The Selective Regulation of α\textsubscript{v}β\textsubscript{1} Integrin Expression Is Based on the Hierarchical Formation of α\textsubscript{v}-containing Heterodimers* 

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The integrin β\textsubscript{1} subunit can form a heterodimer with 12 different α subunits. According to the present model, the expression level of any αβ complex is regulated by the availability of the specific α subunit, whereas β\textsubscript{1} subunit is constantly present in a large excess. The expression of several heterodimers containing the α\textsubscript{v} subunit seems to be regulated by an identical mechanism. The fact that many cells express α\textsubscript{v}β\textsubscript{1}, heterodimer, and that this fibronectin/vitronectin receptor may be selectively regulated, compromises the present model of the regulation of β\textsubscript{1} and α\textsubscript{v} integrins. We have tried to solve this problem by assuming that distinct αβ heterodimers are formed with different tendency. To test the hypothesis, we analyzed WM-266-4 melanoma cells transfected with a cDNA construct coding for an intracellular single-chain anti-α\textsubscript{v} integrin antibody. We could see 70–80% reduction in the cell surface expression of α\textsubscript{v} subunit. However, the only one of the α\textsubscript{v} integrins reduced on the cell surface was α\textsubscript{v}β\textsubscript{1}. This suggests that the cell surface expression level of α\textsubscript{v}β\textsubscript{1} is dependent on the number of α\textsubscript{v} subunits available after the formation of other α\textsubscript{v}-containing heterodimers. Thus, there seems to be a hierarchy in the complex formation between α\textsubscript{v} and its different β-partners. These observations explain how α\textsubscript{v}β\textsubscript{1} can be specifically regulated without concomitant changes in the expression of other α\textsubscript{v} or β\textsubscript{1} integrins.

The members of the integrin family that form receptors for various extracellular matrix proteins can be divided into two major subgroups according to the subunits present in the receptors. Integrin β\textsubscript{1} can form a complex with 12 different α subunits. The β\textsubscript{1}-containing heterodimers are receptors for collagens, laminins, tenascins, and fibronectin. The other subset, the α\textsubscript{v} integrins, are fibronectin and vitronectin receptors, some of which also have the ability to bind various matrix and plasma proteins.

In many cells the two promiscuous subunits, α\textsubscript{v} and β\textsubscript{1}, can form a heterodimer with each other: α\textsubscript{v}β\textsubscript{1} was originally described as a fibronectin or vitronectin receptor (1, 2). It may also have a capacity for binding to osteopontin and to the latent form of transforming growth factor-β (3, 4). Some viruses, including parvovirus B19, adenovirus, and foot-and-mouth disease virus, use α\textsubscript{v}β\textsubscript{1} as their cellular receptor (5–7). The tissue distribution of α\textsubscript{v}β\textsubscript{1} is mostly unknown because of the lack of a specific antibody against α\textsubscript{v}β\textsubscript{1} complex. For the same reason the function of α\textsubscript{v}β\textsubscript{1} integrin in many human cell types is unknown, or the published information is based on cell transfections or on the use of combinations of function blocking antibodies against different α\textsubscript{v} and β\textsubscript{1} integrins. In α\textsubscript{v}-transfected Chinese hamster ovary cells, α\textsubscript{v}β\textsubscript{1} integrin has been found to function as a fibronectin receptor while not supporting cell migration on fibronectin (8). On the other hand, in squamous carcinoma cells derived from head and neck tumors, α\textsubscript{v}β\textsubscript{1} integrin contributes to migration on fibronectin (9). It has been suggested that α\textsubscript{v}β\textsubscript{1} integrin promotes the migration of oligodendrocyte precursors on composite extracellular matrix containing laminin, fibronectin, and vitronectin (10). In avian neural crest cells, α\textsubscript{v}β\textsubscript{1} participates in adhesion to vitronectin, whereas it may have a less important role in cell migration (11). The role of α\textsubscript{v}β\textsubscript{1} as a vitronectin receptor has been emphasized in studies on smooth muscle cells, suggesting that vitronectin-mediated contractility of smooth muscle is mediated by α\textsubscript{v}β\textsubscript{1} integrin (12).

There is some evidence that the cell surface level of α\textsubscript{v}β\textsubscript{1} integrin can be specifically regulated, for example, during development (13). Selective regulation of the number of α\textsubscript{v}β\textsubscript{1} integrins on the cell surface cannot be explained simply by assuming that the expression of α\textsubscript{v} or β\textsubscript{1} genes or the synthesis rate of the corresponding proteins is changed. That would lead to concomitant changes in the numbers of all α\textsubscript{v} or β\textsubscript{1} integrins. The regulation of both α\textsubscript{v} and β\textsubscript{1} integrins has been studied in detail, and their regulation seems to be based on the same principle; the promiscuous subunit (β\textsubscript{1} or α\textsubscript{v}) is synthesized in an excess, and the formation of any αβ heterodimer is dependent on the availability of the other subunit (14, 15). Therefore, the cell surface copy number of, for example, α\textsubscript{v}β\textsubscript{1} and α\textsubscript{v}β\textsubscript{3} integrin is dependent on the synthesis rate of α\textsubscript{v} and β\textsubscript{1} subunits, respectively. The presence of α\textsubscript{v}β\textsubscript{1} heterodimers challenges this model. To address this controversy, we hypothesized that there is a hierarchy in the formation of distinct αβ heterodimers and that α\textsubscript{v}β\textsubscript{1} complex is formed only in the presence of sufficient excesses of α\textsubscript{v} and β\textsubscript{1} subunits in relation to their other partners. To test this hypothesis, we used a previously constructed cDNA coding for an intracellular, single-chain anti-α\textsubscript{v} antibody and analyzed stably transfected WM-266-4 melanoma cell clones. The antibody could significantly reduce the expression level of α\textsubscript{v} subunit and selectively diminish the cell surface expression of α\textsubscript{v}β\textsubscript{1} integrin. However, it could not affect the cell surface expression of another prominent α\textsubscript{v} integrin, namely α\textsubscript{v}β\textsubscript{3}. Thus, the experimental data support our theory, explaining the mechanism of selective regulation of α\textsubscript{v}β\textsubscript{1} integrin expression on cell surface.

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Construction and Transfection of cDNA Coding for Intracellular Single-chain Antibody—Anti-α\textsubscript{V} integrin intracellular single-chain antibody was constructed as described previously (16–19). Briefly, total RNA was isolated from $5 \times 10^8$ cells of hybridoma line L230 expressing anti-α\textsubscript{V} integrin monoclonal antibody (obtained from the ATCC) by using the Ultraspec RNA isolation system (Biotex Laboratories, Inc.). This RNA was used to prepare cDNA by using primers B (TGM GGA GAC GGT GAC CRW GGT CCC T) and D. A DNA segment coding for the interchain linker was amplified from an anti-tat 3 gene (a gift from Wayne A. Marasco, Dana-Farber Cancer Institute, Harvard Medical School, Boston) by PCR using primers A (GGG ACC TGC GTC ACC CTG TCC). The primer sequences were from Richard-son et al. (18). Immunoglobulin heavy and light chain variable domains (VH and VL) were amplified from the cDNA by PCR using primers A (TTT TCT AGA TTA TTA CAG TGG ATC) and B or C (GAG CTC GTG CTC ACM CAR WCT CCA) and D. A DNA segment coding for the interchain linker was amplified from an anti-tat 3 gene (a gift from Wayne A. Marasco, Dana-Farber Cancer Institute, Harvard Medical School, Boston) by PCR using primers E (GGG ACC TGC GTC ACC CTG TCC) and F (TGG AGA CTG GTG GAG CAC GAG CTC AGA).

The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; MMP, matrix metalloproteinase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; FCS, fetal calf serum; ER, endoplasmic reticulum; VH, variable heavy chain; VL, variable light chain.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Melanoma cell line WM-266-4 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM)\textsuperscript{a} supplemented with heat-inactivated 10% fetal calf serum (FCS, Invitrogen), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

**Construction and Transfection of cDNA**—Construction and Transfection of cDNA Coding for Intracellular Single-chain Antibody—Anti-α\textsubscript{V} integrin intracellular single-chain antibody was constructed as described previously (16–19). Briefly, total RNA was isolated from $5 \times 10^8$ cells of hybridoma line L230 expressing anti-α\textsubscript{V} integrin monoclonal antibody (obtained from the ATCC) by using the Ultraspec RNA isolation system (Biotex Laboratories, Inc.). This RNA was used to prepare cDNA by using primers B (TGM GGA GAC GGT GAC CRW GGT CCC T) and D. A DNA segment coding for the interchain linker was amplified from an anti-tat 3 gene (a gift from Wayne A. Marasco, Dana-Farber Cancer Institute, Harvard Medical School, Boston) by PCR using primers A (GGG ACC TGC GTC ACC CTG TCC). The primer sequences were from Richard-son et al. (18). Immunoglobulin heavy and light chain variable domains (VH and VL) were amplified from the cDNA by PCR using primers A (TTT TCT AGA TTA TTA CAG TGG ATC) and B or C (GAG CTC GTG CTC ACM CAR WCT CCA) and D. A DNA segment coding for the interchain linker was amplified from an anti-tat 3 gene (a gift from Wayne A. Marasco, Dana-Farber Cancer Institute, Harvard Medical School, Boston) by PCR using primers E (GGG ACC TGC GTC ACC CTG TCC) and F (TGG AGA CTG GTG GAG CAC GAG CTC AGA).

The single-chain antibody gene was assembled from the VH, VL, and interchain linker fragments by overlap extension (20), followed by PCR amplification with primers A and I (as described under “Experimental Procedures”). The PCR products were separated on 0.8% agarose gel. The 830-bp fragment of anti-α\textsubscript{V} cDNA construct is visible in anti-α\textsubscript{V}-transfected clones (a) but not in vector control clones (v). St means standard marker of 800 bp.

**RESULTS**

**Fig. 1. Intracellular single-chain anti-α\textsubscript{V} integrin antibody, its expression, and function in WM-266-4 melanoma cells.** A, schematic illustration of intracellular single-chain anti-α\textsubscript{V} integrin antibody. Antigen binding sites are in immunoglobulin heavy and light chain variable domains (VH and VL, respectively). Between these domains there is a flexible interchain linker. Amino-terminal pro-sequence leads the antibody to endoplasmic reticulum, and carboxyl-terminal ER retention signal (KDEL) prevents the transport of the antibody outside the ER. B, FACSscan analysis of transfected WM-266-4 cell clones. Sixteen anti-α\textsubscript{V} cDNA construct-transfected (a) and six vector control cell clones (v) were tested for the cell surface expression of α\textsubscript{V} integrin. The α\textsubscript{V} integrin expression of six anti-α\textsubscript{V} and three vector control clones is illustrated in this figure. Monoclonal anti-α\textsubscript{V} antibody L230 was used as a primary antibody. Negative control cells (nc.) were stained with fluorescein isothiocyanate-labeled secondary antibody only. Panel C shows the presence of anti-α\textsubscript{V} cDNA construct in anti-α\textsubscript{V},transfected cell clones. Total RNA of WM-266-4 cell clones was isolated and RT-PCR reaction was performed by using primers A and I (as described under “Experimental Procedures”). The PCR products were separated on 0.8% agarose gel. The 830-bp fragment of anti-α\textsubscript{V} cDNA construct is visible in anti-α\textsubscript{V}-transfected clones (a) but not in vector control clones (v). St means standard marker of 800 bp.

**TABLE**—Summary of α\textsubscript{V} Integrin Expression in WM-266-4 Cell Clones

| Cell clone | St | α3a11 | a15v3 | v3 | v4 | v5 |
|---|---|---|---|---|---|---|
| anti-αV (mRNA) | — | — | — | — | — | — |

\[ \text{Expression of the mRNA coded by the intracellular antibody construct in transfected cells was confirmed by RT-PCR of the total RNA isolated from both anti-αV plasmid and vector control cells. RT-PCR was performed by the GeneAmp® RNA PCR kit (PerkinElmer Life Sciences) using primers A and D. T_m was 88°C.} \]

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Fig. 2. Cell surface expression of α5 and α5-related β subunits of three selected anti-α5 (●) and vector-transfected (○) cell clones. Analysis was made by FACScan. The clones stained with secondary antibody only (○) show the unspecific background.

4 °C or 1 h at 37 °C. Before coating, COL 15 was denatured for 20 min at 56 °C. Bovine serum albumin (BSA, 0.1%) was used to measure the nonspecific binding or spreading. Residual protein absorption sites in all wells were blocked with 0.1% bovine serum albumin in phosphate-buffered saline for 1 h at 37 °C. Confluent cell cultures were detached by using 0.01% trypsin and 0.02% EDTA, rinsed in DMEM containing 10% FCS, and then washed twice with DMEM. Cells were suspended in DMEM, transferred into wells (1.5 × 10^4 cells/well), and incubated for 35–120 min at 37 °C. Saturating concentrations of functional monoclonal antibodies against α5 (BD PharMingen), α6 (mAb 16) (23), β1, β2, β3 (Chemicon), and α5β1 (PIF6) (24) integrins were incubated with cells for 15–30 min at room temperature before adding them to wells. Nondherent cells were removed by rinsing the wells with medium; adherent cells were fixed with 8% formalin and 10% sucrose and then washed with distilled water. A spread cell was characterized as one having a clearly visible ring of cytoplasm around the nucleus. The portion of spread cells was expressed as percentage of the number of adherent cells.

Northern Blot Hybridizations—Cells were cultured in DMEM supplemented with 10% FCS for 24 h. The total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA), and mRNA levels of specific genes were measured by Northern blot hybridization. RNAs were separated in formaldehyde-containing agarose gels, transferred to nylon membranes (Zeta-Probe, Bio-Rad), and hybridized with 32P-labeled (Amersham Biosciences) cDNA probes. The following cDNAs were used: human matrix metalloproteinase-1 (MMP-1; Ref. 25), human matrix metalloproteinase-2 (MMP-2; Ref. 26), and rat glyceraldehyde-3-phosphate dehydrogenase (27). [32P]cDNA-mRNA hybrids were visualized by autoradiography.

Flow Cytometry—Cells were grown to early confluence, detached with trypsin-EDTA, washed with PBS (pH 7.4), and then incubated with PBS containing 1% FCS for 30 min at 4 °C. Cells were collected by centrifugation, exposed to saturating concentration of antibodies against α5 integrin (L230), α1β1 integrin (BD PharMingen), α1 integrin (Endogen, Rockford, IL), α5 integrin (12F1) (28), α5 integrin (BD PharMingen), α5 integrin (mAb 16) (23), β1 integrin (R-322, rabbit polyclonal) (14), β2 integrin (Southern Biotech, Birmingham, UK), α5β1 integrin (PIF6) (24), or β1 integrin (ET76) (24) in 1% FCS/PBS for 30 min at +4 °C. For labeling, cells were incubated with rabbit anti-mouse (1:20 dilution), rabbit anti-rat (1:100 dilution), or swine anti-rabbit (1:20 dilution) IgG coupled to fluorescein (all from DAKO A/S, Glostrup, Denmark) for 30 min at 4 °C, washed twice with PBS, and suspended in the same buffer. Relative amounts of cell surface integrins were determined by comparison of fluorescent emission intensity data as collected using a FACScan apparatus (BD Pharmingen). Control samples were prepared by treating cells without primary antibodies.

Immunoprecipitations and Western Blotting—Cells were metabolically labeled with 50 μCi/ml [35S]methionine (Translabel, ICN Biomedicals Inc., Irvine, CA) for 16 h in methionine-free minimum essential medium. Cell monolayers were rinsed on ice with a solution containing 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 25 mM Tris-HCl (pH 7.4) and then detached by scraping. Cell pellets were obtained by centrifugation at 500 × g for 5 min. Cells were solubilized in 200 μl of the same buffer containing 100 mM n-octyl-β-D-glucopyranoside (Sigma) on ice with occasional vortexing. Insoluble material was removed by centrifugation at 1 × 10^4 × g for 5 min at 4 °C. Radioactivity in cell lysates was counted, and equal amounts of radioactivity was used in each sample. Triton X-100 (0.5% v/v) and bovine serum albumin (0.5 mg/ml) were added to the supernatants, which were then precleared by incubation with 50 μl of packed protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden). Supernatants were immunoprecipitated with α5 integrin or β1 integrin antibody (L230 or R-322, correspondingly) for 12 h at 4 °C. After incubation with secondary antibody (rabbit anti-mouse, DAKO), immune complexes were recovered by binding to protein A-Sepharose and washing the beads four times with 25 mM Tris-buffered isotonic saline (pH 7.4) containing 0.5% Triton X-100 and 1 mg/ml bovine serum albumin and twice with 0.5 M NaCl and 25 mM Tris-HCl (pH 7.4). The immunoprecipitates were separated by electrophoresis on sodium dodecyl sulfate-containing 6% polyacrylamide gels under reducing (immunoprecipitation with anti-β1) or nonreducing (immunoprecipitation with anti-α5) conditions, followed by autoradiography. In pulse-chase assays, cells were metabolically labeled for 1 h and harvested at 0, 2, 4, and 8 h after pulse.

To examine the amount of α5β1 heterodimer, immunoprecipitation was performed with anti-β1 (R-322) (14). Immunoprecipitates were separated on a 7.5% polyacrylamide gel under reducing conditions, and the gel was transferred to a nylon membrane (Hybond ECL, Amersham Biosciences). Nonspecific adsorption sites were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween by incubating
Regulation of αvβ1 Expression and αv-Integrin Heterodimer Formation

the membrane at room temperature for 1 h. A saturating concentration (1:100) of polyclonal αv integrin antibody (Chemicon International Inc., Temecula, CA) was added to the blocking solution. The membrane was rinsed twice, washed twice for 15 min, and washed three times for 5 min. Horseradish peroxidase-linked anti-mouse IgG (Amersham Biosciences) was used as a secondary antibody. The antibody was diluted in Tris-buffered saline containing 0.1% Tween containing 5% milk (1:100), and the membrane was incubated for 1 h. Washing was performed as above. β1 integrin-linked αv integrin was visualized by an enhanced chemiluminescence reaction (ECL kit, Amersham Biosciences).

Migration Assays—Coating of 24-well immunoplates (MaxiSorp) was done by exposing each well to 0.3 ml of phosphate-buffered saline (PBS, pH 7.4) containing 27.0 µg/ml (5 µg/cm²) of fibrinogen, fibronectin (human plasma fibronectin, Chemicon), or COL 15 domain of type XVII collagen (22) for 12 h at 4 °C. Residual protein absorption sites in all wells were blocked with 0.1% bovine serum albumin in phosphate-buffered saline for 1 h at 37 °C. The trypsinized cells were rinsed with DMEM plus 10% FCS or 0.2% soybean trypsin inhibitor and washed twice in DMEM or Opti-MEM® (Invitrogen, Inc.). Steel cylinders were placed in each well, and cells were added (2 × 10⁴ cells/well). After 3 h the cylinders were removed, 1 ml of either DMEM or Opti-MEM® was added, and cells were allowed to migrate for 3 days. Cells were fixed with 10% sucrose and 8% formalin in PBS and stained with 0.1% crystal violet and 0.2 M boric acid. Migration was determined by measuring the increase of the cell colony area.

Invasion Assays—Cell culture inserts (BD PharMingen) contained polyethylene terephthalate membranes of 8 µm pore size. Membranes were coated with human plasma fibronectin (Chemicon), type I collagen gel (Cellon S.A.), or Matrigel® (Collaborative Research, Bedford, MA). In fibronectin invasion assays, a concentration of 20 µg/ml was used, and the total volume was 80 µl/insert. Inserts were allowed to air-dry in a cell culture hood overnight. In Matrigel® and type I collagen invasion assays, 30 µg of Matrigel®, 35 µl of Collagen gel, respectively, were used for each insert. Inserts were used with 24-well cell culture plates (Costar). Cells from cultures in early confluence were trypsinized, and one volume of 0.2% soybean trypsin inhibitor or 10% FCS/DMEM was added to inhibit trypsin activity. Cells were then washed twice in DMEM, resuspended in 0.1% BSA/DMEM, and added to inserts (1 × 10⁴ cells DMEM/µl) in 100 µl of 10% FCS/DMEM. A saturating concentration of chemoattractant in the lower chamber. Cells were allowed to invade at 37 °C for 8–9 h through fibronectin and 2 days through Collen gel and Matrigel®. Upper chamber was washed clean with a swab, and the invaded cells on the lower surface of the membrane were fixed with PBS containing 2% paraformaldehyde and stained with 0.1% crystal violet and 0.2 M boric acid. Invaded cells on the lower side of the membrane were counted under a light microscope with a 10× lens. Three representative fields were counted from each insert. The result was reported as the number of invaded cells in three fields.

RESULTS AND DISCUSSION

Cell Clones Expressing Intracellular Anti-αv Integrin Antibody Show Reduced Cell Surface Expression of αv Subunit without Changes in the β3 Subunit—Sixteen stable cell clones transfected with a cDNA construct coding for an intracellular anti-αv antibody (Fig. 1A) and 6 vector control cell clones were tested for expression levels of αv integrin. In anti-αv cDNA-transfected clones, the cell surface expression level of αv was reduced up to 80% when compared with vector control clones (Fig. 1B). Three anti-αv cDNA-transfected cell clones (a3, a11, and a15) and three vector control clones (v3, v4, and v5) were selected for further experiments. In these clones the presence of mRNA derived from intracellular antibody construct was confirmed by RT-PCR (Fig. 1C). Surprisingly, there were no significant decreases in the expression levels of β3 or β5 integrins (Fig. 2A). A third partner of αv subunit, β6, was not detected on these cells (Fig. 2A). To confirm the unreduced expression level of αv, β3, metabolically labeled cells were immunoprecipitated with an anti-β3 antibody. The results did not indicate a decrease in the number of β3-connected, mature αv integrin subunits in anti-αv-expressing cell clones (Fig. 2A). Immunoprecipitations from anti-αv-expressing cell clones had an extra band with an approximated molecular size of 140 kDa, corresponding to the precursor form of αv integrin described in previous papers (15, 19). The higher molecular mass form represents an integrin with a complex-type N-linked oligosaccharide, whereas the lighter precursor form represents an integrin that is still inside the endoplasmic reticulum with high mannosetype oligosaccharides (14). Here, the maturation process was studied by pulse-chase experiments followed by immunoprecipitations with anti-αv antibody. Maturation occurred in 8 h in vector control clones (data not shown) in accordance with our previous studies with Saos-2 osteosarcoma cells (19). The presence of the larger intracellular pool of precursor αv may indicate a decelerated maturation process of the αv integrins because of the intracellular antibodies.

Intracellular Antibody against αv Integrin Has a Selective Effect on αvβ1 Expression Suggesting a Hierarchy in the Formation of αv-containing Heterodimers—The diminished cell surface expression of the αvβ1 heterodimer was not accompanied by a similar reduction of the β3 subunit, leading us to the hypothesis that the expression of αvβ1 heterodimer must be affected. In the absence of a specific antibody for αvβ1, we first immunoprecipitated the total cell lysate with an anti-β1 antibody and then Western-blotted the immunoprecipitates with anti-αv integrin antibody. Nearly all β1-bound αv integrin was in its precursor form in anti-αv antibody-transfected cell clones, whereas in vector control clones β1-associated αv integrin was in the
mature form only (Fig. 3B). Thus, our data indicate that, when the amount of $\alpha_v$ subunit in the endoplasmic reticulum is reduced, very little $\alpha_v\beta_1$ ends up on the cell surface. Importantly, we have also tested the mechanism of overexpression of $\alpha_v\beta_1$-related binding to fibronectin (9). In these experiments, the $\alpha_v\beta_1$-transfected cell clone derived from cells in which $\alpha_v\beta_1$ was a major fibronectin receptor (9). The obvious hierarchy in

![Diagram](http://www.jbc.org/)

**Fig. 5. Adhesion, spreading, and lateral cell migration experiments on fibronectin.** A and B, three parallel samples of one vector control (v3) and one anti-$\alpha_v$ integrin-transfected (a3) clone were incubated with or without anti-$\alpha_4$, anti-$\alpha_5$, and anti-$\beta_3$ integrin antibodies and plated on fibronectin-coated wells of 96-well plate. C, control. Cells were allowed to attach for 35 min. Cells were washed and fixed, and three representative microscope fields (10×) of adherent cells were counted. The number of spread cells is reported as a percentage from total number of attached cells. C, two independent migration assays were performed. Three anti-$\alpha_v$-transfected (a) and three vector control (v) cell clones were allowed to migrate for 3 days in serum-free conditions, after which the cells were fixed and stained, and increase of the cell colony area was measured. There were three parallel samples of each clone, and the increase is reported as an average of individual cell clones ± S.D. The difference between anti-$\alpha_v$-transfected and vector control cells was statistically significant when the two experiments were analyzed together ($p < 0.0001$; two-way analysis of variance).
the formation of different α5-containing heterodimers suggests that the amount of α5β1 integrin can be regulated selectively and independently of other integrin heterodimers. In simplified terms, this model proposes that the number of α5β1 and α5β2 heterodimers is regulated at the level of the β1 and β2 genes, respectively, but the activity of the α5 gene dictates the number of α5β1, heterodimers (Fig. 3C).

Integrin α5β1 Mediates Melanoma Cell Migration on Fibronectin—Selective reduction of α5β1 levels in experimental clones allowed direct observations about its functions to be made. To control for integrin-mediated characteristics, expression levels of other integrins in the experimental clones were tested (Fig. 4). In one of the antibody-expressing clones (a3), the expression of β3 integrin was higher than in any other clone. This can be attributed to simultaneously higher expression of collagen-binding integrins α1 and α2. The expression of α6, another fibronectin-binding integrin, was slightly reduced in one of the clones when compared with vector control clones. One vector control clone (v4) did not have any α5 integrin on its surface. Platelet integrin αIIbβ3 was not detected on the cell surface of any of the clones. The expression levels of β5 and β3 subunits were equal in all cell clones.

Because of small variations in the levels of α5 and α2 integrins, only clones that had the same numbers of these receptors were selected and tested for adhesion. On fibronectin, cell spreading of the selected anti-α5 and vector control clones was equal (Fig. 5A). The spreading of both clones could be reduced but not completely prevented when the fibronectin receptors β3, α4, and α5 were blocked by monoclonal antibodies. Treatment with antibodies revealed differences in the spreading behavior of the anti-α5-expressing and vector control cells; in the presence of antibodies, their average spreading was 50 and 30%, respectively. The number of attached cells within each well was counted with similar results (in the presence of antibodies: 75 versus 55%) (Fig. 5B). This indicates that α5β1 integrin can contribute to adhesion and spreading on fibronectin but does so preferentially in the absence of other fibronectin receptors.

On vitronectin cell adhesion and spreading was much slower than on fibronectin. No α5β3-related differences in spreading behavior were observed for any of the clones when they were plated on vitronectin. Recently, we have suggested that in keratinocytes α5β1 contributes to cell spreading on type XVII collagen (COL 15 domain) (29). However, we did not find any α5β3-dependent differences in adhesion or spreading of WM-266-4 melanoma cells on COL 15 domain (data not shown). Likewise, similar results were obtained for spreading and adhesion assays on fibrinogen (data not shown). Fibrinogen has previously been reported to function as a ligand for RGD-binding integrins (30).

We also tested whether the lack of α5β1 integrin has an influence on WM-266-4 melanoma cell migration. Three antibody-transfected and three vector control clones were allowed to migrate on fibronectin for 3 days in serum-free DMEM. Two independent experiments were performed, each of which comprised two or three parallel wells of each clone (Fig. 5C). Lateral migration was measured as an increase in the surface area covered by the cells. The area covered by the vector control clones increased on average 75–115% more when compared with anti-α5-transfected clones (p < 0.0001; two-way analysis of variance). These results indicate that α5β1 integrin participates in the lateral migration of WM-266-4 cells on fibronectin. Importantly, the small variation in the expression levels of α4 and α2 integrins did not play a role in these experiments. Similar to adhesion and spreading assays, there were no α5β2-related differences in lateral migration on COL 15 domain of type XVII collagen or fibrinogen (data not shown).

Our finding that α5β1 integrin is a low affinity fibronectin receptor is in accordance with the previous data (8). Cells may try to compensate for this low affinity with a high expression level. Spreading and adhesion on fibronectin were approximately the same in anti-α5 antibody-expressing and control clones. This indicates that α5β1, α5β2, and α5β3 are mainly responsible for spreading and adhesion on fibronectin. When these receptors were blocked, we could see the influence of α5β1 integrin. Moreover, the depletion of α5β1 integrin could markedly reduce the lateral migration of WM-266-4 cells. Previously it has been shown that α5β1-expressing Chinese hamster ovary-B2 cells that lack α5 integrin are unable to migrate on fibronectin (8). Thus, it is probable that α5β1 integrin is involved in cell migration on fibronectin, but, to migrate, the cells need another fibronectin-binding integrin such as α5β3 or α5β2. The low affinity/high expression of α5β1 integrin may be an advantage for cancer cells in such a dynamic process as cell migration, where continuous formation and dissolution of adhesion sites plays a major role.

In addition to fibronectin, α5β1 integrin has been suggested to show some binding to vitronectin. However, in our experiments with melanoma cells, we found that cell spreading on vitronectin was not affected by α5β1 integrin. The main receptor responsible for spreading on vitronectin seemed to be α5β3 integrin. However, on vitronectin α5β2 integrin did not have an effect on primary adhesion, whereas blocking of α5β3 from α5β2-depleted cells clearly reduced the number of adherent cells (data not shown). These results stress the diversity of integrin functions on different substrates.

There are a number of studies in which connections between the expression of specific integrin receptors and the production of MMPs have been proposed (31–34). In the published papers, α5β1 integrin has not been studied. We made several invasion assays with type I collagen gels, the basement membrane analog Matrigel®, and fibronectin, but no α5β1-dependent differences were observed. Furthermore, the mRNA levels of MMP-1 and MMP-2 were not α5β1-dependent. Based on these results, α5β1 does not play a major role in the invasion process or in the expression of metalloproteinases.

To conclude, previous studies have shown that α5β1 might be selectively regulated during development (35). Our study suggests that it is an important fibronectin receptor that is abundant on some cancer cells. Furthermore, we propose a molecular mechanism that explains the selective regulation of α5β1 integrin.

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