PURIFICATION OF GPI8, A SUBUNIT OF GPI-TRANSAMIDASE, FROM ENGINEERED *E. COLI*

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Glycosylphosphatidylinositol (GPI) is a glycolipid membrane anchor for many cell surface proteins. It is synthesized in the endoplasmic reticulum in a complex biosynthetic pathway. The enzyme GPI transamidase (GPIT) attaches GPIs to proteins that contain a signal for GPI attachment. GPIT, a complex of five proteins, removes the C terminus of target proteins and replaces it with GPI. The presumed catalytic portion of GPIT is Gpi8, a monotopic protein, with a single trans-membrane protein. We sought to purify and crystallize Gpi8 for structural and molecular study.

In order to purify Gpi8, *E. coli* are transformed with cDNA that codes for a truncated form of yeast; Gpi8 contains six histidine residues instead of its membrane anchoring sequence. The His_6 sequence is inserted to enable purification of the protein on a metal affinity column. The transformed *E. coli* are allowed to grow, causing Gpi8 protein to accumulate in the cytoplasm. The cells are sonicated and their cytoplasmic contents are retrieved by centrifuging and collecting the supernatant. The supernatant is loaded onto a metal affinity column and Gpi8 binds to the resin while the cytosolic proteins do not. Gpi8 is eluted from the resin with high concentration imidazole and its purity is determined by SDS-PAGE and gel filtration. Concentrated Gpi8 is placed in wells with different reagents to identify conditions that promote crystallization. Elucidating the synthesis of GPI-anchored proteins is important because the molecules are present in many cells and are essential in the functioning of particular enzymes. GPI anchored proteins lace the cell membrane of parasites like *Trypanosoma brucei*, which causes sleeping sickness. Understanding Gpi8, and its role in the biosynthesis of GPI's can facilitate the creation of specific drugs against parasites.

INTRODUCTION

Glycosylphosphatidylinositol (GPIs) are glycolipids (sugar-containing lipids) that are responsible for anchoring certain proteins onto cell membranes. GPI-anchored proteins occur in all eukaryotes where they function in a variety of processes ranging from synaptic transmission to embryo development. GPI-proteins also lace the epithelial walls of kidney cells and, for many parasitic protozoa, they form a dense surface coat important for infectivity. The enzyme, GPI-transamidase (GPIT) is responsible for attaching a GPI onto a protein. GPIT contains 5 subunits, including Gpi8, its catalytic subunit. It is unknown how Gpi8 attaches a GPI to a protein. By crystallizing Gpi8, we can determine its amino acid arrangement through x-ray diffraction. The active site of Gpi8 can then be determined from a 3-dimensional structure, thus allowing the GPI-protein complex reaction to be more clearly understood. GPI-anchored protein's importance in human cells makes discerning its biosynthesis crucial. With such knowledge, GPI-based remedies against kidney and parasitic protozoan diseases can be developed.

MATERIALS/METHOD

Materials and Instrumentation

pET28a/YKH plasmid. BL21-Codon Plus (DE3)-RIPL *E. Coli*. LB media-pH 7.2. Talon resin. Crystallization Sets: Wizard I, II, & III. Crystal Screen I & II. PEG Ion2 Screen. Mass spectrometer (RockU facility).

Buffers

Sonication Buffer (SB): 300 mM NaCl, 1 mM PMSF, 50 mM Na-P, 2 mM °-me. Wash Buffer (WB): 50 mM Na-P, 300 mM NaCl, 5 mM imidazole. Elution Buffer : 50 mM Na-P, 300 mM NaCl, 200 mM imidazole.

Expression of Gpi8

All kanamycin concentrations were 40 μg/mL. *E.coli* cells were transformed with pET28a-YKH plasmid according to protocol from Stratagene. Cells were grown overnight on LB Kan^+ plate. A single colony was selected and grown overnight in 250 mL of medium Kan^+ at 37°C. 20 mL–25 mL of cells was added to fresh medium Kan^+ containing 0.1 M of gly-gly. Cultures were grown until OD_{600} was between 0.3–0.4. Cells were induced with IPTG to a final concentration of 0.4 mM and incubated at 19°C with shaking for 4 hours. Cultures were pelleted at 3000 × g at 4°C for 20 minutes and stored at −80°C.

Purification of Gpi8

All purification steps were conducted at 4°C. Cells were resuspended in sonication buffer (SB). The cells were sonicated for 3 minutes (6 s sonication with 15 s intervals of rest). The lysate was centrifuged (JA-20) at 15000 rpm for 30 min. The supernatant was removed and bound to the resin for 1.5 hours. Column was centrifuged until resin sediments and the flow through was removed. The column was washed with SB (10× resin volume) and twice with WB. Each wash lasted 5 minutes. Contents were centrifuged at 700 rpm for 3 minutes and the supernatant was removed. The resin was eluted by gravity flow with 3–4× resin volume of elution buffer containing 200 mM of imidazole. Collected volume, containing protein, was concentrated to upwards of 10 mg/mL using a Amicon millipore filter (10,000 MW cutoff).

Crystallization

Using a 96 well template, 1 μL of Wizard reagents was mixed with 1 μl of protein and placed in a 20°C incubator. Wells were checked periodically for the following 2–3 weeks for crystals.

RESULTS

Gpi8 was initially purified by IMAC, where an increased concentration of imidazole displaced Gpi8 bound to the resin. Gpi8 was further purified by gel filtration, which showed a high concentration of protein and no contamination. Each of the gel profiles had a shoulder to the left of the main peak. The average elution volume was 16.05 mL based on 4 runs. This average would predict Gpi8's MW to be 28 kDa. Gpi8 samples were run on a 10% SDS-PAGE gel to further confirm their purity. A solid band of protein appeared between the 45 kDa and 31 kDa MWM as expected. On earlier gels, extremely faint bands were present above and below Gpi8. We changed the way we extracted the protein from collecting the supernatant above the resin to gravity flow. As a result, the gels showed greater purity and higher concentration of protein. The fractions from the gel filtration were run on a 10% SDS-PAGE gel, and all bands ran equal distances, including the samples taken from the shoulder. Based on data from standards, the calculated MW of Gpi8 would be 32 kDa. We also analyzed Gpi8 on a native gel to see the protein's behavior in its natural state. On the native gel two strong bands appeared 4.1 mm from each other. The lower band was Gpi8 and the higher band was Gpi8's presumed dimer. Mass spectrometry confirmed the sole presence of Gpi8 in our sample. So far crystallization results (done in collaboration) have been mixed. Some wells show no chemical activity while others have precipitated Gpi8 in different forms. Precipitations were brown and wispy or silvery threads. Of the 428 wells set up, four 6% 2-propanol conditions looked promising, with one showing emergence of crystals.

DISCUSSION

All gel filtration profiles had a distinct sharp peak, indicating that our protein sample was highly concentrated. On every gel profile, there was a bump to the left of the main peak that was Gpi8's dimer. On the 10% SDS-PAGE gels, the fractions corresponding to this bump did not run higher than the fractions taken from the main peak. The faint bands present could not have been Gpi8's dimer because none ran near the area of 68 kDa, the homodimer's MW. The faint bands in the earlier elution steps were likely chaperonins. Other gels appeared similar to Gel 2 where no faint bands existed. A native gel comfirmed the presence of a second protein in the sample. The lower band was Gpi8, and the higher band was Gpi8's dimer. The dimer was detected in the gel filtration and native gel but not on SDS-PAGE. SDS breaks the covalent bonds in proteins and denatures them; therefore dimer formation would not occur. Native gels maintain the sample leaving the homodimers of Gpi8 intact. The mass spectrometry recovered 75% of our protein and confirmed that we did not have extraneous *E. coli* proteins in our sample. After running a comparison of Gpi8's sequence to the yeast protein database the highest scoring hit was a subunit of the GPIT complex as expected. Many of the wells did not have Gpi8 crystals, which can be attributed to many reasons. The PMSF added to the sonication buffer attaches to the active site of Gpi8 and might affect the folding of the protein and its crystallization. Also the negatively charged histidine tags might have interfered with crystallization.

RESOURCES

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