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Production of recombinant soluble human integrin α4β1

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Abstract Integrin α4β1 is a major leukocyte adhesion receptor that is a key target for the development of anti-inflammatory therapeutics. With the dual long-term goals of developing a reagent for use in high-throughput inhibitor screening assays and for crystallisation trials and subsequent structure determination, we have generated a recombinant soluble α4β1 receptor. Both subunits were truncated prior to the transmembrane domains by site-directed mutagenesis and expressed using baculovirus infection of insect cells. The molecular weights of the recombinant subunits were as expected for post-translationally unmodified protein. In addition, as observed for the native subunit, a proportion of the α4 subunit was proteolytically processed into two fragments. ELISA and solid phase ligand-binding assays were performed to investigate the folding and functionality of the soluble integrin. The data suggest that the receptor was correctly folded and that it bound recombinant ligands with similar kinetics to the native molecule.

Key words: Integrin α4β1; Soluble receptor; Structure; Baculovirus; Adhesion

1. Introduction

Integrin α4β1 is a major leukocyte adhesion receptor that binds primarily to the cell surface immunoglobulin vascular cell adhesion molecule-1 (VCAM-1) and the extracellular matrix protein fibronectin [1]. Perturbation of α4β1 function in animal models of inflammation using either monoclonal antibodies or ligand mimetic peptides has indicated a major role for this receptor in conditions such as asthma [2], rheumatoid arthritis [3] and atherosclerosis [4] and made it a key target for the development of anti-inflammatory therapeutics (reviewed in [5]). The development of such therapeutic agents is currently hampered by the lack of tertiary structure information for α4β1, and by the lack of a reagent to permit high-throughput drug screening. One solution to these problems would be the generation of a recombinant soluble form of the receptor.

All integrins are heterodimeric proteins consisting of non-covalently-associated α and β subunits [6]. Since both subunits possess a single transmembrane domain, truncation of each would be needed to generate the soluble receptor. In addition, some integrins, including α4β1 [7], exhibit only weak inter-subunit association, suggesting that soluble receptors may dissociate into their component subunits. Despite these potential difficulties, in recent years, a number of truncated integrins have been produced using both eukaryotic and insect cell hosts. All of the soluble truncated integrins have been produced by deletion or replacement of the integrin transmembrane and cytoplasmic domains. Using a baculovirus expression system, a soluble αIIbβ3 integrin was expressed using Sf9 cells which consisted of the heavy chain of the αIIb subunit and a shortened β3 subunit truncated before the cysteine-rich repeats [8]. The soluble αIIbβ3 produced was a divalent cation-dependent complex which was functionally active in binding its ligand fibrinogen with similar affinity to full-length αIIbβ3 purified from platelets. Rotary shadowing of the full-length and soluble αIIbβ3 revealed similar structures with a globular head and two tails, which were shortened in the soluble integrin [8]. Similar αVβ3 and αVβ5 integrin constructs have been expressed using a baculovirus expression system [9,10], while soluble αIIbβ3 and αVβ3 integrins and an α8β1-alkaline phosphatase chimera have been produced using mammalian COS and HEK293 cells [11–13].

To resolve potential problems with inter-subunit association during the production of soluble integrins, soluble α3β1 integrin has been produced with the cytoplasmic and transmembrane domains replaced by the Jun and Fos dimerisation motifs [14]. The chimeric integrin was produced using a *Drosophila* expression system and subunit association and processing were observed. The soluble α3β1 bound to its ligands, invasin and laminin-5. Soluble αVβ5 has since been expressed using the same Jun-Fos tail dimerisation method, also in insect cells [15]. A majority of the soluble integrins that have been reported were purified on a ligand or peptide ligand mimetic column (an RGD column for αIIbβ3 [8,9,11] and invasin for α3β1 [14]). The only published purification using an epitope tag was for the α8β1-alkaline phosphatase chimera which was purified on a nickel column utilising a His tag added to the α8 subunit [12].

With the dual long-term goals of developing a reagent that might be used for high-throughput inhibitor screening assays and that might serve as a starting point for crystallisation trials and subsequent structure determination, we report here the generation of a recombinant soluble α4β1 receptor (rsα4β1). The α4 and β1 subunits were truncated prior to the transmembrane domains by site-directed mutagenesis and expressed in insect cells. An epitope tag was added at the C-terminus of the α4 subunit to aid isolation by immunoaffinity chromatography.

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2. Materials and methods

2.1. Antibodies

The antibodies used in this study were: the mouse anti-human β1 monoclonals 8E3 [16], 12G10 [16], 15/7 (from Ted Yednock, Athena Neurosciences, Seattle, WA, USA), JB1B (from Serotec, Oxford, UK), K20, 4B4 (both from Coulter Electronics, Luton, UK), TS2/16 (from Francisco Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain), the rat anti-human β1 monoclonals 13 (from Ken Yamada, NIH, Bethesda, MD, USA) and 9EG7 (from Dietmar Vestweber, University of Munster, Germany), the mouse anti-human α4 monoclonals 8F2 (from Chikao Morimoto, Dana-Farber Cancer Institute, Boston, MA, USA), HP2/1, HP1/2, HP1/3 (all three from Francisco Sanchez-Madrid), GG5/3 and 44H6 (both from Ted Yednock), and the mouse anti-Ty epitope monoclonal BB2 (from Jayne Brookman, University of Manchester). Non-immune mouse and rat IgG were purchased from Sigma (Poole, Dorset, UK).

2.2. Mutagenesis of integrin subunit cDNAs and transfer vector construction

Full-length cDNA constructs encoding the human α4 (in pBluescript) and β1 (in pcDNA3) integrin subunits were kindly donated by Martin Hemler (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA) and Erkki Ruoslahti (Burnham Institute, La Jolla, CA, USA), respectively. A *Kpn*I–*Xba*I fragment of α4, which contained the region to be targeted for mutagenesis, was subcloned into pUC118. The β1 cDNA was excised by *Eco*RI digestion and subcloned into pUC118. A shortened β1 construct was produced by removal of an internal *Bgl*II fragment and religation. CJ236 *Escherichia coli* cells were transformed with the pUC118 constructs and infected with M13 phage to produce single-stranded DNA (ssDNA). The ssDNA was isolated and was used for site-directed mutagenesis according to the method of Kunkel [17].

The mutagenic oligonucleotide 5′ GCT ACT TGA AAT AAT CAC TAT GGT CTA ATC AAG GGG ATC TTG GTT TGT ATG AAC TTC ATA ACG TTT GGG TCT TTG ATG ATG 3′ was used to mutate the α4 cDNA to contain a 10 amino acid epitope tag and stop codon (underlined sequence) two amino acids 5′ to the predicted transmembrane domain. The epitope tag was designed from a sequence of the Ty1 surface protein of the virus-like particle of *Saccharomyces cerevisiae* for which a monoclonal antibody had been produced [18]. It was considered unlikely that this monoclonal antibody would cross-react with proteins of other species. The full-length α4 cDNA was reconstructed within the original pBlueScript vector following *Kpn*I–*Xba*I digestion of the mutant fragment and religation. Mutant α4 was excised with *Sal*I and *Xba*I and subcloned into the pFASTBAC transfer vector (Gibco-BRL, Paisley, UK).

The mutagenic oligonucleotide 5′ GGA ATG ATG TCT TAA CCA GTG GG 3′ was used to mutate the β1 cDNA to contain a stop codon (underlined sequence) two amino acids 5′ to the encoded predicted transmembrane domain. The excised β1 *Bgl*II cDNA fragment was reinserted into the mutated β1 cDNA and the ends were sequenced. The β1 cDNA was then subcloned into the original pcDNA3 vector, and re-excised with *Kpn*I and *Xho*I for subcloning into the pFASTBAC transfer vector.

2.3. Translocation and bacmid purification

The transfer vector containing the two mutated cDNA constructs was transformed into DH10BAC *E. coli* cells, which harbour a copy of the baculovirus genome (bacmid), according to the manufacturer's instructions (Gibco-BRL, Paisley, UK). Translocation of the vector region containing the encoded subunits and virus promoters into the bacmid was then induced. Cells containing recombinant bacmid were identified as white colonies due to disruption of an encoded LacZ cassette within the bacmid. Recombinant bacmid was then isolated and analysed by agarose gel electrophoresis to confirm the presence of bacmid DNA.

2.4. Sf9 cell transfection and recombinant virus production

Isolated recombinant bacmid was transfected into Sf9 insect cells by lipofection to produce recombinant virus. 1×10^6 Sf9 cells were seeded into 35 mm plates and left to attach for 1 h. 5 μl of recombinant bacmid DNA was mixed with 100 μl of Grace's medium and a second mixture of 6 μl of Cellfectin reagent (Gibco-BRL, Paisley, UK) in 100 μl Grace's medium was prepared. The two mixtures were combined and gently mixed before incubating for 45 min at room temperature. The lipofection mixture was added onto the cells and incubated at 27°C for 5 h before replacement with culture medium. Cell lysis was monitored by phase contrast microscopy, and culture supernatant containing recombinant virus was harvested four days after transfection. The virus titre was analysed by plaque assay. 1×10^6 cells were seeded into the wells of a 6 well plate and left to attach for 1 h. A series of dilutions of virus were made (10^{-2} to 10^{-9}) in Grace's medium. 1 ml of the diluted virus was added to the cell monolayers before incubation for 1 h at room temperature. The virus inoculum was removed and an overlay mixture of 1×Grace's medium and 1% (w/v) SeaPlaque agarose (Flowgen, Lichfield, UK) was added onto the monolayer. Once the agarose had set, the plates were incubated at 27°C. A neutral red overlay (0.4% (w/v) SeaPlaque agarose, 0.025% (w/v) neutral red) was added after 6 days to assist in plaque visualisation. Plaques appeared as clear circles in a pink background. Initial culture supernatants contained a virus titre of 10^3 to 10^4 pfu/ml. Virus titre was then amplified by reinfection of Sf9 cells with a multiplicity of infection (MOI) of between 0.01 and 0.1 pfu/cell. Culture supernatants were harvested between 7 and 10 days post-infection, and after two rounds of amplification, the virus titre was at least 8×10^7 pfu/ml. DNA isolated from recombinant virus was analysed by PCR using primers within the translocated portion of the pFASTBAC vector and within the inserted subunit cDNAs. Products were produced of the correct size, indicating the presence of both subunit cDNAs within the recombinant virus DNA.

2.5. High5 cell infection and optimisation of recombinant protein production

High titre virus was used to infect suspension-cultured High5 cells with different MOI, and samples were collected from the cultures at 24 h time points. Both cells and culture supernatant samples were collected, cells were extracted with a Triton X-100 extraction buffer (0.1(w/v) Triton X-100, 1 mM PMSF and 0.5 μg/ml leupeptin in TBS (150 mM NaCl, 25 mM Tris–HCl, pH 7.40)). The cells were mixed with a small volume of the extraction buffer and vortexed every 10 min for 45 min whilst incubating on ice. The mixture was centrifuged at 1000×*g* and the supernatant collected. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions and transferred to nitrocellulose. The nitrocellulose was then Western blotted using an anti-human α4 integrin mAb, 8F2, an anti-human β1 integrin mAb, 8E3, and the mAb directed against the epitope tag, BB2.

2.6. Purification of rsα4β1

Culture supernatant was harvested from suspension cultures of infected High5 cells by centrifugation at 14 000×*g*, and was precleared by mixing with mouse IgG-Sepharose for 2 h at 4°C. Soluble integrin was affinity purified using BB2-Sepharose (5 mg BB2 per ml of cyanogen bromide-activated Sepharose beads). The culture supernatant was mixed with the Sepharose for 2 h at room temperature, and then the beads were collected into a column and washed with five column volumes of TBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The soluble protein was eluted from the column by addition of 1 mg/ml peptide containing the sequence of the epitope tag (KEVHTNQDPLDK).

2.7. Solid phase ligand binding assay

Purified integrin was tested for ligand binding activity using a series of solid phase assays. Soluble integrin was coated into half-volume wells of a 96 well ELISA plate (Costar, High Wycombe, UK) at an approximate concentration of 1 μg/ml in PBS^+ for 16 h at room temperature. The coating solution was then removed and non-specific binding blocked by addition of 200 μl per well of 5% (w/v) BSA in TBS and incubated for 2 h at room temperature. A biotinylated fibronectin fragment, H120 [19], or a chimeric protein consisting of the first two domains of human VCAM-1 with a human IgG_1 Fc domain ([20]; 2dVCAM-Fc) were used as ligands. Ligands were diluted in TBS, 1 mM $MnCl_2$, 1 mg/ml BSA (Buffer A) to the required concentration and 50 μl added to each well and incubated at 37°C for 3 h. The plate was washed three times with buffer A before addition of either 50 μl ExtrAvidin-peroxidase (1:500 dilution in buffer A; Sigma, Poole, Dorset, UK), and incubated for 20 min at room temperature, or 50 μl Protein A-peroxidase (1:1000 dilution in buffer A; Sigma, Poole, Dorset, UK) and incubated for 1 h at room temperature,

depending on the ligand used. The plate was washed four times with buffer A and 50 μl of substrate 2,2′-azino-bis (3-ethylbenzthiazoline 6-sulphonic acid) (0.1% (w/v) ABTS in 0.1 M sodium acetate, 0.05 M NaH_2PO_4, pH 5.0, 0.01% (v/v) H_2O_2) was added. Absorbance readings were measured at 405 nm on a Dynatec MR4000 plate reader.

2.8. Identification of epitopes by sandwich ELISA

Antibodies were diluted to 10 μg/ml in PBS^+ and 50 μl aliquots coated into the half-volume assay plate overnight at 4°C. Following aspiration, 200 μl/well of blocking buffer was added to block non-specific binding sites on the well surface, and the incubation continued at room temperature for 2 h. Again after aspiration, 50 μl aliquots of soluble integrin diluted in buffer A were added and the plate was incubated for 2 h at room temperature. Wells were washed three times with buffer A and then 50 μl biotinylated BB2 antibody (0.5 μg/ml in buffer A) was added per well. The plate was incubated for 1 h at room temperature and then washed three times with buffer A. 100 μl of a 1:500 dilution of ExtrAvidin-peroxidase in buffer A was added to the wells, and the plate was incubated for 20 min at room temperature. After four washes with buffer A, the colour reaction was developed and measured as for the solid phase ligand binding assays.

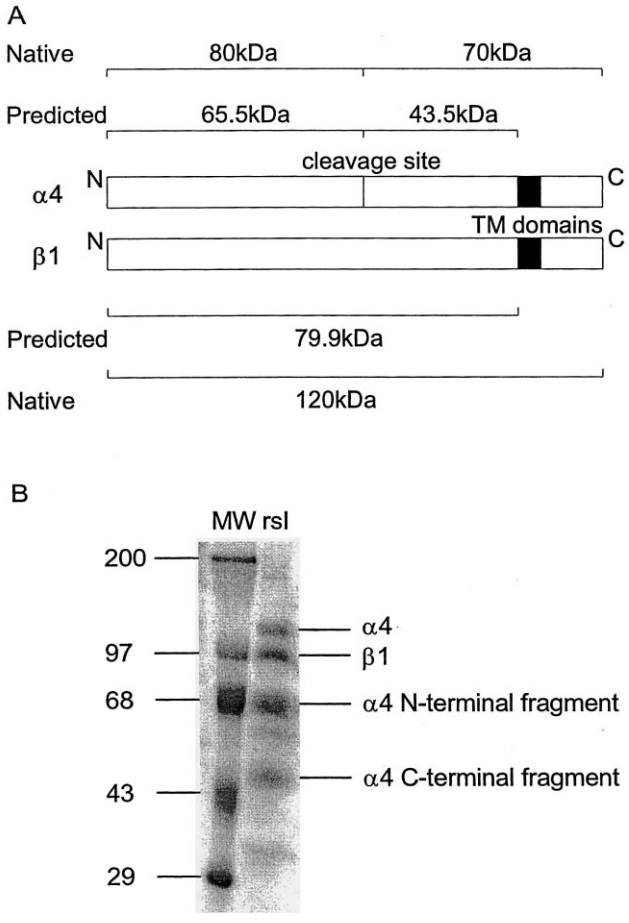

Fig. 1. A: Schematic diagram indicating the molecular weights of the α4 and β1 integrin subunits. The native molecular weights are those observed with the full-length integrin. The predicted molecular weights are those predicted from the length of the amino acid sequence for the soluble integrin. For the soluble α4 subunit, the predicted molecular weight also includes the 10 amino acid epitope tag that was attached to the C-terminus of the subunit. B: SDS–PAGE of rsα4β1. Protein was purified from High5 culture supernatant using BB2-Sepharose, analysed on a 7.5% gel under reducing conditions, and stained with Coomassie Blue. The left hand lane shows molecular weight standards with their molecular mass indicated on the left hand side of the gel. The identity of the bands in the right hand lane were determined by Western blotting and are indicated on the right hand side of the gel.

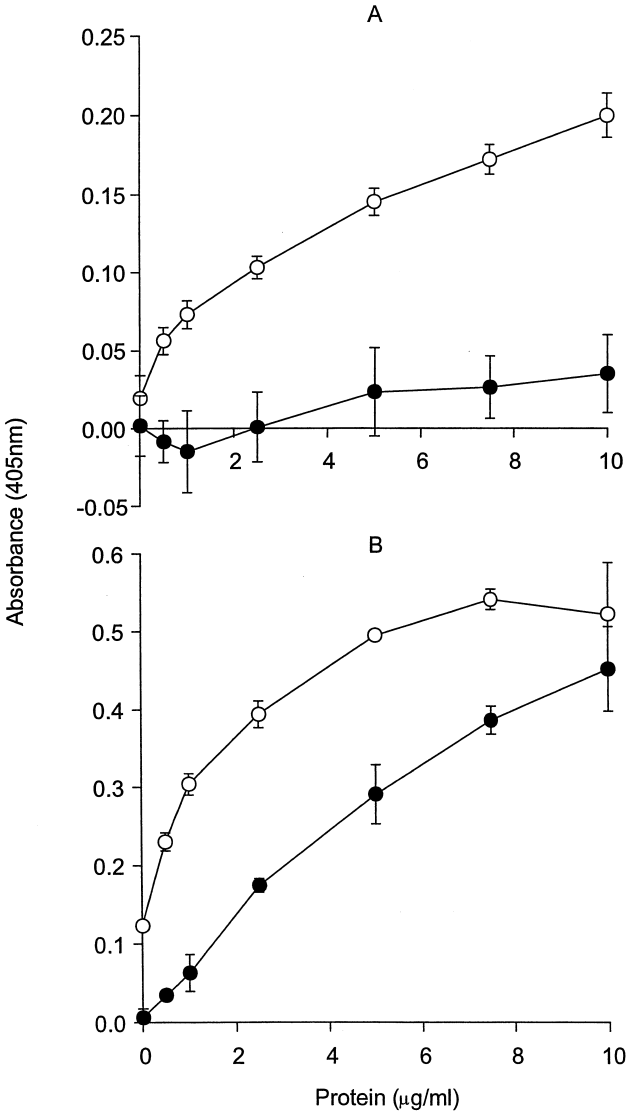

Fig. 2. A: Solid phase assay of the binding of 2dVCAM-Fc (open circles) and D40A-2dVCAM-Fc (closed circles) to rsα4β1. B: Solid phase assay of the binding of 2dVCAM-Fc (open circles) and biotinylated H/120 binding (closed circles) to rsα4β1. Error bars = standard deviation of triplicate values.

3. Results and discussion

cDNA constructs encoding the human α4 and β1 subunits were truncated prior to the transmembrane domains by site-directed mutagenesis and expressed using baculovirus infection of High5 insect cells. A BB2 epitope tag was added to the C-terminus of the α4 subunit to aid isolation by immunoaffinity chromatography. To determine the optimal conditions for expression of soluble α4β1 integrin, High5 cells were infected with recombinant virus at different MOI and the growth medium sampled as a function of time. Samples were analysed by SDS–PAGE under non-reducing conditions, followed by Western-blotting with anti-integrin and anti-epitope tag monoclonal antibodies. The anti-β1 monoclonal antibody, 8E3, recognised a protein band of molecular weight 80 kDa, the anti-α4 monoclonal antibody, 8F2, detected bands of 110 kDa and 65 kDa (the latter probably representing the

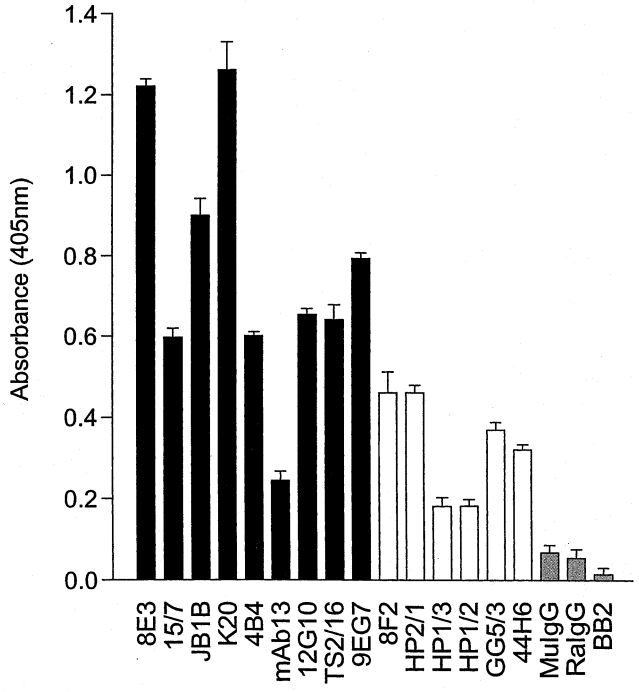

Fig. 3. Sandwich ELISA of rsα4β1 to examine epitope expression. A series of anti-α4 and anti-β1 monoclonal antibodies were tested for their ability to capture rsα4β1 from solution. Error bars = standard deviation of triplicate values.

N-terminal cleavage product of α4), and the antibody directed against the epitope tag, BB2, recognised bands of 110 kDa and 43 kDa (the latter probably representing the C-terminal cleavage product of α4; data not shown). These data are consistent with correct expression of both integrin subunits and with natural post-translational cleavage of the α4 subunit (see scheme in Fig. 1A).

Optimal conditions for protein production were found to be an MOI of 1 and a time of growth medium harvest of 72 h (data not shown). Using these conditions, bulk cultures were established and recombinant soluble α4β1 was purified using immunoaffinity chromatography on a BB2 antibody matrix. The yield of total purified rsα4β1 protein was in the 100–850 μg/l range and was therefore comparable to previous reports of soluble integrin production. The purified protein was analysed by SDS–PAGE and found to exhibit a similar banding pattern to that observed in Western blotting experiments of growth medium supernatant (Fig. 1B). Under reducing conditions (as in Fig. 1B), the apparent molecular weight of the β1 subunit band increased consistent with the presence of a relatively large number of intramolecular disulfide bonds. Under non-reducing conditions, a proportion of the purified protein was observed as a high molecular weight species (> 200 kDa). This high molecular weight protein was probably due either to formation of multimeric integrin complexes or to misfolding of the protein, as it was lost upon reduction.

To test for functional activity, the ability of purified rsα4β1 to bind to its natural ligands, VCAM-1 and fibronectin, was tested using a solid phase protein–protein binding assay. VCAM-1 was found to bind to the integrin in a dose-dependent manner (Fig. 2A). Binding was specific since VCAM-1 containing a point mutation, D40A, in its primary integrin binding site produced negligible levels of binding (Fig. 2A). In other experiments (not shown), EDTA also inhibited VCAM-1 binding. The immobilised receptor was also able to bind a recombinant fragment of fibronectin that contains the α4β1-binding domain (H/120; 19) in a dose-dependent, saturable manner (Fig. 2B); half-maximal binding of VCAM-1 was obtained at ~1 μg/ml, and H/120 at ~4 μg/ml, a similar difference to that observed for the native receptor [21].

To examine whether the soluble integrin was correctly folded, the ability of a library of 15 anti-α4 and -β1 monoclonal antibodies to recognise the receptor was examined. A sandwich ELISA technique was employed in which the receptor was first captured by the BB2 anti-epitope tag antibody (Fig. 3). All of the antibodies were found to bind to the soluble integrin to varying extents, as has been observed previously for native integrin. As expected, the level of BB2 binding was reduced to the level of the control mouse and rat IgG. Thus, the recombinant receptor is both folded and able to bind ligand.

In summary, we have produced a rsα4β1 integrin with ligand binding activity and folding which resembles the native protein. In the future, the generation of larger quantities of receptor will permit an assessment of the product for structural and drug screening studies.

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