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Molecular cloning, overexpression, and an efficient one-step purification of $\alpha 5\beta 1$ integrin

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Abstract

The $\alpha 5\beta 1$ integrin heterodimer is involved in many cellular processes and is an anticancer therapeutic target. Therefore, access to quantities of protein suitable for studies aimed at understanding its biological functions is important. To this end, a large-scale protein expression system, utilizing the recombinant baculovirus/SF9 insect cell expression system, was created to produce the extracellular domain of the $\alpha 5\beta 1$ integrin. An incorporated 8X-histidine tag enabled one-step nickel-column purification. Following sequence confirmation by LC-MS/MS, the conformation of the heterodimer was characterized by native dot blot and negative stain electron microscopy. Cellular transduction inhibition studies confirmed biological activity. The system allows expression and purification of $\alpha 5\beta 1$ integrin in quantities suitable for an array of different experiments including structural biology.

Keywords

$\alpha 5\beta 1$ integrin expression; pFastBac1 vector; recombinant baculovirus; electron microscopy

Introduction

Regulation of cell-cell and cell-matrix interactions is critical in the development of single-cell to multi-cellular organisms. Integrins are a specific class of transmembrane receptors that mediate these interactions in inside-out and outside-in signaling events. Thus, they transduce information from the cell to the extracellular matrix (ECM) and reveal the state of the cell to the outside environment. They are heterodimeric type I proteins consisting of α and β subunits that each have a large extracellular domain, a single transmembrane domain, and short cytoplasmic tail [1]. In mammals, there are 18 α and 8 β subunits, which differ in amino acid sequence and form 24 distinct heterodimer combinations. In addition, integrins are expressed in a multitude of tissues and have various ligand binding specificities [2,3].

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Therefore, tissue location and ligand availability are strong determinants of integrin interactions that promote cellular migration, proliferation, and differentiation [4].

The $\alpha 5\beta 1$ integrin receptor, also called the fibronectin receptor, interacts directly with the extracellular matrix protein, fibronectin. These interactions are involved in embryogenesis, angiogenesis, and wound healing [5]. However, changes in $\alpha 5\beta 1$ integrin expression between normal and tumoral cells suggest involvement in tumor progression and aggressiveness [6]. A homozygous deletion of the $\alpha 5$ integrin in mice results in defects in neural tube development and blood cell leakage during embryogenesis [7], while $\beta 1$ integrin knock outs in mouse endothelial cells exhibit vascular remodeling defects caused by adhesion and migration alteration and reduced survival of endothelial cells [8]. In addition to these functions, the $\alpha 5\beta 1$ integrin is also studied for its roles in cell-surface virus attachment and internalization [9–11], leukocyte rolling [12], and cytoskeletal activation and rearrangement [13]. Thus, $\alpha 5\beta 1$ integrin is an extensively studied receptor due to its roles in many facets of biology. For this, the ability to express and purify this receptor in the quantities needed for numerous assays including structural and cell-based, is crucial for functional annotation.

Here we report the creation of an expression system for producing soluble and functional $\alpha 5\beta 1$ integrin. We utilized the pFastBac vector with an 8X-histidine tag as the backbone for the construction of two recombinant baculoviruses encoding the $\alpha 5$ and $\beta 1$ integrin extracellular domains, in-frame with Fos and Jun dimerization domains, respectively. Co-infections in *Spodopterafrugiperda*9 (SF9) cells and a one-step nickel-column purification yielded pure protein in the amounts ~4 mg per liter of cell culture. The purified $\alpha 5\beta 1$ integrin inhibited cellular transduction by Adeno-associated virus serotype 2 (AAV2), a reported viral ligand [9], consistent with a functional state for the protein.

Materials and Methods

Construct design

The full-length cDNAs for $\alpha 5$ integrin (OpenBiosystems cat. # MHS1011–61377), $\beta 1$ Integrin (OpenBiosystems cat. # MHS 1010–58245), Fos (OpenBiosystems cat. # MHS1011–60754), and Jun (OpenBiosystems cat. # MHS1010–97228128) were purchased for PCR amplification of the appropriate domains. A 3003 bp fragment of $\alpha 5$ integrin encoding only the extracellular domain was PCR amplified (iProof High-Fidelity DNA polymerase; BIORAD cat. # 172–5302) with primers (see Table 1) designed with restriction endonuclease cloning sites *Sal* I and *Not* I. The following thermal cycler parameters were used for all PCR amplification steps: (1) 98°C – 2 min, (2) 98°C – 30 sec, (3) 58°C – 1 min, (4) 72°C – 4 min, and (5) 72°C – 10 min, with 35 cycles run between steps 2 – 4. Following amplification, the cDNA fragment was visualized and excised from an agarose gel (BIO-RAD cat. # 161–3102), extracted (Qiagen cat. # 28706), restriction enzyme digested with *Sal* I and *Not* I, and ligated into the pFastBac1 vector (Invitrogen cat. # 10360-014) using the same cloning sites. A 1:1 insert to vector molar ratio was used for ligation. The ligation reaction was transformed into JM109 competent cells (Stratagene cat. # 200235) and streaked on an agarose plate with ampicillin (50 μ g/ml) for overnight incubation at 37°C. Colonies were selected and grown in 5 ml of LB media containing ampicillin. Clones were purified (Qiagen cat. # 27106) and identified by restriction digest and verified by DNA sequencing. A similar cloning strategy was employed for the remaining cloning procedures. A 160 bp fragment of the Fos gene involved in forming a leucine zipper with Jun was PCR amplified from the full length cDNA with primers (Table 1) designed with restriction sites *Not* I and *Xba* I, and a five glycine linker. This linker region, between the $\alpha 5$ integrin extracellular domain and Fos, was added to incorporate flexibility between the two expressed domains. The amplified Fos fragment was restriction digested, and cloned in-

frame utilizing the same cloning sites in the pFastBac1 vector. An 8X-histidine tag was inserted in-frame using complementary primers (Table 1) designed with restriction sites *Xba* I and *Hind* III. The primers were mixed at equimolar ratios (1 pmol/μl) and incubated in a heat block for 5 min at 95°C. The primers were gradually cooled to room temperature for subsequent ligation using the same cloning sites in the pFastBac1 vector. The completed construct was verified by DNA sequencing.

A second construct was generated for the β1 integrin extracellular domain. A 2208 bp fragment was amplified with primers (see Table 1) designed with restriction sites *Bam* HI and *Sal* I, and inserted into the pFastBac1 vector using the same cloning sites. A 153 bp fragment of Jun was amplified with primers (see Table 1) designed with restriction sites *Sal* I and *Xba* I and a glycine linker, and inserted in frame with the β1 integrin extracellular domain utilizing the same cloning sites in the vector. An 8X-histidine tag was inserted in-frame in the same manner as the α5 integrin construct (see above). The completed construct was verified by DNA sequencing.

Generation of recombinant baculoviruses

The completed α5 and β1 integrin DNA constructs were used to generate recombinant baculoviruses according to the manufacturer's protocol (Invitrogen; Bac-to-Bac Baculovirus Expression System). Individual clones (α5 1–5 and β1 1–5) were plaque-purified and amplified to a passage 2 (P2) stage. Each clone was analyzed for integrin DNA following agarose gel visualization by extraction and PCR amplification using primers (Table 1) for the full length gene.

Small-scale protein expression

SF9 insect cells were cultured in 2800 ml Pyrex flasks and maintained in SF-900 II SFM media (Gibco cat. # 11496-015) supplemented with 10% antibiotic-antimycotic (Gibco Cat. # 15240-062) in a 28°C incubator. All P2 stocks were titered by plaque assays and the clones with the highest titered stock, α5-3 and β1-5, were used to infect 50 ml of 2×10^6 SF9 insect cells at a multiplicity of infection (MOI) of 5. Cells were harvested 72 h post-infection and separated from the media by centrifugation at 1811g at 4°C for 20 min. The cell pellet was discarded and the media fraction, with the addition of an EDTA-free protease inhibitor mix (Roche cat. # 04693159001), was applied to an equilibrated (lysis buffer; 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0) 1 ml Ni-NTA agarose (Qiagen cat. # 30210) gravity-flow column (BIORAD cat. # 731-1550) and the flow-through sample was re-loaded prior to washing and elution. A stepwise wash gradient with increasing concentrations of imidazole (60 mM, 80 mM, 100 mM; three 1 ml fractions were collected for each wash) was used to remove non-specific protein nickel interactions. Bound protein was eluted with a 250 mM imidazole buffer and collected in 300 μl fractions. The lysis, wash, and elution buffers were identical except for varying imidazole concentrations, and the wash and elution buffers were protease inhibitor free. The media, flow-through, wash, and elution fractions were analyzed by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; see below) and, in addition, the elution fractions were analyzed by Western blot (see below).

Large-scale protein expression and purification via nickel column chromatography

Approximately 1 L of SF9 insect cells (2×10^6), in SF-900 II SFM media, were co-infected with recombinant baculovirus stocks of α5-3 and β1-5 at an MOI of 5 for each virus. Cells were harvested 72 h post-infection and separated from the media by centrifugation at 1811g at 4°C for 20 min. The cell pellet was discarded and 10% PEG 8000 (Fisher Scientific Cat. # BP233-1) was added to the media to precipitate protein O/N followed by centrifugation at 17700g at 4°C for 90 min to collect the pellet. This pellet was resuspended in 25–50 ml lysis

buffer (including protease inhibitor) depending on pellet size. Following resuspension, an additional centrifugation step at 1811g at 4°C for 20 min was performed to clarify the supernatant. The pellet was discarded and the supernatant was applied to an equilibrated (lysis buffer) 5 ml Ni-NTA agarose gravity-flow column and the flow-through sample was re-loaded prior to washing and elution. The column was washed separately with 25 ml each of 100 mM and 150 mM imidazole. Bound protein was eluted with 400 mM imidazole and collected in 1 ml fractions. The flow-through, wash, and elution fractions were analyzed by SDS-PAGE.

SDS-PAGE analysis

Nickel column fractions were boiled for 10 min, centrifuged briefly, and loaded onto a 4–20% polyacrylamide gel (Bio-Rad cat. # 345-003). SDS-PAGE was performed at 125 V for 90 min [14]. Gels were stained in a solution consisting of 12.5% Coomassie blue stain stock R-250, 10% acetic acid, and 50% methanol for 1 h. Gels were destained in solution consisting of 10% acetic acid and 50% methanol overnight. Visualized protein bands were cut from the gel and the slices were stored in destain until the samples were prepared for protein verification by mass spectrometry (see below). Based on the SDS-PAGE data, fractions containing only the $\alpha 5$ and $\beta 1$ integrin domains were pooled and concentrated with a 9-kDa cutoff concentrator (Amicon). Folding, assembly, and activation of integrin heterodimers are dependent on divalent cations, which have crucial roles in these processes [15, 16]. For these proteins, Ca^{2+} aids heterodimer formation, while Mg^{2+} and Mn^{2+} support ligand binding. Thus samples were buffer exchanged and concentrated into buffers including 1 mM MgCl_2 and 0.1 mM CaCl_2 for further studies.

Western blot analysis

Fraction samples were prepared as above for SDS-PAGE analysis prior to Western blot analysis. Briefly, gels were transferred to nitrocellulose membranes (Whatman cat. # 10401197) at 1 amp for 1 h 30 min. Transferred membranes were blocked in 5% dry milk/1X PBS, 0.05% Tween for 1 h, incubated with a mouse anti-his primary antibody (Abcam cat. # 18184) in a 1:5,000 dilution in a 1% dry milk solution, and incubated with a secondary anti-mouse IgG antibody (1:5,000; GE Healthcare cat. # NA931V). Three consecutive washes (5min; 1X PBS, 0.05% Tween) were conducted between each step. Integrins were detected using chemiluminescence (Millipore cat. WBKLS0500).

Native dot blot

An un-boiled (none denatured) elution fraction (total 7 μg), previously indicated by SDS-PAGE and Western blot to contain the $\alpha 5$ and $\beta 1$ integrins, was loaded onto a nitrocellulose membrane and treated under the same conditions as a Western blot (see above). A primary monoclonal antibody (MAb), anti-integrin $\alpha 5\beta 1$ (Abcam 6131), which recognizes a conformational epitope for the $\alpha 5$ integrin subunit, was utilized in place of the anti-his antibody for detection. A second primary Mab, anti-integrin $\beta 1$ (Abcam 7168), which recognizes a linear-conformational epitope for the $\beta 1$ integrin subunit was also used.

Trypsin digestion mass spectrometry

A wash solution of 200 L of 50 mM NH_4HCO_3 /50% acetonitrile (pH 8.4) was added to the excised SDS-PAGE gel bands containing proteins believed to be the integrin subunits based on the Western and native dot blot analyses. The contents of each tube (containing $\alpha 5$ and $\beta 1$ separately) were vortexed for 45 min and the solution was discarded. The wash step was repeated until the gel slices were clear, and then they were dried (Speedvac) to remove liquid. A freshly prepared 45 mM dithiothreitol solution (reducing agent) was added to cover the gel slices for 45 min at 55°C. The DTT solution was replaced with approximately

the same volume of freshly prepared 100 mM iodoacetamide followed by incubation at RT for 45 min. The iodoacetamide solution was removed and the gel slices were washed with 200 μ L of 50 mM NH_4HCO_3 /50% acetonitrile (pH 8.4) for 30 min with vortexing. The wash solution was removed and this procedure was repeated 3 times. The gel slices were dried in a Speedvac. Approximately 5–10 μ L of 12.5ng/ μ L Trypsin (Promega) in 25 mM NH_4HCO_3 (pH 8.4) was added to each gel slice and incubated at 4°C for 30 min. Excess enzyme solution was removed, the required volume of 25 mM NH_4HCO_3 (pH 8.4) were added to cover the gel slices and they were incubated at 37°C for 12 to 16 h. The reaction was stopped by adding aqueous 5% acetic acid to a final concentration of 0.5%.

The tubes containing the gel slices were agitated for 15 min and centrifuged at 10000g for 1 min on a bench top microcentrifuge. The supernatant (containing tryptic peptides) was transferred to a clean 0.5 mL tube (BioRad). Approximately 50 μ L of extraction solution (80 % acetonitrile, 1 % TFA) was added to the gel slices followed by sonication in an ultrasonic water bath for 5 min. The supernatant (containing additional tryptic peptides) was transferred to the same 0.5-mL tube. The gel slices were extracted again with an additional 50 μ L of acetonitrile and agitation by gentle vortexing for 10 min and centrifuged at 10000g for 1 min. The supernatant was combined with the previous two extractions in the 0.5 mL tube. The pooled extracted peptides were dried by Speedvac. Approximately 15 μ L of Loading Buffer (3% ACN, 1% acetic acid, and 0.1 % TFA) was added to each tube and gently agitated by vortexing. The supernatants were transferred to a vial for Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis.

LC- MS/MS

The enzymatically-digested integrin samples were injected onto a capillary trap (LC PackingsPepMap) and desalted for 5 min with a flow rate 10 μ L/min of 0.1% v/v acetic acid. The samples were loaded onto a column (LC Packing[®] C18 Pep Map) on the Tempo nanoflow HPLC system. The elution gradient of the HPLC column started at 97% solvent A (0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H_2O), 3% solvent B (0.1% v/v acetic acid, 96.9% v/v ACN), and 3% v/v H_2O and finished at 60% solvent B, 40% solvent A and ran for 20 min. For protein identification LC-MS/MS analysis was conducted using a hybrid quadrupole-linear ion trap mass spectrometer (4000QTRAP, Applied Biosystems). The focusing potential and ion spray voltage was set to 275 V and 2600 V, respectively. The information-dependent acquisition (IDA) mode of operation was employed in which a survey scan from m/z 400–1500 was acquired followed by collision-induced dissociation (CID) of the two most intense ions. Survey and MS/MS spectra for each IDA cycle were accumulated for 1 and 3 s, respectively.

Protein search algorithm for peptide mass identification

Tandem mass spectra were extracted by ABI Analyst version 1.4.1. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.0.01). Mascot was set up to search the IPI Human database (67,524 entries) assuming the enzyme used for protein digestion was trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 1.0 Da. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met were specified in Mascot as variable modifications. Scaffold (version Scaffold-02–03-01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [17]. Protein identifications were accepted if established at greater than 99.0% probability and contain at least 2 identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm [18].

Negative stain electron microscopy of $\alpha 5\beta 1$ integrin

Five micro liters of nickel-column purified $\alpha 5\beta 1$ integrin was pipetted onto glow-discharged copper grids (Ted Pella, Inc, cat. # 01754-F) and washed three times by inversion on 15 μ l drops of diH₂O. The grid was then stained for 30 sec by inversion onto two 15 μ l drops of 2% uranyl formate (Polyscience, Inc. cat. # 24762). The grids were blotted using filter paper after the last wash and staining steps. A negative control grid of uranyl formate only was prepared in the same manner. The samples were visualized by using an FEI Tecnai G² Spirit-TWIN transmission electron microscope operating at 120 kV.

Transduction inhibition assays in vitro

The $\alpha 5\beta 1$ integrin has been reported to serve as a cellular internalization receptor for AAV2 [9]. To determine the capacity of the expressed extracellular domain of the $\alpha 5\beta 1$ integrin to compete for binding by AAV2, five experimental cellular infection conditions were assayed in HeLa cells: 1) Mock (1 $\times$ PBS only), 2) AAV2 alone, and, 3) AAV2 + $\alpha 5\beta$ integrin (200 μ g/ml), 4) AAV2 + $\alpha 5\beta$ integrin (100 μ g/ml), 5) AAV2 + $\alpha 5\beta$ integrin (10 μ g/ml). A highly purified stock of self-complementary AAV2 viruses (5×10^8 viral genomes per μ l) packaging the enhanced green fluorescence gene (scAAV2-EGFP) was used for these studies [27]. The virus and integrin complexes were mixed in 50 μ l of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) with no FBS and no antibiotic/antimycotic and incubated at room temperature for 30 min prior to infection of the HeLa cells. The cells were washed once with 1 $\times$ PBS and infected at MOI of 1250 viral genomes per cell in the various experiments. After 2 h, an additional 150 μ l of DMEM media with 5% FBS and antibiotic/antimycotic (Life Technologies) were added to each well. Seventy-two h after infection, transgene expression was detected by fluorescence microscopy using an Axiovert 25 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Images from three independent visual fields were analyzed quantitatively by the ImageJ analysis software (National Institutes of Health, Bethesda, MD). Transgene expression was assessed as total area of green fluorescence (pixel²) per visual field (mean $\pm$ SD). Analysis of variance (ANOVA) was used to compare test results with the control, and they were determined to be statistically significant.

Results and discussion

Construct design and generation of recombinant baculoviruses

The maps for the plasmid baculovirus transfer vectors (pFastBac1) vectors used for the expression of both $\alpha 5$ and $\beta 1$ integrins, with the primer code to locate the primers used (Table 1) for amplification of the various domains, are shown in Figure 1. A Kozak sequence was inserted into the original pFastBac plasmid vector for translation initiation at the *Sal* I and *Bam* HI sites, directly upstream of the start sites for $\alpha 5$ and $\beta 1$ integrin extracellular domains, which are followed by Fos and Jun domains, respectively. This was done due to numerous reports in the literature suggesting that a Kozak sequence enhances protein expression in insect cells [19, 20]. An 8X-histidine tag, inserted in addition to a stop codon for translation termination in-frame with the Fos and Jun domains, enable a one-step nickel column purification. The Fos and Jun domains, known to self dimerize, were inserted in place of the $\alpha 5$ and $\beta 1$ integrin transmembrane domains to prevent protein aggregation and precipitation which is often observed when expressing and purifying membrane proteins. A restriction enzyme reaction to digest the $\alpha 5$ and $\beta 1$ integrin extracellular domains from the rest of the plasmid showed DNA bands at ~ 3.0 kb for $\alpha 5$ and ~ 2.2 kb for $\beta 1$, both of which were expected (Fig. 2A). Five plaque-purified baculovirus recombinants, isolated by PCR amplification, contained the expected products for the $\alpha 5$ -Fos and $\beta 1$ -Jun DNA (Fig. 2B and C). The completed constructs are similar to those previously published for the expression of αV -Fos and $\beta 5$ -Jun with the exception that they utilized the pQE-

TriVector System [21]. However, overlap between the $\alpha 5$ cDNA and *Nco* I restriction digest sites within the pQE-TriVector precluded its use for cloning/expression in the current study.

Small-scale protein expression, purification, and LC-MS/MS analysis

The use of SF9 insect cells for protein expression ensures high yields and correct post-translational processing [22]. A small volume (50 ml) expression in SF9 cells followed by nickel column purification was used to validate the integrin constructs. Infection of SF9 cells with the highest titered baculovirus recombinant for each integrin subunit, $\alpha 5$ -3 and $\beta 1$ -5, following amplification to a P3 stage, produced soluble integrins in the media fraction that were purified via nickel-column chromatography for $\alpha 5$ integrins (Fig. 3A and C) and $\beta 1$ (Fig. 3B and C). Eluted fractions contained protein bands migrating at ~145 kDa for $\alpha 5$ and ~100 kDa for $\beta 1$ integrins, respectively, when visualized by SDS-PAGE. The bands are higher than the theoretical molecular weights for $\alpha 5$ (~115 kDa) and $\beta 1$ (~85 kDa) integrin. A possible explanation for this observed higher gel migration is glycosylation of the integrin heterodimer which is required for functional activity [23]. The eluted bands (elution 3, Fig. 3A and B) were uniquely identified as $\alpha 5$ and $\beta 1$ integrins by LC-MS/MS (Fig. 4A and B). Based on previous cell pellet purifications (data not shown), the ratio of secreted to cytosolic integrins are typically 75% to 25% despite the lack of secretion signals in the integrin constructs. In addition, integrin purification from PEG-precipitated media samples were more efficient at 90–95% purity compared to the samples from cell lysates isolated from freeze-thawed cell pellets at 60–70% purity. This is most likely due to higher levels of contaminating host cell proteins in cell lysates compared to the PEG-precipitated media.

Large-scale protein expression, purification, and Dot blot characterization

To produce large quantities of $\alpha 5$ and $\beta 1$ integrins (up to 4mg), 1 L volumes of SF9 insect cells were co-infected with $\alpha 5$ and $\beta 1$ integrin baculovirus constructs to produce the $\alpha 5\beta 1$ heterodimer followed by a one-step nickel column purification. Cellular proteins were removed by 100 and 150 mM imidazole concentrations (Fig. 5A, lanes 3–4), while the bound integrins were detected in the 400 mM imidazole elution fractions (Fig. 5A, lanes 5–17). Washing with 60–100 mM imidazole failed to remove cellular contaminants, while stringent washing steps of 100–150 mM imidazole was effective. A yield of ~4 mg of purified $\alpha 5\beta 1$ heterodimer, per 1 L of infected SF9 cells, was obtainable from pooled (Fig. 5A, lanes 5–17) and concentrated (Fig. 5B) elution fractions. A loss of ~10–20% integrins was estimated to occur due to protein adhesion to the surface membrane of the concentrator used at the last purification step.

The conformational state of the purified $\alpha 5\beta 1$ integrin heterodimer was determined by Dot blot analysis (Fig. 5C) with function blocking monoclonal antibodies, anti- $\alpha 5\beta 1$ [24] and anti- $\beta 1$ integrin [25]. The non-denatured (unboiled) sample was recognized by both Mabs, while the non-native (boiled sample) reacted to the anti- $\beta 1$ Mab only. Both antibodies have been reported to recognize conformational epitopes, however, the anti- $\beta 1$ Mab also reported to recognize a linear-epitope consistent with the observed positive signal for the denatured sample. These observations are consistent with the adoption of a native state by the expressed and purified $\alpha 5\beta 1$ integrin heterodimer.

Transmission EM analysis of the $\alpha 5\beta 1$ integrin heterodimer

Negative-stain transmission EM was used to visualize the integrity and conformational state of the purified $\alpha 5\beta 1$ integrin heterodimer. Figure 6 shows images of negative-stain only control (Fig. 6A) and $\alpha 5\beta 1$ integrin heterodimer (Fig. 6B–H). A dilution series enabled the visualization of individual heterodimers in a number of different conformations at a concentration of ~0.003 mg/ml (Fig. 6B and C). The conformation of the integrin molecules in the inset (Fig. 6G and H) were observed to be in the V-shape or bent conformation,

similar to the those previously reported for integrins not bound to ligands [26]. Thus the purified heterodimer appears to be in a native state.

Transduction inhibition assays

Previous studies have shown that $\alpha 5\beta 1$ integrin is a co-receptor for AAV2 and serves to promote virus internalization into the cell [9]. Thus to further demonstrate that the expressed and purified extracellular domain of the $\alpha 5\beta 1$ integrin exists in a native functional state, the ability of the purified heterodimer to compete for receptor attachment by AAV2 was tested in a transduction assay. HeLa cell transduction efficiency by scAAV2-EGFP with and without pre-incubating the virus with $\alpha 5\beta 1$ integrin showed that the protein was capable of inhibiting cellular internalization (Fig. 7), presumably by competing with endogenous integrin binding. Mock-infected HeLa cells showed no GFP transgene expression, the virus alone sample showed efficient GFP expression, while pre-incubation with the integrin heterodimer at 10 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, and 200 $\mu\text{g/ml}$ resulted in a 29%, 54%, and 99%, respectively, decrease in transduction as previously reported [9]. This capacity of the expressed $\alpha 5\beta 1$ integrin to inhibit AAV2 transduction is consistent with a functional state with the ability to mimic a reported virus-receptor interaction.

Summary

In this study, a robust system for expressing and purifying the $\alpha 5\beta 1$ integrin heterodimer in SF9 insect cells was developed. Recombinant baculoviruses packaging the genes for $\alpha 5$ -Fos and $\beta 1$ -Jun integrin were created and used for co-infections. Using a one-step nickel column purification, ~4 mg of conformationally and biologically active integrin were purified. The integrin quantities obtained are suitable for an array of different studies including those requiring higher protein concentrations such as X-ray crystallography. An X-ray crystal structure for an $\alpha 5\beta 1$ integrin extracellular fragment was previously solved, however, the integrin constructs for expression lacked full length extracellular domains and were utilized for establishing stable cells lines, which can be time-consuming [27]. The large-scale protein production strategy presented here may be applied to other proteins with a specific interest on heterodimers.

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Highlights

$\alpha 5 \beta 1$ integrin expression system for large-scale protein production.

Offers a simple purification method for folded protein.

High-level expression in quantities suitable for biochemical and biophysical assays.

Provides biologically active protein confirmed by transduction inhibition assays.

Figure 1.

Diagram of plasmids used for expression of $\alpha 5\beta 1$ integrin. The cDNA fragments encoding the $\alpha 5$ (A) and $\beta 1$ (B) extracellular domains were inserted in frame with a glycine linker, Fos and Jun dimerization domains, respectively, and an 8X histidine-tag. The positions of the primers (Pr, see Table 1) with their unique enzyme restriction sites used for plasmid assembly are shown.

Figure 2. Verification of $\alpha 5$ and $\beta 1$ integrin genes by restriction enzyme digestions and PCR screening of recombinant baculoviruses for packaged $\alpha 5$ and $\beta 1$ integrin DNA. (A) Uncut (U) and restriction enzyme-digested plasmid DNA for $\alpha 5$ (~3.0 kb) and $\beta 1$ (~2.2 kb) integrin are shown. (B and C) PCR amplified bands verify that all five clones for each respective recombinant baculovirus packaged the appropriate DNA. The sizes of the amplified products are the same as in (A).

A

B

C

Figure 3.

Small-scale insect cell expression and nickel column purification of $\alpha 5$ and $\beta 1$ integrin subunits. (A and B) SDS-PAGE visualization of expressed and purified proteins: Lanes: (1) Molecular weight marker (M), (2) Media (M), (3) flow through (FT), (4) 100 mM imidazole wash 3 (W3), (5–9) elutions 1–5. (C) Western blot of eluted samples shown in A and B probed with an anti-his antibody.

A

IPI00306604 (100%), 114,538.5 Da

Integrin alpha-5

16 unique peptides, 17 unique spectra, 20 total spectra, 179/1049 amino acids (17% coverage)

MGSRTPEsplHAVQLRWGPRRRPPLLPLLLLLPPPPRVGGFNLDAAEAPVLSGPPGSFFGFSVEFYRPGTDGVSVLV
 GAPKANTSQPGVLQGGAVYLCWPWGASPTQCTPIEFDSKGSRLLESSLSSEGEPEVEYKSLQWFGATVRAHGSSILAC
 APLYSWRTEKEPLSDPVGTCYLSTDNFTRILEYAPCRSDFSWAAGQGYCQGGFSAEFTKTGRVVLGGPGSYFWQQQI
 LSATQEIQIAESYYPEYLINLVQGGQLQTRQASSIYDDSYLGYSAVGEFSGDDTEDFVAGVPKGNLTGYVTILNGSDIRS
 LYNFSGEQMASYFGYAVAATDVNGDGLDDLLVGAPLLMDRTPDGRPQEVGRVYVYLQHPAGIEPTPTLTTLTGHDEFGR
 FGSSLTPLGLDQDQGYNDVAIGAPFGGETQQGVVFVFPGGPGGLGSKPSQVLQPLWAASHTPDFFGSALRGGRDLG
 NGYPDLVGSFGVDKAVVYRGRPIVSASASLTIFPAMFNPEERSCSLEGNPVACINLSFCLNASGKHVADSIGFTVELQLD
 WQKQKGGVRRALFLASRQATLTQTLLIQNGAREDCREMKIYLRNESEFRDKLSPIHIALNFSLDPAQVDSHGLRPAHY
 QSKSRIEDKAQILLDCGEDNICVPDLQLEVFGEQNHVYLGDKNALNLTFAQNVGEGGAYEAELRVTAPEAEYSGGLVR
 HPGNFSSLCDYFAVNQSRLLVCDLGNPMKAGASLWGGLRFTVPHLRDTKKTQDFQILSKNLNNSQSDVVSFRLSVE
 AQAQVTLNGVSKPEAVLFPVSDWHPRDQPQKEEDLGPVHHVYELINQGPSSISQGVLELSCPQALEGQQLLYVTRVT
 GLNCTTNHPINPKGLELDPEGLHHQKREAPSRSSASSGPQILKCPAEACFRRLCELGPLHQQESQSLQLHFRVWAK
 TFLQREHQPFSLQCEAVYKALKMPYRILPRQLPQKERQVATAVQWTKAEGSYGVPLWIIILAILFGLLLLGLLIYILYKLGFF
 KRSLPYGTA MEKAQLKPPATSDA

B

IPI00217561 (100%) 91,620.0 Da

Isoform Beta-1C of integrin beta-1

26 unique peptides, 27 unique spectra, 34 total spectra, 256/825 amino acids (31% coverage)

MNLQPIFWIGLISSVCCVFAQTDENRCLKANAKSCGECIQAGPNCGWCTNSTFLQEGMPTSARCCDDLEALKKKKGCPDD
 DIENPRGSKDIKKNKNVTNRSGTAELKLPEDITQIQPQQLVLRRLRSGEPQTFTLKFKAEDYPIDLYYLMDSLYSMKDDL
 ENVKSLGTDLMNEMRRITSDFRIGFGSFVEKTVMPYISTTPAKLRNPCTSEQNCTSPFSYKNVLSLTNKGVEFVNLVGKQ
 RISGNLDSPEGGFDAIMQVAVCGSLIGWRNVTRLLVFSTDAGFHAFAGDGKLGIVLPNDGQCHLENNMYTMSHYDYDYP
 SIAHLVQKLSENNIQTIFAVTEEFQPVYKELKNLIPKSAVGTLSSANNVLIIDAYNSLSSEVILENGKLSSEGVTSISYSYC
 KNGVNGTGENGRKCSNISIGDEVQFEISITSNKCCKDSDSFKRPLGFTEEVEVILQYICECECQSEGIPESPKCHEGNG
 TFECGACRCNEGRVGRHCECSTDEVNSEDMDAYCRKENSSEICSNNGECVCGQCVCRKRDNTEIYSGKFCECDNF
 NCDRSNGLICGGNGVCKCRVCECNPNYTGSAACDCSLDTSTCEASNGQICNGRGICECGVCKCTDPKFQGGQTCCEMCQT
 CLGVCAEHKECVQCRAFNKGEKDTCTQECYSFNITKVESRDKLPQPVQPDVPVSHCKEKDVDDCWFFYFTYSVNGNNE
 VMVHVVENPECPTGPDIIPIVAGVAVGIVLIGLALLIWKLLMIHIDRREFAKFEKEKMNAKWDTSLSVAQPGVQWCDISSL
 QPLTSRFQFQFSLSLPSTWDYRVKILFIRVP

Figure 4.

Tandem MS/MS verification of expressed integrin domains. (A) $\alpha 5$ and (B) $\beta 1$ integrins, showing the amino acid sequence for the proteins with the unique peptides identified highlighted in yellow.

Figure 5.

Large-scale insect cell expression and nickel column purification of $\alpha 5 \beta 1$ integrin. (A) SDS-PAGE visualization of expressed and purified proteins: Lanes: (1) Molecular weight marker (M), (2) flow through (FT), (3) 100 mM imidazole wash 1 (W1), (4) 150 mM imidazole wash 2 (W2), (5–18) elutions 1–14. (B) Lanes: (1) SDS-PAGE of pooled and concentrated elution fractions 1–14 (shown in lanes 5–18 in (A)), (2) Western blot of same sample shown in lane 1 probed with an anti-his antibody. (C) Native dot blot of boiled (denatured) and unboiled native $\alpha 5 \beta 1$ integrin probed with anti- $\alpha 5 \beta 1$ integrin and anti- $\beta 1$ integrin antibodies.

Figure 6.

A dilution series of $\alpha 5 \beta 1$ integrin visualized by negative stain EM. (A) Control grid with uranyl formate stain only. (B) Purified $\alpha 5 \beta 1$ integrin stock (0.6 mg/ml). (C) 2 $\times$ dilution. (D) 10 $\times$ dilution. (E) 20 $\times$ dilution. (F) 100 $\times$ dilution. (G) 200 $\times$ dilution. (H) Close up view of (G). Samples were stained with uranyl formate and images were taken at 50,000 $\times$ magnification. Scale bar is 100 nm.

A

B

Figure 7.

Analyses of AAV2-mediated transduction of HeLa cells. (A) Cells mock-infected, infected with scAAV2-EGFP alone, and infected with scAAV2-EGFP and 10 µg/ml, 100 µg/ml, and 200 µg/ml of $\alpha 5\beta 1$ integrin at a MOI of 1250 viral genomes/cell. Transgene expression was detected by fluorescence microscopy 72 h post-infection. A representative visual field view is shown from each triplicate experiment. Magnification was $\times 100$. (B) Quantitative analyses of AAV2 transduction efficiency in HeLa cells. Images from three visual fields were analyzed quantitatively by ImageJ analysis software. Transgene expression was assessed as total area of green fluorescence (pixel²) per visual field (mean $\pm$ SD). ANOVA was used to compare test results with the control, and they were determined to be statistically significant. **p<0.01 vs scAAV2-EGFP alone.

Table 1

Primer Sequences

Primer Name	Sequences	<i>a</i> P _r
α 5_F5-RE ^b	5' <i>gagg</i> <u><i>gtc</i></u> <i>gac</i> gccaccatggggagccggacgccag 3'	1
α 5_R5-RE	5' <i>gagg</i> <u><i>cg</i></u> <i>gccgc</i> atttctgccttggtccattgc 3'	2
Fos_F4-RE	5' <i>gagg</i> <u><i>cg</i></u> <i>gccgc</i> ctgggtggtggtggtgctgactgatacactccaagcg 3'	3
Fos_R3-RE	5' <i>gagt</i> ctagagt <i>gag</i> ctgccaggatgaactc 3'	4
His_F1-RE	5' ctagacatcatcatcatcatcattaaa 3'	5
His_R1-RE	5' agcttttaatgatgatgatgatgatgt 3'	6
β 1_F5-RE	5' <i>gagg</i> <u><i>gat</i></u> <i>ccg</i> ccaccatgggtaattacaaccaatttc 3'	7
β 1_R7-RE	5' <i>gagg</i> <u><i>tc</i></u> <i>gac</i> gtctggaccagtgggacac 3'	8
Jun_F2-RE	5' <i>gagg</i> <u><i>tc</i></u> <i>gac</i> gggtggtggtggtgtagaatgccggctggagg 3'	9
Jun_R2-RE	5' <i>gagt</i> ctagagtgggtcatgactttctg 3'	10

^a Indicates the primers used as shown in Figure 1;

^b restriction sites are italicized and glycine linkers are underlined.