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Construction, expression, and purification of recombinant α V β 5 integrin

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Abstract

A recombinant integrin expression system has been created for the large-scale production of α V β 5 integrin extracellular domains that take advantage of Fos and Jun dimerization for expression in bacterial, insect, and mammalian cells. This utilizes an all-in-one vector, pQE-TriSystem, with molecular machinery for parallel expression without the need of additional subcloning. Optimal expression in HEK293 cells was determined by a time course analysis. The heterodimer was purified in a one-step nickel column purification scheme, and the sequence and functional state were confirmed by mass spectrometry and inhibition assays, respectively. The yields of α V β 5 integrin obtained are in quantities suitable for multiple applications including structural biology and functional assays.

Keywords

α V β 5 integrin expression; pQE-TriSystem vector; and HEK293 cell protein expression

Introduction

Integrins are cell adhesion receptors that transmit information via inside-out and outside-in signaling mechanisms that link both intracellular and extracellular environments [1,2]. They are heterodimeric proteins that comprise α and β subunits each containing extracellular, transmembrane, and cytoplasmic domains. In mammals, 18 α and 8 β subunits exist that heterodimerize to form 24 distinct combinations that have a multitude of biological functions including fertilization, embryonic development, immune responses, platelet aggregation, and receptor attachment [3,4]. Furthermore, integrin defects have been implicated in various cancers and numerous human diseases, including genetic and autoimmune disorders [5].

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The $\alpha V\beta 5$ integrin is one of the most widely studied and serves as a receptor for extracellular matrix protein vitronectin, implicated in neural crest cell migration, angiogenesis, and tumor progression [6]. Furthermore, mutations in $\alpha V\beta 5$ integrin, specifically the αV subunit, has deleterious effects on placental and cerebral vasculature that lead to embryonic lethality and neuroepithelial defects, respectively [1]. This receptor is thus widely studied in the field of receptor biology in efforts to understand its function. The identification of the key roles of $\alpha V\beta 5$ integrin from a structural, cell biology, and signaling standpoint will elucidate the underlying mechanisms involved in integrin biology and provide information for the development of therapeutics.

The goal of this study was to produce highly purified, soluble recombinant integrin for structure-function studies. This effort was undertaken because currently available commercial sources provide limited amounts of samples, which are often lyophilized leading to decreased protein activity, and are reconstituted with additional ingredients, for example, Octyl- β -D-glucopyranoside, Triton X-100, and sodium azide that are inhibitory to many experimental procedures. Furthermore, there are only a few example studies in the literature that focus solely on expression of the extracellular domains of integrins for ligand-receptor interaction characterization where the insoluble transmembrane domains are removed [7]. The approach utilized here aimed to create an expression system that would ensure posttranslational modifications of the $\alpha V\beta 5$ integrin and yield suitable quantities of the dimerized domains for biophysical and structural experiments. We report the overexpression of $\alpha V\beta 5$ integrin in HEK293 cells using the pQE-TriSystem vector, an 8X histidine-tagged plasmid that possesses the necessary molecular components to facilitate expression in bacteria, insect, and mammalian cells. The protein was successfully purified in a one-step method employing nickel column chromatography with yields up to 2 mg per cell factory (equivalent to forty, 15 cm plates). The sequence of the protein and its functional state were verified by mass spectrometry and inhibition assays, respectively.

Materials and methods

Construct design

The full-length cDNAs for αV Integrin (GeneService Ltd cat. # 40146451), $\beta 5$ Integrin (OpenBiosystems cat. # MHS1011-169792), Fos (OpenBiosystems cat. # MHS1011-60754), and Jun (OpenBiosystems cat. # MHS1010-97228128) were purchased for PCR amplification of the appropriate domains. A 2,971bp fragment of integrin αV 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 *Nco*I and *Eco*RI. 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 excised from an agarose gel (BIO-RAD cat. # 161-3102), extracted (Qiagen cat. # 28706), restriction enzyme digested with *Nco*I and *Eco*RI, and ligated into the pQE-TriSystem Vector (Qiagen cat. # 33903) using the same cloning sites. A 3:1 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 153 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 *Eco*RI and *Xho*I, and a five glycine linker. This linker region between the αV 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 pQE-TriSystem vector. The completed construct was verified by DNA sequencing.

A second construct was generated for the integrin $\beta 5$ extracellular domain. A 2,173 bp fragment was amplified with primers (see Table 1) designed with restriction sites *Pci*I and *Hind*III, and inserted into the pQE-TriSystem vector using *Nco*I and *Hind*III cloning sites. A 153 bp fragment of Jun was amplified with primers (see Table 1) designed with restriction sites *Hind*III and *Xho*I and a glycine linker, and inserted in frame with the integrin $\beta 5$ extracellular domain utilizing the same cloning sites in the vector. The completed construct was verified by DNA sequencing.

Small-scale protein expression/time course analysis

Human Embryonic Kidney 293 cells (HEK293; American Type Culture Collection) were cultured in 15 cm plates (BD Falcon cat. # 353025) and maintained in Dulbecco's modified Eagle's medium (Gibco 11995) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5% fetal bovine serum (SAFC Cat. #123030C) at 37°C in a 5% CO₂: 95% air humidifier incubator. Cells were grown to 80% confluency and transfected with equimolar ratios of the α V and $\beta 5$ DNA plasmids by calcium phosphate precipitation [8]. Briefly, 45 μ g of each construct were transfected per 15 cm plate ($\sim 2.5 \times 10^7$ cells) for a total of 90 μ g DNA. Cells were harvested at various time points post-transfection: 0 h, 12 h, 24 h, 32 h, 40 h, and 48 h. Additional time points were not taken since most of the cells lost adherence to plates 48 h post-transfection. The cells were separated from the media (supernatant) by centrifugation at 1,811 $\times$ g at 4°C for 20 min. The cell pellet (CP) was resuspended in SDS loading dye and both the CP and supernatant were boiled at 100°C for 10 min and analyzed by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; see below).

Large scale protein expression and purification via nickel column chromatography

A cell factory of HEK293 cells was transfected with equimolar amounts of both the α V and $\beta 5$ plasmids as described above and harvested 48 h post-transfection. The cells were separated from the media after centrifugation and resuspended in 20 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20, pH 8.0) that included an EDTA-free protease inhibitor mix (Roche cat. # 04693159001). The transfected cells were lysed by three/freeze thaw cycles with the addition of benzonase nuclease (Novagen cat. # 70664) after the last thaw followed by incubation at 37°C for 30 min. The cell lysate was clarified by centrifugation at 1,811 $\times$ g at 4°C for 20 min. The cellular debris was discarded and the supernatant was applied to an equilibrated (in lysis buffer) 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 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 and collected in 300 μ l fractions. The cell lysis, wash, and elution buffers were identical except for varying imidazole concentrations, and the wash and elution buffers were protease inhibitor free. The load, wash, and elution fractions were analyzed by SDS-PAGE, Western blots, and Native dot blots (see below).

SDS-PAGE analysis

Eluted fraction samples were boiled for 10 min, centrifuged briefly, and loaded onto a 4–% polyacrylamide gel (Bio-Rad cat. # 345-003). SDS-PAGE was performed at 125 V for 90 min [9]. 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 SDSPAGE data, fractions containing only the α V and β 5 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 [10,11]. 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 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 elution fraction (total 5 μ g) that has not been denatured, previously indicated by SDS-PAGE and Western blot to contain the α V and β 5 integrins was loaded onto a nitrocellulose membrane and treated under the same conditions as a Western blot. A primary monoclonal antibody (MAb), anti-integrin α V β 5 (Abcam P1F6), which recognizes a conformational epitope for the integrin heterodimer, was utilized in place of the anti-his antibody for detection.

Trypsin digestion mass spectrometry

A wash solution of 200 μ 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. The contents of each tube were vortexed for 45 minutes 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) to cover the gel slices were added and they were incubated at 37°C for 12 to 16 hrs. 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 10,000 g for 1 min on a benchtop 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 $\times 10,000$ g 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 (LCMS/ MS) analysis.

LC- MS/MS

The enzymatically-digested samples were injected onto a capillary trap (LC Packings PepMap) 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 H₂O), 3% solvent B (0.1% v/v acetic acid, 96.9% v/v ACN), and 3% v/v H₂O 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 quadrupolelinear 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 [12]. 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 [13].

Co-immunoprecipitation

HEK 293 cells were transfected with plasmids for α V integrin, β 5 integrin, and both α V and β 5 integrins. Cell lysates were obtained as described earlier. Approximately 2.5 μ l of anti-integrin α V β 5 MAb (P1F6) and 30 μ l of protein A Sepharose beads (GE Healthcare) were incubated at 4°C with gentle rocking for 1 h. Cell lysates from the three different transfections (~500 μ l each) were added to individual microcentrifuge tubes that included the MAb and protein A Sepharose. A fourth tube devoid of the MAb that included only protein A and the α V β 5 integrin cell lysate was utilized as a negative control. The four tubes were incubated at 4°C O/N with gentle rocking. Samples were spun at $1,811 \times g$ for 5 min for pelleting the protein A beads followed by removal of unbound proteins in cell lysates, which were designated flow-through. Beads were washed three times with 1 ml of cell lysis buffer and centrifuged at $1,811 \times g$ for 5 min. The beads were resuspended in 60 μ l of 2X SDS loading dye, boiled for 5 minutes to dissociate the immunocomplexes, collected by centrifugation, and SDS-PAGE was performed with the supernatant.

Transduction inhibition assays in vitro

To determine the capacity of the expressed extracellular domain of $\alpha V\beta 5$ integrin to compete for cell surface receptor binding by Adeno-Associated Virus Serotype 2 (AAV2), four experimental conditions were assayed: 1) Mock (1 $\times$ PBS only), 2) virus alone, 3) virus + $\alpha V\beta 5$ integrin (100 $\mu\text{g/ml}$), and 4) virus + $\alpha V\beta 5$ integrin (10 $\mu\text{g/ml}$). Highly purified stocks of self-complementary AAV2 viruses (5×10^7 viral genomes per microliter) packaging the enhanced green fluorescence gene (scAAV2-EGFP) was a kind gift provided by Drs. Arun Srivastava and Chen Ling (University of Florida, Gainesville, FL). The virus and integrin complexes were mixed in 50 μl of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) with no FBS and antibiotic/antimycotic and incubated at room temperature for 30 min prior to infection of Human cervical cancer (HeLa) cells. The cells were washed once with 1 X PBS and infected at a multiplicity of infection (MOI) of 1,250 viral genomes per cell in the various experiments. After 2 h, an additional 150 μl of DMEM media with 5% FBS and antibiotic/antimycotic (Life Technologies) were added to each well. Seventy-two hours 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 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 development rationale

The maps for the plasmid vector used for the expression of both αV and $\beta 5$ integrins, with the primer code to locate the primers used (Table 1) for amplification of the various domains is shown in figure 1. Since the pQE-TriSystem vector system incorporates a eukaryotic Kozak sequence for translation initiation at the *Nco*I restriction site, primers were designed for in-frame insertion in this region. The size of the DNA products obtained by restriction digests of the vectors following PCR amplification of the extracellular, Fos, and Jun domains, and ligation into the vector were of the expected sizes (Fig. 2). The two extracellular integrin domains for the αV and $\beta 5$ were observed at ~ 2.2 kb and ~ 3.0 kb, respectively, and the Fos and Jun were each observed at ~ 150 bp (Fig. 2A). The size of the fragments from the restriction digests were ~ 5.9 kb and ~ 3.0 kb for αV , and ~ 5.9 kb and 2.2 kb for $\beta 5$ (Fig. 2B and C). The Fos and Jun digestion product bands were less intense in the agarose gels compared to the integrin domains possibly due to reduced incorporation of ethidium bromide in the small DNA products. In these constructs the Fos and Jun dimerization domains were inserted in place of the transmembrane domains.

Integrin expression in HEK 293 mammalian cells and purification

HEK 293 cells were selected for the expression of the $\alpha V\beta 5$ integrin; a physiologically relevant system in which the proteins will undergo post-translational modifications, such as glycosylation, as reported to be important for integrin receptors [14,16]. An expression time course analysis determined that optimal protein expression was at 48 h post-transfection (Fig. 3, lane 11). His-tagged integrins were identified in cell lysate fractions obtained from the cell pellet and have not been secreted into the media.

In the one – step purification of the expressed integrins using a nickel column, cellular proteins were removed by the 60–100 mM imidazole concentrations (Fig. 4A, lanes 4–8), while the bound integrin, migrating at apparent molecular weights of ~ 150 kDa (αV) and ~ 85 kDa ($\beta 5$), were detected in the 250 mM elution fractions (Fig. 4A, lanes 9–17). The

theoretical molecular weights for αV and $\beta 5$ are 115 kDa and 85 kDa, respectively. However, the αV integrin typically migrates higher on SDS-PAGE, probably due to post-translational modifications seen with other integrin heterodimers [17]. Thus the slightly higher molecular weight of the expressed αV integrin suggests that it is post-translationally modified. The yields are 2–3 mg per cell factory based on the pooled elution fractions (Fig. 4A, lanes 10–17 and Fig. 4B & C). These are consistent with those estimated for recombinant protein expression in mammalian cells that range from 0.01% - 1% of the total protein content given our starting material of ~500 mg. The one-step purification method reduces recombinant protein losses that are typically seen when multiple rounds of purification are necessary. However, we do estimate ~10% loss of $\alpha V\beta 5$ integrin to the concentrator. LC-MS/MS analysis of the two integrin bands (Fig. 4B) confirmed the presence of the multiple unique peptides for the αV and $\beta 5$ integrin sequences (Fig. 5) and combined with SDS-PAGE analysis we estimate purity greater than 90–95% (Fig. 4B).

In approximately 20% of the purification runs, a contaminating ~50 kDa protein was eluted within the first few fractions (data not shown). This protein was sequenced by LC-MS/MS and identified as the mammalian transcription factor, 'Non-POU domain-containing octamer-binding protein'. This protein contained numerous patches of basic amino acid residues predicted to have mediated the interactions with the nickel resin. Concentrators with a 150-kDa cutoff were employed for removal of the contaminating protein.

Conformational analyses of $\alpha V\beta 5$ integrin

The conformational integrity of the expressed integrins was investigated with a function blocking $\alpha V\beta 5$ integrin MAb (P1F6), which has been utilized in a wide range of studies including endocytosis of crocidolite fibers in human epithelial cells [18], integrin binding in photoreceptor rods in human retinal pigment epithelium [19], adenoviral gene therapy in bladder carcinoma cells [20]. Coimmunoprecipitation experiments showed that P1F6 interacts with the $\alpha V\beta 5$ integrin heterodimer (Fig. 6A, lane 8) in comparison to the no-antibody control (Fig. 6A, lane 4). Non-specific binding, albeit very minimal, of $\alpha V\beta 5$ integrin to the protein A beads was also observed, however, the positive signal for integrin $\alpha V\beta 5$ was greatly enhanced (Fig. 6A, compare lanes 4 and 8). The singly transfected αV and $\beta 5$ integrins (Fig. 6A, lanes 9–16) did not interact with the P1F6 MAb with the majority of the sample in the unbound fraction (Fig. 6A, lanes 10 and 14) and the lack of positive signal in the elution fractions (Fig. 6A, lanes 12 and 16). The interaction between $\alpha V\beta 5$ and the P1F6 MAb was further substantiated by a Native dot blot in which the non-denatured (un-boiled) sample was recognized while the denatured (boiled) sample was not (Fig. 6B). These observations support a conclusion that the expressed and purified $\alpha V\beta 5$ integrin heterodimer exists in a native folded state. This verifies that the use of the Fos and Jun dimerization domains as a molecular zipper was effective in compensating for the function of the transmembrane domain of this heterodimer. The Fos and Jun dimerization domains fold into two parallel coiled-coil heterodimers and have been used in constructing other heterodimers, for example, $\alpha 3\beta 1$ integrin, major histocompatibility complex (MHC) HLA-DR2 proteins in yeast, and T-cell receptor [21,22]. As observed in these other studies, this utility decreased protein aggregation, which is essential for functional studies.

Transduction inhibition assays

A previous study identified $\alpha V\beta 5$ integrin as a co-receptor for AAV2 and its role in mediating viral entry into the cell [23]. To further substantiate our claims that $\alpha V\beta 5$ integrin is folded properly and functionally active, we tested the transduction efficiency of scAAV2-EGFP with and without preincubating the virus with $\alpha V\beta 5$ integrin in HeLa cells (Fig. 7). From the results shown in Fig. 7a, it is evident that whereas the mock infected HeLa cells showed no green fluorescent protein signal, the transduction efficiency of scAAV2-EGFP

alone was the highest compared to the pre-incubations of virus with $\alpha V\beta 5$ integrin. Specifically, the transduction efficiency of scAAV2-EGFP was inhibited by 75% and 23% when pre-incubated with 100 $\mu\text{g/ml}$ and 10 $\mu\text{g/m}$ of $\alpha V\beta 5$ integrin, respectively, compared to scAAV2-EGFP alone (Fig. 7B). In summary, this study clearly demonstrates that we expressed and purified a functionally active $\alpha V\beta 5$ integrin that inhibits a known virus-receptor interaction in a true biological assay.

Conclusions

Protein production in the typical quantities required for biophysical and structural biology purposes is often a bottleneck for structure-function correlation efforts. With the advent of new DNA vectors that facilitate multi-system expression, numerous proteins can now be expressed with the specific research interests of the lab in mind. For example, heterologous protein expression in *E.coli* yield higher quantities than insect and mammalian cells, however, at the expense of post-translational modifications. The pQE-TriSystem vector used in this study provides the flexibility of protein expression in all three systems, which the researcher can troubleshoot, optimize, and determine the most appropriate for the protein of interest. In this study, we expressed, purified in an efficient one-step scheme, and characterized the conformational and functional state of the extracellular domain of $\alpha V\beta 5$ integrin. Our expression system has the capability to produce sufficient quantities of $\alpha V\beta 5$ integrin, which circumvents quantity limitation and the necessity to eliminate unwanted additives prior to use.

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Highlights

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

Offers one-step nickel column purification of folded protein.

Expression in quantities suitable for biochemical and biophysical assays.

Figure 1.

Diagram of plasmids used for expression of $\alpha V\beta 5$ integrin. The cDNA fragments encoding the αV (A) and $\beta 5$ (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. PCR amplification of cDNAs and verification of assembled plasmids by restriction enzyme digestion. (A) PCR fragments amplified: αV (2.17 kb), $\beta 5$ (2.97 kb), Fos (0.153 kb), and Jun (0.153 kb). (B and C) Restriction enzyme digestions for αV -Fos and $\beta 5$ -Jun, respectively. DNA bands verify that the correct plasmid sizes were present.

Figure 3.

Time course analysis of HEK293 expression of integrin $\alpha V\beta 5$. Lane 1: 0 h – CP, (2) 0 h – S, (3) 12 h – CP, (4) 12 h – S, (5) 24 h – CP, (6) 24 h – S, (7) 32 h – CP, (8) 32 h – S, (9) 40 h – CP, (10) 40 h – S, (11) 48 h – CP, (12), 48 h – S. Cell pellet and supernatant are abbreviated CP and S, respectively. The Western blot was probed with an anti-His antibody.

Figure 4.

Nickel column purification of HEK293-expressed integrin. (A) SDS-PAGE, Lanes: (1) Molecular weight marker (M), (2) cell lysate (CL), (3) flow through (FT), (4) 60 mM imidazole wash 1 (W1), (5) 80 mM imidazole wash 1 (W4), (6) 100 mM imidazole wash 1 (W7), (7) 100 mM imidazole wash 2 (W8), (8) 100 mM imidazole wash 3 (W9), (9–17) elutions 1–9. Three wash steps were performed for each imidazole concentration; however, only representative washes are shown. (B) SDS-PAGE of pooled and concentrated elution fractions 2–9 (shown in lanes 10–17 in (A)). (C) Western blot of sample shown in B probed an anti-his antibody.

A

□ IP100027505 (100%), 116,041.0 Da

Isoform 1 of Integrin alpha-V precursor

20 unique peptides, 20 unique spectra, 22 total spectra, 208/1048 amino acids (20% coverage)

MAFP PRRRLR	LGP RGLPLLL	SGL LPLLCRA	FNLDV DSPA E	YSGPEGSYFG
FAVDFFVPSA	SSRM FLLVGA	PKANTTQPGI	VEGGQVLKCD	WSSTRR CQPI
EFDATGNRDY	AKDDPLEFKS	HQWFGASVRS	KQDK ILACAP	LYHWR TEMKQ
ERE PVGTCFL	QDGTKTVEYA	PCRSQDIDAD	GQGFCCQGGFS	IDFTKADRVL
LGGPGSFYWL	QQLISDQVAE	IVSK YDPNVY	SIKYNNQLAT	RTAQAI FDDSL
YLGYSVAVGD	FNGDGI DD FV	SGVPRAARTL	GMVYIYDGKN	MSSLYNFTGE
QMAAYFGFSV	AATDINGDDY	ADVFIGAPLF	MDRGSDGKLQ	EVGQVSVSLQ
RASGDFQTTK	LNGFEVFARF	GSAIAPLGLD	DQDGFNDIAI	AAPYGGEDKK
GIVYIFNGRS	TGLNAVPSQI	LEGQWAARSM	PSSFYGYSMKG	ATDIDKNGYP
DLIVGAFGVD	RAILYRARPV	ITVNAGLEVY	PSILNQDNKT	CSLPGTALKV
SCFNVR FCLK	ADGKGVLPRK	LN FQVELLLD	KLKQKGAIRR	ALFLYSR SPS
HSKNMTISRGL	GLMQCEEELIA	YLRDESEFRD	K LTPITIFME	YRLDYRTAAD
TTGLQPIILNQ	FTPANISRQA	HILLDCGEDN	VCKPK LEVSV	DSDQK KIYIG
DDNPLTLIVK	AQNQGEGAYE	AELIVSIPLO	ADFI GVVRRN	EALARLSCAF
KTENQTR QVV	CDLGNPMKAG	TQLLAGLRFS	VHQQSEMDTS	VKFDLQIQSS
NLFDKVSPPV	SHKVDLAVLA	AVEIRGVSSP	DHVFLPIPNW	EHK ENPETEE
DVGPPVQHIIY	ELRNNNGPSSF	SKAMLHLQWP	YKYNNNTLLY	ILHYDIDGPM
NCTSDMEINP	LRIKISSLQT	TEKNDTVAGQ	GERDHLITKR	DLALSEGD IH
TLGCGVAQCL	KIVCQVGRLD	RGKSAILYVK	SLLWTEETFMN	KENQNHSSSL
K SSASFNVE	FPYK NLPIDF	ITNSTLVTTN	VTWGIQPA PM	PVPVWVILLA
VLAGL LLLAV	LVFV MYRMGF	FKRVRPPQEE	QEREQLQ PHE	N GEGNSET

B

□ IP100029741 (100%), 88,054.8 Da

Integrin beta-5 precursor

5 unique peptides, 5 unique spectra, 5 total spectra, 45/799 amino acids (6% coverage)

MPRAPAPLYA	CLLGLCALLP	RLAGLNICT S	GSATSCEECL	LIHPKCAWCS
KEDFGSPRSI	TSRCDLRANL	VKNGCGGEIE	SPASSFHVLR	SLPLSSKGS G
SAGWDVIQMT	PQEI AVNLRP	GDK TTFQLQV	RQVEDY PVDL	YYLMDLSLSM
KDDLDNIRSL	GTKLAEEMRK	LTSNFR LGFG	SFVDKD ISPF	SYTAPRYQTN
PCIGYK LFPN	CVPSFGFRHL	LPLTDRVDSF	NEEVRRKQ RVS	RNRDAPEGGF
DAVLQA AAVCK	EKIGWRKDAL	HLLVFTTDDV	PHIALDGKLG	GLVQPHDGGC
HLNEANEYTA	SNQMDYPSLA	LLGEKLAENN	INLI FAVTKN	HYMLYKNFTA
LIPGTTVEIL	DGDSKNIIQL	IINAYNSIRS	KVELSVWDQP	EDLNLFFFTAT
CQDGVSYPGQ	RKCEGLKIGD	TASF EVSLEA	RSCPSRHTES	VFALRPVGF R
DSLEVGVTYN	CTCGCSVGL E	PNSARCN GSG	TYVCGLC ECS	PGYLGTRCEC
QDGENQSVYQ	NLCREAE GKP	LCSGRGDCSC	NQCSCFESEF	GKIYGPFCEC
DNFSCARNKG	VLCSGHGEC H	CGECKCHAGY	IGDNCNCST D	ISTCRGRDGG
ICSERGHCLC	GQCQCTEPGA	FGEMCEK CPT	CPDACSTKR D	CVECLLLHSG
KPDNQ TCHSL	CRDEVITWVD	TIVKDDQEA V	LCFYKTAKDC	VMMFTYVELP
SGKSNLTVLR	EPECGNTPNA	MTILLAVVGS	ILLVGLALLA	IWKLLVTIHD
RREFAKFQSE	RSRARYEMAS	NPLYRKPIST	HTVDFTFNKF	NKSYNGTVD

Figure 5.

Tandem MS/MS verification of expressed integrin domains. (A) α V and (B) β 5 integrins, showing the amino acid sequence for the proteins with the unique peptides identified highlighted in yellow. Cysteines and methionines are shown in green.

A

B

Figure 6.

Characterization of integrin $\alpha V\beta 5$ using a conformational anti- $\alpha V\beta 5$ antibody. (A) Western blot of fractions from a co-immunoprecipitation of cell lysate (CL) with anti- $\alpha V\beta 5$ integrin antibody (P1F6) showing cell lysate (CL), unbound (U), wash (W), and elution (E) for different transfections as labeled at the bottom of the figure. Asterisks indicate the heavy and light chains of the antibody of the P1F6. The western blot was probed with an anti-his antibody. (B) Native dot blot of unboiled and boiled (denatured) integrin $\alpha V\beta 5$ probed with the anti- $\alpha V\beta 5$ antibody.

A

B

Figure 7.

Comparative analyses of AAV2-mediated transduction of HeLa cells with α V β 5 integrin inhibition. (A) Cells were mock-infected, infected with scAAV2-EGFP alone, and infected with scAAV2-EGFP and 100 μ g/ml or 10 μ g/ml of α V β 5 integrin at an MOI of 1,250 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. Original magnification X100. (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 <0.05 or ** p <0.01 vs scAAV2-EGFP alone.

Primer Sequences

^aIndicates the primers used as shown in Figure 1;

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