cDNA cloning reveals two mouse β5 integrin transcripts distinct in cytoplasmic domains as a result of alternative splicing

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The integrin β5 subunit has only been found to form a heterodimer with subunit αv which acts as a vitronectin receptor. Integrin αvβ5 has been implicated in cell migration and growth factor-induced angiogenesis. In the present study, a mouse liver cDNA library was screened using a human β5 cDNA fragment obtained by reverse transcriptase PCR (RT-PCR). Three of the clones (MB5, MB15 and MB17) overlapped to give an open reading frame, called β5A, which is homologous to the human β5 subunit. The sequence of another clone (MB26), called β5B, was identical with β5A, except for a deletion of 29 bp near the 3′ end of the open reading frame. The 29 bp deletion resulted in an open-reading-frame shift and a completely different C-terminal sequence in β5B. β5A and β5B were shown, by RT-PCR, to be co-expressed in most mouse tissues tested, although β5B mRNA was detected at much lower levels than β5A. β5A and β5B mRNAs were also detected in the mouse monocytic cell line, J774, and in isolated mouse peritoneal macrophages. Adhesion of peritoneal macrophages has been shown to up-regulate the expression of both β5A and β5B mRNAs. The 29 bp sequence begins with a putative intron-splicing donor site (GTGAT …). A 3′ fragment of the mouse integrin β5 gene was cloned by PCR and sequenced showing that the 29 bp sequence was also immediately followed by an intron. Therefore, the 29 bp sequence was apparently expressed as part of the β5A mRNA but was spliced out as part of the downstream intron in β5B. Since the cytoplasmic domains of the integrin β subunits are important in cytoskeleton attachment and signalling, the two alternatively spliced β5 isoforms may have distinct roles in cell adhesion and other cellular functions.

INTRODUCTION

Integrins are a family of cell-adhesion receptors that mediate cell adhesion to extracellular matrix (ECM), plasma proteins (vitronectin, fibrinogen, fibronectin, etc) and to other cells [1]. Integrins are non-covalent α/β heterodimers in which each subunit consists of a large N-terminal extracellular domain, a single transmembrane domain and, in most integrins, a short C-terminal cytoplasmic tail. The extracellular domains of the α and β subunits bind to ECM components and other ligands while the cytoplasmic tails attach to the cytoskeleton via cytoplasmic adapter proteins [2,3]. To date, 14 α and 8 β subunits have been characterized which together form more than 20 different integrin heterodimers. Some integrins, e.g. β1 integrins, are widely expressed in different tissues and cells whereas others, e.g. β2 and β7 integrins, are restricted to certain cell types [4]. Integrins are not merely mechanical anchors linking the cytoskeleton to extracellular structures or ligands. They are also signalling receptors that can co-ordinate cell adhesion and migration with many other cellular functions, such as cell proliferation, differentiation and apoptosis, and play key roles in many biological processes, such as embryonic development, wound healing, tumour metastasis and inflammation [1,5,6].

Integrin αvβ3 is a vitronectin receptor and has been implicated in tumour-associated angiogenesis, an essential step for tumour growth beyond a certain size [7,8]. This integrin also binds to the endothelial adhesion receptor PECAM-1 (platelet-endothelial cell-adhesion molecule-1) and is potentially involved in leucocyte recruitment [9]. The subunit β5 was initially identified as a novel β subunit that forms a heterodimer with subunit αv [10]. Like integrin αvβ3, αvβ5 also mediates cell adhesion to vitronectin [10,11]. However, studies have shown that it is distinct from αvβ3 in that, on certain cells, it only acquires significant affinity for vitronectin upon stimulation with phorbol ester or insulin-like growth factor [12,13]. This is similar to β2 integrins, which need to be activated to acquire higher affinity for counter-receptors on endothelial cells and leucocytes [14,15]. Platelet integrin αIIbβ3 only binds to soluble fibrinogen upon activation of platelets, e.g. by thrombin [16]. Incorporation of integrins into focal-adhesion plaques requires integrin occupation with ligands and the intrinsic signal in the cytoplasmic tails of the β subunits [2,3]. Unlike wild-type β1 integrins, chimaeric β1 integrins, having β5 cytoplasmic tails when expressed on Chinese hamster ovary cells, could not be recruited into focal-adhesion sites [17]. Also, αvβ5 has been implicated in growth factor- or phorbol ester-initiated angiogenesis [18].

Human β5 has been detected on many cell types, but not lymphocytes [19]. It has also been detected in a variety of embryonic organs [20]. Our recent work showed that expression of β5 mRNA could be induced on monocytes immediately after adhesion and serves as a marker of monocyte differentiation into macrophages (S. M. Tan and J. Lu, unpublished work). In the present study, two types of mouse β5 cDNA sequences have been identified which are totally divergent in their C-terminal cytoplasmic domains as a result of alternative splicing.

MATERIALS AND METHODS

A mouse liver λ ZAP cDNA library was purchased from Stratagene (La Jolla, CA, U.S.A). α-[^{32}P]dCTP and a random-primed DNA-labelling kit were obtained from Amersham (Amersham, Bucks, U.K.). Oligonucleotide primers were synthesized in the National University Medical Institute, Singapore.

Abbreviations used: RT-PCR, reverse transcriptase PCR; ECM, extracellular matrix; MMR, macrophage mannose receptor.

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The cDNA nucleotide sequence data for integrin β5A and β5B presented in this paper will appear in the GenBank database under the accession numbers AF043256 and AF043257, respectively.

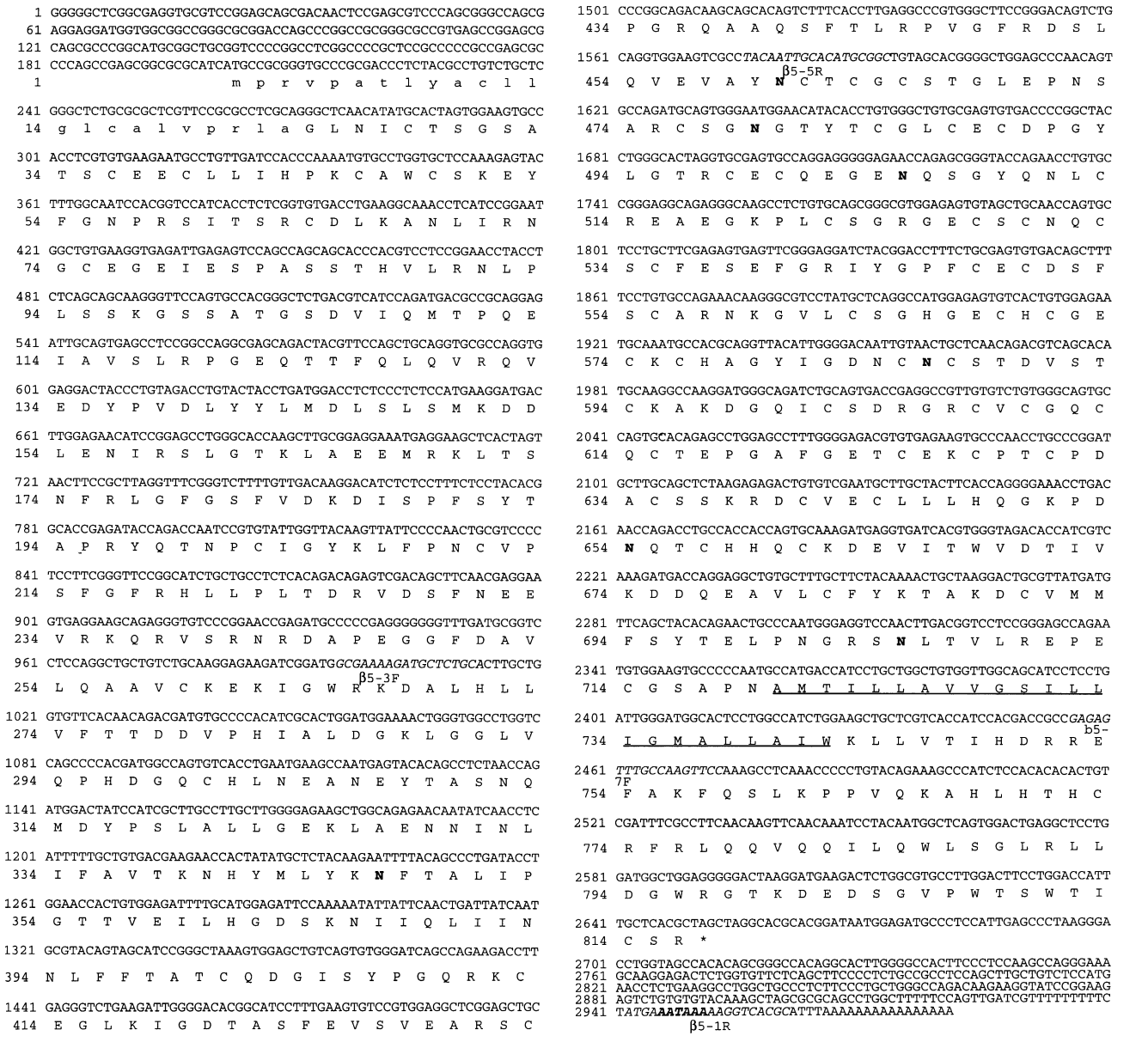

Figure 1 cDNA and derived amino acid sequences of mouse integrin subunit β5B

The sequence was obtained from a single mouse β5 cDNA clone, MB26. The derived amino acid sequence is in single-letter code. The putative 23 residue leader peptide is in lower-case letters. The putative 23 residue transmembrane domain is underlined. Potential Asn-linked glycosylation sites are in bold. The putative polyadenylation signal, AATAAA, is also in bold. Oligonucleotide primers synthesized according to this cDNA sequence are highlighted in italic and labelled with names of the primers, i.e. β5-3F, β5-5R, β5-7F and β5-1R.

Isolation and sequencing of mouse integrin β5 cDNA clones

Two primers, hβ5c-F (5′-GACCACCTTCCAGCTACA-3′) and hβ5c-R (5′-AGGCTGATCCCAGACTGA-3′), were synthesized based on published human integrin β5 sequences [19,21], to amplify a cDNA fragment of approx. 800 bp from a human liver cDNA library (results not shown). The 800 bp human β5 cDNA fragment was labelled with ^{32}P and used to screen approx. 5×10^5 plaques from a mouse liver λ ZAP cDNA library. Positive clones were purified and phagemid pBS SK$^-$ in the λ vectors that contained the cDNA inserts was excised from the phage vectors using the Exassist/SOLR system provided by the manufacturer (Stratagene). Each clone was sequenced from both ends using T3 and T7 primers, which flank the cloning site. Selected clones were completely sequenced from both directions. Sequencing was performed on the automated ABI 373 sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.)

Cloning and sequencing of the 3′ fragments of mouse and human integrin β5 genes

Two primers were synthesized, based on the mouse integrin β5 cDNA sequences obtained, and were designed to span the alternative-splicing sites in the mouse β5 gene, i.e. β5-7F (5′-GAGAGTTTGCCAAGTTCC-3′) and β5-1R (5′-GCGTGAC-CTTTTTATTTCAT-3′) (Figure 1). The primers were phos-

phorylated and PCR was performed using mouse genomic DNA as template. The PCR (100 μl) was carried out for 35 cycles, each consisting of 94 °C, 30 s; 51 °C, 30 s; and 72 °C, 3 min. The PCR product was purified and cloned into pBS KS$^+$ by blunt-end ligation and sequenced. The corresponding region of the human integrin β5 gene was similarly amplified by PCR from human genomic DNA using two primers, hβ5g-F (5′-CTGCTTGTCACCATCCAC-3′) and hβ5g-R (5′-TCTGATGAAAAGGATCAGAC-3′), designed on the basis of published sequences [19,21]. The PCR product was cloned into pBS KS$^+$ and sequenced.

Detection of β5A and β5B mRNAs in mouse tissues

Figure 2 shows 5′ primers designed to be specific for β5A and β5B. Primers β5A-as (5′-TTGCCAAGTTCCAAAGTGA-3′) and β5B-as (5′-TGCCAAGTTCCAAAGCCT-3′) were different only in 3 nucleotides at their 3′ ends. RT-PCRs (reverse transcriptase PCRs) were carried out with a common 3′ anti-sense primer, β5-1R. The primer pairs were shown to be specific in the amplification of β5A and β5B cDNA sequences since the β5A-specific primer pair did not amplify the expected PCR product from the β5B cDNA clone, MB26, and vice versa. RT-PCRs were carried out on RNAs from 9 different mouse tissues, i.e. liver, brain, colon, kidney, lung, spleen, uterus, skeletal muscle and heart. Total RNA was isolated from these mouse tissues according to the method of Chomczynski and Sacchi [22]. First strand cDNAs were synthesized from each RNA sample (1 μg) using a cDNA synthesis kit and both oligo-dT and random primers. The cDNA reactions (20 μl) were diluted to 100 μl with water and 5 μl of each dilution was used in PCR. The PCRs were carried out for 40 cycles, each consisting of 94 °C, 30 s; 51 °C, 30 s; and 72 °C, 30 s, and the reactions (10 μl) were examined on a 1% (w/v) agarose gel. PCRs were also carried out using primers common to both β5A and β5B (Figure 1), i.e. β5-3F (5′-GCGAAAAGATGCTCTGCA-3′) and β5-5R (5′-GCCGCATGTGCAATTGTA-3′), which amplify the β5 cDNA sequence encoding part of the extracellular domain of mouse β5 (Figure 1). As indications of cDNA input in each PCR reaction, PCRs were also carried out on each cDNA sample for the levels of mouse β-actin cDNA using two primers, mβac-F (5′-ATCCTGTGGCATCCATGA-3′) and mβac-R (5′-ACGCAGCTCAGTAACAGT-3′), synthesized based on published mouse β-actin cDNA sequence [23].

Detection of β5A and β5B mRNAs in mouse peritoneal macrophages and in the mouse monocytic cell line J774

The mouse monocytic cell line, J774, was cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. Total RNA was isolated from the cells for RT-PCR. RNA was also isolated from J774 cells that had been treated with PMA (25 ng/ml) for 24 h. Mouse peritoneal macrophages were collected following a previously described method without using an eliciting agent [24]. RNA was isolated from approx. 1×10^6 cells harvested while the remaining cells (approx. 1×10^7) were plated in six-well tissue-culture plates. After incubation for 1 h at 37 °C in the presence of 5% CO_2, the plates were washed three times with PBS and adherent macrophages were then harvested 0, 2, 4, 8 and 24 h after washing. RNAs were similarly isolated from these macrophage samples for RT-PCR. RT-PCRs with primer pairs β5A-as/β5-1R, β5B-as/β5-1R and β5-3F/β5-5R (which amplifies both β5A and β5B) were carried out as described above. β-Actin cDNA was also similarly measured in the macrophage samples as an indication of cDNA input in each PCR. RT-PCR was also carried out on the macrophage samples to assay

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     K  L  L  V  T  I  H  D  R  R  E  F  A  K  F  Q  S  E  R  S
β5A ..........................................(β5A-as)GTGAGCGCTCC
β5B AAGCTGCTCGTCACCATCCACGACCGCCGAGAGTTTGCCAAGTTCCAAA(β5B-as)---
     K  L  L  V  T  I  H  D  R  R  E  F  A  K  F  Q

     R  A  R  Y  E  M  A  S  N  P  L  Y  R  K  P  I  S  T  H  T
β5A AGGGCCCGTTATGAAATG.....................................
β5B ------------------GCCTCAAACCCCCTGTACAGAAAGCCCATCTCCACACACACT
                    S  L  K  P  P  V  Q  K  A  H  L  H  T  H

    V  D  F  A  F  N  K  F  N  K  S  Y  N  G  S  V  D  *
β5A ..........................................................
β5B GTCGATTTCGCCTTCAACAAGTTCAACAAATCCTACAATGGCTCAGTGGACTGAGGCTCC
    C  R  F  R  L  Q  Q  V  Q  Q  I  L  Q  W  L  S  G  L  R  L

β5A ..........................................................
β5B TGGATGGCTGGAGGGGACTAAGGATGAAGACTCTGGCGTGCCTTGGACTTCCTGGACCA
    L  D  G  W  R  G  T  K  D  E  D  S  G  V  P  W  T  S  W  T

β5A ..........................................................
β5B TTTGCTCACGCTAGCTAGGCACGCACGGATAATGGAGATGCCCTCCATTGAGCCCTAAGG
    I  C  S  R  *

β5A ..........................................................
β5B GACCTGGTAGCCACACAGCGGGCCACAGGCACTTGGGGCCACTTCCCTCCAAGCCAGGGA

β5A ..........................................................
β5B AAGCAAGGAGACTCTGGTGTTCTCAGCTTCCCCTCTGCCGCCTCCAGCTTGCTGTCTCCA

β5A ..........................................................
β5B TGAACCTCTGAAGGCCTGGCTGCCCTCTTCCCTGCTGGGCCAGACAAGAAGGTATCCGGA

β5A ..........................................................
β5B AGAGTCTGTGTGTACAAAGCTAGCGCGCAGCCTGGCTTTTTCCAGTTGATCGTTTTTTTT

β5A′.........................ATGGTGTaaaaaaaaaaaaaaaaaaaaaaaaa
 β5A ........................--------aaaaaaaaaaaaaaaaaaaaa
β5B TCTATGAAATAAAAAGGTCACGCATTT-------aaaaaaaaaaaaaaa
          (β5-1R)

β5A′aaaaa
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Figure 2 Alignment of the cDNA and derived amino acid sequences of the cytoplasmic domains of mouse integrin subunits, β5A and β5B

The β5A- and β5B-specific 5′ primers (β5A-as and β5B-as) and the common 3′ primer (β5-1R) used in RT-PCRs are in bold. The NPLY sequence is underlined. … Denotes cDNA sequences identical with the aligned sequence, and --- denotes gaps introduced for the maximum alignment of the sequences. The sequence of a β5A cDNA clone, MB15 (β5A′), which deviated from the sequences of MB17 (β5A) and MB26 (β5B) immediately in front of the poly A tail by having an extra 7–8 nucleotides, is shown in italic.

for the mRNA of a macrophage-specific protein, the macrophage mannose receptor (MMR). The MMR primers, mMR-F (5′-GGCCTTTGGAATAATATCCA-3′) and mMR-R (5′-GCTCATTCTGCTCGATGTT-3′), were synthesized based on published cDNA sequence for mouse MMR [25]. MMR is not synthesized in circulating monocytes but can be induced to express after monocyte adhesion acting as a monocyte differentiation marker [26]. All the PCR products were examined on 1% (w/v) agarose gels.

Quantification of β5A and β5B mRNA expression by GeneScan

RT-PCR was performed, with primers β5-7F and β5-1R, using RNA isolated from different mouse tissues and peritoneal macrophages as templates. PCR was carried out for 30 cycles, each consisting of 94 °C, 30 s; 51 °C, 30 s; and 72 °C, 45 s. Fluorescence-labelled dCTP, [R110]dCTP (Applied BioSystems), was used in a 1:200 ratio to unlabelled dCTP. The PCR products (in 1 μl) were applied on to a 4.25% polyacrylamide sequencing gel and separated on the Applied BioSystems Prism 377 DNA sequencer. The separated PCR products were analysed with GeneScan 2.1 software (Applied BioSystems) for sizes and fluorescence intensities and the β5B/β5A ratio in each sample was calculated.

RESULTS

cDNA and derived amino acid sequences of mouse integrin β5

Screening of the mouse liver cDNA library with an 800 bp human β5 integrin cDNA fragment identified 30 positive clones, MB1–MB30. The λ ZAP clones were purified and the pBS SK⁻ phagemid, which is part of the λ vector and contained the cDNA inserts, was excised from each clone and purified for sequencing analysis. These clones were initially sequenced using the T3 and T7 primers which flank the cloning site in pBS KS, and mouse β5 sequences were identified by homology with human β5 cDNA sequences [19,21]. Clones MB5, MB15 and MB17 were partial clones which were sequenced from both directions and whose sequences overlapped to give a complete open reading frame. The assembled cDNA sequence encodes an amino acid sequence, called β5A, which shares 93 % sequence identity with human β5.

The longest clone, MB26, was also completely sequenced from both directions (Figure 1). The sequence of clone MB26, named β5B, is identical with that of β5A except for a 29 bp deletion (Figure 2). The 29 bp sequence is close to the 3′ end of the open reading frame and encodes part of the cytoplasmic domain of β5A. The deletion in β5B also led to an open-reading-frame shift and, therefore, to complete divergence of the cytoplasmic C-terminus of the β5B polypeptide which is consequently 18 residues longer than that of β5A (Figure 2). The sequence divergence occurs 16 residues from the transmembrane domain (Figure 2).

The entire open reading frames of β5A and β5B give rise to two polypeptide sequences of 798 and 816 amino acid residues, respectively, including a putative 23 residue leader peptide, a 696 residue extracellular domain, a putative 23 residue transmembrane domain, a 56 residue cytoplasmic domain for β5A and a 74 residue cytoplasmic domain for β5B (Figures 1 and 2). Fifty six cysteine residues are conserved in the extracellular domains of known integrin β subunits and these are also conserved in the mouse β5 sequences (Figure 1). Of the potential Asn-linked glycosylation sites found in the extracellular domain of human β5, six of the eight are conserved in the mouse β5 sequences (Figure 1). The potential Asn-linked glycosylation site in human β5, N_{552}FS, is lost in mouse β5 as a result of the replacement of N_{552} by a serine residue (S_{552}FS) in the mouse sequence (Figure 1) [19,21]. Another potential Asn-linked glycosylation site in mouse β5 (CSGN_{479}GT) deviates from a similar glycosylation site in human β5 (CN_{477}GSGT) (Figure 1). The β5B cytoplasmic domain showed no obvious homology with that of any known receptor and is also characterized by the presence of two cysteine residues (Figures 1 and 2). There is very little sequence divergence between mouse and human β5 sequences over the N-terminal 410 residues, which contain ligand binding sites, especially between residues T_{124} and T_{355}. These are identical between the mouse and human sequences. An NPxY sequence is conserved in the cytoplasmic domains of many integrin β subunits and, in β3, it has been shown to be essential for melanoma cell metastasis and migration on vitronectin [27]. This NPxY sequence is conserved in mouse β5A, but it is absent in β5B as a result of an open-reading-frame shift caused by the 29 bp deletion (Figure 2).

Alternative splicing of the mouse integrin β5 gene

The lack of sequence divergence over other parts of β5A and β5B suggested that the two sequences were probably derived from a single gene and, like some other integrins [1,28], the 29 bp deletion in MB26 was probably the result of alternative splicing. In fact, the 29 bp deletion does begin with a putative intron splicing donor sequence (GTGAG …) (Figure 2). A fragment of the mouse integrin β5 gene spanning a region containing the 29 bp deletion was amplified from mouse genomic DNA by PCR using a 5′ primer immediately upstream of the deletion (β5-7F) and a 3′ primer downstream of the deletion (β5-1R) (Figure 1). A 1.4 kb product was amplified and cloned into the plasmid pBS KS⁺ vector. Sequencing of the genomic fragment showed that the 29 bp sequence was followed immediately by an intron of approx. 700 bp (Figure 3A). Therefore, the 29 bp sequence is flanked by two intron-splicing donor sites and it is apparently expressed in β5A when the 3′ donor site is utilized, whereas it is spliced out in β5B as part of the downstream intron when the 5′ donor site is employed (Figure 3A).

The corresponding region of the human β5 gene was also cloned and sequenced. An intron of similar size was found in the genomic fragment at a position identical with that in the mouse β5 gene (Figure 3). However, the corresponding 29 bp sequence in human β5 does not begin with a typical intron-splicing donor

(A)

```
R  E  F  A  K  F  Q  S  E  R  S  R  A  R  Y  E  M (β5A)
                β5-7F
GAGAGTTTGCCAAGTTCCAAAGTGAGCGCTCCAGGGCCCGTTATGAAATGgtaagagggg
R  E  F  A  K  F  Q  S (β5B)

gtggttaaaaaagaggttgctggcttagagccagggtcaaaggctaaaaacaatcaatca
aacaaacaaaaaaaagagctaggtgtggtagtattcacctataatcccagctgctttgga
ggctgagacaggagtttgaagttcaaagcttagcctgggttacttaccaagcccttgtct
ggaaataaatgaccaaatttttgagtgtagttcaatggtagtgtgcttgcctaacatgcgc
a..approx.400 bp..aattccacagatgaaaagtaagagctcagagaaggaaaggga
ttgggggagagcacagaacaagtcagaagcaggcatccctgcaggtgctggacgggcctg

aggctgaactaacacgtgtgcgctcctctcttgtgctcgcagGCCTCAAACCCCCTGTAC
AGAAAGCCCATCTCCACACACACTGTCGATTTCGCCTTCAACAAGTTCAACAAATCCTAC
AATGGCTCAGTGGACTGAGGCTCCTGGATGGCTGGAGGGGGACTAAGGATGAAGACTCTG
GCGTGCCTTGGACTTCCTGGACCATTTGCTCACGCTAGCTAGGCACGCACGGATAATGGA
GATGCCCTCCATTGAGCCCTAAGGGACCTGGTAGCCACACAGCGGGCCACAGGCACTTGG
GGCCACTTCCCTCCAAGCCAGGGAAAGCAAGGAGACTCTGGTGTTCTCAGCTTCCCCTCT
GCCGCCTCCAGCTTGCTGTCTCCATGAACCTCTGAAGGCCTGGCTGCCCTCTTCCCTGCT
GGGCCAGACAAGAAGGTATCCGGAAGAGTCTGTGTGTACAAAGCTAGCGCGCAGCCTGGC
TTTTTCCAGTTGATCGTTTTTTTTTCTATGAAATAAAAAGGTCACGC
                                        β5-1R
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(B)

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 L  L  V  T  I  H  D  R  R  E  F  A  K  F  Q  S  E  R  S  R
    hβ5g-F
CTGCTTGTCACCATCCACGACCGGAGGGAGTTTGCAAAGTTTCAGAGCGAGCGATCCAGG

 A  R  Y  E  M
GCCCGCTATGAAATGgtaagcacgtgggaaatgggaagcagaggagacttcaagctcaga
ggcgtggttgagttcagcggtatgttagcaatagaggctcactaatgtctttggcattaa
aaacaaaagaatcaacaccagaaacgctgtgcccagttctggacagagagaattgcacag
ccccagtgcagagtgagctgcacagatccctgttccagacccaggtcaggaaggtcatgg
gggcagaagcgctgggcgtgcctcaggaagagtctgaccagtctcagagagagcacagac
caacaggaaagctcagattcttatggtccattacaacactcacacctgcagtctggtcac
cccaggcagactgcaaatgcccagaagactgagcgcagtcagtttctgctgaacctgggc
ttggtttctgttaaagctggaagggacttcagagaccttctgacccaactcctccttcca
gaggtggatagtgaaagcttatgaacctgggcttggtttctgttaaagctggaagggact
tcagagactttctgacccaattcttctttccagaggtggatagtgaaagcttagagaagg
cgatggctttcctggggaacacagagctgtcctagagcccacggccgtctcctcctgagt
gctctgcctgctgctgtcgcagggggctgacctgggaagggatcctagggcctgcgtctg
tcggtttgagtgtgtgagctaacatg

                                        A  S  N  P  L  Y  R  K  P
tgtcctcatcctcttccccgccgtgttctgtagGCTTCAAATCCATTATACAGAAAGCCT

 I  S  T  H  T  V  D  F  T  F  N  K  F  N  K  S  Y  N  G  T
ATCTCCACGCACACTGTGGACTTCACCTTCAACAAGTTCAACAAATCCTACAATGGCACT

 V  D  *
GTGGACTGATGTTTCCTTCTCCGAGGGGCTGGAGCGGGGATCTGATGAAAAGGATCAGAC
                                        hβ5g-R
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Figure 3 Intron/exon organizations of the regions of mouse (A) and human (B) β5 genes encoding the cytoplasmic domains

(**A**) The 29 bp sequence that is absent in mouse β5B, is underlined. The 29 bp sequence begins with a putative intron-splicing donor sequence that is probably utilized in the maturation of β5B mRNA and is in bold capital letters. The beginning of the 3′ intron is highlighted in bold and lower-case letters. (**B**) The 29 bp sequence in the human β5 genomic sequence is underlined. The primers used to amplify the mouse and human β5 genomic fragments are in italic, i.e. β5-7F, β5-1R, hβ5g-F and hβ5g-R.

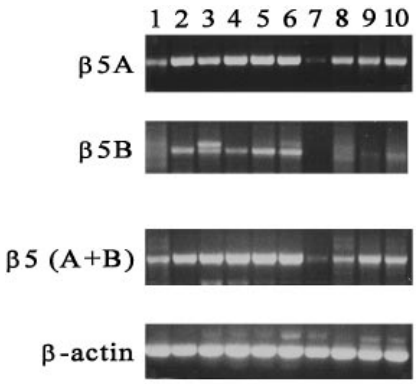

Figure 4 Detection of β5 mRNA expression in mouse tissues

Total RNA was isolated from 9 different mouse tissues for RT-PCR using primer pairs β5A-as/β5-1R (β5A), β5B-as/β5-1R (β5B), β5-3F/β5-5R [β5(A+B)], and β-actin primer. Tissues examined include liver (lane 2), brain (lane 3), colon (lane 4), kidney (lane 5), lung (lane 6), spleen (lane 7), uterus (lane 8), skeletal muscle (lane 9) and heart (lane 10). Lane 1 contains RT-PCR products amplified from RNA isolated from the mouse monocytic cell line J774. The PCR products (10 μl) were examined on a 1% (w/v) agarose gel.

sequence and such a signal is not found in the immediate 5′ region. Therefore, a similar alternative splicing event may not occur in human β5 gene expression. In fact, no human β5 cDNA sequence has been found to bear the 29 bp deletion [19,21]. However, human β5 cDNA sequences are known to differ, the main difference being the presence of either an FNK or an FNKFNK sequence near the C-terminus of the cytoplasmic domains [19,21]. Sequencing of the human β5 gene fragment showed no evidence that this was due to alternative splicing (Figure 3B). Ten independent clones containing the genomic fragment were sequenced and all have the FNKFNK sequence (results not shown). The origin and functional implication of the FNK sequence variation is not known. Mouse β5B again does not contain any FNK sequences due to open-reading-frame shift (Figure 2).

Expression of β5A and β5B in different mouse tissues

To determine the tissue distribution of mouse integrin subunit β5, 9 different mouse tissues were examined with RT-PCR, with β5A- and β5B-specific primer pairs (Figure 2). The β5A-specific RT-PCR, using the β5A clone, MB5, as a template, amplified an expected product of approx. 550 bp, which is slightly larger than that amplified from the β5B clone MB26 with the β5B-specific primer pair (results not shown). The β5A-specific primers also amplified a similarly larger product than the β5B-specific primers from a mouse liver cDNA library. However, β5B cDNA was detected at a much lower level than β5A in the library. Similar RT-PCRs were then carried out on 9 different mouse tissues. As seen in Figure 4, β5A was detected at similar levels in most tissues except the spleen, in which very little β5A mRNA was detected (Figure 4). β5B mRNA was detected in most mouse tissues except spleen and its expression in uterus and skeletal muscle was also very low (Figure 4, lanes 7, 8 and 9). The low level of β5 mRNA in spleen has also been shown with primers β5-3F and β5-5R, which detect the extracellular domain of both β5A and β5B (Figure 4, lane 7). Therefore, whereas the cell(s) of origin of mouse β5 integrin cannot be defined simply by these RT-PCR assays, the cell(s) must be scarce in spleen compared with many other tissues. It has been reported that human β5 was not detectable in human lymphocytes [19]. The low level of β5 mRNA detected in mouse spleen could simply reflect the abundance of lymphocytes in this lymph organ. Immunohistochemical studies may help to define more accurately the β5-synthesizing cells in spleen. A second, slightly larger, PCR product was amplified from mouse brain and lung using the β5B-specific primers, and this was shown by sequencing to be a novel sequence unrelated to β5 and was probably amplified as PCR artifact.

To determine the relative abundance of β5A and β5B, fluorescence-assisted RT-PCR was carried out on mouse-tissue and macrophage RNA samples using a pair of primers common to both β5A and β5B. The expected β5A and β5B PCR products were 536 bp and 507 bp, respectively, and the fluorescence intensities associated with the corresponding PCR products were measured using the GeneScan 2.1 software. Figure 5 shows a typical presentation of the GeneScan results in which the fluorescence intensities of the PCR products are represented by the areas of the peaks. The β5B PCR product is generally 20–50-fold lower than that of β5A in liver, brain, colon, kidney and lung, and is not detectable by this method in spleen, uterus, skeletal muscle and heart (results not shown).

Expression of mouse β5 in monocytic cells

Human β5 is known to be expressed in monocytes as a differentiation marker. It is not expressed in circulating monocytes and is only induced to express upon monocyte adhesion (S. M. Tan and J. Lu, unpublished work). PMA stimulation has been shown to up-regulate β5 expression in the human monocytic cell line, the THP-1 cells. RT-PCR was then performed on RNA isolated from the mouse monocytic cell line, J774 cells in which β5A mRNA was detected (Figure 4, lane 1). The expression of β5A mRNA in J774 cells was not clearly up-regulated by PMA,

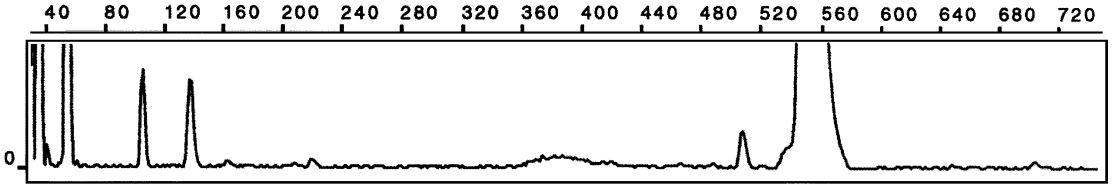

Figure 5 Quantification of β5A and β5B mRNA expression by GeneScan

RT-PCR was carried out for 30 cycles using primers β5-7F and β5-1R, which are common to both β5A and β5B. Fluorescence-labelled dCTP, [R100]dCTP, was incorporated into the reactions in a ratio of 1:200 to unlabelled dCTP. PCR was performed in 15 μl volume and 1 μl was loaded on to a 4.25% gel and separated on an Applied BioSystems DNA sequencer. The electrophoresis profiles were analysed by the GeneScan 2.1 software for the fluorescence intensities of each PCR product. The overlining scale represents an electrophoresis standard to indicate the sizes of the fluorescence-labelled PCR products.

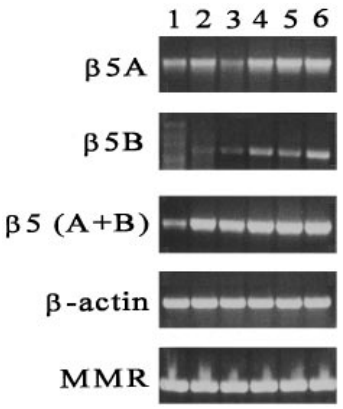

Figure 6 Detection of β5 mRNA expression in mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated and cultured in tissue-culture plates as described in Materials and methods. Non-adherent cells were removed by washing after incubation in tissue-culture plates for 1 h and macrophages adherent to tissue-culture plastic were harvested at serial time points after washing, i.e. 0 (lane 2), 2 (lane 3), 4 (lane 4), 8 (lane 5) and 24 h (lane 6). RNA was isolated from these macrophage samples for RT-PCR. RNA was also isolated from freshly isolated, non-adherent macrophages for similar RT-PCRs (lane 1). RT-PCRs were carried out using primer pairs β5A-as/β5-1R (β5A), β5B-as/β5-1R (β5B), β5-3F/β5-5R [β5 (A + B)], MR-F/MR-R (MMR), and β-actin primers (β-actin). The PCR products (10 μl) were examined on a 1% (w/v) agarose gel.

probably because these cells are well differentiated (results not shown). β5B mRNA was not detected in J774 cells at a significant level (Figure 4, lane 1), even after PMA stimulation for 24 h (results not shown).

β5A mRNA was found to be highly expressed in mouse peritoneal macrophages. However, β5B mRNA was not detectable in non-adherent peritoneal macrophages unless the cells were adhered to plastic tissue-culture plates, which also up-regulated β5A mRNA expression (Figure 6). The mRNA of the macrophage-specific protein, MMR, was highly expressed in peritoneal macrophages. Adhesion of the macrophages did not further up-regulate the expression of the MMR mRNA (Figure 6), showing that these macrophages were well differentiated. β5A mRNA in peritoneal macrophages was, as in other mouse tissues, detected at much higher levels than β5B mRNA (Figure 6). This study has, for the first time, shown the expression of β5 mRNA in natural macrophages. The up-regulation of β5B mRNA expression after macrophage adhesion was also determined by GeneScan using RNA samples stated in Figure 6 (results not shown). β5B PCR product was not detectable in non-adherent macrophages. It was detectable 2 h after adhesion and the expression increased by approx. 6-fold 24 h after adhesion (results not shown).

DISCUSSION

The isolated mouse β5 cDNA clones revealed two types of sequence, β5A and β5B, which are otherwise identical except that a 29 bp deletion is present in the β5B cDNA (clone MB26). This deletion resulted in a cytoplasmic C-terminus in mouse β5B that diverged from those of mouse β5A and human β5 sequences. Sequencing of a mouse β5 gene fragment showed that the 29 bp sequence is positioned at an exon/intron boundary, which was retained as part of the 5′ exon in β5A but was alternatively spliced out as part of the 3′ intron in β5B (Figure 3A).

Alternatively spliced variants have been described for both the α and β subunits of integrins. Sites of alternative splicing are found in both the extracellular and cytoplasmic domains [28–35]. Some of the variants are expressed in a tissue-specific and/or developmentally regulated manner and have been shown to have distinct functions [30–33]. Four β1 variants have been characterized, i.e. β1, β1B, β1C and β1D, which are different in their cytoplasmic C-termini as results of alternative splicing [32–35]. β1D is only expressed in cardiac and skeletal muscle and β1C has been shown to inhibit cell proliferation [36,37]. Integrin subunits α3, α6 and α7 are alternatively spliced in the cytoplasmic domains following a similar pattern which yields two isoforms, A and B, for each subunit [38], whereas in addition, α7 has a third similarly spliced variant (α7C) [31]. Both the α7 and β7 subunits are also alternatively spliced in the ligand-binding extracellular domains [29,38]. This is the first report of alternative splicing of the integrin β5 mRNA, although cDNA cloning of the baboon β5 subunit indicated that human β5 mRNA sequences contained a putative alternative splicing site in the 3′ untranslated region [39]. The three mouse β5 cDNA clones are indeed heterogeneous in polyadenylation sites by having 1–8 extra nucleotides at the 3′ end that are clearly not due to alternative splicing (Figure 2).

The integrins comprise a large family of heterodimers as a result of pairing between different α and β subunits, and alternative splicing has further diversified this family of adhesion receptors in both structure and functions. Alternative splicing in the extracellular domains may yield variants with different affinities and/or specificities for ECM and other ligands, which may be important in the regulation of cell migration. Variation in the cytoplasmic domains could allow the same type of integrin to trigger different cellular responses in the same extracellular environment. Together with tissue-specific and/or developmentally regulated expression of the different variants, alternative splicing has provided a dynamic mechanism for the fine adjustment of integrin functions in many biological processes.

Mouse β5 mRNA was detected in all the 9 tissues examined (Figure 4). However, β5B mRNA was detected at much lower levels in these tissues than β5A and was not detectable in spleen. This was not due to the β5B-specific RT-PCR being less sensitive than that for β5A, since both PCR assays showed similar sensitivity on cloned β5A and β5B cDNA. β5A mRNA was also detected in the mouse monocytic cell line J774, but no specific PCR product was detected for β5B in these cells (Figure 4). β5A, but not β5B, was detected in freshly isolated mouse peritoneal macrophages (Figure 6). Adhesion of these macrophages to tissue-culture plastic up-regulated β5A, and also induced β5B, mRNA expression. Similar up-regulation of human β5 mRNA has been observed in PMA-stimulated THP-1 cells, a human monocytic cell line (S. M. Tan and J. Lu, unpublished work). In general, the β5A and β5B isoforms are co-expressed in almost all mouse tissues, although β5B is expressed at much lower levels. Both β5 isoforms are expressed in adherent peritoneal macrophages. However, there is no evidence as to whether the two isoforms may be preferentially expressed on different cell types in certain mouse tissues.

The alternative splicing event in mouse integrin β5 has, however, not been reported in human β5 despite the presence of an intron at an identical position in the human β5 gene (Figure 3). Differences have indeed been reported in known human β5 cDNA sequences [19,21]. The most prominent is the presence of either one FNK sequence or two tandem FNK sequences in the cytoplasmic domains. Sequencing of the human β5 gene fragment showed no evidence that this sequence variation was due to alternative splicing (Figure 3B). The functional impact(s) of sequence divergency in the cytoplasmic domains of mouse and human integrin subunits β5 require further investigation of the

different variants. The characterization of the mouse β5 cDNA sequences should assist studies on the tissue/cell origin(s) of the integrin subunit in adult and embryonic mouse tissues and on the establishment of mouse models to assess the functions of this integrin subunit. The role of β5 in monocyte/macrophage functions also deserves further investigation.

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