycan is a selectively enriched and widespread focal adhesion component. *Mol. Biol. Cell.* 5:183-192.

Yamada, K. M. 1989. Fibronectins: structure, functions, and receptors. *Curr. Opin. Cell Biol.* 1:956-963.

Yang, J. T., H. Rayburn, and R. O. Hynes. 1993. Embryonic mesodermal defects in α5 integrin-deficient mice. *Development.* 119:1093-1105.

Zambruno, G., P. C. Marchisio, A. Melchiori, S. Bondanza, R. Cancedda, and M. De Luca. 1993. Expression of integrin receptors and their role in adhesion, spreading and migration of normal human melanocytes. *J. Cell Sci.* 105:179-190.