# The size of the intracellular β1-integrin precursor pool regulates maturation of β1-integrin subunit and associated α-subunits

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A large pool of precursor β1-integrin subunits is frequently found intracellularly. During malignant transformation this pool often disappears. Concomitantly, integrin-mediated cell-adhesion functions are disturbed, even though no change in the number of β1-integrin receptors on the cell surface can be observed. Here, we have studied the role of an intracellular pre-β1-integrin pool by transfecting human MG-63 osteosarcoma cells with plasmid construction producing an antisense RNA for the β1-integrin subunit. Stable cell clones expressing β1-integrin antisense RNA were shown to have a reduced intracellular pool of pre-β1-integrin subunits. In the antisense-transfected cells, the synthesis of the β1-integrin chain was reduced by 65% compared with non-transfected or vector-transfected MG-63 cells. The decreased synthesis of the β1-integrin chain was associated with accelerated maturation of the β1-integrin chain (half-maturation time about 5 h in antisense-transfected cells compared with about 10.5 h in control cells), whereas maturation of the α-integrin chain slowed down. The amount of β1-integrins on the cell surface, however, remained unaltered. Cell clones with the largest decrease in the relative amount of the pre-β1-integrin subunit also showed altered integrin function. They were found to synthesize fibronectin, but were unable to assemble it into a fibronectin matrix on the cell surface. Thus we conclude that the repression of biosynthesis of the β1-integrin chain leads to alterations in receptor maturation and may be connected with altered receptor function.

## INTRODUCTION

Integrins are a family of heterodimeric cell-surface adhesion receptors which mediate cell attachment to extracellular matrix proteins as well as cell–cell interactions [for reviews see Ruoslahti (1991) and Hynes (1992)]. Integrin-dependent cell adhesion is involved in many important cellular events, including cell proliferation, differentiation and gene expression.

Integrins are composed of two distinct transmembrane glycoprotein subunits, α and β, which are non-covalently linked to each other. Up to 14 different α-subunits and eight β-subunits are known today (Hynes, 1992). The large extracellular domains of both α- and β-chains associate to form a binding site for extracellular matrix proteins. The short intracellular domains of integrins interact with actin-containing cytoskeleton via α-actinin and talin (Buck and Horwitz, 1987; Burridge et al., 1988; Otey et al., 1990). Once bound to the ligand, integrins typically promote the organization of the actin cytoskeleton, leading to clustering of receptors into focal contacts. As well as coupling both intracellular and extracellular ligands, integrins seem to be also involved in signal-transduction events.

Integrin heterodimers can be divided into subfamilies on the basis of their common subunit. Those of the β1 subfamily include at least nine different heterodimers sharing the same β1-integrin subunit. They function primarily as receptors for collagens, laminins and fibronectin. An individual integrin can often bind to more than one ligand, and each ligand is usually recognized by more than one integrin. Many integrin subunits also have variable cytoplasmic domains produced by alternative splicing of the mRNA molecules giving integrin heterodimers more versatility.

Our previous studies (Heino et al., 1989) have shown that the regulation of β1-integrin heterodimer expression resembles that of other multisubunit membrane protein complexes, e.g. T-cell receptors (Lippincott-Schwartz et al., 1988) and influenza virus haemagglutinin subunits (Copeland et al., 1988). Many cell lines, including fibroblasts and osteosarcoma cells, synthesize an excess of the pre-β1-integrin subunit in endoplasmic reticulum (ER) (Akiyama and Yamada, 1987; Heino et al., 1989). The maturation rate of the pre-β1-integrin pool seems to be dependent on the number of pre-α-subunits available, suggesting that further segregation of integrin precursors from ER requires αβ-complex formation (Heino et al., 1989). The size of the free pre-β1-subunit pool is not stable, but can be regulated by growth and differentiation factors (Ignotz and Massagué, 1987; Heino et al., 1989) and is decreased during malignant transformation (Akiyama et al., 1990). In both cases, maturation of the pre-β1-integrin pool is accelerated. This phenomenon can be explained by the altered α/β ratio in ER. Interestingly, in WI-38 human lung fibroblasts, viral transformation altered not only the rate of maturation of the pre-β1-integrin pool but also integrin distribution, whereas the number of cell-surface integrin heterodimers remained relatively unchanged (Akiyama et al., 1990).

Here we have transfected human MG-63 osteosarcoma cells with a plasmid driving antisense β1-integrin RNA expression to reduce the synthesis of the β1-integrin chain. In the transfected cell lines, the intracellular pool of free pre-β1-subunits was clearly decreased. Thus we were able to create a model system to study the role of pre-β1-chain excess in the integrin maturation process. The reduction of β1-integrin subunit expression was associated with concomitant alterations in the cell-adhesion system: faster maturation of β1-integrin polypeptides and decelerated maturation rate of the β1-integrin-associated α-subunits. In two subclones with the largest reduction in the size of the pre-

Abbreviations used: DMEM, Dulbecco's modification of Eagle's medium; ER, endoplasmic reticulum; FCS, fetal calf serum; PBS+, PBS containing $Ca^{2+}$ and $Mg^{2+}$.

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β1-integrin pool, a loss of fibronectin matrix from the cell surface was observed suggesting altered receptor function.

## MATERIALS AND METHODS

### Antibodies

For immunoprecipitation, polyclonal rabbit antisera raised against the cytoplasmic domain of human β1-integrin subunit (Larjava et al., 1990) and against human α5-integrin cytoplasmic domain (LaFlamme et al., 1992) were used. Polyclonal antisera against human α2 and α3-integrin cytoplasmic domains (amino acid sequences of synthetic peptides KLGFFKRKYEKMTKN-PDEIDETTELSS and KRARTRALYEAKRQKAEMKSQPS-ETERLTDDY respectively) were also raised in rabbits (Santala et al., 1994). For immunoprecipitation of fibronectin, polyclonal antibody against human plasma fibronectin was used (Chen et al., 1986).

For Western blots, the same anti-(β1-integrin) antibody was used as for immunoprecipitation. The α5-integrin chain was detected with polyclonal antiserum against a synthetic peptide corresponding to α5-integrin subunit (Larjava et al., 1990).

For immunostaining experiments, polyclonal antibodies against human plasma fibronectin (Chen et al., 1986) and human placental fibronectin receptor (Roberts et al., 1988) were used. The anti-(fibronectin receptor) antibody recognizes the β1-integrin subunit in the receptor complex.

### Cell culture

Human osteosarcoma MG-63 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Irvine, U.K.) supplemented with 23 mM $NaHCO_3$, 20 mM Hepes (Gibco Biocult, Paisley, U.K.), antibiotics (50 μg/ml streptomycin sulphate, 100 units/ml penicillin) and 10% (v/v) fetal calf serum (FCS). For subculturing, the cells were detached with cell-culture-grade trypsin/EDTA (0.05% trypsin, 0.02% EDTA). Trypsin activity was inhibited by washing the detached cells with the complete growth medium.

### Antisense construction

Plasmid pLK/B1 was constructed by ligating the 1.1 kb *Nsi*I–*Hin*dIII fragment of human β1-integrin cDNA from plasmid pGEM1-P32 [nucleotides 1227–2357 in the published sequence (Argraves et al., 1987)] to *Hin*dIII–*Nsi*I-digested pcDNAI/neo (Invitrogen, San Diego, CA, U.S.A.) in antisense orientation. The antisense orientation of the β1-integrin cDNA fragment was confirmed using digestions and DNA sequencing. All DNA manipulations were carried out by standard methods (Sambrook et al., 1989).

### Transfections

MG-63 cells were transfected using Lipofectin reagent (Gibco–BRL Life Technologies, Gaithersburg, MD, U.S.A.). Vector pcDNAI/neo was introduced into cells as a control. Briefly, cells were cultured to 50% confluence in six-well plates (Costar, Cambridge, MA, U.S.A.) and washed twice with PBS. Optimem I medium (Gibco–BRL Life Technologies) containing 55 μM 2-mercaptoethanol was added to wells (1 ml per well). In transfections, 3 μg of plasmid DNA and 12 μg of Lipofectin were used. Cells were incubated with DNA and Lipofectin for 8 h. Then an equal volume of DMEM supplemented with 20% (v/v) FCS was added, and incubation was continued overnight. Next day, cells were changed back to DMEM + 10% (v/v) FCS and selected with G-418 (250 μg/ml) (Sigma, St. Louis, MO, U.S.A.) for about 2 weeks until all non-transfected cells on control plates had died. Individual clones were subcloned with cloning rings and propagated for analysis. Transfected MG-63 clones were routinely grown in medium supplemented with 50 μg/ml G-418.

### Immunoprecipitation

To analyse the amount of β1-integrins synthesized, MG-63 clones were cultured to confluence, washed twice with PBS and metabolically labelled with 100 μCi/ml [$^{35}$S]methionine (Tran$^{35}$S-label; specific radioactivity > 1000 Ci/mmol; ICN Radiochemicals, Irvine, U.S.A.) in Optimem I for 24 h. Cells were washed three times with PBS, and cell-membrane fractions were extracted with precooled 2% (w/v) Triton X-100 in PBS+ (PBS with $Ca^{2+}$ and $Mg^{2+}$) containing proteinase inhibitors (20 μg/ml leupeptin, 3 mM phenylmethanesulphonyl fluoride) for 10 min on ice. For immunoprecipitation, $2 \times 10^6$ c.p.m. of cell extracts were used. Immunoprecipitations were performed basically as previously described (Roberts et al., 1988). Briefly, cell extracts were diluted with the extraction buffer to 500 μl. BSA (0.5 mg/ml) was added to samples, which were then precleaned with 40 μl of 50% (v/v) Protein A–Sepharose (Pharmacia LKB Biotechnology) at 4 °C for 30 min. The supernatants were immunoprecipitated with an antibody against the given integrin subunit at 4 °C for 16 h. Immune complexes were recovered by binding to Protein A–Sepharose at 4 °C for 6 h and by washing three times with the extraction buffer, once with 50 mM Tris/HCl (pH 8.0) containing 500 mM NaCl and 0.1% (w/v) Tween 20 and once with 5 mM Tris/HCl (pH 8.0) containing 20 mM NaCl and 0.05% Tween 20. The immunoprecipitated samples were separated by SDS/PAGE with 4% stacking and 7.5% resolving gels, intensified and exposed for fluorography. Integrin bands were quantified by densitometric scanning using LKB UltroScan XL apparatus (Pharmacia, Uppsala, Sweden).

Pulse–chase experiments were carried out to study the maturation rates of different integrin chains. Cells were cultured to confluence and incubated in methionine-free DMEM for 2 h before labelling with [$^{35}$S]methionine for 30 min. Cells were then washed twice with PBS and incubated further in their complete growth medium. To analyse β1-integrin chain maturation, the cell-membrane proteins were extracted after 0, 2, 4, 7.5, 13 and 24 h of chase and immunoprecipitated as above. For analysis of maturation of α-integrin chains, cells were harvested either 0, 1, 2, 3, 4 and 5 h or 0, 1, 1.5, 2, 2.5 and 3 h after the pulse.

For analysis of cell-surface integrins, confluent cells were labelled as monolayers by iodination as described elsewhere (Gullberg et al., 1990) using 400 μCi/ml $^{125}$I (Amersham Corp.). Cell-membrane fractions were extracted and immunoprecipitated with the anti-(β1-integrin) antibody as above.

For calculation of the amount of the pre-β1-integrin chain synthesized, the MG-63 clones were grown to confluence, preincubated in methionine-free DMEM for 2.5 h and labelled with [$^{35}$S]methionine (100 μCi/ml) for 1 h. The cell-membrane fractions were extracted as above. Equal amounts of trichloroacetic acid-precipitable radioactivity were immunoprecipitated with the anti-(β1-integrin) antibody as above.

To study the synthesis of fibronectin, cells were plated on six-well dishes (200000 cells per well) in 1 ml of serum-free Optimem I or in an equal volume of DMEM + 10% (v/v) FCS. The cells

were allowed to attach at 37 °C for 3 h or grown to confluence; then 100 μCi/ml [$S^{35}$]methionine was added for 24 h. Media from wells were collected, and equal volumes of each sample were immunoprecipitated with the anti-fibronectin antibody as above. Before electrophoresis, the samples were treated with 0.1 M 2-mercaptoethanol.

### Western blots

Western immunoblotting was performed as described previously (Burnette, 1981) using unlabelled cell-membrane extracts. Briefly, the samples were separated by SDS/PAGE as above and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 5% milk powder in PBS at 37 °C for 1.5 h. The anti-integrin antibodies were added at room temperature for 2 h. Then the membrane was rinsed three times with milk/PBS, and peroxidase-conjugated pig anti-(rabbit IgG) antibody (DAKO-Immunoglobulins) in milk/PBS was added for 1 h. The membrane was again rinsed three times with milk/PBS, equilibrated in 50 mM Tris/HCl (pH 7.6) and stained with diaminobenzidine [0.06% in 50 mM Tris/HCl (pH 7.6) containing 0.03% $H_2O_2$].

### Northern-blot hybridization

Total cellular RNAs were extracted by the guanidine thiocyanate procedure (Chomczynski and Sacchi, 1987). For Northern blotting, 15 μg samples of total RNA were separated in 1% formaldehyde/agarose gel and transferred to Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, CA, U.S.A.). For detection of the β1-integrin mRNA and the transfected antisense RNA, a 1.1 kb *NsiI–HindIII* fragment of pGEM-P32 (Argraves et al., 1987) was used as a probe. For the control hybridization, a 0.5 kb *BglI* fragment of rat glyceraldehyde-3-phosphate dehydrogenase cDNA (Fort et al., 1985) was also labelled. Labelling of probes was performed by random priming with DIG DNA Labelling Kit (Boehringer–Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Prehybridization, hybridization and stringency washes were carried out according to the manufacturer's recommendations for the membrane (Zeta-Probe). For hybridization, 20 ng/ml labelled probe was used. Blots were detected with DIG Luminescent Detection Kit (Boehringer–Mannheim) mainly as described in the manufacturer's manual, except that the chemiluminescent substrate 3-(2′-spiroadamantane)-4-methoxy-4(-3″-phosphoryloxy)phenyl-1,2-dioxetane was diluted 1:1000. The membrane was stripped for reprobing by rinsing with distilled water, incubating in a large volume of 2 mM EDTA/0.1% SDS solution at 80 °C for 15 min and finally rinsing with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate).

### Growth curves

Cells were plated in their normal growth medium on to 96-well microtitre dishes (2000 or 5000 per well; Nunclon). They were allowed to grow for 1, 2, 3, 4 or 5 days, washed once with PBS and fixed with formaldehyde fixative (4% formaldehyde and 5% sucrose in PBS+) for 30 min. Fixed cells were washed three times with water and stained using the Crystal Violet method (Kueng et al., 1989). Briefly, cells were stained with 0.1% Crystal Violet in 200 mM boric acid (pH 6.0) for 20 min. Extra colour was washed away with water. After plates had dried, the colour was extracted with 10% acetic acid. Absorbance was measured in Labsystems Multiskan PLUS apparatus (Eflab, Finland) at 540 nm.

### Immunostaining

For immunostaining, cells (50000) were plated on to glass coverslips in 24-well culture dishes (Costar) in either DMEM+ 10% (v/v) FCS or serum-free Optimem I. Cells were allowed to attach for 24 h and then fixed as above. Fixed cells were permeabilized for 4 min in PBS+ containing 0.5% Triton X-100. Immunostaining was carried out as described previously (Larjava et al., 1990) using rhodamine-conjugated goat anti-(rabbit IgG) antiserum as a secondary antibody. Immunofluorescence was documented with a Leitz Aristoplan immunofluorescence microscope.

Fibronectin fibril formation in transfected MG-63 cell clones was estimated using a visual analogue scale (Huskisson, 1983; Christensen, 1988) of cells immunostained as above with anti-fibronectin antibody. In every cell clone, 20 individual cells were estimated on a scale where one end represented the cells totally without fibronectin on them and was indicated as 0, and the other end represented cells where all the fibronectin cables were long and well-formed and was indicated as 10. Numerical values were calculated for each of the cells, and the average of these values was considered to represent the amount and quality of fibronection fibrils on the cell clones studied.

### Cell-spreading assay

For the cell-spreading assay, a 96-well plate was coated with various extracellular matrix ligands: laminin (from mouse EHS tumours; Collaborative Research Inc., Bedford, MA, U.S.A.), fibronectin (from human plasma; Sigma) and collagen (Vitrogen 100; Celtrix Laboratories, Palo Alto, CA, U.S.A.). Fibronectin and laminin (10 μg/ml in PBS+) were added to wells at room temperature for 60 min. Unoccupied sites were blocked with heat-denatured BSA (10 mg/ml in PBS) at room temperature for 30 min. Control wells were treated with BSA only. Unbound proteins were removed by washing five times with PBS+. Collagen (2.4 mg/ml; 50 μl/well) was added to wells, allowed to gelatinize at 37 °C for 60 min and air-dried. Before plating cells, collagen films were washed and rehydrated with sterile water.

Cells were detached, and trypsin was inactivated with trypsin inhibitor (25 mg/ml in Optimem I; Sigma T-1928) for 10 min. Cells were plated on to the coated wells in Optimem I (5000 cells per well) and allowed to attach and spread at 37 °C for 60 min. Cell spreading was quantified basically as described previously (Hahn and Yamada, 1979). Briefly, cells were fixed by adding 2 × formaldehyde fixative (see above). Then the culture wells were filled with PBS to the top and covered with a flat glass plate for observation with a phase-contrast microscope equipped with a 10 × objective. Ten randomly selected fields from three replicate wells were studied. Cells that were surrounded by a lamellar cytoplasm were considered as spread. After evaluation, the cells were stained with Crystal Violet (see above) for documentation.

## RESULTS

### Expression of β1-integrin antisense RNA leads to a relative reduction in the intracellular β1-integrin precursor pool

Plasmid pLK/B1 was obtained by ligating a 1.1 kb fragment from the 3′ coding region of human β1-integrin cDNA (Argraves et al., 1987) in antisense orientation into a mammalian expression vector (pcDNAI/neo) under the regulation of the cytomegalo-

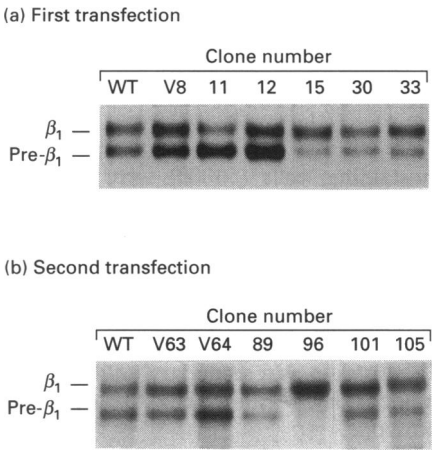


**Figure 1 β1-integrins in antisense-transfected cell clones**

Cell clones were metabolically labelled with [$^{35}$S]methionine for 24 h. The cell membranes were extracted and analysed by immunoprecipitation with anti-(β1-integrin) antibody. WT, wild-type MG-63 cells; V8, V63, V64, vector-transfected control clones; 11, 12, 15, 30, 33, 89, 96, 101, 105, β1-integrin antisense-transfected clones.

**Table 1 Comparison of β1-integrin antisense-transfected cell clones**

Five cell clones transfected with antisense β1-integrin cDNA (B1/11, 12, 15, 30, 33), wild-type MG-63 osteosarcoma cells (WT) and one vector-transfected control clone (V/8) were analysed by immunoprecipitation with anti-(β1-integrin) antibody and by Northern-blot hybridization with β1-integrin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. The values presented are calculated from densitometric scans of autoradiograms from Figures 2 and 3. ND, not determined; —, not observed; +, faint expression; + + +, strong expression.

| Cell clone | mRNAs β1/GAPDH | 0.8 and 1.2 kb transcripts | Pre-β1-integrin (% of total β1) |
|---|---|---|---|
| WT | 0.79 | — | 47 |
| V/8 | ND | ND | 48 |
| B1/11 | 1.00 | — | 70 |
| B1/12 | 0.96 | — | 57 |
| B1/15 | 0.88 | + + + | 22 |
| B1/30 | 1.00 | + | 38 |
| B1/33 | 0.67 | + | 36 |

virus immediate early gene promoter/enhancer sequences to obtain stable expression. This construction was designed to produce an antisense RNA covering the cysteine-rich domain and the transmembrane domain regions in the β1-integrin gene, but not the cytoplasmic domain. pLK/B1, and pcDNAI/neo as a control were introduced into human MG-63 osteosarcoma cells. The β1-integrin antisense-transfected cell clones (22 clones together) were analysed after steady-state metabolic labelling by immunoprecipitation with the anti-(β1-integrin) antibody.

The cell clones obtained could be classified into two categories: (i) clones with a similar or larger pre-β1-integrin pool than control cells and (ii) clones with a smaller pre-β1-integrin pool than control cells (Figure 1a; Table 1). To measure β1-integrin mRNA levels, seven clones representing both subtypes were further analysed by Northern-blot hybridization (Figure 2; Table 1). The cell clones with reduced pre-β1-integrin expression exhibited similar mRNA levels to the others (Figure 2; Table 1).

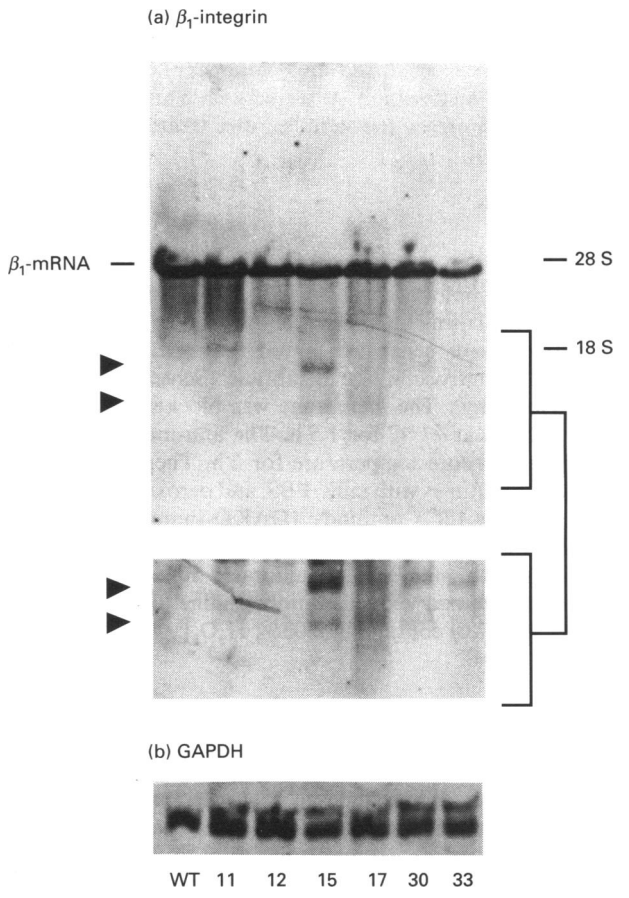


**Figure 2 Analysis of β1-integrin antisense-transfected cell clones by Northern-blot hybridization**

(**a**) Hybridization with β1-integrin cDNA probe; small transcripts are marked with arrowheads. Lower panel, small transcripts, longer exposure. (**b**) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization. WT, wild-type MG-63 cells; 11, 12, 15, 17, 30, 33, β1-integrin antisense-transfected clones.

All clones showed, however, two extra transcripts (about 1.2 kb and 0.8 kb) in addition to the normal 4.2 kb β1-integrin mRNA (Figure 2). These extra RNAs were not seen in cell clones with normal or increased pre-β1-integrin synthesis. The cell clone exhibiting the most reduced pre-β1-chain pool and the highest level of extra transcripts (clB1/15) was selected for a detailed analysis of integrin maturation and function, together with non-transfected MG-63 cells and a vector-transfected control clone (clV/8).

### Reduced size of the β1-integrin precursor pool leads to accelerated maturation of β1-integrin subunits but slower maturation of α-subunits

Synthesis and maturation of the β1-integrin subunit was studied using a short [$^{35}$S]methionine pulse (30 min) followed by a chase and immunoprecipitation. The rates of pre-β1-chain synthesis and maturation were the same in both controls (wild-type MG-63 and clV/8), whereas the synthesis of pre-β1-integrin was reduced by about 65% in the clB1/15 cell clone. Furthermore, the half-maturation of the β1-integrin chain took about 5 h, whereas in the control clones the half-maturation time was about 10.5 h (Figure 3). Thus antisense transfection did not only

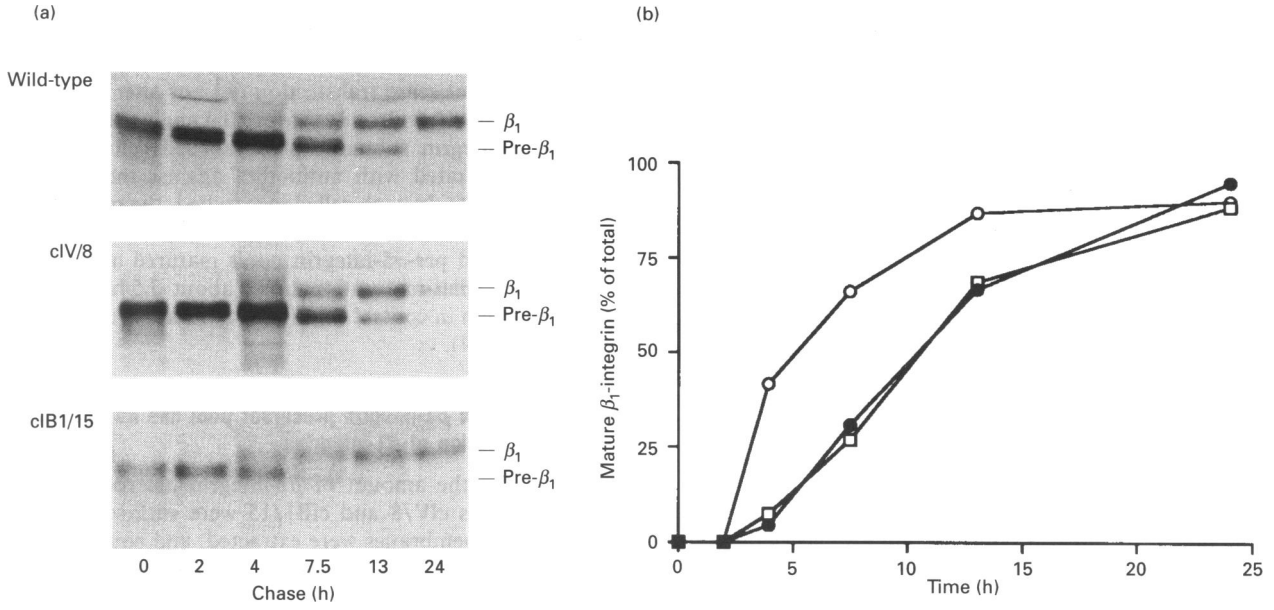


**Figure 3 Maturation of β1-integrin subunit**

Wild-type MG-63 cells, vector-transfected cells (clV/8) and β1 antisense-transfected cells (clB1/15) were labelled with [$^{35}$S]methionine for 30 min. Cell membranes were collected 0, 2, 4, 7.5, 13 and 24 h after the chase and analysed by immunoprecipitation with anti-(β1-integrin) antibody (**a**). (**b**) Maturation curves showing mature β1-integrin as a percentage of the total β1-integrin subunit were drawn from densitometric scans of the autoradiograms shown in (**a**). ●, Wild-type; □, clV/8; ○, clB1/15.

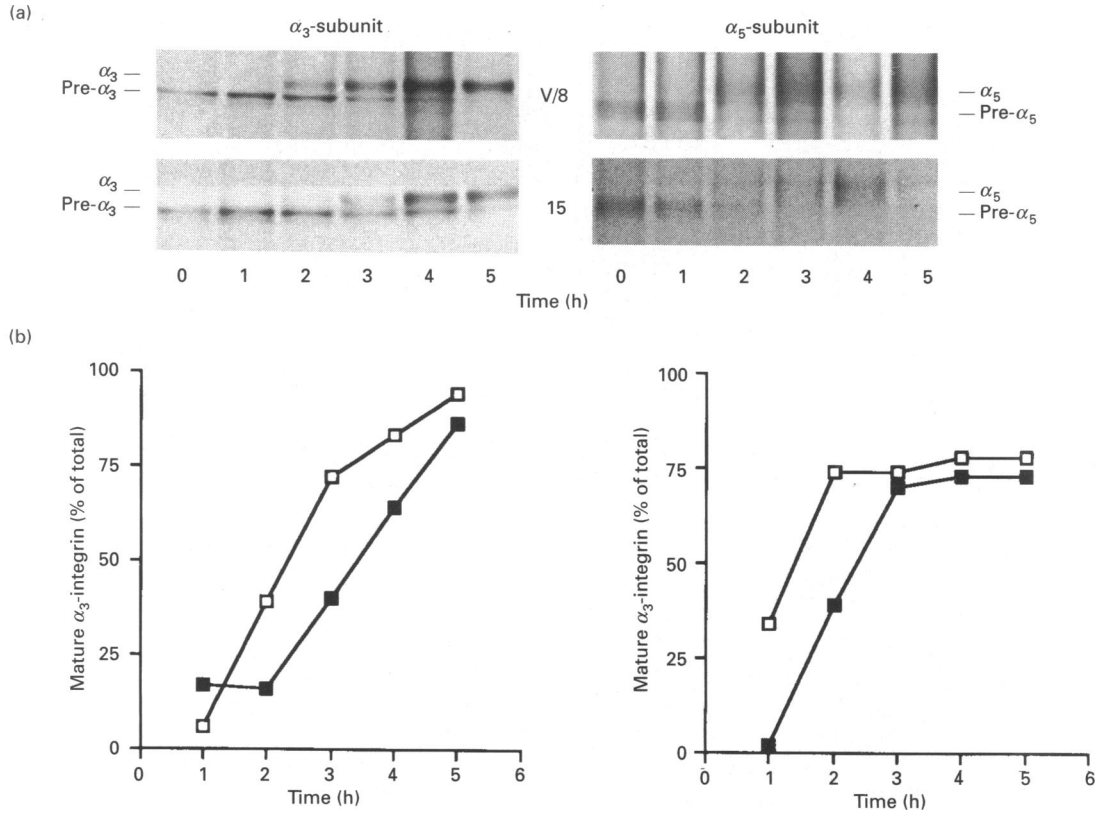


**Figure 4 Maturation of β1-integrin-associated α-chains**

Vector-transfected cells (clV/8) and β1-integrin antisense-transfected cells (clB1/15; 15) were labelled with [$^{35}$S]methionine for 30 min. Cell membranes were collected 0, 1, 2, 3, 4 and 5 h after the chase and analysed by immunoprecipitation with anti-α3- and anti-α5-integrin antibodies (**a**). (**b**) Maturation curves showing mature α-subunits as a percentage of the total amount of corresponding α-subunit were drawn from densitometric scans of autoradiograms seen in (**a**). □, clV/8; ■, clB1/15.

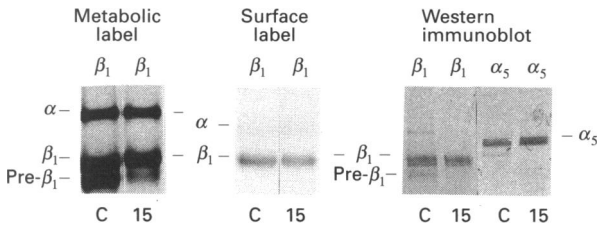


**Figure 5 Expression of β1-integrin heterodimers**

β1-integrins in vector-transfected cells (clV/8; C) and β1-integrin antisense-transfected cells (clB1/15; 15) were analysed by immunoprecipitation after metabolic labelling (24 h) with [$^{35}$S]methionine and after cell-surface iodination with $^{125}$I with anti-(β1-integrin) antibody, and by Western immunoblotting with anti-(β1-integrin) and anti-(α5-integrin) antibodies. The label α represents all those integrin α-chains that are co-precipitated with β1-integrin subunit with anti-(β1-integrin) antibody, namely α2, α3 and α5.

reduce the rate of β1-integrin synthesis, but also accelerated the maturation rate of the pre-β1-integrin pool.

The α-integrin subunits associated with the β1-integrin chain in MG-63 cells have been previously characterized (Takada et al., 1987; Heino and Massagué, 1989; Hynes et al., 1989). Here, in accordance with previous reports, the most abundant α-subunit was α3, and α2- and α5-subunits were expressed at much lower levels. Antisense transfection did not alter cell-surface α-chain expression (results not shown). To analyse the maturation of these α-integrin subunits, pulse-labelled cell extracts were immunoprecipitated with antibodies against the α3- and α5-integrin subunits. In each cell clone studied, the maturation rate of α5 was somewhat faster than that of α3. In the clB1/15 clone the pre-α3- and pre-α5-integrin pools matured about 1.5 times more slowly (half-maturation times about 3.5 h and 2.5 h respectively) than in control cells (half-maturation times 2.5 h and 1.5 h) (Figure 4).

### Reduction of the β1-integrin precursor pool has no effect on cell-surface expression of β1-integrins

To determine the amount of β1-integrin heterodimers on cell surfaces, clones clV/8 and clB1/15 were surface-labelled with $^{125}$I. The cell membranes were extracted, and equal amounts of total radioactivity were immunoprecipitated with the anti-(β1-integrin) antibody. The proportion of total cellular protein present as mature β1-integrins was not altered by transfection

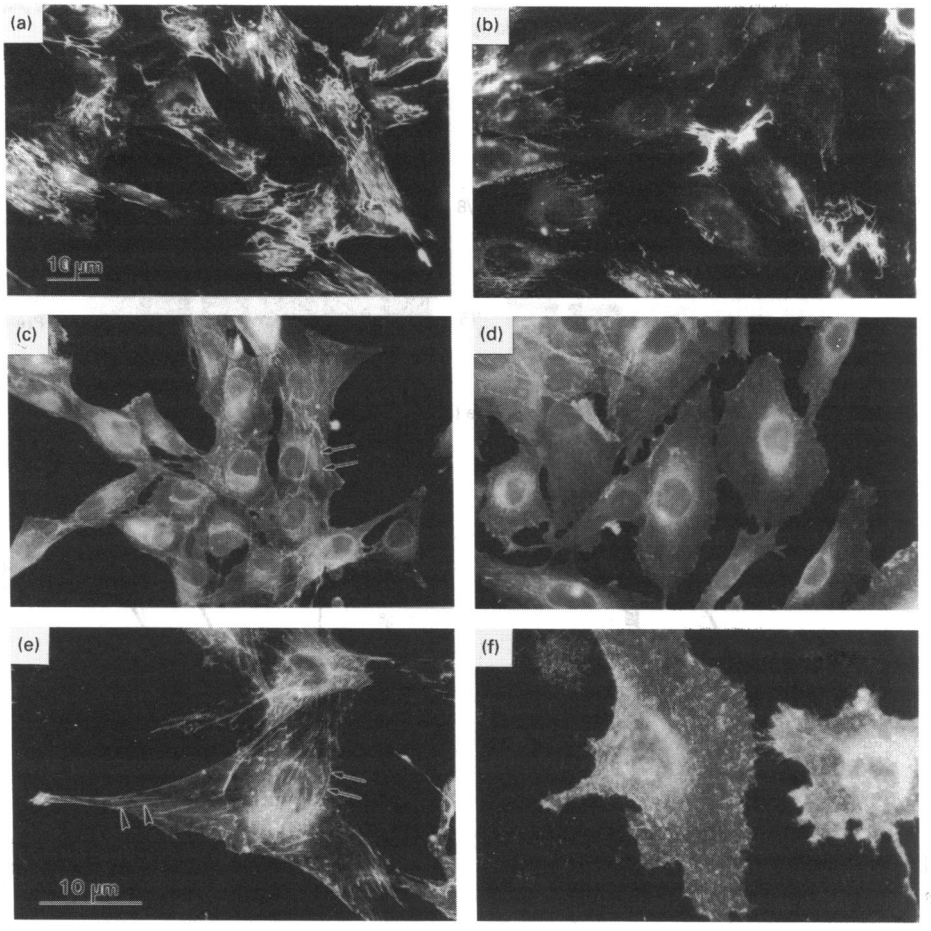


**Figure 6 Immunolocalization of fibronectin and β1-integrins**

Vector-transfected cells (**a**, **c** and **e**) and β1-integrin antisense-transfected cells (**b**, **d** and **f**) were seeded for 24 h and immunostained with anti-fibronectin antibody (**a** and **b**) or anti-(β1-integrin) antibody (**c** and **d** in the presence of serum, **e** and **f** in the absence of serum). (**a**) to (**d**) and (**e**) and (**f**) represent the same magnification respectively. Staining patterns resembling focal adhesion sites and extracellular matrix-binding sites identified by optical focusing are marked with arrowheads and arrows respectively.

**Table 2 Fibronectin matrix formation in antisense-transfected cell clones**

Fibronectin matrix formation on wild-type MG-63 cells, four vector-transfected control clones, five β1-integrin antisense-transfected cell clones with decreased intracellular pre-β1-integrin pool and two β1-integrin antisense-transfected cell clones with almost no free pre-β1-integrin subunit was analysed by use of the visual analogue scale. Amount and quality of fibronectin cables in each cell clone were estimated on a scale ranging from 0 to 10.

| | Number of clones | Amount of fibronectin | Range |
|---|---|---|---|
| Wild-type | 1 | 6.2 | |
| Vector controls | 4 | 5.8±1.0* | 4.6–7.2 |
| Antisense-transfected (pre-β1 pool reduced) | 5 | 4.7±0.6* | 4.0–5.8 |
| Antisense-transfected (pre-β1 pool missing) | 2 | 3.3 | 3.2–3.5 |

* Means ± S.D.

with β1-integrin antisense RNA (Figure 5). This result was consistent with those obtained with other techniques: i.e. immunoprecipitation of cells metabolically steady-state labelled with [$^{35}$S]methionine and by Western blotting (Figure 5).

### Reduction of the β1-integrin precursor pool may be associated with reduced cellular ability to bind fibronectin

To analyse the distribution of β1-integrins and fibronectin on the cell surface, clV/8 and clB1/15 cells were immunostained with the corresponding antibodies (Figure 6). In the control cells, anti-fibronectin antibodies decorated a network of fibronectin cables on the cell surface. In contrast, in the clB1/15 cells, fibronectin was found to be almost absent from the cell surface; only some unorganized streak-like patterns were seen, often at the cell–cell boundaries. β1-integrin receptors on control cells were localized in structures resembling focal adhesion and extracellular matrix contact sites identified by optical focusing according to the morphological criteria given in Singer et al. (1988). In the clB1/15 cells, the β1-integrins were localized diffusely along the cell surface showing some accumulation on cell-membrane projections.

No clear changes in growth rate between the clB1/15 cells and the control cells could be detected (results not shown). Each clone also adhered and spread on collagen, fibronectin and laminin (results not shown). Neither was the capacity for fibronectin synthesis decreased when compared with the control cells (results not shown). Thus the only changes observed were in β1-integrin maturation, localization and function.

To study further the possibility that the reduced size of the pre-β1-integrin pool could be associated with the decreased ability to bind fibronectin, we made and analysed 71 new β1-integrin antisense clones and 22 new vector-transfected clones. Of the antisense-transfected clones, we found nine representing reduced pre-β1-integrin expression, but only one (clB1/96) in which the pre-β1-integrin pool had almost completely disappeared (Figure 1b). Fibronectin fibril formation on wild-type MG-63 control cells, four vector-transfected control clones, five antisense clones with decreased free pre-β1-integrin subunit and two antisense clones (clB1/15 and new clB1/96) with hardly any free pre-β1-integrin subunit was studied using the visual analogue scale. The antisense clones with decreased pre-β1-integrin pool were found to bind, on average, less fibronectin and to have shorter fibronectin cables than the wild-type and vector-transfected MG-63 control cells (Table 2). The two clones with almost no pre-β1-integrin also showed the weakest binding of fibronectin on the cell surface. Thus the data suggest an association between these two phenomena.

## DISCUSSION

Regulation of the synthesis of multisubunit membrane protein complexes frequently follows the same model: one or several of the subunits is produced in excess compared with the others. The excess subunit pool stays in the ER and does not mature until the complex has formed. The cell-surface expression of the complex is therefore regulated by the availability of the rate-limiting subunits. The maturation of β1-integrin heterodimers is believed to be regulated in a similar manner (Heino et al., 1989). Many cell types have a large intracellular pre-β1-integrin pool in their ER (Heino et al., 1989; De Strooper et al., 1991), and part of this pool is degraded intracellularly instead of forming a complex with an α-chain. Furthermore, co-precipitation experiments have indicated that the precursor proteins already form αβ-heterodimers, probably in the ER (Heino et al., 1989). Finally, in different cell types and after growth-factor stimulation, the maturation rate of the pre-β1-integrin pool seems to correlate with the pre-α/pre-β ratio (Heino et al., 1989).

Here, we wanted specifically to down-regulate the synthesis of the β1-integrin subunit to investigate whether the relative absence of the β1-integrin precursor pool directly controls the maturation rates of integrin subunits. Furthermore, it was possible to study whether reduction of the pre-β1-integrin pool correlates with changes in integrin distribution and function as it does in certain transformed phenotypes (Akiyama et al., 1990). The approach chosen was to construct a plasmid expressing β1-integrin antisense RNA and to introduce it into cells to obtain stable cell lines. After screening several subclones, we were able to find some with reduced β1-integrin chain synthesis. These clones expressed β1-integrin RNA species that were presumed to represent antisense products responsible for the reduced pre-β1-integrin synthesis. The presence of the two smaller transcripts could be explained by the fact that the β1-integrin cDNA fragment used contains an extra signal sequence for polyadenylation when expressed in antisense orientation.

Previous studies have suggested several mechanisms for antisense RNA action, including inhibition of mRNA splicing, mRNA transport and translation. An antisense RNA molecule can also affect the stability of the targeted mRNA molecule [for reviews see Inoye (1988) and Krystal (1992)]. In this study, antisense transfection did not decrease the β1-integrin mRNA level, suggesting that mRNA stability was not affected. The exact molecular mechanism by which the antisense transfection decreased β1-integrin translation was not studied.

In some of the cell clones selected, β1-integrin synthesis was reduced, leading to a decline in the intracellular pre-β1-integrin pool. In these circumstances, pre-β1-chain stayed for noticeably shorter and pre-α-chains for longer times in the precursor form (and probably in the ER) than normal after translation. Thus the results strongly support the idea that a negative correlation exists between the amount of β1 precursor and the maturation rate of the β1-integrin chain. This kind of model has been suggested previously (Heino et al., 1989). Interestingly, the excess of pre-β1-integrin chain seems to be essential for the maximum speed of α-subunit maturation. Furthermore, the excess of pre-β1-chain might guarantee that all pre-α-subunits can form a heterodimer with the pre-β1-subunit. An interesting hypothesis is that under conditions in which only a limited number of pre-β1-chains are available, the α-subunit with the strongest affinity for the pre-β1-

chain mainly forms the complex. Our experimental data showing equally changed maturation times for pre-α3- and pre-α5-subunits do not support this idea.

In the present study, we did not observe any changes in the β1-integrin surface expression in transfected cells, as measured by surface labelling and direct measurement of cell-surface β1-integrins. This fits well with the observations (Akiyama et al., 1990) that the intracellular β1-integrin precursor pool is not involved in the regulation of integrin cell-surface expression. In fact, most of the precursor β1-integrin molecules are degraded intracellularly (Roberts et al., 1988; De Strooper et al., 1991). On several occasions, expression of β1-integrins on the cell surface remained unaltered whether the receptor function was activated or inactivated (see Hynes, 1992).

In transformed cell lines, a significant decrease in the β1-integrin precursor pool has been described (Akiyama et al., 1990). Analogously to observations made in the present study, this reduction occurred concomitantly with the accelerated maturation rate of the β1 precursor molecules. In transformed cell lines, cell-surface expression of β1-integrins was comparable with that in control cell lines even though integrin localization was altered (Akiyama et al., 1990). We therefore analysed the surface localization of β1-integrins in our cell clones expressing β1-integrin antisense RNA. We did not detect focal contact-like structures; instead receptors were diffusely distributed along the cell surface. This closely resembles the situation seen in transformed cell lines (Akiyama et al., 1990). These findings suggest that the size of the pre-β1-integrin pool, the speed of maturation of integrin subunits and the receptor distribution on the cell surface may be mutually regulated.

A typical feature of transformed cell lines is that they do not assemble fibronectin matrix, even though they synthesize fibronectin [for reviews see Ruoslahti and Giancotti (1989) and Vaheri et al. (1989)]. The presence of the α5β1 heterodimer on the cell surface is proposed to be the prerequisite for fibronectin deposition (Fogerty et al., 1990; Akiyama et al., 1991). However, many transformed cell lines seem to express normal amounts of β1-integrins on the cell surface, suggesting that the number of receptors is sufficient for fibronectin binding. Interestingly, in the present study, β1-integrin antisense-transfected cell clones, in which the pre-β1-integrin pool was greatly reduced, were unable to assemble fibronectin on the cell surface, even though they were able to synthesize it and attach to it when it served as a stabilized ligand on the cell-culture plate. It is possible that the diffuse distribution of β1-integrins in these cells prevents fibronectin fibril formation (Ruoslahti and Giancotti, 1989). We would like cautiously to hypothesize that, in transformed cell lines, the altered maturation process of β1-integrins can be correlated with inappropriate surface location and the inability of cells to deposit fibronectin.

There are a number of possibilities of how receptors can be directed to an unfavourable surface location or how their function may be altered. The number and structure of N-linked oligosaccharides is one regulator of integrin function (Akiyama et al., 1989). Furthermore, changes in integrin glycosylation are commonly seen after transformation of cells (Van de Water et al., 1988; Dedhar and Saulnier, 1990). It is possible that by altering the length of time for which the integrin precursors are exposed to enzymes that modify their oligosaccharides in the ER, their glycoconjugates can also be altered. However, in the present study we were unable to find any signs, such as altered electrophoretic mobility of integrins, to support this hypothesis.

Integrin function seems to be sensitive to conformational changes. Indeed, several recent observations support the idea that integrins can be in both an 'inactive' and 'activated' state. The activation or inactivation of an integrin heterodimer can be induced by external factors including synthetic peptides (Du et al., 1991) and cations (Grzesiak et al., 1992) or by phosphorylation (Tapley et al., 1989; Haimovich et al., 1991). However, often the molecular basis of integrin activation has remained obscure (Hynes, 1992), suggesting the existence of unknown regulatory mechanisms.

To conclude, we have established permanent cell clones with reduced β1-integrin synthesis caused by specific antisense RNA. This approach has confirmed our previous model of integrin maturation. The results also suggest the putative importance of the intracellular pre-β1-integrin pool for normal integrin maturation and subsequent function.

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