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Cloning, Expression, and Purification of the Glycosylated Transmembrane Protein, Cation-Dependent Mannose 6-Phosphate Receptor, from Sf9 Cells Using the Baculovirus System

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Abstract

The cation-dependent mannose 6-phosphate receptor (CD-MPR) is a single-pass type I membrane protein. This protein functions to transport lysosomal enzymes displaying phosphomannosyl residues from the Golgi complex and the cell surface to the lysosome. This glycosylated protein contains three disulfide bridges in its 159-residue extracytoplasmic domain. One of the problems with studying eukaryotic membrane proteins is isolating sufficient quantities. Structural studies typically require several hundred milligrams of highly purified protein. Here we present a method to isolate milligram quantities of CD-MPR/Asn81 suitable for structural studies.

Keywords

CD-MPR; Transmembrane protein; Sf9 cells; Purification; Glycoprotein

1 Introduction

The P-type lectin family of receptors is comprised of the cation-independent mannose 6-phosphate receptor (CI-MPR) as well as the significantly smaller cation-dependent mannose 6-phosphate receptor (CD-MPR) [1]. These transmembrane receptors are involved not only in delivering newly synthesized lysosomal enzymes to the lysosome but are also themselves trafficked to the cell surface where they bind to proteins displaying phosphomannosyl-modified carbohydrates. These receptors are trafficked throughout the cell by several sorting signals present in their cytoplasmic tails [2]. Although multiple structural studies have been conducted on the soluble cytosolic region of CD-MPR, studies on the full-length protein have been limited.

Insect cells, and in particular Sf9 cells, have long been used to express recombinant glycosylated proteins. We have now used this expression system to generate the full-length transmembrane protein, CD-MPR, at high enough quality and in quantities sufficient to be utilized in structural studies. To aid in crystallization, the same single glycosylation site

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mutant, termed CD-MPR/ Asn81, used to obtain the soluble structure of the receptor has been maintained [3].

In this chapter, we briefly describe the modification of a standard transfer vector so that high levels of expression can be achieved in Sf9 cells. This information is provided to users who may not have access to an appropriate vector suitable for targeting recombinant proteins through the secretory pathway in Sf9 cells. The subsequent cloning step, which should be standard in any molecular biology laboratory, is only briefly described and serves as an introduction to novices planning on using this system. We then describe the use of the baculovirus system to generate functional recombinant CD-MPR in quantities sufficient for structural studies. All steps involving Sf9 cells are described in detail. The result is a protein that is glycosylated and retained in the membrane. As presented here, the protocol is divided into seven steps: cloning the gene of interest (Subheading 3.1); culturing (Subheading 3.2) and transfection of Sf9 cells (Subheading 3.3); generation (Sub-heading 3.4) and titering (Subheading 3.5) of viral stocks; expression and purification of the full-length protein (Subheading 3.6); and characterization of the purified protein. This recombinant approach, while described specifically for CD-MPR, can be applied with modifications to other glycosylated proteins that are trafficked to the cell surface.

2 Materials

2.1 Instruments and Other General Materials

1. MaxQ™ 4000 orbital benchtop shaker (Thermo Fisher Scientific, Waltham, MA, USA) or similar.
2. Standard tissue culture hood.
3. Inverted microscope.
4. EVOS® FL Auto Imaging System.
5. ÄKTA Start chromatography system (GE Healthcare Life Sciences, Pittsburgh, PA, USA).
6. NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA).
7. Branson 450 or similar sonicator .
8. Low speed centrifuge capable of holding a JLA8.1000 rotor and a JA-20 rotor or similar (Beckman Coulter, Indianapolis, IN, USA).
9. Ultracentrifuge capable of holding a Ti-75 rotor or similar (Beckman Coulter).
10. 20 mL syringe and large bore (20G) needle.

2.2 Insect Cells

Sf9 Expression Systems (Expression Systems, Davis, CA, USA)
(www.ExpressionSystems.com) (see Note 1).

2.3 Media and Transfection Reagents

1. ESF 921 Medium: This is a protein-free insect cell medium appropriate for cell propagation as well as protein production (www.ExpressionSystems.com).
2. BestBac 2.0 v-cath/chiA Deleted Baculovirus Cotransfection Kit: This kit contains the viral DNA backbone, transfection medium and transfection reagent (www.ExpressionSystems.com).

2.4 Vectors and Plasmid Purification

1. pVL1392: This transfer vector may be obtained from several sources including Expression Systems (www.ExpressionSystems.com).
2. PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA): Transfection efficiency is dependent on quality of the plasmid DNA (*see Note 2*).

2.5 Plasticware for Cell Transfection, Propagation, and Protein Expression

1. Propagation and protein expression were done in Corning® disposable sterile bottles.
2. Transfections and immunofluorescent studies were done in 24-well Falcon™ plates.

2.6 Chemicals and Miscellaneous Reagents

1. Lysis buffer: 20 mM Tris base pH 7.8 at 4 °C, 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF).
2. Membrane wash buffer: 20 mM Tris base pH 7.8 at 4 °C, 150 mM NaCl.
3. Membrane solubilization buffer: 20 mM Tris base pH 7.8 at 4 °C, 150 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100.
4. Ni-wash and elution buffers: 20 mM Tris base pH 7.8 at 4 °C, 150 mM NaCl, 0.05% Triton X-100 and either 20, 50, 100, or 400 mM imidazole pH 7.5.
5. HisTrap™ excel 1 mL columns prepacked with Ni Sepharose excel affinity media for capture and purification of histidine-tagged proteins (GE Healthcare Life Sciences, Pittsburgh, PA, USA) (www.gelifesciences.com).
6. Bio-Rad protein assay solution (www.bio-rad.com) (Bio-Rad, Hercules, CA, USA).
7. Borosilicate glass tubes, 13 × 100 mm or similar.
8. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 15 mM Na₂HPO₄, adjust pH to 7.4 with KH₂PO₄.
9. PBS with 0.1% bovine serum albumin (BSA): add 0.1% (w/v) BSA to PBS.
10. CD-MPR antibody: provided by NMD [4].
11. Chicken anti-rabbit Alexa Fluor® 594 antibody (Thermo Fisher Scientific).

3 Methods

3.1 Cloning Gene of Interest

3.1.1 Modification of pVL1392—Although companies have developed different vectors for expressing foreign proteins in Sf9 cells, we have chosen to remain with one of the early transfer vectors, pVL1392. This transfer vector contains features for growth and maintenance in standard *E. coli* strains such as DH5 α and JM109. This 9.8 kb plasmid contains the pMB1 origin of replication from pUC19 for high copy number growth. Sandwiched between the 5' and 3'-viral sequences required for homologous recombination is the very strong polyhedron promoter, which drives recombinant protein expression. This vector is lacking one key feature needed for recombinant protein expression: a cleavable signal sequence which directs the recombinant protein to the endoplasmic reticulum for entry into the secretory pathway, an essential targeting signal for secreted and membrane-bound proteins. To overcome this limitation, we have chosen to use the honeybee melittin signal peptide: 5'-MKFLVNVALVFMVVYISYIYA-3' (Fig. 1) [6].

3.1.2 Cloning CD-MPR into Modified pVL1392—The DNA encoding the signal peptide was synthesized and digested as indicated in Fig. 1. Following successful incorporation of the DNA encoding the signal sequence, the plasmid was digested with EcoRI and XbaI and treated with FAST-alkaline phosphatase (AP). The purified PCR product encoding CD-MPR/Asn81 was also digested with the above two restriction enzymes. The digests were subjected to standard electrophoresis on a 1% low melting point agarose gel and the desired bands excised. Standard cloning procedures were followed and final clones were subjected to DNA sequencing to verify the sequence.

3.2 Culturing Sf9 Insect Cells

Sf9 cells obtained in suspension culture were propagated and frozen as directed by the company. Cells were propagated by thawing a 1 mL vial of 50×10^6 cells at 37 °C and diluting the cells into a 250 mL sterile storage bottle containing 30 mL ESF 921 medium.

1. Cells are grown at 27 °C in suspension cultures while shaking at 140 rpm in a MaxQ™ 4000 orbital benchtop shaker.
2. Cells were seeded in ESF 921 medium at a density of $0.75\text{--}1 \times 10^6$ cells/mL in either 250 mL or 1 L sterile Corning® storage bottles. The 250 mL bottles contained 30–100 mL of liquid culture while the 1 L bottles contained 400 mL suspension culture (see **Note 3**).
3. Cells were passaged when they reached a density of $4\text{--}6 \times 10^6$ cells/mL (typically every 2–3 days).

3.3 Transfection of Sf9 Cells

The transfection protocol is based on the published protocol from Expression Systems with modifications to utilize a 24-well Falcon plate. All steps are carried out in a standard tissue culture hood using sterile technique.

1. Seed cells at a density of 1.8×10^5 cells/well of a 24-well Falcon plate. Allow the cells to adhere to the plate ~30 min while transfection solutions are made (*see Note 4*).
2. Combine gently in a polypropylene tube (solution A) 0.4 μ g of CD-MPR/pVL1392, 1 μ L BestBac 2.0, v-cath/chiA Deleted linearized viral DNA, 20 μ L Transfection Medium.
3. Combine together in a polypropylene tube (solution B) 1–2 μ L supplied transfection reagent and 20 μ L Transfection Medium.
4. Let the solutions sit for 5 min in the hood.
5. Combine solution B into A and incubate in the tissue culture hood for ~30 min at room temperature.
6. Add 160 μ L Transfection Medium to the tube containing the combined solutions A and B.
7. Gently remove the medium from the plated adherent cells and replace with the solution from **step 6**.
8. Place tray in a tight-sealing, small plastic container and incubate at 27 °C 4–5 h (*see Note 5*).
9. Remove the transfection mix and feed cells with 600 μ L ESF 921.
10. Place tray back in plastic container being sure it is sealed and incubate at 27 °C without shaking.
11. Collect medium containing P-0 virus after 5 days, centrifuge 5 min at $500 \times g$ to remove any cells (*see Note 6*).

3.4 Generation of Virus Stocks

1. Seed the cells at a density of 1×10^6 cells/mL in 30 mL of ESF 921 medium.
2. Inoculate these seeded cells with the 600 μ L P-0 viral stock from **step 11** above.
3. Incubate with shaking (140 rpm) at 27 °C 4 days.
4. Cells should stop multiplying before day 3 (4×10^6 cells/mL) and should appear larger in size compared to noninfected cells.
5. On day 4, count cells and take note of cell appearance. Cell counts should be between 3 and 6×10^6 cells/mL depending on the transfection efficiency (*see Note 7*).
6. Pellet cells using a swinging bucket centrifuge ($500 \times g$ for 5 min) in a sterile 50 mL conical bottom centrifuge tube.
7. Sterile filter the supernatant. This supernatant is the P-1 virus stock and is stored at 4 °C in the dark.

3.5 Titering of Virus Stocks

Determination of the optimal infection conditions for protein expression is critical. The amount of virus stock required to infect a given number of cells will vary from batch to batch of virus as well as with the age of the viral stock. The amount of virus stock needed to infect a given number of Sf9 cells is determined by infecting a series of 30 mL cultures each containing 1×10^6 cells/mL.

1. Seed four 250 mL Corning® bottles with 30 mL of culture containing 1×10^6 cells/mL.
2. Leave one bottle uninfected as a control and infect the other three bottles with increasing volumes of virus stock: 25, 50 and 250 μ L is typical.
3. Count the cells 3 and 4 days post infection (*see Note 3*) and determine which flask(s) did not grow after infection. This determination will indicate how much virus needs to be added to arrest cell growth (*see Note 8*).

3.6 Expression and Purification of Full Length CD-MPR

The key to protein production in large quantities is having a large number of cells to infect at the same time (*see Note 9*). This procedure involves two independent steps involving infection of Sf9 cells and protein purification.

1. For the infection of Sf9 cells, count cells and infect with the amount of virus determined above to allow one doubling (*see Note 10*).
2. Cultures are grown as before with shaking at 140 rpm at 27 °C. It is important to monitor cell density, determined through counting, until the culture has stopped growing.
3. Cells are ready for harvest 48 h after they have stopped dividing, which typically occurs on the third day post-infection. Infected cells appear larger, but cell death should be less than 10% (Fig. 2).
4. Cells are harvested by centrifugation at $1000 \times g$ for 15 min in a JLA8.1000 rotor. Cell pellets can be either frozen at -80 °C for future use or processed immediately.
5. For protein purification, cell pellets from 1.2 L of Sf9 culture are resuspended in 150 mL of cold lysis buffer: 20 mM Tris, pH 7.8 at 4 °C, 300 mM NaCl, and 0.1 mM PMSF.
6. Cells are lysed by three 10 s bursts of sonication on ice (*see Note 11*).
7. Cell debris is removed by centrifugation at $500 \times g$ for 10 min in a JA-20 rotor.
8. The supernatants are collected and centrifuged at $80,000 \times g$ for 60 min in a Ti-75 rotor (Beckman Coulter).
9. The membrane pellets are resuspended in 100 mL of wash buffer: 20 mM Tris, pH 7.5 at 22 °C, 150 mM NaCl.
10. The pellets are centrifuged again as in **step 4** above.

11. The membrane pellets are resuspended in 100 mL of fresh cold (4 °C) wash buffer (*see step 5*). To ensure pellets are fully resuspended, the solution is passed several times through a large bore (20G) needle attached to a 20 mL syringe.
12. Triton X-100 (10%) stock solution is added to a final concentration of 1% (v/v) along with 0.1% (w/v) of a 20% sodium deoxycholate solution. The mixture is stirred in a cold room at 4 °C for 1 h.
13. After 1 h, half (50 mL) of the mixture from **step 12** is transferred to centrifuge tubes.
14. The membrane extracts are collected by centrifugation at $12,000 \times g$ in a JA20 rotor.
15. The extracts are loaded using a ÄKTA Start system at 2 mL/ min on a 1 mL HisTrap excel column preequilibrated with 10 column volumes of buffer: 20 mM Tris, pH 7.5 at 22 °C, 150 mM NaCl, 0.05% Triton X-100.
16. The column is washed until the baseline returned to 0 milliabsorbance units (mAU).
17. The column is then washed with buffer containing increasing concentrations of imidazole: 25, 50, 100, and 400 mM in 20 mM Tris, pH 7.5 at 22 °C, 150 mM NaCl, 0.05% Triton X-100. The baseline is allowed to approach 0 mAU before the next concentration of imidazole was introduced.
18. The remaining 50 mL of membrane solution is allowed to stir overnight (~18 h) at 4 °C before being subjected to **steps 13–17** above.

3.7 Preliminary Characterization of Purified Protein

We have used several standard techniques to evaluate if the length of solubilization and subsequent longer exposure to higher detergent concentrations had any effect on the quality or quantity of the transmembrane protein, CD-MPR/Asn81, being isolated. As described here, four steps are involved: (1) SDS-PAGE analysis, (2) Protein quantification, (3) Treatment with PNGaseF, and (4) SPR analysis of binding affinity.

1. Following elution from the HisTrap excel Ni column, the results of the purification are analyzed by SDS-PAGE. As this is a standard laboratory protocol, details are not provided.
2. Proteins were visualized after staining with Coomassie Blue (Fig. 3) (*see Note 12*).
3. For Protein Quantification, the Bio-Rad protein assay solution is used to measure total protein concentration.
4. For this, water (800 µL) is aliquoted into borosilicate glass tubes.
5. An aliquot (10 µL) of the 100 mM eluate (Subheading 3.6, **step 17**) is added to the water.

6. Bio-Rad Protein Assay solution (200 μ L) is added to each sample followed by vortexing of the sample.
7. Samples are read on a NanoDrop 2000c spectrophotometer.
8. Concentrations are determined relative to a standard curve determined from known amounts of bovine serum albumin.
9. The amount of total protein obtained from 1 h solubilization compared to overnight should be comparable if solubilization was complete within an hour (1.6 mg and 2.1 mg, 1 h versus overnight).
10. To verify the presence of an N-linked oligosaccharide, protein from the 100 mM imidazole eluates are incubated with PNGa-seF (follow manufacturer's recommendation for amount) over-night at room temperature.
11. Samples are subjected to SDS-PAGE (4%/12%) and gels stained with Coomassie Blue (Fig. 4) (*see* **Note 12**).
12. Surface Plasmon Resonance (SPR) Analysis of Binding Affinity should then be performed, as CD-MPR binds to *N*-glycans containing terminal mannose 6-phosphate residue(s) to traffic lysosomal enzymes.
13. Following previously published procedures, SPR studies can be used to characterize the interaction between CD-MPR and the lysosomal enzyme acid α -glucosidase (GAA) (Fig. 5) [7]. Details are not provided, as this technique may be more apt for this glycoprotein.

4 Notes

1. Sf9 cells isolated from the ovarian tissue of the fall army worm, *Spodoptera frugiperda*, were selected for their faster growth rate and ability to reach higher densities than some of the other established lepidopteran insect cell lines. This cell line is able to be maintained in monolayer cultures for transfections as well as in shake culture for protein production. They are suitable for use in all phases of protein generation from transfection, to virus propagation, to protein production [8].
2. This kit from Promega has provided very reliable results.
3. Cells are counted using a standard hemocytometer. Cells are split on Mondays and Wednesdays at 1×10^6 cells/mL. Cells are split at a lower density of 0.75×10^6 cells/mL on Fridays to avoid overgrowth by Monday.
4. In order to ensure cells are at ~75% confluence, plate wells at the calculated number of cells and also 1.5 and 2 times the calculated number of cells.
5. If nonshaking incubator is not available to incubate the plate, a shaking floor incubator can be utilized provided some type of stable, nonmoving platform can be installed. In the MaxQ™ 4000 orbital benchtop shaker, enough stationary floor space is provided to rest the legs of a homemade metal shelf. This shelf is tall enough to allow bottles to rest on the shaking platform underneath.

6. Failure to remove cells may result in higher cell densities and lower infection rates.
7. In our hands, viral stocks grown in this manner and having these final cell counts will yield a virus stock of 1×10^9 virus units/mL.
8. Also note which flask doubled in cell number since it is often advantageous to allow the cells to double once after infection in order to increase cell numbers and accordingly protein yields.
9. If six bottles, each with 400 mL of cells at 4×10^6 cells/mL, are needed on Friday and today is Monday, counting back, halving the volume each day until the current day will determine how many cells are needed (Friday: 6×400 , Thursday: 3×400 , Wednesday: 1.5×400 , Tuesday: 300 mL, Monday: 150 mL at 4×10^6 cells/mL today).
10. If your 25 μ L virus inoculum resulted in no cell growth then $(30 \text{ mL } (1 \times 10^6 \text{ cells/mL}))/0.025 \text{ mL} = 1.25 \times 10^9$ infecting units/mL.
11. The extent of cell disruption was evaluated by pipetting 20 μ L onto a transparent surface and viewing by a light microscope.
12. Samples were resolved on a standard SDS-PAGE gel using a 12% resolving gel overlaid with a 4% stacking gel. Gels were electrophoresed at 190 V for 45 min in standard running buffer (24.8 mM Tris base, 192 mM glycine, 0.1% SDS).

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Fig. 1.

Schematic diagram depicting modifications made to the standard pVL1392 transfer vector. The DNA sequence encoding the melittin signal peptide (translated DNA sequence shown in red) along with a octa-His tag and GS from an added restriction site (blue) and CD-MPR (black) has been modified to remove glycosylation at all but one (N81Q) position that was previously determined to be critical for proper folding [5]

Fig. 2.

Immunodetection of CD-MPR in Sf9 cells following infection with recombinant baculovirus. Infected and uninfected control Sf9 cells grown in suspension culture are allowed to adhere 30 min to a 24-well plate. After attachment, cells are washed with PBS and fixed by incubation on ice for 20 min in the presence of 10% formalin in PBS. Cells were rinsed with PBS plus 0.1% BSA before being incubated overnight at 4 °C in the dark in a solution of PBS plus 0.1% BSA and a 1:100 dilution of anti-CD-MPR (NMD) [4]. Cells were then rinsed with PBS plus 0.1% BSA two times prior to incubation with a 1:500 chicken anti-rabbit Alexa 864 antibody. Control, uninfected cells showed no detectable levels of fluorescence and are not shown. Images were taken using a 40× objective, the bar representing 100 μm

Fig. 3.

Purification of full-length CD-MPR. Ni-NTA column purification results comparing solubilization for 1 h at 4 °C compared to overnight (~18 h). A 12% resolving/4% stacking SDS-PAGE gel was loaded with 4 μ L of unstained Benchmark™ Protein Ladder (lane 1) and 12 μ L of the corresponding eluates: buffer wash (lane 2), 20 mM imidazole (lane 3), 50 mM imidazole (lane 4), 100 mM imidazole (lane 5), and 400 mM imidazole (lane 6). Monomer (~28 kDa) and dimer (~60 kDa) species are marked with arrows

Fig. 4.

PNGaseF treatment of proteins to detect the presence of an N-linked oligosaccharide. An aliquot of each 100 mM imidazole eluate was incubated overnight at room temperature in the absence or presence of PNGaseF. The cleavage reaction products were then resolved on a 12% resolving/4% stacking SDS-PAGE gel. For reference, PNGaseF is loaded in the right lane marked with an asterisk. Monomer (~28 kDa) and dimer (~60 kDa) species of CD-MPR are indicated with arrows

Fig. 5. SPR studies characterizing the interaction between CD-MPR/Asn81 and GAA. Increasing concentrations (10, 20, 40, 80, 120, 200, and 400 nM) of the CD-MPR/Asn81 protein are flowed over the GAA monoester coupled surface for 120 s followed by buffer and regeneration with 10 mM M6P. The response at equilibrium (Req) of the sensorgrams are plotted versus the concentration of protein and fit to a 1:1 binding isotherm (inset). Data from the 1 h detergent solubilization protocol are shown. The time of solubilization with 1% Triton X-100/0.1% sodium deoxycholate did not significantly affect binding affinity, and resulted in similar k_D values of 100 and 81 nM, respectively, for 1 h and overnight incubation times