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Molecular cloning, overexpression, and an efficient one-step purification of α5β1 integrin

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ABSTRACT

The α5β1 integrin heterodimer is involved in many cellular processes and is an anti-cancer 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 α5β1 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 α5β1 integrin in quantities suitable for an array of different experiments including structural biology.

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)[1] 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 a short cytoplasmic tail [1]. In mammals, there are 18α and 8β 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]. Therefore, tissue location and ligand availability are strong determinants of integrin interactions that promote cellular migration, proliferation, and differentiation [4].

The α5β1 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 α5β1 integrin expression between normal and tumoral cells suggest involvement in tumor progression and aggressiveness [6]. A homozygous deletion of the α5 integrin in mice results in defects in neural tube development and blood cell leakage during embryogenesis [7], while β1 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 α5β1 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, α5β1 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 α5β1 integrin. We utilized the pFastBac1 vector with an 8X-histidine tag as the backbone for the construction of two recombinant baculoviruses encoding the α5 and β1 integrin extracellular domains, in-frame with Fos and Jun dimerization domains, respectively. Co-infections in *Spodoptera frugiperda* 9 (SF9) cells and a one-step nickel-column purification yielded pure protein in the amount of ~4 mg per liter of cell culture. The purified α5β1 integrin inhibited cellular transduction by Adeno-associated virus serotype 2 (AAV2), a reported viral ligand [9], consistent with a functional state for the protein.

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[1] *Abbreviations used:* ECM, extracellular matrix; SF9, *spodoptera frugiperda* 9; AAV2, adeno-associated virus serotype 2; MOI, multiplicity of infection, MAb, monoclonal antibody; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC–MS/MS, liquid chromatography tandem mass spectrometry; IDA, information-dependent acquisition; CID, collision-induced dissociation.

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Materials and methods

Construct design

The full-length cDNAs for α5 integrin (OpenBiosystems cat. # MHS1011-61377), β1 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 α5 integrin encoding only the extracellular domain was PCR amplified (iProof High-Fidelity DNA polymerase; BIORAD cat. # 172-5302) with primers (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 s, (3) 58 °C–1 min, (4) 72 °C–4 min, and (5) 72 °C–10 min, with 35 cycles run between steps 2 and 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 μg/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 α5 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 (RT) 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 (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 (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.

Table 1
Primer sequences.

Primer Name	Sequences	[a]Pr
α5_F5-RE[b]	5′ gag*gtcgac*gccaccatggggagccggacgccag 3′	1
α5_R5-RE	5′ gag*gcggccgca*tttctgccttggtccattgc 3′	2
Fos_F4-RE	5′ gag*gcggccgct*ggtggtggtggtggtctgactgatacactccaagcg3′	3
Fos_R3-RE	5′ gagtctagagtgagctgccaggatgaactc 3′	4
His_F1-RE	5′ ctagacatcatcatcatcatcattaaa 3′	5
His_R1-RE	5′ agcttttaatgatgatgatgatgatgtgt 3′	6
β1_F5-RE	5′ gag*ggatcc*gccaccatgggtaatttacaaccaattttc 3′	7
β1_R7-RE	5′ gag*gtcgac*gtctggaccagtgggacac 3′	8
Jun_F2-RE	5′ gag*gtcgac*ggtggtggtggtggtagaatcgccggctggagg 3′	9
Jun_R2-RE	5′ gag*tctaga*gtggttcatgacttttctg 3′	10

[a] Indicates the primers used as shown in Fig. 1.
[b] Restriction sites are italicized and glycine linkers are underlined.

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 1811*g* 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 NaH_2PO_4, 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 step-wise wash gradient with increasing concentrations of imidazole (60, 80, 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 1811*g* 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 17700*g* at 4 °C for 90 min to collect the pellet. This pellet was resupsended in 25–50 ml lysis buffer (including protease inhibitor) depending on pellet size. Following resuspension, an additional centrifugation step at 1811*g* 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 and 150 mM imidazole. Bound protein was eluted with 400 mM imidazole and collected in 1 ml