# Identification of β1C-2, a novel variant of the integrin β1 subunit generated by utilization of an alternative splice acceptor site in exon C

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A new splice variant of the human integrin subunit β1 has been identified and designated β1C-2. It differs from the previously reported β1C (in this report designated β1C-1) by 18 nucleotides, and is generated by splicing from exon 6 to an alternative splice acceptor site within exon C, causing an in-frame deletion of six amino acids of the cytoplasmic region of β1C-1. The β1C-2 mRNA is present in several human cell lines and tissues at low levels, similarly to β1C-1. In peripheral T-lymphocytes, β1C-2 is the selectively expressed isoform. Neither β1C-1 nor β1C-2 mRNA could be detected in mouse tissues, and Southern hybridization of a mouse genomic β1 clone with a human exon-C-specific probe failed to identify a corresponding mouse exon. The antisense orientation of exon C is highly homologous to an Alu element. Since Alu elements are restricted to primates, the β1C-1 and β1C-2 variants of the integrin subunit β1 are specific for these species. The protein coded for by the β1C-2 cDNA can be expressed and localized to the surface of β1 deficient mouse cells. However, while stable transformed clones expressing high levels of the β1A were commonly found, the β1C-1 and β1C-2 expressing clones expressed barely detectable amounts of the β1 protein. Hence, high levels of β1C-2 may be incompatible with cell proliferation, as previously suggested for β1C-1.

## INTRODUCTION

The integrin family of transmembrane cell surface receptors mediates adhesive contacts of cells with the extracellular matrix and in some instances with other cells [1]. The interactions between integrins and extracellular ligands trigger organization of the cytoskeleton, which in turn regulates various biochemical responses within the cell. Integrins can establish connections with actin filaments through bridging proteins present in focal contacts including talin, vinculin, tensin and α-actinin. In addition, activation of integrins can lead to activation of the $Na^+/H^+$ antiporter resulting in elevated intracellular pH, increased intracellular $Ca^{2+}$, altered pattern of tyrosine phosphorylation and altered gene expression [2,3]. The cytoplasmic domains of integrins are necessary for initiation of these signalling events and it has been demonstrated that integrins are able to activate several signal transduction pathways. A second functional role of the intracellular domains is to regulate the extracellular ligand binding activity of the receptor. Interactions with intracellular protein(s) or chemical modification of the cytoplasmic integrin tails are thought to control the conformation of the extracellular domain [2,3].

Integrins are heterodimeric proteins composed of α and β subunits. At present, 16 α and eight β subunits have been identified, which can combine to form at least 22 different receptors. There is considerable overlap in the ligand binding specificities among the known integrins but specific cellular responses may still be generated, since each integrin has unique cytoplasmic parts [1]. The potential for generating or receiving different signals within the cell is further increased by the occurrence of splice variants of these proteins. Alternative intracellular domains, resulting from the processing of the pre-mRNA, have been described for the subunits α3 [4], α6 [4,5], α7 [6], β1 [7–10], β3 [11] and β4 [12]. The β1 subunit is of particular interest since it is a constituent of ten different integrins and is expressed in all mammalian cell types tested, except for mature erythrocytes. Four alternatively spliced variants of the cytoplasmic domain have been reported and designated β1A, β1B [7], β1C [8] (designated β1C-1 in the present paper) and β1D [9,10]. They are all derived by alternative splicing from exactly the same position in the cytoplasmic tail, whereas the extracellular, the transmembrane and the membrane proximal 26 amino acids are common to all four isoforms.

The β1A isoform is widely expressed and the cytoplasmic domain is fully conserved at the amino-acid level between all mammalian species tested, chicken and *Xenopus* [13]. The β1B subunit has been found mainly in keratinocytes and hepatocytes [7,14]. When expressed in cultured cells, β1B integrins have been reported not to localize to focal contacts [14] and appear to be unable to bind extracellular ligands (A. Armulik, G. Svineng, K. Wennerberg, R. Fässler and S. Johansson, unpublished work). The β1B subunit exhibits a dominant negative effect on β1A-containing integrins by unknown mechanisms [15]. The β1D is limited to striated muscles, where it is found in large amounts [9,10]. In mature myotubes of heart and skeletal muscle, β1D is the only detectable form of β1 [16]. The β1C-1 protein has been detected at low levels in human erythroleukaemia (HEL) cells [8], in epithelial cells [17], and in injured liver and tumour necrosis factor (TNF) α stimulated human umbilical vein endothelial cells (HUVEC) [18]. In addition, transcripts of β1C-1 have been identified in placenta, normal platelets and several blood cell lines [8]. Over-expression of β1C-1 in mouse fibroblasts and in Chinese-hamster ovary (CHO) cells led to inhibition of DNA synthesis and decreased proliferation of the cells [18,19].

In the present report we describe a new splice variant of the integrin β1 subunit, named β1C-2, that is closely related to β1C-1. The expression of β1C-2 in several human cell lines and tissues is compared with that of β1C-1. The physiological relevance of the two related integrin subunits is discussed in the light of their similarity with Alu elements.

Abbreviations used: TNF, tumour necrosis factor; HUVEC, human umbilical vein endothelial cells; RT-PCR, reverse-transcriptase PCR; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; SR-proteins, serine-/arginine-rich proteins; WGA, wheat-germ agglutinin.

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## EXPERIMENTAL

### Total RNA from cell lines and tissues

Total RNA was isolated using the RNeasy Total RNA Kit (Qiagen Inc., Chatsworth, CA, U.S.A.) or the guanidine thiocyanate method [20] from the following human cell lines: HL-60 (promyelocyte-derived from acute promyelocytic leukaemia), U-937 (monocyte-like cell derived from histocytic lymphoma), THP-1 (monocyte derived from acute monocytic leukaemia), Monomac-6 (monocyte), KU812 (basophil), K-562 (erythroleukaemia cell derived from chronic myelogenous leukaemia), BJAB (B-lymphocyte), Jurkat (T-lymphocyte derived from acute T-cell leukaemia), Eol-1 (eosinophil), LAMA-84 (basophil/megakaryocyte), HMC-1 (mast cell), HeLa (epithelial-like cell from epithelioid carcinoma), AG1518 (fibroblast), 293 (embryonal kidney) and HUVEC stimulated with TNF$\alpha$ (100 units/ml for 48 h). Total RNA was isolated also from human peripheral T-lymphocytes, monocytes, B-cells from a patient with chronic lymphocyte leukaemia, whole liver and kidney, and from mouse liver and testis. In addition, a cDNA library made from human embryonic myoblast RNA was used in the RT-PCR experiments.

### Isolation of primary T-lymphocytes

Primary human T-lymphocytes were isolated by T-cell rosetting and density gradient centrifugation. Briefly, peripheral blood lymphocytes were separated from other blood cells by centrifugation through Ficoll, washed in 1 × PBS, and mixed with 2-aminoethylisothiouronium bromide-activated sheep erythrocytes. The sheep erythrocyte-bound T-cells were then separated from macrophages and B-cells by a second centrifugation through Ficoll. Finally, the sheep erythrocytes were removed from the T-lymphocytes by incubation in pure water for 1 min. FACS analysis showed that the preparation contained 95% CD3-positive cells, where 35% were CD4-positive and 50% CD8 positive.

### Oligonucleotides

The following oligonucleotides were used as primers in PCR: GS1 (5′-GAAAGACACGTGCACACAGGA), GS3 (5′-ATTACAGGGTGAAAATCCTAT), GS5 (5′-CATACTTCGGATTGACCACA), GS6 (5′-TCTCTCTCTGTCGCCCAG), GS8 (5′-ATTTTCACCCTGTAATCCCAGGTACTC), GS9 (5′-TTCATGACAGAAGGGAGTTTGCTAA), GS21 (5′-GCTCTAGATCACGGCACTCTTATAAATAGG) and MB1-1 (5′-ATGCTGCTGCTGTGAGCT) (Figure 1A). The GS4 (5′-GCCAACAAATCTAACATTC) primer is localized to the pBluescript region of the pBS$\beta$1A vector (see below) downstream of the $\beta$1 cDNA insert. Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were sense 5′-ACCACAGTCCATGCCATCAC and antisense 5′-TCCACCACCCTGTTGCTGTA (Clontech Laboratories, Inc., CA, U.S.A.). The oligonucleotides were synthesized on a Gene Assembler Plus and purified by the OligoPrep Kit (Pharmacia Biotech, Uppsala, Sweden), or purchased from Pharmacia.

### Reverse transcriptase (RT) and PCR

Total RNA was precipitated and dissolved in RNase-free water and the cDNA was synthesized using the RNA PCR kit (Perkin–Elmer, Norwalk, CT, U.S.A.). The RT reaction was performed in 10 $\mu$l containing 5 mM $MgCl_2$, 1 × reaction buffer, 1 mM of each of the dNTPs, 1 unit/$\mu$l of RNase inhibitor, 2.5 units/$\mu$l of MuLV RT, 2.5 $\mu$M of random hexamer or 0.75 $\mu$M of downstream primer and 0.5 $\mu$g of total RNA. Synthesis of the cDNA was performed at 42 °C for 20 min and was terminated by incubation at 95 °C for 5 min. The PCR reaction following the cDNA synthesis was performed in a Thermocycler (MJ Research Inc., Watertown, MA, U.S.A.) equipped with a hot lid, after the addition of $MgCl_2$ to a final concentration of 2.5 mM, 1 × reaction buffer and 1.25 units of AmpliTaq (Perkin–Elmer) in a total volume of 50 $\mu$l.

### DNA sequencing

All plasmids subjected to sequencing were isolated by the EasyPrep™ Plasmid Prep kit (Pharmacia) or by QIAGEN-tip100 (Qiagen). Sequencing reactions were performed using the Cy-5 AutoRead™ sequencing kit (Pharmacia) and the electrophoresis and subsequent analysis of the samples were performed on an ALF*express*™ (Pharmacia). All sequences were further analysed using the DNAStar software (DNASTAR Inc., Madison, WI, U.S.A.).

### Screening for $\beta$1A, $\beta$1C-1 and $\beta$1C-2 transcripts by RT-PCR

RT-PCR was performed by using a random hexamer or a gene specific (GS5) primer in the cDNA synthesis, and GS1 and GS5 in the first PCR to obtain a mix of $\beta$1A, $\beta$1C-1 and $\beta$1C-2 PCR-products (Figure 1A). After purification of the PCR products on MicroSpin columns (Pharmacia), 1 $\mu$l of the total 50 $\mu$l was used as a template in a second nested PCR using one primer (GS9) located within exon 6 and the downstream primer (GS8) spanning the border of exon C and exon 7 (Figure 1A). This enabled the amplification of both $\beta$1C-1 and $\beta$1C-2 transcripts in the same reaction, and the location of the primers in different exons ensured that the PCR products of the expected sizes were amplification of mature mRNA and not contaminating genomic DNA. All pipetting was done using tips with aerosol filters, and controls without template were included at all stages to check for cross-contamination between the PCR reactions. The PCR products were 186 bp for $\beta$1C-1 and 168 bp for $\beta$1C-2, and were separated by electrophoresis on a 4% NuSieve gel (FMC BioProducts, Rockland, ME, U.S.A.) together with the 1 Kb DNA Ladder (Gibco-BRL) and were visualized with ethidium bromide.

### Southern-blot analysis of a mouse $\beta$1 genomic cosmid clone

Two probes were made, one specific for exon C and one specific for exon 7. The exon-C-specific probe was made by [$\alpha$-$^{32}$P]dCTP-labelling of an isolated GS6–GS8 PCR product, using the Klenow-enzyme (Boehringer-Mannheim GmbH, Mannheim, Germany) together with the primers GS6 and GS8 (Figure 1A). The exon-7-specific probe was made in the same way using GS3 and MB1-1 as primers and a GS3-MB1-1 PCR product as template (Figure 1A). Cosmid DNA (2.4 $\mu$g) containing the mouse integrin $\beta$1 gene was digested with *Hin*dIII (Figure 4, lane 1) and *Bam*HI (Figure 4, lane 2) (Promega, Madison, WI, U.S.A.) respectively. This integrin $\beta$1 cosmid clone contains exons 2–5 [21], exon 6 (results not shown) and exon 7 (Figure 4C). As a positive control, the pBS$\beta$1C-1 plasmid (see below) containing the human $\beta$1C-1 cDNA was digested with *Eco*RV and *Csp*45I (Promega) and loaded on to the gel in different amounts; 30 ng, 110 ng and 280 ng (Figure 4, lanes 3, 4 and 5). *Eco*RV cuts both within the C-specific region and in the vector, and *Csp*45I cuts downstream of exon 7. This gives three fragments of approx. 4500 bp, 3200 bp and 1400 bp respectively. Both the 3200 bp fragment and the 1400 bp band contain sequences complementary to the exon C probe, whereas the exon 7 probe is only complementary to the

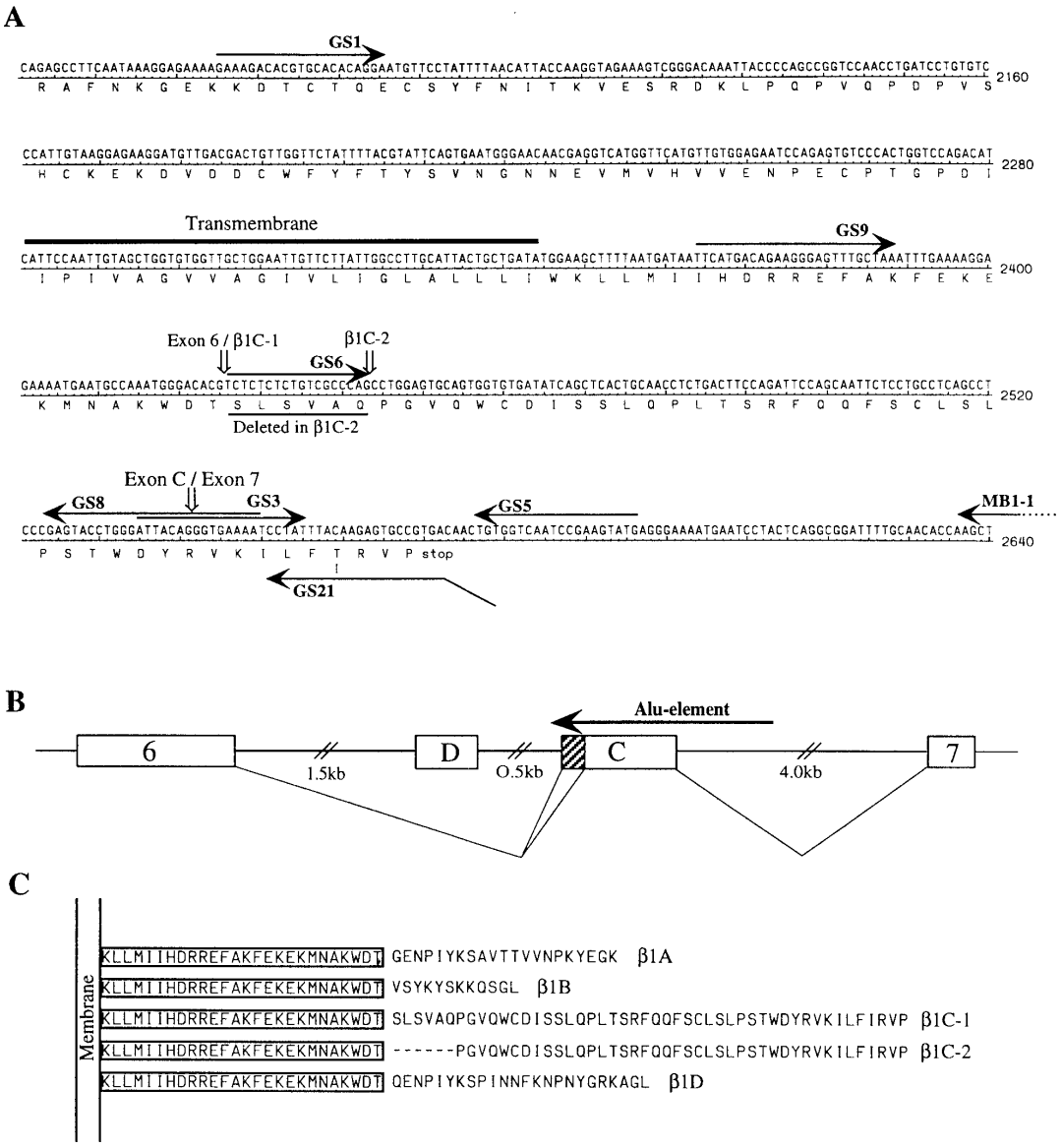


**Figure 1 Partial cDNA and amino acid sequence of $\beta$1C-1 and $\beta$1C-2, the genomic organization, and amino-acid sequence of the $\beta$1 splice variants**

(**A**) Partial cDNA and amino-acid sequence of $\beta$1C-1 and $\beta$1C-2. The exon–exon borders are indicated with vertical arrows. The 18 nucleotides that are excluded in $\beta$1C-2 are underlined. The predicted transmembrane region is indicated with a solid line. The horizontal arrows indicate the primers used in PCR (see the Experimental section). (**B**) Schematic representation of the genomic sequence covering exon 6, exon D, exon C and exon 7. Exon sequences are shown as boxes and introns as solid lines, and the approximate length of the introns is indicated. The splicing pattern used to generate $\beta$1C-1 or, alternatively, $\beta$1C-2 transcripts are depicted, and the position and orientation of the Alu element is indicated with a horizontal arrow. (**C**) The predicted amino-acid sequences of the cytoplasmic tails of the different splice variants of integrin $\beta$1. The common membrane-proximal regions are boxed, and the six amino acids deleted in $\beta$1C-2 compared with $\beta$1C-1 are indicated with dashes.

1400 bp band. The digested DNA, together with the 1 Kb DNA ladder (Gibco-BRL), was separated on a 0.7% (w/v) agarose gel and transferred to a Hybond$^{TM}$-N$^{+}$ (Amersham International, Little Chalfont, Bucks., U.K.) nylon membrane by vacuum blotting in 0.4 M NaOH using blotting apparatus from Pharmacia. After blotting, the membrane was washed in 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate) and subjected to UV cross-linking. Prehybridization was done in 6 × SSC, 0.5% (w/v) SDS, 3 × Denhart's containing 100 $\mu$g/ml of denatured salmon sperm DNA at 42 °C for 3 h. The exon C probe was denatured by heating to 95 °C for 3 min, and was then added to 2 × SSC/0.5% (w/v) SDS and hybridized to the filter overnight at 42 °C. The membrane was washed at 42 °C in 2 × SSC/0.5% (w/v) SDS, exposed to a GS-525 Molecular Imager screen (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for 1 h, and analysed using the Molecular Analyst Software (Bio-Rad). The membrane was stripped by pouring boiling 0.5% (w/v) SDS on to the membrane. Complete removal of the probe was verified by re-exposure of the membrane to the screen. After another pre-hybridization step, the membrane was hybridized to the denatured exon-7 probe in 2 × SSC, 0.5% (w/v) SDS at 60 °C overnight. The membrane was washed in 0.1 × SSC/0.1% (w/v) SDS at 60 °C, exposed for 1 h and analysed using the phosphorimager.

### Constructs containing the integrin β1A, β1C-1 and β1C-2 cDNAs

The construction of the expression vector pBSβ1A has been described in detail [22]. Briefly, the vector contains the complete cDNA of mouse integrin β1A and a puromycin-resistance cassette. Both genes are driven by a phosphoglycerate kinase promoter. The construct contains a unique *Xba*I site located between the two cassettes, enabling linearization of the plasmid before electroporation into mammalian cells. An ampicillin-resistance gene makes it possible to select for the plasmid when grown in bacteria.

A PCR product containing the unique β1C-2 region was introduced into a *Sma*I-digested pUC19 vector by standard blunt-end cloning [23]. In order to introduce the β1C-2 region into the pBSβ1A vector [22] we employed a modified spliced-overlap-extension PCR method (SOE-PCR) [24]. Briefly, the pUC19-vector containing the GS1–GS8 PCR product of β1C-2 was used as template in PCR, where the downstream GS8 primer was phosphorylated and the upstream GS1 primer was not (Figure 1A). This PCR product (GS1–PGS8) was then treated with a λ-exonuclease enzyme (Pharmacia) that selectively digests the 5′-phosphorylated strand of double-stranded DNA, leaving the unphosphorylated strand intact. Using the pBSβ1A vector as template and the primers GS4 and phosphorylated GS3, a PCR-product of 1453 bp was obtained. The GS4 primer is located in the vector sequence at position 3967–3987 and GS3 is complementary to 16 bp of the GS8 primer (Figure 1A). After removal of the 5′ phosphorylated strand, the two single-stranded DNAs (GS1–GS8 and GS3–GS4) were annealed to each other at the 16 bp overlap and subsequent extension and PCR with the GS1 and GS4 primers gave the final β1C-2-containing product of 1824 bp. Both the 1824 bp PCR product and the pBSβ1A vector were digested with *Eco*72I and *Csp*45I (Promega), purified with the Wizard DNA Purification Kit (Promega), ligated and electroporated into *Escherichia coli* XL-1 Blue. Positive clones were identified by restriction enzyme mapping and sequencing of the PCR product and all regions involved in the cloning verified that the sequence was as expected. The same procedure was used to generate the pBSβ1C-1 vector. For transient expression of β1A and β1C-2, the cDNAs were transferred from the pBSβ1A and pBSβ1C-2 vectors, respectively, to the pUHD10-3 vector [25] by blunt-end cloning of an *Acc*III/*Sca*I (Promega) fragment containing the complete β1 cDNA into an *Eco*RI (Promega)-digested pUHD10-3 vector after treatment with T4-DNA polymerase (Promega). Positive clones were identified by restriction enzyme mapping and sequencing was used to verify that they had the correct sequence. These expression vectors were named pTetβ1A and pTetβ1C-2 respectively. The pTetβ1C-2 was further modified by replacing the *Eco*72I–*Xba*I region with a shorter fragment generated by PCR using the GS1 primer containing an *Eco*72I site and the GS21 primer containing an *Xba*I site. The GS21 primer contains a tail with an *Xba*I site and a point mutation which renders the mouse-encoded threonine to the human-encoded isoleucine (Figure 1A). Again, positive clones were identified by restriction enzyme mapping and sequencing was used to verify presence of the correct sequence.

### Cell lines and transfection

The GD25 cells are derived from a integrin-β1 deficient mouse embryonic-stem-cell clone and have been described in detail previously [21,22,26]. The GD25 cells were cultured in Dulbecco's modified Eagle's medium/10 % (v/v) fetal calf serum/2 mM L-glutamine containing 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone (Gibco-BRL). In order to generate stable clones by electroporation of the GD25 cells, the pBSβ1 constructs containing the different splice variants (β1A, β1C-1 and β1C-2) were linearized by digestion with *Xba*I (Promega), precipitated in ethanol and resuspended in water at a concentration of 1 μg/μl. GD25 cells were harvested by trypsin/EDTA treatment and washed twice with 1 × PBS. The cells were resuspended in 1 × PBS and $3 \times 10^6$ cells were mixed with 20 μg of linearized plasmid and electroporated at 2.0 kV and 0.9 μF. After electroporation, the cells were kept on ice for 10 min, resuspended in 20 ml of medium and plated on to two 96-well plates (100 μl/well). After three days, selection was begun by addition of puromycin to a final concentration of 5 μg/ml. Surviving clones were chosen after 1–3 weeks and further cultured in medium containing 10 μg/ml of puromycin. The β1 expression levels of the individual clones were determined by FACS analysis (see below). For transient expression, the constructs pTetβ1A and pTetβ1C-2 were introduced into GD25 cells stably expressing the Tet repressor (GD25T cells) [25]. The GD25T cell line was generated by transfection of the GD25 cells with the Tet repressor-encoding vector, pUHD15-1, containing a hygromycin cassette. Clones resistant to hygromycin (200 μg/ml) were isolated and the clone expressing the highest levels of the Tet repressor was used in further experiments. For transient transfection, 17 μg of pTetβ1A and pTetβ1C-2, respectively, were mixed with 100 μl of Superfect transfection reagent (Qiagen) and added to 70 % confluent 125-cm² cell-culture flasks (Falcon) of GD25T cells. After transfection, the cells were maintained in Dulbecco's modified Eagle's medium/10 % (v/v) fetal calf serum/2 mM L-glutamine containing 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone (Gibco-BRL) for 48 h before analysis by immunoblotting (see below).

### FACS analysis

The expression levels of the β1 proteins on the surface of the isolated GD25-β1 clones were determined by FACS analysis. The stably-transfected β1 clones were harvested by trypsin/EDTA treatment and resuspended in 1 × PBS containing 10 % (v/v) goat serum for 30 min at 4 °C. A total of $1 \times 10^6$ cells were incubated for 20 min, on ice, with primary antibody (polyclonal rabbit anti-β1) [27] diluted 1:100 in 1 × PBS containing 2 % (v/v) goat serum and 0.001 % $NaN_3$ (FACS-PBS). After being washed twice with ice-cold FACS-PBS, the cells were incubated, on ice, for 20 min with secondary antibody (fluorescein-labelled goat anti-rabbit IgG) (Jackson Immuno Research, West Grove, PA, U.S.A.) diluted 1:20 in FACS-PBS. The cells were then washed twice and resuspended in 500 μl of ice-cold FACS-PBS. The cells (5000 cells/sample) were analysed at 488 nm in a FACScan (Becton Dickinson Co., Mountain View, CA, U.S.A.) equipped with a 5-W argon laser.

### Immunoblotting of integrins

Cells were washed three times with 1 × PBS and solubilized with 1 % (v/v) Triton X-100/10 mM Tris/HCl, pH 7.4, containing protease inhibitors (PI) (2 mM EDTA/2 mM PMSF/2 mM N-ethylmaleimide containing 1 μg/ml of pepstatin A). After centrifugation at 20000 ***g*** for 30 min at 4 °C, the cleared cell lysate was incubated with 100 μl of a 50 % (w/v) slurry of wheat-germ agglutinin (WGA)–Sepharose for 1 h end-over-end, at 4 °C. The WGA–Sepharose was washed three times with TBS (Tris-buffered saline, pH 7.4) containing protease inhibitors (as above) and 0.1 % (v/v) Triton X-100, and the bound material was released from the WGA–Sepharose by boiling for 3 min in sample buffer containing 4 % (w/v) SDS under non-reducing conditions. The WGA bound material was resolved by SDS/PAGE (7 % gel) and transferred to a cellulose nitrate filter

(Schleicher and Schuell, Germany). The filter was blocked in 5% non-fat milk in TBS/0.1% (v/v) Tween 20 (TBS-T) and subsequently incubated with primary antibody (polyclonal rabbit anti-$\beta$1) [27] diluted 1:200 in TBS-T. Protein detection was done using a horseradish peroxidase-conjugated anti-rabbit antibody (Amersham) diluted 1:5000 in TBS-T followed by the enhanced chemiluminescence (ECL) detection system (Amersham). Prestained SDS/PAGE standards (Bio-Rad) with molecular masses of 197, 117, 89, and 52 kDa were used as markers.

## RESULTS

### Identification of a novel splice variant of integrin $\beta$1

During our cloning of the $\beta$1C-1 splice variant of integrin $\beta$1 by RT-PCR from human HL-60 cells, we identified a new splice variant differing from the known $\beta$1C-1 by 18 nucleotides. The new splice variant of $\beta$1, named $\beta$1C-2, is generated by alternative splicing from exon 6 into exon C, 18 nucleotides downstream of the splice-acceptor site used for $\beta$1C-1 (Figure 1). This alternative splicing does not change the reading frame of the transcript compared with $\beta$1C-1, thus $\beta$1C-2 differs from $\beta$1C-1 by only six amino acids (SLSVAQ). This novel splice product implies that the $\beta$1C-1-specific sequence contains the splice-acceptor site used for $\beta$1C-2 (Figures 1 and 2). A consensus acceptor site in higher eukaryotes is composed of a branch point, a pyrimidine stretch followed by an N, a pyrimidine and the almost invariant AG dinucleotide (Figure 2A). A consensus branch-point sequence (YNCTRAY) [28] is located 84 bp and 102 bp upstream of $\beta$1C-1 and $\beta$1C-2 respectively (Figure 2B), and is probably used for splicing of both variants. Further, the $\beta$1C-2 splice-acceptor site consists of a pyrimidine stretch which is interrupted by two Gs, and an AG dinucleotide at the end. The first position of the downstream exon is usually a G or an A, whereas it is a C in $\beta$1C-2 (Figure 2A). The presence of the hallmarks of a splice-acceptor site indicates that the splicing, resulting in the $\beta$1C-2 mRNA, is possible.

### Distribution of $\beta$1C-2 transcript in different cell lines and tissues

The discovery of the new splice variant $\beta$1C-2, led us to search for cell types where this splicing event takes place. For this purpose we used RT-PCR with total RNA from different human cell lines and tissues as templates. Since it has been reported that $\beta$1C-1 is present in blood cells [8], 11 different human blood cell lines, normal monocytes and T-lymphocytes, and B-cells from a chronic lymphocyte leukaemia patient were screened for the presence of transcripts of $\beta$1C-1 and $\beta$1C-2 (Figure 3). Non-

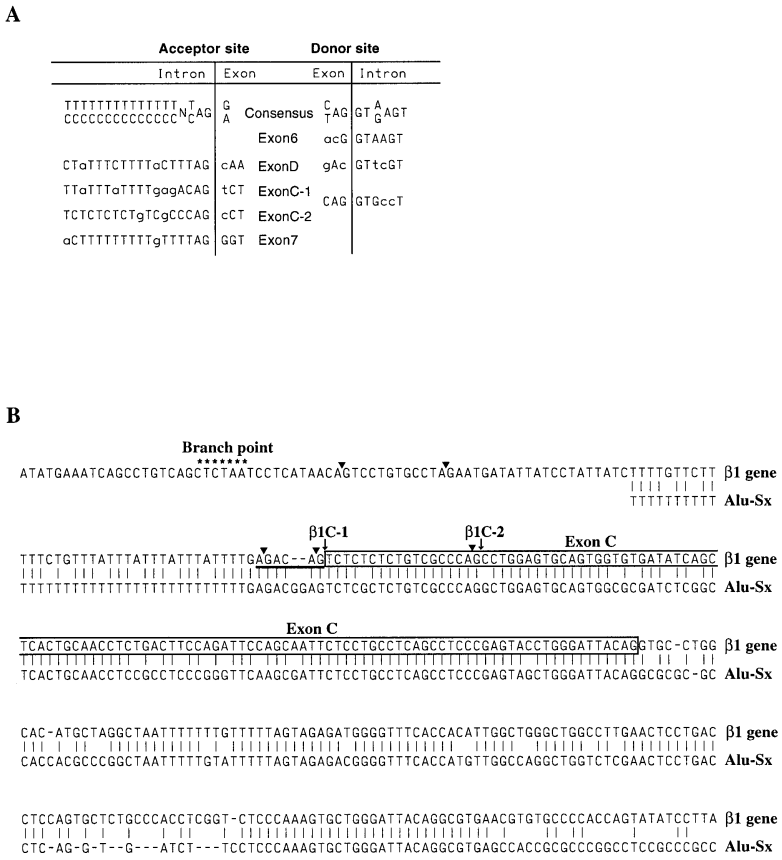


**Figure 2 Splice sites and the genomic sequence of exon C and its surrounding introns compared with the consensus Alu Sx sequence**

(**A**) A comparison of a consensus splice-donor and acceptor site for higher eukaryotes with the splice-acceptor sites of exon D, exon 7, the two alternative acceptor sites in exon C, and the splice donor sites of exon 6, exon D and exon C. The nucleotides differing from the consensus are in lower case letters. Only 14 nucleotides of the polypyrimidine tracts are included in this comparison. (**B**) Alignment of the genomic sequence of exon C and its surrounding intron sequence with the complementary Alu Sx consensus sequence. Exon C is boxed and identical nucleotides between the $\beta$1 genomic sequence and the Alu sequence are indicated with vertical bars. A consensus branch point sequence (YNCTRAY) is indicated with asterisks, and potential splice-acceptor site AG dinucleotides are indicated with arrow heads. The splice acceptor sites used to generate $\beta$1C-1 and $\beta$1C-2 are indicated with vertical arrows. A potential SR protein-binding site (GAGACAG) is underlined.

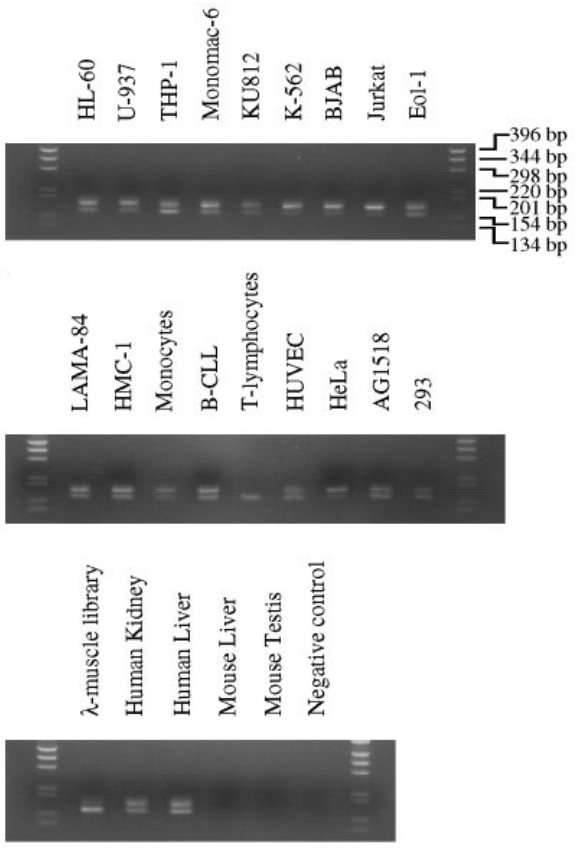


**Figure 3 Identification of $\beta$1C-1 and $\beta$1C-2 transcripts in cell lines and tissues**

Total RNA was used as template in the cDNA synthesis with a $\beta$1 specific primer (GS5) as downstream primer. The following PCR was performed with GS1 and GS5 primers in order to amplify a mixture of both $\beta$1A, $\beta$1C-1 and $\beta$1C-2 transcripts. The primers were removed and a second PCR reaction was performed with an exon 6 specific upstream primer (GS9) and a downstream primer positioned at the junction of exon C and exon 7 (GS8). The two PCR products representing $\beta$1C-1 (186 bp) and $\beta$1C-2 (168 bp), respectively, were separated on a 4% (w/v) NuSieve gel by electrophoresis and stained with ethidium bromide. The size marker is the 1 Kb DNA ladder (Gibco-BRL).

blood cell lines such as HeLa, AG1518 (fibroblast), 293 (embryonal kidney cells), HUVEC, liver and kidney tissues, and a cDNA library derived from myoblasts, were also analysed for the presence of the same transcripts (Figure 3). In all samples studied, the mRNA levels of $\beta$1A were considerably higher than the levels of both $\beta$1C-1 and $\beta$1C-2 (results not shown). Primers designed to amplify both $\beta$1C-1 and $\beta$1C-2 in the same reaction were used so that a semiquantitative comparison of the transcription levels of the two variants could be made. The upstream primer (GS9) is located in exon 6 and the GS8 primer is spanning the junction between exon C and exon 7 to ensure that the PCR product was not an amplification of contaminating genomic DNA (Figure 1A). Priming of the RT reaction with either a random hexamer or an integrin $\beta$1 specific primer (GS5) gave identical results. Comparison of the $\beta$1C-2 mRNA levels with the levels of $\beta$1C-1 showed a variation of the expression between the two variants in different samples (Figure 3). However, no correlation between cell type or between transformed versus non-transformed cells and the expression pattern of $\beta$1C-1 and $\beta$1C-2 could be identified.

In a previous report, peripheral lymphocytes were found not

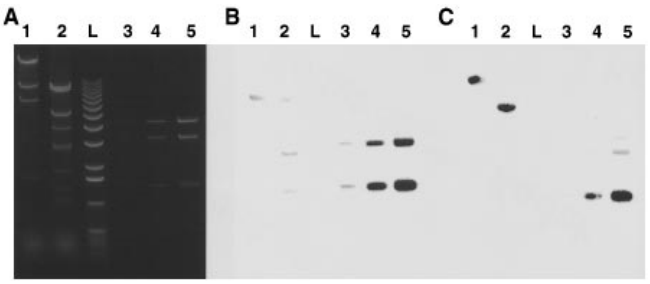


**Figure 4 Hybridization of a cosmid clone containing the mouse integrin $\beta$1 gene to probes specific for exon C and exon 7**

The cosmid clone containing the mouse $\beta$1 gene was digested with *Hin*dIII (lane 1) and *Bam*HI (lane 2). The vector pBS$\beta$1C-1 containing the human $\beta$1C-1 cDNA was digested with *Eco*RV and *Csp*45I and loaded on to a 0.7% (w/v) agarose gel in different amounts (lane 3, 30 ng; lane 4, 110 ng and lane 5, 280 ng). Cleavage using *Eco*RV and *Csp*45I gave two fragments containing $\beta$1C sequences of 38 bp (upper 3200 bp band) and 78 bp (lower 1700 bp band). The 1700 bp band also contained the exon 7 sequence. Lane L contained the 1 Kb DNA ladder (Gibco-BRL). (**A**) An agarose gel showing the DNA before blotting to the membrane. (**B**) Hybridization to an exon-C-specific probe under low stringency conditions. (**C**) Rehybridization to an exon-7-specific probe under conditions of high stringency, after removal of the exon-C probe.

to express $\beta$1C mRNA [8]. However, our study on isolated T-lymphocytes showed that the $\beta$1C-1 mRNA was barely detectable, even with nested PCR; the $\beta$1C-2 mRNA was present at normal levels in T-lymphocytes (Figure 3). Primary T-cells are, therefore, one example of selective expression of $\beta$1C-2 compared with the $\beta$1C-1 isoform. PCR products from HL60 cells, peripheral T-lymphocytes and HUVEC were cloned and sequenced to verify that the PCR products represented $\beta$1C-1 and $\beta$1C-2 mRNAs.

### Attempts to identify the exon C equivalent in mouse

Of those human cell lines and tissues tested, all expressed $\beta$1C-1 and/or $\beta$1C-2, whereas no transcripts for $\beta$1C-1 or $\beta$1C-2 could be detected by RT-PCR in either mouse testis or mouse liver RNA under the same conditions (Figure 3). Several attempts were made to find the equivalent of exon C in mouse. Different RT-PCR strategies were employed, including usage of three different degenerated primers for the C-specific region, giving no positive results (results not shown). Further, Southern-blot hybridization was performed on a genomic cosmid clone known to contain exons 2–7 of the mouse $\beta$1 gene (Figure 4) [21]. A 124-bp probe containing the 116 bp exon-C-specific part of human $\beta$1 failed to identify a C-related sequence, even at very low stringency (Figure 4B). Rehybridization of the same blot with an 119 bp exon-7-specific probe at high stringency demonstrated that the clone contained exon 7 (Figure 4C), which is located downstream of exon C in humans (Figure 1B). The fact that the low stringency conditions allowed the 124-bp exon-C probe to hybridize with the 38 bp complementary region of the upper band in the positive control plasmid (Figure 4, lanes 3, 4 and 5), further strengthens the conclusion that there is no specific hybridization to the mouse cosmid clone. Taken together, these results indicate that the C-isoforms found in humans do not exist in mice.

### Exon C is part of an antisense Alu element

As previously mentioned, exon C is homologous to a segment of an Alu element [8]. Comparison of exon C and the surrounding genomic sequence (Accession no. U33881) with an Alu database

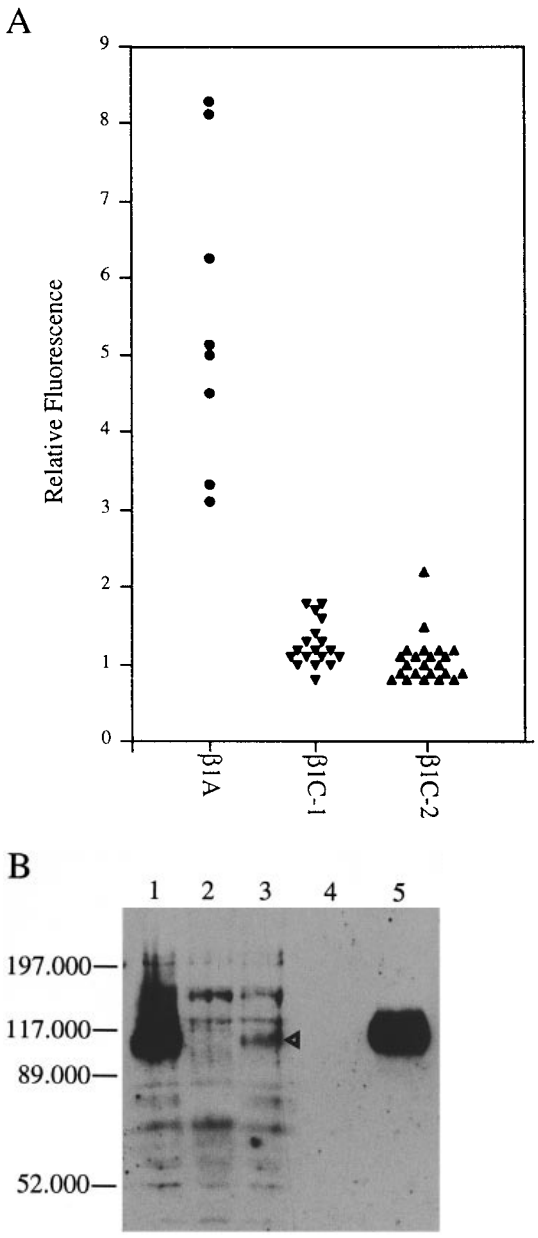


**Figure 5 Expression of β1A, β1C-1 and β1C-2 in integrin-β1-deficient cells**

(**A**) The puromycin-resistant, stable clones of the β1A, β1C-1 and β1C-2 variants were analysed for β1 expression on the surface of β1-deficient GD25 cells by FACS analysis. Each dot represents one clone, which was analysed using a polyclonal rabbit anti-β1 antibody and a secondary FITC-labelled anti-rabbit antibody. Each clone ($5 \times 10^3$ cells) was analysed and the relative fluorescence was calculated as the median fluorescence level divided by the median fluorescence level of the negative control (GD25) cells. Eight β1A, 18 β1C-1 and 23 β1C-2 clones were analysed. (**B**) GD25T cells, untransfected (lane 2) or transiently transfected with β1A (lane 1) or β1C-2 (lane 3) were lysed and the WGA-purified material was immunoblotted with a polyclonal anti-β1 antibody. Purified rat α5β1 integrin (lane 5) was included as a positive control and lane 4 was left empty. The β1C-2 band in lane 3 is indicated with an open arrow-head.

(Pythia version 2.5) [29], shows that the sequence contains a complete Alu element which belongs to the Sx subfamily [29]. The Alu element is in the antisense orientation compared with exon C, and it is the 'right' monomer [30] that is spliced into the β1C-1 and β1C-2 transcripts (Figure 2B). Using the Martinez–Needleman-Wunsch method in the Align program (DNASTAR), the homology at the nucleotide level between a consensus Alu-Sx sequence and the complete exon C-Alu element was calculated to be 81%, although it was as high as 91% within the 116 bp exon C region (Figure 2B). Several cases have been reported in which the 'right' monomer of an antisense Alu is being transcribed and alternatively spliced into mature mRNAs, such as in the human natural-resistance-associated macrophage protein [31], the α-3 subunit of a neuronal acetylcholine receptor [32], the serine/threonine kinase STK2 [33], the complement pro-C5 [34], the decay-accelerating factor [35] and in the cytoplasmic domain of an interferon receptor subunit ($IFN_1R$-2) [36]. A comparison of these similar cases with the exon C–Alu element and a consensus Alu-Sx sequence reveals considerable homology at the nucleotide level. In addition, exon C, natural-resistance-associated macrophage protein, neuronal acetylcholine receptor and decay-accelerating factor are all translated in the same reading frame, and a comparison of the predicted amino-acid sequence of the natural-resistance-associated macrophage protein and β1C-1 show that they are highly similar (81%), whereas the decay-accelerating factor and the neuronal acetylcholine receptor are less similar to β1C. Pro-C5 and STK2 are translated in a different reading frame and the amino-acid similarity between them is 77%. All calculated amino-acid comparisons are made on fragments of the same length using the Lipman–Pearson Protein Alignment method in the Align program (DNASTAR).

### Expression of integrin β1-subunits in β1 deficient cells

In order to investigate the function of β1C-2 compared with β1A and β1C-1, the splice variants were expressed in integrin β1 deficient cells (GD25 cells). Several stable clones were established for each of β1A, β1C-1 and β1C-2 and the levels of protein expression on the cell surface were determined by FACS analysis. For β1A, most clones showed relatively high expression of the β1 integrin, whereas only low expressing clones of β1C-1 and β1C-2 were obtained (Figure 5A). A total of 23 β1C-2 stable clones were analysed, and their expression levels were compared with 18 β1C-1 and 8 β1A clones. The clones were generated at separate rounds of electroporations and with different plasmid purifications, ruling out the possibility that this is a phenomenon of one single experiment. In addition, immunoblotting of WGA-purified cell lysates showed that the β1C-1 and β1C-2 clones had barely detectable levels of the β1 protein compared with the β1A clones, confirming the results of the FACS analysis. This was done using both the stable clones of pBSβ1-vectors (results not shown) and transiently-expressed pTetβ1-vectors (Figure 5B) which utilize the phosphoglycerate kinase and the cytomegalovirus promoter respectively. The levels of mRNA expression in the different stable pBSβ1 clones were investigated by RT-PCR using primers common for β1A, β1C-1 and β1C-2, together with primers for the housekeeping gene G3PDH. In samples with identical amounts of G3PDH mRNAs, the levels of β1A were significantly higher than those of both β1C-1 and β1C-2 mRNAs (results not shown). From these results, we conclude that the low level of protein on the surface of the stable clones is a result of low mRNA expression. In addition, similarly to β1C-1, high expression levels of the β1C-2 protein are not attainable by either stable or transient expression of the cDNA.

## DISCUSSION

The identification of a new splice variant of the integrin subunit β1 adds one more variant to the four isoforms reported previously (β1A, β1B, β1C-1 and β1D). Sequencing of PCR products amplified from total RNA of one established cell line (HL60), one primary cell line (HUVEC) and normal peripheral T-lymphocytes

verifies the existence of β1C-2 as a splice variant of integrin β1, not only in transformed cells but also in normal cells. This novel variant is named β1C-2 because it results from usage of an alternative downstream splice-acceptor site in exon C. Thus, the β1C-2 variant lacks 6 amino acids (SLSVAQ) in the cytoplasmic tail compared to β1C-1 (Figure 1). A comparison of the splice site used to generate β1C-2 with a consensus splice site shows that the acceptor site contains a suboptimal polypyrimidine tract and a conserved AG dinucleotide (Figure 2A). The β1C-2 exon starts with a C, compared with the A or G which occurs in the consensus sequence, although variation at this position appears to be acceptable (Figure 2A). Comparing the splice site of β1C-2 with those of the other alternatively-spliced exons in β1 (Figure 2A) reveals some variations in the sequences and this could be the clue to the different expression patterns of the different variants.

A consensus branch-point (YNCTRAY) [28] is located upstream of exon C (Figure 2B), and according to the model where the splice acceptor site is identified by a scanning mechanism from the branch point, the first AG dinucleotide is preferred as the splice acceptor site [37]. Examination of the genomic sequence downstream of the branch point for exon C reveals several AG dinucleotides (Figure 2B), posing the question of what factors determine the choice of splice-acceptor site in the case of exon C. Some important aspects for the choice of AG sites have been identified [28,38]. The first two downstream AG (11 bp) and AG (23 bp) are probably not used because they are located too close to the branch point and upstream of the polypyrimidine tract (Figure 2B) [37]. The third AG (82 bp) is preceded by a G which is thought to make the AG less attractive for the spliceosome [37], leaving the closely positioned β1C-1 AG (86 bp) available (Figure 2B). In addition, the possibility that a suboptimal branch point, located closer to the exon C splice-acceptor sites, rather than the consensus branch point, is being used cannot be excluded. Another factor that seems to affect the choice of splice-acceptor site is the binding of serine-/arginine-rich proteins (SR-proteins) to surrounding regions [28]. SR-proteins have been shown to bind purine boxes [38] and a potential SR protein binding site (GAGACAG) is located immediately upstream of the β1C-1 acceptor site (Figure 2B). Binding to this site would probably interfere with the recognition of the β1C-1 acceptor site, and favour the β1C-2 AG (104 bp) instead (Figure 2B).

A large number of cell lines and a few normal cell types and tissues were tested for the presence of β1C-2 transcripts. The mRNA levels of β1C-2 were compared with the levels for β1C-1 and were found to vary between the different samples. Both transcripts were present in transformed cells (blood-cell derived and non-blood-cell derived) as well as in normal monocytes, kidney and liver tissues, indicating that there is a low level of expression of both variants in most cells regardless of cell type and whether they are transformed or not. A notable exception was isolated normal peripheral T-lymphocytes, which were found to express essentially no β1C-1, whereas the levels of β1C-2 were as high as in the other cell types. This result is partly in agreement with a previous report where no β1C-1 mRNA could be detected in peripheral blood lymphocytes [8], although the primers used in that study should have been able to amplify β1C-2 transcripts. The reason for this discrepancy is not known. Our analysis of two separate T-cell preparations from two different donors gave identical results, and negative controls exclude the possibility of contamination from previously amplified PCR products. This splicing event is differentially regulated in normal T-lymphocytes compared with the T-cell line (Jurkat) where β1C-1 is expressed at a higher level than β1C-2 (Figure 3), but the reason for this is presently unknown. The existence of binding sites for splice-regulatory factors and the variation of the expression pattern for β1C-1 and β1C-2 do not prove any regulation of this particular splicing event. Nevertheless, these questions need to be investigated in more detail in order to determine if different cells have controlled splicing machinery for this particular splicing of the β1 pre-mRNA.

Investigation of exon C and the surrounding genomic sequence revealed the existence of a nearly complete Alu Sx element [29,39] located in the antisense orientation compared with exon C (Figure 1 and Figure 2B). Alu elements represent the most abundant family of short, dispersed, repetitive DNA in humans with about 500000 copies per haploid genome [30]. They are small transposable elements of approximately 300 bp and consist of two directly repeated monomeric units with homology to 7SL RNAs [40,41]. The two monomeric units are linked with an adenine-rich sequence and the whole element ends in a polyadenyl tail. The Alu Sx elements are thought to be one of the 'middle-age' subfamilies, indicating that this particular insertion into the integrin β1 gene took place before the divergence of the New World monkeys about 40 million years ago [42]. The fact that Alu elements are found only in primates supports our results from RT-PCR and hybridization studies that the C isoforms do not exist in mice. This conclusion is consistent with a recently published study by Baudoin et al. [43]. Mice do have a repetitive element called B1 that has derived from 7SL RNA [44], however, the B1 elements have some resemblance to the 'left' Alu monomer, whereas the exon C is part of the 'right' Alu monomer. It is believed that the B1 and Alu elements appeared independently in rodents and primates after the divergence from their common ancestor [40,45], and that a B1 element could have integrated into the equivalent intron in the mouse β1 gene and, thereby, given rise to a mouse exon C is not very likely.

When looking more closely at consensus Alu elements, it is evident that the antisense Alu element contains several potential splice sites, and the requirement for a polypyrimidine tract upstream of splice acceptor sites is fulfilled both by the complementary adenine-rich linker region and the complementary polyadenyl tail of the Alu elements [46]. The branch point, in the case of β1C-1 and β1C-2, is located upstream of the Alu element (Figure 2B). The need for the sequence outside the Alu elements to contain a suitable branch point is probably the reason why relatively few antisense Alu elements actually become spliced into mRNA compared with the number of elements existing in intron sequences. Nevertheless, the existence of several cases where an Alu element is part of the coding region of human mRNAs [31–36,46,47], suggests that Alu elements can be a source of protein variability. Most of the antisense Alu elements giving rise to variant mRNAs have been incorporated by alternative splicing, as is also the case for β1C-1 and β1C-2. The antisense consensus Alu element, when translated, contains long stretches of open reading frames, indicating that they are able to generate protein domains. However, it is still unclear whether these Alu insertions give rise to any particular protein domain that has any common feature. At present, no function has been found for most of the protein isoforms resulting from Alu exons and there is no obvious connection between the various proteins reported today which have alternative Alu exons. In addition, the fact that most of the known examples (including β1C-1 and β1C-2) are expressed at very low levels compared with the 'normal' transcripts [31,32,34,36] may indicate that these splicing events occur accidentally by usage of inefficient splice sites. Absence of β1C-1 and β1C-2, as well as other proteins translated from Alu exons, in other species except primates may argue that these proteins are not functionally important. In contrast to this view are the results presented for β1C-1 where over-expression

led to down-regulation of DNA synthesis and inability of the cells to pass the G1 to S phase transition check-point [18,19]. In addition, several recent reports have shown that Alu elements integrated into control regions have adapted roles as enhancers, silencers and promoter elements, supporting the hypothesis that some Alu elements have evolved into functional units [46,48].

Our attempt to obtain stable clones of $\beta$1C-1- and $\beta$1C-2-expressing cells gave only clones with barely detectable levels of the protein on the cell surface. This is in agreement with other studies reported previously in which it was not possible to isolate any stably transfected high expressing clones of $\beta$1C-1 in either hamster or mouse cells [18,19]. The mouse $\beta$1-deficient cells (GD25 cells) allowed us to detect even a very low $\beta$1 expression using a polyclonal $\beta$1 antiserum in both FACS analysis and immunoblotting. RT-PCR on total RNA from these clones showed that the $\beta$1-mRNA levels of the $\beta$1C-1 and $\beta$1-2 stable clones were much lower than for the $\beta$1A clones. Transient expression of $\beta$1A and $\beta$1C-2 showed also that the levels of the $\beta$1A protein were higher than for $\beta$1C-2, further supporting the results of the FACS analysis and excluding the possibility that the results were due to clonal selection. Together, these results indicate that high expression of $\beta$1C-2 proteins cannot be obtained but the actual cause for this is currently unknown. Other studies have shown that the C-terminal of $\beta$1C-1 is responsible for its growth inhibitory effects [18,19]. Therefore the significance of the six amino acids differing between $\beta$1C-1 and $\beta$1C-2 with regard to growth inhibition or interference with other signalling pathways needs to be investigated in more detail and these experiments have been initiated. The $\beta$1C protein has been shown to be present in different tissues [8,17,18], however, the antibodies used in those studies would not distinguish between $\beta$1C-1 and $\beta$1C-2 proteins. Our results on the distribution of the mRNA for $\beta$1C-1 and $\beta$1C-2 suggests that both variants are expressed in most tissues, and further experiments are needed in order to determine if both variants are being translated *in vivo* and where they are expressed. The possibility that up-regulation of $\beta$1C-1 and $\beta$1C-2 may induce pathological conditions should also be considered. In addition, the recent study showing that the expression of a $\beta$1C protein is down-regulated in prostate carcinoma compared with normal tissue [17] further prompts the need for information about the regulation of expression and the physiological and pathophysiological relevance of both $\beta$1C-1 and $\beta$1C-2.

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## REFERENCES

1 Hynes, R. O. (1992) Cell **69**, 11–25
2 Clark, E. A. and Brugge, J. S. (1995) Science **268**, 233–239
3 Schwartz, M. A., Schaller, M. D. and Ginsberg, M. H. (1995) Ann. Rev. Cell. Dev. Biol. **11**, 549–599
4 Tamura, R. N., Cooper, H. M., Collo, G. and Quaranta, V. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10183–10187
5 Hogervorst, F., Kuikman, I., van, Kessel, A. G. and Sonnenberg, A. (1991) Eur. J. Biochem. **199**, 425–433
6 Collo, G., Starr, L. and Quaranta, V. (1993) J. Biol. Chem. **268**, 19019–19024
7 Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G. and Silengo, L. (1990) Gene **95**, 261–266
8 Languino, L. R. and Ruoslahti, E. (1992) J. Biol. Chem. **267**, 7116–7120
9 van der Flier, A., Kuikman, I., Baudoin, C., van der Neut, R. and Sonnenberg, A. (1995) FEBS Lett. **369**, 340–344
10 Zhidkova, N. I., Belkin, A. M. and Mayne, R. (1995) Biochem. Biophys. Res. Commun. **214**, 279–285
11 van Kuppevelt, T., Languino, L. R., Gailit, J. O., Suzuki, S. and Ruoslahti, E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5415–5418
12 Tamura, R. N., Rozzo, C., Starr, L., Chambers, J., Reichardt, L. F., Cooper, H. M. and Quaranta, V. (1990) J. Cell Biol. **111**, 1593–1604
13 Marcantonio, E. E. and Hynes, R. O. (1988) J. Cell Biol. **106**, 1765–1772
14 Balzac, F., Belkin, A. M., Koteliansky, V. E., Balabanov, Y. V., Altruda, F., Silengo, L. and Tarone, G. (1993) J. Cell Biol. **121**, 171–178
15 Balzac, F., Retta, S. F., Albini, A., Melchiorri, A., Koteliansky, V. E., Geuna, M., Silengo, L. and Tarone, G. (1994) J. Cell Biol. **127**, 557–565
16 Belkin, A. M., Zhidkova, N. I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E. and Burridge, K. (1996) J. Cell Biol. **132**, 211–226
17 Fornaro, M., Tallini, G., Bofetiado, C. J., Bosari, S. and Languino, L. R. (1996) Am. J. Pathol. **149**, 765–773
18 Fornaro, M., Zheng, D. Q. and Languino, L. R. (1995) J. Biol. Chem. **270**, 24666–24669
19 Meredith, Jr., J., Takada, Y., Fornaro, M., Languino, L. R. and Schwartz, M. A. (1995) Science **269**, 1570–1572
20 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
21 Fässler, R., Pfaff, M., Murphy, J., Noegel, A. A., Johansson, S., Timpl, R. and Albrecht, R. (1995) J. Cell Biol. **128**, 979–988
22 Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S. and Fässler, R. (1996) J. Cell Biol. **132**, 227–238
23 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
24 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. and Pease, L. R. (1989) Gene **77**, 61–68
25 Gossen, M. and Bujard, H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5547–5551
26 Fässler, R. and Meyer, M. (1995) Genes Dev. **9**, 1896–1908
27 Bottger, B. A., Hedin, U., Johansson, S. and Thyberg, J. (1989) Differentiation **41**, 158–167
28 Krämer, A. (1996) Annu. Rev. Biochem. **65**, 367–409
29 Jurka, J. and Milosavljevic, A. (1991) J. Mol. Evol. **32**, 105–121
30 Schmid, C. W. and Jelinek, W. R. (1982) Science **216**, 1065–1070
31 Cellier, M., Govoni, G., Vidal, S., Kwan, T., Groulx, N., Liu, J., Sanchez, F., Skamene, E., Schurr, E. and Gros, P. (1994) J. Exp. Med. **180**, 1741–1752
32 Mihovilovic, M., Mai, Y., Herbstreith, M., Rubboli, F., Tarroni, P., Clementi, F. and Roses, A. D. (1993) Biochem. Biophys. Res. Commun. **197**, 137–144
33 Levedakou, E. N., He, M., Baptist, E. W., Craven, R. J., Cance, W. G., Welcsh, P. L., Simmons, A., Naylor, S. L., Leach, R. J., Lewis, T. B. et al. (1994) Oncogene **9**, 1977–1988
34 Haviland, D. L., Haviland, J. C., Fleischer, D. T., Hunt, A. and Wetsel, R. A. (1991) J. Immunol. **146**, 362–368
35 Caras, I. W., Davitz, M. A., Rhee, L., Weddell, G., Martin, Jr., D. W. and Nussenzweig, V. (1987) Nature (London) **325**, 545–549
36 Mullersman, J. E. and Pfeffer, L. M. (1995) J. Interferon Cytokine Res. **15**, 815–817
37 Smith, C. W., Chu, T. T. and Nadal-Ginard, B. (1993) Mol. Cell. Biol. **13**, 4939–4952
38 Kanopka, A., Muhlemann, O. and Akusjarvi, G. (1996) Nature (London) **381**, 535–538
39 Batzer, M. A., Deininger, P. L., Hellmann-Blumberg, U., Jurka, J., Labuda, D., Rubin, C. M., Schmid, C. W., Zietkiewicz, E. and Zuckerkandl, E. (1996) J. Mol. Evol. **42**, 3–6
40 Deininger, P. L. and Daniels, G. R. (1986) Trends Genet. **2**, 76–80
41 Sinnett, D., Richer, C., Deragon, J. M. and Labuda, D. (1991) J. Biol. Chem. **266**, 8675–8678
42 Kapitonov, V. and Jurka, J. (1996) J. Mol. Evol. **42**, 59–65
43 Baudoin, C., van der Flier, A., Borradori, L. and Sonnenberg, A. (1996) Cell Adhesion Communication **4**, 1–11
44 Zietkiewicz, E. and Labuda, D. (1996) J. Mol. Evol. **42**, 66–72
45 Schmid, C. W. (1996) Prog. Nucleic Acid Res. Mol. Biol. **53**, 283–319
46 Makalowski, W., Mitchell, G. A. and Labuda, D. (1994) Trends Genet. **10**, 188–193
47 Barnett, T. R., Drake, L. and Pickle, W. D. (1993) Mol. Cell. Biol. **13**, 1273–1282
48 Britten, R. J. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 9374–9377

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