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# Cross Talk between $\beta_1$ and $\alpha_V$ Integrins: $\beta_1$ Affects $\beta_3$ 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  $\beta_1$ -null GD25 cells ectopically expressing the  $\beta_1A$  integrin subunit, we provide evidence for the existence of a cross talk between  $\beta_1$  and  $\alpha_V$  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  $\beta_1A$  expression. Moreover, with the use of GD25 cells expressing the integrin isoforms  $\beta_1B$  and  $\beta_1D$ , as well as two  $\beta_1$  cytoplasmic domain deletion mutants lacking either the entire cytoplasmic domain ( $\beta_1TR$ ) or only its "variable" region ( $\beta_1COM$ ), we show that the effects of  $\beta_1$  over  $\alpha_V$  integrins take place irrespective of the type of  $\beta_1$  isoform, but require the presence of the "common" region of the  $\beta_1$  cytoplasmic domain. In an attempt to establish the regulatory mechanism(s) whereby  $\beta_1$  integrins exert their *trans*-acting functions, we have found that the down-regulation of  $\alpha_V\beta_3$  is due to a decreased  $\beta_3$  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  $\alpha$  and  $\beta$  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  $\beta$  subunit has been shown to play a critical role in focal adhesion and actin stress fiber organi-

zation 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.

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

Most integrins belong to one of two major subfamilies defined by the  $\beta_1$  and  $\alpha_V$  subunits. The  $\beta_1$  subunit pairs with at least 12 different  $\alpha$  subunits ( $\alpha_1$ - $\alpha_{11}$ ,  $\alpha_V$ ) 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  $\beta_1$  integrins in mediating important cell adhesion and signal transduction events. Four different  $\beta_1$  isoforms have been identified ( $\beta_1A$ ,  $\beta_1B$ ,  $\beta_1C$ , and  $\beta_1D$ ), 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  $\alpha_V$  subunit is known to associate with at least five different  $\beta$  subunits ( $\beta_1$ ,  $\beta_3$ ,  $\beta_5$ ,  $\beta_6$ , and  $\beta_8$ ). Among these  $\alpha_V$  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  $\beta_1$  and  $\alpha_V$  integrins play a primary role in important physiological and pathological processes (reviewed in Varnier and Cheresch, 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  $\beta_1$  and  $\alpha_V$  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.*,

*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  $\beta_1$ -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  $\beta_1$  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  $\beta_1$  integrin subunit or two  $\beta_1$  mutants lacking either the entire cytoplasmic domain ( $\beta_1TR$ ) or only the cytoplasmic domain "variable" region that characterizes each isoform ( $\beta_1COM$ ) (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  $\beta_1$  integrin subunit, showing the existence of a functional cross talk between these two  $\beta_1$  isoforms and the endogenous  $\alpha_V$  integrins. In particular, both  $\beta_1B$  and  $\beta_1D$  expression prevented different fibronectin (FN)-dependent  $\alpha_V$  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  $\beta_1$  and  $\alpha_V$  integrins is mainly based on the regulation of  $\beta_3$  and  $\beta_5$  integrin subunit expression exerted by  $\beta_1$  integrins. In fact, the ectopic expression of either  $\beta_1A$ ,  $\beta_1B$ , or  $\beta_1D$  in GD25 cells induces a drastic down-regulation of  $\beta_3$  and an up-regulation of  $\beta_5$  integrin cell surface levels. Moreover, analysis of GD25 cells expressing  $\beta_1$  integrins lacking either the entire  $\beta_1$  cytoplasmic domain ( $\beta_1TR$ ) or only its variable region ( $\beta_1COM$ ) demonstrate that the "common" region of the  $\beta_1$  cytoplasmic domain is required for these effects. We further demonstrate that  $\beta_1$  exerts its control over  $\alpha_V\beta_3$  expression level by modulating the  $\beta_3$  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  $\beta_5$  subunit at the cell surface.

## MATERIALS AND METHODS

### Antibodies and Reagents

The mouse anti-human  $\beta_1$  monoclonal antibody (mAb) TS2/16 was obtained from American Type Culture Collection (Manassas, VA). The rat anti-mouse  $\alpha_6$  mAb GoH3 was a gift from A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The rabbit polyclonal antisera to  $\alpha_V$ ,  $\alpha_3$ , and  $\alpha_5$  integrin cytoplasmic domains, produced in our laboratory, were previously described (Retta *et al.*, 1998). The polyclonal antisera to  $\beta_3$  and  $\beta_5$  were produced with the use of a previously described protocol (Defilippi *et al.*, 1995). Briefly, rabbits were immunized against a GST- $\beta_3$  fusion protein containing the cytoplasmic domain of the mouse  $\beta_3$  integrin subunit and against a synthetic peptide reproducing an amino acid sequence from the carboxy terminus of mouse  $\beta_5$  integrin subunit, respectively. The  $\beta_5$  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  $\beta_1$  integrin heterodimers because of disruption of the  $\beta_1$  gene by homologous recombination, was established after differentiation of  $\beta_1$ -null embryonic stem cells and immortalization with simian virus 40 large T antigen (Wennerberg *et al.*, 1996). GD25 cells expressing the human  $\beta_1A$ ,  $\beta_1B$ , or  $\beta_1D$  integrin isoforms or the  $\beta_1TR$  and  $\beta_1COM$  human  $\beta_1$  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  $\beta_1$  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  $\mu$ g/ml streptomycin. The  $\beta_1$ -expressing GD25 cells were cultured in the same medium plus 300  $\mu$ g/ml hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany). Cell populations expressing high levels of the  $\beta_1$  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  $\mu$ g/ml), leupeptin (10  $\mu$ g/ml), phenylmethylsulfonyl fluoride (1 mM), and benzamide (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 pre-cleared 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 pre-cleared 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-polyacrylamide (7.5%) gel electrophoresis under non-reducing 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  $1 \times 10^7$  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  $\mu$ M monensin, and plated for 2 h on tissue culture dishes that had been coated with 10  $\mu$ g/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  $\beta_3$  and  $\beta_5$  or  $\beta_1$  integrin mRNAs. cDNA was synthesized from 5  $\mu$ g of cytoplasmic RNA with the use of the 1st Strand cDNA Synthesis kit (Roche Molecular Biochemicals), and subjected to 28 ( $\beta_3/\beta_5$ ) or 32 ( $\beta_1$  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  $\mu$ l of cDNA, 1 $\times$  reaction buffer (Amersham Pharmacia Biotech AB), 1.5 mM  $MgCl_2$ , 200  $\mu$ M dNTP, 0.5  $\mu$ M of each primer, and 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech AB) in a total volume of 50  $\mu$ l. 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  $\beta_3$  and  $\beta_5$  and the human  $\beta_1$  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.

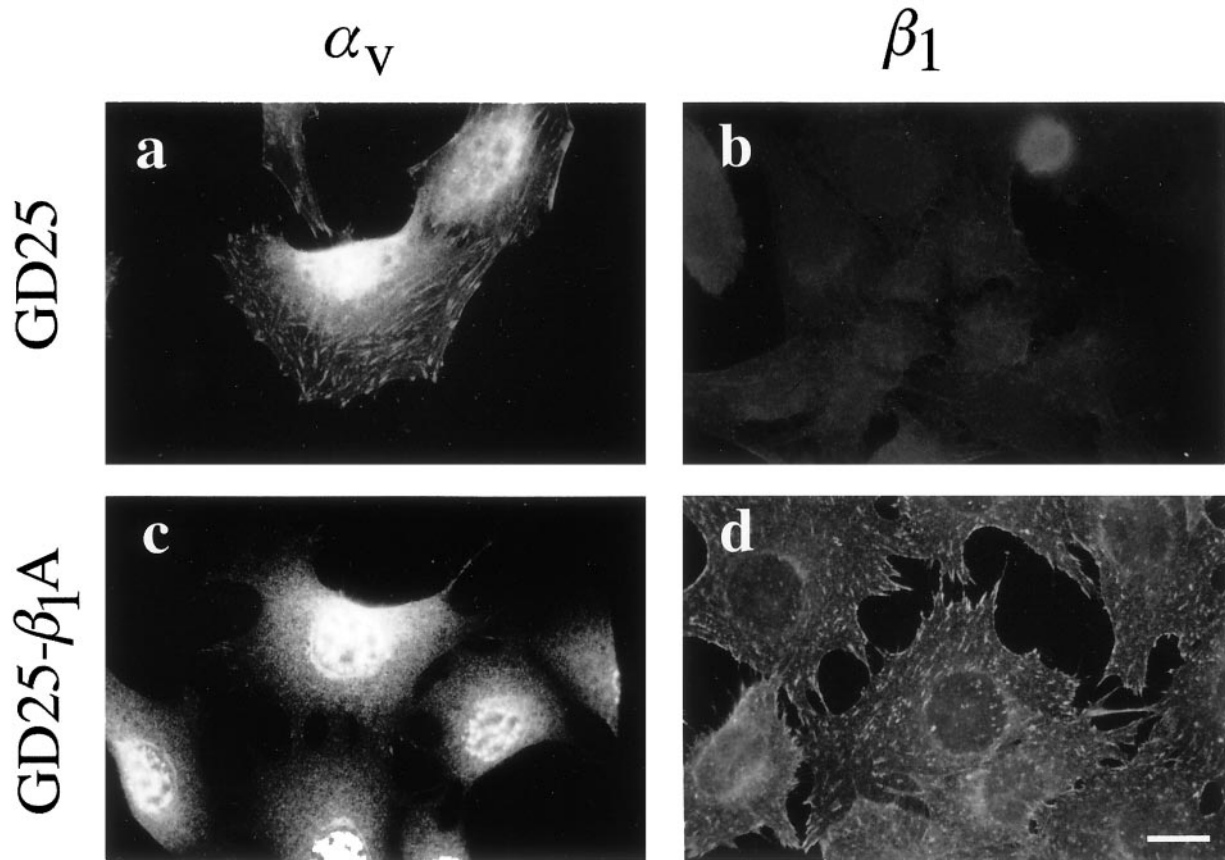
For Northern blot hybridization, equal amounts of the purified total RNA (25  $\mu$ g/lane) were separated by electrophoresis on a 1.2% agarose gel containing 1.8% formaldehyde and 1 $\times$  FA Gel buffer [20 mM 3-(N-morpholino)propanesulfonic acid, 5 mM NaAc, 1 mM EDTA, pH 7.0], transferred to a Nytran 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  $\beta_3$  and  $\beta_5$  integrin subunits amplified by PCR and cloned in our laboratory. The same blots were rehybridized with a probe of the housekeeping gene  $\beta$ -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  $\mu$ M 5,6-dichloro-1- $\beta$ -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  $\alpha_V$  and  $\beta_1$  integrins on GD25 and GD25- $\beta_1A$  cells plated on fibronectin. GD25 (a and b) and GD25- $\beta_1A$  (c and d) cells were allowed to attach and spread on coverslips coated with fibronectin (30  $\mu\text{g}/\text{ml}$  in PBS) for 3 h at 37°C. Cells were then fixed, permeabilized, and incubated with primary antibodies against  $\alpha_V$  (rabbit anti- $\alpha_V$ ) and  $\beta_1$  (mAb TS2/16) integrin subunits. The  $\alpha_V$  and  $\beta_1$  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  $\beta_1$  integrins displace  $\alpha_V$  integrins from focal adhesions. Bar, 15  $\mu\text{m}$ .

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  $\alpha_V$  and  $\beta_1$  integrins, the cells were stained with the rabbit antiserum to  $\alpha_V$  (1:200 in PBS/1% BSA) or the mAb TS2/16 to  $\beta_1$  (10  $\mu\text{g}/\text{ml}$  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.

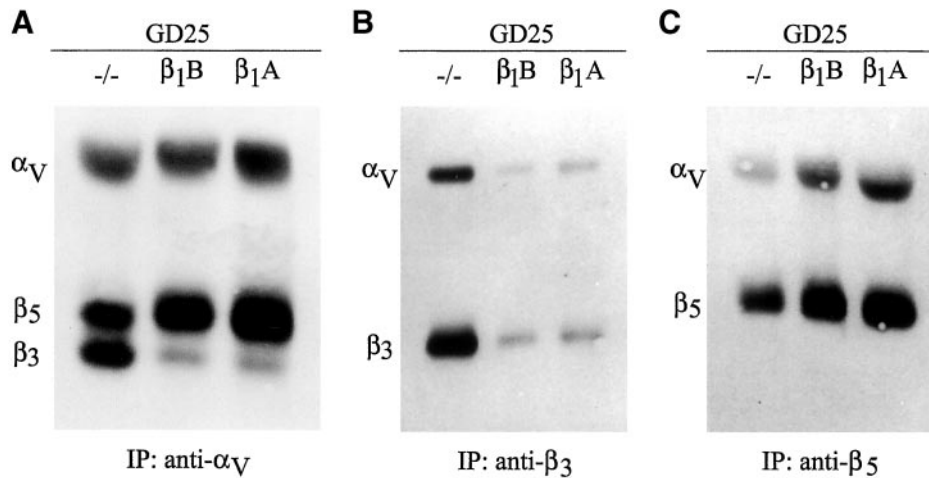
## RESULTS

### *Expression of $\beta_1$ Integrins Affects Subcellular Localization of $\alpha_V$ Integrins*

To determine the distribution of  $\alpha_V$  and  $\beta_1$  integrin heterodimers on GD25 and GD25- $\beta_1A$  cells attached to fibronectin, indirect immunofluorescence experiments with specific antibodies were performed. GD25 cells, which do not express  $\beta_1$  integrin heterodimers (Figure 1b), formed

$\alpha_V$ -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  $\alpha_V$ -containing focal adhesions was consistently reduced on GD25- $\beta_1A$  cells attached to fibronectin (Figure 1c), whereas  $\beta_1A$ -containing focal adhesions were abundant (Figure 1d).

Thus,  $\beta_1A$ , by localizing to focal adhesions, displaces the  $\alpha_V$ -containing heterodimers from these structures. Interestingly, we have previously shown that the expression of two other human  $\beta_1$  isoforms, namely,  $\beta_1B$ , that does not localize to focal adhesions, and  $\beta_1D$ , that is efficiently targeted to focal adhesions, also causes the delocalization of  $\alpha_V$  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  $\beta_1$  integrins in mediating focal adhesion assembly; however, when expressed,  $\beta_1$  integrins behave as *trans*-dominant molecules with respect to  $\alpha_V$  integrins.



**Figure 2.** Surface expression of  $\alpha_v$ ,  $\beta_3$  and  $\beta_5$  integrins in GD25 and GD25- $\beta_1$  cells. Integrin heterodimers were immunoprecipitated from surface biotinylated untransfected (-/-) or  $\beta_1A$ - and  $\beta_1B$ -transfected GD25 cells with polyclonal antibodies specific for  $\alpha_v$  (A),  $\beta_3$  (B), and  $\beta_5$  (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  $\beta_1A$  or  $\beta_1B$  isoforms does not alter significantly the expression level of the  $\alpha_v$  integrin subunit, it induces a net change of the  $\alpha_v\beta_3/\alpha_v\beta_5$  ratio.

### Expression of the $\beta_1$ Integrin Subunit in GD25 Cells Induces Drastic Reduction of Surface Level of $\alpha_v\beta_3$ and an Up-Regulation of $\alpha_v\beta_5$

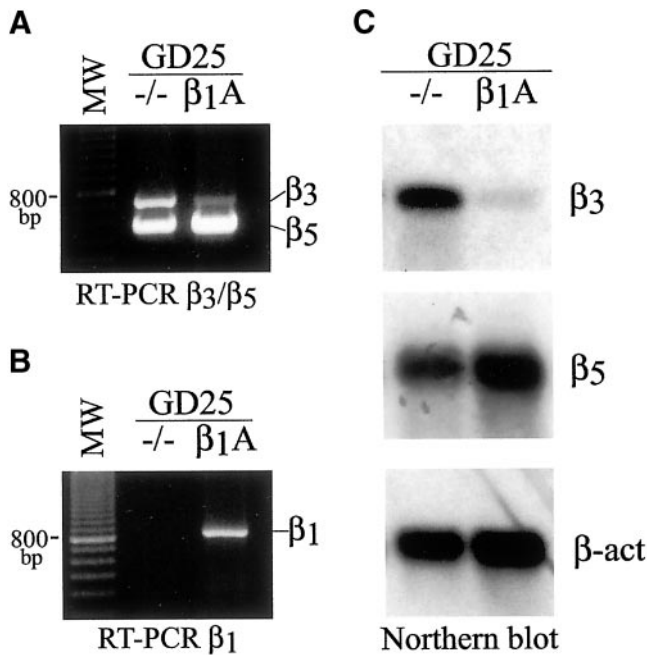
Previous results showed that transfection of GD25 cells with cDNA constructs of human  $\beta_1$  integrin led to surface expression of the  $\beta_1$  integrin subunit associated with the endogenous  $\alpha_3$ ,  $\alpha_5$ , and  $\alpha_6$  subunits but not with the  $\alpha_v$  subunit (Retta *et al.*, 1998). In addition, no obvious differences in  $\alpha_v$  integrin expression were seen by immunoprecipitation from  $^{125}I$ -surface-labeled GD25 and GD25- $\beta_1A$  cells with an anti- $\alpha_v$  antiserum (Retta *et al.*, 1998). To understand the cellular mechanism(s) controlling the effect of  $\beta_1$  over  $\alpha_v$  integrins, we analyzed more in detail GD25 and GD25- $\beta_1A$  cells for the expression levels of their  $\alpha_v$  integrin heterodimers, namely,  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ . Untransfected or  $\beta_1A$ -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  $\alpha_v$  integrin subunit coimmunoprecipitated  $\alpha_v$  together with its associated  $\beta_3$  and  $\beta_5$  subunits (Figure 2A). The biotinylated  $\alpha_v$ ,  $\beta_3$ , and  $\beta_5$  proteins resulted as distinct bands in Western blots and, surprisingly, we noticed that, whereas expression of the  $\alpha_v$  subunit did not change significantly, the relative amounts of  $\beta_3$  and  $\beta_5$  proteins in  $\beta_1A$ -expressing GD25 cells were clearly different from those of untransfected GD25 cells (Figure 2A). With the use of antibodies specific for  $\beta_3$  and  $\beta_5$  subunits, we confirmed this evidence:  $\beta_3$  protein levels were much lower in GD25- $\beta_1A$  than in GD25 cells, whereas the opposite was true for  $\beta_5$  protein levels (Figure 2, B and C). Thus, although the expression of the human  $\beta_1A$  integrin isoform in GD25 cells did not modify the surface expression level of the  $\alpha_v$  integrin subunit, it led to a down-regulation and an up-regulation of the levels of its associated  $\beta_3$  and  $\beta_5$  subunits, respectively. These data suggest that the *trans*-dominant effect of  $\beta_1$  integrin isoforms over the subcellular localization of  $\alpha_v$  integrins in GD25- $\beta_1$  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 $\beta_1$ Integrins Differentially Regulates mRNA Steady-State Levels of $\beta_3$ and $\beta_5$ Integrin Subunits

To address at what level the control of  $\beta_3$  and  $\beta_5$  protein expression in GD25- $\beta_1$  cells was exercised, we first compared mRNA steady-state levels of these two integrin subunits in GD25- $\beta_1A$  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  $\beta_3$  and  $\beta_5$  integrin subunit mRNAs. As shown in Figure 3A, a great difference in  $\beta_3$  mRNA steady-state levels was observed between GD25 and GD25- $\beta_1A$  cells. Interestingly, the lower mRNA levels of  $\beta_3$  in GD25- $\beta_1A$  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  $\beta_5$  mRNA levels among untransfected and  $\beta_1$ -transfected GD25 cells, with a small elevation observed in GD25- $\beta_1A$  cells (Figure 3A). RT-PCR for  $\beta_1$  mRNA was performed as control (Figure 3B). Thus, the presence of  $\beta_1$  integrins in GD25 cells differentially modulates  $\beta_3$  and  $\beta_5$  mRNA expression.

Northern blot analysis demonstrated that our cDNA probes to  $\beta_3$  and  $\beta_5$  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  $\beta_3$  was much lower in GD25- $\beta_1A$  than in GD25 cells (Figure 3C,  $\beta_3$ ), thus reflecting the difference observed by RT-PCR and protein analysis (see above). In addition, the Northern blot analysis confirmed that the difference in  $\beta_5$  mRNA steady-state levels between GD25 and GD25- $\beta_1A$  cells (Figure 3C,  $\beta_5$ ) did not fully correlate with the difference in  $\beta_5$  cell-surface expression level (compare with Figure 2, A and C). Thus, in GD25- $\beta_1A$  cells the down-regulation of  $\alpha_v\beta_3$  cell-surface expression strictly correlates with the down-regulation of  $\beta_3$  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  $\beta_5$  subunit cell-surface recruitment.



**Figure 3.**  $\beta_1$  integrins differentially regulate mRNA steady-state levels of  $\beta_3$  and  $\beta_5$  integrin subunits. Total RNA was isolated from  $1 \times 10^7$  cultured GD25 (-/-) and GD25- $\beta_1A$  cells, and  $\beta_3$  and  $\beta_5$  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  $\beta_3$  and  $\beta_5$  mRNAs. (B) RT-PCR for  $\beta_1$  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  $\mu$ g/lane) were probed sequentially by  $^{32}$ P-labeled mouse integrin  $\beta_3$  and  $\beta_5$  cDNA fragments, and by a  $^{32}$ P-labeled  $\beta$ -actin probe as a control for RNA loading. Notice that the presence of  $\beta_1$  integrins causes a marked down-regulation of  $\beta_3$  and a little up-regulation of  $\beta_5$  mRNA expression levels.

### $\beta_1$ Effect over $\alpha_V$ Integrins Occurs Irrespective of the Type of $\beta_1$ Isoform and Is Dependent on the Presence of the $\beta_1$ Cytoplasmic Domain Common Region

We have previously characterized some of the functional properties of  $\beta_1B$  and  $\beta_1D$  integrin isoforms, comparing these properties with those of the common  $\beta_1A$  isoform. In particular, we have shown that the unique cytoplasmic sequences of  $\beta_1B$  and  $\beta_1D$  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; Cali *et al.*, 1998; Retta *et al.*, 1998).

To analyze more in detail the effects of  $\beta_1$  over  $\beta_3$  and  $\beta_5$  integrins, we tested GD25 cells expressing  $\beta_1B$  and  $\beta_1D$  isoforms as well as two  $\beta_1$  deletion mutants lacking almost the entire cytoplasmic domain ( $\beta_1TR$ ) or the cytoplasmic domain variable region ( $\beta_1COM$ ) (Retta *et al.*, 1998). Untransfected or  $\beta_1$ -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  $\alpha_V$  subunit was expressed at a constant level in all the examined cells (Figures

2A and 4A), whereas a down-regulation of  $\beta_3$  and an up-regulation of  $\beta_5$  cell-surface levels were seen in either  $\beta_1B$ - (Figures 2 and 4, B and C) or  $\beta_1D$ -expressing GD25 cells (Figure 4, A-C). Moreover, the expression of the  $\beta_1COM$  cytoplasmic domain mutant induced similar effects, although to a lower extent (Figure 4, A-C). However, GD25- $\beta_1TR$  cells, expressing a  $\beta_1$  mutant carrying the deletion of the cytoplasmic domain ( $\beta_1TR$ ), behaved equivalently to the  $\beta_1$ -deficient GD25 cells (Figure 4, A-C).

The spectrum of relative  $\beta_3$  integrin levels in untransfected or  $\beta_1$ -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  $\beta_3$  subunit in GD25 cells expressing either  $\beta_1B$ ,  $\beta_1D$ ,  $\beta_1COM$  or  $\beta_1TR$ , we found that in GD25- $\beta_1B$  and GD25- $\beta_1D$  cells it was as low as in GD25- $\beta_1A$  cells, whereas in GD25- $\beta_1COM$  cells it was also reduced but to a lower extent (Figure 5, A-C). On the contrary, the  $\beta_3$  mRNA level in GD25- $\beta_1TR$  was higher and similar to that of  $\beta_1$ -deficient GD25 cells (Figure 5, A-C). On the other hand, although a little increase of  $\beta_5$  mRNA steady-state level was observed in GD25 cells expressing  $\beta_1B$ ,  $\beta_1D$ , or  $\beta_1COM$  (Figure 5, B and D), it did not fully reflect the high increase observed at the  $\beta_5$  protein level in the same cells.

These results indicate that the  $\beta_1$ -dependent modulation of  $\beta_3$  and  $\beta_5$  integrin subunit expression was not confined to GD25- $\beta_1A$  cells, but that it was also present in GD25 cells expressing two other  $\beta_1$  isoforms. In addition, the fact that a down-regulation of  $\beta_3$  and an up-regulation of  $\beta_5$  were also observed in GD25- $\beta_1COM$ , but not in GD25- $\beta_1TR$  cells, strongly suggests that the control of the expression level of  $\beta_3$  and  $\beta_5$  integrin subunits was dependent on the presence of the  $\beta_1$  cytoplasmic domain common region.

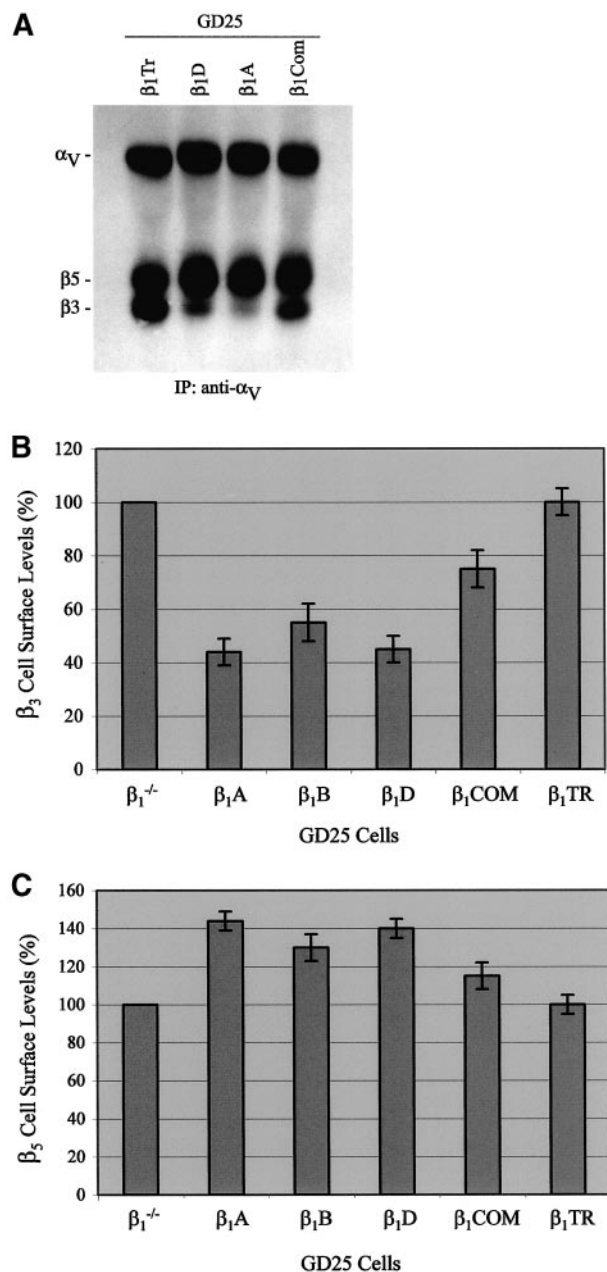
### Cell Adhesion to ECM Proteins Is not Required for $\beta_1$ Effect on $\beta_3$ mRNA Steady-State Level

To determine whether cell adhesion to ECM proteins was required for  $\beta_1$  effect over  $\beta_3$  expression levels, we performed Northern blot analysis of  $\beta_3$  mRNA steady-state level in cells plated on tissue culture dishes coated with either polylysine or two ECM proteins, namely, fibronectin and vitronectin. GD25- $\beta_1TR$  and GD25- $\beta_1COM$  cells were cultured to confluence in complete culture medium and then resuspended in serum-free medium, containing 1  $\mu$ M 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  $\beta_3$  mRNA steady-state level was constitutively low in GD25- $\beta_1COM$  cells compared with that of GD25- $\beta_1TR$  cells. Similar results were obtained by comparing  $\beta_3$  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  $\beta_1$  effect over  $\beta_3$  expression.

### De Novo Surface Expression of $\beta_1$ -associated $\alpha$ Subunits Does Not Affect the Level of $\beta_3$ Integrin Subunit

Because human  $\beta_1$  expression in GD25 cells leads to the assembly and cell-surface recruitment of integrin complex



**Figure 4.** Comparative analysis of surface expression of  $\alpha_v$  integrins in GD25 cells expressing different integrin  $\beta_1$  forms. (A)  $\alpha_v$  integrin heterodimers were immunoprecipitated from surface biotinylated GD25 cells expressing either the  $\beta_1$ A or  $\beta_1$ D isoforms or two  $\beta_1$  deletion mutants, lacking the entire cytoplasmic domain ( $\beta_1$ TR) or the cytoplasmic domain variable region ( $\beta_1$ COM), with the use of a polyclonal antibody against the  $\alpha_v$  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  $\beta_3$  (B) and  $\beta_5$  (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  $\beta_1$  effect over the  $\alpha_v\beta_3/\alpha_v\beta_5$  ratio occurs irrespective of the type of the  $\beta_1$  isoform and is dependent on the presence of the  $\beta_1$  cytoplasmic domain common region.

with endogenous  $\alpha_3$ ,  $\alpha_5$ , and  $\alpha_6$  subunits (Retta *et al.*, 1996), it was possible that these  $\beta_1$ -associated  $\alpha$  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- $\beta_1$ TR cells by extending our observations with this cell line. The expression of the  $\beta_1$ TR mutant at the surface of GD25- $\beta_1$ TR cells was comparable with that of the  $\beta_1$ A isoform in GD25- $\beta_1$ A 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  $\alpha$  subunits associated with  $\beta_1$ TR in GD25 cells compared with GD25- $\beta_1$ A cells (Figure 7). Nevertheless, the presence of  $\beta_1$ TR 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  $\beta_3$  is not directly due to de novo surface expression of the  $\beta_1$ -associated  $\alpha$  subunits and confirm that for the effect of  $\beta_1$  integrins over  $\alpha_v\beta_3/\alpha_v\beta_5$  integrin ratio a  $\beta_1$  subunit carrying, at least, the common region of the cytoplasmic domain is required.

#### Expression of $\beta_1$ Integrins Induces a Marked Decrease in $\beta_3$ mRNA Stability

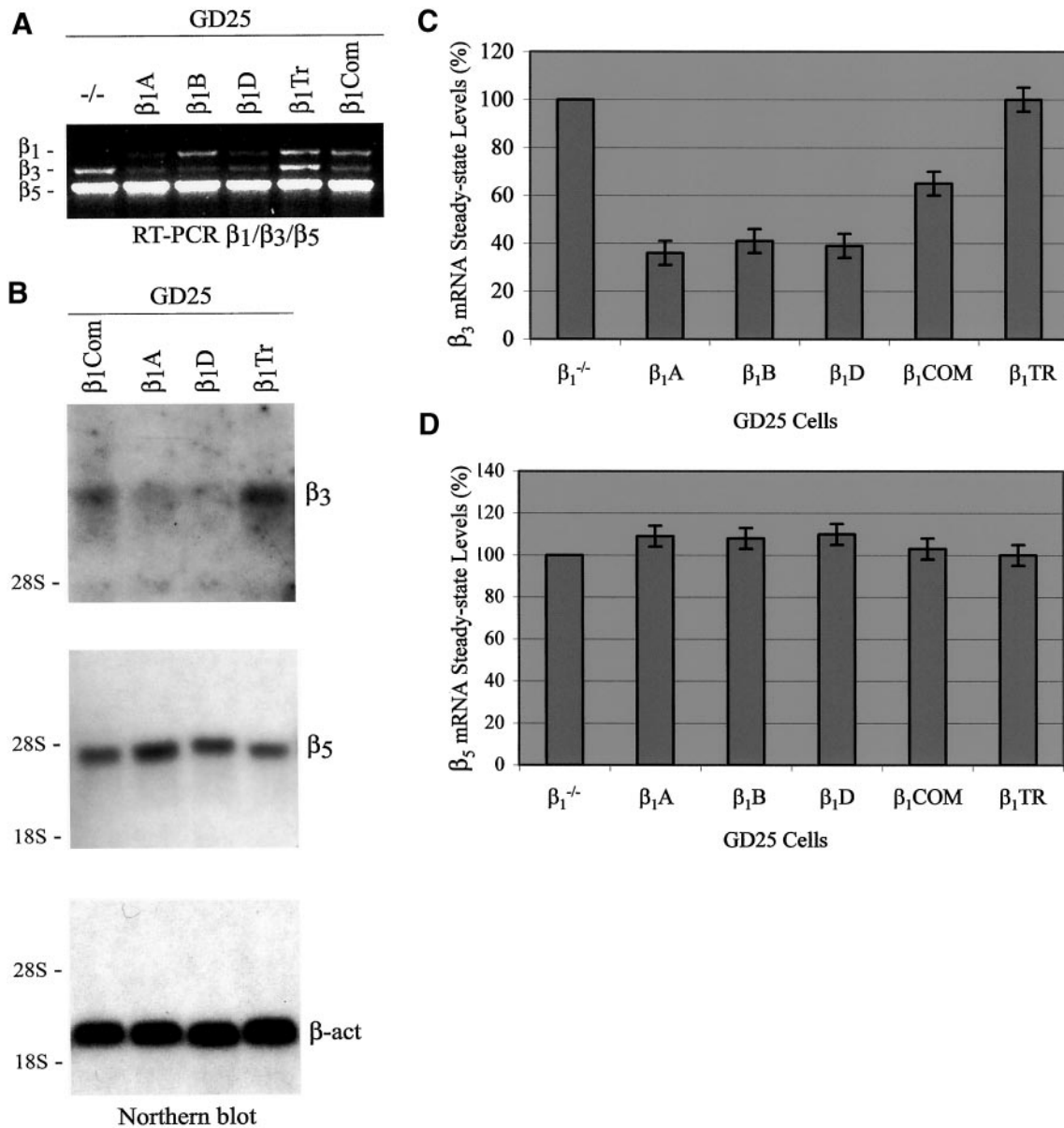
Because modulation of mRNA stability is a potential regulatory mechanism for integrin expression (Sachs, 1993; Feng *et al.*, 1999), we next asked whether the changes in mRNA steady-state levels were due to changes in integrin mRNA stability. The rate of turnover of  $\beta_3$  and  $\beta_5$  mRNAs was determined by inhibition of RNA synthesis with 60  $\mu$ M DRB followed by quantitative blot hybridization analysis of  $\beta_3$  and  $\beta_5$  mRNA as a function of time. In GD25- $\beta_1$ A cells grown on tissue culture dishes a clear decrease of  $\beta_3$  mRNA stability was detected compared with GD25 cells (Figure 8A). The  $\beta_3$  mRNA decayed with an apparent half-life of >8 h in GD25 cells but <4 h in GD25- $\beta_1$ A cells (Figure 8, A and B). In contrast, the stability of  $\beta_5$  mRNA was much higher than that of  $\beta_3$ , and no significant difference was observed when GD25 and GD25- $\beta_1$ A cells were compared. Therefore, the effects of  $\beta_1$  expression over  $\beta_3$  and  $\beta_5$  mRNA levels clearly involve a regulation of  $\beta_3$ , but not  $\beta_5$ , 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  $\beta_1$ B or  $\beta_1$ D integrin isoforms in  $\beta_1$ -null GD25 cells prevented different FN-dependent functions of endogenous  $\alpha_v$  integrins, including their ability to mediate cell adhesion, to localize to focal adhesions, and to assemble an FN matrix, thus indicating the existence of a functional cross talk between these two  $\beta_1$  isoforms and  $\alpha_v$  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  $\beta_1$  integrins exert their *trans*-acting functions. The main findings are that 1) de novo expression of the  $\beta_1$  integrin subunit in  $\beta_1$ -null GD25 cells induces a

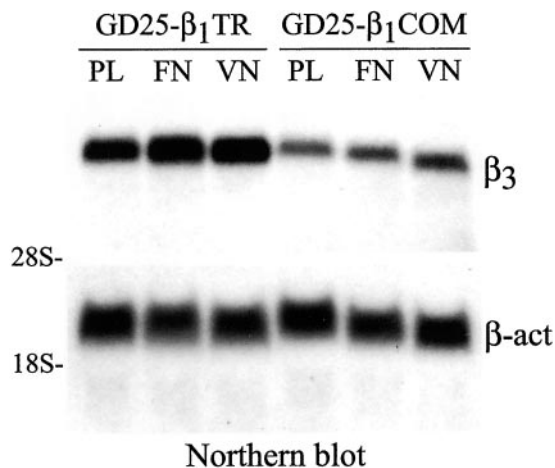




**Figure 5.** Comparative analysis of  $\beta_3$  and  $\beta_5$  mRNA steady-state levels in GD25 cells expressing different integrin  $\beta_1$  forms. Total RNA was isolated from  $1 \times 10^7$  cultured cells as described in MATERIALS AND METHODS. (A) Multiplex RT-PCR assay for the simultaneous detection of  $\beta_1$ ,  $\beta_3$ , and  $\beta_5$  mRNAs in GD25 cells expressing either the  $\beta_1A$ ,  $\beta_1B$ , or  $\beta_1D$  isoforms or two  $\beta_1$  deletion mutants lacking the entire cytoplasmic domain ( $\beta_1TR$ ) or the cytoplasmic domain variable region ( $\beta_1COM$ ). (B) Northern blot: equal amounts of total RNA (25  $\mu$ g/lane) were probed sequentially by  $^{32}P$ -labeled mouse integrin  $\beta_3$  and  $\beta_5$  cDNA fragments and by a  $^{32}P$ -labeled  $\beta$ -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  $\beta_3$  and  $\beta_5$  mRNA levels as detected by Northern blot. Northern signals were normalized to  $\beta$ -actin and displayed as percentage of the control (GD25). Data are representative of three independent experiments. Notice that the down-regulation of  $\beta_3$  mRNA steady-state level occurs irrespective of the type of the  $\beta_1$  isoform and is dependent on the presence of the  $\beta_1$  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  $\beta_1$  effect occurs irrespective of the type of  $\beta_1$  isoform but is dependent on the presence of the common region of the  $\beta_1$  cytoplasmic domain; and 3) the down-regulation of  $\alpha_v\beta_3$  is due to a de-

creased mRNA stability of the  $\beta_3$  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  $\beta_1$  and  $\alpha_v$  integrins based on mechanisms of control of mRNA and protein levels.



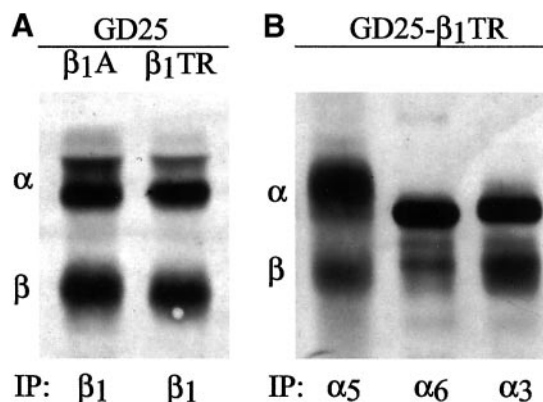
**Figure 6.** Cell adhesion to ECM proteins is not required for  $\beta_1$  effect on  $\beta_3$  mRNA steady-state level. GD25- $\beta_1$ TR and GD25- $\beta_1$ COM cells were cultured to confluence in complete culture medium. Cells were then resuspended in serum-free medium containing 1  $\mu$ M monensin 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  $\mu$ g/lane) were analyzed for  $\beta_3$  mRNA steady-state level by Northern blot hybridization with the use of  $^{32}$ P-labeled mouse integrin  $\beta_3$  cDNA fragments as probe. Equal loading was confirmed by hybridization of the same blot with a  $^{32}$ P-labeled probe for  $\beta$ -actin. The positions of 28s and 18s rRNAs are indicated as markers for RNA sizes. Notice that the  $\beta_3$  mRNA steady-state level is constitutively low in GD25- $\beta_1$ COM compared with that of GD25- $\beta_1$ TR cells.

#### Expression of the $\beta_1$ Integrin Subunit in GD25 Cells Induces a Drastic Reduction of Surface Level of $\alpha_v\beta_3$ and an Up-Regulation of $\alpha_v\beta_5$

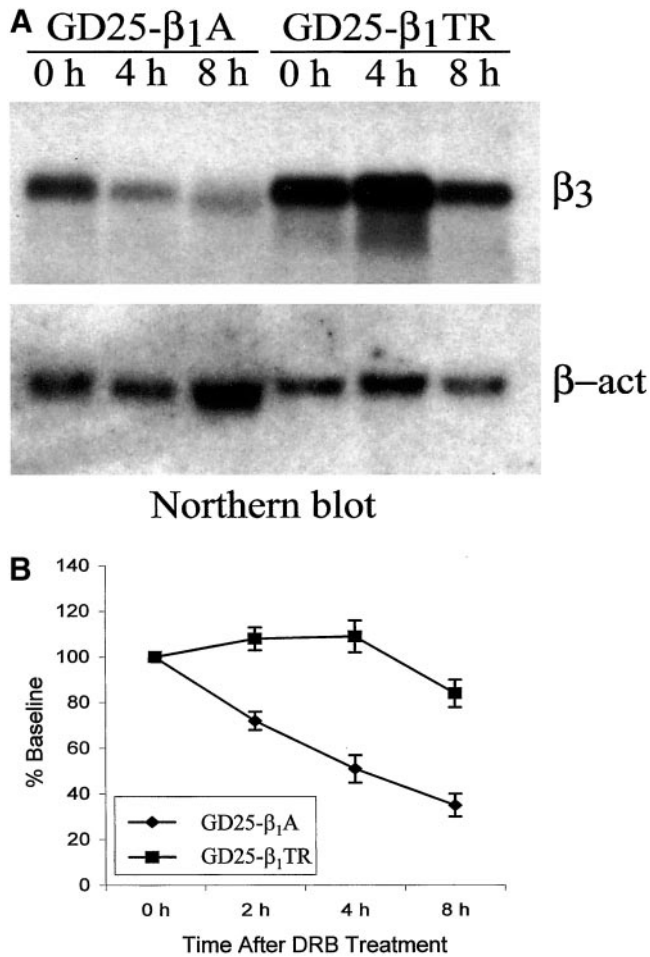
Despite the apparent high degree of integrin-ligand binding redundancy (Hynes, 1992), the localization of distinct integrins to focal adhesions is usually very restricted (Fath *et al.*, 1989). In GD25 cells two integrins are believed to be able to localize to focal adhesions on fibronectin, namely,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

differential temporal and spatial regulation of its  $\beta$  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  $\beta_1$  or  $\alpha_v$  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  $\alpha_v$  and  $\alpha_5$  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  $\alpha_v$  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  $\alpha_v$  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  $\alpha_v$  and  $\beta_1$  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  $\alpha$  subunits associated with the  $\beta_1$ TR cytoplasmic domain deletion mutant. (A)  $\beta_1$  Integrins immunoprecipitated from surface biotinylated  $\beta_1$ A- and  $\beta_1$ TR-transfected GD25 cells with the mAb TS2/16. (B) Integrin heterodimers immunoprecipitated from surface biotinylated GD25- $\beta_1$ TR cells with the mAb GoH3 against  $\alpha_6$  and polyclonal antibodies against  $\alpha_3$  and  $\alpha_5$  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  $\beta_1$ TR mutant correctly associates with three major  $\alpha$  subunits at the surface of GD25- $\beta_1$ TR cells.



**Figure 8.** The presence of the  $\beta_1$  cytoplasmic domain doubles the decay rate of the  $\beta_3$  mRNA in GD25 cells. GD25- $\beta_1$ A and GD25- $\beta_1$ TR 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  $\mu$ M 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  $\mu$ g/lane) were probed sequentially by  $^{32}$ P-labeled mouse integrin  $\beta_3$  and  $\beta$ -actin cDNA fragments. (B) Scanning densitometry analysis of  $\beta_3$  mRNA levels as detected by Northern blot.  $\beta_3$  Northern signals were normalized to  $\beta$ -actin and displayed as percentage of the baseline (time 0). Data presented are the mean values  $\pm$  SE of three independent experiments. Notice the lower stability of  $\beta_3$  integrin subunit mRNA in GD25- $\beta_1$ A than in GD25- $\beta_1$ TR cells.

tion of  $\alpha_V$  integrins for the lack of certain  $\beta_1$  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  $\beta_1$  integrin expression. Taken together, these observations suggest that the cross talk between  $\beta_1$  and  $\alpha_V$  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  $\alpha_7$ B 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  $\beta_1$  splice variants during mouse muscle development: genetic analysis by homologous recombination has demonstrated that mice lacking  $\beta_1$ D, the only  $\beta_1$  isoform in adult muscles, develop normally without gross apparent defects; in this case, however, the  $\beta_1$ A isoform is not down-regulated as in wild-type muscles and can apparently compensate both in function and in level for the absence of  $\beta_1$ D (Baudoin *et al.*, 1998). A similar phenomenon might likely occur during  $\beta_1$ -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  $\beta_1$ -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 $\beta_1$ Integrins Differentially Regulates mRNA Steady-State Levels of $\beta_3$ and $\beta_5$ 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- $\beta_1$ , 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- $\alpha$ , interferon- $\gamma$  (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  $\beta_1$  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- $\beta_1$ A cells clearly involves an increased decay rate of  $\beta_3$  subunit mRNA. On the contrary, because the up-regulation of the  $\beta_5$  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  $\beta_5$  subunit. This is consistent with previous reports showing the presence of intracellular pools of  $\beta$  subunits and indicating the

availability of the  $\alpha$  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  $\beta_3$  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  $\beta_1$  integrins, highlighting the usefulness of stabilizing specific integrin mRNA only when it is needed.

### **$\beta_1$ Effect over $\beta_3$ Expression Is Dependent on the Presence of the $\beta_1$ Cytoplasmic Domain Common Region**

Our results clearly demonstrate that the control of  $\beta_3/\beta_5$  ratio is  $\beta_1$ -dependent, and neither confined to a particular cell population nor restricted to a specific  $\beta_1$  isoform. Instead, it requires the presence of the common region of the  $\beta_1$  cytoplasmic domain. In addition, the fact that the  $\beta_1$ COM mutant does not contribute to cell adhesion (Retta *et al.*, 1998), together with the observation that the  $\beta_3$  mRNA steady-state level is constitutively low in GD25- $\beta_1$ COM cells compared with GD25- $\beta_1$ TR cells, indicates that the binding of extracellular ligands is not required for  $\beta_1$  to regulate  $\beta_3$  expression. On the other hand, it is noteworthy that, although in long-term adherent cells the  $\beta_1$ COM mutant was less effective than  $\beta_1$ A in inducing an effect over the  $\beta_3$  mRNA steady-state level, no significant difference was observed when we compared  $\beta_3$  mRNA levels in GD25- $\beta_1$ COM and GD25- $\beta_1$ A 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  $\beta_1$ A, the expression of  $\beta_1$ COM in GD25 cells does not entirely prevent the localization of  $\alpha_v$  integrins to FN-dependent focal adhesions (Retta *et al.*, 1998). Thus, a likely possibility is that the common region of the  $\beta_1$  cytoplasmic domain is able to constitutively induce a down-regulation of  $\beta_3$  mRNA steady-state level; however, the  $\alpha_v\beta_3$  integrin, due to its ability to localize to focal adhesions in GD25- $\beta_1$ COM cells, counteracts this  $\beta_1$  constitutive action

leading to a mild effect on  $\beta_3$  expression level. Interestingly, in accordance with a recent report (Feng *et al.*, 1999), the higher  $\beta_3$  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  $\beta_3$  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  $\beta_1$ -dependent events regulating the  $\beta_3$  mRNA steady-state levels.

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