# The integrin $\alpha_v\beta_3$ is a receptor for the latency-associated peptides of transforming growth factors $\beta_1$ and $\beta_3$

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The integrins $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ have all recently been shown to interact with the RGD motif of the latency-associated peptide (LAP$\beta_1$) of transforming growth factor $\beta_1$ (TGF$\beta_1$), with binding to $\alpha_v\beta_6$ and $\alpha_v\beta_8$ leading to TGF$\beta_1$ activation. Previously it has been suggested that the remaining $\alpha_v$ integrin, $\alpha_v\beta_3$, does not interact with LAP$\beta_1$. However, here we show clearly that $\alpha_v\beta_3$ does indeed interact with the LAP$\beta_1$ RGD motif. This interaction is similar to other $\alpha_v\beta_3$ ligands in terms of the cations required for adhesion, the concentrations of LAP$\beta_1$ required for binding and the ability of a small-molecule inhibitor of $\alpha_v\beta_3$, SB223245, to block the interaction. Using glutathione S-transferase fusion proteins we have mapped a minimal integrin-binding loop in LAP$\beta_1$ and then used this approach to probe the integrin-binding properties of the equivalent loops in LAP$\beta_2$ and LAP$\beta_3$. We show that the RGD motif of LAP$\beta_3$ also interacts with $\alpha_v\beta_3$, in addition to $\alpha_v\beta_6$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$, whereas the corresponding loop in LAP$\beta_2$ does not interact with these integrins. These observations therefore correct a previously reported inaccuracy in the literature. Furthermore, they are important as they link $\alpha_v\beta_3$ and TGF$\beta$, which may have implications in cancer and a number of inflammatory and fibrotic diseases where expression of both proteins has been documented.

Key words: adhesion, binding, cell, fibronectin, vitronectin.

## INTRODUCTION

The transforming growth factor $\beta$ (TGF$\beta$) family of cytokines affect a variety of cellular processes, including cell proliferation, extracellular-matrix synthesis, integrin expression, immune function and development [1–4]. The mammalian family consists of three members, namely TGF$\beta_1$, TGF$\beta_2$ and TGF$\beta_3$, which are secreted as heterotrimeric complexes derived from two genes. Each TGF$\beta$ gene encodes a protein of 390–414 amino acids which is processed into two polypeptide chains, a 249–282-amino-acid N-terminal subunit and a 112-amino-acid C-terminal subunit. The C-terminal subunit forms the active TGF$\beta$ cytokine, which is a 25 kDa homodimer. The N-terminal subunit is known as the latency-associated protein (LAP) and forms a homodimer of approx. 90 kDa. Each LAP dimer forms a non-covalent complex with a TGF$\beta$ dimer termed the small latent complex (SLC), retaining the TGF$\beta$ cytokine in an inactive conformation. The SLC is usually complexed with a protein called the latent TGF$\beta$-binding protein (LTBP), encoded by a separate gene, forming a large latent complex (LLC), predominantly found in the extracellular matrix. The sequestering of TGF$\beta$ into these inactive complexes provides a mechanism for the fine regulation of the many biological effects mediated by these cytokines [3,4].

There has been much interest in delineating the mechanisms which liberate TGF$\beta$ activity from the latent complexes. Both proteolytic and non-proteolytic mechanisms for activating latent TGF$\beta_1$ have been described. Proteases implicated in TGF$\beta_1$ activation include plasmin [5,6], urokinase-type and tissue-type plasminogen activators [7,8], matrix metalloproteases 2 and 9 (MMP-2 and MMP-9) [9] and cathepsin [10], which elicit effects by proteolytic degradation of LAP$\beta_1$. Non-proteolytic activation mechanisms involve interactions with LAP$\beta_1$, inducing a conformational change and exposing the receptor-binding site in TGF$\beta_1$. Both thrombospondin 1 [11–13] and the integrins $\alpha_v\beta_6$ [14], $\alpha_v\beta_1$ [15], $\alpha_v\beta_8$ [16] and, weakly, $\alpha_v\beta_5$ [15] bind to LAP$\beta_1$, and the binding of thrombospondin 1, $\alpha_v\beta_6$, or $\alpha_v\beta_8$ to LAP$\beta_1$ has been shown to result in TGF$\beta_1$ activation *in vitro*. The thrombospondin and $\alpha_v\beta_6$ mechanisms have been further validated *in vivo* by analysis of thrombospondin 1- [17] and $\beta_6$- [18,19] knockout mice, which show features that may be attributable to a loss of TGF$\beta_1$ activity.

Both LAP$\beta_1$ and LAP$\beta_3$ contain RGD motifs, whereas LAP$\beta_2$ does not. As LAP$\beta_1$ has been shown to bind to a number of RGD-dependent integrins (all members of the $\alpha_v$-containing family except $\alpha_v\beta_3$) [14–16], we examined whether it bound to two other RGD-dependent integrin receptors, $\alpha_5\beta_1$ and $\alpha_v\beta_3$. This analysis clearly showed that the remaining $\alpha_v$-containing integrin, $\alpha_v\beta_3$, bound to LAP$\beta_1$, despite evidence in the literature to the contrary [15]. We demonstrate that the binding of LAP$\beta_1$ by $\alpha_v\beta_3$ is RGD-dependent, and has similar characteristics to other $\alpha_v\beta_3$ ligands; namely the cations required for adhesion, the concentrations of LAP$\beta_1$ required for binding and the ability of a small-molecule inhibitor of $\alpha_v\beta_3$, SB223245, to block the interaction. We also show that $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ bind to LAP$\beta_3$ in an RGD-dependent manner, but not to LAP$\beta_2$, presumably due to the absence of an RGD site. These data are important, particularly given that $\alpha_v\beta_3$ has previously been discounted as a receptor for LAP$\beta_1$ [15]. Biologically, the $\alpha_v\beta_3$ interactions with LAP$\beta_1$ and LAP$\beta_3$ may be clinically important

Abbreviations used: GST, glutathione S-transferase; LAP, latency-associated peptide; MMP, matrix metalloprotease; SLC, small latent complex; TGF$\beta$, transforming growth factor $\beta$.

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in several diseases including cancer, inflammatory and fibrotic diseases.

## EXPERIMENTAL

### Materials

Antibodies were obtained from Chemicon (LM609, P1F6, 10D5), Immunotech (4B4, SAM1, AMF7, 69-6-5) and Sigma (MOPC21, UPC10). The $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor SB223245 [20], the $\alpha_4\beta_1$ inhibitor BIO1211 [21] and the $\alpha_v\beta_6$ inhibitor GW603365A (acetyl-RTDLDSLRT-$NH_2$) [22] were obtained from within GlaxoSmithKline. All oligonucleotides were obtained from Life Technologies. Fibrinogen was purchased from Calbiochem and baculovirus-expressed full-length LAP$\beta_1$ was from Sigma. Vitronectin was purified from out-dated plasma as described in [23,24], whereas $\alpha_v\beta_3$ protein was obtained from Chemicon. The K562, A549 and A375M cell lines were originally obtained from the A.T.C.C., whereas DX3 human melanoma cells were available within GlaxoSmithKline [25]. K562-derived cells were cultured in a 1:1 mixture of RPMI 1640 (Hepes modification; Life Technologies)/Dulbecco's modified Eagle's medium (Hepes modification; Sigma), supplemented with L-glutamine (Life Technologies) and 10% fetal calf serum (Gibco). Geneticin (Life Technologies) was added to 1 mg/ml for all transfectants. A375M and A549 cells were cultured in RPMI 1640 (Hepes modification), whereas DX3 cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Life Technologies), all supplemented with L-glutamine and 10% fetal calf serum.

### Cloning of integrin subunits

The integrin chains $\alpha_v$, $\beta_3$, $\beta_5$ and $\beta_6$ were all cloned by reverse transcriptase PCR of RNA prepared from tissue sources using the Promega RNAgents total RNA isolation system. Oligo(dT) chromatography was subsequently used to purify polyadenylated RNA from the total RNA. $\alpha_v$, $\beta_3$ and $\beta_5$ were cloned using RNA from DX3 melanoma cells, and $\beta_6$ from HT29 colon carcinoma cells. The primers for the $\alpha_v$ and $\beta_6$ cDNAs contained a *Bam*HI restriction site proximal to the initiation codon and an *Eco*RI restriction site distal to the stop codon. For $\beta_3$, a 5′ *Hin*dIII site was included together with a 3′ *Xba*I site. For $\beta_5$, *Eco*RI sites were included at both the 5′ and 3′ ends. For sequence verification, the $\alpha_v$, $\beta_3$ and $\beta_5$ cDNAs were cloned into pSP64 (Promega), whereas the $\beta_6$ cDNA was inserted into PCR-SCRIPT (Stratagene). All sequences were in agreement with the EMBL/Genbank accession numbers for these cDNAs.

### Generation of K562 cell lines stably expressing $\alpha_v\beta_3$, $\alpha_v\beta_5$ or $\alpha_v\beta_6$

Integrin subunit cDNAs were cloned into pCDNA3 (Invitrogen) using the restriction sites incorporated during cloning. The pcDNA3-$\alpha_v$ plasmid was co-transfected with pcDNA3-$\beta_3$, pcDNA3-$\beta_5$ or pcDNA3-$\beta_6$ into $2\times10^6$ K562 cells using LIPOFECTAMINE™ plus (Life Technologies) according to the manufacturer's instructions. After a 48 h recovery Geneticin was added to the culture medium at 1 mg/ml to select for stably transfected cells. K562-$\alpha_v\beta_3$ and K562-$\alpha_v\beta_5$ transfectants both adhered to the flask plastic during selection, probably due to vitronectin and other matrix components in the serum coating the plastic. Analysis of K562-$\alpha_v\beta_3$ adherent cells by flow cytometry [26] showed high $\alpha_v\beta_3$ expression on this population (Table 1), which was then used for all experiments. K562-$\alpha_v\beta_5$ adherent cells showed biphasic $\alpha_v\beta_5$ expression on flow-cytometric analysis, so were further selected by clonal dilution to yield single clones with reproducible $\alpha_v\beta_5$ expression, of which one clone (clone 19) is shown for all experiments. Two further clones showed similar results in adhesion assays [clones 6 and FF (results not shown)]. K562-$\alpha_v\beta_6$ cells in suspension displayed low $\alpha_v\beta_6$ expression by flow-cytometric analysis, so were also selected by clonal dilution with one clone (clone 3) shown for all experiments. A further clone (clone 22) showed similar results in adhesion assays (results not shown). All three cell types were characterized, and displayed expected ligand-binding activities relative to the integrin repertoire expressed at the cell surface (results not shown). Flow cytometry analysis of all cell lines used in this study is shown in Table 1. Further analysis examined $\alpha_v\beta_1$ expression on the transfected lines by immunoprecipitation and Western blot analysis (as no $\alpha_v\beta_1$-heterodimer-specific antibody is available). This showed the presence of $\alpha_v$ in the $\beta_1$ immunoprecipitate from the K562-$\alpha_v\beta_5$, but not the K562-$\alpha_v\beta_3$ or K562-$\alpha_v\beta_6$ cell lines (results not shown), indicating the presence of the $\alpha_v\beta_1$ integrin heterodimer in K562-$\alpha_v\beta_5$ cells only.

**Table 1 Analysis of integrin expression on cell lines by flow cytometry**

Antibody clones used were 4B4 ($\beta_1$), AMF7 ($\alpha_v$), LM609 ($\alpha_v\beta_3$), P1F6 ($\alpha_v\beta_5$), 10D5 ($\alpha_v\beta_6$) and SAM-1 ($\alpha_5$), with a goat anti-mouse IgG secondary antibody labelled with Alexa 488, as described in [26]. Data are presented as mean relative fluorescence values. Antibody isotype controls used were MOPC21 (mouse $IgG_1$) and UPC10 (mouse $IgG_{2a}$), and were given mean fluorescence values of 1.

| | Mean relative fluorescence | | | | | |
|---|---|---|---|---|---|---|
| Cell line | $\beta_1$ | $\alpha_v$ | $\alpha_v\beta_3$ | $\alpha_v\beta_5$ | $\alpha_v\beta_6$ | $\alpha_5$ |
| K562 | 13 | 2.6 | 1.2 | 0.95 | 0.83 | 11 |
| K562-$\alpha_v\beta_3$ | 14 | 106 | 93 | 0.96 | 2.2 | 13 |
| K562-$\alpha_v\beta_5$ | 12 | 6.2 | 0.91 | 4.9 | 0.69 | 7.3 |
| K562-$\alpha_v\beta_6$ | 21 | 8.3 | 0.71 | 0.97 | 8.2 | 19 |
| A549 | 352 | 90 | 2.2 | 28 | 0.68 | 15 |
| A375M | 178 | 83 | 85 | 30 | 0.74 | 23 |
| DX3 | 397 | 86 | 45 | 46 | 0.75 | 34 |

### Cloning and expression of matrix proteins

Glutathione S-transferase (GST) fusion proteins corresponding to the RGD cell-adhesion domains of tenascin (amino acids 802–891) [27], fibronectin (fibronectin type III repeats 8–10, amino acids 1266–1540), LAP$\beta_1$ (amino acids 242–252), LAP$\beta_2$ (amino acids 259–269) and LAP$\beta_3$ (amino acids 259–269) were generated. Tenascin and fibronectin fragments were generated by reverse-transcriptase PCR from HT29 and DX3 total RNA respectively; other constructs were generated by oligonucleotide insertion. All constructs were cloned into pGEX-2T via the *Bam*HI/*Eco*RI sites except fibronectin, which had a 5′ *Xba*I site. Details of all the oligonucleotides used in this study are available from S.B.L. on request. Recombinant proteins were over-expressed in, and purified from, *Escherichia coli* XL1-Blue cells according to standard protocols and as described previously [26], and stored in PBS at −70 °C. Protein concentrations were determined using the Coomassie plus protein assay reagent (Pierce). The integrity and purity of each preparation were analysed by SDS/PAGE and shown to be greater than 95% for all proteins except GST-tenascin, which was subject to some apparent proteolytic fragmentation, but was still > 70% pure intact protein. Most proteins were also verified by MS.

### Adhesion assays

These assays were performed broadly as described in [26]. Either $3\times10^5$ cells (all K562-derived cells) or $1\times10^5$ cells (DX3, A549

and A375M cells) were used per well. Desired cations (either 2 mM $MgCl_2$ or 0.5 mM $MnCl_2$ as indicated in the Figure legends) and other additions were made as indicated. Cells were visualized and quantified using Crystal Violet staining (Sigma). Each Figure is representative of at least three identical experiments and all results are presented as means ± S.D. from duplicate determinations. Data were fitted to a four-parameter logistic equation for $IC_{50}$ determinations.

### OR*IGEN*® assays

Epoxy beads (Dynal) were pre-coated with $\alpha_v\beta_3$ protein as per the manufacturer's recommendations. Protein ligands were biotinylated with NHS-Biotin (Perbio) as per the manufacturer's protocol. Streptavidin tag was obtained from *IGEN*. The assays were performed in a buffer containing 25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 % Tween-20 and 0.5 % BSA in a 150 $\mu$l total well volume. Plates were shaken for 4 h and read on an *IGEN* M8 Analyser. Each Figure is representative of at least three identical experiments and all results are presented as means ± S.D. from duplicate determinations. Data were fitted to a four-parameter logistic equation for $IC_{50}$ determinations.

## RESULTS

### $\alpha_v\beta_3$ interacts with LAP$\beta_1$

As LAP$\beta_1$ has been shown to bind to a number of RGD-dependent integrins (all members of the $\alpha_v$-containing family except $\alpha_v\beta_3$) [14–16], we examined whether it bound to two other RGD-dependent integrin receptors, $\alpha_5\beta_1$ and $\alpha_v\beta_3$. The study of specific integrin–ligand interactions is often complicated by the presence of multiple integrins on cell lines. To alleviate this problem we have used K562 cells, which only express the $\alpha_5\beta_1$ integrin endogenously. Wild-type K562 cells were transfected with $\alpha_v\beta_3$ (K562-$\alpha_v\beta_3$), $\alpha_v\beta_5$ (K562-$\alpha_v\beta_5$) and $\alpha_v\beta_6$ (K562-$\alpha_v\beta_6$) to generate stable cell lines. Figure 1 shows that in the presence of magnesium ions K562-$\alpha_v\beta_3$ and K562-$\alpha_v\beta_6$ cells bound well to LAP$\beta_1$, and the adhesion was inhibited by specific function-blocking antibodies to the transfected integrin heterodimer, demonstrating adhesion via $\alpha_v\beta_3$ and $\alpha_v\beta_6$ integrins respectively. K562-$\alpha_v\beta_5$ cells only adhered well to LAP$\beta_1$ in the presence of manganese ions, and this binding was mediated by both $\alpha_v\beta_5$ and $\alpha_v\beta_1$ integrins, consistent with small amounts of $\alpha_v\beta_1$ present on the K562-$\alpha_v\beta_5$ cell line (results not shown). Untransfected K562 cells (K562-WT on Figure 1) failed to adhere to LAP$\beta_1$ in the presence of either magnesium or manganese ions, confirming that $\alpha_5\beta_1$ does not recognize LAP$\beta_1$, despite it being an RGD-binding integrin. These results indicate that $\alpha_v\beta_3$ is a novel receptor for LAP$\beta_1$, and the inhibition data with antibodies for each cell line suggest that the adhesion event is not the result of the transfected integrin(s) modulating the activity of the endogenous $\alpha_5\beta_1$ integrin ([26,28] and S. B. Ludbrook, unpublished work).

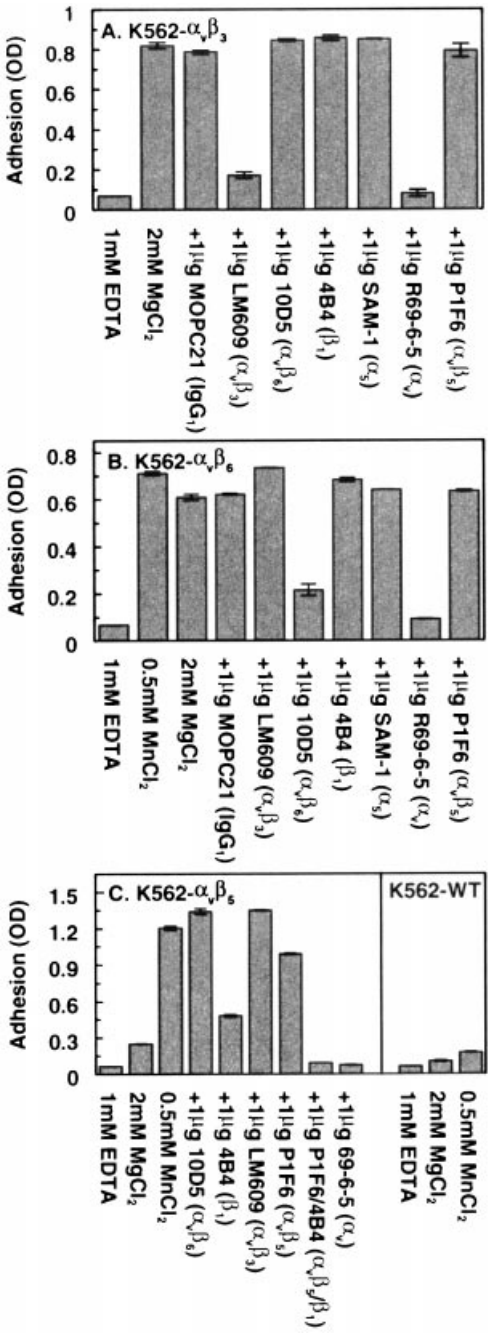


**Figure 1 Adhesion of K562-$\alpha_v\beta_3$, K562-$\alpha_v\beta_5$, K562-$\alpha_v\beta_6$ and wild-type K562 cells to LAP$\beta_1$**

K562-$\alpha_v\beta_3$ (**A**), K562-$\alpha_v\beta_6$ (**B**) and K562-$\alpha_v\beta_5$ and wild-type K562-WT (**C**) cells were allowed to attach to wells coated with 0.5 $\mu$g of LAP-$\beta_1$ under conditions as indicated (antibodies added at 1 $\mu$g/well). Antibody-inhibition data were generated using 2 mM $MgCl_2$ (K562-$\alpha_v\beta_3$ and K562-$\alpha_v\beta_6$) or 0.5 mM $MnCl_2$ (K562-$\alpha_v\beta_5$) as the cation.

### Comparison of LAP$\beta_1$ with other $\alpha_v\beta_3$ ligands

$\alpha_v\beta_3$ interacts with a wide variety of ligands, including fibrinogen [29,30], vitronectin [31–33], tenascin [27], fibronectin [34], osteopontin [35], L1 [36], cyr61 [37] and connective tissue growth factor [38]. Ligand recognition by $\alpha_v\beta_3$ can be mediated via both RGD [29,32] and non-RGD [30] motifs; in addition, the $\beta_3$-containing integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ contain a high-affinity calcium-binding site on the $\beta_3$ subunit that down-regulates ligand recognition [39,40]. Therefore, to examine the relevance of the interaction of K562-$\alpha_v\beta_3$ with LAP$\beta_1$, and to determine whether the RGD sequence in LAP$\beta_1$ was responsible, three different experiments were performed. Firstly, to determine the amounts of ligands required to support adhesion via $\alpha_v\beta_3$, we compared the adhesion of K562-$\alpha_v\beta_3$ cells to LAP$\beta_1$, vitronectin, fibrinogen, tenascin and a GST-LAP$\beta_1$ fragment fusion protein (LAP$\beta_1$

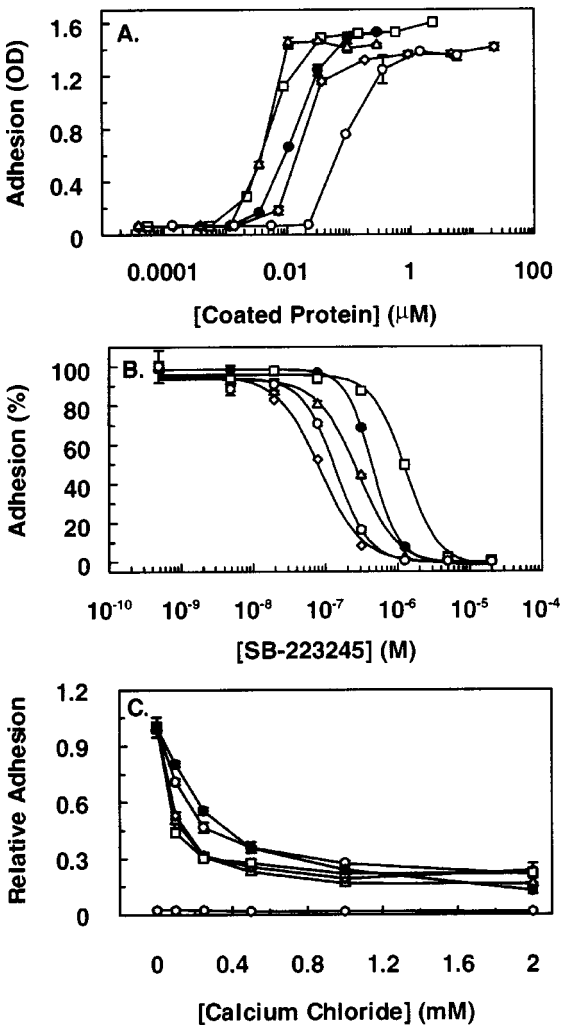


**Figure 2 Comparison of LAP$\beta_1$, fibrinogen, vitronectin and tenascin ligands for $\alpha_v\beta_3$**

(**A**) K562-$\alpha_v\beta_3$ cells were allowed to attach to wells coated with a range of concentrations of LAP$\beta_1$ (●), fibrinogen (◇), vitronectin (□), tenascin (△) and GST-LAP$\beta_1$ (○) in the presence of 2 mM $MgCl_2$. (**B**) K562-$\alpha_v\beta_3$ cells were allowed to attach to wells coated with LAP$\beta_1$ (0.5 $\mu$g/well), fibrinogen (2 $\mu$g), vitronectin (1 $\mu$g), tenascin (0.5 $\mu$g) or GST-LAP$\beta_1$ (0.5 $\mu$g) (symbols as in **A**) in the presence of 2 mM $MgCl_2$. The dose–response relationship with SB223245 was tested. Calculated $IC_{50}$ values were LAP$\beta_1$ (442 nM), vitronectin (1283 nM), fibrinogen (83 nM), tenascin (279 nM) and GST-LAP$\beta_1$ (139 nM). (**C**) As panel (**B**) with the inclusion of GST (0.5 $\mu$g; open hexagons). A dose–response relationship with $CaCl_2$ was performed. Adhesion levels were normalized, but were similar to those shown in (**A**), with adhesion to GST-coated wells of $0.074 \pm 0.004$ absorbance unit (i.e. no adhesion).

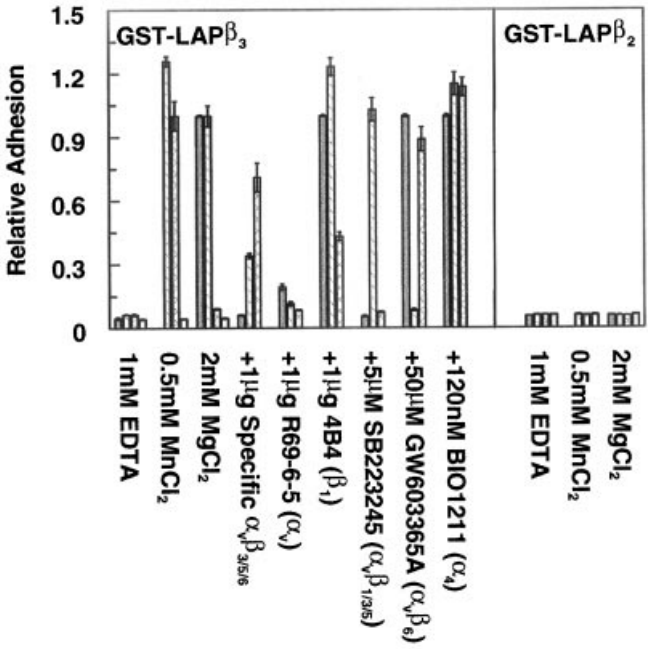


**Figure 3 Adhesion of K562-$\alpha_v\beta_3$, K562-$\alpha_v\beta_5$, K562-$\alpha_v\beta_6$ and wild-type K562 cells to GST-LAP$\beta_2$ and GST-LAP$\beta_3$**

K562-$\alpha_v\beta_3$ (shaded bars), K562-$\alpha_v\beta_6$ (hatched bars), K562-$\alpha_v\beta_5$ (cross-hatched bars) and wild-type K562-WT (white bars) cells were allowed to attach to wells coated with 0.5 $\mu$g of GST-LAP$\beta_3$ amino acids 259–269 or GST-LAP$\beta_2$ amino acids 259–269 under conditions as indicated (antibodies added at 1 $\mu$g/well). Antibody-inhibition data were generated using 2 mM $MgCl_2$ (K562-$\alpha_v\beta_3$ and K562-$\alpha_v\beta_6$) or 0.5 mM $MnCl_2$ (K562-$\alpha_v\beta_5$) as the cation. Specific $\alpha_v\beta_{3/5/6}$ indicates the addition of clone LM609 (anti-$\alpha_v\beta_3$), P1F6 (anti-$\alpha_v\beta_5$) or 10D5 (anti-$\alpha_v\beta_6$) to the specific K562-transfected cell line. SB223245 inhibits $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ at the concentration used. The complete absence of a bar indicates no test under that condition.

amino acids 242–252; $^{242}$GRRGDLATIHG$^{252}$) in a matrix titration experiment (Figure 2A). This analysis showed that both the full-length and GST fusion protein of LAP$\beta_1$ supported K562-$\alpha_v\beta_3$ cell adhesion at similar levels of protein coating to the other ligands used. Secondly, the interaction was analysed by testing the ability of a small-molecule inhibitor of $\alpha_v\beta_3$, SB223245 [20], to block the interaction of K562-$\alpha_v\beta_3$ cells with these ligands. SB223245 blocked $\alpha_v\beta_3$–ligand interactions with a range of $IC_{50}$ values. It was most active against the $\alpha_v\beta_3$–fibrinogen interaction ($IC_{50}$, 83 nM), relative to GST-LAP$\beta_1$ (139 nM), tenascin (279 nM), LAP$\beta_1$ (442 nM) and vitronectin (1283 nM; Figure 2B). Thirdly, we assessed the effects on adhesion to each of these ligands of increasing concentrations of $CaCl_2$. This showed that $CaCl_2$ markedly inhibited K562-$\alpha_v\beta_3$ cell adhesion to all ligands tested (tenascin, fibrinogen, LAP$\beta_1$, GST-LAP$\beta_1$, vitronectin) in a dose-dependent manner in agreement with the literature [39,40]. Collectively these data demonstrate that $\alpha_v\beta_3$ recognizes LAP$\beta_1$ with similar characteristics to those observed for other ligands such as fibrinogen, vitronectin and tenascin. By this analysis, of these four ligands, vitronectin is the best ligand for $\alpha_v\beta_3$ followed by LAP$\beta_1$ and tenascin, with fibrinogen being the weakest ligand. In addition, as the GST-LAP$\beta_1$ protein, which only contains an 11-amino acid fragment of LAP$\beta_1$ surrounding the RGD motif, has similar adhesion properties to intact LAP$\beta_1$, it is likely that this RGD-containing region of LAP$\beta_1$ is the sole determinant of $\alpha_v\beta_3$ binding.

## Integrin binding to LAP$\beta_2$ and LAP$\beta_3$

The majority of studies analysing the cell-adhesion properties of TGF$\beta$ LAPs have focused on TGF$\beta_1$, and hence LAP$\beta_1$ [14,15]. However, LAP$\beta_3$ also contains an RGD motif in the same region, whereas LAP$\beta_2$ contains SGD. Therefore we predicted that, like LAP$\beta_1$, LAP$\beta_3$ may interact with RGD-dependent integrins whereas LAP$\beta_2$ should not. Given the success of our minimized adhesion peptide approach with LAP$\beta_1$, we adopted a similar strategy to test the adhesion properties of LAP$\beta_3$ and LAP$\beta_2$. The same regions of LAP$\beta_2$ (amino acids 259–269 $^{259}$YTSGDQKTIKS$^{269}$) and LAP$\beta_3$ (amino acids 259–269

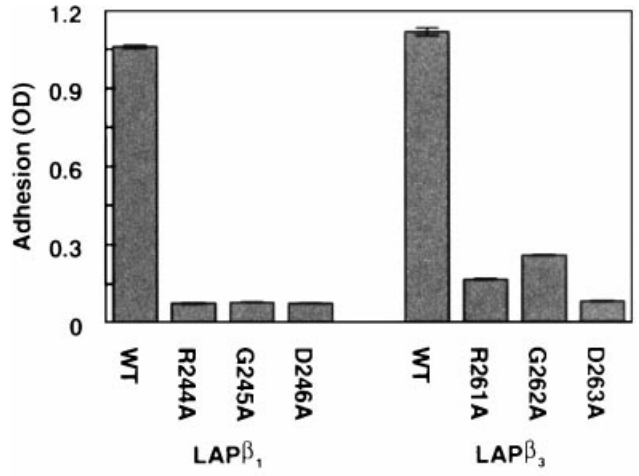


**Figure 4 Importance of the LAP$\beta_1$ and $\beta_3$ RGD sequences for K562-$\alpha_v\beta_3$ binding**

K562-$\alpha_v\beta_3$ cells were allowed to attach, in the presence of 2 mM $MgCl_2$, to wells coated with 0.5 $\mu$g of either wild-type GST-LAP$\beta_1$ or GST-LAP$\beta_3$ proteins (amino acids 242–252 and 259–269 respectively), or site-specific mutants in which individual residues of the RGD motif had been mutated to alanine.

$^{259}$HGRGDLGRLKK$^{269}$) were expressed as GST fusion proteins and tested for their ability to support adhesion of K562-$\alpha_v\beta_3$, K562-$\alpha_v\beta_5$ and K562-$\alpha_v\beta_6$ cells. The GST-LAP$\beta_3$ protein (amino acids 259–269) had similar adhesion properties to LAP$\beta_1$ in that it supported adhesion of K562-$\alpha_v\beta_3$ and K562-$\alpha_v\beta_6$ cells in the presence of magnesium via $\alpha_v\beta_3$ and $\alpha_v\beta_6$ respectively (Figure 3). K562-$\alpha_v\beta_5$ cells required manganese to further activate the integrins and adhered via a mixture of $\alpha_v\beta_5$ and $\alpha_v\beta_1$ integrins, whereas wild-type K562 cells failed to adhere under either cation condition. As predicted, there was no adhesion of any cell type tested to the GST-LAP$\beta_2$ protein (amino acids 259–269; Figure 3).

### LAP$\beta_1$ and LAP$\beta_3$ RGD mutants fail to support $\alpha_v\beta_3$ binding

To confirm the RGD-dependence of integrin binding to LAP$\beta_1$, GST fusion proteins were generated where $Arg_{244}$, $Gly_{245}$ or $Asp_{246}$ were separately exchanged for alanine and the mutant proteins tested for their ability to support adhesion of K562-$\alpha_v\beta_3$ cells (Figure 4). Mutation of any of the three RGD residues abolished binding, clearly demonstrating that $\alpha_v\beta_3$ interacts with LAP$\beta_1$ at the RGD motif. Similar results were obtained for LAP$\beta_3$ RGD mutant proteins (Figure 4). The LAP$\beta_1$ and LAP$\beta_3$ mutant proteins also failed to support adhesion of either K562-$\alpha_v\beta_6$ or K562-$\alpha_v\beta_5$ cells (results not shown).

### The interactions between $\alpha_v\beta_3$ and LAP$\beta_1$/$\beta_3$ are direct

Cell-adhesion data strongly suggested that the interactions between $\alpha_v\beta_3$ and LAP$\beta_1$/$\beta_3$ were direct, as the interaction was cation-dependent, susceptible to inhibition by specific integrin-blocking antibodies, RGD-dependent and inhibited by $CaCl_2$ addition. To confirm this we assessed the interaction between purified $\alpha_v\beta_3$ protein and LAP$\beta_1$, $\beta_2$ and $\beta_3$ using OR*IGEN*® assay technology. Firstly we tested whether $\alpha_v\beta_3$ protein could interact with the GST fusion proteins for LAP$\beta_1$, $\beta_2$ and $\beta_3$. A cation-dependent signal was observed with both LAP$\beta_1$ and

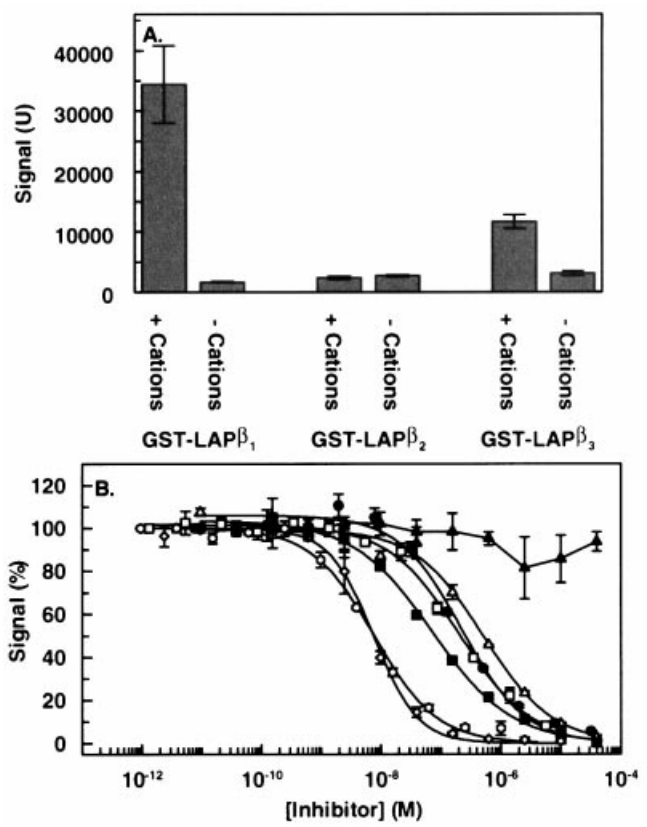


**Figure 5 Direct $\alpha_v\beta_3$ protein–protein interaction with GST-LAP$\beta_1$/$\beta_3$**

(**A**) $\alpha_v\beta_3$ protein (0.4 nM), immobilized to beads, was incubated with 10 nM biotinylated GST-LAP$\beta_1$, GST-LAP$\beta_2$ and GST-LAP$\beta_3$ proteins, together with 20 nM streptavidin tag, in OR*IGEN* assay buffer. The assay was performed in either the presence of cations (1 mM $MnCl_2$, 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) or in their absence (without cations but with 10 mM EDTA). The electrochemiluminescent signal represents the signal obtained in the instrument on binding. (**B**) Competing protein and small-molecule ligands were tested for inhibitory potencies in the $\alpha_v\beta_3$–LAP$\beta_1$ OR*IGEN* assay (0.4 nM $\alpha_v\beta_3$, 10 nM biotinylated GST-LAP$\beta_1$, 20 nM streptavidin tag) in a dose–response experiment. The following competitors were used: GST-tenascin (△; $IC_{50}$, 511 nM), GST-fibronectin (open hexagons; 7.6 nM), vitronectin (□; 201 nM), GST-LAP$\beta_1$ (●; 222 nM), GST-LAP$\beta_2$ (▲; $> 40\ \mu$M), GST-LAP$\beta_3$ (■; 72 nM) and SB 223245 (◇; 7.5 nM).

LAP$\beta_3$ but not LAP$\beta_2$ (Figure 5A), demonstrating specific direct binding between $\alpha_v\beta_3$ and LAP$\beta_1$/$\beta_3$. Secondly, as the amplitude of the signal at a given concentration of ligand is a poor indicator of differences in binding affinities (as it is influenced by ligand biotinylation levels and site of biotinylation), and to compare LAP$\beta_1$ and $\beta_3$ with other $\alpha_v\beta_3$ ligands in this cell-free system, we tested $\alpha_v\beta_3$ ligands as competing proteins in the $\alpha_v\beta_3$–LAP$\beta_1$ OR*IGEN*® assay (Figure 5B). Fibronectin was the most active competitor by this analysis ($IC_{50}$, 7.6 nM) and was of similar potency to the SB223245 small-molecule inhibitor (7.5 nM). The remaining ligands tested all had $IC_{50}$ values in a relatively narrow range, where LAP$\beta_1$ (222 nM) and LAP$\beta_3$ (72 nM) compared well with vitronectin (201 nM) and tenascin (511 nM), and LAP$\beta_2$ was inactive ($> 40\ \mu$M). These protein-based studies demonstrate firstly that the $\alpha_v\beta_3$ interaction with LAP$\beta_1$ and LAP$\beta_3$ is direct, secondly that LAP$\beta_1$ and LAP$\beta_3$ have similar binding affinities for the isolated $\alpha_v\beta_3$ receptor as other $\alpha_v\beta_3$ ligands, and thirdly that $\alpha_v\beta_3$ interacts with soluble LAP$\beta_1$ and LAP$\beta_3$, a form that may be encountered *in vivo*.

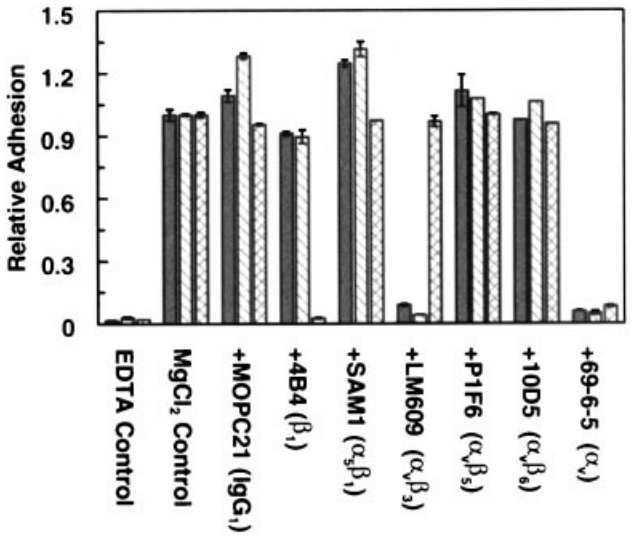


**Figure 6 Adhesion characteristics of DX3, A375M and A549 cell lines to LAP$\beta_1$**

DX3 (shaded bars), A375M (hatched bars) or A549 (cross-hatched bars) cells were allowed to attach to wells coated with 0.5 $\mu$g of LAP$\beta_1$. Adhesion occurred in 2 mM $MgCl_2$ or with the antibodies that are indicated under the histogram (1 $\mu$g/well).

### Native $\alpha_v\beta_3$-expressing cell lines interact with LAP$\beta_1$ via $\alpha_v\beta_3$

The K562-$\alpha_v\beta_3$ cell line used in this study expresses very high levels of $\alpha_v\beta_3$ integrin (see Table 1), relative to the levels of the $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrins on the K562-$\alpha_v\beta_5$ and K562-$\alpha_v\beta_6$ cell lines respectively. It is possible that the adhesion observed was an artifact of the extremely high levels of $\alpha_v\beta_3$ on the K562-$\alpha_v\beta_3$ cell line. To address this, we investigated the binding profile of cell lines reported to endogenously express the $\alpha_v\beta_3$ integrin to LAP$\beta_1$ and LAP$\beta_3$. The cell lines used were the human melanoma cell lines DX3 [25] and A375M [41], and the epithelial lung carcinoma cell line A549 [42,43]. Analysis of integrin expression on these cell lines showed expression of $\alpha_v\beta_3$ on the DX3 and A375M cells, but negligible levels on the A549 cells (Table 1). Analysis of the adhesion properties of these three cell lines to LAP$\beta_1$, using specific integrin-blocking antibodies, showed that A375M and DX3 cells adhered to LAP$\beta_1$ via $\alpha_v\beta_3$, whereas A549 cells adhered to LAP$\beta_1$ via $\alpha_v\beta_1$ (Figure 6). These results demonstrate firstly that cell lines which endogenously express the $\alpha_v\beta_3$ integrin (DX3 and A375M) do adhere to LAP$\beta_1$ via $\alpha_v\beta_3$. Secondly, the A549-binding data to LAP$\beta_1$ agree with the previous report as being mediated via $\alpha_v\beta_1$ [15], with the absence of an $\alpha_v\beta_3$ component being due to negligible levels of $\alpha_v\beta_3$ being expressed by this cell line. Similar results were obtained with cell adhesion to GST-LAP$\beta_1$ and GST-LAP$\beta_3$ (results not shown).

## DISCUSSION

Here we demonstrate that $\alpha_v\beta_3$ interacts with LAP$\beta_1$. This increases the number of integrins known to bind LAP$\beta_1$ to five, the entire $\alpha_v$ family ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$). K562-$\alpha_v\beta_3$ cells bind to LAP$\beta_1$ with similar characteristics to other known $\alpha_v\beta_3$ ligands, such as tenascin, fibrinogen and vitronectin, in terms of the ligand concentrations required for adhesion, the cations required for binding and the ability of a small-molecule inhibitor of $\alpha_v\beta_3$ to block the interaction. Like the other $\alpha_v$ integrin heterodimers, $\alpha_v\beta_3$ interacts with LAP$\beta_1$ via the RGD motif, as mutant proteins in which any of these residues are mutated to alanine fail to support adhesion of K562-$\alpha_v\beta_3$ cells. We have also characterized the integrin-binding properties of the corresponding regions in LAP$\beta_2$ and LAP$\beta_3$. We show that LAP$\beta_3$ is also a ligand for $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$, where binding also occurs through the RGD motif. Conversely, LAP$\beta_2$ is not a ligand for any of these integrins, presumably due to the lack of an RGD motif, which has been replaced by SGD. Further analysis of the interaction of $\alpha_v\beta_3$ with LAP$\beta_1$ and LAP$\beta_3$ demonstrated that, in a protein–protein assay, $\alpha_v\beta_3$ interacted with LAP$\beta_1$ and $\beta_3$, but not $\beta_2$, in a cation-dependent manner. This confirmed not only that the $\alpha_v\beta_3$–LAP$\beta_1/\beta_3$ interactions are direct, but also that $\alpha_v\beta_3$ can bind to LAP$\beta_1/\beta_3$ proteins when they are presented in a soluble form, a form that can be encountered *in vivo* in addition to matrix-associated LAP$\beta_1/\beta_3$. During the preparation of this manuscript, a report demonstrated that $\alpha_v\beta_6$ interacts with LAP$\beta_3$ [44]. Our data confirm this, and also establish that the LAP$\beta_3$ RGD motif possesses similar integrin-recognition properties to the LAP$\beta_1$ RGD sequence. Therefore $\alpha_v$ integrins interact with both LAP$\beta_1$ and LAP$\beta_3$, and have the potential to modulate the localization and possibly activation of TGF$\beta_1$ and TGF$\beta_3$, but not directly TGF$\beta_2$.

Although these data clearly show an interaction between $\alpha_v\beta_3$ and LAP$\beta_1$ and LAP$\beta_3$, a previous study [15] excluded $\alpha_v\beta_3$ as a receptor for LAP$\beta_1$, as A549 cells, which reportedly express $\alpha_v\beta_3$ [42,43], adhered to LAP$\beta_1$ via $\alpha_v\beta_1$. We examined the binding of the DX3, A375M and A549 cell lines, which are all reported to endogenously express $\alpha_v\beta_3$, to LAP$\beta_1$ and LAP$\beta_3$. All three cell lines adhered to LAP$\beta_1$ and LAP$\beta_3$. However, analysis using specific integrin-blocking antibodies showed that DX3 and A375M cells interacted via $\alpha_v\beta_3$, whereas A549 cells interacted via $\alpha_v\beta_1$, as reported in [15]. Flow cytometry analysis of these three cell lines clearly showed high expression levels of the $\alpha_v\beta_3$ integrin on both DX3 and A375M cell lines, but negligible levels on the A549 cells. These data demonstrate that cell lines which endogenously express the $\alpha_v\beta_3$ integrin use $\alpha_v\beta_3$ to interact with LAP$\beta_1$ and LAP$\beta_3$. They also indicate that A549 cells do not use $\alpha_v\beta_3$ to adhere to LAP$\beta_1$ and LAP$\beta_3$ because of very low $\alpha_v\beta_3$ expression levels. The previous report [15] did not show $\alpha_v\beta_3$ expression levels on the A549 cells used and furthermore $\alpha_v\beta_3$ binding to known ligands was not confirmed. However, it is clear in the literature that A549 cells have been shown to adhere to fibrinogen via $\alpha_v\beta_3$ [42,43]. Our A549 cells adhered very poorly to fibrinogen in an $\alpha_5\beta_1$-dependent manner (results not shown), suggestive of either $\alpha_5\beta_1$ being the fibrinogen receptor [45] or the adhesion resulting from the low levels of contaminating fibronectin in the fibrinogen preparation.

$\alpha_v\beta_3$ plays an important role in a variety of physiological and patho-physiological processes, including tumour angiogenesis, rheumatoid arthritis and a number of inflammatory and repair processes [46]. This is reflected by the extensive repertoire of known $\alpha_v\beta_3$ ligands, which include the matrix proteins vitronectin [31–33], fibronectin [34] and tenascin [27], and the cytokines osteopontin [35], cyr61 [37] and connective tissue growth factor [38]. The interactions between $\alpha_v\beta_3$ and distinct ligands may drive different aspects of these biological processes. For example, the $\alpha_v\beta_3$–osteopontin interaction has been shown to be important in mediating the Th1 response via the regulation of interleukin-12 expression from macrophages [47]. In many diseases involving $\alpha_v\beta_3$, up-regulation of TGF$\beta$ protein or activity has also been described, such as vascular disorders [48], diabetic retinopathy [49], scleroderma [50] and rheumatoid arthritis [51,52]. It will therefore be interesting to determine what role, if any, the $\alpha_v\beta_3$ interactions with LAP$\beta_1$ and LAP$\beta_3$ play in these diseases.

The interaction between $\alpha_v\beta_3$ and LAP$\beta_1$ is important, as it provides a mechanism through which TGF$\beta_1$ and TGF$\beta_3$ can be localized to the surface of a number of cell types. For example, $\alpha_v\beta_3$ is expressed on angiogenic/activated endothelium, fibroblasts, macrophages, T-cells and smooth-muscle cells [46]. This interaction may be sufficient to activate TGF$\beta_1$ and TGF$\beta_3$ via a conformational change in LAP, as shown previously for $\alpha_v\beta_6$ [14]. Alternatively, binding to $\alpha_v\beta_3$ may localize LAP$\beta_1$ and LAP$\beta_3$ to a proteolytically rich environment at the cell surface, resulting in TGF$\beta$ activation. A similar function has been ascribed to the binding of LAP$\beta_1$ to thrombospondin [53] and the mannose 6-phosphate receptor [54]. Moreover, $\alpha_v\beta_8$ has recently been shown to generate TGF$\beta_1$ activity by localizing the LAP$\beta_1$–TGF$\beta_1$ SLC to the cell surface, thereby permitting membrane-type 1–MMP proteolytic cleavage of LAP$\beta_1$ to liberate the TGF$\beta_1$ cytokine [16]. Interestingly, $\alpha_v\beta_3$ expression is associated with enhanced cell-surface proteolytic activity by MMP-2 [55], for which LAP$\beta_1$ has been shown to be a substrate [9], thereby presenting a potential mechanism to generate TGF$\beta_1$ activity from the interaction described here. In addition, the interaction of LAP$\beta_1$ and LAP$\beta_3$ with $\alpha_v\beta_3$ may directly initiate $\alpha_v\beta_3$-specific intracellular signalling events commonly associated with integrin–ligand ligation [56]. All of these possibilities are currently the subject of an ongoing study, particularly whether $\alpha_v\beta_3$ either directly activates TGF$\beta$ by a conformational change in LAP$\beta_1/\beta_3$, or indirectly via protease activity. For the $\alpha_v\beta_6$ integrin there is clear evidence of the importance of TGF$\beta$ activity regulation from observations of $\beta_6$-knockout mice [18,19]. There is no such evidence from similar studies in $\alpha_v\beta_3$-knockout mice [57,58]. However, for the $\alpha_v\beta_3$-knockout mice the situation is complex as deletion of the $\beta_3$ subunit results in the absence of two integrins, $\alpha_v\beta_3$ and the platelet integrin $\alpha_{IIb}\beta_3$. Likewise, deletion of the $\alpha_v$ subunit results in the loss of five integrins, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$. Individuals with the $\alpha_v$ deletion fail to reach birth due to a placental defect [59], but the data would clearly fail to suggest $\alpha_v\beta_3$ activation of TGF$\beta$ activity, in part because $\alpha_v\beta_6$ is also deleted. Research on the $\beta_3$-knockout mice has shown thus far that the mice have a bleeding disorder phenotypically similar to human Glanzmann thrombasthenia, resulting predominantly from the loss of the platelet integrin $\alpha_{IIb}\beta_3$ [57]. In addition it has been shown that $\beta_3^{-/-}$ mice osteoclasts are dysfunctional in bone resorption, resulting from the loss of $\alpha_v\beta_3$ [58]. It would be of interest to determine in these mice if there is a defect in TGF$\beta$ activation, using appropriate models, for example the bleomycin model of pulmonary fibrosis. Alternatively, to our knowledge investigations of TGF$\beta$ activity in animal models treated with $\alpha_v\beta_3$ small molecule inhibitors or blocking antibodies have not been performed.

In summary, in contrast with the literature, we have identified the RGD motif of LAP$\beta_1$ as a new ligand for $\alpha_v\beta_3$. Furthermore we have shown that the RGD motif of LAP$\beta_3$ is recognized by $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$, whereas the non-RGD-containing LAP$\beta_2$ does not recognize any of these integrins. These interactions may be important in a number of aspects of TGF$\beta$ and $\alpha_v\beta_3$ biology, particularly the many disease processes associated with both $\alpha_v\beta_3$ and TGF$\beta$ up-regulation, such as cancer, rheumatoid arthritis and a variety of other diseases involving inflammation and repair.

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## REFERENCES


1 Massague, J. (1990) The transforming growth factor $\beta$ family. Annu. Rev. Cell Biol. **6**, 597–641

2 Blobe, G. C., Schiemann, W. P. and Lodish, H. F. (2000) Role of transforming growth factor $\beta$ in human disease. N. Engl. J. Med. **342**, 1350–1358

3 Piek, E., Heldin, C. H. and Ten Dijke, P. (1999) Specificity, diversity, and regulation in TGF-$\beta$ superfamily signaling. FASEB J. **13**, 2105–2124

4 Massagué, J., Blain, S. W. and Lo, R. S. (2000) TGF$\beta$ signaling in growth control, cancer, and heritable disorders. Cell **103**, 295–309

5 Lyons, R. M., Gentry, L. E., Purchio, A. F. and Moses, H. L. (1990) Mechanism of activation of latent recombinant transforming growth factor $\beta_1$ by plasmin. J. Cell Biol. **110**, 1361–1367

6 Sato, Y., Tsuboi, R., Moses, H. and Rifkin, D. B. (1990) Characterization of the activation of latent TGF-$\beta$ by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. J. Cell Biol. **111**, 757–763

7 Nunes, I., Shapiro, R. L. and Rifkin, D. B. (1995) Characterization of latent TGF-$\beta$ activation by murine peritoneal macrophages. J. Immunol. **155**, 1450–1459

8 Chu, T. M. and Kawinski, E. (1998) Plasmin, substilisin-like endoproteases, tissue plasminogen activator, and urokinase plasminogen activator are involved in activation of latent TGF-$\beta_1$ in human seminal plasma. Biochem. Biophys. Res. Commun. **253**, 128–134

9 Yu, Q. and Stamenkovic, I. (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-$\beta$ and promotes tumor invasion and angiogenesis. Genes Dev. **14**, 163–176

10 Lyons, R. M., Keski-Oja, J. and Moses, H. L. (1988) Proteolytic activation of latent transforming growth factor-$\beta$ from fibroblast-conditioned medium. J. Cell. Biol. **106**, 1659–1665

11 Schultz-Cherry, S. and Murphy-Ullrich, J. E. (1993) Thrombospondin causes activation of latent transforming growth factor-$\beta$ secreted by endothelial cells by a novel mechanism. J. Cell Biol. **122**, 923–932

12 Schultz-Cherry, S., Chen, H., Mosher, D. F., Misenheimer, T. M., Krutzsch, H. C., Roberts, D. D. and Murphy-Ullrich, J. E. (1995) Regulation of transforming growth factor-$\beta$ activation by discrete sequences of thrombospondin 1. J. Biol. Chem. **270**, 7304–7310

13 Ribeiro, S. M., Poczatek, M., Schultz-Cherry, S., Villain, M. and Murphy-Ullrich, J. E. (1999) The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-$\beta$. J. Biol. Chem. **274**, 13586–13593

14 Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J. D., Dalton, S. L., Wu, J., Pittet, J.-F., Kaminski, N., Garat, C., Matthay, M. A. et al. (1999) The integrin $\alpha_v\beta_6$ binds and activates latent TGF $\beta_1$: a mechanism for regulating pulmonary inflammation and fibrosis. Cell **96**, 319–328

15 Munger, J. S., Harpel, J. G., Giancotti, F. G. and Rifkin, D. B. (1998) Interactions between growth factors and integrins: latent forms of transforming growth factor-$\beta$ are ligands for the integrin $\alpha_v\beta_1$. Mol. Biol. Cell **9**, 2627–2638

16 Mu, D., Cambier, S., Fjellbirkeland, L., Baron, J. L., Munger, J. S., Kawakatsu, H., Sheppard, D., Broaddus, V. C. and Nishimura, S. L. (2002) The integrin $\alpha_v\beta_8$ mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-$\beta_1$. J. Cell Biol. **157**, 493–507

17 Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P. and Bouck, N. (1998) Thrombospondin-1 is a major activator of TGF-$\beta_1$ *in vivo*. Cell **93**, 1159–1170

18 Huang, X. Z., Wu, J. F., Cass, D., Erle, D. J., Corry, D., Young, S. G., Farese, R. V. and Sheppard, D. (1996) Inactivation of the integrin $\beta_6$ subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. J. Cell. Biol. **133**, 921–928

19 Huang, X., Wu, J., Zhu, W., Pytela, R. and Sheppard, D. (1998) Expression of the human integrin $\beta_6$ subunit in alveolar type II cells and bronchiolar epithelial cells reverses lung inflammation in $\beta_6$ knockout mice. Am. J. Respir. Cell Mol. Biol. **19**, 636–642

20 Keenan, R. M., Miller, W. H., Kwon, C., Ali, F. E., Callahan, J. F., Calvo, R. R., Hwang, S.-M., Kopple, K. D., Peishoff, C. E., Samanen, J. M. et al. (1997) Discovery of potent nonpeptide vitronectin receptor ($\alpha_v\beta_3$) antagonists. J. Med. Chem. **40**, 2289–2292

21 Lin, K. C., Ateeq, H. S., Hsiung, S. H., Chong, L. T., Zimmerman, C. N., Castro, A., Lee, W. C., Hammond, C. E., Kalkunte, S., Chen, L. L. et al. (1999) Selective, tight-binding inhibitors of integrin $\alpha4\beta_1$ that inhibit allergic airway responses. J. Med. Chem. **42**, 920–934

22 Kraft, S., Diefenbach, B., Mehta, R., Jonczyk, A., Luckenbach, G. A. and Goodman, S. L. (1999) Definition of an unexpected ligand recognition motif for $\alpha_v\beta_6$ integrin. J. Biol. Chem. **274**, 1979–1985

23 Yatohgo, T., Izumi, M., Kashiwagi, H. and Hayashi, M. (1988) Novel purification of vitronectin from human plasma by heparin affinity chromatography. Cell Struct. Funct. **13**, 281–292

24 Bittorf, S. V., Williams, E. C. and Mosher, D. F. (1993) Alteration of vitronectin. Characterization of changes induced by treatment with urea. J. Biol. Chem. **268**, 24838–24846

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

26 Barry, S. T., Ludbrook, S. B., Murrison, E. and Horgan, C. M. T. (2000) A regulated interaction between $\alpha 5\beta_1$ integrin and osteopontin. Biochem. Biophys. Res. Commun. **267**, 764–769

27 Yokosaki, Y., Monis, H., Chen, J. and Sheppard, D. (1996) Differential effects of the integrins $\alpha_9\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ on cell proliferative responses to tenascin. Roles of the $\beta$ subunit extracellular and cytoplasmic domains. J. Biol. Chem. **271**, 24144–24150

28 Blystone, S. D., Slater, S. E., Williams, M. P., Crow, M. T. and Brown, E. J. (1999) A molecular mechanism of integrin crosstalk: $\alpha_v\beta_3$ suppression of calcium/calmodulin-dependent protein kinase II regulates $\alpha_5\beta_1$ function. J. Cell Biol. **145**, 889–897

29 Cheresh, D. A. (1987) Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. Proc. Natl. Acad. Sci. U.S.A. **84**, 6471–6475

30 Yokoyama, K., Erickson, H. P., Ikeda, Y. and Takada, Y. (2000) Identification of amino acid sequences in fibrinogen gamma-chain and tenascin C C-terminal domains critical for binding to integrin $\alpha_v\beta_3$. J Biol. Chem. **275**, 16891–16898

31 Horton, M. A. (1997) The $\alpha_v\beta_3$ integrin ''vitronectin receptor''. Int. J. Biochem. Cell Biol. **29**, 721–725

32 Smith, J. W. and Cheresh, D. A. (1990) Integrin ($\alpha_v\beta_3$)-ligand interaction. Identification of a heterodimeric RGD binding site on the vitronectin receptor. J. Biol. Chem. **265**, 2168–2172

33 Kieffer, N., Fitzgerald, L. A., Wolf, D., Cheresh, D. A. and Phillips, D. R. (1991) Adhesive properties of the $\beta$ 3 integrins: comparison of GP IIb-IIIa and the vitronectin receptor individually expressed in human melanoma cells. J. Cell Biol. **113**, 451–461

34 Charo, I. F., Nannizzi, L., Smith, J. W. and Cheresh, D. A. (1990) The vitronectin receptor $\alpha_v\beta_3$ binds fibronectin and acts in concert with $\alpha_5\beta_1$ in promoting cellular attachment and spreading on fibronectin. J. Cell Biol. **111**, 2795–2800

35 Miyauchi, A., Alvarez, J., Greenfield, E. M., Teti, A., Grano, M., Colucci, S., Zambonin-Zallone, A., Ross, F. P., Teitelbaum, S. L., Cheresh, D. and Hruska, K. A. (1991) Recognition of osteopontin and related peptides by an $\alpha_v\beta_3$ integrin stimulates immediate cell signals in osteoclasts. J. Biol. Chem. **266**, 20369–20374

36 Felding-Habermann, B., Silletti, S., Mei, F., Siu, C. H., Yip, P. M., Brooks, P. C., Cheresh, D. A., O'Toole, T. E., Ginsberg, M. H. and Montgomery, A. M. (1997) A single immunoglobulin-like domain of the human neural cell adhesion molecule L1 supports adhesion by multiple vascular and platelet integrins. J. Cell Biol. **139**, 1567–1581

37 Kireeva, M. L., Lam, S. C. and Lau, L. F. (1998) Adhesion of human umbilical vein endothelial cells to the immediate-early gene product Cyr61 is mediated through integrin $\alpha_v\beta_3$. J. Biol. Chem. **273**, 3090–3096

38 Babic, A. M., Chen, C.-C. and Lau, L. F. (1999) Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin $\alpha_v\beta_3$, promotes endothelial cell survival, and induces angiogenesis *in vivo*. Mol. Cell. Biol. **19**, 2958–2966

39 Hu, D. D., Hoyer, J. R. and Smith, J. W. (1995) $Ca^{2+}$ suppresses cell adhesion to osteopontin by attenuating binding affinity for integrin $\alpha_v\beta_3$. J. Biol. Chem. **270**, 9917–9925

40 Cierniewska-Cieslak, A., Cierniewski, C. S., Blecka, K., Papierak, M., Michalec, L., Zhang, L., Haas, T. A. and Plow, E. F. (2002) Identification and characterization of two cation binding sites in the integrin $\beta_3$ subunit. J. Biol. Chem. **277**, 11126–11134

41 Gehlsen, K. R., Davis, G. E. and Sriramarao, P. (1992) Integrin expression in human melanoma cells with differing invasive and metastatic properties. Clin. Exp. Metastasis **10**, 111–120

42 Falcioni, R., Cimino, L., Gentileschi, M. P., D'Agnano, I., Zupi, G., Kennel, S. J. and Sacchi, A. (1994) Expression of $\beta_1$, $\beta_3$, $\beta_4$, and $\beta_5$ integrins by human lung carcinoma cells of different histotypes. Exp. Cell Res. **210**, 113–122

43 Odrljin, T. M., Haidaris, C. G., Lerner, N. B. and Simpson-Haidaris, P. J. (2001) Integrin $\alpha_v\beta_3$-mediated endocytosis of immobilized fibrinogen by A549 lung alveolar epithelial cells. Am. J. Respir. Cell Mol. Biol. **24**, 12–21

44 Annes, J. P., Rifkin, D. B. and Munger, J. S. (2002) The integrin $\alpha_v\beta_6$ binds and activates latent TGF$\beta_3$. FEBS Lett. **511**, 65–68

45 Suehiro, K., Gailit, J. and Plow, E. F. (1997) Fibrinogen is a ligand for integrin $\alpha 5\beta_1$ on endothelial cells. J. Biol. Chem. **272**, 5360–5366

46 Miller, W. H., Keenan, R. M., Willette, R. N. and Lark, M. W. (2000) Identification and *in vivo* efficacy of small-molecule antagonists of integrin $\alpha_v\beta_3$ (the vitronectin receptor). Drug Disc. Today **5**, 397–408

47 Ashkar, S., Weber, G. F., Panoutsakopoulou, V., Sanchirico, M. E., Jansson, M., Zawaideh, S., Rittling, S. R., Denhardt, D. T., Glimcher, M. J. and Cantor, H. (2000) Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science **287**, 860–864

48 Coleman, K. R., Braden, G. A., Willingham, M. C. and Sane, D. C. (1999) Vitaxin, a humanized monoclonal antibody to the vitronectin receptor ($\alpha_v\beta_3$), reduces neointimal hyperplasia and total vessel area after balloon injury in hypercholesterolemic rabbits. Circ. Res. **84**, 1268–1276

49 Spirin, K. S., Saghizadeh, M., Lewin, S. L., Zardi, L., Kenney, M. C. and Ljubimov, A. V. (1999) Basement membrane and growth factor gene expression in normal and diabetic human retinas. Curr. Eye Res. **18**, 490–499

50 Ludwicka, A., Ohba, T., Trojanowska, M., Yamakage, A., Strange, C., Smith, E. A., Leroy, E. C., Sutherland, S. and Silver, R. M. (1995) Elevated levels of platelet derived growth factor and transforming growth factor-$\beta_1$ in bronchoalveolar lavage fluid from patients with scleroderma. J. Rheumatol. **22**, 1876–1883

51 Lettesjo, H., Nordstrom, E., Strom, H., Nilsson, B., Glinghammar, B., Dahlstedt, L. and Moller, E. (1998) Synovial fluid cytokines in patients with rheumatoid arthritis or other arthritic lesions. Scand. J. Immunol. **48**, 286–292

52 Buckley, C. D., Amft, N., Bradfield, P. F., Pilling, D., Ross, E., Arenzana-Seisdedos, F., Amara, A., Curnow, S. J., Lord, J. M., Scheel-Toellner, D. and Salmon, M. (2000) Persistent induction of the chemokine receptor CXCR4 by TGF-$\beta_1$ on synovial T cells contributes to their accumulation within the rheumatoid synovium. J. Immunol. **165**, 3423–3429

53 Yehualaeshet, T., O'Connor, R., Green-Johnson, J., Mai, S., Silverstein, R., Murphy-Ullrich, J. E. and Khalil, N. (1999) Activation of rat alveolar macrophage-derived latent transforming growth factor $\beta$-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. Am. J. Pathol. **155**, 841–851

54 Godar, S., Horejsi, V., Weidle, U. H., Binder, B. R., Hansmann, C. and Stockinger, H. (1999) M6P/IGFII-receptor complexes urokinase receptor and plasminogen for activation of transforming growth factor-$\beta_1$. Eur. J. Immunol. **29**, 1004–1013

55 Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P. and Cheresh, D. A. (1996) Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha_v\beta_3$. Cell **85**, 683–693

56 Schlaepfer, D. D. and Hunter, T. (1998) Integrin signalling and tyrosine phosphorylation: just the FAKs? Trends Cell. Biol. **8**, 151–157

57 Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F. P., Coller, B. S., Teitelbaum, S. and Hynes, R. O. (1999) $\beta_3$-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival J. Clin. Invest. **103**, 229–238

58 McHugh, K. P., Hodivala-Dilke, K., Zheng, M.-H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, F. P., Hynes, R. O. and Teitelbaum, S. L. (2000) Mice lacking $\beta_3$ integrins are osteosclerotic because of dysfunctional osteoclasts. J. Clin. Invest. **105**, 433–440

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


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