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Cross Talk between β_1 and α_V Integrins: β_1 Affects β_3 mRNA Stability

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There is increasing evidence that a fine-tuned integrin cross talk can generate a high degree of specificity in cell adhesion, suggesting that spatially and temporally coordinated expression and activation of integrins are more important for regulated cell adhesive functions than the intrinsic specificity of individual receptors. However, little is known concerning the molecular mechanisms of integrin cross talk. With the use of β_1-null GD25 cells ectopically expressing the β_1A integrin subunit, we provide evidence for the existence of a cross talk between β_1 and α_V integrins that affects the ratio of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin cell surface levels. In particular, we demonstrate that a down-regulation of $\alpha_V\beta_3$ and an up-regulation of $\alpha_V\beta_5$ occur as a consequence of β_1A expression. Moreover, with the use of GD25 cells expressing the integrin isoforms β_1B and β_1D, as well as two β_1 cytoplasmic domain deletion mutants lacking either the entire cytoplasmic domain (β_1TR) or only its "variable" region (β_1COM), we show that the effects of β_1 over α_V integrins take place irrespective of the type of β_1 isoform, but require the presence of the "common" region of the β_1 cytoplasmic domain. In an attempt to establish the regulatory mechanism(s) whereby β_1 integrins exert their *trans*-acting functions, we have found that the down-regulation of $\alpha_V\beta_3$ is due to a decreased β_3 subunit mRNA stability, whereas the up-regulation of $\alpha_V\beta_5$ is mainly due to translational or posttranslational events. These findings provide the first evidence for an integrin cross talk based on the regulation of mRNA stability.

INTRODUCTION

Integrins form one family of cell adhesion receptors that play a prominent role in the adhesive interactions between cells and their surrounding extracellular matrix (ECM) (Hynes, 1992). All integrins are heterodimers composed of noncovalently linked α and β subunit transmembrane glycoproteins containing large extracellular domains, short transmembrane domains, and carboxyl-terminal cytoplasmic domains of variable length (Hynes, 1992). These adhesive receptors are endowed with both structural and regulatory functions, linking extracellular matrix to the actin cytoskeleton at focal adhesion sites and providing bidirectional transmission of signals across the plasma membrane (Schoenwaelder and Burridge, 1999; Critchley, 2000). The cytoplasmic domain of the β subunit has been shown to play a critical role in focal adhesion and actin stress fiber organization and both outside-in and inside-out integrin signaling (Liu *et al.*, 2000).

Through their molecular interactions integrins regulate a number of critical cellular processes, including proliferation, differentiation, survival, migration, and gene expression (Giancotti, 1997; Giancotti and Ruoslahti, 1999). It is now clear that altered, modulated, or regulated adhesive interactions can change the way cells interact with their environment with dramatic consequences for both normal and pathological conditions. Cells can vary their adhesive properties by selectively expressing different integrins and by modulating their integrin specificity and affinity for ligands (Hynes, 1996). However, cells often display multiple integrins capable of interacting with a particular ECM protein and, conversely, individual integrins can recognize several extracellular matrix molecules (Hynes, 1992). Thus, integrin expression and ligand specificity are often apparently redundant, at least in terms of simple adhesion. The biological significance of this phenomenon is not clear yet; nevertheless, there is increasing evidence that individual integrin

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Abbreviations used: ECM, extracellular matrix; FN, fibronectin.

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receptors mediate distinct functions and can convey unique information (Giancotti, 2000).

Most integrins belong to one of two major subfamilies defined by the β_1 and α_V subunits. The β_1 subunit pairs with at least 12 different α subunits (α_1-α_{11}, α_V) to comprise receptors for a variety of ECM proteins, including collagen, laminin, fibronectin, and vitronectin (Hynes, 1992). A large body of literature (Brakebusch *et al.*, 1997; Giancotti, 1997; reviewed in Schoenwaelder and Burridge, 1999) has addressed the role of β_1 integrins in mediating important cell adhesion and signal transduction events. Four different β_1 isoforms have been identified (β_1A, β_1B, β_1C, and β_1D), which differ in their cytoplasmic domains and differentially affect many integrin functions (Belkin *et al.*, 1997; Fornaro and Languino, 1997; Belkin and Retta, 1998; Pfaff *et al.*, 1998; Retta *et al.*, 1998).

The α_V subunit is known to associate with at least five different β subunits (β_1, β_3, β_5, β_6, and β_8). Among these α_V integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ have been extensively studied. The $\alpha_V\beta_3$ integrin, in particular, has a relatively limited cellular and tissue distribution (Yamada *et al.*, 1995), but its expression and activity are tightly regulated during a variety of biological processes, including cell proliferation and survival (Montgomery *et al.*, 1994), wound healing (Clark *et al.*, 1996a), angiogenesis (Brooks *et al.*, 1994), bone remodeling (McHugh *et al.*, 2000), tumor progression (Albelda *et al.*, 1990) and metastasis (Yun *et al.*, 1996). This integrin can bind to a variety of ECM proteins, including vitronectin, fibronectin, fibrinogen, thrombospondin, Von Willebrand factor, and denatured collagen (Kühn and Eble, 1994), and it is able to recruit cytoskeletal and signaling proteins to focal adhesion sites (Lewis *et al.*, 1996). In addition, $\alpha_V\beta_3$ is one of the integrins that promotes the assembly of fibronectin matrix (Wennerberg *et al.*, 1996; Wu *et al.*, 1996; Retta *et al.*, 1998).

In contrast to $\alpha_V\beta_3$, $\alpha_V\beta_5$ is among the most widely expressed integrins. This receptor can specifically and efficiently bind its ligand vitronectin but remains randomly distributed over the surface of the cells and does not trigger the assembly of focal adhesion structures (Wayner *et al.*, 1991; Leavesley *et al.*, 1992). Moreover, $\alpha_V\beta_5$ integrin has different requirements than $\alpha_V\beta_3$ for mediating adhesive events, such as cell spreading and migration, to the common ligand vitronectin (Klemke *et al.*, 1994; Lewis *et al.*, 1996), and it can induce differential biological responses (Friedlander *et al.*, 1995).

Perturbation experiments with antibodies, blocking peptides, and antisense oligonucleotides demonstrated that both β_1 and α_V integrins play a primary role in important physiological and pathological processes (reviewed in Varner and Cheresh, 1996; Brakebusch *et al.*, 1997; Bader *et al.*, 1998). However, recent genetic analyses have clearly increased questions as to the primacy of these integrins, and instead have pointed to a cross talk model where spatiotemporal regulation, combinatorial expression, and activation of several integrin receptors generate a high degree of specificity in cell adhesion (Fassler *et al.*, 1996; Hynes, 1996, 1999; Brakebusch *et al.*, 1997; Bader *et al.*, 1998; Hodivala-Dilke *et al.*, 1999; McHugh *et al.*, 2000). Several observations indicate the existence of a cross talk between β_1 and α_V integrins, which usually takes the form of one integrin influencing the functional behavior of another integrin expressed on the same cell (Yang and Hynes, 1996; Belkin *et al.*, 1997; Retta *et al.*, 1998; Blystone *et al.*, 1999; Corbett and Schwarzbauer, 1999). However, in most cases, the mechanistic basis of this receptor cross talk is not completely understood, and it is unknown whether and how the integrin cross talk can regulate the ratio of integrin cell-surface expression levels.

GD25 cells, derived from β_1-null mouse embryonic stem cells (Wennerberg *et al.*, 1996), are a valuable model for examination of integrin cross talk. In fact, these cells express $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_6\beta_4$ as major integrin complexes but do not express integrins of the β_1 subfamily, thus permitting a variety of genetic experiments exploring the basis of integrin cross talk. We have previously transfected GD25 cells with cDNAs encoding for the isoform A, B, or D of the human β_1 integrin subunit or two β_1 mutants lacking either the entire cytoplasmic domain (β_1TR) or only the cytoplasmic domain "variable" region that characterizes each isoform (β_1COM) (Retta *et al.*, 1998). With the use of these cells, we investigated the specific functional properties of the isoform B and D of the human β_1 integrin subunit, showing the existence of a functional cross talk between these two β_1 isoforms and the endogenous α_V integrins. In particular, both β_1B and β_1D expression prevented different fibronectin (FN)-dependent α_V integrin functions, including its ability to mediate cell adhesion, to localize to focal adhesions, and to assemble an FN matrix (Belkin *et al.*, 1997; Retta *et al.*, 1998).

In the present study, we show that the cross talk between β_1 and α_V integrins is mainly based on the regulation of β_3 and β_5 integrin subunit expression exerted by β_1 integrins. In fact, the ectopic expression of either β_1A, β_1B, or β_1D in GD25 cells induces a drastic down-regulation of β_3 and an up-regulation of β_5 integrin cell surface levels. Moreover, analysis of GD25 cells expressing β_1 integrins lacking either the entire β_1 cytoplasmic domain (β_1TR) or only its variable region (β_1COM) demonstrate that the "common" region of the β_1 cytoplasmic domain is required for these effects. We further demonstrate that β_1 exerts its control over $\alpha_v\beta_3$ expression level by modulating the β_3 mRNA stability, whereas the up-regulation of $\alpha_V\beta_5$ is mainly due to translational or posttranslational events leading to an increased recruitment of the β_5 subunit at the cell surface.

MATERIALS AND METHODS

Antibodies and Reagents

The mouse anti-human β_1 monoclonal antibody (mAb) TS2/16 was obtained from American Type Culture Collection (Manassas, VA). The rat anti-mouse α_6 mAb GoH3 was a gift from A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The rabbit polyclonal antisera to α_V, α_3, and α_5 integrin cytoplasmic domains, produced in our laboratory, were previously described (Retta *et al.*, 1998). The polyclonal antisera to β_3 and β_5 were produced with the use of a previously described protocol (Defilippi *et al.*, 1995). Briefly, rabbits were immunized against a GST-β_3 fusion protein containing the cytoplasmic domain of the mouse β_3 integrin subunit and against a synthetic peptide reproducing an amino acid sequence from the carboxy terminus of mouse β_5 integrin subunit, respectively. The β_5 peptide EKAQLKPPATSDA was synthesized by solid phase methods with the use of an LKB Biolynx synthesizer (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and coupled to keyhole limpet hemocyanin with the use of glutaraldehyde. The mouse anti-paxillin mAb was purchased from Transduction Laboratories (Nottingham, United Kingdom). The affinity-purified rhodamine-labeled goat anti-mouse and goat anti-rabbit IgG were from Sigma (St. Louis, MO). Poly-L-lysine and monensin were from

Sigma. Vitronectin and fibronectin were purified from human plasma as previously described (Balzac *et al.*, 1994; Retta *et al.*, 1999). Protein A-Sepharose and protein G-Sepharose were from Amersham Pharmacia Biotech AB.

Cells and Culture Conditions

The mouse GD25 fibroblast line, which lacks expression of β_1 integrin heterodimers because of disruption of the β_1 gene by homologous recombination, was established after differentiation of β_1-null embryonic stem cells and immortalization with simian virus 40 large T antigen (Wennerberg *et al.*, 1996). GD25 cells expressing the human β_1A, β_1B, or β_1D integrin isoforms or the β_1TR and β_1COM human β_1 mutants, lacking the entire cytoplasmic domain and the cytoplasmic domain variable region, respectively, were obtained as previously described (Belkin *et al.*, 1997; Retta *et al.*, 1998). To avoid selection for anomalous functional traits, no efforts were made to establish clonal cell lines; instead, bulk cell populations expressing β_1 integrins were selected. Cells were cultured in DMEM (Invitrogen Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The β_1-expressing GD25 cells were cultured in the same medium plus 300 μg/ml hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany). Cell populations expressing high levels of the β_1 forms used were selected by the panning method and monitored by flow cytometry as described previously (Retta *et al.*, 1998).

Biotinylation of Cell Surface Proteins

Adherent cells, grown to 80–90% confluence in 90-mm tissue culture dishes, were washed twice with ice-cold buffer A (1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 5 mM KCl, 138 mM NaCl, 5,6 mM D-glucose, 25 mM HEPES, pH 7.4) and incubated with 0.5 mg/ml membrane-impermeable biotinylation reagent Sulfo-NHS-Biotin (Sigma) in buffer A at 4°C for 30 min. The reaction was quenched with DMEM containing 0.6% bovine serum albumin (BSA) and 25 mM HEPES, pH 7.4. The cells were then washed four times with ice-cold buffer A and lysed on ice in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 0.5% Triton X-100 and the protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and benzamidine (1 mM) (all from Sigma). Cell lysates were centrifuged at 12,000 $\times$ *g* for 30 min at 4°C, and total protein concentration in the supernatants was determined with the use of a bicinchoninic acid protein assay (Pierce, Rockford, IL). Supernatants containing equal amounts of proteins were precleared with a mixture of protein A-Sepharose and protein G-Sepharose and used in immunoprecipitation experiments.

Immunoprecipitation and Analysis of Integrins

Integrins were immunoprecipitated from precleared cell lysate supernatants by incubation with appropriate dilutions of specific antibodies and a mixture of protein A-Sepharose and protein G-Sepharose beads for 1 h at 4°C. Complexes were washed four times with the lysis buffer then the proteins were eluted with Laemmli's sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS) and subjected to SDS-polyacrilamide (7.5%) gel electrophoresis under nonreducing conditions. To visualize the biotinylated proteins, the gel was electroblotted onto Hybond-C transfer membrane (Amersham Pharmacia Biotech AB). The blot was then blocked with 5% BSA in phosphate-buffered saline (PBS) for 1 h at 42°C, incubated with streptavidin-peroxidase (Sigma) (1:10.000 in PBS/1% BSA) for 1 h at room temperature, and further processed by the Western blotting enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech AB).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis

Total RNA was isolated from 1×10^7 cultured cells with the use of the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Where indicated, before RNA isolation confluent cells were detached by treatment with 5 mM EDTA in PBS, washed twice with serum-free DMEM, resuspended in the same medium containing 1 μM monensin, and plated for 2 h on tissue culture dishes that had been coated with 10 μg/ml polylysine, fibronectin, or vitronectin as previously described (Retta *et al.*, 1998). A multiplex semiquantitative RT-PCR was used to detect the relative levels of β_3 and β_5 or β_1 integrin mRNAs. cDNA was synthesized from 5 μg of cytoplasmic RNA with the use of the 1st Strand cDNA Synthesis kit (Roche Molecular Biochemicals), and subjected to 28 (β_3/β_5) or 32 (β_1 and $\beta_1/\beta_3/\beta_5$) PCR cycles. The reaction conditions and oligonucleotide PCR primers used were optimized so that the amplification products fell within the range of PCR amplification linearity. PCR was performed with each reaction mixture containing 5 μl of cDNA, 1$\times$ reaction buffer (Amersham Pharmacia Biotech AB), 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM of each primer, and 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech AB) in a total volume of 50 μl. The following stages were used for each PCR cycle: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a prolonged extension stage of 72°C for 5 min after the final cycle. The primers were derived from nonhomologous regions of the mouse β_3 and β_5 and the human β_1 cDNA sequences, and led to 705-, 570-, and 857-bp PCR products, respectively. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Gels were photographed under UV light and intensities of the amplified cDNA fragments were quantitated with the use of a densitometric software (Molecular Analyst; Bio-Rad, Hemel Hempstead, United Kingdom). Molecular size standards (123-bp DNA ladder) were from Sigma.

For Northern blot hybridization, equal amounts of the purified total RNA (25 μg/lane) were separated by electrophoresis on a 1.2% agarose gel containing 1.8% formaldehyde and 1$\times$ FA Gel buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid, 5 mM NaAc, 1 mM EDTA, pH 7.0], transferred to a Nytran SuPerCharge transfer membrane (Schleicher & Schuell, Dassel, Germany) with the use of the TurboBlotter blotting device accordingly to manufacturer's instructions (Schleicher & Schuell), and UV cross-linked to the membrane. The membrane was prehybridized by incubation in Church's buffer (0.5 M Na-phosphate buffer, 10 mg/ml BSA, 7% SDS, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, pH 7.4) for 8 h at 65°C and hybridized with ^{32}P-labeled probes overnight at 65°C in Church's buffer. After hybridization, the membrane was washed once in 2$\times$ SSC + 0.1% SDS, once in 1$\times$ SSC + 0.1% SDS, once in 0.2$\times$ SSC + 0.1% SDS, and once in 0.1$\times$ SSC + 0.1% SDS for 15 min each at 65°C. The membrane was then exposed to x-ray film for 24–72 h at −80°C with an intensifying screen. Probes were synthesized by random priming with cDNA fragments of mouse β_3 and β_5 integrin subunits amplified by PCR and cloned in our laboratory. The same blots were rehybridized with a probe of the housekeeping gene β-actin to ensure equal loading.

Measurement of mRNA Stability

The measurement of mRNA stability was performed as described by Xu and Clark (1996). Briefly, cells were divided into three plates and cultured in 10% FBS/DMEM for 24 h before the addition of 60 μM 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB; Sigma), an inhibitor of transcription initiation. After addition of DRB, the cells were collected at 0, 4, 8, 12, and 24 h for RNA analysis. Total RNA isolation and Northern analysis were performed as described above.

Immunofluorescence Microscopy

Immunofluorescence studies were performed as described previously (Retta *et al.*, 1996). Briefly, cells were seeded onto fibronectin-

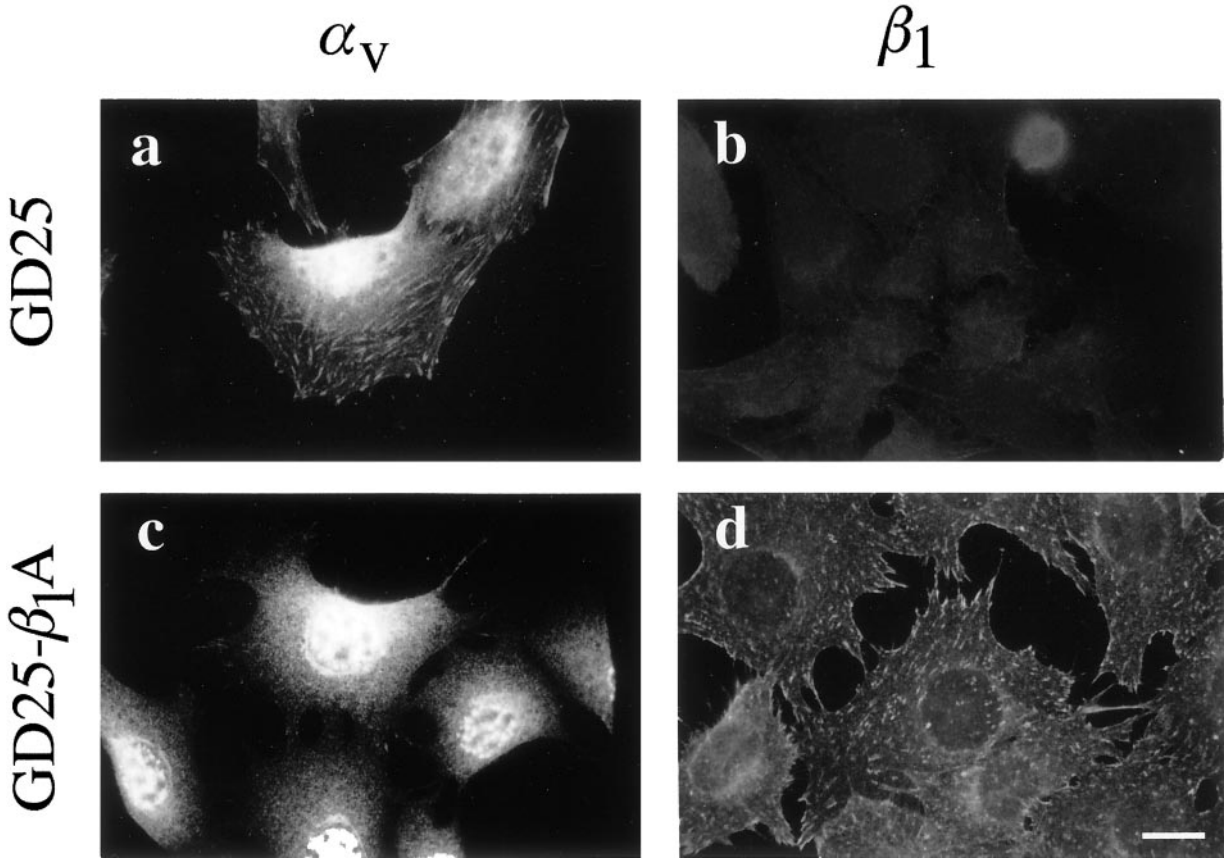

Figure 1. Subcellular localization of α_V and β_1 integrins on GD25 and GD25-β_1A cells plated on fibronectin. GD25 (a and b) and GD25-β_1A (c and d) cells were allowed to attach and spread on coverslips coated with fibronectin (30 μg/ml in PBS) for 3 h at 37°C. Cells were then fixed, permeabilized, and incubated with primary antibodies against α_V (rabbit anti-α_V) and β_1 (mAb TS2/16) integrin subunits. The α_V and β_1 antibody–antigen complexes were then detected with rhodamine-conjugated anti-rabbit and anti-mouse secondary antibodies, respectively. Representative fields were photographed with the use of an Olympus BM11 microscope fitted with epifluorescence. Notice that β_1 integrins displace α_V integrins from focal adhesions. Bar, 15 μm.

coated glass coverslips and allowed to spread for 3 h in complete culture medium. Cells were then washed with cold PBS, fixed for 10 min with 3.7% paraformaldehyde in PBS, permeabilized with ice-cold 0.5% Triton X-100, 3.7% paraformaldehyde in PBS for 5 min, and incubated with 1% BSA in PBS for 30 min. To localize α_V and β_1 integrins, the cells were stained with the rabbit antiserum to α_V (1:200 in PBS/1% BSA) or the mAb TS2/16 to β_1 (10 μg/ml in PBS/1% BSA). Bound primary antibodies were visualized by appropriate rhodamine-labeled secondary antibodies (1:100). Photographs were taken on an Olympus BX-60 epifluorescence microscope.

RESULTS

Expression of β_1 Integrins Affects Subcellular Localization of α_V Integrins

To determine the distribution of α_V and β_1 integrin heterodimers on GD25 and GD25-β_1A cells attached to fibronectin, indirect immunofluorescence experiments with specific antibodies were performed. GD25 cells, which do not express β_1 integrin heterodimers (Figure 1b), formed α_V-containing prominent focal adhesions when allowed to attach and spread on coverslips coated with fibronectin (Figure 1a), consistent with the reported ability of $\alpha_V\beta_3$ to localize to focal adhesions in these cells (Wennerberg *et al.*, 1996; Retta *et al.*, 1998). In contrast, the amount of α_V-containing focal adhesions was consistently reduced on GD25-β_1A cells attached to fibronectin (Figure 1c), whereas β_1A-containing focal adhesions were abundant (Figure 1d).

Thus, β_1A, by localizing to focal adhesions, displaces the α_V-containing heterodimers from these structures. Interestingly, we have previously shown that the expression of two other human β_1 isoforms, namely, β_1B, that does not localize to focal adhesions, and β_1D, that is efficiently targeted to focal adhesions, also causes the delocalization of α_V heterodimers on the cell surface (Belkin *et al.*, 1997; Retta *et al.*, 1998). Taken together, these data indicate that in GD25 cells cultured on fibronectin $\alpha_V\beta_3$ takes over the function of β_1 integrins in mediating focal adhesion assembly; however, when expressed, β_1 integrins behave as *trans*-dominant molecules with respect to α_V integrins.

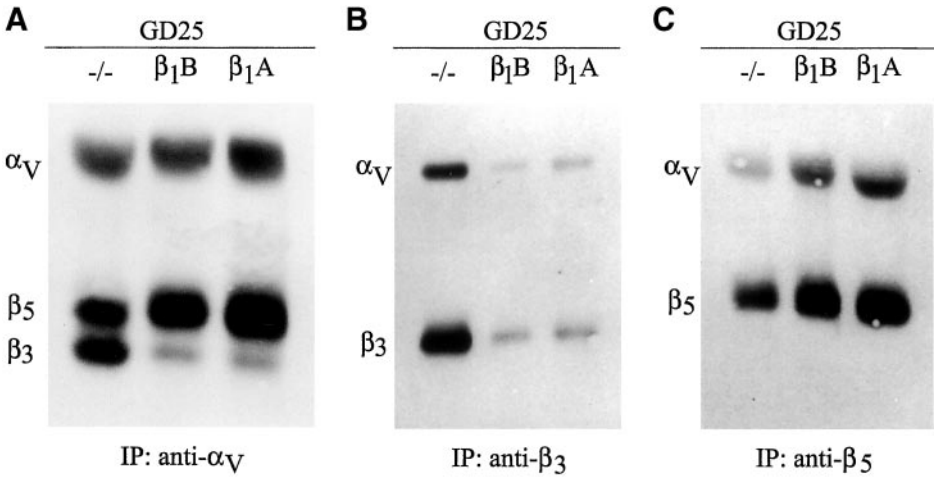

Figure 2. Surface expression of α_v, β_3 and β_5 integrins in GD25 and GD25-β1 cells. Integrin heterodimers were immunoprecipitated from surface biotinylated untransfected (−/−) or β_1A- and β_1B-transfected GD25 cells with polyclonal antibodies specific for α_v (A), β_3 (B), and β_5 (C) integrin subunits, respectively. After separation by nonreducing SDS-PAGE and Western blot, the immunoprecipitated proteins were detected with the use of peroxidase-conjugated streptavidin and ECL as described in MATERIALS AND METHODS. Notice that, although the expression of either integrin β_1 A or B isoforms does not alter significantly the expression level of the α_V integrin subunit, it induces a net change of the $\alpha_V\beta_3/\alpha_V\beta_5$ ratio.

Expression of the β_1 Integrin Subunit in GD25 Cells Induces Drastic Reduction of Surface Level of $\alpha_V\beta_3$ and an Up-Regulation of $\alpha_V\beta_5$

Previous results showed that transfection of GD25 cells with cDNA constructs of human β_1 integrin led to surface expression of the β_1 integrin subunit associated with the endogenous α_3, α_5, and α_6 subunits but not with the α_V subunit (Retta *et al.*, 1998). In addition, no obvious differences in α_V integrin expression were seen by immunoprecipitation from ^{125}I-surface–labeled GD25 and GD25-β_1A cells with an anti-α_V antiserum (Retta *et al.*, 1998). To understand the cellular mechanism(s) controlling the effect of β_1 over α_V integrins, we analyzed more in detail GD25 and GD25-β_1A cells for the expression levels of their α_V integrin heterodimers, namely, $\alpha_V\beta_3$ and $\alpha_V\beta_5$. Untransfected or β_1A-transfected GD25 cells were surface-labeled with Sulfo-NHS-Biotin then $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins were immunoprecipitated from nonionic detergent cell extracts and analyzed by Western blot. As expected, a polyclonal serum to the α_V integrin subunit coimmunoprecipitated α_V together with its associated β_3 and β_5 subunits (Figure 2A). The biotinylated α_V, β_3, and β_5 proteins resulted as distinct bands in Western blots and, surprisingly, we noticed that, whereas expression of the α_V subunit did not change significantly, the relative amounts of β_3 and β_5 proteins in β_1A-expressing GD25 cells were clearly different from those of untransfected GD25 cells (Figure 2A). With the use of antibodies specific for β_3 and β_5 subunits, we confirmed this evidence: β_3 protein levels were much lower in GD25-β_1A than in GD25 cells, whereas the opposite was true for β_5 protein levels (Figure 2, B and C). Thus, although the expression of the human β_1A integrin isoform in GD25 cells did not modify the surface expression level of the α_V integrin subunit, it led to a down-regulation and an up-regulation of the levels of its associated β_3 and β_5 subunits, respectively. These data suggest that the *trans*-dominant effect of β_1 integrin isoforms over the subcellular localization of α_V integrins in GD25-β_1 cells is due to a switching of the relative amounts of the cell-surface expression levels of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins.

Expression of β_1 Integrins Differentially Regulates mRNA Steady-State Levels of β_3 and β_5 Integrin Subunits

To address at what level the control of β_3 and β_5 protein expression in GD25-β_1 cells was exercised, we first compared mRNA steady-state levels of these two integrin subunits in GD25-β_1A with those of untransfected GD25 cells, with the use of both RT-PCR and Northern blot procedures.

A duplex RT-PCR assay with two sets of primers was developed for the simultaneous detection of the relative levels of β_3 and β_5 integrin subunit mRNAs. As shown in Figure 3A, a great difference in β_3 mRNA steady-state levels was observed between GD25 and GD25-β_1A cells. Interestingly, the lower mRNA levels of β_3 in GD25-β_1A compared with those in GD25 cells reflected what we observed at the level of protein cell-surface expression (compare with Figure 2, A and B). On the contrary, there was little difference in β_5 mRNA levels among untransfected and β_1-transfected GD25 cells, with a small elevation observed in GD25-β_1A cells (Figure 3A). RT-PCR for β_1 mRNA was performed as control (Figure 3B). Thus, the presence of β_1 integrins in GD25 cells differently modulates β_3 and β_5 mRNA expression.

Northern blot analysis demonstrated that our cDNA probes to β_3 and β_5 specifically recognized mRNAs of ~6.6 and 3.5 kb, respectively, consistent with what has been previously described (Yamada *et al.*, 1995). As resulted from this analysis, the mRNA steady-state level of β_3 was much lower in GD25-β_1A than in GD25 cells (Figure 3C, β_3), thus reflecting the difference observed by RT-PCR and protein analysis (see above). In addition, the Northern blot analysis confirmed that the difference in β_5 mRNA steady-state levels between GD25 and GD25-β_1A cells (Figure 3C, β_5) did not fully correlate with the difference in β_5 cell-surface expression level (compare with Figure 2, A and C). Thus, in GD25-β1A cells the down-regulation of $\alpha_V\beta_3$ cell-surface expression strictly correlates with the down-regulation of β_3 mRNA steady-state level, whereas the up-regulation of $\alpha_V\beta_5$ is mainly due to translational or posttranslational events leading to an increase of β_5 subunit cell-surface recruitment.

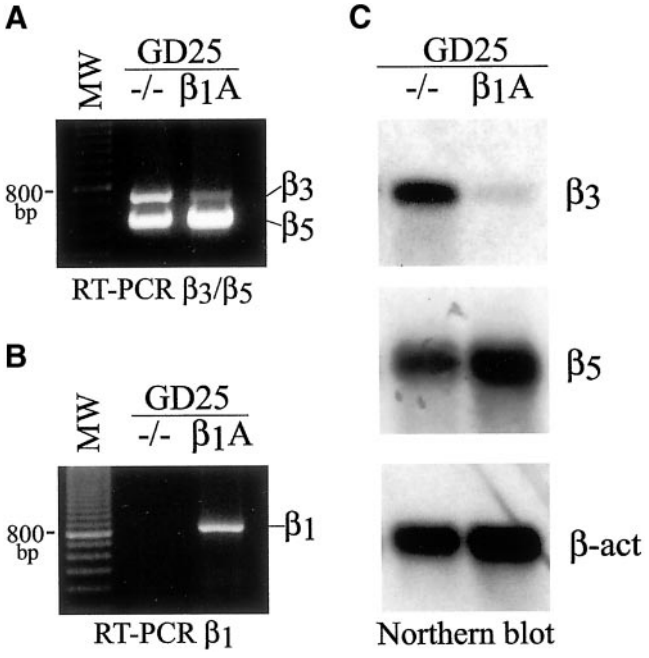

Figure 3. β_1 integrins differentially regulate mRNA steady-state levels of β_3 and β_5 integrin subunits. Total RNA was isolated from 1×10^7 cultured GD25 (−/−) and GD25-β_1A cells, and β_3 and β_5 mRNAs were evaluated by RT-PCR and Northern blot analyses as described in MATERIALS AND METHODS. (A) Duplex RT-PCR assay for the simultaneous detection of β_3 and β_5 mRNAs. (B) RT-PCR for β_1 mRNA performed as control. Molecular size standards (123-bp DNA ladder) are shown on the left. (C) Northern blot: equal amounts of total RNA (25 μg/lane) were probed sequentially by ^{32}P-labeled mouse integrin β_3 and β_5 cDNA fragments, and by a ^{32}P-labeled β-actin probe as a control for RNA loading. Notice that the presence of β_1 integrins causes a marked down-regulation of β_3 and a little up-regulation of β_5 mRNA expression levels.

β_1 Effect over α_V Integrins Occurs Irrespective of the Type of β_1 Isoform and Is Dependent on the Presence of the β_1 Cytoplasmic Domain Common Region

We have previously characterized some of the functional properties of β_1B and β_1D integrin isoforms, comparing these properties with those of the common β_1A isoform. In particular, we have shown that the unique cytoplasmic sequences of β_1B and β_1D endow these molecules with distinctive functional properties with respect to a number of cellular functions (Balzac *et al.*, 1994; Belkin *et al.*, 1997; Belkin and Retta, 1998; Calì *et al.*, 1998; Retta *et al.*, 1998).

To analyze more in detail the effects of β_1 over β_3 and β_5 integrins, we tested GD25 cells expressing β_1B and β_1D isoforms as well as two β_1 deletion mutants lacking almost the entire cytoplasmic domain (β_1TR) or the cytoplasmic domain variable region (β_1COM) (Retta *et al.*, 1998). Untransfected or β_1-transfected GD25 cells were surface-labeled with Sulfo-NHS-Biotin, and the expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin heterodimers was examined by immunoprecipitation and Western blot analysis as described (see MATERIALS AND METHODS). The α_V subunit was expressed at a constant level in all the examined cells (Figures 2A and 4A), whereas a down-regulation of β_3 and an up-regulation of β_5 cell-surface levels were seen in either β_1B- (Figures 2 and 4, B and C) or β_1D-expressing GD25 cells (Figure 4, A–C). Moreover, the expression of the β_1COM cytoplasmic domain mutant induced similar effects, although to a lower extent (Figure 4, A–C). However, GD25-β_1TR cells, expressing a β_1 mutant carrying the deletion of the cytoplasmic domain (β_1TR), behaved equivalently to the β_1-deficient GD25 cells (Figure 4, A–C).

The spectrum of relative β_3 integrin levels in untransfected or β_1-transfected GD25 cells compared well with our subsequent mRNA analysis. In fact, when we analyzed by RT-PCR and Northern blot the mRNA steady-state level of the β_3 subunit in GD25 cells expressing either β_1B, β_1D, β_1COM or β_1TR, we found that in GD25-β_1B and GD25-β_1D cells it was as low as in GD25-β_1A cells, whereas in GD25-β_1COM cells it was also reduced but to a lower extent (Figure 5, A–C). On the contrary, the β_3 mRNA level in GD25-β_1TR was higher and similar to that of β_1-deficient GD25 cells (Figure 5, A–C). On the other hand, although a little increase of β_5 mRNA steady-state level was observed in GD25 cells expressing β_1B, β_1D, or β_1COM (Figure 5, B and D), it did not fully reflect the high increase observed at the β_5 protein level in the same cells.

These results indicate that the β_1-dependent modulation of β_3 and β_5 integrin subunit expression was not confined to GD25-β_1A cells, but that it was also present in GD25 cells expressing two other β_1 isoforms. In addition, the fact that a down-regulation of β_3 and an up-regulation of β_5 were also observed in GD25-β_1COM, but not in GD25-β_1TR cells, strongly suggests that the control of the expression level of β_3 and β_5 integrin subunits was dependent on the presence of the β_1 cytoplasmic domain common region.

Cell Adhesion to ECM Proteins Is not Required for β_1 Effect on β_3 mRNA Steady-State Level

To determine whether cell adhesion to ECM proteins was required for β_1 effect over β_3 expression levels, we performed Northern blot analysis of β_3 mRNA steady-state level in cells plated on tissue culture dishes coated with either polylysine or two ECM proteins, namely, fibronectin and vitronectin. GD25-β_1TR and GD25-β_1COM cells were cultured to confluence in complete culture medium and then resuspended in serum-free medium, containing 1 μM monensin, and allowed to attach and spread on polylysine-, fibronectin-, and vitronectin-coated dishes for 2 h at 37°C before RNA isolation for Northern blot analysis.

The results, shown in Figure 6, indicate that the β_3 mRNA steady-state level was constitutively low in GD25-β_1COM cells compared with that of GD25-β_1TR cells. Similar results were obtained by comparing β_3 mRNA steady-state levels in cells kept in suspension in serum-free medium for up to 2 h with those of long-term adherent cells. Thus, these data suggest that ligation of ECM proteins is not required for β_1 effect over β_3 expression.

De Novo Surface Expression of β_1-associated α Subunits Does Not Affect the Level of β_3 Integrin Subunit

Because human β_1 expression in GD25 cells leads to the assembly and cell-surface recruitment of integrin complex

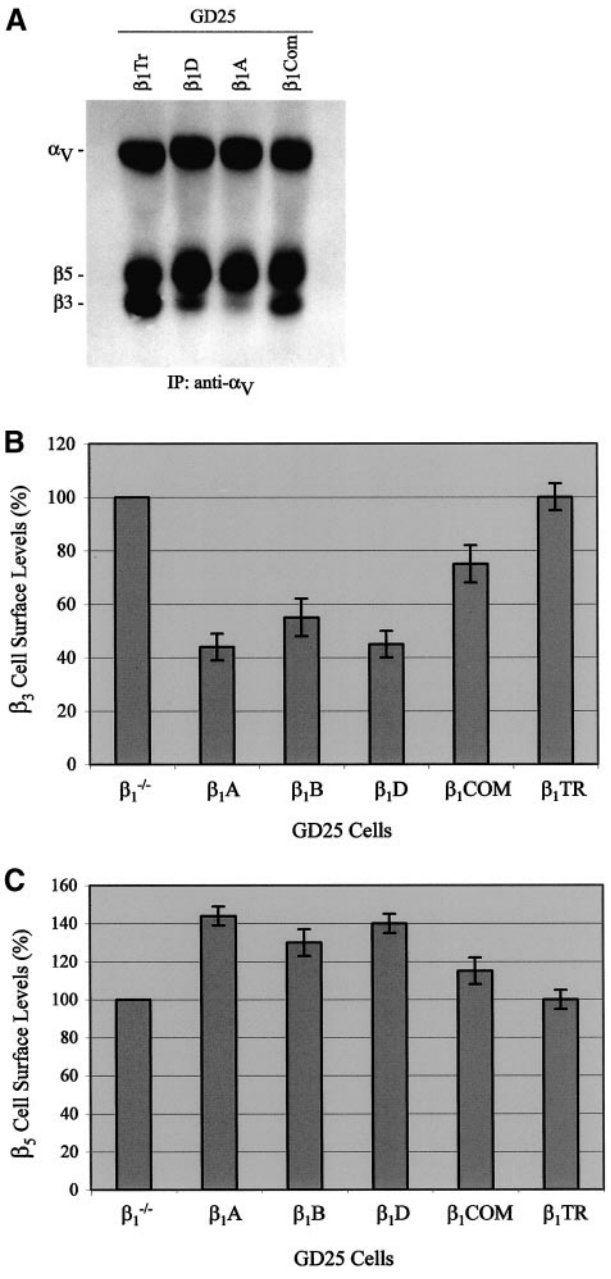

Figure 4. Comparative analysis of surface expression of α_v integrins in GD25 cells expressing different integrin β_1 forms. (A) α_v integrin heterodimers were immunoprecipitated from surface biotinylated GD25 cells expressing either the β_1A or β_1D isoforms or two β_1 deletion mutants, lacking the entire cytoplasmic domain (β_1TR) or the cytoplasmic domain variable region (β_1COM), with the use of a polyclonal antibody against the α_v subunit. After separation by nonreducing SDS-PAGE and Western blot, the immunoprecipitated proteins were detected with the use of peroxidase-conjugated streptavidin and ECL as described in MATERIALS AND METHODS. (B and C) Scanning densitometry analysis of β_3 (B) and β_5 (C) integrins as detected by Western blot. Data are displayed as percentage of the control (GD25) and are representative of three independent experiments. Notice that the β_1 effect over the $\alpha_V\beta_3/\alpha_V\beta_5$ ratio occurs irrespective of the type of the β_1 isoform and is dependent on the presence of the β_1 cytoplasmic domain common region.

with endogenous α_3, α_5, and α_6 subunits (Retta *et al.*, 1996), it was possible that these β_1-associated α subunits could play a direct role in the down-regulation of $\alpha_V\beta_3$ integrin.

To exclude this possibility we took advantage of GD25-β_1TR cells by extending our observations with this cell line. The expression of the β_1TR mutant at the surface of GD25-β_1TR cells was comparable with that of the β_1A isoform in GD25-β_1A cells, as previously determined by flow cytometry analysis (Retta *et al.*, 1998). In addition, by immunoprecipitation experiments we did not see any detectable change in the pattern of α subunits associated with β_1TR in GD25 cells compared with GD25-β_1A cells (Figure 7). Nevertheless, the presence of β_1TR integrin heterodimers did not lead to any detectable effect over $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins (Figures 4, A–C, and 5, A–D). Taken together, these data strongly suggest that the down-regulation of β_3 is not directly due to de novo surface expression of the β_1-associated α subunits and confirm that for the effect of β_1 integrins over $\alpha_V\beta_3/\alpha_V\beta_5$ integrin ratio a β_1 subunit carrying, at least, the common region of the cytoplasmic domain is required.

Expression of β_1 Integrins Induces a Marked Decrease in β_3 mRNA Stability

Because modulation of mRNA stability is a potential regulatory mechanism for integrin expression (Sachs, 1993; Feng *et al.*, 1999), we next asked whether the changes in mRNA steady-state levels were due to changes in integrin mRNA stability. The rate of turnover of β_3 and β_5 mRNAs was determined by inhibition of RNA synthesis with 60 μM DRB followed by quantitative blot hybridization analysis of β_3 and β_5 mRNA as a function of time. In GD25-β_1A cells grown on tissue culture dishes a clear decrease of β_3 mRNA stability was detected compared with GD25 cells (Figure 8A). The β_3 mRNA decayed with an apparent half-life of >8 h in GD25 cells but <4 h in GD25-β_1A cells (Figure 8, A and B). In contrast, the stability of β_5 mRNA was much higher than that of β_3, and no significant difference was observed when GD25 and GD25-β_1A cells were compared. Therefore, the effects of β_1 expression over β_3 and β_5 mRNA levels clearly involve a regulation of β_3, but not β_5, mRNA stability

DISCUSSION

There is increasing evidence that a coordinated cross talk between integrin receptors is crucial for an integrated and functional response of a single cell to the extracellular environment (Porter and Hogg, 1997; Blystone *et al.*, 1999; Hynes, 1999). However, the molecular mechanisms of integrin cross talk remain mostly undetermined.

Previously, we showed that the expression of either β_1B or β_1D integrin isoforms in β_1-null GD25 cells prevented different FN-dependent functions of endogenous α_V integrins, including their ability to mediate cell adhesion, to localize to focal adhesions, and to assembly an FN matrix, thus indicating the existence of a functional cross talk between these two β_1 isoforms and α_V integrins (Belkin *et al.*, 1997; Retta *et al.*, 1998). The present study was undertaken to examine this integrin cross talk and establish the regulatory mechanism(s) whereby β_1 integrins exert their *trans*-acting functions. The main findings are that 1) de novo expression of the β_1 integrin subunit in β_1-null GD25 cells induces a

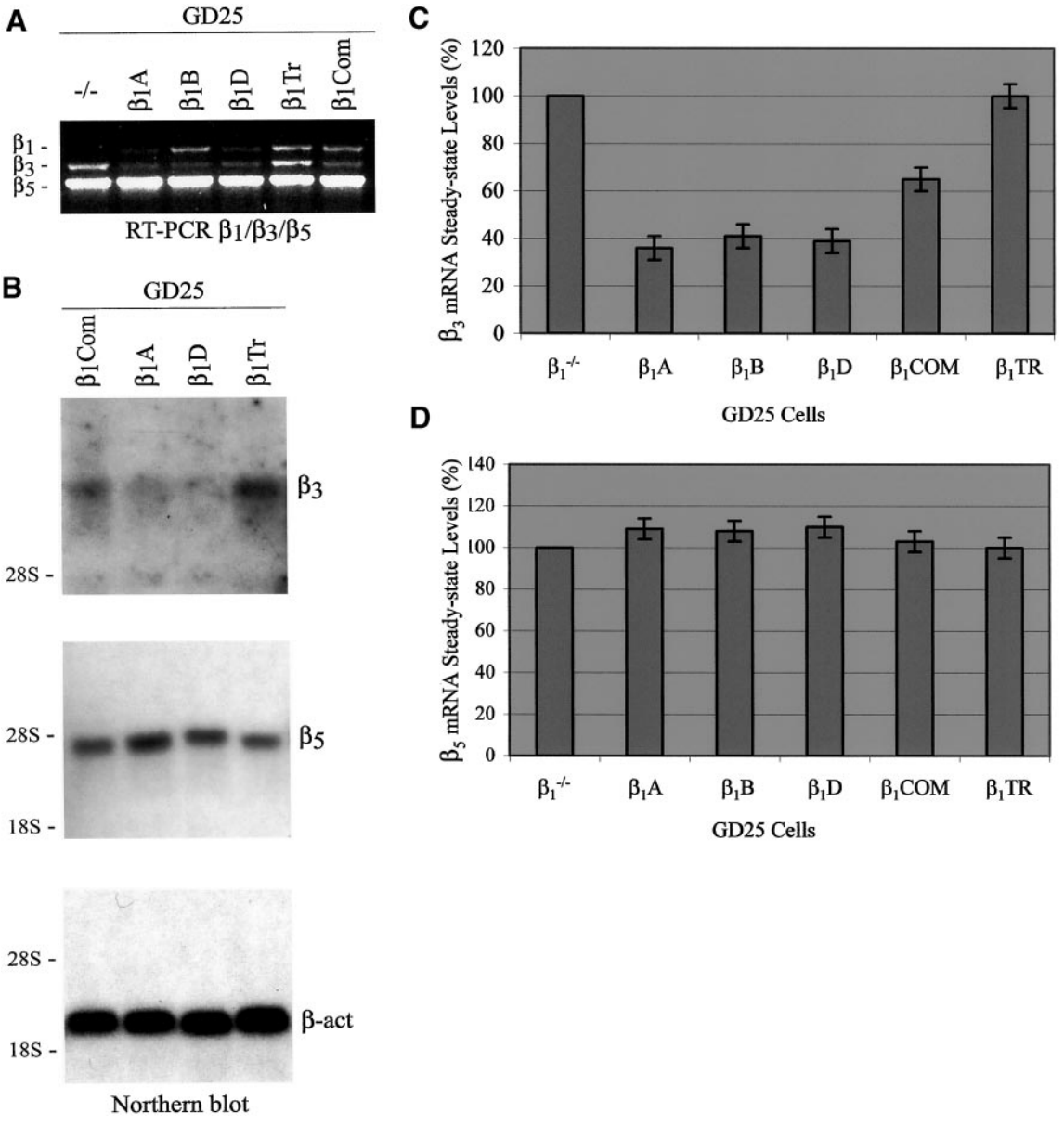

Figure 5. Comparative analysis of β_3 and β_5 mRNA steady-state levels in GD25 cells expressing different integrin β_1 forms. Total RNA was isolated from 1×10^7 cultured cells as described in MATERIALS AND METHODS. (A) Multiplex RT-PCR assay for the simultaneous detection of β_1, β_3, and β_5 mRNAs in GD25 cells expressing either the β_1A, β_1B, or β_1D isoforms or two β_1 deletion mutants lacking the entire cytoplasmic domain (β_1TR) or the cytoplasmic domain variable region (β_1COM). (B) Northern blot: equal amounts of total RNA (25 μg/lane) were probed sequentially by ^{32}P-labeled mouse integrin β_3 and β_5 cDNA fragments and by a ^{32}P-labeled β-actin probe as a control for RNA loading. The positions of 28s and 18s rRNAs are indicated as markers for RNA sizes. (C and D) Scanning densitometry analysis of β_3 and β_5 mRNA levels as detected by Northern blot. Northern signals were normalized to β-actin and displayed as percentage of the control (GD25). Data are representative of three independent experiments. Notice that the down-regulation of β_3 mRNA steady-state level occurs irrespective of the type of the β_1 isoform and is dependent on the presence of the β_1 cytoplasmic domain common region.

drastic down-regulation of $\alpha_V\beta_3$ and an up-regulation of $\alpha_V\beta_5$ integrin cell surface levels; 2) this β_1 effect occurs irrespective of the type of β_1 isoform but is dependent on the presence of the common region of the β_1 cytoplasmic domain; and 3) the down-regulation of $\alpha_V\beta_3$ is due to a decreased mRNA stability of the β_3 subunit, whereas the up-regulation of $\alpha_V\beta_5$ is mainly due to translational or posttranslational events. These findings provide the first evidence of a cross talk between β_1 and α_V integrins based on mechanisms of control of mRNA and protein levels.

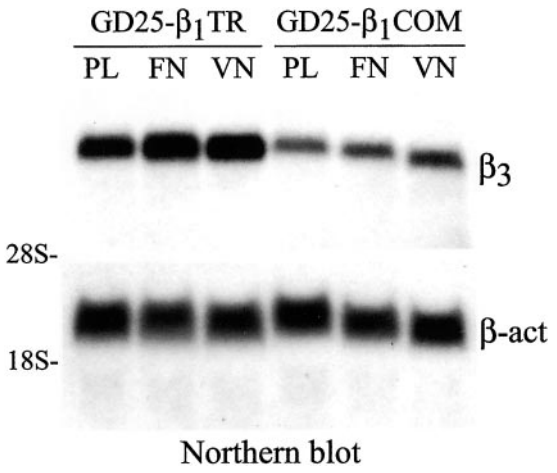

Figure 6. Cell adhesion to ECM proteins is not required for β_1 effect on β_3 mRNA steady-state level. GD25-β_1TR and GD25-β_1COM cells were cultured to confluence in complete culture medium. Cells were then resuspended in serum-free medium containing 1 μM monensin and allowed to attach and spread on polylysine-(PL), FN-, or vitronectin (VN)-coated tissue culture dishes for 2 h at 37°C before lysis. Total RNA was isolated as described in MATERIALS AND METHODS, and equal amounts (25 μg/lane) were analyzed for β_3 mRNA steady-state level by Northern blot hybridization with the use of ^{32}P-labeled mouse integrin β_3 cDNA fragments as probe. Equal loading was confirmed by hybridization of the same blot with a ^{32}P-labeled probe for β-actin. The positions of 28s and 18s rRNAs are indicated as markers for RNA sizes. Notice that the β_3 mRNA steady-state level is constitutively low in GD25-β_1COM compared with that of GD25-β_1TR cells.

Expression of the β_1 Integrin Subunit in GD25 Cells Induces a Drastic Reduction of Surface Level of $\alpha_v\beta_3$ and an Up-Regulation of $\alpha_v\beta_5$

Despite the apparent high degree of integrin-ligand binding redundancy (Hynes, 1992), the localization of distinct integrins to focal adhesions is usually very restricted (Fath *et al.*, 1989). In GD25 cells two integrins are believed to be able to localize to focal adhesions on fibronectin, namely, $\alpha_V\beta_3$ and, upon β_1 ectopic expression, $\alpha_5\beta_1$ (Wennerberg *et al.*, 1996; Belkin *et al.*, 1997; Retta *et al.*, 1998). However, whereas α_V integrins can take over some FN-dependent functions in the absence of β_1 integrins (Wennerberg *et al.*, 1996; Retta *et al.*, 1998), immunofluorescence analyses of β_1A-transfected and untransfected GD25 cells plated on fibronectin show that β_1 integrins clearly dominate upon α_V integrins in localizing to focal adhesions. This phenomenon is mainly due to a cross talk between β_1 and α_V integrins that occurs at the level of expression on the cell surface. In fact, although the presence of β_1 integrins in GD25 cells does not affect the total amount of α_V integrins, it causes a clear rearrangement of the relative cell surface levels of β_3 and β_5 subunits, leading to a marked down-regulation of $\alpha_V\beta_3$ and a correspondent up-regulation of $\alpha_V\beta_5$.

A great deal of experimental work has shown that integrin expression is highly dynamic during development (reviewed in Darribere *et al.*, 2000; Tarone *et al.*, 2000). In particular, it has been suggested that the presence and functions of the α_V integrins are developmentally controlled by differential temporal and spatial regulation of its β subunits (Yamada *et al.*, 1995), whereas there are reports showing that a balanced ratio of integrin receptors is crucial for the maintenance of the differentiation state of a particular cell (Carroll *et al.*, 1995; Sastry *et al.*, 1996). However, genetic ablation experiments have shown that the absence of some widely expressed integrins that were believed to be key regulators of development and differentiation has resulted into mild or late phenotypes. In particular, it has come out that processes such as myogenesis, vasculogenesis, and angiogenesis, which through antibody or peptide perturbation experiments were shown to be dependent on specific β_1 or α_V integrins, can actually proceed without these integrins (Bader *et al.*, 1998; Hirsch *et al.*, 1998), suggesting that there might be some overlapping or compensatory functions between different integrins. A very striking example of this point comes from a thorough study of compound mutations showing that gene knockouts of α_V and α_5 integrin subunits have synergistic effects when combined pairwise and result in a phenotype similar to that of FN-null mutation, suggesting that $\alpha_5\beta_1$ and α_V integrins normally overlap or can compensate each other in mesodermal development (Yang *et al.*, 1999). Other examples derive from in vitro experiments showing that in cultured cells α_V integrins are able to compensate for the loss of the $\alpha_5\beta_1$ fibronectin receptors (Wennerberg *et al.*, 1996; Yang and Hynes, 1996; Retta *et al.*, 1998). On the other hand, $\alpha_V\beta_3$ has previously been shown to negatively regulate $\alpha_5\beta_1$-mediated cell migration (Bilato *et al.*, 1997; Simon *et al.*, 1997; Blystone *et al.*, 1999) and phagocytosis (Blystone *et al.*, 1994, 1999). Notably, the above-cited reports are all examples of functional cross talk between α_V and β_1 integrins, apparently without quantitative up-regulation of integrin levels. Our present report now demonstrates that, besides the existence of a functional compensa-

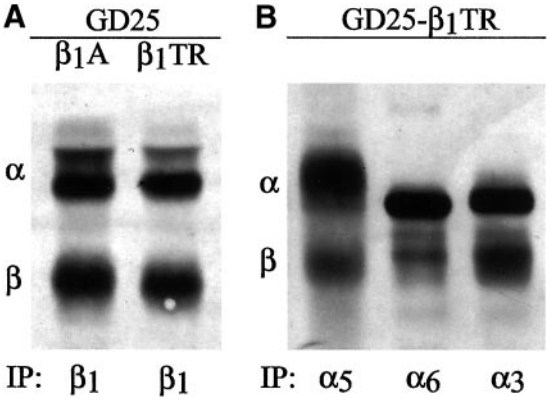

Figure 7. Pattern of α subunits associated with the β_1TR cytoplasmic domain deletion mutant. (A) β_1 Integrins immunoprecipitated from surface biotinylated β_1A- and β_1TR-transfected GD25 cells with the mAb TS2/16. (B) Integrin heterodimers immunoprecipitated from surface biotinylated GD25-β_1TR cells with the mAb GoH3 against α_6 and polyclonal antibodies against α_3 and α_5 integrin subunits. After separation by nonreducing SDS-PAGE and Western blot, the immunoprecipitated proteins were detected with the use of peroxidase-conjugated streptavidin and ECL as described in MATERIALS AND METHODS. Notice that the β_1TR mutant correctly associates with three major α subunits at the surface of GD25-β_1TR cells.

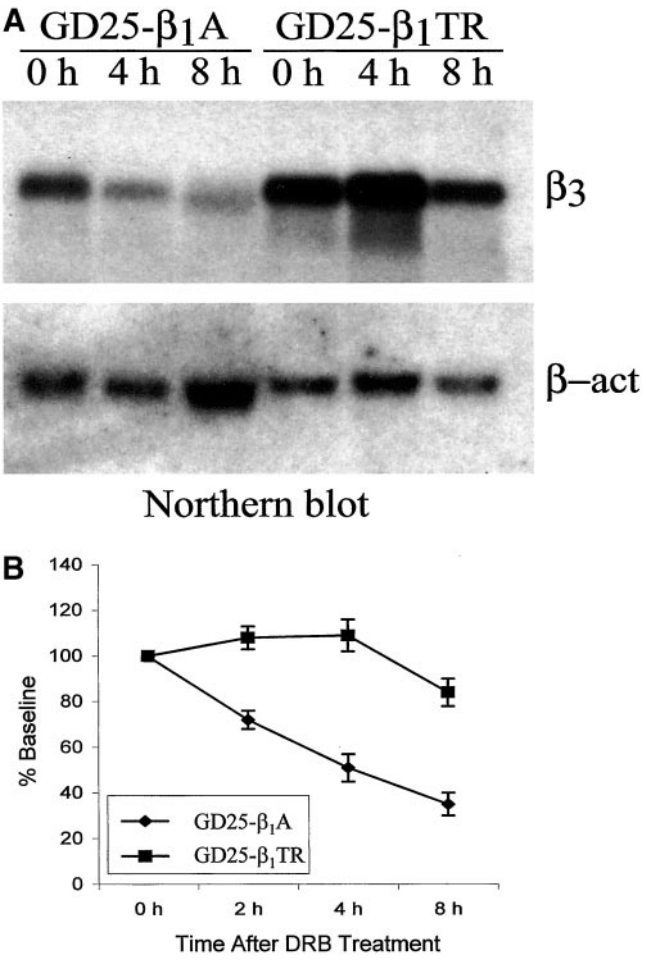

Figure 8. The presence of the β_1 cytoplasmic domain doubles the decay rate of the β_3 mRNA in GD25 cells. GD25-β_1A and GD25-β_1TR cells grown to confluence were divided into three 10-cm Petri dishes and cultured in DMEM containing 10% FBS for 24 h before the addition of 60 μM DRB, an inhibitor of transcription initiation. After addition of DRB, the cells were collected at 0, 4, and 8 h for RNA analysis. Total RNA isolation and Northern analysis were performed as described in MATERIALS AND METHODS. (A) Northern blot: equal amounts of total RNA (25 μg/lane) were probed sequentially by ^{32}P-labeled mouse integrin β_3 and β-actin cDNA fragments. (B) Scanning densitometry analysis of β_3 mRNA levels as detected by Northern blot. β_3 Northern signals were normalized to β-actin and displayed as percentage of the baseline (time 0). Data presented are the mean values ± SE of three independent experiments. Notice the lower stability of β_3 integrin subunit mRNA in GD25-β_1A than in GD25-β_1TR cells.

tion of α_V integrins for the lack of certain β_1 integrin functions, a cross talk mechanism occurs that regulates the ratio of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ on the cell surface upon β_1 integrin expression. Taken together, these observations suggest that the cross talk between β_1 and α_V integrins must be bidirectional, articulate, and based on integrated and tightly regulated mechanisms. Several examples support the existence of such a composite cross talk between integrins. One example comes from our recent report showing that ectopic expression of α_7B integrin subunit in Chinese hamster ovary cells leads to down-regulation of both cell surface expression and ligand binding affinity of the integrin $\alpha_5\beta_1$ (Tomatis *et al.*, 1999). Another straightforward example derives from analysis of the expression of β_1 splice variants during mouse muscle development: genetic analysis by homologous recombination has demonstrated that mice lacking β_1D, the only β_1 isoform in adult muscles, develop normally without gross apparent defects; in this case, however, the β_1A isoform is not down-regulated as in wild-type muscles and can apparently compensate both in function and in level for the absence of β_1D (Baudoin *et al.*, 1998). A similar phenomenon might likely occur during β_1-null myoblast differentiation where an up-regulation of $\alpha_V\beta_3$, both in level and function, could give a reason for the unexpected mild phenotype (Hirsch *et al.*, 1998). Interestingly, $\alpha_V\beta_3$ has been recently shown to be up-regulated in β_1-null cardiac cells compared with wild-type cells (Guan *et al.*, 2001). On the other hand, a switch from $\alpha_V\beta_5$ to $\alpha_V\beta_3$ integrins has been observed to occur in important biological processes, including wound healing (Clark *et al.*, 1996a,b) and tumor progression in situ (Marshall *et al.*, 1991; Li *et al.*, 1998). Thus, taken together with the above-mentioned observations, our results support the hypothesis that the enigma of the recurrent discrepancy between blocking experiments and genetic analyses (Hynes, 1996; Brakebusch *et al.*, 1997; Bader *et al.*, 1998; Hirsch *et al.*, 1998; McHugh *et al.*, 2000) could be explained by taking into account that a compensatory up-regulation both in level and function between integrins can occur.

Expression of β_1 Integrins Differentially Regulates mRNA Steady-State Levels of β_3 and β_5 Integrin Subunits

The expression of integrins can be modulated by a variety of agents, including proinflammatory cytokines, growth factors, hormones, extracellular matrix components, and pharmacological agents (Delcommenne and Streuli, 1995; Kim and Yamada, 1997). In particular, the integrin $\alpha_V\beta_3$ has been shown to be up-regulated by transforming growth factor-β_1, platelet-derived growth factor-BB (Janat *et al.*, 1992), basic fibroblast growth factor (Sepp *et al.*, 1994), vitamin D (Medhora *et al.*, 1993), fibronectin (Feng *et al.*, 1999), and phorbol esters (Swerlick *et al.*, 1992), and down-regulated by tumor necrosis factor-α, interferon-γ (Defilippi *et al.*, 1991), and collagen (Feng *et al.*, 1999). Mechanisms regulating integrin expression include regulation of protein levels by transcriptional or posttranscriptional events, alternative splicing of mRNA, and mobilization of preexisting intracellular stores (Xu and Clark, 1996; Kim and Yamada, 1997). However, little is known regarding integrin cross talk mechanisms that modulate integrin ratios in individual cells. Here, we show that the expression of β_1 integrins in GD25 cells regulates the cell surface levels of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ by two distinct mechanisms. In particular, the decreased expression of integrin $\alpha_V\beta_3$ in GD25-β_1A cells clearly involves an increased decay rate of β_3 subunit mRNA. On the contrary, because the up-regulation of the β_5 protein level we observed was not reflected at the mRNA level, the enhanced cell surface expression of $\alpha_V\beta_5$ is probably mainly due to the mobilization to the cell surface of intracellular stores of the β_5 subunit. This is consistent with previous reports showing the presence of intracellular pools of β subunits and indicating the

availability of the α subunit as a rate-limiting step in integrin complex assembly and cell-surface expression (Swerlick *et al.*, 1992).

The regulation of mRNA stability is a very important mechanism of posttranscriptional regulation of gene expression, and evidence exists for both a wide range of half-lives for different mRNAs in the same cells and different half-lives for the same mRNA in the same cell under different circumstances (Sachs, 1993). Interestingly, it has been previously suggested that coordinate signals from ECM molecules and growth factors can modulate the mRNA decay rate of specific integrins (Xu and Clark, 1996). In addition, a recent report shows that the β_3 mRNA stability can be increased by cell interaction with fibrin but not with collagen (Feng *et al.*, 1999). Because most extracellular matrix proteins signal through integrins, which have also been shown to physically associate and act synergistically with growth factor receptors (Giancotti and Ruoslahti, 1999), it is possible to hypothesize a scenario where the expression of a specific integrin can influence the expression of another integrin by affecting its mRNA stability, either directly or with the cooperation of an associated growth factor receptor. This could be a way for a rapid change of integrin ratios in response to a variation of the extracellular environment, as it occurs during tissue formation or repair. Interestingly, $\alpha_V\beta_3$ expression has been shown to increase focally and transiently during cutaneous wound repair (Feng *et al.*, 1999). On the other hand, the existence of this regulatory integrin cross talk could explain the compensatory up-regulation of $\alpha_V\beta_3$ in the absence of β_1 integrins, highlighting the usefulness of stabilizing specific integrin mRNA only when it is needed.

β_1 Effect over β_3 Expression Is Dependent on the Presence of the β_1 Cytoplasmic Domain Common Region

Our results clearly demonstrate that the control of β_3/β_5 ratio is β_1-dependent, and neither confined to a particular cell population nor restricted to a specific β_1 isoform. Instead, it requires the presence of the common region of the β_1 cytoplasmic domain. In addition, the fact that the β_1COM mutant does not contribute to cell adhesion (Retta *et al.*, 1998), together with the observation that the β_3 mRNA steady-state level is constitutively low in GD25-β_1COM cells compared with GD25-β_1TR cells, indicates that the binding of extracellular ligands is not required for β_1 to regulate β_3 expression. On the other hand, it is noteworthy that, although in long-term adherent cells the β_1COM mutant was less effective than β_1A in inducing an effect over the β_3 mRNA steady-state level, no significant difference was observed when we compared β_3 mRNA levels in GD25-β_1COM and GD25-β_1A cells either plated on polylysine or kept in suspension for 2 h (unpublished). These results could be explained by taking into account that, in contrast with the expression of β_1A, the expression of β_1COM in GD25 cells does not entirely prevent the localization of α_V integrins to FN-dependent focal adhesions (Retta *et al.*, 1998). Thus, a likely possibility is that the common region of the β_1 cytoplasmic domain is able to constitutively induce a down-regulation of β_3 mRNA steady-state level; however, the $\alpha_V\beta_3$ integrin, due to its ability to localize to focal adhesions in GD25-β_1COM cells, counteracts this β_1 constitutive action leading to a mild effect on β_3 expression level. Interestingly, in accordance with a recent report (Feng *et al.*, 1999), the higher β_3 mRNA level in cells cultured on fibronectin or vitronectin than on polylysine (Figure 6) suggests that ECM proteins that are ligands for $\alpha_V\beta_3$ can sustain β_3 mRNA steady-state level.

In conclusion, our results indicate a novel mechanism of integrin cross talk where one integrin can regulate the expression of another by modulating the decay rate of its mRNA. The biological implications of this integrin cross talk are potentially of high functional significance as a fine-tuned mechanism for selective and transient integrin expression in different extracellular contexts. Our attempt now will be to uncover β_1-dependent events regulating the β_3 mRNA steady-state levels.

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REFERENCES

Albelda, S.M., Mette, S.A., Elder, D.E., Stewart, R., Damjanovich, L., Herlyn, M., and Buck, C.A. (1990). Integrin distribution in malignant melanoma: association of the β_3 subunit with tumor progression. Cancer Res. *50*, 6757–6764.

Bader, B.L., Rayburn, H., Crowley, D., and Hynes, R.O. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all α_v integrins. Cell *95*, 507–519.

Balzac, F., Retta, S.F., Albini, A., Melchiorri, A., Koteliansky, VE., Geuna, M., Silengo, L., and Tarone, G. (1994). Expression of β1B integrin isoform in CHO cells results in a dominant negative effect on cell adhesion and motility. J. Cell Biol. *127*, 557–565.

Baudoin, C., Goumans, M.J., Mummery, C., and Sonnenberg, A. (1998). Knockout and knockin of the β1 exon D define distinct roles for integrin splice variants in heart function and embryonic development. Genes Dev. *12*, 1202–1216.

Belkin, A.M., and Retta, S.F. (1998). β1D integrin inhibits cell cycle progression in normal myoblasts and fibroblasts. J. Biol. Chem. *273*, 15234–15240.

Belkin, A.M., Retta, S.F., Pletjushkina, O.Y., Balzac, F., Silengo, L., Fassler, R., Koteliansky, VE., Burridge, K., and Tarone, G. (1997). Muscle β1D integrin reinforces the cytoskeleton-matrix link: modulation of integrin adhesive function by alternative splicing. J. Cell Biol. *139*, 1583–1595.

Bilato C, Curto KA, Monticone RE, Pauly RR, White AJ, Crow MT. (1997). The inhibition of vascular smooth muscle cell migration by peptide and antibody antagonists of the $\alpha_v\beta_3$ integrin complex is reversed by activated calcium/calmodulin-dependent protein kinase II. J. Clin. Invest. *100*, 693–704.

Blystone, S.D., Graham, IL., Lindberg, F.P., and Brown, E.J. (1994). Integrin $\alpha_v\beta_3$ differentially regulates adhesive and phagocytic functions of the fibronectin receptor $\alpha_5\beta_1$. J. Cell Biol. *127*, 1129–1137.

Blystone, S.D., Slater, S.E., Williams, M.P., Crow, M.T., and Brown, E.J. (1999). Molecular mechanism of integrin crosstalk: $\alpha_v\beta_3$ sup-

pression of calcium/calmodulin-dependent protein kinase II regulates $\alpha_5\beta_1$ function. J. Cell Biol. *145*, 889–897.

Brakebusch, C., Hirsch, E., Potocnik, A., and Fassler, R. (1997). Genetic analysis of β1 integrin function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. J. Cell Sci. *110*, 2895–2904.

Brooks, P.C., Clark, R.A., and Cheresh, D.A. (1994). Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. Science *264*, 569–571.

Calì G, Retta SF, Negri R, Damiano I, Gentile R, Tarone G, Nitsch L, Garbi C. (1998). β1B integrin interferes with matrix assembly but not with confluent monolayer polarity, and alters some morphogenetic properties of FRT epithelial cells. Eur. J. Cell Biol. *75*, 107–117.

Carroll, J.M., Romero, M.R., and Watt, F.M. (1995). Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. Cell *83*, 957–968.

Clark, R.A., Ashcroft, G.S., Spencer, M.J., Larjava, H., and Ferguson, M.W. (1996b). Re-epithelialization of normal human excisional wounds is associated with a switch from $\alpha_v\beta_5$ to $\alpha_v\beta_6$ integrins. Br. J. Dermatol. *135*, 46–51.

Clark, R.A., Tonnesen, M.G., Gailit, J., and Cheresh, D.A. (1996a). Transient functional expression of $\alpha_v\beta_3$ on vascular cells during wound repair. Am. J. Pathol. *148*, 1407–1421.

Corbett, S.A., and Schwarzbauer, J.E. (1999). β3 integrin activation improves $\alpha_v\beta_3$-mediated retraction of fibrin matrices. J. Surg. Res. *83*, 27–31.

Critchley, D.R. (2000). Focal adhesions—the cytoskeletal connection. Curr. Opin. Cell Biol. *12*, 133–139.

Darribere, T., Skalski, M., Cousin, H.L., Gaultier, A., Montmory, C., and Alfandari, D. (2000). Integrins: regulators of embryogenesis. Biol. Cell *92*, 5–25.

Defilippi, P., Retta, S.F., Olivo, C., Palmieri, M., Venturino, M., Silengo, L., and Tarone, G. (1995). p125FAK tyrosine phosphorylation and focal adhesion assembly: studies with phosphotyrosine phosphatase inhibitors. Exp. Cell Res. *221*, 141–152.

Defilippi, P., Truffa, G., Stefanuto, G., Altruda, F., Silengo, L., and Tarone, G. (1991). Tumor necrosis factor α and interferon gamma modulate the expression of the vitronectin receptor (integrin β3) in human endothelial cells. J. Biol. Chem. *266*, 7638–7645.

Delcommenne, M., and Streuli, C.H. (1995). Control of integrin expression by extracellular matrix. J. Biol. Chem. *270*, 26794–26801.

Fassler, R., Georges-Labouesse, E., and Hirsch, E. (1996). Genetic analyses of integrin function in mice. Curr. Opin. Cell Biol. *8*, 641–646.

Fath, K.R., Edgell, C.J., and Burridge, K. (1989). The distribution of distinct integrins in focal contacts is determined by the substratum composition. J. Cell Sci. *92*, 67–75.

Feng, X., Clark, R.A., Galanakis, D., and Tonnesen, M.G. (1999). Fibrin and collagen differentially regulate human dermal microvascular endothelial cell integrins: stabilization of αv/β3 mRNA by fibrin1. J. Invest. Dermatol. *113*, 913–919.

Fornaro, M., and Languino, L.R. (1997). Alternatively spliced variants: a new view of the integrin cytoplasmic domain. Matrix Biol. *16*, 185–193.

Friedlander, M., Brooks, P.C., Shaffer, R.W., Kincaid, C.M., Varner, J.A., and Cheresh, D.A. (1995). Definition of two angiogenic pathways by distinct α_v integrins. Science *270*, 1500–1502.

Giancotti, F.G. (1997). Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Opin. Cell Biol. *9*, 691–700.

Giancotti, F.G. (2000). Complexity and specificity of integrin signaling. Nat. Cell Biol. *2*, E13–E14.

Giancotti, F.G., and Ruoslahti, E. (1999). Integrin signaling. Science *285*, 1028–1032.

Guan, K., Czyz, J., Furst, D.O., and Wobus, A.M. (2001). Expression and cellular distribution of α(v) integrins in β(1) integrin-deficient embryonic stem cell-derived cardiac cells. J. Mol. Cell Cardiol. *33*, 521–532.

Hirsch, E., Lohikangas, L., Gullberg, D., Johansson, S., and Fassler, R. (1998). Mouse myoblasts can fuse and form a normal sarcomere in the absence of β1 integrin expression. J. Cell Sci. *111*, 2397–2409.

Hodivala-Dilke, K.M., McHugh, K.P., Tsakiris, D.A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F.P., Coller, B.S., Teitelbaum, S., and Hynes, R.O. (1999). β3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J. Clin. Invest. *103*, 229–238.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Hynes, R.O. (1996). Targeted mutations in cell adhesion genes: what have we learned from them? Dev. Biol. *180*, 402–412.

Hynes, R.O. (1999). Cell adhesion: old and new questions. Trends Cell Biol. *9*, 33–37.

Janat, M.F., Argraves, W.S., and Liau, G. (1992). Regulation of vascular smooth muscle cell integrin expression by transforming growth factor β1 and by platelet-derived growth factor-BB. J. Cell Physiol. *151*, 588–595.

Kim, L.T., and Yamada, K.M. (1997). The regulation of expression of integrin receptors. Proc. Soc. Exp. Biol. Med. *214*, 123–131.

Klemke, R.L., Yebra, M., Bayna, E.M., and Cheresh, D.A. (1994). Receptor tyrosine kinase signaling required for integrin $\alpha_v\beta_5$-directed cell motility but not adhesion on vitronectin. J. Cell Biol. *127*, 859–866.

Kühn, K., and Eble, J. (1994). The structural bases of integrin-ligand interactions. Trends Cell Biol. *4*, 256–261.

Leavesley, D.I, Ferguson, G.D., Wayner, E.A., and Cheresh, D.A. (1992). Requirement of the integrin β3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. J. Cell Biol. *117*, 1101–1107.

Lewis, J.M., Cheresh, D.A., and Schwartz, M.A. (1996). Protein kinase C regulates $\alpha_v\beta_5$-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. J. Cell Biol. *134*, 1323–1332.

Li, X., Chen, B., Blystone, S.D., McHugh, K.P., Ross, F.P., and Ramos, D.M. (1998). Differential expression of αv integrins in K1735 melanoma cells. Invasion Metastasis *18*, 1–14.

Liu, S., Calderwood, D.A., and Ginsberg, M.H. (2000). Integrin cytoplasmic domain-binding proteins. J. Cell Sci. *113*, 3563–3571.

Marshall, J.F., Nesbitt, S.A., Helfrich, M.H., Horton, M.A., Polakova, K., and Hart, IR. (1991). Integrin expression in human melanoma cell lines: heterogeneity of vitronectin receptor composition and function. Int. J. Cancer *49*, 924–931.

McHugh, K.P., Hodivala-Dilke, K., Zheng, M.H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, F.P., Hynes, R.O., and Teitelbaum, S.L. (2000). Mice lacking β3 integrins are osteosclerotic because of dysfunctional osteoclasts. J. Clin. Invest. *105*, 433–440.

Medhora, M.M., Teitelbaum, S., Chappel, J., Alvarez, J., Mimura, H., Ross, F.P., and Hruska, K. (1993). 1 α,25-dihydroxyvitamin D3 upregulates expression of the osteoclast integrin $\alpha_v\beta_3$. J. Biol. Chem. *268*, 1456–1461.

Montgomery, A.M., Reisfeld, R.A., and Cheresh, D.A. (1994). Integrin $\alpha_v\beta_3$ rescues melanoma cells from apoptosis in three-dimensional dermal collagen. Proc. Natl. Acad. Sci. USA *91*, 8856–8860.

Pfaff, M., Liu, S., Erle, D.J., and Ginsberg, M.H. (1998). Integrin β cytoplasmic domains differentially bind to cytoskeletal proteins. J. Biol. Chem. *273*, 6104–6109.

Porter, J.C., and Hogg, N. (1997). Intern cross talk: activation of lymphocyte function-associated antigen-1 on human T cells alters $\alpha_4\beta_1$- and $\alpha_5\beta_1$-mediated function. J. Cell Biol. *138*, 1437–1447.

Retta, S.F., Balzac, F., Ferraris, P., Belkin, A.M., Fassler, R., Humphries, M.J., De Leo, G., Silengo, L., and Tarone, G. (1998). β1-Integrin cytoplasmic subdomains involved in dominant negative function. Mol. Biol. Cell *9*, 715–731.

Retta, S.F., Barry, S.T., Critchley, D.R., Defilippi, P., Silengo, L., and Tarone, G. (1996). Focal adhesion and stress fiber formation is regulated by tyrosine phosphatase activity. Exp. Cell Res. *229*, 307–317.

Retta, S.F., Ferraris, P., and Tarone, G. (1999). Purification of fibronectin from human plasma. Methods Mol. Biol. *96*, 119–124.

Sachs, A.B. (1993). Messenger RNA degradation in eukaryotes. Cell. *74*, 413–421.

Sastry, S.K., Lakonishok, M., Thomas, D.A., Muschler, J., and Horwitz, A.F. (1996). Integrin α subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. J. Cell Biol. *133*, 169–184.

Schoenwaelder, S.M., and Burridge, K. (1999). Bidirectional signaling between the cytoskeleton and integrins. Curr. Opin. Cell Biol. *11*, 274–286.

Sepp, N.T., Li, L.J., Lee, K.H., Brown, E.J., Caughman, S.W., Lawley, T.J., and Swerlick, R.A. (1994). Basic fibroblast growth factor increases expression of the $\alpha_v\beta_3$ integrin complex on human microvascular endothelial cells. J. Invest. Dermatol. *103*, 295–299.

Simon, K.O., Nutt, E.M., Abraham, D.G., Rodan, G.A., and Duong, L.T. (1997). The $\alpha_v\beta_3$ integrin regulates $\alpha_5\beta_1$-mediated cell migration toward fibronectin. J. Biol. Chem. *272*, 29380–29389.

Swerlick, R.A., Brown, E.J., Xu, Y., Lee, K.H., Manos, S., and Lawley, T.J. (1992). Expression and modulation of the vitronectin receptor on human dermal microvascular endothelial cells. J. Invest. Dermatol. *99*, 715–722.

Tarone, G., Hirsch, E., Brancaccio, M., De Acetis, M., Barberis, L., Balzac, F., Retta, F., Botta, C., Altruda, F., and Silengo, L. (2000). Integrin function and regulation in development. Int. J. Dev. Biol. *44*, 725–731.

Tomatis, D., Echtermayer, F., Schober, S., Balzac, F., Retta, S.F., Silengo, L., and Tarone, G. (1999). The muscle-specific laminin receptor $\alpha_7\beta_1$ integrin negatively regulates $\alpha_5\beta_1$ fibronectin receptor function. Exp. Cell Res. *246*, 421–432.

Varner, J.A., and Cheresh, D.A. (1996). Integrins and cancer. Curr. Opin. Cell Biol. *8*, 724–730.

Wayner, E.A., Orlando, R.A., and Cheresh, D.A. (1991). Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J. Cell Biol. *113*, 919–929.

Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996). β1 Integrin-dependent and -independent polymerization of fibronectin. J. Cell Biol. *132*, 227–238.

Wu, C., Hughes, P.E., Ginsberg, M.H., and McDonald, J.A. (1996). Identification of a new biological function for the integrin $\alpha_v\beta_3$: initiation of fibronectin matrix assembly. Cell Adhes. Commun. *4*, 149–158.

Xu, J., and Clark, R.A. (1996). Extracellular matrix alters PDGF regulation of fibroblast integrins. J. Cell Biol. *132*, 239–249.

Yamada, S., Brown, K.E., and Yamada, K.M. (1995). Differential mRNA regulation of integrin subunits αV, β1, β3, and β5 during mouse embryonic organogenesis. Cell Adhes. Commun. *3*, 311–325.

Yang, J.T., Bader, B.L., Kreidberg, J.A., Ullman-Cullere, M., Trevithick, J.E., and Hynes, R.O. (1999). Overlapping and independent functions of fibronectin receptor integrins in early mesodermal development. Dev. Biol. *215*, 264–277.

Yang, J.T., and Hynes, R.O. (1996). Fibronectin receptor functions in embryonic cells deficient in $\alpha_5\beta_1$ integrin can be replaced by α_V integrins. Mol. Biol. Cell *7*, 1737–1748.

Yun, Z., Menter, D.G., and Nicolson, G.L. (1996). Involvement of integrin $\alpha_v\beta_3$ in cell adhesion, motility, and liver metastasis of murine RAW117 large cell lymphoma. Cancer Res. *56*, 3103–3111.