Transmembrane-truncated $\alpha v\beta 3$ integrin retains high affinity for ligand binding: evidence for an ‘inside-out’ suppressor?

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The molecular mechanisms of $\alpha v\beta 3$ integrin affinity regulation have important biological implications in tumour development, wound repair and angiogenesis. We expressed, purified and characterized recombinant forms of human $\alpha v\beta 3$ (r- $\alpha v\beta 3$) and compared the activation state of these with $\alpha v\beta 3$ in its cellular environment. The ligand specificity and selectivity of recombinant full-length and double transmembrane truncations of r- $\alpha v\beta 3$ cloned in BacPAK6 vectors and expressed in Sf9 and High Five insect cells were compared with those of native placental $\alpha v\beta 3$ and the receptor *in situ* on the cell surface. r- $\alpha v\beta 3$ integrins were purified by affinity chromatography from detergent extracts of cells (full-length), and from the culture medium of cells expressing double-truncated r- $\alpha v\beta 3$. r- $\alpha v\beta 3$ had the same epitopes, ligand-binding specificities, bivalent cation requirements and suscep-

tibility to RGD-containing peptides as native $\alpha v\beta 3$. On M21-L4 melanoma cells, $\alpha v\beta 3$ mediated binding to vitronectin, but not to fibrinogen unless activated with Mn^{2+} . Non-activated $\alpha IIB\beta 3$ integrin as control in M21-L-IIB cells had the opposite profile, mediating binding to fibrinogen, but not to vitronectin unless activated with Mn^{2+} . Thus these receptors had moderate to low ligand affinity. In marked contrast, purified $\alpha v\beta 3$ receptors, with or without transmembrane and cytoplasmic domains, were constitutively of high affinity and able to bind strongly to vitronectin, fibronectin and fibrinogen under physiological conditions. Our data suggest that, in contrast with the positive regulation of $\alpha IIB\beta 3$ *in situ*, intracellular controls lower the affinity of $\alpha v\beta 3$, and the cytoplasmic domains may act as a target for negative regulators of $\alpha v\beta 3$ activity.

INTRODUCTION

The molecular mechanisms of integrin affinity regulation have important biological implications and are the subject of intense current research and debate. We have been studying the ‘inside-out’ regulation of integrin $\alpha v\beta 3$. Integrins are cell-surface heterodimeric transmembrane glycoproteins consisting of α - and β -subunits that mediate cell–cell and cell–extracellular matrix interactions. Ligand binding to integrins leads to the generation of intracellular signals which, in concert with other signals, coordinate cell adhesion with cell migration, growth and differentiation [1]. The affinity of integrins for their ligands is regulated from both inside and outside the cell [1], as has been best characterized in the platelet fibrinogen receptor integrin $\alpha IIB\beta 3$ [2]. The affinity regulation of other integrins, including $\alpha v\beta 3$ ‘the vitronectin receptor’ [3], is less well understood. $\alpha v\beta 3$ has recently emerged as a potential therapeutic target in pathologies such as tumour growth, metastasis and angiogenesis [4–6]. In common with $\alpha IIB\beta 3$, $\alpha v\beta 3$ recognizes Arg-Gly-Asp (RGD) sequences in its target ligands [1,3]. In this study we assess the role of the cytoplasmic domains of $\alpha v\beta 3$ in modulating its activity and specificity for RGD-containing ligands.

Integrins can display at least three states of affinity for a ligand: low, in which no ligands bind; medium, in which the favoured ligands bind; high, in which non-favoured ligands can also bind. It is not clear whether all integrins can attain all states in all cellular backgrounds. $\alpha IIB\beta 3$ regulates fibrinogen-mediated platelet aggregation. On resting platelets, $\alpha IIB\beta 3$ has low ligand affinity and is unable to bind soluble fibrinogen, but it is rapidly converted into a high-affinity state after activation [2]. Extra-

cellular stimuli trigger a signal inside platelets which increases the affinity of the $\alpha IIB\beta 3$ extracellular domains for their ligands. This activation process is termed ‘inside-out’ signalling [7]. In common with other type-I membrane receptors, the molecular details of this transmembrane process are unknown [8]. This normal path can be experimentally circumvented by adding high levels of extracellular Mn^{2+} or activating antibodies which activate $\alpha IIB\beta 3$ independently of an inside-out event [2,9,10]. In normal signalling, the cytoplasmic domain of the αIIB chain is crucial and mutation of the αIIB juxta-membrane cytoplasmic sequence-GFFKR converts $\alpha IIB\beta 3$ from low into high affinity [11]. Soluble extracellular domains of $\alpha IIB\beta 3$ also have high ligand affinity [12], supporting the negative regulatory effect of the cytoplasmic domains. Recent studies have identified $\beta 3$ -endoneurin as a molecule that binds $\alpha IIB\beta 3$ cytoplasmic domains and triggers the switch from a low- to a high-affinity state [13]. These cellular studies concur that the default state of the $\alpha IIB\beta 3$ integrin in its cellular environment is low affinity and that modification by protein association is necessary to increase its affinity.

In marked contrast, with few exceptions [14,15], reports on the cellular activity of $\alpha v\beta 3$ suggest that it is in a constitutively moderate affinity state [16,17]. By analogy with $\alpha IIB\beta 3$, this implies that the cellular environments frequented by $\alpha v\beta 3$ contain constitutively expressed activators, that $\alpha v\beta 3$ is activated by a mechanism wholly distinct from that for $\alpha IIB\beta 3$, or that the default state of $\alpha v\beta 3$ is high affinity and is suppressed by intracellular regulators. We set out to test these possibilities by engineering recombinant $\alpha v\beta 3$ molecules (r- $\alpha v\beta 3$) and analysing the ligand-binding activity and specificity of full-length (FL) and transmembrane truncated (Δ TM) forms, in comparison with

Abbreviations used: r- $\alpha v\beta 3$, recombinant integrin $\alpha v\beta 3$; FL, full-length integrin chain; Δ TM, transmembrane and cytoplasmic domain-truncated integrin chain; OG, *n*-octyl β -D-glucoside; HRP, horseradish peroxidase.

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$\alpha v\beta 3$ expressed on cells. Recombinant integrins have often been expressed in eukaryotic systems, but the purification of biochemical amounts has rarely been achieved. Full-length $\alpha v\beta 3$ has been expressed in and purified from CHO cells [18] and such recombinant molecules have been used in ligand-binding assays [18,19]. Purified transmembrane truncated $\alpha v\beta 6$ expressed in CHO cells [20], $\alpha v\beta 8$ expressed in 293 cells [21] and $\alpha IIb\beta 3$ expressed using baculovirus [12] are soluble and can bind ligands. Significant amounts of an active truncated form of $\alpha IIb\beta 3$ have been produced [12]. There have been no reports on the biological activity of purified truncated $\alpha v\beta 3$. Here, we describe the high-level expression and purification of biologically competent soluble truncated $\alpha v\beta 3$ using the baculovirus expression system [22]. The soluble molecule retains the ligand-binding activity and specificity of the native placental and recombinant full-length receptors. All three receptor forms are in a high-affinity state for ligand binding. This suggests that as yet unknown inside-out regulators of the $\alpha v\beta 3$ integrin, in contrast with $\alpha IIb\beta 3$, may reduce its affinity for ligands.

EXPERIMENTAL

Materials

Cell culture of the lines M21-L4 and M21-L-IIb, and their use in cell-attachment assays, have been detailed elsewhere [6]. M21-L is a human melanoma line selected for null expression of cell-surface αv integrins [3]. Full-length human integrin αv was transfected into M21-L to construct the αv -expressing stable transformant M21-L4 [4]. M21-L-IIb is M21-L similarly transfected with full-length human integrin αIIb [23,24]. Human vitronectin, fibronectin and fibrinogen were purified from human plasma [6]. Native integrin $\alpha v\beta 3$ was purified from human placenta by affinity chromatography [6,25]. Proteins were more than 95% pure as assessed by SDS/PAGE and ELISA.

Integrin αv and $\beta 3$ cDNAs were provided by Dr. D. Cheresh (Scripps Institute, La Jolla, CA, U.S.A.). Restriction and DNA-modifying enzymes were from Boehringer-Mannheim. The baculovirus expression system was from Clontech Laboratories (supplied by Cambridge Bioscience). Sf9 cells (Invitrogen) were maintained in TC100 supplemented with 10% insect-cell-qualified foetal bovine serum (Life Technologies), penicillin (50 IU/ml; Life Technologies) and streptomycin (50 μ g/ml; Life Technologies). High Five cells (BTI-TN-5B1-4; Invitrogen) were maintained in Express Five medium (Life Technologies) supplemented with 16.5 mM L-glutamine (Life Technologies), penicillin (50 IU/ml) and streptomycin (50 μ g/ml).

AP3 (anti- $\beta 3$), LM609 (anti- $\alpha v\beta 3$), P4C10 (anti- $\beta 1$), P1F6 (anti- $\alpha v\beta 5$) and 17E6 (anti- αv) have been described in detail elsewhere [6]. Cyclic peptides {EMD 66203 (cyclic Arg-Gly-Asp-[D-Phe]-Val), EMD 73286 (cyclic Arg-Gly-Glu-[D-Phe]-Val), EMD 82734 (cyclic Lys-Gly-Asp-[D-Phe]-Val), EMD 69601 (cyclic Arg-[β -Ala]-Asp-[D-Phe]-Val), EMD 85189 (cyclic Arg-Gly-Asp-[D-Phe]-[N-Me-Val])} and linear peptides EMD 56574 (GRGDSPK) and EMD 73453 (GRGESPK) were synthesized in-house [26,27]. Echistatin was from Bachem.

Construction of recombinant transfer vectors

Where not explicitly stated, standard molecular-biology protocols were used [28]. αv cDNA was excised as an *EcoRI* fragment from αv pcDNA-1Neo [4] and cloned into the transfer plasmid pBacPAK8 (Clontech). The resulting recombinant was termed αv FL(pBac8). The integrin $\beta 3$ cDNA was excised as a *XbaI* fragment from $\beta 3$ pcDNA-1Neo and cloned into the transfer plasmid pBacPAK9 (Clontech). The transmembrane truncated

αv and $\beta 3$ cDNAs were constructed by PCR using *Pfu* Thermo-stable DNA polymerase (Stratagene). Truncated αv cDNA coding for amino acids 1–987 of mature αv was generated using oligonucleotide primers 5'-GAC CAG CAT TTA CAG TGA-3' (forward) and 5'-CA CAG GTC TAC ACT ATG GCT GAA TGC CCC AGG-3' [reverse primer, containing translation stop codon (bold) after that coding for Pro⁹⁸⁷ followed by *XbaI* restriction site (underlined)]. The PCR product was digested with *SalI* and *XbaI* restriction enzymes and the fragment was cloned into *SalI*-*XbaI*-digested αv pcDNA-1Neo. The truncated αv cDNA was subcloned into *EcoRI*-*XbaI* sites of pBacPAC9 and the resulting clone was termed $\alpha v\Delta TM$ (pBac9). Truncated $\beta 3$ cDNA coding for amino acids 1–718 of mature $\beta 3$ was generated using oligonucleotide primers 5'-GCG CGC AAG CTT GCC GCC ACC ATG CGA GCG CGG CCG-3' [forward primer containing the translation start codon (bold) and *HindIII* restriction site (underlined)] and 5'-GAT CGA TCT AGA CTA GGT CAG GGC CCT TGG GAC ACT-3' [reverse primer also containing translation stop codon (bold) after that coding for Asp⁷¹⁸ and *XbaI* restriction site (underlined)]. The PCR product was digested with *HindIII* and *XbaI* and cloned into *HindIII*-*XbaI* sites of pSK+ (Stratagene). The resulting clone, termed $\beta 3\Delta TM$ (pSK+), was digested with *HindIII* and the ends were subsequently polished by filling in with Klenow fragment of DNA polymerase I. The blunt-end linearized plasmid was then digested with *XbaI* restriction enzyme and the fragment was subcloned into *SmaI*-*XbaI* sites of pBacPAK9. The resulting clone was termed $\beta 3\Delta TM$ (pBac9). The PCR-amplified regions of both $\alpha v\Delta TM$ (pBac) and $\beta 3\Delta TM$ (pBac9) were verified by DNA sequencing and all the constructs were tested for expression of correct sized protein by *in vitro* transcription and translation from bacteriophage T7 promoter using TNT Coupled Reticulocyte Lysate System (Promega). The site of transmembrane truncation is shown in Figure 2(A).

Generation of recombinant baculovirus

BacPAK6 (Clontech) baculovirus genomic DNA was prepared from high-titre virus stock and linearized with *Bsu36I*. Recombinant baculovirus clones expressing full-length and truncated αv and $\beta 3$ integrins were prepared according to the Clontech BacPAK product protocol. A null recombinant baculovirus clone was also prepared using pBacPAK9 as transfer plasmid for use as negative control. Recombinant viral clones were identified by PCR and the virus was propagated and titred in Sf9 cells [29].

Metabolic pulse-labelling of proteins expressed in Sf9 cells

[³⁵S]Methionine metabolic labelling of Sf9 cells was performed as described [30]. Where indicated, tunicamycin (10 μ g/ml; Sigma) was present for 1 h before and during pulse-labelling. Cells were lysed [100 μ l:1% Nonidet P40, 1 mM CaCl₂, 150 mM NaCl, 0.4 mM Pefabloc (Merck), 10 μ g/ml leupeptin and E64 (Sigma), 10 mM Tris/HCl, pH 7.4]. The lysate was centrifuged in an Eppendorf Microfuge (14000 g; 4 °C; 10 min) and resolved by denaturing SDS/PAGE (8% gel) under reducing conditions, fixed and dried before autoradiography [28].

Protein expression in insect cells

Recombinant proteins were expressed in monolayer or suspension cultures of High Five cells. Cells were co-infected with αv - and $\beta 3$ -expressing recombinant baculovirus, each at a multiplicity of infection of 5–20, in a minimum amount of

medium with gentle agitation. After 2 h the viral inoculum was removed and replaced with fresh Express Five medium, and the infected cells were incubated at 27 °C for 48–64 h. For isolation of membrane-bound recombinant integrin, the cells were harvested by centrifugation (1000 *g*; 10 min) and the cell pellet was used. For isolation of the soluble recombinant integrin, the cell-free supernatant was used without further treatment.

Immunoprecipitation

Immunoprecipitation of surface-biotinylated cell extract and biotinylated cell conditioned medium was performed with monoclonal antibody LM609 linked to Affigel beads (Bio-Rad) [6]. After SDS/PAGE and Western blotting on to Hybond PVDF membrane [31], biotinylated proteins were detected by enhanced chemiluminescence using ECL Western Blotting Detection (Amersham) according to the manufacturers instructions.

Studies of cell shape change

Six well-plates were coated with purified vitronectin (5 μ g/ml in PBS; 2 h) followed by BSA [3 % (w/v) in PBS; 2 h], or with BSA alone. Sf9 cells (1×10^6 /well) were added and then infected with various virus combinations. The cells were observed 48 h after infection. Where indicated, the medium was supplemented with 10 μ M cyclic peptides as competitive inhibitors of αv -integrin–ligand interaction [cyc(RGDfV), cyc(R[β -A]DfV), where small letters are D-amino acids].

Purification of recombinant integrins

High Five cells expressing full-length $\alpha v\beta 3$ were harvested, washed with PBS, and lysed by rotation in 10 vol. of ice-cold lysis buffer [100 mM *n*-octyl β ,D-glucoside (OG), 1 mM CaCl_2 , 2 mM Pefabloc (Merck) in PBS, pH 7.4] for 1 h at 4 °C. The lysate was centrifuged (10000 *g*; 45 min at 4 °C) and the supernatant recirculated overnight at 4 °C over a LM609-antibody affinity column pre-equilibrated with lysis buffer. After a washing (20 bed volumes; 50 mM OG, 2 mM Pefabloc, 2 mM CaCl_2 in PBS, pH 7.4) bound protein was eluted (50 mM OG, 2 mM Pefabloc, 2 mM CaCl_2 , 50 mM sodium acetate, pH 3.1); the eluate was monitored at 280 nm, and the peak fractions were immediately neutralized (1:50 volume 3M Tris/HCl, pH 8.8). Peak fractions were pooled, dialysed (20 mM OG, 1 mM CaCl_2 in PBS, pH 7.4) and concentrated, then analysed by SDS/PAGE. Aliquots at approx. 1 mg/ml protein were shock-frozen and stored at –80 °C. Protein concentration was determined against BSA standards with the BCA protein assay (Pierce). Soluble recombinant $\alpha v\beta 3$ was purified by recirculating the cell-free medium of infected High Five cells over an LM609 affinity column at 4 °C. The washing, elution, analysis and storage were as described for the full-length $\alpha v\beta 3$ except that no detergent was present in the buffers. ELISA of the purified receptors used goat anti-mouse IgG (H+L) horseradish peroxidase (HRP) conjugates (Bio-Rad) and 3,3',5,5'-tetramethylbenzidine dihydrochloride (Sigma) as substrate.

Integrin ligand-binding and competition assays

Integrin-binding assays have been detailed elsewhere [32]. They are summarized below.

Biotinylation of ligands and antibodies

Proteins in PBS were diluted with 5-fold concentrated ligation buffer (500 mM NaCl, 500 mM NaHCO_3) to 1 mg/ml protein.

Freshly prepared *N*-hydroxysuccinimidobiotin (Sigma; 1 mg/ml in DMSO) was added to 0.1 mg/ml and the mixture incubated for 2 h at 20 °C. After dialysis (PBS, 0.025 % NaN_3) protein concentration was determined. The biotinylated proteins were stored at 4 °C.

Ligand-binding assay

Integrins were diluted to 1 μ g/ml in coating buffer (150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 μ M MnCl_2 , 20 mM Tris/HCl, pH 7.4) and adsorbed overnight at 4 °C to 96-well microtitre plates. The plates were washed and blocked with blocking buffer [coating buffer containing 3 % (w/v) BSA; 2 h at 37 °C]. After the plates had been rinsed, serially diluted biotinylated ligands were added. After incubation (3 h; 37 °C), unbound ligand was washed from the plate and bound biotin was detected by incubation with anti-biotin–alkaline phosphatase conjugated antibody and detection of bound antibody with *p*-nitrophenyl phosphate substrate.

Bivalent cation assay

Integrins were immobilized as described above. All buffers used were made without bivalent cations and stripped by overnight incubation with bivalent-cation-chelating resin, Chelex-100 (1:50, w/v; Sigma). The resin was removed by filtration, and defined cation concentrations were added. Biotinylated ligand was incubated with immobilized integrin in defined cation concentrations for 3 h and detected as described for the ligand-binding assay.

Competition assay

Integrins were immobilized as described above. Serially diluted peptides in binding buffer were added followed immediately by biotinylated vitronectin (to 1 μ g/ml). After 3 h incubation at 37 °C, bound ligand was detected as described above. Assays were performed in triplicate and repeated several times. Results of typical experiments are shown.

RESULTS

Cellular $\alpha v\beta 3$ and $\alpha IIb\beta 3$ are in a moderate affinity state

The affinity of $\alpha v\beta 3$ and $\alpha IIb\beta 3$ is defined by their ability to bind ligands. Thus platelets use $\alpha IIb\beta 3$ to bind to immobilized fibrinogen but not to fibrinogen in solution or to vitronectin, whereas cellular $\alpha v\beta 3$ mediates adhesion to vitronectin but not to fibrinogen at physiological concentrations of the bivalent cations Mg^{2+} ($\cong 0.8$ mM) and Ca^{2+} ($\cong 1.4$ mM). We confirmed these data by using αv and αIIb chains transfected on to the same M21-L cellular background and showed that by activating with Mn^{2+} the integrins were converted into a high-affinity state, as measured by their ability to bind both vitronectin and fibrinogen (Figure 1). The ion concentrations necessary for half-maximal stimulation of binding are shown (Table 1). High levels of Mg^{2+} had weaker activity, and Ca^{2+} was inactive. Antibody studies have shown that the binding of M21-L4 to both vitronectin and fibrinogen is mediated by $\alpha v\beta 3$, with $\alpha v\beta 5$ making a minor contribution on vitronectin, whereas M21-LIIB uses only $\alpha IIb\beta 3$ [23,33].

Generation of recombinant baculovirus

αv and $\beta 3$ cDNAs truncated at positions coding for the transmembrane and extracellular interface (Figure 2A) were prepared

Figure 1 Effect of bivalent cations on the attachment of M21-L4 and M21-L-Ilb cells

M21-L4 and M21-L-Ilb cells attached to vitronectin and fibrinogen in physiological saline containing increasing concentrations of Ca^{2+} (●), Mg^{2+} (■) or Mn^{2+} (▲) ions. Maximal attachment was defined independently for each cell and substrate combination as the attachment in 10 mM Mn^{2+} (e.g. 64% of added cells for M21-L-Ilb on fibrinogen). Results are means $\pm$ S.E.M. ($n = 3$).

Table 1 Concentrations of bivalent cations stimulating half-maximal attachment in cell adhesion mediated by $\alpha\nu\beta 3$ and $\alpha\text{IIb}\beta 3$

M21-L4 and M21-L-Ilb cells attached to vitronectin- or fibrinogen-coated substrates in the presence of Ca^{2+} , Mg^{2+} or Mn^{2+} (see Figure 1).

Cell line	Bivalent cation (μM)		
	Ca^{2+}	Mg^{2+}	Mn^{2+}
M21-L4–vitronectin	$> 10\,000$	900	20
M21-L4–fibrinogen	$\geq 10\,000$	$\geq 10\,000$	200
M21-L-Ilb–vitronectin	$\geq 10\,000$	$\geq 10\,000$	200
M21-L-Ilb–fibrinogen	$\geq 10\,000$	2000	30

by PCR. The $\alpha\nu$ -truncated cDNA coded for amino acids 1–987 and the $\beta 3$ for amino acids 1–718. The full-length ($\alpha\nu\text{FL}$ and $\beta 3\text{FL}$) and truncated ($\alpha\nu\Delta\text{TM}$ and $\beta 3\Delta\text{TM}$) cDNAs were subcloned into baculovirus transfer vector pBacPAK9. Recombinant baculovirus expressing either full-length or truncated $\alpha\nu$ or $\beta 3$ chains were prepared by co-transfection with linearized baculovirus BacPAK6 genomic DNA followed by enrichment by plaque purification and virus amplification in Sf9 cells. Accurate integration of the transfer plasmids was confirmed by PCR and Southern blotting of the recombinant viral genomic DNA (results not shown).

Metabolic labelling of Sf9 cells expressing full-length $\alpha\nu$ chain showed two major recombinant proteins of approx. 110 and 150 kDa (Figure 2B, lane 3). Similarly, Sf9 cells expressing full-length $\beta 3$ showed two bands of approx. 90 and 125 kDa (Figure 2B, lane 4). Comparison of mock-infected (lane 1) and null-recombinant-virus-infected cells (lane 2) also showed the degree of suppression of the host cell genome during the very late phase

Figure 2 Expression of recombinant $\alpha\nu\beta 3$ in insect cells

(A) Partial protein sequence of the human $\alpha\nu\beta 3$ heterodimer at the membrane interface showing the sites for truncation of the $\alpha\nu$ and $\beta 3$ chains. N-terminal extracellular domain to the left. Underlined are putative transmembrane sequences. (B) Mock-infected Sf9 cells (lane 1) or Sf9 cells infected with null recombinant (lane 2), $\alpha\nu\text{FL}$ (lane 3) or $\beta 3\text{FL}$ (lane 4) were pulse-labelled with ^{35}S -labelled amino acids. The cell extracts were resolved by SDS/PAGE under reducing conditions. The molecular masses (kDa) of standard protein markers are indicated on the right. (C) Sf9 cells infected with $\alpha\nu\text{FL}$ (lanes 1 and 2), $\alpha\nu\Delta\text{TM}$ (lanes 3 and 4), $\beta 3\text{FL}$ (lanes 5 and 6) or $\beta 3\Delta\text{TM}$ (lanes 7 and 8) were treated with either tunicamycin (lanes 1, 3, 5 and 7) or carrier (lanes 2, 4, 6 and 8). The infected cells were pulse-labelled as above. The migration positions of standard proteins are indicated on the right.

of the viral life cycle. Treatment of cells expressing either individual full-length or truncated forms of $\alpha\nu$ or $\beta 3$ with tunicamycin (Figure 2C, lanes 1, 3, 5 and 7) inhibited the production of the slower migrating band, indicating that the 150/110 kDa (Figure 2C, lanes 2 and 4) and 125/90 kDa (Figure 2C, lanes 6 and 8) bands correspond to N-glycosylated and unglycosylated forms of the recombinant proteins. This confirms several reports in which the proportion of recombinant protein in baculovirus expression systems that undergo post-translation modifications, such as N-glycosylation, decreases during later stages of the virus life cycle [34]. The relatively large difference in size between the glycosylated and unglycosylated forms of $\alpha\nu$ and $\beta 3$ indicates substantial glycosylation, which is in keeping with the presence in $\alpha\nu$ of 13 and in $\beta 3$ of 10 potential N-glycosylation sites.

Studies of change in cell shape

Integrins modulate cell shape by linking extracellular matrix and cytoskeleton. In later phases of a baculovirus infection, Sf9 cells are usually rounded and loosely attached, as were Sf9 cells infected with either the $\alpha\nu$ - or $\beta 3$ -expressing recombinant baculovirus alone or with the null recombinant virus. However, Sf9 cells co-infected with $\alpha\nu\text{FL} + \beta 3\text{FL}$ or $\alpha\nu\Delta\text{TM} + \beta 3\text{FL}$ were firmly attached and spread. No shape change was observed in cells co-infected with $\alpha\nu\text{FL} + \beta 3\Delta\text{TM}$ or $\alpha\nu\Delta\text{TM} + \beta 3\Delta\text{TM}$. This supports the concept of the cytoplasmic domain of the α chain being responsible for negative regulation of cytoskeletal interactions with the β chain cytoplasmic domain [35–37]. $\alpha\nu\beta 3$ recognizes the amino acid sequence Arg-Gly-Asp (RGD) in target ligands. Cell shape change of Sf9 cells infected with $\alpha\nu$ and $\beta 3$ virus on vitronectin-coated plates was inhibited by RGD-

Table 2 Effect of RGD-containing peptide on transfected Sf9 cell spreading

Sf9 cells transfected with the indicated constructs were incubated on vitronectin in the absence (Control) or presence of 10 μ M cyc(RGDfV) or cyc(R[β -A]DfV). The morphology of the cells was scored on a scale from rounded (–) (as for the null recombinant) to well spread (+ + +).

Transfectant	Control	cyc(RGDfV)	cyc(R[β -A]DfV)
αv FL + $\beta 3$ FL	+ +	–	+ +
αv FL + $\beta 3\Delta$ TM	±	–	±
$\alpha v\Delta$ TM + $\beta 3$ FL	+ + +	–	+ + +
$\alpha v\Delta$ TM + $\beta 3\Delta$ TM	–	–	–
Null recombinant	–	–	–

Figure 3 Immunoprecipitation of cell-surface and soluble $\alpha v \beta 3$

Cell extracts (lanes 1–5) and cell-conditioned medium (lanes 6–10) prepared from High Five cells co-infected with αv FL + $\beta 3$ FL (lanes 1 and 6), αv FL + $\beta 3\Delta$ TM (lanes 2 and 7), $\alpha v\Delta$ TM + $\beta 3$ FL (lanes 3 and 8), $\alpha v\Delta$ TM + $\beta 3\Delta$ TM (lanes 4 and 9) or null recombinant (lanes 5 and 10). $\alpha v \beta 3$ complex was immunoprecipitated using monoclonal antibody LM609 and resolved by SDS/PAGE under non-reducing conditions, transferred on to PVDF membrane and detected with streptavidin–HRP conjugate. α_v DelTM, $\alpha_v\Delta$ TM; β_3 DelTM, $\beta_3\Delta$ TM.

containing but not by cyc(R[β -A]DfV) peptides (Table 2). Cyc(RGDfV) is a specific inhibitor of $\alpha v \beta 3$ integrins, effects on α Ib $\beta 3$ being 10000-fold less [26,27].

Co-expression of recombinant αv and $\beta 3$

High Five cells support higher levels of production and proteins than Sf9 cells and were co-infected with various combinations of recombinant baculovirus expressing full-length and truncated αv and $\beta 3$. Immunoprecipitation from cell extracts or from cell-conditioned medium of the $\alpha v \beta 3$ complex with the antibody LM609 (Figure 3) suggested that a recombinant $\alpha v \beta 3$ heterodimer formed on the cell surface when either one or both chains were full length (lanes 1–3). In addition, co-expression of truncated αv with truncated $\beta 3$ resulted in secretion of a soluble heterodimer (Figure 3, lane 9).

Purification and characterization of recombinant full-length and soluble $\alpha v \beta 3$

Antibody-affinity chromatography was used to purify recombinant $\alpha v \beta 3$ integrins (r- $\alpha v \beta 3$). Full-length recombinant $\alpha v \beta 3$ (r- $\alpha v \beta 3$ FL) purified from detergent extracts of High Five cells yielded 940 μ g/l of cell suspension ($\cong 2 \times 10^8$ cells; 15 ml cell pellet). Soluble recombinant $\alpha v \beta 3$ (r- $\alpha v \beta 3\Delta$ TM) from the supernatant of High Five cells co-infected with $\alpha v\Delta$ TM + $\beta 3\Delta$ TM yielded 850 μ g/l. For comparison, preparations from human

placenta typically yield $\cong 2$ mg/kg of wet tissue (B. Diefenbach and S. L. Goodman, unpublished work). In ELISA, r- $\alpha v \beta 3$ integrins were recognized by the same antibodies (LM609, 17E6 and AP3) as, and with similar affinity (as judged by ELISA titres) to, placental $\alpha v \beta 3$. This, and the lack of reaction with anti- $\beta 1$ (P4C10) or anti- $\alpha v \beta 5$ -specific (P1F6) antibodies, indicated conservation of epitopes and low contamination with other integrins (Figure 4A).

Purified placental $\alpha v \beta 3$ dissociated into two bands when resolved by SDS/PAGE under non-reducing conditions: the α chain at 160 kDa and the β chain at 95 kDa (Figure 4B, lane 2). Purified r- $\alpha v \beta 3$ FL (lane 3) dissociated into an αv chain of approx. 140 kDa and a $\beta 3$ chain at 90 kDa. The lower molecular masses may be due to variant glycosylation [34]. Purified r- $\alpha v \beta 3\Delta$ TM dissociated into a truncated αv chain of approx. 120 kDa and a truncated $\beta 3$ chain of 75 kDa.

The αv chain undergoes post-translational cleavage into heavy and light chains, linked by a disulphide bridge. Therefore, under reducing conditions, the α chain of placental $\alpha v \beta 3$ dissociates into a heavy chain of 140 kDa and a light chain of 25 kDa (Figure 4B, lane 6) and the β chain migrates at 110 kDa. As the transmembrane truncation in the α chain lies within the light chain, both full-length and truncated recombinant α heavy chains should migrate under reducing conditions at about 140 kDa. However, the αv chains of full length and soluble receptors exhibited the same apparent molecular masses under both reducing and non-reducing conditions (compare lane 3 with lane 7 and lane 4 with lane 8), suggesting that there had only been partial post-translational cleavage of the αv chain. The faint band of approx. 120 kDa in lanes 7 and 8 may represent the αv heavy chain. This discrepancy between the placental and recombinant $\alpha v \beta 3$ may reflect inefficient proteolytic processing during the very late phase of the baculovirus infection [29].

Biological activity of recombinant truncated $\alpha v \beta 3$

Recombinant $\alpha v \beta 3$ and placental $\alpha v \beta 3$ were immobilized, and binding of biotinylated ligands was examined. RGD-containing ligands, vitronectin, fibrinogen and fibronectin, which are known to bind to $\alpha v \beta 3$, were used. The ligands bound to both the full-length and soluble receptor preparations in a concentration-dependent manner with overlapping concentration-dependencies, and with saturation concentrations similar to those of placental $\alpha v \beta 3$ (Figure 5A). Remarkably, the removal of the transmembrane and cytoplasmic domains had no significant effect on the affinity or specificity of $\alpha v \beta 3$ for its ligands. Furthermore, the high specificity for vitronectin over fibrinogen seen in cell-adhesion assays was missing in the isolated receptor assays, in which vitronectin, fibrinogen and fibronectin all bound $\alpha v \beta 3$ strongly. This is consistent with each form of the receptor being maximally activated (see Figure 1). Irrelevant ligands, such as intact laminin 1 and collagen type 1, did not bind $\alpha v \beta 3$ over background (results not shown).

Vitronectin was used to test the bivalent-cation-dependence of the $\alpha v \beta 3$ receptors. Recombinant and native $\alpha v \beta 3$ showed similar cation-dependence (Figure 5B). At 1 μ M, Mn^{2+} induced most binding. At 1000 μ M Ca^{2+} , Mg^{2+} , Mn^{2+} , Co^{2+} and Ni^{2+} also activated the receptors, whereas 1000 μ M Cu^{2+} and Fe^{2+} had little activating effect. In the presence of 2 mM EDTA there was no ligand binding (results not shown).

To test the specificity of interaction of vitronectin with recombinant $\alpha v \beta 3$, the effects on the binding of biotinylated vitronectin to $\alpha v \beta 3$ of different peptidic inhibitors were tested. All three forms of receptor showed similar sensitivity to each

Figure 4 Analysis of purified receptors

(A) Epitope and purity analysis of full-length and truncated $\alpha\text{v}\beta\text{3}$ by monoclonal antibodies. Increasing receptor concentrations were immobilized and analysed with antibodies LM609 (anti- $\alpha\text{v}\beta\text{3}$), 17E6 (anti- αv), AP3 (anti- β3), P4C10 (anti- β1) and P1F6 (anti- $\alpha\text{v}\beta\text{5}$). ○, placental $\alpha\text{v}\beta\text{3}$; ■, $\text{r-}\alpha\text{v}\beta\text{3FL}$; ◆, $\text{r-}\alpha\text{v}\beta\text{3}\Delta\text{TM}$. (B) Analysis of purified receptors by SDS/PAGE. Lanes 1 and 5, molecular-mass markers; lanes 2 and 6 placental $\alpha\text{v}\beta\text{3}$; lanes 3 and 7, $\alpha\text{vFL} + \beta\text{3FL}$; lanes 4 and 8, $\alpha\text{v}\Delta\text{TM} + \beta\text{3}\Delta\text{TM}$. Lanes 1–4 were under non-reducing conditions, and lanes 5–8 were under reducing conditions.

peptide. GRGDSPK inhibited vitronectin binding to each receptor with an IC_{50} value of between 0.1 and 0.4 μM , whereas GRGESPK had no effect at 100 μM (Figure 5C). The more active cyclic peptides all inhibited vitronectin binding to the receptors. However, cyc(RGDfV) and cyc(RGDf[N-Me-V]) had IC_{50} values of between 0.5 and 5 nM, whereas the peptides that did not contain RGD had IC_{50} values of over 100 nM. Echistatin inhibited vitronectin binding with an IC_{50} of 2 nM on each $\alpha\text{v}\beta\text{3}$. Thus each form of the receptor was fully inhibited by RGD peptides and with similar inhibition characteristics and specificity.

DISCUSSION

The affinity of αv integrins for ligands is modulated by mechanisms that are not well understood. In this study we have expressed and purified functional recombinant human $\alpha\text{v}\beta\text{3}$ integrins, both intact and as soluble deletion constructs, and have compared their properties with native $\alpha\text{v}\beta\text{3}$, both purified and in a cellular context. Our principle findings are that (a) $\alpha\text{v}\beta\text{3}$ and $\alpha\text{IIb}\beta\text{3}$ on a transformed cell background are in a state of intermediate activation, (b) transmembrane truncated human

Figure 5 Activity test of purified r- $\alpha v\beta 3$

(A) Ligand binding to immobilized receptors. Biotinylated vitronectin, fibrinogen and fibronectin were allowed to bind to immobilized receptors: $\circ$, placental $\alpha v\beta 3$; $\blacksquare$, r- $\alpha v\beta 3$ FL; $\blacklozenge$, r- $\alpha v\beta 3\Delta\text{TM}$. Bound ligand was detected using an anti-biotin antibody. (B) Bivalent-cation-dependence of vitronectin binding. Vitronectin was allowed to bind to immobilized receptors using binding buffer containing the indicated bivalent cations at 1 μM or 100 μM . (C) Specific inhibition of vitronectin binding by RGD peptides. Biotinylated vitronectin was incubated with immobilized receptors (symbols as in A) in parallel with increasing concentrations of peptides. Values are converted to percentage of control (no inhibitor).

$\alpha v\beta 3$ (r- $\alpha v\beta 3\Delta TM$) forms a soluble stable active heterodimer, (c) both r- $\alpha v\beta 3FL$ and r- $\alpha v\beta 3\Delta TM$ bind ligands, maintain epitopes and are inhibited in the same manner as native $\alpha v\beta 3$ and (d) the ligand binding of purified recombinant and native $\alpha v\beta 3$ correspond to a most highly activated cellular form of $\alpha v\beta 3$.

Decisions about the activation state of an integrin are complicated by the cellular background. Extracellular stimuli such as antibodies or Mn^{2+} ions [10,38] can induce high affinity. $\beta 1$ integrins are moderately active on many cells, but on T-cells they are switched off until T-cell induction [39]. $\beta 2$ integrins on lymphocytes are switched off until the lymphocytes are induced, when they are switched on [40]. On circulating platelets, $\alpha IIb\beta 3$ is switched off, but immobilized fibrinogen, collagen, thrombin or ADP rapidly trigger a high activity state [2]. $\alpha v\beta 3$, in contrast, is usually found to be moderately active [24]. To minimize such effects we examined the activation states of $\alpha v\beta 3$ and $\alpha IIb\beta 3$ in the same background, the M21-L cell line. We confirmed that, with this background, both integrins could bind only their favoured ligands and were thus in a moderately activated state [4,6,23].

Several integrins have been successfully engineered as secreted soluble recombinants by truncating α and β chain N-terminal of the membrane, including $\alpha IIb\beta 3$, $\alpha v\beta 6$ and $\alpha v\beta 8$, but, to our knowledge, there has been no previous characterization of a soluble $\alpha v\beta 3$. Co-expression of full-length αv and $\beta 3$ chains results in heterodimeric $\alpha v\beta 3$ on the cell surface. Cells co-infected with recombinant virus with one full-length and one truncated α and β chain ($\alpha vFL + \beta 3\Delta TM$ or $\alpha v\Delta TM + \beta 3FL$) express heterodimeric receptor on their surface. However, only cells containing an α -chain construct and full-length β chain spread on vitronectin. RGD-containing peptides blocked the spreading, indicating that constructs function in insect cells. Cells with full-length α chain and truncated β chain did not spread, and here our data corroborate evidence that the integrin β chain is a site of cytoskeletal interaction [41–43].

r- $\alpha v\beta 3\Delta TM$ is secreted as a complex into the medium as shown by precipitation and purification with the $\alpha v\beta 3$ -complex-specific antibody LM609. Both r- $\alpha v\beta 3\Delta TM$ and r- $\alpha v\beta 3FL$ could be purified in high yield and purity. r- $\alpha v\beta 3\Delta TM$ was rapidly isolated from serum-free culture medium without added detergents or the lengthy and hazardous protocols used for placental $\alpha v\beta 3$. We used LM609-affinity columns, but purification using antibodies against the individual chains (e.g. 17E6, 14D9.F8, AP3) also works (B. Diefenbach, unpublished work), as should ligand-mimetic chromatography [18,44]. Recombinant purification is much less destructive of antibody-affinity columns than purification of placental preparations, which degrade columns within three runs. Columns for r- $\alpha v\beta 3$ purification showed no reduction in performance after 10 runs. In comparison with other published procedures for isolating r- $\alpha v\beta 3FL$ [18], the insect system uses serum-free media, does not need a CO_2 incubator, and minimizes problems of contaminating endogenous integrins. Yields appear to be similar in the two systems. r- $\alpha v\beta 3\Delta TM$ is secreted therefore the use of detergents is not necessary. We found no differences in extracellular epitopes between native, full-length and truncated receptors. The rapid production of a soluble $\alpha v\beta 3$ in biochemical amounts opens up the way for its high-resolution structural analysis.

It was remarkable that ligand binding of r- $\alpha v\beta 3\Delta TM$ and r- $\alpha v\beta 3FL$ showed the same cation-dependence and response to RGD-based inhibitors as the placental receptor, and that they bound equally strongly to both vitronectin and fibrinogen, as judged by the concentrations of ligand needed for 50% saturation. r- $\alpha v\beta 3FL$ has been described as binding from solution to immobilized vitronectin, but not to fibrinogen [18]. In contrast,

we and others find that immobilized placental $\alpha v\beta 3$ can bind fibrinogen [45]. This indicates that the full-length and truncated receptors are highly activated, in contrast with their moderately activated state on M21-L4 cells, and suggests that the affinity state of the $\alpha v\beta 3$ receptor is suppressed from within the cell. Such inside-out signalling has been most extensively studied on $\alpha IIb\beta 3$, a receptor closely related to $\alpha v\beta 3$ [7]. Here, the cytoplasmic domains act as negative regulators of affinity, the negative regulation being relieved by cytoplasmic activating proteins [13].

By comparing $\alpha v\beta 3$ in M21-L4 cells and as purified recombinant molecules, the inside-out affinity modulation of $\alpha v\beta 3$ could be addressed. The discussion hinges on whether the binding state of an integrin in the absence of perturbers is high, medium or low. Our data clearly suggest that the answer is high. In cellular assays, $\alpha v\beta 3$ bound vitronectin and $\alpha IIb\beta 3$ bound fibrinogen, and both were induced to bind less favoured ligands on addition of Mn^{2+} . Thus the receptors on the cells are moderately activated. Cytoplasmic accessory proteins interacting or modifying $\alpha IIb\beta 3$ trigger the switch from low to high affinity. $\beta 3$ -endonexin binds near the sequence N⁷⁵⁹ITY in the $\beta 3$ chain and activates $\alpha IIb\beta 3$ [13,46,47]. Mutations in this region characterize Glanzmann's thrombasthenia and prevent $\alpha IIb\beta 3$ activation [48]. Mutation analysis suggests that a salt bridge between the α chain cytoplasmic sequence GFFKR and the adjacent β chain sequence keeps $\alpha IIb\beta 3$ at low affinity, and disruption or removal of the salt bridge activates it [11].

Here we investigated how the $\alpha v\beta 3$ receptor is modulated by its cytoplasmic domains both in the presence and absence of cellular influences. The various $\alpha v\beta 3$ isolates were very similar in their ligand-binding and inhibition characteristics: native, r- $\alpha v\beta 3FL$ and r- $\alpha v\beta 3\Delta TM$ molecules were all highly activated. We were surprised by this finding: in a cellular context in M21-L4 cells, r- $\alpha v\beta 3FL$ was only moderately activated. The GFFKR sequence is conserved between αIIb and αv chains and both have the same partner, $\beta 3$; we had predicted that similar activation processes may occur, and that a cellular accessory protein would be necessary to activate isolated r- $\alpha v\beta 3FL$ fully, with the r- $\alpha v\beta 3\Delta TM$ molecule defaulting to fully active. However, both were highly activated. There are few reports that $\alpha v\beta 3$ can be activated by inside-out signalling [14], and whether $\beta 3$ -endonexin can activate $\alpha v\beta 3$ remains unanswered [13]. Perhaps the precise geometry of the α and β chains is critical for negative regulation, and this is irrevocably disrupted during purification of r- $\alpha v\beta 3FL$, giving constitutively active receptors. In support of this idea, full-length $\alpha IIb\beta 3$ isolated by ligand-affinity chromatography is also constitutively active, but Kunicki and colleagues [49] argue that this may be due to the presence of multiple populations of $\alpha IIb\beta 3$; only the fully activated form can be isolated. This active state is somehow fixed during and after isolation in the absence of subsidiary proteins. A similar phenomenon has been suggested for $\alpha 2\beta 1$ [50]. Certainly it seems anomalous that subtle physiological and artificial mutations of the $\alpha IIb\beta 3$ cytoplasmic domains inactivate ligand binding in cells, whereas any putative damage we cause here to disrupt the interaction (in an unsubtle manner) between $\beta 3$ and αv activates the receptor. The low-resolution analysis of the extracellular domains we present here suggests that the r- $\alpha v\beta 3$ ligand-binding regions are similar to those of native $\alpha v\beta 3$. Unfortunately, no similar high-resolution probes are yet available for the cytoplasmic domains. An alternative explanation is that there exist negative regulators of $\alpha v\beta 3$ activity within the cell, which suppress $\alpha v\beta 3$ activity by binding at the cytoplasmic domains, and these regulators are lost during purification. This is clearly in conflict with current experimental evidence for $\alpha IIb\beta 3$, but would explain our results with purified and cellular $\alpha v\beta 3$. Both the salt bridge

and $\beta 3$ -endoneurin activation hypotheses can be explained by a constitutively active receptor, which is held in an inactive state by unknown subsidiary proteins or by modifications which are relieved by molecules such as endoneurin. Which, if any, of these hypotheses is correct awaits further experimental evidence.

In summary, we have developed a baculovirus expression system for production of biochemical amounts of a soluble form of integrin $\alpha v\beta 3$. The activity and specificity of both the transmembrane truncated soluble and full-length recombinants match native $\alpha v\beta 3$, but both the r- $\alpha v\beta 3$ and native placental molecules are constitutively highly activated in comparison with $\alpha v\beta 3$ in a cellular environment. This supports the concept that the default state of $\alpha v\beta 3$ is highly activated. The high levels of expression and activity, and the ease of purification of the soluble receptor may speed up both the investigation of the activation process and the molecular details of the ligand-receptor interaction.

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